"In vitro pharmacological investigations (cytotoxic, anti-microbial and anti-oxidant activity) fraction of dichloromethane of *Ficus racemosa"*

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF PHARMACY, EAST WEST UNIVERSITY IN THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF PHARMACY

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Declaration by the Research Candidate

I, Nishat Tasnim, hereby declare that the dissertation entitled **"*In vitro* pharmacological investigations (cytotoxic, anti-microbial and anti-oxidant activity) fraction of dichloromethane of *Ficus racemosa*"** submitted by me to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the award of the degree Bachelor of Pharmacy is a complete record of original research work carried out by me during 2014-2015, under the supervision and guidance of Abdullah-Al-Faysal, Lecturer, Department of Pharmacy, East West University and the thesis has not formed the basis for the award of any other degree/diploma/fellowship or other similar title to any candidate of any university.

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Dedication

DEDICATED TO

MY PARENTS

Abstract

The plant *Ficus racemosa* has been used for the general promotion of health and longevity by Asian tribal. It is used as a traditional medicine for the treatment of various diseases like asthma, piles, diarrhea etc. The aim of the present study was to evaluate the cytotoxicity activity, antimicrobial activity and antioxidant activity of dichloromethane extract of *Ficus racemosa*. The powdered leaves of *Ficus racemosa* were extracted with methanol and then partitioned with n-hexane, ethyl acetate and dichloromethane consecutively. The dichloromethane fraction was used to evaluate cytotoxic, antimicrobial and antioxidant activities. The cytoxic activity was measured by brine shrimp lethality bioassay. Dichloromethane fraction showed cytotoxic activity with LC_{50} value 17.64µg/ml in brine shrimp lethality test. The antimicrobial activities of dichloromethane solvent extract of *Ficus racemosa* plant were tested against the Gram-positive and Gram-negative bacterial strains by observing the zone of inhibition. The antimicrobial test was performed by Disc diffusion method. The crude dichloromethane extract of *Ficus racemosa* plant showed low to moderate antimicrobial activities against the microorganisms at concentrations of 400 µg/disc. The fraction contained 33.632mgAAE/g of total phenolic content and 715mg AAE/g total flavonoid content. The results of study clearly indicate the presence of cytotoxic, antimicrobial and antioxidant properties of dichloromethane extract. The obtained results provide a support for the use of this plant in traditional medicine and its further investigation.

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1.1 General Introduction

Plants extract or pure compounds or standardized extracts those are natural product which provides unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity (Sasidharan*et al*,. 2011).According to the WHO, "A medicinal plants 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 used as a drug or therapeutic agent or an active ingredient of a medicinal preparation (Behera, 2006).

The therapeutic treatment of disease with the use of herbs began long ago. Methods of folk healing throughout the world commonly used herbs as part of their tradition. The practice of using herbs to treat diseases is very common among many non-developed societies. It is sometimes more easier to get than purchasing expensive modern pharmaceuticals. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, men turned to ethnopharmacognosy. They found literally thousands of phytochemicals from plants as safe and broadly effective alternatives with less adverse effect. Many beneficial biological activity such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity were reported. In many cases the people claim the good benefit of certain natural or herbal products (Sasidharan*et.al*,. 2011).

1.1.2 Medicinal plant

Medicinal plants are plants which have a recognized medical use. It's containing essential bioactive ingredients are used to cure disease or disorder since time immortal. One of the aims of medicinal plant research is the isolation and identification of markers/ bioactive compounds. Isolation of the markers compounds and bioactive plant constituents has always been a challenging task for the researchers. Separation of these components from the medicinal plants

includes the use of combination of chromatographic techniques such as column chromatography, preparative thin layer chromatography, preparative high performance liquid chromatography, droplet counter current chromatography, centrifugal thin layer chromatography, etc. Centrifugal thin layer chromatography which makes use of centrifugal force for separation of multi-component system offers extensive platform for the isolation of phytoconstituents from medicinal plants. This review focuses on basic principle, instrumentation and advantages of centrifugal thin layer chromatography (Agrawal& Desai, 2015).

1.1.3 Characteristic of Medicinal plant

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

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

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

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

1.1.4 Importance of medicinal plant

The importance of medicinal plants becomes more patent at the present time in developing countries.in Pakistan it is estimated that, 80% of its population depend on plants to cure themselves, 40% in china. In technologically advanced countries as the United States, it is estimated that, 60% of the population use medicinal plants habitually to fight certain aliments. In japan there is more demand of medicinal plants than of official medicines (website 1).

1.1.5 Necessity of Studying medicinal plant

- A future medicine bank to discover. There are approximately half a millon plants with flowers, most of which have not been investigated and which princicles could be decisive in the treatment of present or future diseases.
- Many of the modern medicines are produced indirectly from medicinal plants, for example aspirin.
- Plants are directly used as medicines by a majority of cultures around the world, for example Chinese medicine and Indian medicine.
- Many food crops have medicinal effects, for example garlic.
- Medicinal plants are resources of new drugs. It is estimated there are more than 250, 000 flower plant species.
- Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons.
- Cultivation and preservation of medicinal plants protect biological diversity, for example metabolic engineering of plants (website 1).

1.1.6 Classification of medicinal plant

Of the 2,50,000 higher plant species on earth, more than 80,000 species are reported to have at least some medicinal value and around 5000 species have specific therapeutic value. They are classified according to the part used, habit, habitat, therapeutic value etc, besides the usual botanical classification (Joy*et.al,*. 1998).

Table-1.1: Based on part used(Joy *et.al*,. 1998).

Part	Name of plant
Whole plant	Boerhaaviadiffusa, Phyllanthusneruri
Root	Dasamula
Stem	Tinosporacordifolia, Acoruscalamus
Bark	Saracaasoca
Leaf	Indigoferatinctoria, Lawsoniainermis, Aloe vera
Flower	Biophytumsensityvum, Mimusopselenji
Fruit	Solanum species
Seed	Daturastramonium

Table-1.2: Based on habit(Joy *et.al*,. 1998).

Part	Name of plant
Grasses	Cynodondactylon
Sedges	Cyperusrotundus
Herbs	Vernoniacineria
Shrubs	Solanum species
Climbers	Asparagus racemosus
Trees	Azadirachtaindica

Table-1.3: Based on habitat(Joy *et.al*,. 1998).

Habitat	Name of plant
Tropical	Andrographispaniculata
Sub-tropical	Menthaarvensis
Temperate	Atropabelladona

Table-1.4: Based on therapeutic value(Joy *et.al*,. 1998).

Therapeutic value	Name of plant
Antimalarial	Cinchona officinalis, Artemisia annua
Anticancer	Catharanthusroseus, Taxusbaccata
Antiulcer	Azadirachtaindica, Glycyrrhizaglabra
Antidiabetic	Catharanthusroseus, Momordicacharantia
Anticholesterol	Allium sativum
Antiinflammatory	Curcumadomestica, Desmodiumgangeticum
Antiviral	Acacia catechu
Antibacterial	Plumbagoindica
Antifungal	Allium sativum
Antiprotozoal	Ailanthus sp., Cephaelisipecacuanha
Antidiarrhoeal	Psidiumgujava, Curcuma domestica
Hypotensive	Coleus forskohlii, Aliumsativum
Tranquilizing	Rauvolfiaserpentina
Anaesthetic	Erythroxylum coca
Spasmolytic	Atropabelladona, Hyoscyamusniger
Diuretic	Phyllanthusniruri, Centellaasiatica
Astringent	Piper betle, Abrusprecatorius
Anthelmentic	Quisqualisindica, Punicagranatum
Cardiotonic	Digitalis sp., Thevetiasp
Antiallergic	Nandinadomestica, Scutellariabaicalensis
Hepatoprotective	Silybummarianum, Andrographispaniculata

1.1.7 Cultivation of natural product

Most of medicinal plants, even today, are collected from wild. The continued commercial exploitation of these plants has resulted in receding the population of many species in their natural habitat. Vacuum is likely to occur in the supply of raw plant materials that are used extensively by the pharmaceutical industry as well as the traditional practitioners. Consequently, cultivation of these plants is urgently needed to ensure their availability to the industry as well as to people associated with traditional system of medicine. If timely steps are not taken for their conservation, cultivation and mass propagation, they may be lost from the natural vegetation forever. In situ conservation of these resources alone cannot meet the ever increasing demand of pharmaceutical industry. It is, therefore, inevitable to develop cultural practices and propagate these plants in suitable agro climatic regions. Commercial cultivation will put a check on the continued exploitation from wild sources and serve as an effective means to conserve the rare floristic wealth and genetic diversity.
It is necessary to initiate systematic cultivation of medicinal plants in order to conserve biodiversity and protect endangered species. In the pharmaceutical industry, where the active medicinal principle cannot be synthesized economically, the product must be obtained from the cultivation of plants. Systematic conservation and large scale cultivation of the concerned medicinal plants are thus of great importance. Efforts are also required to suggest appropriate cropping patterns for the incorporation of these plants into the conventional agricultural and forestry cropping systems. Cultivation of this type of plants could only be promoted if there is a continuous demand for the raw materials. It is also necessary to develop genetically superior planting material for assured uniformity and desired quality and resort to organized cultivation to ensure the supply of raw material at growers end. Hence, small scale processing units too have to be established in order that the farmer is assured of the sale of raw material. Thus, cultivation and processing should go hand in hand in rural areas.
In order to initiate systematic cultivation of medicinal and aromatic plants high yielding varieties have to be selected. In the case of wild plants, their demonstration would require careful development work. Sometimes high yielding varieties have also to be developed by selective breeding or clonal micro propagation. The selected propagation materials have to be distributed to the farmer either through nurseries or seed banks. Systematic cultivation needs

specific cultural practices and agronomical requirements. These are species specific and are dependent on soil, water and climatic conditions. Hence research and development work has to be done to formulate Good Agricultural Practices (GAP) which should include proper cultivation techniques, harvesting methods, safe use of fertilizers and pestisides and waste disposal (Joy *et.al,*. 1998).

1.1.8 Tribal medicine

In different localities of Rangamati and Bandarban Districts of Bangladesh a survey was carried out between 2001 and 2002 to document medicinal plants. Kaukhali proper is about 10 km west to Rangamati town. Betbunia is a Union under Kaukhali P.S. situated about 9 km south of Kaukhali proper and about 18 km south-west to Rangamati town. Being a hilly area they are rich in floral diversity. Inhabitants of those areas are mostly tribal, dominated by Chakma and Marma. Many of them still depend on local medicinal plants for the treatment of different diseases. A good number of Bangali families are also living there. They also use quite a good number of medicinal plants for the treatment of different diseases. In recent years due to development of good communication, modern doctors and medicines have reached there, resulting decline in the use of traditional medicine. Therefore the knowledge of traditional use of medicinal plants by the local people is likely to be lost in near future, and for this it is necessary to document as much as possible the existing available information. Keeping this in mind this survey was done to document those valuable ethno-medico-botanical knowledge. The survey was carried out for about a year. During this work 34 species representing 23 genera and 17 families were documented which are used for the treatment of 31 diseases. Local names of those plants, locality and method of use are mentioned(Yusuf *et.al,*. 2006).

Table-1.5 : Some tribal medicinal plants & their uses(Yusuf *et.al*,. 2006).

Scientificname	Tribal name	Locality	Disease
Alstoniascholaris **(Apocynaceae)**	MarmaChailoi	Betbunia	Arthritic pain
Leeaindica ***(Leeacea)***	Chakma Haskura	Toolaban Marissa	Sore, leprosy, eczema, itching, bone fracture
Eupatorium odoratum ***(Asteraceae)***	Tonchongya Demrapata gach	Naramuk Rajsthali	Bleeding

1.1.9 Resource of natural product for establishing a new drug

Now-a-days, approximately 40% of the modern drugs have been developed from natural source. More precisely, 39% of the 520 new approved drugs between 1983 and 1994 were natural products or their derivatives, and 60-80% of antibacterial and anticancer drugs were from natural origin. In 2000, approximately 60% of all drugs in clinical trials for the multiplicity of cancer had natural origin. In 2001, eight (simvastatin, pravastatin, amoxicillin, clavulanic acid, ciprofloxacin, ceftriaxone, cyclosporine and paclitaxel) of the 30 top - selling medicines were natural products or their derivatives (David*et.al*,. 2007)

1.2Phytochemistry

Phytochemistry can be defined as the biochemical study of plants which is concerned with the identification, biosynthesis, and metabolism of chemical constituents of plants, especially used in regard to natural products. Phytochemistry is considered as one of the early subdivisions of organic chemistry. It has been of great importance in the identification of plant substances of medicinal importance (Website 2).

Phytochemistry is the study of phytochemicals produced in plants, describing the isolation, purification, identification, and structure of the large number of secondary metabolic compounds found in plants. Effect of extracted plant phytochemicals depends on:

- The nature of the plant materials
- Its origin
- Degree of processing
- Moisture content (Tiwari& Kumar, 2011)

1.3 Antimicrobial screening

Antimicrobial screening is performed to determine the susceptibility of the pathogenic microorganisms to test compound which, in turn is used to selection of the compound as a therapeutic agent. In general, antimicrobial screening in-vitro is undertaken in following two steps**:**

i) Primary assay

It is essentially a qualitative or semi qualitative test that indicates the sensitivity or resistance of microorganisms to the compound. However this technique cannot be used to distinguish between bacteriostatic and bactericidal agents (Reiner *et.al,*. 1982). The primary assay can be performed in vitro by disk diffusion assay method**,** which includes

- Plate diffusion test
- Streak test

The plate diffusion test utilizes different concentrations of a test compound absorbed on sterile filter paper disks on the same plate whereas the streak test permits the determination of the antibacterial effect of a test compound on several microorganisms simultaneously and is suitable for the estimation of the spectrum of the activity. However, the plate diffusion test is commonly used (Reiner *et.al*,. 1982).

ii) Secondary assay

It quantifies the relative potency such as minimum inhibitory concentration (MIC). The lowest concentration of an antimicrobial agent required to inhibit the growth of the microorganisms in vitro is referred to as minimum inhibitory concentration (MIC). It is done by serial dilution technique (Reiner *et.al*,. 1982).

1.4 Antioxidant activity

The main goal of antioxidant activity test is to find the oxidation reducing power of the plant extract.Oxidation in living organisms is essential for the acquirement of energy in catabolism. However,oxygen-centered free radicals and other reactive oxygen species, which are continuously, produced in vivo result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging, and diseases such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell&Gutteridge, 1999).

Free radicals are natural by-products of human metabolism. These are charged molecules which attack cells, breaking cellular membranes and reacting with the nucleic acids, proteins, and enzymes present in the cells. These attacks by free radicals, collectively known as oxidative stress, are capable of causing cells to lose their structure, function and eventually result in cell dysfunction. They are continuously produced by our body's use of oxygen, such as in respiration and some cell-mediated immune functions. Free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust, radiation, air pollution, pesticides, etc (Li &Trush, 1994).

Normally, there is a balance between the quantity of free radicals generated in the body and the antioxidant defense systems which scavenge these free radicals preventing them from causing deleterious effects in the body. The antioxidant defense systems in the body can only protect the body when the quantity of free radicals is within the normal physiological level. But when this balance is shifted towards more free radicals, increasing their burden in the body either due to environmental conditions or infections, it leads to oxidative stress (Finkel& Holbrook, 2000).

When the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the system, oxidative stress occurs in cellular system, including the superoxide anion radical, the hydroxyl radical, hydrogen peroxide and the peroxyl are greatly reactive molecules, which consequently generate metabolic products that attack lipids in cel1 membrane or DNA (Halliwell&Gutteridge, 1999).

Oxidative stress, involves a series of free radical chain reaction processes, 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 lead to an increase in the amount of free radicals in the body beyond its capacity to control them and cause irreversible oxidative damage (Tseng *et.al*,. 1997).

Improved antioxidant status helps to minimize the oxidative damage and thus can delay or decrease the risk for developing many chronic age related, free radical induced diseases (Karuna*et al*,. 2009). The interest in natural antioxidants, especially of plant origin, has greatly increased in recent years as the possibility of toxicity of synthetic antioxidants has been criticized (Jayaprakash&Rao, 2000).

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, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity (Zheng& Wang 2001).

Epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent (Owen *et al*,. 2000).

1.4.1 Types of Antioxidants

There are two types of antioxidants**.**

➢**Natural antioxidants** : Ascorbic acid, Gallic acid, Vitamin C & E, Coenzyme Q 10, Lipoic acid, Gluthatione

➢**Synthetic antioxidants** :Butylatedhydroxyanisole (BHA), Butylatedhydroxytoluene (BHT), Tertbutylhydroquinone Propyl gallate

1.5 Plant information

Figure 1.1: ***Ficusracemosa***

1.5.1 Synonyms

Ficusglomerata Roxb.

1.5.2 Scientific name

Ficusracemosa

1.5.3 Family

Moraceae

1.5.4Bengali/vernacular name

Jagadumur, Gulangdumur, Yajnadumbar.

1.5.5Tribal name

Jabuna, Sapai (Murong); Zoiggadumur (Chakma); Sanak (Marma).

1.5.6English name

Fig.

1.5.7 Description of the plant

A medium-sized to large deciduous, sometimes evergreen tree with spreading crown and white latex. Leaves 7.5-15 cm long, ovate-oblong or elliptic-lanceolate, entire, tapering to a bluntish point at the apex. Receptacles shortly pedunculate, on short leafless warted branches which issue from the stem and larger branches, subglobose, pyriform or subturbinate, 3.2 cm across, red when ripe.

1.5.8 Using information

- The fruits are considered astringent, stomachic and carminative; given in menorrhagia, haemoptysis, bronchitis, dry cough, diseases of kidney and spleen.
- The unripe fruit is astringent to the bowels, tonic and styptic; allays thirst, useful in leucorrhoea.
- The ripe fruit is acrid and cooling; useful in biliousness, burning sensation, fatigue, urinary discharges, thirst, leprosy, menorrhagia and nose bleeding.
- The fresh juice of the ripe fruit is used as an adjunct to a metallic preparation, which is given in diabetes.
- Fruits are used for rheumatic pain in Khagrachari by the Chakma. Bark is cooling, astringent and galactagogue; useful in asthma, piles and gravid uterus; as an infusion it is given for menorrhagia.
- The leaves are astringent to the bowels and good for bronchitis and bilious affections.
- Latex is aphrodisiac and vulnerary, useful in inflammations, piles, diarrhoea and in combination with sesamum oil in cancer. Roots are used in dysentery; sap is tonic and used in diabetes.

1.5.9 Chemical constituents

- Leaves contain glycosides, gluanol acetate, β-amyrin and β-sitosterol.
- Bark contains cerylbehanate, lupeol, lupeol acetate, α & β- amyrin, gluanol acetate, β-sitosterol, stigmasterol and a ketone. Gluanol acetate and β-sitosterol have also been isolatated from the heartwood.
- An alkaloid, dumurin has been isolated from the stem bark. Fruits contain lupeol acetate, β-sitosterol, hentriacontane, gluanol acetate and tiglic acid ester of taraxasterol and glucose.
- New tetracyclic triterpene-glauanol acetate has been isolated from leaves, bark and heartwood.(Website 3)

1.5.10 Distribution

Throughout Bangladesh, near streams and canals.

1.5.11 Taxonomy

Kingdom: Plantae

Phylum:Magnoliophyta

Class:Magnoliopsida

Order:Rosales

Family:Moraceae

Genus:Ficus

Species:*F. racemosa* (Shiksharthi& Mittal, 2011)

2.1 Literature review

2.1.1 Studies on antibacterial activity of *Ficusracemosa* Linn. Leaf extract

This study was investigated on the extracts of *Ficusracemosa* Linn. Leaves were tested for antibacterial potential against *Escherichia coli* ATCC 10536, *Basilluspumilis* ATCC 14884, *Bacillus subtilis* ATCC 6633, and *Staphylococcus aureus* ATCC 29737. The effects produced by the extracts were significant and were compared with chloramphenicol. The petroleum ether extract was the most effective against the tested organisms (Mandal*et.al,*. 2000)

2.1.2 Anti-bacterial activity of *Ficusracemosa* Linn. Leaves on actinomycesviscosus

The present study was undertaken with an objective to find out the antibacterial activity of *Ficusracemosa* Linn. Leaves against Actinomycetsviscosus.The hydro alcoholic extract of *Ficusracemosa* Linn. Was found effective against Actinomycesvicosus. The minimum inhibitory concentration (MIC) was determined using Broth dilution technique and found to be 0.08mg/ml. The zone of inhibition was measured using Cup plate diffusion technique (Shaikh*et.al,*. 2010).

2.1.3Antibacterial activities of various sequential extracts of *Ficusracemosa*stem bark

The present study evaluated the antibacterial activity of sequential extracts of *Ficusracemosa* stem-bark against *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, Escherichia coli* and *Bacillus subtilis* by disk-diffusion and agar-diffusion methods. In disk-diffusion assay chloroform, acetone and methanol extracts showed moderate antibacterial against

Staphylococcus aureus, *Bacillus cereus*, *Bacillus subtilis* compared to the positive control, while petroleum ether extract did not exhibit antibacterial activity against any of the organisms tested. Aqueous extract inhibited only *Bacillus subtilis*, while none of the extracts inhibited *Pseudomonas aeruginosa*. In agar-diffusion assay, both petroleum ether and aqueous extract did not show any inhibitory activity against any of the test organisms, while methanol extract showed moderate activity against *Staphylococcusaureus*, *Bacillus subtilis*, and *Escherichia coli*. Acetone extract showed moderate inhibition of *Staphylococcus aureus*, *Bacillus cereus* and FRSCE inhibited *Bacillus subtilis* and *Escherichia coli* to some extent (Ahmed *et.al,*. 2010).

2.1.4 *Ficusracemosa* Stem Bark Extract: A Potent Antioxidant and a Probable Natural Radioprotector

This study was conducted byEthanol extract (FRE) and water extract (FRW) of *Ficusracemosa* (family: Moraceae) were subjected to free radical scavenging both by steady state and time resolved methods such as nanosecond pulse radiolysis and stopped-flow spectrophotometric analyses. FRE exhibited significantly higher steady state antioxidant activity than FRW. FRE exhibited concentration dependent DPPH, $ABTS^{\bullet-}$, hydroxyl radical and superoxide radical scavenging and inhibition of lipid peroxidation with IC_{50} comparable with tested standard compounds. In vitro radioprotective potential of FRE was studied using micronucleus assay in irradiated Chinese hamster lung fibroblast cells (V79). Pretreatment with different doses of FRE 1h prior to 2 Gy γ-radiation resulted in a significant ($P < 0.001$) decrease in the percentage of micronucleated binuclear V79 cells. Maximum radioprotection was observed at 20 µg/ml of FRE. The radioprotection was found to be significant ($P < 0.01$) when cells were treated with optimum dose of FRE (20 µg/ml) 1 h prior to 0.5, 1, 2, 3 and 4 Gy γ-irradiation compared to the respective radiation controls. The cytokinesis-block proliferative index indicated that FRE does not alter radiation induced cell cycle delay. Based on all these results we conclude that the ethanol extract of *F. racemosa* acts as a potent antioxidant and a probable radio protector (Veerapur*et.al,*. 2009).

2.1.5 Hypoglycaemic and antioxidant activities of *Ficusracemosa* Linn. Fruits

In this study, the effects of *Ficusracemosa* Linn. Fruit extract and fraction on fasting serum glucose levels of normal, type 1 and type 2 diabetic model rats are presented. The aqueous 80% EtOH extract and its water soluble fraction of *F. racemosa* fruit did not show any serum glucose lowering effect on non-diabetic and type 2 diabetic rats at the fasting condition, whereas the extract showed significant hypoglycemic effect on the type 1 diabetic model rats. Both the extract and fraction were consistently active in both non-diabetic and types 1 and 2 diabetic model rats when fed simultaneously with glucose load. On the contrary, they were ineffective in lowering blood glucose levels when fed 30 min prior to glucose load. The 1-butanol soluble part of the ethanol extract exhibited significant antioxidant activity in DPPH free radical scavenging assay. 3-*O*-(*E*)-Caffeoylquinate (**1**) was isolated for the first time from this plant, which also showed significant antioxidant activity (Jahan*et.al,*. 2009)

2.1.6 Phyto-pharmacological properties of *Ficusracemosa* Linn

This literature review is based on *Ficcusracemosa* is a moderate sized avenue tree found throughout India. It is popular in indigenous system of medicine like ayurveda, siddha, unani and homoeopathy. In the traditional system of medicine various plant parts such as bark, root, leaves, fruits and latex are used in dysentery, diarrhea, diabetics, stomachache, piles and as carminative and astringent and also as antioxidant and anticancer agent. The present review is therefore, an effort to give a detailed survey of the literature on its pharmacological properties (Joseph & Raj, 2010).

2.1.7 Traditional uses,medicinalproperties,and Phyto-pharmacology of *Ficusracemosa*

Ficusracemosa Linn. (Moraceae) is a popular medicinal plant in India, which has long been used in Ayurveda, the ancient system of Indian medicine, for various diseases/disorders including diabetes, liver disorders, diarrhea, inflammatory conditions, hemorrhoids, respiratory, and urinary diseases. *F. racemosa* is pharmacologically studied for various activities including ant diabetic, antipyretic, anti-inflammatory, antitussive, hepatoprotective, and antimicrobial activities. A wide range of phytochemical constituents have been identified and isolated from various parts of *F. racemosa*. In this review, a comprehensive account of its traditional uses, phytochemical constituents, and pharmacological effects is presented in view of the many recent findings of importance on this plant (Ahmed &Urooj, 2010)

3.1 Collection & Preparation of Plant Material

Plant sample (Leaves) of *FicusRacemosa*was collected from Gopalgongin January 2014. Then proper identification of plant sample was done by an expert taxonomist. The leaves of the plant were sun dried for several days. The plant materials were then oven dried for 24hours at considerably low temperature for better grinding. The dried leaves was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Department of Pharmacy, East West University.

3.2 Extraction ofthe Plant Material

About 650gm of the powdered material was taken in separate clean, round bottomed flask (5 liters) and soaked in 3.5 liter of methanol. The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at 39^{o}C with a Heidolph rotary evaporation.

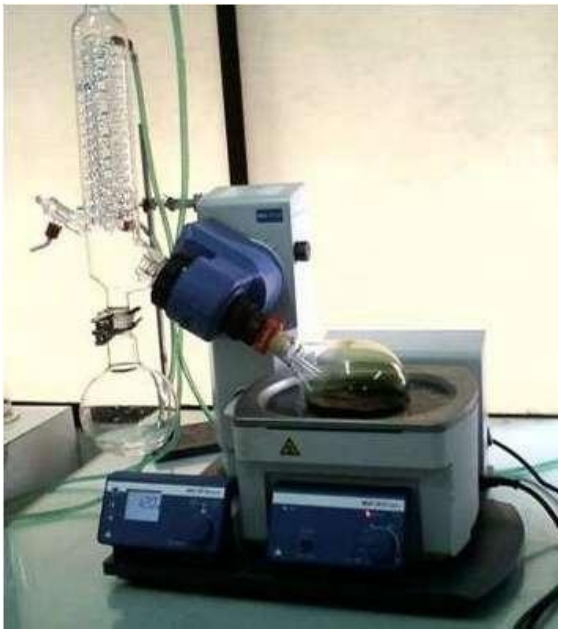


Figure 3.1: Drying of extract using rotary evaporator.

The concentrated extract was then air dried to solid residue. The weight of the crude methanol extract obtained from the powdered whole plant was 25.18 gm respectively.

3.3 Preparation of Mother Solution

5gm of methanol extract was triturated with 90ml of methanol containing 10ml of distilled water. The crude extract was dissolved completely. This is the mother solution.

3.4 Partition of Mother Solution

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

3.4.1 Partition with n-Hexane

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

3.4.2 Partition with Dichloromethane

To the mother solution left after partitioning with n-hexane, 12.5ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with dichloromethane (DCM). The process was repeated thrice (100ml X 3). The DCM fraction was then air dried for solid residue.

3.4.3 Partition with Ethyl Acetate

To the mother solution that left after washing with n-Hexane and DCMwas then taken in a separating funnel and extracted with Ethyl acetate (100ml X 3). The Ethyl acetate soluble fractions were collected together and air dried.

3.4.4 Collection of Ethyl Acetate Fraction

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

3.5 Brine Shrimp Lethality Bioassay

3.5.1 Principle

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

Nuneza, 2013; Rishikesh*et.al*,.2013).

3.5.2 Apparatus & Reagents

Table 3.1: Apparatus and reagents for Brine shrimp lethality bioassay

***Artemiasalina* leach (brine shrimp eggs)**	**Pipettes & Micropipette**
Sea salt (NaCl)	**Glass vials**
Small tank with perforated dividing dam to hatch the shrimp	**Magnifying glass**
Lamp to attract shrimps	**Test samples**

3.5.3 Procedure

3.5.3.1 Preparation of Sea Water

To hatch the brine shrimp nauplii for the assay, sea water representing brine should be prepared at first. To prepare sea water 38gm of pure NaCl was dissolved in distilled water and then the volume made up to 1000ml by distilled water in a 1000ml beaker for *Artemiasalina* hatching. 1-2 drops of NaOH solution of 1N was added with a dropper to obtain the pH 8.4 as sea water.

A rectangular tank was divided in to two unequal compartments by a porous separator. The larger compartment was darkened while the smaller one was kept illuminated. Then a dry preserved egg of *Artemiasalina* Leach was added in the artificial sea water. Oxygen was supplied through an air pump and a table lamp was placed near the beaker. The eggs of *Artemiasalina* were hatched at room temperature (25-30ºC) for 18-24hr. The larvae (nauplii) were attracted by the light and moved to the smaller compartment through the holes. 10 living shrimps were then collected by a pipette and then added to each of the test tubes containing 5ml of seawater. Those freshly hatched free-swimming nauplii were used for the bioassay Hatching of Brine Shrimp

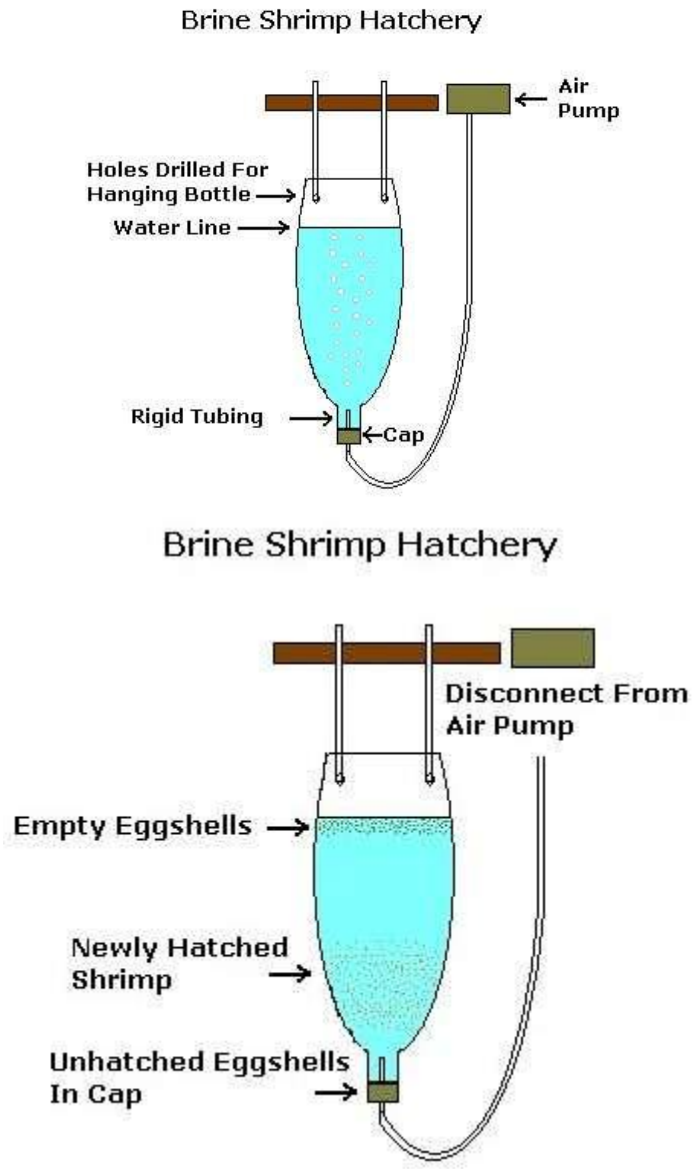


Figure 3.2: Brine shrimp Hatchery

3.5.3.2 Preparationof Test Solutions

Clean test tubes were taken. These test tubes were used for ten different concentrations (one test tube for each concentration) of test samples and ten test tubes were taken for standard drug tamoxifen for ten concentrations of it and another one test tube for control test.

All the test samples of 4mg were taken and dissolved in 200µl of pure dimethyl sulfoxide (DMSO) in vials to get stock solutions. Then 100µl of solution was taken in test tube each containing 5ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400µg/ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. In each case 100µl sample was added to test tube and fresh 100µl DMSO was added to vial. Thus the concentrations of the obtained solution in each test tube were 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml for 10 dilutions.

3.5.3.3 Preparation ofthe Test Samples Of Experimental Plant

All the test samples of 4mg were taken and dissolved in 200µl of pure dimethyl sulfoxide (DMSO) in vials to get stock solutions. Then 100µl of solution was taken in test tube each containing 5ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400µg/ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. In each case 100µl sample was added to test tube and fresh 100µl DMSO was added to vial. Thus the concentrations of the obtained solution in each test tube were 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml for 10 dilutions.

3.5.3.4 Preparation ofthe Positive Control Group

In the present study tamoxifen is used as the positive control. Measured amount of the tamoxifen is dissolved in DMSO to get an initial concentration of 20µg/ml. From that stock solution serial dilutions are made using DMSO to get 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml. Then ten living brine shrimp nauplii in 5ml simulated seawater are added to the positive control solutions in the pre-marked test-tubes to get the positive control groups.

3.5.3.5 Preparation ofthe Negative Control Group

100µl of DMSO was added to the pre-marked test tube containing 5ml of simulated seawater and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

3.5.3.6 Counting of Nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

3.6 Antimicrobial Activity by Disc Diffusion Method

3.6.1 Principle

The disk diffusion susceptibility method is simple and well-standardized. Bacterial inoculums are applied to the surface of a large agar plate. Antibiotic discs and disc of test materials are placed on the inoculated agar surface. Plates are incubated for 16–24hr at 35°C prior to determination of results. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The zones of growth inhibition are measured to the nearest millimeter around each of the antibiotic disks. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium (Barry, 1976).

3.6.2 Apparatus &Reagents

Table 3.2: Apparatus and reagents for antimicrobial test

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

3.6.3 Test Sample of *Ficusracemosa*

Dichloromethane fraction of methanolic extract of *Ficusracemosa*leaves were taken as test sample.

3.6.4 Test Organisms

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

Table 3.3: List of micro-organisms

Type of Bacteria	Name of Bacteria
Gram +ve	*Streptococcusaureaus* *Bacillus subtilis* *Bacillus ceresus* *Sarcinalutea*
Gram –ve	*Escherichia coli* *Salmonella paratyphi* *Vibrio parahaemolyticus* *Shigelladysenteriae*
Fungi	*Candida albicans* *Aspergillusniger*

3.6.5 Procedure

3.6.5.1 Preparation ofthe Medium

To prepare required volume of this medium, 5.6gm of agar medium was taken in a bottle with a cap and distilled water was added to it to make 200ml volume. The contents were then autoclaved to make a clear solution.

Figure 3.3: Autoclave machine

3.6.5.2 Sterilization Procedure

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petri dishes and other glassware were sterilized by autoclaving at a temperature of 121^0C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

Figure 3.4: Laminar hood

3.6.5.3 Preparation ofthe Test Plate

The test organisms were transferred from the subculture to petridish containing about 10 ml of melted and sterilized agar medium. The bacterial and fungal suspension was taken by a loop mixed with normal saline with the help of vortex machine. Then a sterilized cotton bud was taken and dipped into the bacterial suspension. Then the bacterial sample is applied to the petridish with the help of this cotton bud.

3.6.5.4 Preparation of Discs

Three types of discs were used for antimicrobial screening.

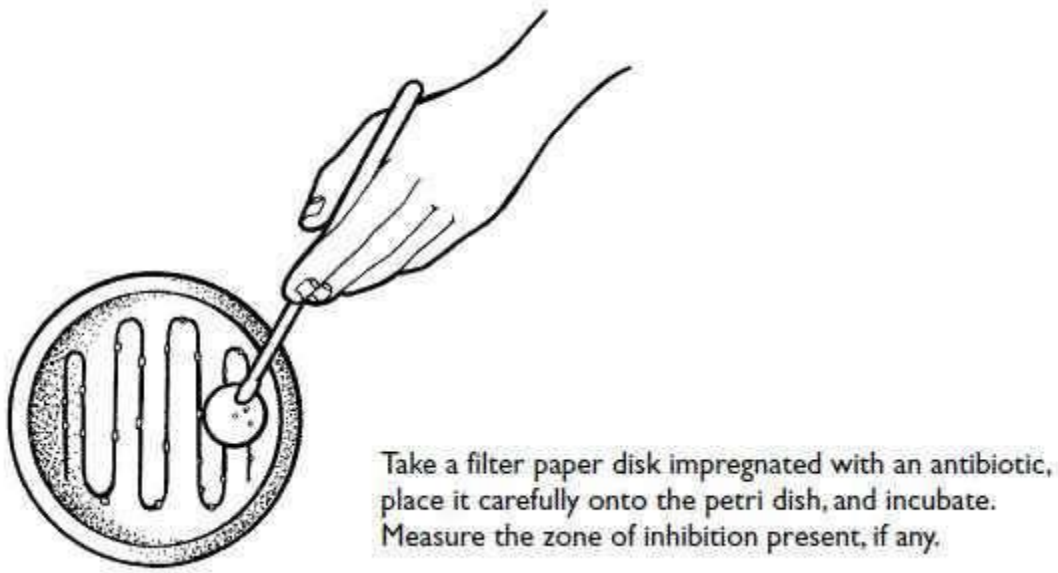


Figure 3.5: Preparation of filter paper discs

 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 investigation, ciprofloxacin (30µg/disc) disc was used as the reference.

 Blank Discs: These were used as negative controls which ensure that the residual solvent (left over the discs even after air-drying) and the filter paper were not active themselves.

 Sample Discs: These discs were soaked with solutions of test samples of known concentration, dried and used to determine the anti-activity of the samples.

3.6.5.5 Preparation of Test Sample

Measured amount of test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. Sterilized metrical filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

3.6.5.6 Application of Test Samples

Standard ciprofloxacin discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. Methanol discs were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

3.6.5.7 Diffusion & Incubation

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

Figure 3.6: Incubator

3.6.5.8 Determination of Antimicrobial Activity by Measuring the Zone Of Inhibition

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

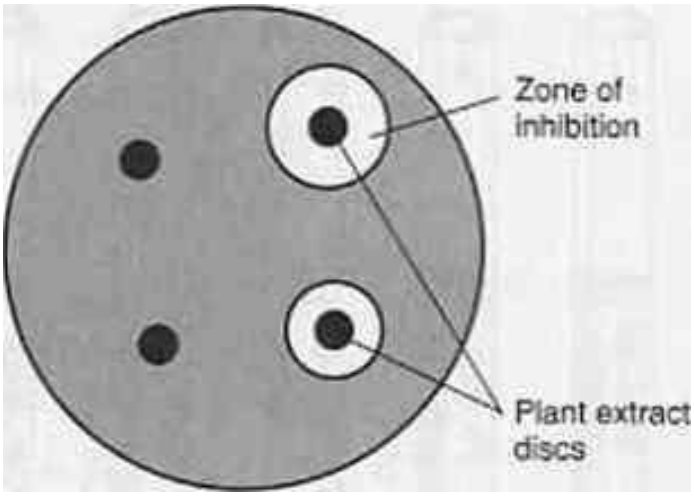


Figure 3.7: Clear zone of inhibition

3.7 Antioxidant Activity

3.7.1 Total Phenolic Content

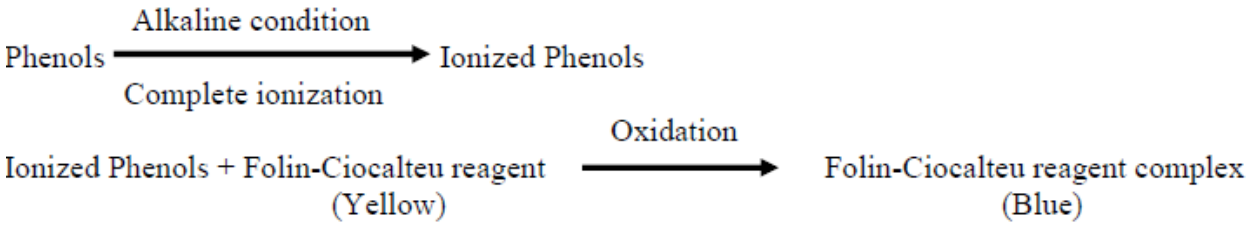
3.7.1.1 Principle

The content of total phenolic compounds in plant methanolic extracts was determined by Folin–Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. In the alkaline condition phenols ionize completely.

Table 3.4: Composition of 100mg Folin-Ciocalteu Reagent

Ingredient	Amount
Water	57.5ml
Lithium Sulfate	15.0mg
Sodium Tungstate Dihydrate	10.0mg
Hydrochloric Acid>=25%	10.0mg
Phosphoric Acid 85% solution in water	5.0mg
Molybdic Acid Sodium Dihydrate	2.5mg

When Folin-Ciocalteu reagent is used in this ionized phenolic solution the reagent will readily oxidize the phenols. Usual color of Folin-Ciocalteu reagent is yellow and after the oxidation process the solution become blue. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates - molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})^{-4}$.The intensity of the color change is measured in a spectrophotometer at 765 nm. The absorbance value will reflect the total phenolic content of the compound(Singleton *et.al,.*, 1999; Vinson *et.al,.*, 2005).



3.7.1.2 Apparatus & Reagents

Table 3.5: Apparatus and reagents used for total phenolic content

Folin-Ciocalteu reagent (10 fold diluted)	UV-spectrophotometer
Ascorbic acid	Beaker (100 & 200ml)
Na_2CO_3 solution (7.5%)	Test tube
Methanol	Micropipette (50-200µl)
Distilled water	Cuvette

3.7.1.3 Procedure

Standard curve preparation:

Ascorbic acid was used here as standard. Different ascorbic acid solutions were prepared having a concentration ranging from 120µg/ml to 80µg/ml. 5ml of FCR (diluted 10 times with water) and4ml of Na_2CO_3 (7.5%w/v) solution was added to ascorbic acid solution. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 765nm. After plotting the absorbance in ordinate against the concentration in abscissa a linear relationship was obtained which was used as a standard curve for the determination of the total phenolic content of the test samples.

Sample preparation:

2mg of the*Ficusracemosa* ethyl acetate fraction was taken and dissolved in 1ml of distilled water to get a sample concentration of 2mg/ml.

Determination of total phenol content:

- 1.0ml of plant extract (200µg/ml) of different concentrations (120µg/ml, 110µg/ml, 100µg/ml, 90µg/ml and 80µg/ml) was taken in test tubes.
- 5ml of Folin–ciocalteu (Diluted 10 fold) reagent solution was added into the test tube.
- 4ml of Sodium carbonate solution was added into the test tube.

- The test tubes containing the samples were incubated for 1hr at the room temperature to complete the reaction.
- Then the absorbance of the solution was measured at 765nm using a spectrophotometer against blank.
- A typical blank solution containing methanol was taken.

3.7.2 Total Flavonoid Content

3.7.2.1 Principle

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

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

3.7.2.2 Apparatus & Reagents

Table 3.6: Apparatus and reagents used for total flavonoid content

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

3.7.2.3 Procedure

Preparation of 10% Aluminium Chloride ($AlCl_3$) Solution: 10mg of $AlCl_3$ was taken into a 100ml of a volumetric flask and the volume was adjusted by distilled water.

Preparation of 4% NaOH Solution: 4mg of NaOH was taken into a 100ml volumetric flask and the volume was adjusted by distilled water.

Preparation of 5% (W/V) $NaNO_2$ Solution: 5mg of $NaNO_2$was taken into a 100ml of a volumetric flask and the volume was adjusted by distilled water.

Preparation of Standard Solution: The stock solution was prepared by taking 0.025gm of ascorbic acid and dissolved into 5ml of ethanol. The concentration of this solution was 5µg/µl of ascorbic acid. The experimental concentrations from this stock solution were prepared by the following manner.

Table 3.7: Preparation of standard solution

Concentration (µg/ml)	Solution taken from stock solution (µl)	Volume adjusted by ethanol (ml)	Final volume (ml)
250	250	4.75	5
200	200	4.80	5
150	150	4.85	5
100	100	4.90	5
50	50	4.95	5

Preparation of Extract Solution:5ml of each plant extracts were taken and dissolved into 5ml of methanol. The concentration of the solution was 1mg/ml of plant extracts. Then the following steps were carried out. 1.5ml extract was taken in a test tube and then 6ml of distilled water was added. Then 5% of $NaNO_2$ was added and incubated for 6 minutes. 10% $AlCl_3$ was added and incubated for 6 minutes. 4% NaOH and 0.6ml distilled water was added. Then it was incubated for 15 minutes. For blank solution 1.5ml methanol was taken and the same procedure was

repeated. Then the absorbance of the solution was measured at 510nm using a spectrophotometer against blank.

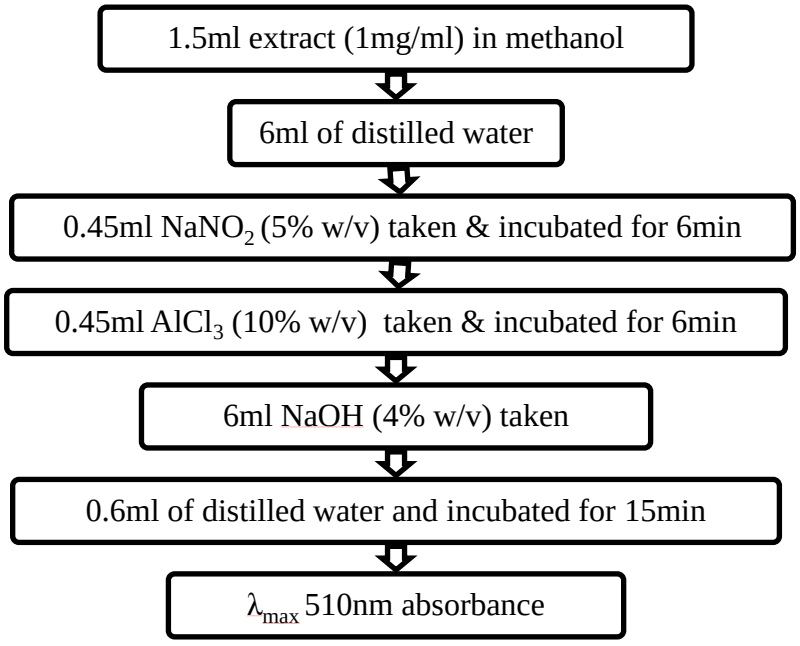


Figure 3.8: Schematic diagram of preparation of extract solution

Preparation of blank solution:

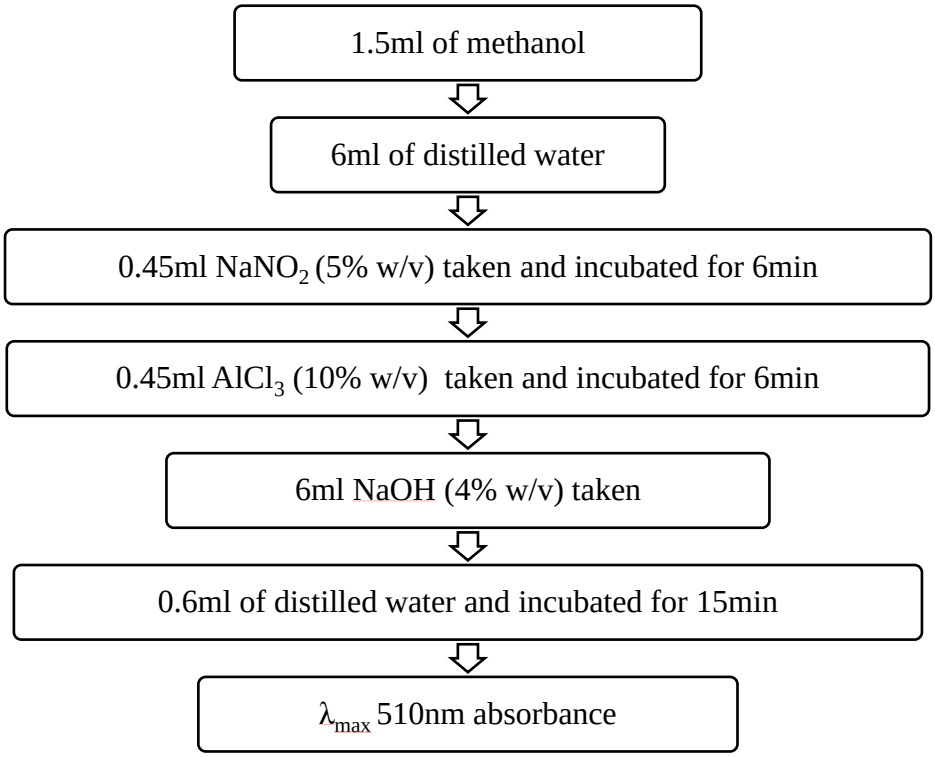


Figure 3.9: Schematic diagram of preparation of blank solution

4.1 Result of Brine Shrimp Lethality Bio-Assay

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

$$\% \text{ Mortality} = \frac{(\text{Number of dead nauplii}) \times 100}{\text{Total number of nauplii}}$$

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

4.1.1 Preparation of Curve for Standard

Here, Tamoxifen was used as reference standard

Table 4.1: Results of the bioassay of Tamoxifen (standard)

Test tube no.	Concentration (C) (µg/ml)	LogC	Number of Naupliialive	Number of Naupliidead	% Mortality	LC_{50} (µg/ml)
1	400	2.602	0	10	100	
2	200	2.301	1	9	90	
3	100	2.000	2	8	80	
4	50	1.699	3	7	70	
5	25	1.398	4	6	60	13.38
6	12.5	1.097	5	5	50	
7	6.25	0.796	6	4	40	
8	3.125	0.495	7	3	30	
9	1.5625	0.194	8	2	20	
10	.078125	-0.107	9	1	10	

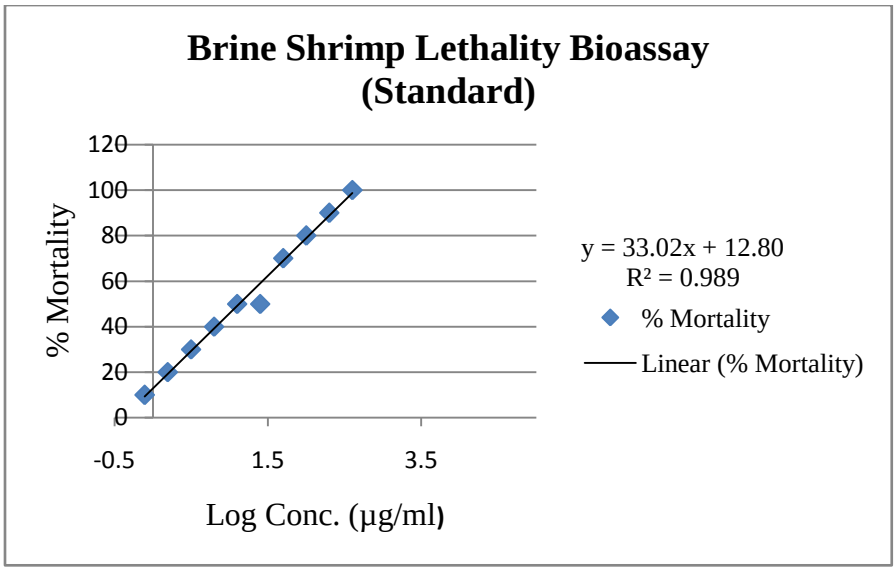


Figure 4.1: % mortality and predicted regression line of Tamoxifen (standard)

4.1.2 Preparation of Dichloromethane Fraction Curve

Table 4.2: Results of the bioassay of dichloromethane fraction (extract)

Test tube no.	Concentration (C) (µg/ml)	LogC	Number of nauplii alive	Number of naupliidead	% Mortality	LC_{50} (µg/ml)
1	400	2.602	0	10	100	
2	200	2.301	0	10	100	
3	100	2.000	1	9	90	
4	50	1.699	3	7	70	
5	25	1.398	4	6	60	
6	12.5	1.097	6	4	40	17.64
7	6.25	0.796	6	4	40	
8	3.125	0.495	7	3	30	
9	1.5625	0.194	8	2	20	
10	.078125	-0.107	8	2	20	

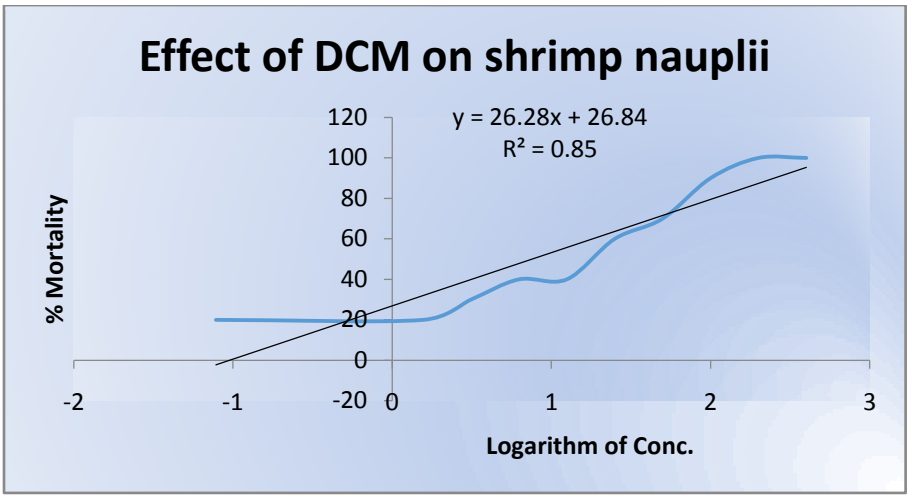


Figure 4.2: % mortality and predicted regression line of dichloromethane fraction (extract).

4.1.3Discussion

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

Table 4.3: Cytotoxic activity of Tamoxifen and dichloromethane fraction of *Ficusracemosa*leaves

Sample	Linear regression equation	R^2 value	LC_{50} (µg/ml, 24hr)
Standard (Tamoxifen)	y = 33.021x + 12.806	0.989	13.38
Extract (DCM fraction)	y = 26.28x + 26.84	0.85	17.64

In this investigation, standard and dichloromethane fraction exhibited cytotoxic activities with the LC_{50} values 13.38µg/ml and 17.64µg/ml respectively as shown in Table 4.3. For dichloromethane fraction R^2 value is less than the standard which indicates that the extract has less potent activity than standard against brine shrimp nauplii.

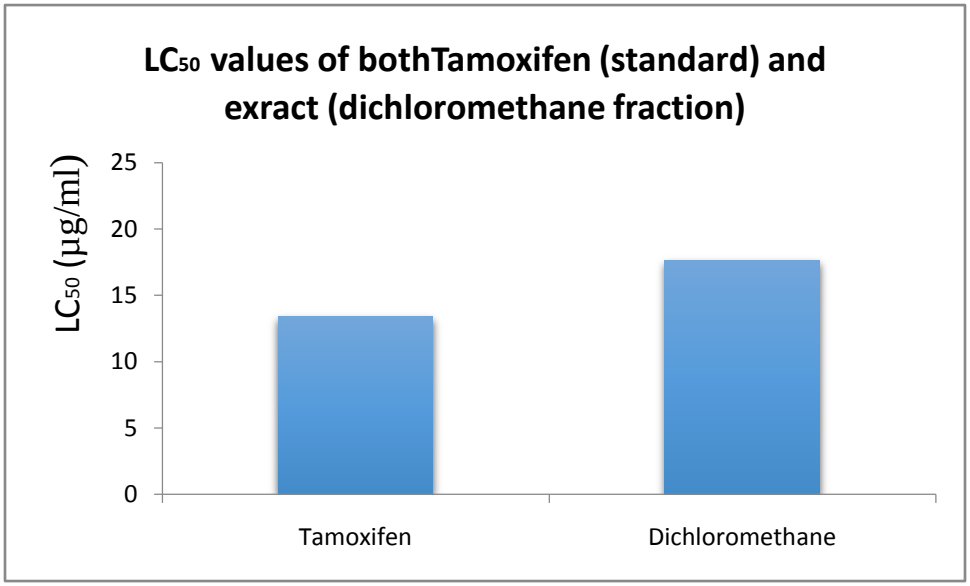


Figure 4.3: Comparison between LC_{50} values of standard and extract

From the above figure it can be concluded that for dichloromethane fraction the lethal concentration required to kill 50% of the sample population is higher than the standard. So the extract is less potent than Tamoxifen (Standard) at lower concentration.

4.2Result of Antimicrobial screening test

4.2.1The result of Antimicrobial Test

The antimicrobial activities of aqueous fraction of *Ficusracemosa* leaves extract were subjected in the study against various Gram positive bacteria, Gram negative bacteria and fungi. The

aqueous fraction was subjected to the various bacterial and fungal cultures and from that zones of inhibition were measured. Ciprofloxacin was used as standard reference.

4.2.2Zone Of Inhibition of Standard and Dichloromethane Fraction

Table 4.4: Antimicrobial activity of standard (Ciprofloxacin) and dichloromethane fraction

Types of microorganism		Zone of inhibition(mm)	
		Standard sample (mm)	Dichloromethane Fraction (mm)
Gram positive bacteria	*Streptococcusaureaus*	8	3
	Bacillus subtilis	3	1
	Bacillus ceresus	1	1
	Sarcinalutea	12	8
Gram negative bacteria	*Escherichia coli*	9	4
	Vibrio parahaemolyticus	6	2
	Shigelladysenteriae	10	7
	Salmonella paratyphi	4	2
Fungi	*Candida albicans*	15	5
	Aspergillusniger	16	6

4.2.3Discussion

Dichloromethane fraction of *Ficusracemosa* leaves extract showed low to moderate antimicrobial activity when compared toCiprofloxacin reference standard drug. None of the zone of inhibition of dichloromethane fraction is equal toCiprofloxacinagainst any bacteria or fungi as shown in the Figure: 4.4. Among all the microbiological cultures, the fraction showed the best antimicrobial activity against *Sarcinalutea* (8mm)and *Shigelladysenteriae*(7mm)comparable to the standard (18mm).

4.3Antioxidant Test Results

Antioxidant tests are classified by various methods. Samples were subjected to various standard methods to determine various scavenging capacity and amount that is equivalent to the standard like ascorbic acids. Antioxidant property of the aqueous fraction of *Ficusracemosa*leaves extract was determined by following methods-

- Determination of total phenolic content
- Determination of total flavonoids content

4.3.1Result of Total Phenolic Content

The dichloromethane extract of leaves and the aqueous fractions of the ethanol extract of *Ficusracemosa*leaves were subjected to determine total phenolic content. Ascorbic acid was used as reference standard.

4.3.1.1 Preparation of Standard Curve

Table 4.5: Total phenol content of ascorbic acid

Concentration (µg/ml)	Absorbance (at 765 nm)	Regression line	R^2value
80	2.406		
90	2.473		
100	2.767	y = 0.019x+0.824	0.937
110	3.057		
120	3.080		

A linear relationship was observed when the absorbances were plotted against concentrations, as shown in Figure 4.4. This linear curve was considered as a standard curve.

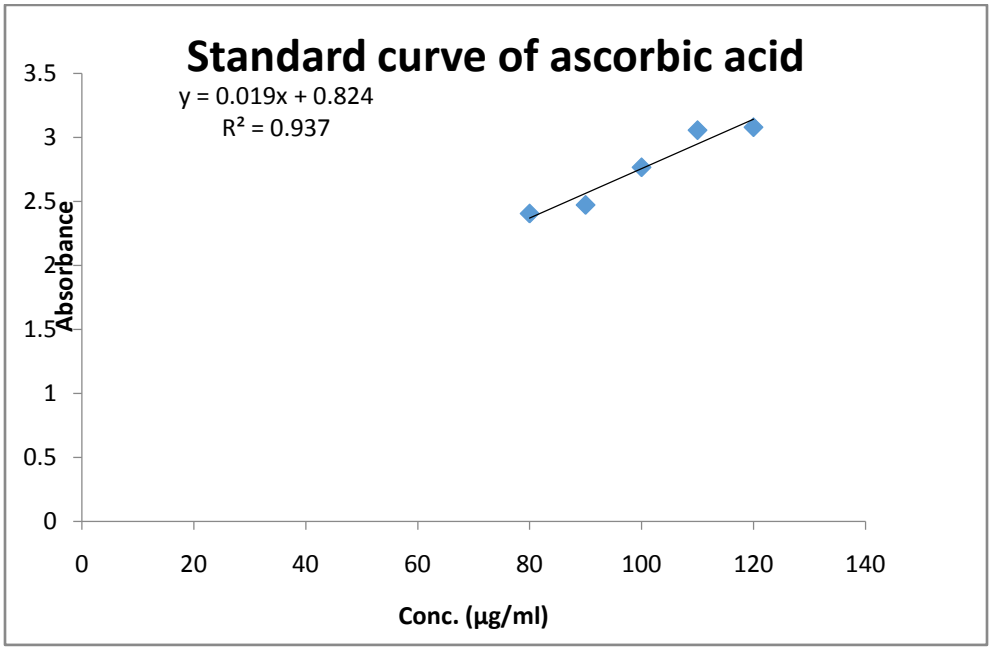


Figure 4.4: Graphical representation of assay of phenolic content of ascorbic acid

4.3.1.2Total Phenol Content Present in Extract

Based on the absorbance values of the extract solution, reacted with Folin-Ciocalteu reagent and compared with the standard solutions of ascorbic acid equivalents (AAE), the total phenolic content present in the extract is calculated and given in the table below.

Table 4.6: Total phenolic content of dichloromethane fraction of leaves of *Ficusracemosa*

Concentration (mg/ml)	Absorbance	mg AAE/g
2	1.463	33.632

4.3.1.3Discussion

The Total Phenolic content of Ficusracemosawas found. The plant extract contain phenolic content. The total phenolic content of methanolic crude extract, dichloromethane fraction 33.632 mg AAE/g. Further research is needed on the determination of the correlation between the antioxidant capacity and the chemical components of the plant.

4.3.2Result of Total Flavonoid Content

The dicholoromethane fractions of *Ficusracemosa*leaves were subjected to determine total flavonoid content. Ascorbic acid was used as reference standard.

4.3.2.1Preparation of Standard Curve

Table 4.7: Total flavonoid content of ascorbic acid

Concentration (µg/µl)	Absorbance (At 510 nm)	Regression line	R^2 value
50	0.05	y = 0.0017x-0.042	0.991
100	0.13		
150	0.19		
200	0.29		
250	0.39		

After absorbances were taken of different solution of ascorbic acid of concentrations ranging from 50µg/µl to 250µg/µl, a linear relationship was observed when the absorbances were plotted against concentrations, as shown in Figure 4.10 This linear curve was considered as a standard curve.

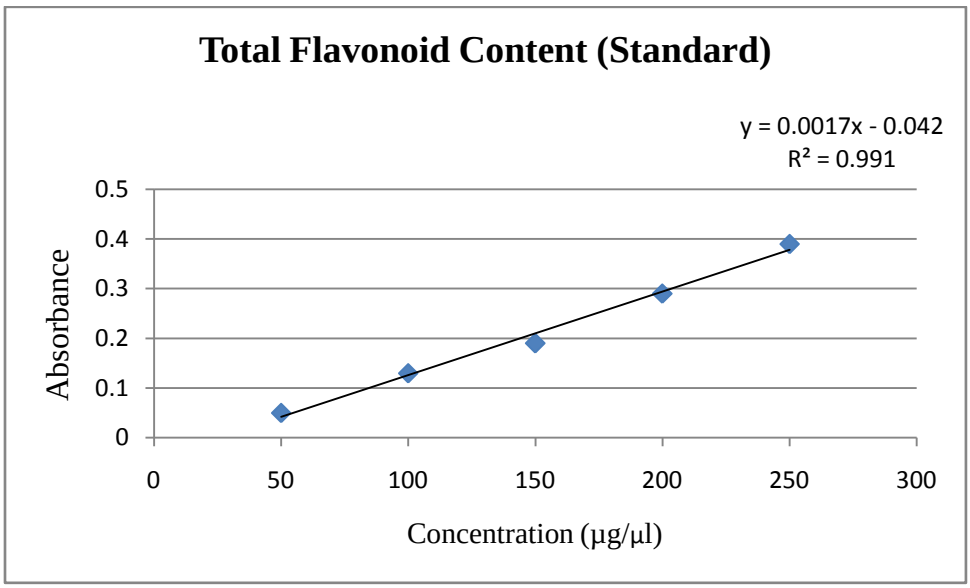


Figure 4.5: Graphical representation of assay of flavonoid content of ascorbic acid

4.3.2.2Total Flavonoid Content Present in Extract

Based on the absorbance value of extract solution and using the regression line equation of the standard curve, the total flavonoid present in the extract is calculated and is given in Table 4.8.

Table 4.8: Total flavonoid content of dichloromethane fraction of *Ficusracemesa*leaves extract

Sample	Concentration (mg/ml)	Absorbance	Total flavonoid content (mg of AAE/g of dried extract)
Dichloromethane fraction of *Ficusracemosa*	1	0.673	715

4.3.2.3Discussion

To determine the total flavonoid content of the test samples the standard curve was used. For 1mg/ml concentration of dichloromethane fraction of *Ficusracemosa* (leaves)715mg of AAE/gm of dried extract of flavonoid content was found. So it can be said that, the extract contains antioxidative compounds.

5.1 Conclusion

As the literature review suggests, the presence of several phytochemical compounds in *Ficusracemosa* makes the plant pharmacologically active. The present study showed that it has very good antioxidant activity that could make it a potent drug against free radical mediated diseases. The dichloromethane extract possesses cytotoxic activity that could be a better treatment in tumor as well as cancer. The study also showed that, the extract showed low to moderate antimicrobial activity that could be a better treatment in antimicrobial infections. However, studies are required on higher animal model and subsequently on human subjects to prove efficacy as an antioxidant, cytotoxic and antimicrobial agent.

The medicinal values of the leaves of this plant may be related to their phytochemical constituent. So, further investigations are needed to isolate and identify the active compounds present in the plant extract and its various fractions and their efficacy need to be tested. It will help in the development of new novel and safe drugs for the treatment of various diseases.

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