Phytochemical and Pharmacological Investigations of

Coccinia cordifolia

SUBMITTED BY

Tasneem Nayla Mredula

ID: 2007-1-70-005

SUBMISSION DATE: 19-06-2012

DEPARTMENT OF PHARMACY
EAST WEST UNIVERSITY

Phytochemical and Pharmacological Investigations of

Coccinia cordifolia

A Thesis Paper submitted to the Department of Pharmacy, East West University in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy

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In the name of ALLAH

The most Gracious

The most Merciful

CERTIFICATE

This is to certify that the thesis paper Phytochemical and Pharmacological Investigations of *Coccinia cordifolia* submitted to the Department of Pharmacy, East West University, Aftabnagar, Dhaka in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy was carried out by Tasneem Nayla Mredula, ID-2007-1-70-005.

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This is to certify that the thesis Phytochemical and Pharmacological Investigations of *Coccinia cordifolia* submitted to the Department of Pharmacy, East West University, Aftabnager, Dhaka in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy was carried out by Tasneem Nayla Mredula ID-2007-1-70-005 under our guidance and supervision and that no part of the thesis has been submitted for any other degree. We further certify that all the sources of information, laboratory facilities availed of this connection is dully acknowledged.

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Abstract

The study was designed for phytochemical investigation of methanol extract of the whole plant of *Coccinia cordifolia* (Family: Cucurbitaceae) and screening of their biological activities like antimicrobial cytotoxic or other activities. The powdered whole plant part of *Coccinia cordifolia* was extracted with methanol. The concentrated extract was then partitioned with *n*-hexane, chloroform and methanol.

All fractions for *Bacillus sereus* is showing the low antimicrobial activity and the growth of Gram (+ve) bacteria for *Bacillus sereus* is not inhibited properly. But for the *S. aureus* the methanol fraction shows activity where the diameter of zone of inhibition is 14.75 mm.

The Gram (-ve) bacteria *Escherichia coli and Shigella boydii* are also not inhibited by the fractions but for *V. mimicus* the N- hexane fraction shows activity where the diameter of zone of inhibition is 12.75 mm.

Methanolic extract of stem bark and different fractions was screened for cytotoxic properties using brine shrimp lethality bioassay where Vincristine sulfate LC_{50} value 0.323 μg/ml was used as a positive control. From the results of the brine shrimp lethality bioassay, it can be predicted that the crude extract and Dichloromethane (DCM), N-Hexane (NHE), Ethyl acetate (EAE), Methanol (MEL) fractions of methanolic extract possess cytotoxic principles and have considerable cytotoxic potency having LC_{50} values to be 0.041μg/ml, 0.063 μg/ml, 0.14 μg/ml and 0.36 μg/ml respectively. The degree of lethality of brine shrimp increases in a dose dependent manner. From the results of the brine shrimp lethality bioassays it can be well predicted that the chloroform fraction of metabolic crude extracts possess cytotoxic principles and have considerable cytotoxic potency.
Comparison with positive control vincristine signifies that cytotoxicity exhibited by the crude extracts and further bioactivity guided investigation can be done to find out potent antitumor and pesticidal compounds.

The plant extract contain phenolic content. From the results of the total phenolic test it can be predicted that the crude extract of Dichloromethane (DCM), N-Hexane (NHE), Ethyl acetate

(EAE), Methanol (MEL) fractions contain 26.50 mg GAE/g, 27.02 mg GAE/g, 8.40 mg GAE/g and 11.24 mg GAE/g respectively.

Different fraction of *Coccinia cordifolia* is subjected to free radical scavenging activity developed by the method of Brand-Williams. Here, *tert*-butyl-1-hydroxytoluene (BHT) was used as reference standard. The IC_{50} value found for Dichloromethane (DCM), N-Hexane (NHE), Ethyl acetate (EAE), Methanol (MEL) fractions are 4.81µg/ml, 6.20µg/ml, 2.34 µg/ml and 2.07 µg/ml respectively.

CHAPTER-01
INTRODUCTION

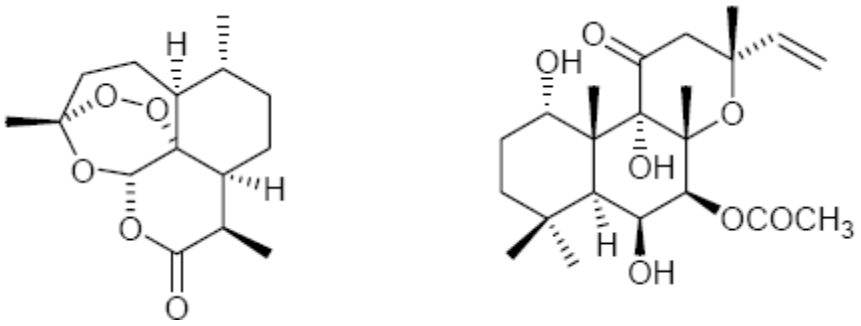
1.1 Introduction

Throughout the ages humans have relied on nature for their basic needs for the production of foodstuffs, shelter, clothing, means of transportation, fertilizers, flavors and not least, medicines. Nature has been a source of several medicines for treating various types of diseases in humans and animals for many years.

Plants are the important sources of a diverse range of chemical compounds. Some of these compounds possessing a wide range of pharmacological activities are either impossible or to difficult to synthesize in the laboratory. A phytochemist uncovering these resources is producing useful materials for screening programs for drug discovery. Emergence of newer disease also leading the scientists to go back to nature for newer effective molecules.

Plants have formed the basis for traditional medicine systems which have been used for thousands of years in countries such as China (Chang et al., 1986) and India (Kapoor et al., 1990). The use of plants in the traditional medicine of many other cultures has been extensively documented. These plant-based systems continue to play an essential role in health care, and it has been estimated by the World Health Organization that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care (Schultes et al., 1990). Plant products also play an important role in the health care systems of the remaining 20% of the population, mainly residing in developed countries (Arvigo et al., 1993). In a study it has been shown that at least 119 chemical substances, derived from 90 plant species, can be considered as important drugs that are in use in one or more countries. Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine (Arvigo et al., 1993).

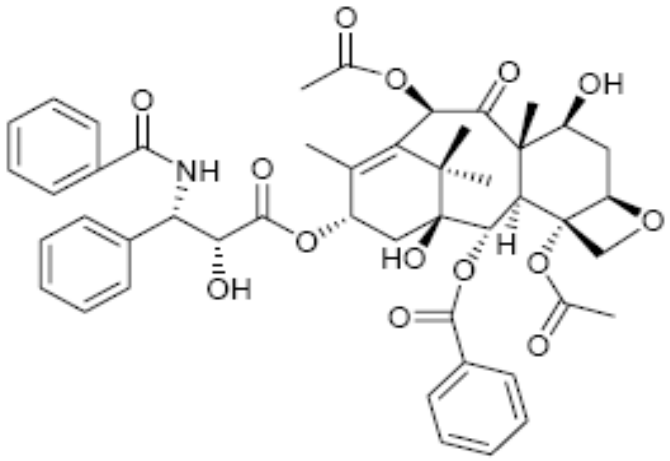
Examples of traditional medicine providing leads to bioactive natural products abound. Suffice it to point to some recent confirmations of the wealth of this resource. Artemisine (qinghaosu) (1, Figure 1.1) is the antimalerial sesquiterpene from a Chinese medicinal herb *Artemisia annua* (wormwood) used in herbal remedies since ancient times (Klaymann, 1985, Clark, 1996). Forskolin (2, Figure 1.1) is the antihypertensive agent from *Coleus forskohlii* Briq. (Labiatae), a plant whose use was described in ancient Hindu Ayurvadic texts (Bhat et al., 1977).



1 **2**

Figure 1.1: **Artemisinin (1) and Forskolin (2)**

Paclitaxel (3, Figure 1.2) is the most recent example of an important natural product that has made an enormous impact on medicine. It is interact with tubulin during the mitotic phase of the cell cycle, and thus prevents the disassembly of the microtubules and their by interrupts the cell division (Wani et al., 1991). The original target diseases for the compound were ovarian and breast cancers, but now it is used to treat a number of other human tissue proliferating diseases as well (Strobel et al., 2004).

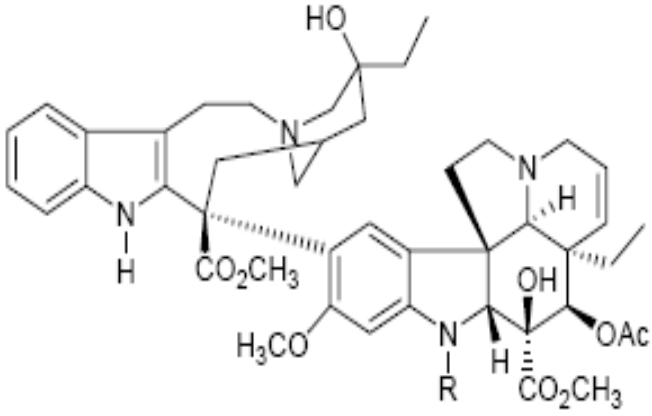


3

Figure 1.2: **Paclitaxel (3)**

A case of serendipity is the discovery of the so called vinca alkaloids, vincristine (4) and vinblastin (5), in *Catharanthus roseus*. A random screening programme (conducted at Eli Lilly and Company) of plants with antineoplastic activity found these anticancer agents in the 40th of

200 plants examined. Ethnomedicinal information attributed an anorexigenic effect (i.e. causing anorexia) to an infusion from plant (Tyler, 1986).



4 R=CHO **5** R=CH_3

Figure 1.3: **Vincristine (4) and Vinblastin (5)**

Within the next quarter century, the achievements of science and technology will be so great that, when brought to bear upon the mysteries of nature that have long puzzled us those mysteries will yield their secrets with amazing rapidity. It will be a fascinating and eventful period. We will not know only the causes of disease but the cures for most. Significant new drugs of plant origin and new methods of producing them will continue to be important parts of that service and thus Plants are considered as are of the most important and interesting subjects that should be explored for the discovery and development of newer and safer drug candidates.

1.2 Objective of the work

Bangladesh is blessed with a large number of medicinal plants belonging to various families including Cucurbitaceae. Cucurbitaceae family consists of about 300 genuses with 6000 species (Trease & Evans, 1996). The species contain a wide range of pharmacologically active compounds which are very useful and effective against various diseases like asthma, bronchitis, paralysis, ulcer, piles, sinus, scabies, eczema, Herpes, Cancer, warts and CNS depression. Although uses of some of these species are based on old and new experiences and clinical data,

many of them have no foundation what so ever. Therefore an attempt has been taken to study the phytochemical activity of *Coccinia cordifolia*, a member of the Cucurbitaceae family, growing in Bangladesh and evaluate its pharmacological profiles.So, the main objective is to explore the possibility of developing new drug candidates from *Coccinia cordifolia* for the treatment of various diseases.

A survey conducted in 1990 in different villages of Bangladesh shows that on average of 14% of people suffering illness approach qualified allopathic doctors, 29% contact unqualified village doctors, 10% contact mullahs, 29% contact quack and 19% contact homeopaths. The survey indicates an extensive use of medicinal plants, most of which are served in crude and substandard form, by our people. Traditional medicines are still manufactured in our country by following the age-old unscientific, traditional methods. Hundreds of indigenous medicinal plants are employed in different Ayurvedic and Unani commercial preparations without proper standardization, quality control, evaluation and determination of the chemical nature, pharmacological and toxicological studies of the active components which are essential to utilize their therapeutic potential fully. Toxicity of the plants or plant extracts is coming to light with the advancement of science. Since Bangladesh is a country of low economic growth, a proper health care system can be established by supplying low cost medicines to its population. This may be possible only by developing standard drugs from our natural resources of medicinal plants. In order to achieve this goal research and development of traditional medicines should be given the due priority.

Besides, Bangladesh imports a large quantity of pharmaceutical raw materials including medicinal plants and semi-processed plant products to manufacture drugs. Each year a great deal of money is spent on this purpose.

1.3 Research on medicinal plants: Bioactivity guided approach

Plants, the molecular architect, still offer a great potentiality for drug research. Plants contain compounds having interesting skeletons. Bioactivity guided phytochemical investigation of medicinal plants may yield newer chemical constituents of remarkable therapeutic interest. Occasionally, native

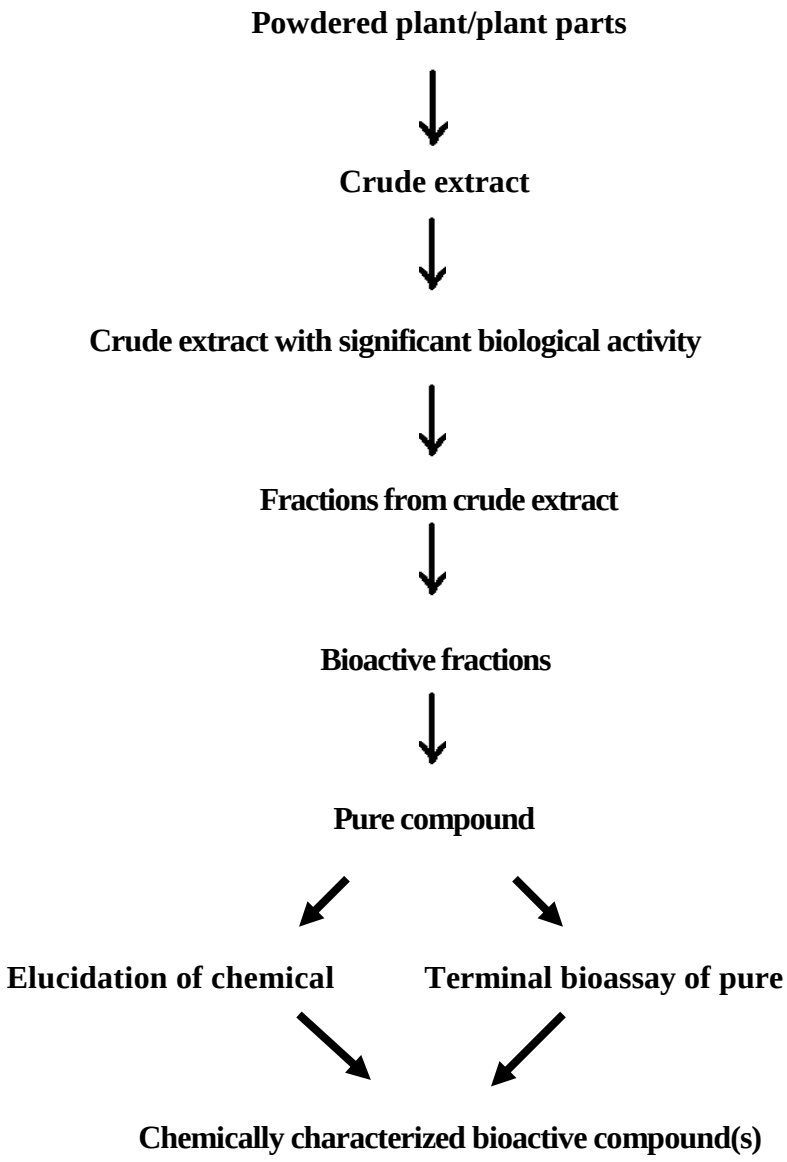
lore provides clue to plants with pharmacological activity. Extensive phytochemical investigation and isolation of active component(s) in the pure form thus become necessary to avoid untoward effects and to ensure safe use of herbal medicines. With technological advancement, phytochemical studies of medicinal plants got a rapid pace and the presence of many chemical compounds came into light. These plant-derived compounds often played an important role in directing laboratory synthesis of many new classes of drug molecules.

Bioactivity guided phytochemical approach, has three phases of investigation. **First,** biological activity is detected in crude material, and a bioassay system is set up to permit the identification of active fractions and discarding the inactive ones. **Second**, the crude material is fractionated by the most appropriate chemical procedures, all fractions are tested, and active fractions are further fractionated, and so on, until pure compounds are obtained. **Third**, the chemical structures of pure compounds are determined.

Sometimes phytochemical analysis of plants may yield such chemical constituents having no remarkable therapeutic interest. The crude drug containing several constituents may be found to be ineffective in case of therapy for which it was used traditionally. For example, *Vinca rosea*, once used traditionally as antidiabetic drug was found to contain hypoglycemic alkaloid principles in minute but it was found to contain anticancer principle vinca alkaloid in a high yield. *Rauwolfia serpentina* which was traditionally used for a variety of illnesses, revealed the presence of an antihypertensive and tranquilizing agent reserpine. So systemic research on medicinal plants may open the door of many unknown therapeutic tools.

It is evident from the above discussion that the search of plant constituents having therapeutic interest requires bioactivity studies with the crude extracts prior to phytochemical analysis. Only the bioactive extracts or fractions would be of interest for next phytochemical analysis. Without prior pharmacological studies, the phytochemical studies alone can provide the chemical constituents of the plant, but these may or may not have therapeutic value. So in medicinal plants research, bioactivity guided phytochemical approach might be a rational approach.

Flow chart of Bioactivity Guided Phytochemical Approach



CHAPTER-02
PLANT REVIEW

2.1 The plant family: Cucurbitaceae

The family Cucurbitaceae includes a large group of plants which are medicinally valuable. It is a family of about 130 genera and about 800 species. Seeds or fruit parts of some cucurbits are reported to possess purgatives, emetics and antihelmintics properties due to the secondary metabolite cucurbitacin content. A number of compounds of this group have been investigated for their cytotoxic, hepatoprotective, anti-inflammatory and cardiovascular effects. Cucurbitacins constitute a group of diverse triterpenoid substances which are well known for their bitterness and toxicity. They are highly oxygenated, tetracyclic triterpenes containing a cucurbitane skeleton characterized. The cucurbitacins are arbitrarily divided into twelve categories, incorporating cucurbitacins A-T. A lot of work has been done by the researchers throughout the world on various plants of the family Cucurbitaceae. Some of the important plants that have been extensively studied are ***Momordica charantia,*** *Cucurbita pepo, Cucurbita andreana, Cucurbita ficifolia, Cucumis sativus, Cucumis melo, Citrullus colocynthis, Luffa echinata* etc.

2.2 Broader classification of Cucurbitaceae

Domain	*Eukaryota*
Kingdom	*Plantae*
Subkingdom	*Viridaeplantae*
Phylum	*Tracheophyta*
Subphylum	*Euphyllophytina*
Class	*Spermatopsida*
Order	*Cucurbitiales*
Family	*Cucurbitaceae*
Genus	*Coccinia*
Botanical Name	*Coccinia cordifolia*

Table 2.1: **Classification of Cucurbitaceae**

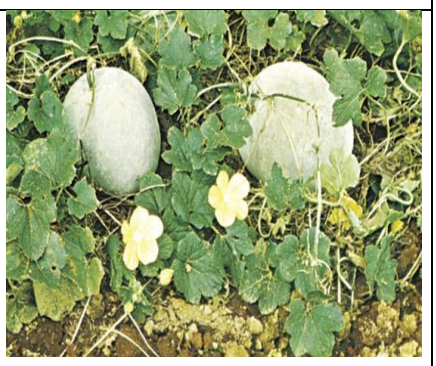
2.3 Species of Cucurbitaceae available in Bangladesh

Cucurbitaceae plants grow well in Bangladesh. They are found in plain areas as well as in hilly areas like Sylhet and Chittagong. According to the recent reports of Bangladesh National Harberium, the following Cucurbitaceae plants are available in Bangladesh as shown in Table.

Genus	Species
1. **Cucurbita**	*Cucurbita maxima*
2. **Lagenaria**	*Lagenaria siceraria*
3. **Benincasa**	*Benincasa hispida*
4. **Cucumis**	*Cucumis sativus*
5. **Luffa**	*Luffa echinata* *Luffa cylindrical*
6. **Momordica**	*Momordica charantia* *Momordica cochinchinensis*
7. **Trichosanthes**	*Trichosanthes cucumerina* *Trichosanthes kirilowii*
8. **Cucurbita**	*Cucurbita pepo* *Cucumis melo*
9. **Citrullus**	*Citrullus colocynthis*

Table 2.2: **Available species of Cucurbitaceae in Bangladesh**

2.4 Medicinal Importance of Cucurbitaceae

Genus/ Species	Plant parts	Medicinal or other uses	Picture
1. **Cucurbita** *Cucurbita maxima*	• Seeds	• Treatment for Tapeworms.	
2. **Lagenaria** *Lagenaria siceraria*	• Fruits	• Cardioprotective effect against doxorubicin induced cardiotoxicity, diuretic activity, anti hyperlipidemic activity, cures pain, ulcers and fever and used for pectoral cough, asthma and other bronchial disorders	
3. **Benincasa** *Benincasa hispida*	• Fruits • Seeds	• Antidiarrheal, renoprotective activity on ischemia/reperfusion induced renal damage, management of peptic ulcer • antifungal activity, anti-angiogenic effect,	
4. **Cucumis** *Cucumis sativus*	• Fruits • Seeds	• Removing constipation and aid indigestion, has demulcent property • cooling, tonic, diuretic and anthelmintic	

***5.* <u>Luffa</u>** *Luffa echinata*	• Fruits	• Treatment of liver ailments	
***6.* <u>Momordica</u>** *Momordica charantia*	• Fruits • Seeds	• Anthelmintic, antiemetic, carminative, purgative and for the treatment of anaemia, jaundice, malaria, cholera • Anti-diabetic, anti-oxidant property	
***7.*<u>Trichosanthes</u>** *Trichosanthes cucumerina*	• Roots • Fruits • Seeds	• Cure for bronchitis, headache and boils, considered as cathartic • Used as an anthelmintic • Used for stomach disorders and are also considered as antifebrile and anthelmintic	
***8.* <u>Cucurbita</u>** *Cucurbita pepo*	• Fruits • Seeds	• Astringent to the bowels, increases appetite, cures leprosy and purifies the blood • cure sore chests, haemoptysis, bronchitis and fever	

9. **Citrullus** *Citrullus colocynthis*	• Fruits • Roots, Seeds	• Used in hypoglycemia, tumors, ascites, leucoderma, ulcers, asthma, bronchitis and constipation • Analgesic and anti-inflammatory activities	

Table 2.3: **The medicinal importance and other uses of Cucurbitaceae plants**

2.5 Botanical features of Cucurbitaceae family

2.5.1 Leaves: Leaves are exstipulate alternate simple palmately lobed or palmately compound

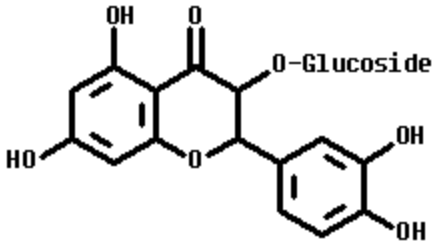
2.5.2 Flowers: Many species have large, yellow or white flowers. The flowers are unisexual, with male and female flowers on different plants (dioecious) or on the same plant (monoecious).

- Male flowers: The male flowers typically have 5 petals fused together surrounding 3-5 stamens.
- Female flowers: The female flowers consist of 3 stigma lobes with an inferior ovary.

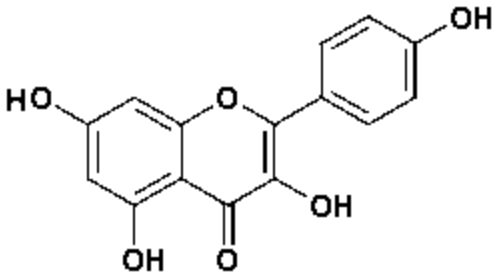
2.6 Chemitry of Cucurbitaceae

The major chemical constituents present in this species were Alkaloids, Flavonols, kaempferol and quercetin, or quercetin.

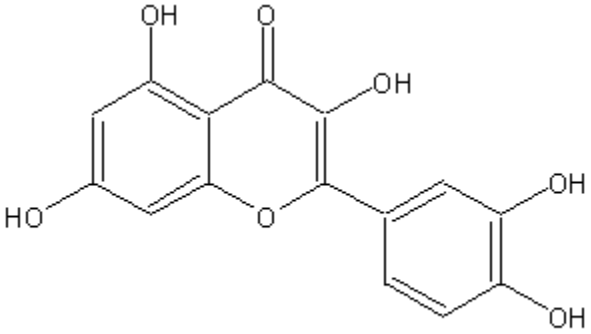
Some of the structures are given below-



Flavonol



Kaemferol



Quercetin

2.7 Phytochemical review of plant *Coccinia cordifolia*.

Figure 2.1: **Whole plant of *Coccinia cordifolia***

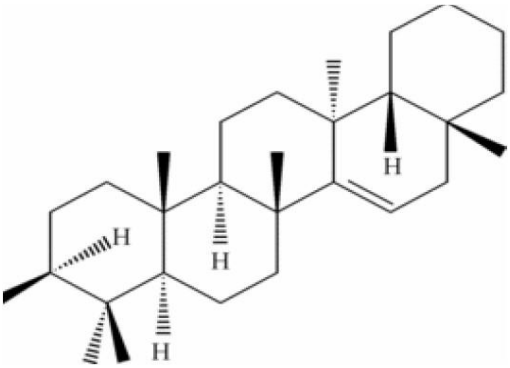
2.8 Traditional uses of C*occinia cordifolia*

- Antidiabetic activity
- Antioxidant activity
- Anti-inflammatory activity
- Post- and pre- treatment anti-inflammatory activity
- Analgesic activity
- Antipyretic activity
- Hypolipidemic activity
- Hepatoprotective activity
- Antituberculosis activity

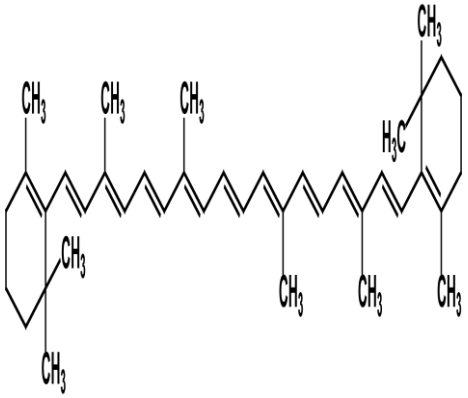
Plant part	Constituent reported
Roots (Vaishnav et al., 2001; Vaishnav and Gupta, 1996; Vaishnav and Gupta, 1995; Khastgir et al.,1958; Sucrow and Reimerdes, 1968)	Triterpenoid, saponin coccinioside – k(i). $C_{41}H_{66}O_{12}$ Flavonoid glycoside ombuin 3-o- arabinofuranoside 3- o- β- (α-l- arabinopyranosyl)-(1→2) –β-d-glucopyranosyl- (1→3)- β- hydroxylup – 20(29)- en-28- oic acid. Lupeol, β-amyrin, and β- sitosterol. Stigmast -7- en-3-one,
Fruits (Kundu and Ray,1987; Basu and Ghosh, 1972; Bhakuni et al., 1962)	Taraxerone, taraxerol, and (24R)-24- ethylcholest- 5- en- 3β- ol glucoside. B- carotene, lycopene, cryptoxanthin, and apo- 6'- lycopenal B- sitosterol and taraxerol
Aerial parts (Qudrat-i-Khuda et al., 1965; Dhargalkar and Guha,1959)	Heptacosane Cephalandrol, $C_{29}H_{58}O$ tritriacontane $C_{33}H_{68}$ B- sitosterol alkaloids Cephalandrine a and Cephalandrine b.
Whole plant (Rahman et al., 1990)	Aspartic acid, Glutamic Acid, Asparagine, Tyrosine, Histidine, Phenylalanine And Threonine Valine Arginine

Table 2.4: **Chemical Constituent of C*occinia cordifolia***

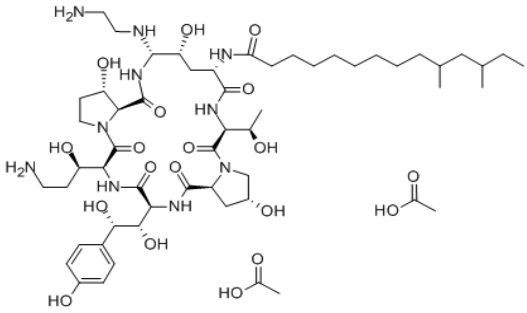
2.9 Structure of *Coccinia cordifolia* plant containing chemical constituent



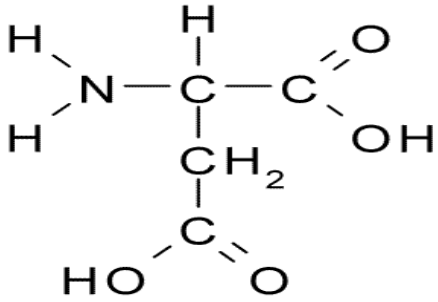
Triterpenoid



B- carotene



Heptacosane



Aspartic acid

CHAPTER-03
PLANT
LITERATURE REVIEW

3.1 Antibacterial Activity of the Leaves of *Coccinia cordifolia* (W and A) of India

H. Arshad, W. Shadma, Z. Iffat and H. Sarfaraj

Abstract: The aim of the present research was focused on investigating the antibacterial and preliminary phytochemical properties of *Coccinia cordifolia* (W.A.) via in vitro approach. The aqueous and organic solvent (Petroleum ether, chloroform and ethanol) extracts from the leaves of *Coccinia cordifolia* (Cucurbitaceae) were tested against *Enterobacter aerogenes, Pseudomonas aeruginosa, Staphylococcus epidermidis, Bacillus subtilis* and *Salmonella typhimurium* by agar well diffusion method and broth dilution method. Results showed promising antibacterial activity against the bacteria tested. Among these, ethanol and aqueous extracts were found to have a more potent inhibitory effect comparing with the other extracts. Which prove the potentiality of the plant extracts for the treatment of various skin and gastrointestinal infections in humans.

3.2 Pharmacology and phytochemistry of *Coccinia cordifolia*

U. A. Deokate and S. S. Khadabadi

Abstract: *Coccinia cordifolia* (Bimba, kanduri, Cucurbitaceae) is famous for its hypoglycemic and antidiabetic properties in Ayurvedic system of medicine. Other applications include the therapy of various conditions such as skin diseases and gonorrhoea. The present review highlights the phytochemistry and pharmacology of *Coccinia cordifolia*. There are many patented formulations derived from *C. cordifolia* which are now distributed increasingly all over the world. This has given rise to a concomitant increase in research on the phytochemical constituents and biological activity of *C. cordifolia*.

3.3 Antihepatotoxic acticity of *Coccinia cordifolia*

V. Gopalakrishnan, K.N.V. Rao, M. Devi, N. Padmaha, P. Manju Lakshmi, T. Srividya, and G. Vadivukarasi

Abstract: Aqueous, light petroleum, chloroform, alcohol, benzene and acetone extracts of the leaves of *Coccinia cordifolia*. (Family: Cucurbitaceae) were screened for antihepatotoxic activity. The extracts were given after the liver was damaged with Ccl4 Liver function was assessed based on liver to body weight ratio pentobarbitone sleep time, serum levels of transaminase (SGPT, SGOT), alkaline phosphatase (SALP and bilirubin. Alcohol and light petroleum was found to have good anti-hepatotoxic activity.

3.4 Antimicrobial activity of the fruit extracts of *Coccinia cordifolia*

SZ Shaheen, K Bolla, K Vasu, MAS Charya

Abstract: The bioactive compounds of fruits of *Coccinia cordifolia* were investigated for antibacterial activity against some pathogenic bacteria. The aqueous extracts did not show much significant activity, while the organic extracts (petroleum ether and methanol) showed the highest activity against the test bacteria. The activity was more pronounced on gram-positive organisms with Staphylococcus aureus being more susceptible and Salmonella paratyphi A being more resistant. Phytochemical analysis showed that the extracts contain alkaloids, tannins, saponins, flavonoids, glycosides and phenols.

3.5 Anti-inflammatory, analgesic and antipyretic activity of aqueous extract of fresh leaves of *Coccinia cordifolia*

N. Junaid, S. Parabhdeep, B. Yogita and R. K. Goel

Abstract: This study was aimed to evaluate both post- and pre-treatment anti-inflammatory activities of the aqueous extract of fresh leaves of *Coccinia cordifolia* in rats using the

carrageenan-induced paw oedema method at various dose levels. Analgesic and antipyretic properties were evaluated using tail flick model and yeast-induced hyperpyrexia, respectively. Ceiling effect of the extract was observed at 50 mg/kg in pre-treatment carrageenan test. In post-treatment studies, a dose-dependent anti-inflammatory effect was observed in the dose range of 25–300 mg/kg. The effect was equivalent to diclofenac (20 mg/kg) at 50 mg/kg but it was significantly pronounced at higher doses. Effectiveness of extract in the early phase of inflammation suggests the inhibition of histamine and serotonin release. The extract produced marked analgesic activity comparable to morphine at 300 mg/kg, which suggests the involvement of central mechanisms. A significant reduction in hyperpyrexia in rats was also produced by all doses of extract with maximum effect at 300 mg/kg comparable to paracetamol. In conclusion, this study has established the anti-inflammatory activity, analgesic and antipyretic activity of *C. cordifolia* and, thus, justifies the ethnic uses of the plant.

3.6 Evaluation of Anti-inflammatory activity of *Coccinia cordifolia* leaves extracts

S. Niranjan, G. Ranju, S. K. Umesh, S. S. Uma, J. K. Gautam and S. Abhishek

Abstract: The effects of *Coccinia cordifolia* leaves extracts on different phases of acute inflammation were examined. Investigations were performed using different phlogistic agents-induced paw edema *viz*.Carrageenan-induced paw oedema and Dextran- induced paw oedema in rats. Various extracts (ethanol and aqueous) of *Coccinia cordifolia* leaves extracts at a dose of 250 mg/kg and 500 mg/kg orally were tested. Diclofenac sodium at the dose of 10mg/kg was used as standard. Both the extracts showed significant activity (*$p<0.0$ & **$p<0.01$) compared with the control in both of these models. The dry powdered leaves were found to contain alkaloids, glycosides, saponins, tannins and carbohydrates. Thus it is revealed from the screening model used that the ethanol extract and aqueous fraction of this plant possesses acute anti-inflammatory activity.

3.7 *Coccinia cordifolia* in the treatment of patients with diabetes mellitus

A. K. Khan, S. Akhtar, H. Mahtab

Abstract: *Coccinia cordifolia* is a creeper which grows wildly in Bangladesh and in many parts of the Indian sub-continent. The plant has been used since ancient times as an antidiabetic drug by physicians who practice the Indian system of medicine known as Ayurvedha. We have conducted a double blind control trial with preparation from the leaves of the plant on uncontrolled, maturity onset diabetics. The trial lasted for six weeks for an individual patient. Out of the 16 patients who received the experimental preparations 10 showed marked improvement in their glucose tolerance while none out of the 16 patients in the dummy group showed such a marked improvement. This difference is highly significant (kappa 2 with Yates' correction = 11.7, $P < 0.001$).

3.8 Hypoglycemic Activity of *Coccinia cordifolia* (Cucurbitaceae) Leaves

S.S. AJAY

Abstract: In view of suggested anti diabetic potential, effect of aqueous and cold extracts of *Coccinia cordifolia* (Cucurbitaceae) leaves on fasting blood sugar levels and serum biochemical analysis in streptozotocin induced diabetic rats was investigated. All the extracts of *Coccinia cordifolia* produced a significant anti diabetic activity at dose levels of 1/5th of their lethal doses.

3.9 Indian medicinal herbs as sources of antioxidants

S. A. Shahin, K. Naresh, L. Abhinav, S. Angad, S. Hallihosur, S. Abhishek and B. Utpal

Abstract: Currently there has been an increased interest globally to identify antioxidant compounds that are pharmacologically potent and have low or no side effects for use in preventive medicine and the food industry. As plants produce significant amount of antioxidants to prevent the oxidative stress caused by photons and oxygen, they represent a potential source of

new compounds with antioxidant activity. Traditional herbal medicines form an important part of the healthcare system of India. Ayurveda, supposed to be the oldest medical system in the world, provides potential leads to find active and therapeutically useful compounds from plants. Considering the growing interest in assessing the antioxidant capacity of herbal medicine in this review we discuss about rarely reviewed 24 plants reported to have antioxidant properties. Some of the plants reviewed are part of multi-herbal preparations while others are used singly. Certain herbs like *Amaranthus paniculatus*, *Aerva lanata*, *Coccinia cordifolia* and *Coriandrum sativum* are used as vegetables indicating that these plants could be source of dietary antioxidant supplies, which is another emerging area of research.

CHAPTER-04
STUDY PROTOCOL

4.1 Study Protocol

Our present study was designed to isolate pure compounds as well as to observe biological activities of the isolated pure compounds with crude extract and their different fractions of the plant of *Coccinia cordifolia* (Family: Cucurbitaceae). The study protocol consisted of the following steps:

- Cold extraction of the powdered whole plant of the plant with methanol at room temperature.
- Filtration and solvent evaporation of the methanolic crude extract.
- Partitioning of methanolic crude extract with n-hexane, dicholoro methane and ethyl acetate.
- Performing antimicrobial test and phenolic content test.
- Brine shrimp lethality bioassay and determination of LC_{50} value for methanolic crude extract and different fractions.

CHAPTER-05

MATERIAL REQUIRED

5.1 Materials for chemical investigation

5.1.1Glass wares

	Materials	Source
1.	Quick fit flasks	-
2.	Conical flasks	Laboratory Equipments
3.	Beakers	Laboratory Equipments
4.	Test tubes	Laboratory Equipments
5.	Funnels	Laboratory Equipments
6.	Measuring cylinders	Laboratory Equipments
7.	Pipettes etc.	Laboratory Equipments
8.	Pasteur pipettes	Laboratory Equipments

5.1.2 Solvents

	Materials	Source
1.	N-Hexane	Merck, Germany and Sigma, USA
2.	Di-Chloro Methane	Merck, Germany and Sigma, USA
3..	Methanol	Merck, Germany and Sigma, USA
4.	Ethyl acetate	Merck, Germany and Sigma, USA

5.1.3 Equipments

	Equipments	Source
1.	Rotary vacuum evaporator	University Instruments Lab
2.	Electronic balance	Denver Instruments M-220
5.	Grinding machine	University

		Instruments Lab
6.	Oven (0°C-210°C)	Gallen Kamp Hotbox
7.	Solvent distillation plant	University Instruments Lab
8.	Distilled water plant	University Instruments Lab

5.2 Material for microbial investigation

Apparatus and Reagents
▪ Filter paper discs
▪ Petri dishes
▪ Inoculating loop
▪ Sterile cotton
▪ Sterile forceps
▪ Spirit burner
▪ Micropipette
▪ Screw cap test tubes
▪ Nose mask and Hand gloves
▪ Laminar air flow hood
▪ Autoclave
▪ Incubator
▪ Refrigerator

▪ Nutrient Agar Medium

5.3 Material for brine shrimp lethality bioassay

Apparatus and Reagents
• *Artemia salina* leach (brine shrimp eggs)
• Sea salt (NaCl)
• Small tank with perforated dividing dam to hatch the shrimp
• Lamp to attract shrimps
• Pipettes
• Micropipette
• Magnifying glass
• Test samples.
• Glass vials

CHAPTER-06
PHYTOCHEMICAL INVESTIGATION

6.1 Phytochemical Investigation:

The Phytochemical investigation of a plant can be divided roughly into the following major steps:

a) Collection and proper identification of the plant material

b) Preparation of plant sample

c) Extraction of the plant material

6.2 Collection and proper identification of the plant material

Leaves of *Coccinia cordifolia* were collected from Noakhali Bangladesh, in October 2011 and authentification of the sample has been confirmed by the taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka. A voucher specimen has been deposited **(Accession Number 37518)** in the Herbarium for further reference.

6.3 Preparation of plant sample

The leaves were sun dried for several days. After complete drying the dried leaves were then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, East West University. The coarse powder was then stored in air-tight container with marking for identification and kept in cool, dark and dry place for future use.

6.4 Extraction of the plant material

About 900 gm of powdered leave material was taken in clean, round bottomed flask (5 liters) and macerated at room temperature in 3 liters of methanol for 10 days with occasional shaking for better extraction. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper. After filtration the filtrate was concentrated at 40°C with a Heidolph rotary evaporation. The concentrated extract was then air dried to solid residue. The weight of the crude methanolic extract of leaves was obtained 56 gm.

6.5 Solvent-solvent partitioning and isolation of compounds

Solvent–solvent partitioning was done using the protocol designed by Kupchan and modified by Wagenen *et al.* (1993). The crude extract (35g) was dissolved in 10% aqueous methanol and partitioned between n-hexane, Di-choloro Methane (DCM) and Ethyl Acetate fractions. All the four fractions were evaporated to dryness. These were collected for further analysis. The extraction and fractionation process is shown below:

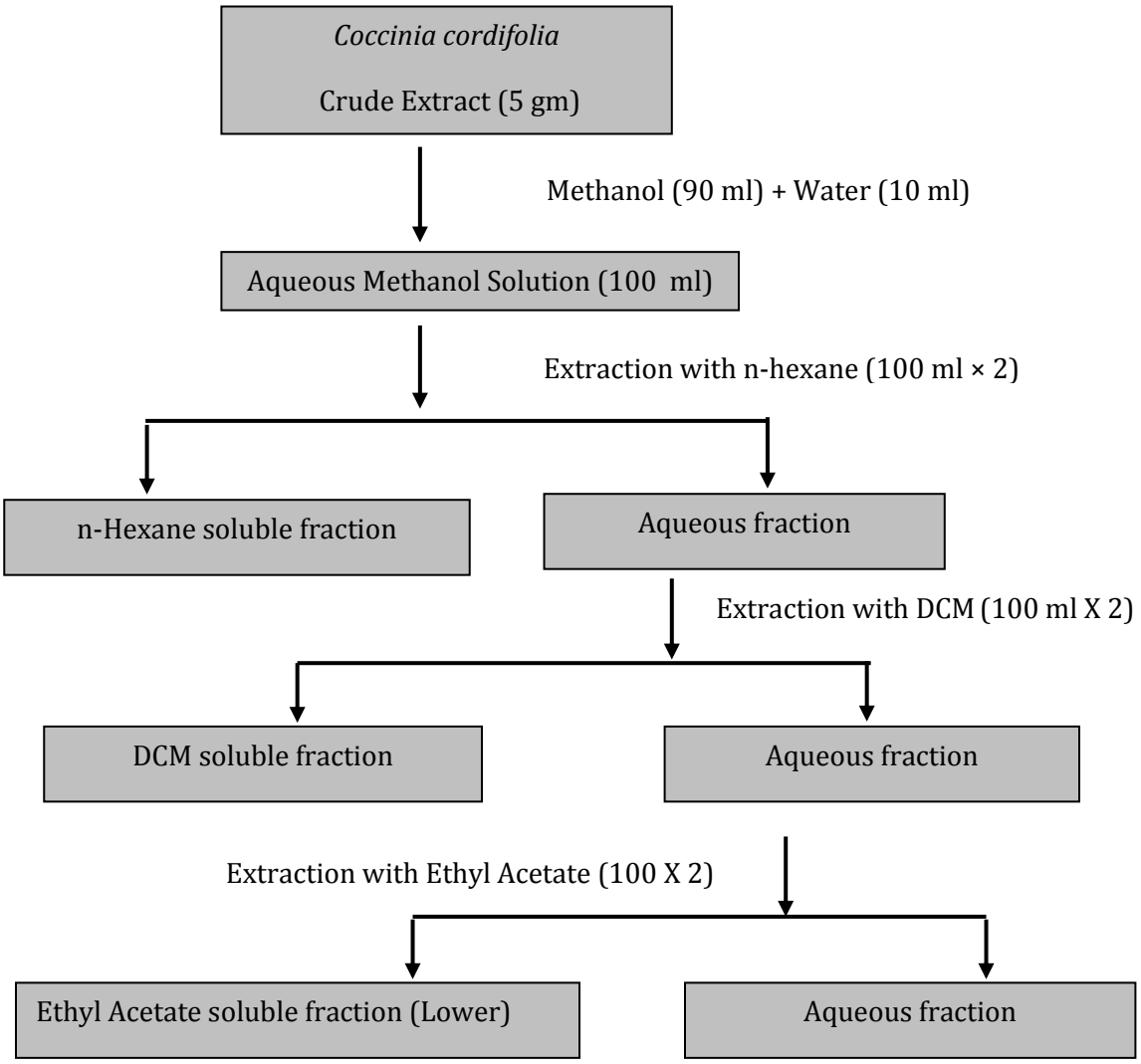


Figure 6.1: **Schematic representation of the modified kupchan partitioning of methanolic crude extract of *Coccinoia cordifolia***

Plant	Part	Extract and Fraction	Weight
Coccinia cordifolia	Whole plant	Crude of methanolic extract	35.0 g
Coccinia cordifolia	Whole plant	n-Hexane fraction	2.5 g
		Ethyl acetate fraction	4.5 g
		Methanol fraction	2.54g
		Di chloro methane	2.7 g

Table 6.1: **After evaporation the weight of the different fractions obtained**

CHAPTER-07
PHARMACOLOGICAL INVESTIGATIONS
ANTIMICROBIAL ACTIVITY

7.1 Introduction

Worldwide, infectious disease is one of main causes of death accounting for approximately one-half of all deaths in tropical countries. Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is that infectious disease mortality rates are actually increasing in developed countries, such as the United States. It is estimated that infectious disease is the underlying cause of death in 8% of the deaths occurring in the US (Pinner *et al*., 1996). This is alarming given that it was once believed that we would eliminate infectious disease by the end of the millennium. The increases are attributed to increases in respiratory tract infections and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosicomial and community acquired infections. Furthermore, the most dramatic increases are occurring in the 25–44 year old age group.

These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. It is this last solution that would encompass the development of new antimicrobials (Fauci, 1998).

The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth. This ability may be estimated by any of the following three methods.

- Disc diffusion method
- Serial dilution method
- Bioautographic method

Among all, disc diffusion (Bayer *et al*., 1966) is a widely accepted for screening of antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials.

7.2 Principle of disc diffusion method

Solutions of known concentration (μg/ml) of the test samples are made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) are then impregnated with known amounts of the test substances using

micropipette. Discs containing the test material are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs and blank discs (impregnated with solvents) are used as positive and negative control. These plates are then kept at low temperature (4 ^{0}C) for 24 hours to allow maximum diffusion. During this time dried discs absorb water from the surrounding media and then the test materials are dissolved and diffused out of the sample disc. The diffusion occurs according to the physical law that controls the diffusion of molecules through agar gel (Barry, 1976). As a result there is a gradual change of test materials concentration in the media surrounding the discs.

The plates are then incubated at 37 ^{0}C for 24 hours to allow maximum growth of the organisms. If the test materials have any antimicrobial activity, it will inhibit the growth of the microorganisms and a clear, distinct zone of inhibition will be visualized surrounding the medium. The antimicrobial activity of the test agent is determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment is carried out more than once and the mean of the readings is required (Bayer et al., 1966).

In the present study the crude extract, some column fractions as well as five purified compounds were tested for antimicrobial activity by disc diffusion method.

Figure 7.1: **Disc diffusion method**

7.3 Apparatus, reagent and test sample

Apparatus and reagents			
1.	Filter paper discs	8.	Autoclave
2.	Nutrient Agar Medium	9.	Laminar air flow hood
3.	Petridishes	10.	Spirit burner
4.	Sterile cotton	11.	Refrigerator
5.	Sterile forceps	12.	Incubator and Micropipette
6.	Inoculating loop	13.	Nosemask and Hand gloves
7.	Screw cap test tubes	14.	Chloroform and Ethanol

Table 7.1: **Apparatus and reagents**

7.4 Test materials of *Coccinia Cordifolia*

- Methanolic crude extract of the leaves of plant, ***Coccinia Cordifolia***
- Dichloromethane fraction of crud extraction
- N-Hexane fraction of crud extraction
- Ethylacetade fraction of crud extraction
- Methanol fraction of crud extraction

7.5 Test organisms

The bacterial and fungal strains used for the experiment were collected as pure cultures from the Department of Pharmacy, East West University. Both gram positive and gram-negative organisms were taken for the test. Test organisms are given below.

Name of Bacteria	Type of Bacteria
Shigella boydii	Gram -ve
Bacillus aureus	Gram +ve
Staphycoccus aureus	Gram +ve
Escherichia coli	Gram -ve
Vibrio mimicus	Gram -ve

Table 7.2: **List of test bacteria and their type**

7.6 Culture medium and their composition

The following media is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms.

Ingredients	Amounts
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml
pH	7.2 ± 0.1 (at 25^0C)

Table 7.3: **Composition of nutrient agar medium**

7.7 Experimental procedure

7.7.1 Preparation of the medium

To prepare required volume of this medium, calculated amount of each of the constituents was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The pH (at 25 ^{0}C) was adjusted at 7.2 – 7.6 using NaOH or HCl. 10 ml and 5 ml of the medium was then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15-lbs. pressure/sq. inch at 121^{0}C for 20 minutes. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

7.7.2 Sterilization procedures

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. Petridishes and other glassware's were sterilized by autoclaving at a temperature of 121 ^{0}C and a pressure of 15-lbs./sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

7.7.3 Preparation of subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37 ^{0}C for their optimum growth. These fresh cultures were used for the sensitivity test.

7.7.4 Preparation of the test plates

The test organisms were transferred from the subculture to the test tubes containing about 10 ml of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial and fungal suspension was immediately transferred to the sterilized petridishes. The

petridishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

7.7.5 Preparation of discs

Three types of discs were used for antimicrobial screening.

7.7.6 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, kanamycin (30μg/disc) disc was used as the reference.

7.7.7 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.

7.8 Preparation of sample discs with test samples

Measured amount of each test sample

- Dichloromethane fraction of crud extraction (DCM)
- N-Hexane fraction of crud extraction (NHE)
- Ethylacetade fraction of crud extraction (EAE)
- Methanol fraction of crud extraction (MEL)

This samples dissolved in specific volume of solvent (Chloroform) 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.

Sample	Dose (μg/disc)	Required amount for 20 disc (mg)
DCM	200	5
NHE	200	5
EAE	200	5
MEL	200	5

Table 7.4: **Amount of test sample taken**

7.9 Application of the test samples

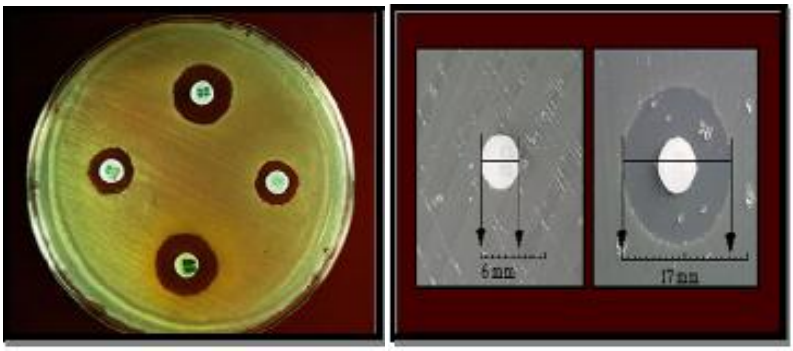
Standard Kanamycin (30 mg/disc) discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. Blank discs were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

7.10 Diffusion and incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4 ^{0}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 ^{0}C for 24 hours.

7.11 Determination of antimicrobial activity

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.



(A) **(B)**

Figure 7.2: **(A) Clear zone of inhibition and (B) Determination of clear zone of inhibition**

7.12 Results

Test microorganisms	**Diameter of zone of inhibition (mm)**				
	DCM	**EAE**	**NHE**	**MEL**	**Kanamycin**
Gram positive bacteria					
Bacillus sereus	9.5	-	9.75	-	-
Staphylococcus aureus	-	-	-	14.75	34

Test microorganisms	**Diameter of zone of inhibition (mm)**				
	DCM	**EAE**	**NHE**	**MEL**	**Kanamycin**
Gram negative bacteria					
Escherichia coli	10.25	12.5	10.75	-	37
Shigella boydii	6.25	11	11.5	-	37
Vibrio mimicus	7	13	12.75	10.5	37

CHAPTER-08
PHARMACOLOGICAL INVESTIGATIONS
BRINE SHRIMP LETHALITY BIOASSAY

8.1 Introduction

Brine shrimp lethality bioassay (Meyer *et.al,* 1982; Persoone, 1980) is a rapid general bioassay for the bioactive compounds of the natural and synthetic origin. Bioactive compounds are almost always toxic at high doses. Thus it justifies the statement that **'Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at a higher dose'**. Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, anti-microbial and pharmacological activities of natural products and it is a recent development in the bioassay for the bioactive compounds. By this method, natural product extracts, fractions as well as the pure compounds can be tested for their biosphere- activity. Here, *in vivo* lethality in a simple zoologic organism (Brine shrimp nauplii) is used as a convenient monitor for screening and in the discovery of new bioactive natural products.

This bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal & anti-tumor etc. of the compounds (Meyer, 1982; McLaughlin, 1988).

Brine shrimp lethality bioassay stands superior to other cytotoxicity testing procedures because it is a rapid method utilizing only 24 hours, inexpensive and requires no special equipment. Unlike other methods, it does not require animal serum. Furthermore, it utilizes a large number of organisms for statistical validation and a relatively small amount of sample.

8.2 Principle

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 vials containing 5 ml of simulated sea water. Then samples of different concentrations are added to the premarked vials 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.

Plant Part	Test samples	Measured amount (mg)
Whole plant	Dichloromethane fraction of crud extraction	**0.008**
	N-Hexane fraction of crud extraction	**0.008**
	Ethyl acetate fraction of crud extraction	**0.008**
	Methanol fraction of crud extraction	**0.008**

Table 8.1: **Test samples of experimental plant (*Coccinia Cordifolia*)**

8.3 Materials

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

8.4. Procedure

8.4.1 Preparation of sea water

38 gm sea salt (pure NaCl) was weighed, dissolved in one liter of distilled water and filtered off to get clear solution.

8.4.2 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. Two days were 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 5 ml of seawater.

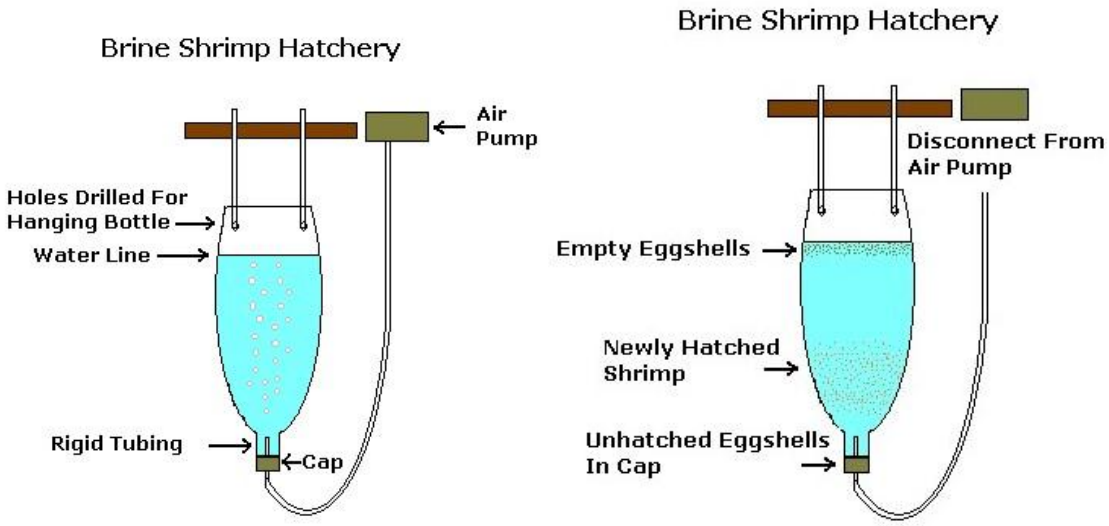


Figure 8.1: **Brine shrimp Hatchery**

8.4.3 Preparation of test solutions with samples of experimental plant

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 Vincristine for ten concentrations of it and another one test tube for control test.

All the test samples of 4 mg were taken and dissolved in 200 µl 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 400 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, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.5625 µg/ml and 0.78125 µg/ml for 10 dilutions.

8.4.4 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 vincristine sulfate is used as the positive control. Measured amount of the vincristine sulfate is dissolved in DMSO to get an initial concentration of 20 µg/ml from which serial dilutions are

made using DMSO to get 10 μg/ml, 5 μg/ml, 2.5μg/ml, 1.25 μg/ml, 0.625 μg/ml, 0.3125 μg/ml, 0.15625 μg/ml, 0.078125 μg/ml, 0.0390 μg/ml. Then the positive control solutions are added to the pre-marked vials 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 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.

8.4.5 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.

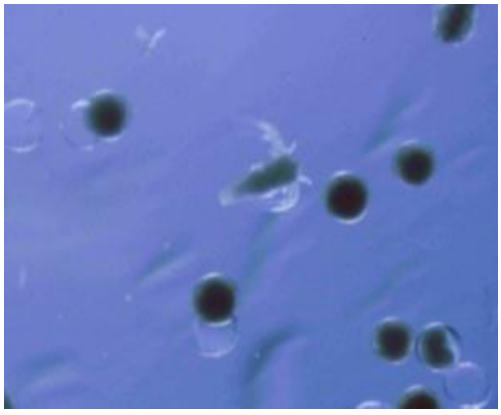


Figure 8.2: **Brine shrimp nauplii**

8.5 Analysis of data

The concentration-mortality data were analyzed statistically by using probit analysis and linear regression using a simple IBM-PC program. The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal concentration (LC_{50}) value. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

Test tube No.	**Concentration (C) (µg/ml)**	**LogC**	**% Mortality**	**LC_{50} (µg/ml)**
1	400	2.602	100	3.23
2	200	2.301	100	
3	100	2.000	100	
4	50	1.699	90	
5	25	1.398	80	
6	12.5	1.097	80	
7	6.250	0.796	60	
8	3.125	0.495	50	
9	1.563	0.194	40	
10	0.781	-0.107	20	

Table 8.2: **Effect of Vincristine Sulphate (positive control) on brine shrimp nauplii**

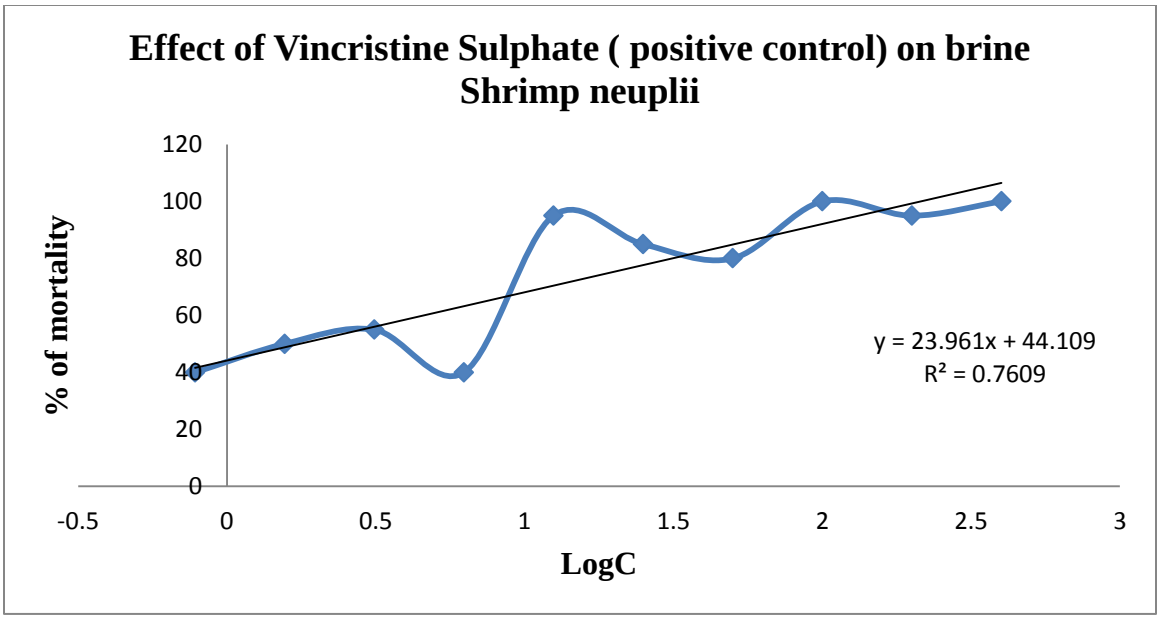


Figure 8.3: **Effect of Vincristine Sulphate (positive control) on brine shrimp nauplii**

Test tube No.	Concentration (C) (µg/ml)	LogC	% Mortality	LC_{50}
1	400	2.602	100	0.041
2	200	2.301	100	
3	100	2.000	100	
4	50	1.699	100	
5	25	1.398	90	
6	12.5	1.097	95	
7	6.250	0.796	65	
8	3.125	0.495	50	
9	1.563	0.194	85	
10	0.781	-0.107	75	

Table 8.3: **Effect of Dichloromethane fraction of crud extraction of *Coccinia cordifolia* on brine shrimp nauplii**

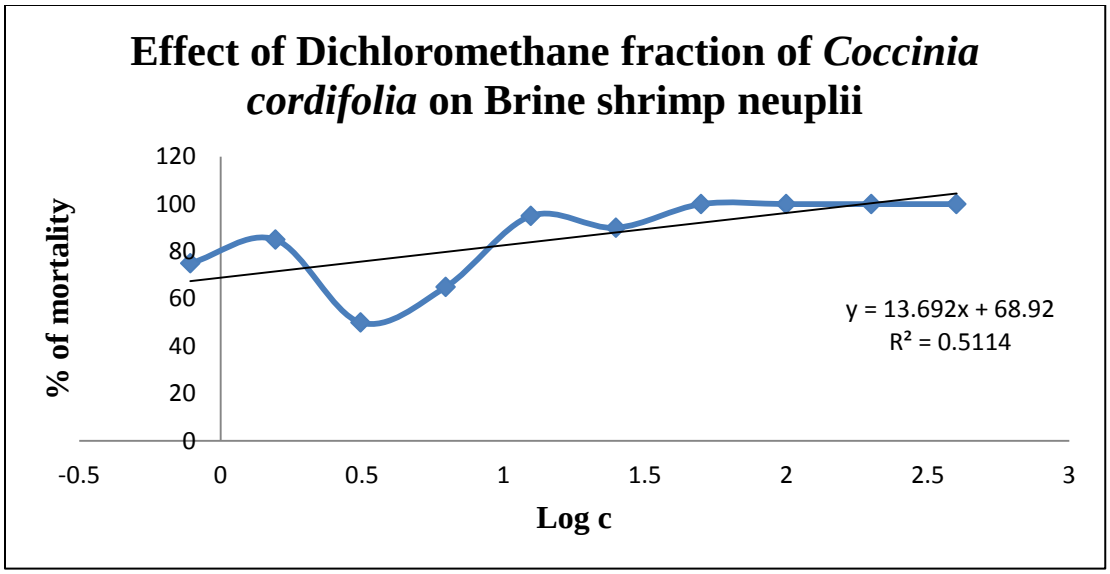


Figure 8.4: **Effect of Dichloromethane on brine shrimp nauplii**

Test tube No.	**Concentration (C) (µg/ml)**	**LogC**	**% Mortality**	LC_{50}
1	400	2.602	100	
2	200	2.301	100	
3	100	2.000	80	
4	50	1.699	85	
5	25	1.398	80	0.063
6	12.5	1.097	90	
7	6.250	0.796	85	
8	3.125	0.495	90	
9	1.563	0.194	50	
10	0.781	-0.107	60	

Table 8.4: **Effect of N-Hexane fraction of crud extraction of *Coccinia cordifolia* on brine shrimp nauplii**

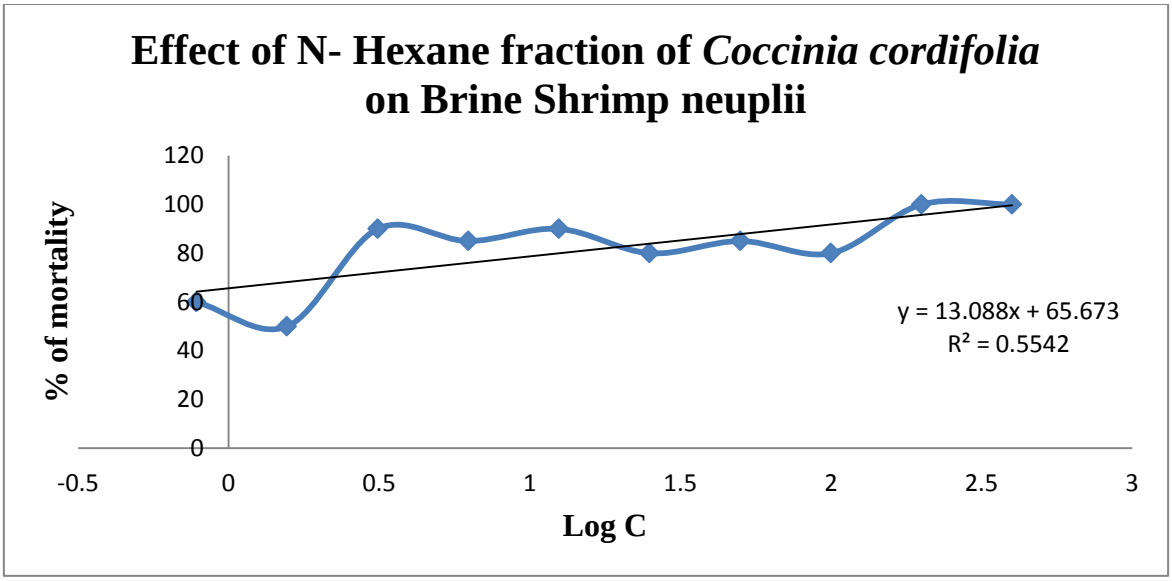


Figure 8.5: **Effect of N- Hexane on brine shrimp nauplii**

Test tube No.	**Concentration (C) (µg/ml)**	**LogC**	**% Mortality**	LC_{50}
1	400	2.602	100	
2	200	2.301	100	
3	100	2.000	90	
4	50	1.699	95	
5	25	1.398	90	0.14
6	12.5	1.097	90	
7	6.250	0.796	50	
8	3.125	0.495	85	
9	1.563	0.194	65	
10	0.781	-0.107	60	

Table 8.5: **Effect of Ethyl acetate fraction of crud extraction of *Coccinia Cordifolia* on brine shrimp nauplii**

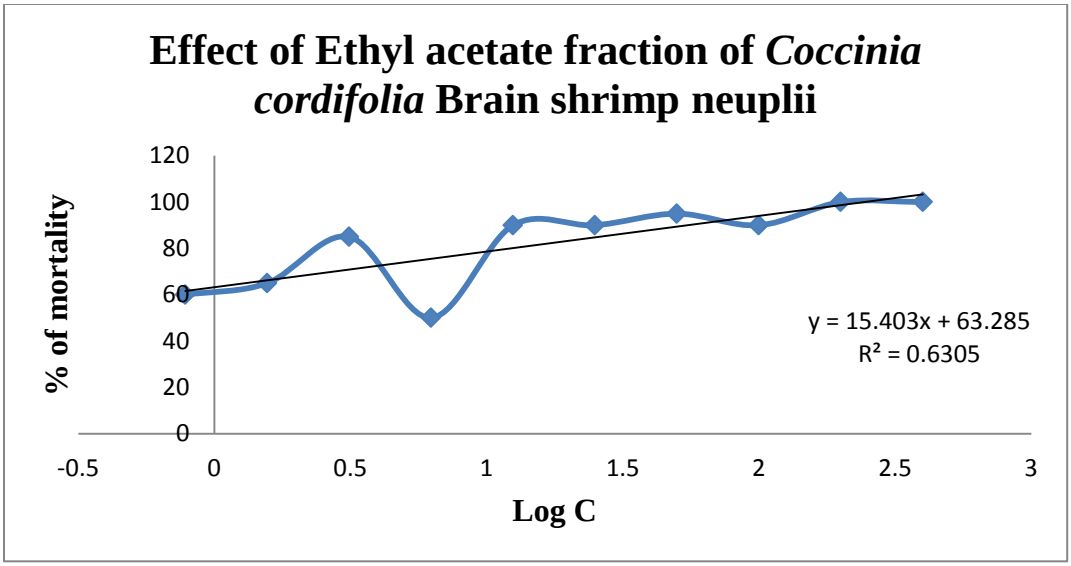


Figure 8.6: **Effect of Ethyl acetate on brine shrimp nauplii**

Test tube No.	Concentration (C) (µg/ml)	LogC	% Mortality	LC_{50}
1	400	2.602	100	0.36
2	200	2.301	100	
3	100	2.000	95	
4	50	1.699	95	
5	25	1.398	90	
6	12.5	1.097	85	
7	6.250	0.796	85	
8	3.125	0.495	60	
9	1.563	0.194	60	
10	0.781	-0.107	50	

Table 8.6: **Effect of Methanol fraction of crud extraction of *Coccinia Cordifolia* on brine shrimp nauplii**

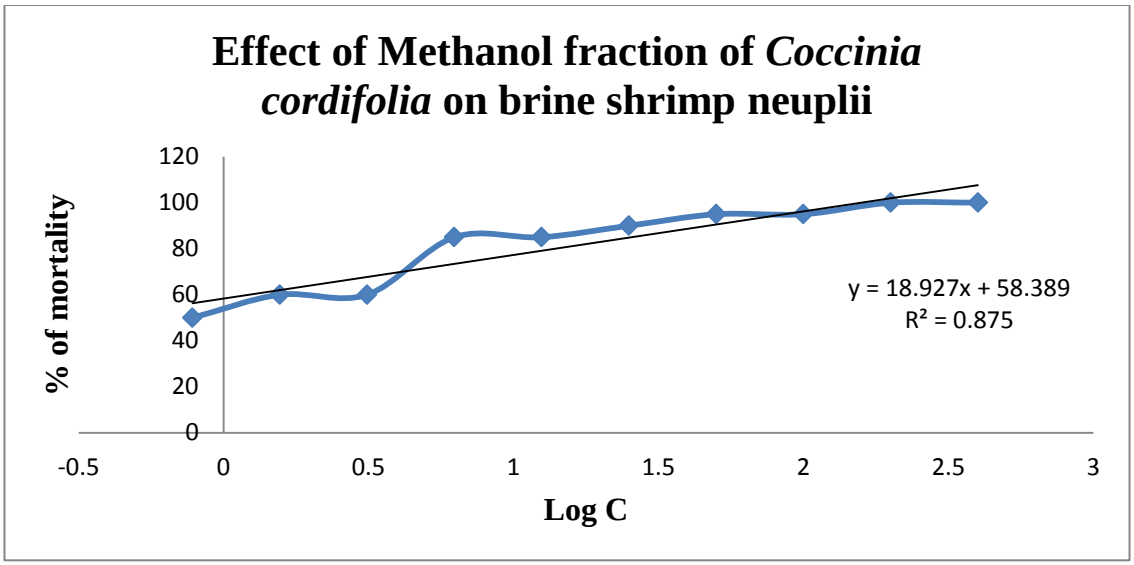


Figure 8.7: **Effect of Methanol on brine shrimp nauplii**

CHAPTER-09

TOTAL PHENOLICS CONTENT

9.1 Introduction

The antioxidative effect is mainly due to phenolic components, such as flavonoids (Pietta, 1998), phenolic acids, and phenolic diterpenes (Shahidi, Janitha, & Wanasundara, 1992). The antioxidant activity of phenolic compounds is mainly due to their redox properties,

It has been reported that there is an inverse relationship between the antioxidative status occurrence of human diseases (Rice,Evans, Sampson, Bramley, & Holloway,1997). In addition, antioxidant compounds which are responsible For Such antioxidants activity, could be isolated and then used as antioxidants for the prevention and treatment of free radical-related disorders (Middleton,Kandaswami, & Theoharides, 2000; Packer, Rimbach,& Virgili, 1999). Therefore, research to identify antioxidative compounds is an important issue. Although it remains unclear which of the compounds, of medical plants are the active ones, polyphenols recently have received increasing attention because of some interesting new findings regarding their biological activities. From pharmacological and therapeutic points of view, the antioxidant properties of polyphenols, such as free radical scavenging and inhibition of lipid per oxidation, are the most crucial. Even though a variety of herbs are known to be sources of phenolic compounds, studies isolating polyphenols and evaluating their antioxidative effects have rarely been carried out.

The purpose of this study was to evaluate extractives of ***Coccinia Cordifolia*** -as new potential sources of natural antioxidants and phenolic compounds. This study also demonstrates a possible relationship between phenolic content and antioxidant activity.

9.2 Principle

In the alkaline condition phenols ionize completely. 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 intensity of the color change is measured in a spectrophotometer at 760 nm. The absorbance value will reflect the total phenolic content of the compound (Harbertson and Spayd, 2006).

9.3 Materials & Methods

Total phenolic content of ***Coccinia Cordifolia*** extractive was measured employing the method as described by Skerget *et al*.(2005) involving Folin-Ciocalteu reagent as oxidizing agent and gallic acid as standard (Majhenic *et al*., 2007).

SL. No.	Component	Percent
1	Water	57.5
2	Lithium Sulfate	15.0
3	Sodium Tungstate Dihydrate	10.0
4	Hydrochloric Acid>=25%	10.0
5	Phosphoric Acid 85 % solution in water	5.0
6	Molybdic Acid Sodium Dihydrate	2.5

Table 9.1: **Composition of Folin-Ciocalteu Reagent**

9.4 Standard curve preparation

Gallic acid was used here as standard. Different Gallic acid solution were prepared having a concentration ranging from 100 µg / ml to 10 µg / ml. 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of Na_2CO_3 (7.5 % w/v) solution was added to 0.5 ml of Gallic acid solution. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 760 nm. 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.

9.5 Sample preparation

2 mg of the ***Coccinia Cordifolia*** extractives was taken and dissolved in 1ml of distilled water to get a sample concentration of 2mg / ml in every case.

Name of fraction	Concentration (mg/ml)
N-Hexane (NHE)	2.0
Dichloromethane (DCM)	2.0
Ethylacetade (EAE)	2.0
Methanol (MEL)	2.0

Table 9.2: **Test samples for total phenolic content determination**

9.6 Total phenolic compound analysis

To 0.5 ml of extract solution (conc. 2 mg/ml), 2.5 ml of **Folin-Ciocalteu** reagent (diluted 10 times with water) and 2.0 ml of Na_2CO_3 (7.5 % w/v) solution was added. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 760 nm by UV-spectrophotometer and using the standard curve prepared from Gallic acid solution with different concentration, the total phenols content of the sample was measured. The phenolic contents of the sample were expressed as mg of GAE (Gallic acid equivalent) / gm. of the extract.

9.7 Flow diagram for Total Phenolic Content Assay

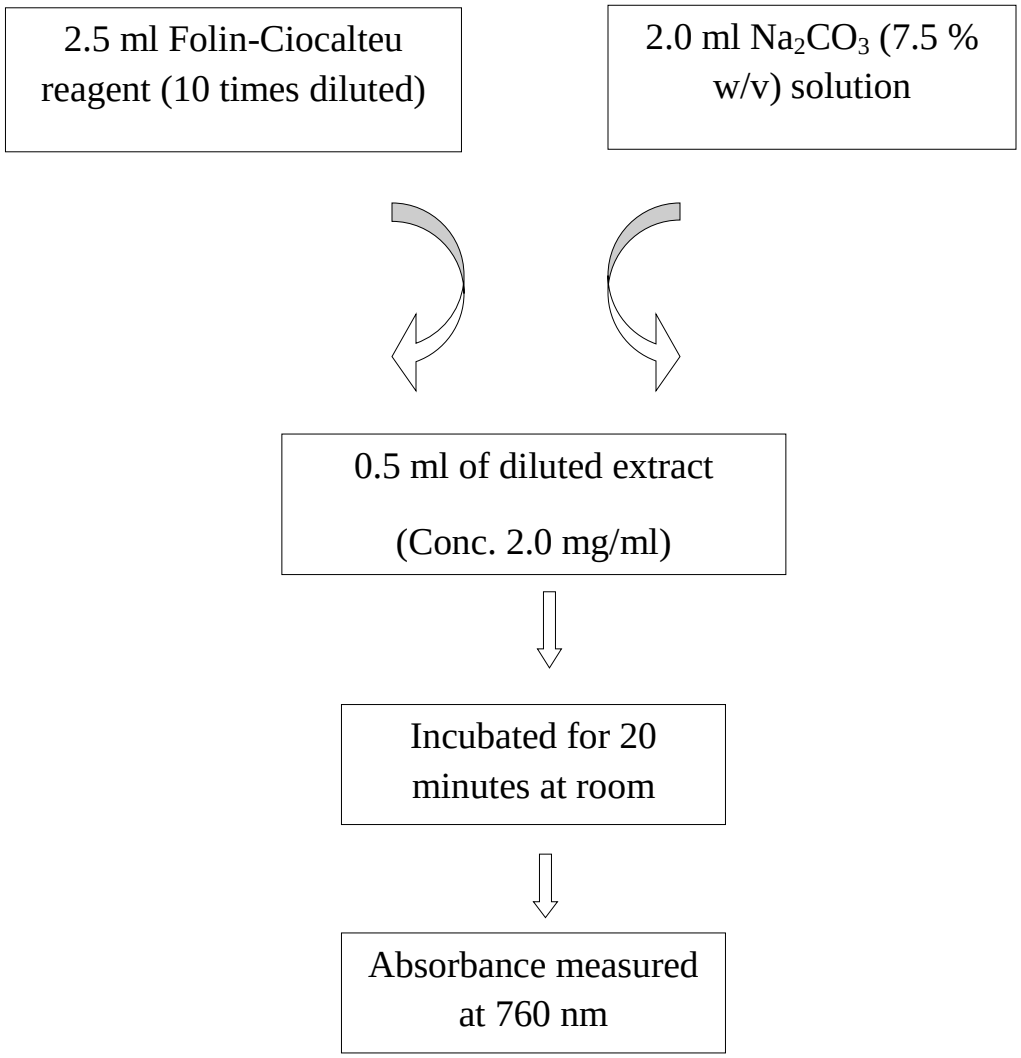


Figure 9.1: **Schematic representation of the total phenolic content determination**

9.8 Result of Total Phenolic Content

The amount of total phenolic content in *Coccinia Cordifolia*

SL. No.	Conc. Of the Standard (µg / ml)	Absorbance	Regression line	R^2
1	10	0.234	y=0.012x1.226	0.991
2	20	0.487		
3	30	0.718		
4	40	1.140		
5	50	1.521		
6	60	2.294		
7	70	2.285		
8	80	2.711		
9	90	3.188		
10	100	3.665		

Table 9.3: **Standard curve preparation by using gallic acid**

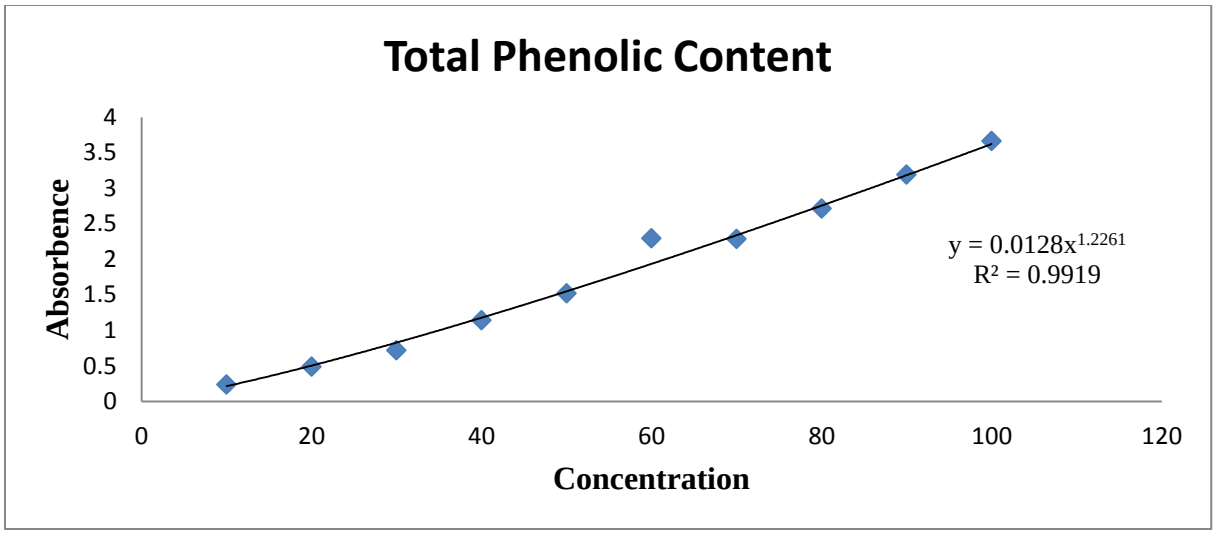


Figure 9.2: **Graphical representation of assay of Phenolic compound in Methanolic extract of gallic acid**

Name of fraction	Absorbance	mgGAE/g
NHE	0.683	27.02
DCM	0.667	26.50
EAE	0.163	8.40
MEL	0.233	11.24

Table 9.4: **Total phenolic content of fraction of whole plant of *Coccinia Cordifolia***

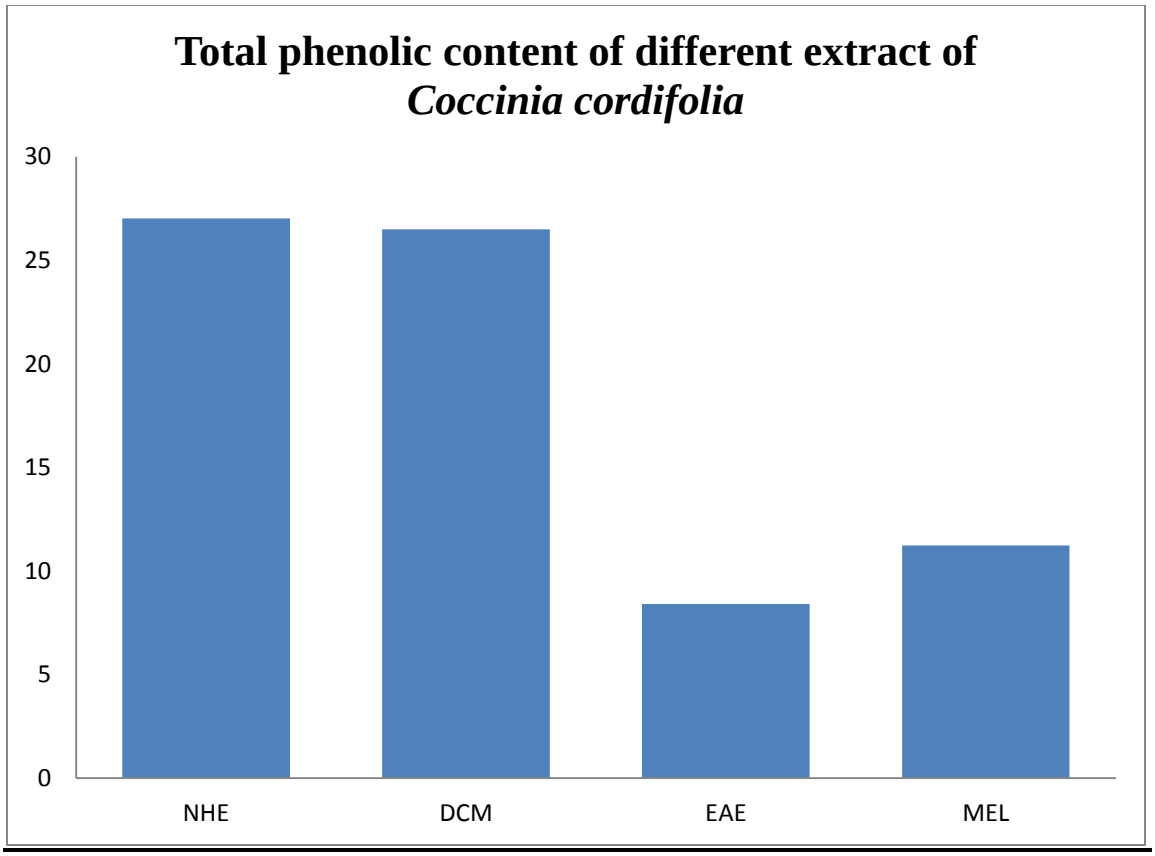


Figure 9.3: **Total phenolic content of different extract of *Coccinia cordifolia***

9.5 Discussion

The Total Phenolic content of ***Coccinia Cordifolia*** was found. The plant extract contain phenolic content. Further research is needed on the determination of the correlation between the antioxidant capacity and the chemical components of the plant.

CHAPTER-10
PHARMACOLOGICAL INVESTIGATIONS
FREE RADICAL
SCAVENGING ACTIVITY

10.1 Introduction

There is a considerable recent evidence that free radical induce oxidative damage to biomolecules. This damage causes atherosclerosis, aging, cancer and several other pathological events in living organisms (Halliwell *et al.*, 1992). Antioxidants which scavenge free radicals are known to posses an important role in preventing these free radical induced-diseases. There is an increasing interest in the antioxidants effects of compounds derived from plants, which could be relevant in relations to their nutritional incidence and their role in health and disease (Steinmetz and potter, 1996; Arouma, 1998; Bandonience *et al.,* 2000; Pieroni *et al.,* 2002; Couladis *et al.,* 2003). A number of reports on the isolation and testing of plants derived antioxidants have been described during the past decade. Natural antioxidants constitute a broad range of substances including phenolic or nitrogen containing compounds and carotenoids (Shahidi *et al.,* 1992; Velioglu *et al.,* 1998; Pieta *et al.,* 1998).

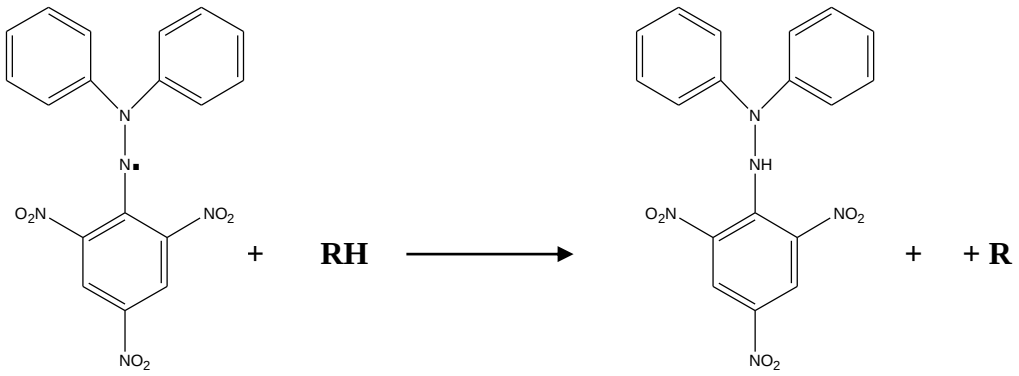
Dietary food contains a wide variety of free radical-scavenging antioxidants; for example, flavonoids and antioxidant vitamins such as ascorbic acid and ∞-tocopherol. These compounds are particularly rich in vegetables, fruits, tea, and wine. Epidemiological studies have shown that higher intake of fresh vegetables, fruits, tea and wine is associated with lower risk of mortality from cancer and coronary heart diseases. There is currently strong interest in natural antioxidants and their role in human health and nutrition.

Lipid peroxidation is one of the main reasons for deterioration of food products during processing and storage. Synthetic antioxidant such as butylated hdroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ) are widely used as food additives to increase self life, especially lipid and lipid containing products by retarding the process of lipid peroxidation. However, BHT and BHA are known to have not only toxic and carcinogenic effects and humans (Ito *et al*., 1986; Wichi, 1988), but abnormal effects on enzyme systems (Inatani, Nakatani & Fuwa *et al.*, 1983). Therefore, the interest in natural antioxidant, especially of plant origin, has greatly increased in recent years (Jayaprakasha & Jaganmohan Rao., 2000).

10.2 Principle

The present study was aimed at evaluating the *in vitro* free radical scavenging activity of *Coccinia cordifolia* using 1,1-diphenyl-2-picrylhydrazyl (DPPH) by the method of Brand-Williams *et al.,* 1995. 2.0 ml of a methanol solution of the extract at different concentration were mixed with 3.0 ml of a DPPH methanol solution (20 µg/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 *tert*-butyl-1-hydroxytoluene (BHT) 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 IC_{50} which is the concentration of samples to produce 50% reduction of the DPPH.



DPPH **DPPH-H**

Figure 10.1: **Mechanism of free radical scavenging activity**

10.3 Materials and Methods

DPPH was used to evaluate the free radical scavenging activity of various compounds and medicinal plants (Choi *et al.,* 2000; Desmarchelier *et al.,* 1997).

10.3.1 Materials

- 1,1-diphenyl-2-picrylhydrazyl (DPPH)
- *tert*-butyl-1-hydroxytoluene (BHT)
- Methanol
- Chloroform
- Carbon tetrachloride
- n-Hexane
- UV-Spectrophotometer
- Beaker (100 & 200 ml)
- Test tube
- Light-proof box
- Pipette (5 ml)
- Micropipette (50 – 200 ml)
- Amber reagent bottle
- Distilled water

10.3.2 Methods

- 50µl of various concentrations of the extracts in methanol was added to 5 ml of a 0.004% methanol solution of DPPH.
- After 30 min incubation period at room temperature the absorbance was read against a blank at 517 nm.
- Inhibition free radical DPPH in percent *(I%)* was calculated as follows: *(I%) = (1 – A_{sample}/A_{blank}) X 100*
    - Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test material).

- Extract concentration providing 50% inhibition *(IC_{50})* was calculated from the graph plotted inhibition percentage against extract concentration.
- BHT was used as positive control.
- Tests carried out in triplicate and average value was taken.

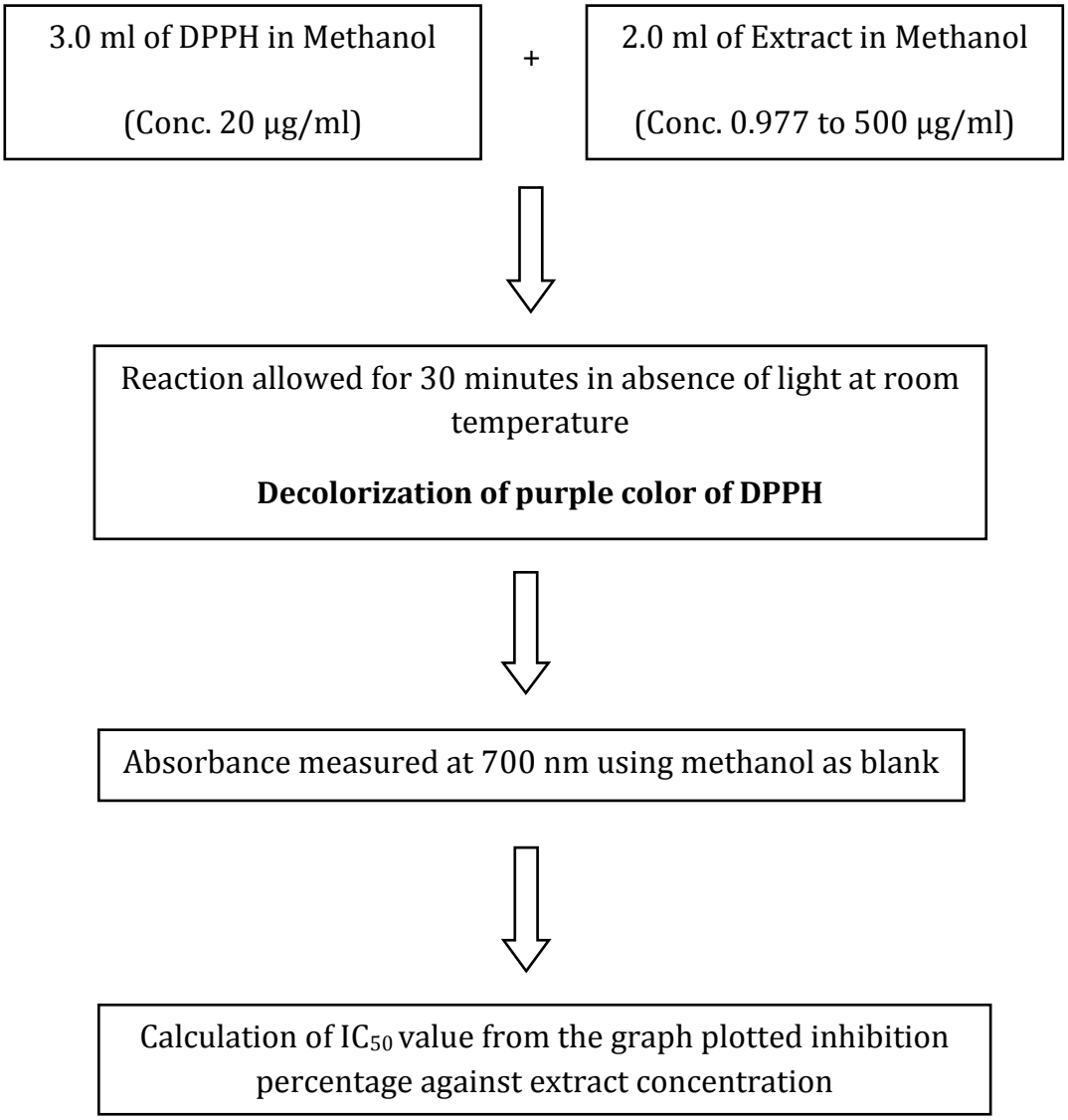


Figure 10.2: **Schematic representation of the method of assaying free radical scavenging activity**

10.4 Results and Discussion

Different partitionates of methanolic extract of *Cocinia cordifolia* were subjected to free radical scavenging activity developed by the method of Brand-Williams *et al.*, 1995. Here, *tert*-butyl-1-hydroxytoluene (BHT) was used as reference standard.

10.4.1 Results and Discussion of the Test Samples of *Coccinia cordifolia*

The tert-butyl-1-hydroxytoluene (BHT) was used as reference standard. And IC_{50} value of other fraction is is 18.48 µg/ml

Code	Sample	IC_{50} (µg/ml)
NHE	N-Hexane Crude Extract	6.20
DCM	Dichloromethane Crude Extract	4.81
EAE	Ethyl acetate Crude Extract	2.34
MEL	Methanol Crude Extract	2.07

Table 10.1: **IC_{50} values of standard and different fractions of *Coccinia cordilfolia***

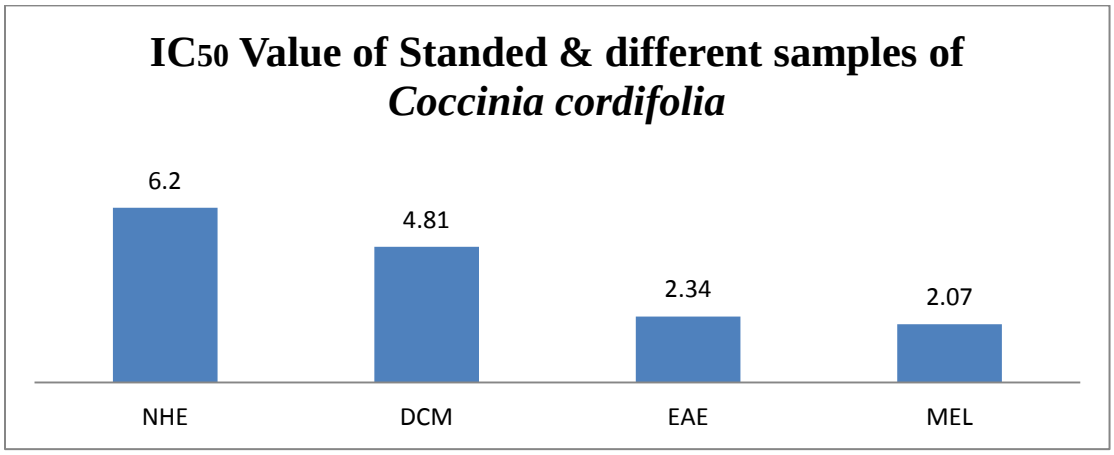


Figure 10.3: **IC_{50} values of standard and different partitionates of *Coccinia cordifolia***

12	Absorbance of Blank	Concentration (µg/ml)	Absorbance of sample	% Inhibition	IC_{50}
1	0.674	500	0.145	78.487	6.20
2		250	0.074	89.021	
3		125	0.048	92.878	
4		62.5	0.161	76.113	
5		31.25	0.257	61.870	
6		15.625	0.338	50.593	
7		7.813	0.387	42.581	
8		3.906	0.390	42.136	
9		1.953	0.406	39.763	
10		0.977	0.391	41.988	

Table 10.2: **IC_{50} value of N-Hexane**

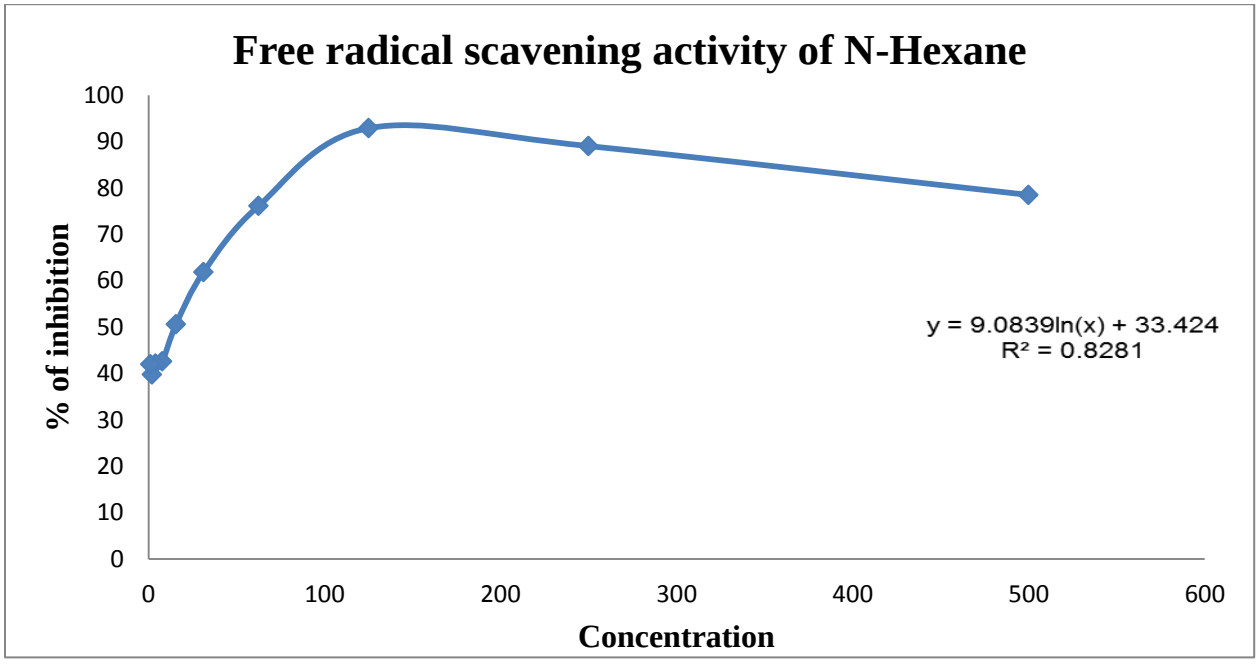


Figure 10.4: **Free radical scavenging activity of N-Hexane**

Test tube No.	Absorbance of Blank	Concentration (µg/ml)	Absorbance of sample	% Inhibition	IC_{50}
1	0.674	500	0.071	89.029	4.81
2		250	0.039	94.213	
3		125	0.029	95.627	
4		62.5	0.024	96.439	
5		31.25	0.134	80.119	
6		15.625	0.279	58.605	
7		7.813	0.347	48.813	
8		3.906	0.390	42.136	
9		1.953	0.436	35.311	
10		0.977	0.432	35.905	

Table 10.3: **IC_{50} value of Di Chloro Methane (DCM)**

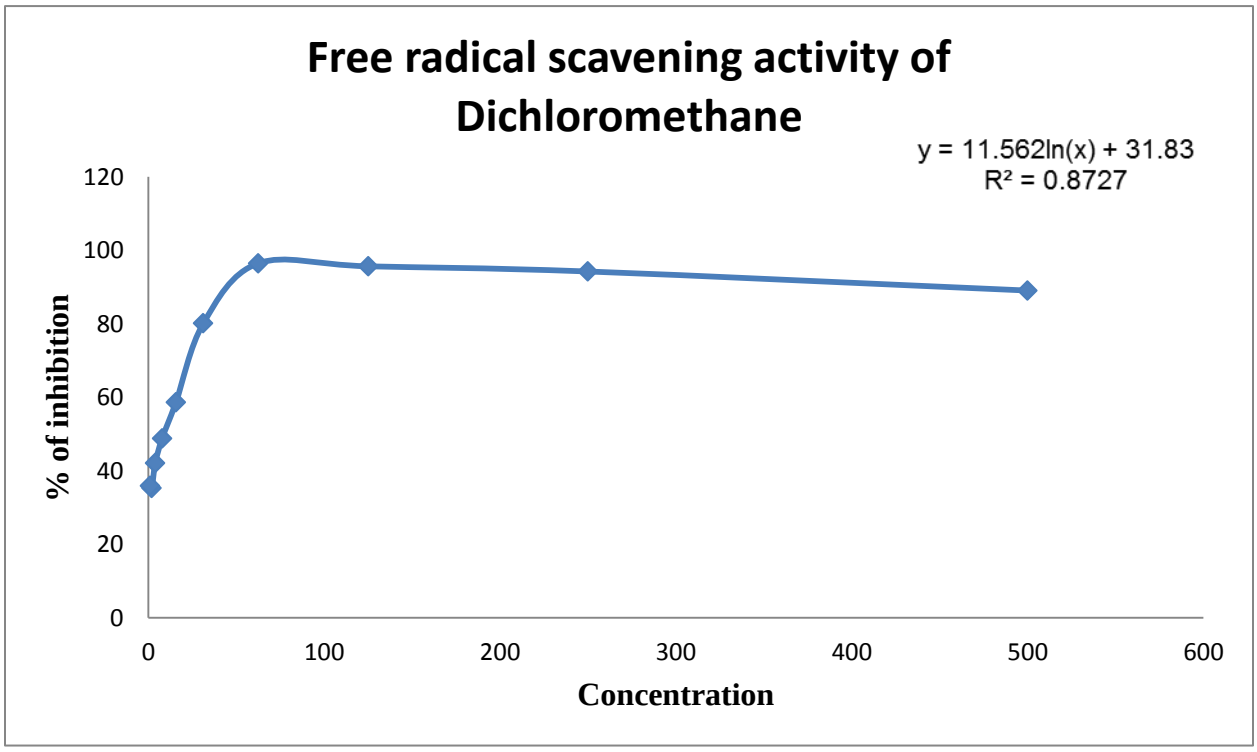


Figure 10.5: **Free radical scavenging activity of Di Chloro Methane**

Test tube no	Absorbance of blank	Concentration (µg/ml)	Absorbance of sample	% Inhibition	IC_{50}
1	0.674	500	0.045	93.323	2.34
2		250	0.026	96.142	
3		125	0.023	96.588	
4		62.5	0.020	97.032	
5		31.25	0.018	97.329	
6		15.625	0.111	83.531	
7		7.813	0.298	56.,047	
8		3.906	0.361	46.439	
9		1.953	0.390	42.136	
10		0.977	0.414	38.576	

Table 10.4: **IC_{50} value of Ethyl Acetate**

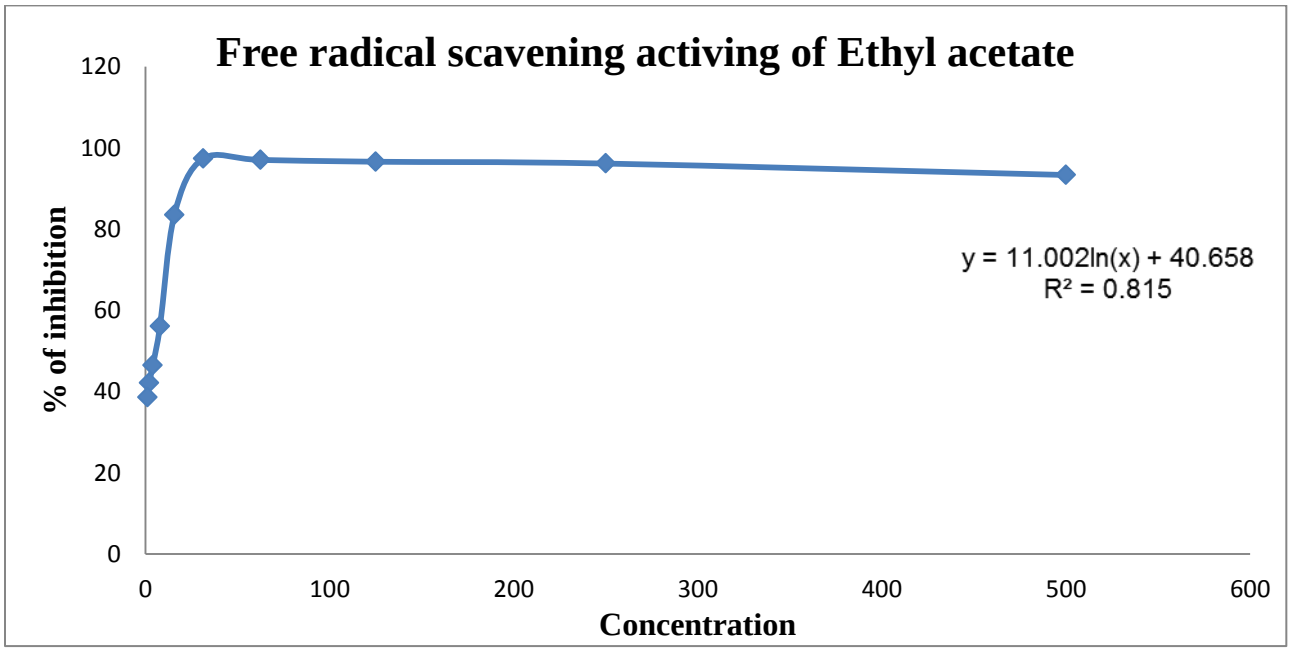


Figure 10.6: **Free radical scavenging activity of Ethyl Acetate**

Test tube No.	Absorbance of Blank	Concentration (µg/ml)	Absorbance of sample	% Inhibition	IC_{50}
1	0.674	500	0.018	97.329	2.07
2		250	0.010	98.516	
3		125	0.014	97.923	
4		62.5	0.010	98.516	
5		31.25	0.010	98.516	
6		15.625	0.101	85.015	
7		7.813	0.251	62.760	
8		3.906	0.344	48.961	
9		1.953	0.418	37.982	
10		0.977	0.393	41.691	

Table 10.5: **IC_{50} value of Methanol**

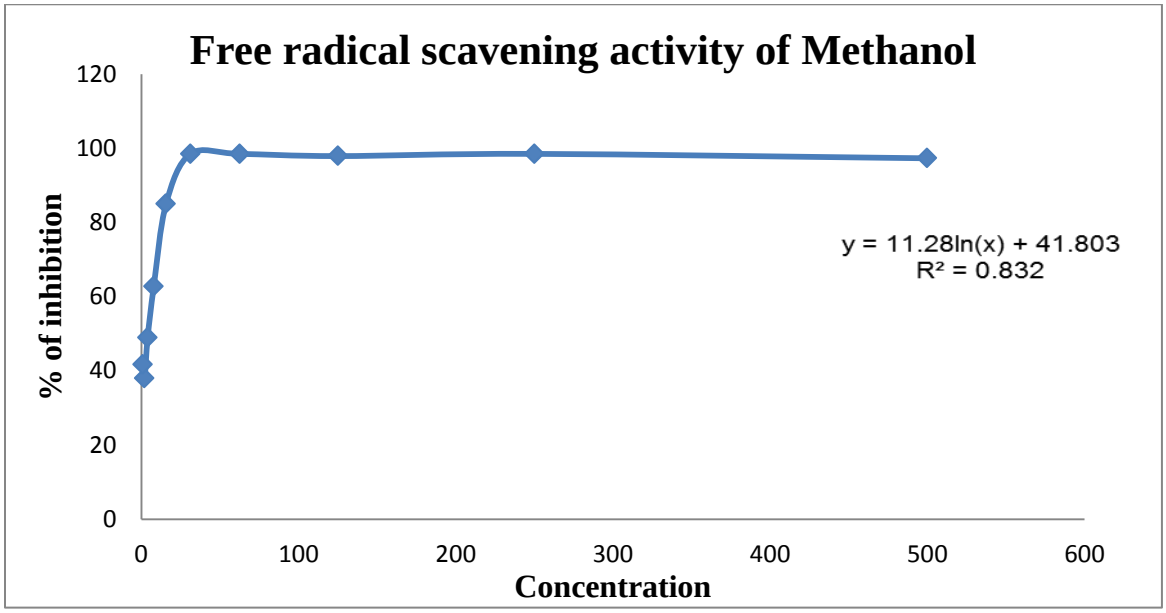


Figure 10.7: **Free radical scavenging activity of Methanol**

CHAPTER-11
RESULTS

Results:

Pharmacological Investigations:

11.1 Screening of in-vitro antimicrobial activity

The antimicrobial activities of different fractions of *Coccinia cordifolia* were examined in the study. Different fractions are

- Dichloromethane fraction of crud extraction (DCM)
- N-Hexane fraction of crud extraction (NHE)
- Ethylacetade fraction of crud extraction (EAE)
- Methanol fraction of crud extraction (MEL)

The results are given in table compare with Kanamycin Standard.

Test microorganisms	**Diameter of zone of inhibition (mm)**				
	DCM	**EAE**	**NHE**	**MEL**	**Kanamycin**
Gram positive bacteria					
Bacillus sereus	9.5	-	9.75	-	-
Staphylococcus aureus	-	-	-	14.75	34

Test microorganisms	**Diameter of zone of inhibition (mm)**				
	DCM	**EAE**	**NHE**	**MEL**	**Kanamycin**
Gram negative bacteria					
Escherichia coli	10.25	12.5	10.75	-	37
Shigella boydii	6.25	11	11.5	-	37
Vibrio mimicus	7	13	12.75	10.5	37

11.1.1 Result and Discussion of Antimicrobial Activity

All fractions for *Bacillus sereus* is showing the low antimicrobial activity and the growth of Gram (+ve) bacteria for *Bacillus sereus* is not inhibited properly. But for the *S. aureus* the methanol fraction shows activity where the diameter of zone of inhibition is 14.75 mm.

The Gram (-ve) bacteria *Escherichia coli and Shigella boydii* are also not inhibited by the fractions but for *V. mimicus* the N- hexane fraction shows activity where the diameter of zone of inhibition is 12.75 mm.

11.2 Brine shrimp lethality Bioassay

Bioactive compounds are almost always toxic at higher dose. Thus, *in vivo* lethality in a simple zoological organism can be used as a convenient informant for screening and fractionation in the discovery of new bioactive natural products.

In the present bioactivity study all the crude extracts of *Coccinia cordifolia* showed positive results indicating that the test samples are biologically active. Each of the test samples showed different mortality rates at different concentrations. Plotting of log of concentration versus percent mortality for all test samples showed an approximate linear correlation. From the graphs, the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) was determined for the samples. The positive control groups showed non linear mortality rates at lower concentrations and linear rates at higher concentrations. There was no mortality in the negative control groups indicating the test as a valid one and the results obtained are only due to the activity of the test agents. The amounts of test samples taken are given below:

Plant Part	Test samples	Measured amount (mg)
Whole plant	Dichloromethane fraction of crud extraction	0.008
	N-Hexane fraction of crud extraction	0.008
	Ethylacetade fraction of crud extraction	0.008
	Methanol fraction of crud extraction	0.008

Table 11.1: **Test samples of experimental plant (*Coccinia cordifolia*)**

11.2.1 Results and discussion of brine shrimp lethality

Following the procedure of Meyer (Meyer *et al*., 1982) the lethality of

- Dichloromethane fraction of crud extraction (DCM)
- N-Hexane fraction of crud extraction (NHE)
- Ethylacetade fraction of crud extraction (EAE)
- Methanol fraction of crud extraction (MEL)

However, varying degree of lethality to ***Coccinia cordifolia*** extracts was observed with exposure to different dose levels of the test samples. The degree of lethality was directly proportional to the concentration of the extract ranging from significant with the lowest concentration (0.78125µg/ml) to highly significant with the highest concentration (400µg/ml). Maximum mortalities took place at concentration 400 µg/ml, whereas least mortalities were at 0.781 µg/ml concentration. In other words, mortality increased gradually with the increase in concentration of the test samples. From the results of the brine shrimp lethality bioassays it can be well predicted that the chloroform fraction of metabolic crude extracts possess cytotoxic principles and have considerable cytotoxic potency.

Comparison with positive control vincristine signifies that cytotoxicity exhibited by the crude extracts and further bioactivity guided investigation can be done to find out potent antitumor and pesticidal compounds.

Sample	**LC_{50} (µg/ml)**	**Regression equation**	**R^2**
Vincristine sulphate **(VIS)**	3.23	y = 23.961x + 44.109	0.7609
Dichloromethane fraction of crud extraction **(DCM)**	0.041	y = 13.692x + 68.92	0.5114
N-Hexane fraction of crud extraction **(NHE)**	0.063	y = 13.088x + 65.673	0.5542
Ethyl acetate fraction of crud extraction **(EAE)**	0.14	y = 15.403x + 63.285	0.6305
Methanol fraction of crud extraction **(MEL)**	0.36	y = 18.927x + 58.389	0.875

Table 11.2: **Results of the test samples of *Coccinia cordifolia***

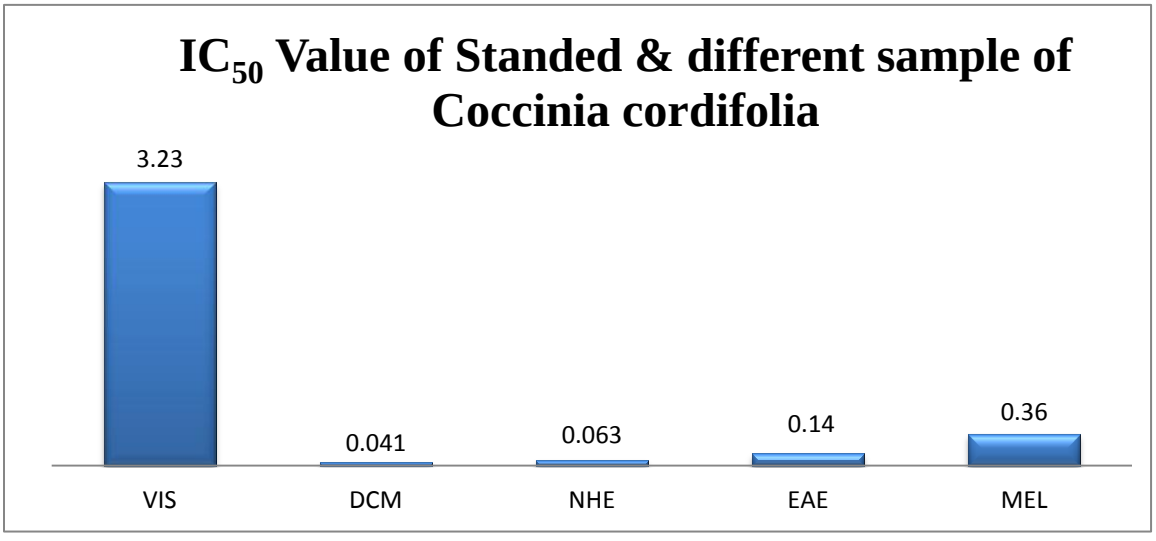


Figure 11.1: **IC_{50} values of standard and different partitionates of *Coccinia cordifolia***

11.3 Result of Total Phenolic Content

The amount of total phenolic content in *Coccinia Cordifolia*

The Total Phenolic content of ***Coccinia Cordifolia*** was found. The plant extract contain phenolic content. Further research is needed on the determination of the correlation between the antioxidant capacity and the chemical components of the plant.

Name of fraction	**Absorbance**	**mgGAE/g**
NHE	0.683	27.02
DCM	0.667	26.50
EAE	0.163	8.40
MEL	0.233	11.24

Table 11.3: **Total phenolic content of fraction of whole plant of *Coccinia Cordifolia***

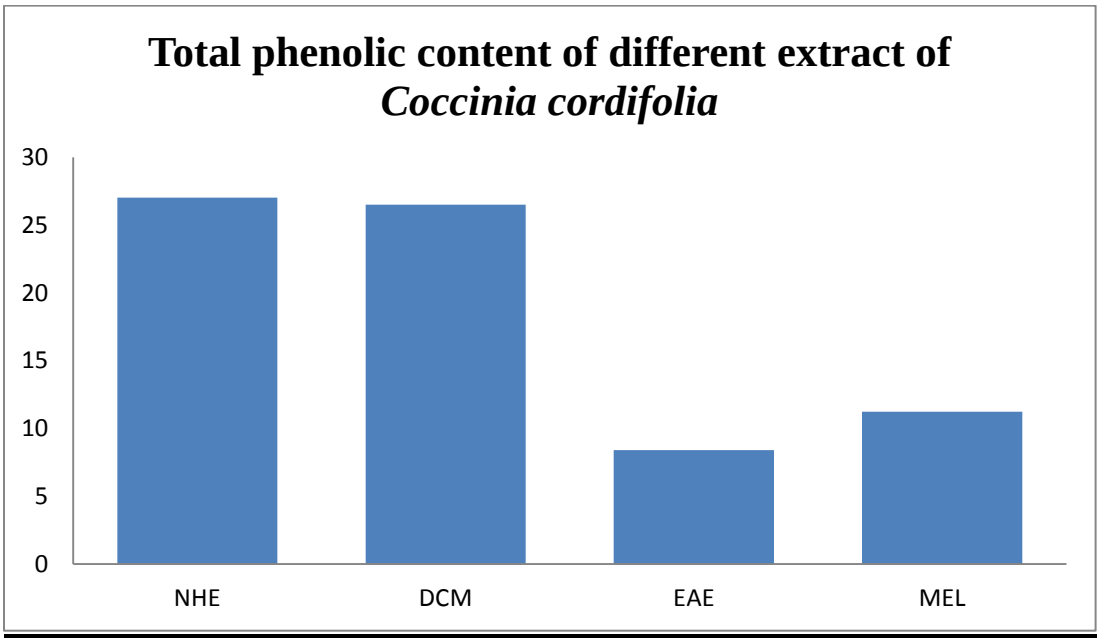


Figure 11.2: **Total phenolic content of different extract of *Coccinia cordifolia***

11.4 Free Radical Scavenging Activity

Different fraction of *Coccinia cordifolia* is subjected to free radical scavenging activity developed by the method of Brand-Williams *et al.,* 1995. Here, *tert*-butyl-1-hydroxytoluene (BHT) was used as reference standard.

Code	Sample	IC_{50} (µg/ml)
NHE	N-Hexane Crude Extract	6.20
DCM	Dichloromethane Crude Extract	4.81
EAE	Ethylacetade Crude Extract	2.34
MEL	Methanol Crude Extract	2.07

Table 11.4: **IC_{50} values of standard and different fractions of *Coccinia cordifolia***

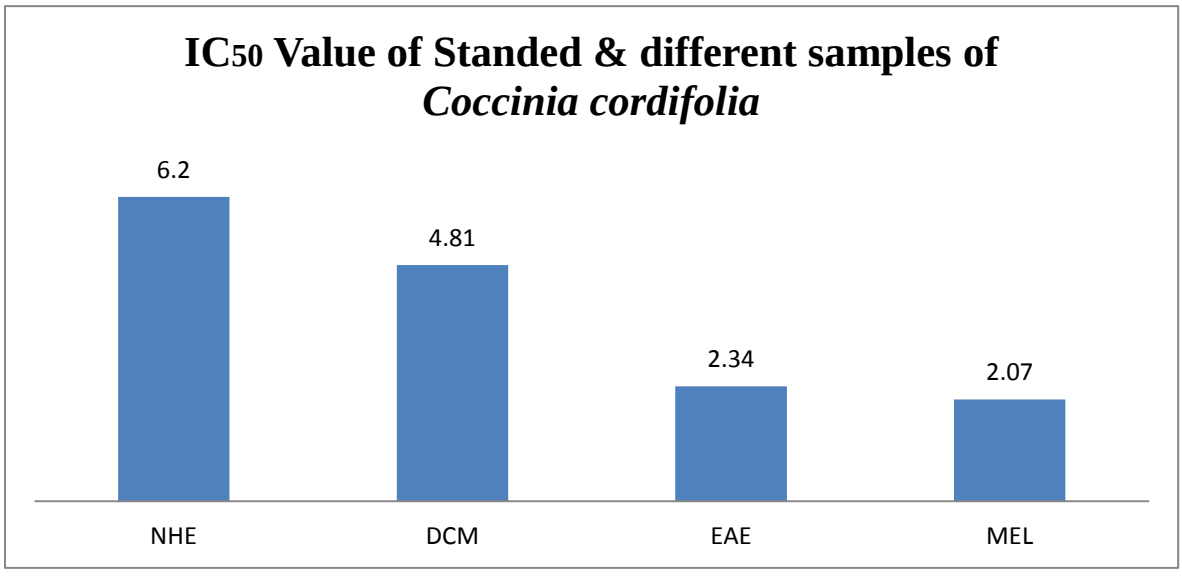


Figure 11.3: **IC_{50} values of standard and different partitionates of *Coccinia cordifolia***

CHAPTER-12
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