Pharmacological Investigation of Leaves of *Streblus asper*

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

Submitted by:

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ID#2007-1-70-061

East West University
Department of Pharmacy

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A Dissertation Submitted to:

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This thesis paper is dedicated to Almighty Allah

CERTIFICATE

This is to certify that, the research work on "Pharmacological Investigation of Leaves of "***Streblus asper*"** submitted to the department of pharmacy, East West University, Aftabnagor, Dhaka, in partial fulfillment of the requirement for the degree of bachelor of pharmacy (B.Pharm) was carried out by Afra Anjum Ami, ID# 2007-1-70-061 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 resources of the information in thus connection are duly acknowledged.

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Acknowledgement

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

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

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LIST OF ABBREVIATION

BNH	Bangladesh National Herbarium
BHA	Butylated hydroxyanisole
BHT	Butylated hdroxytoluene
CAM	Complementary and alternative medicine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DCM	Dichloromethane
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EAE	Ethyla acetade
Fig	Figure
FCR	Folin-Ciocalteu Reagent
G	Gram
GAE	Gallic acid Equivalent
h	Hour
HCl	Hydrochloric acid
IC_{50}	Inhibitory Concentration with 50% scavenging
LC_{50}	Lethal Concentration with 50% mortality
µg	Microgram
Mg	Milligram
MIC	Minimum Inhibitory Concentration
MEL	Methanol
ml	Millilitre
mol wt	Molecular weight

NHE	n-hexane
NO	Nitric Oxide
NaOH	Sodium hydroxide.
NaCl	Sodium Chloride
PG	Propyl gallate
Sec	Second
Tab	Table
TBHQ	tert-butyl hydroquinone
UV	Ultra violet

Abstract

The study was designed for pharmacological investigation of different extract of leaves of *Streblus asper* (Family: Moracea, Bengali name – Shaora (শেওড়া)) and screening of their biological activities like, antimicrobial, cytotoxicity and free radical scavenging activities. The powdered leaves of ***Streblus asper*** was extracted with methanol. The concentrated extract was then partitioned with N-hexane, Dichloromethane, and Ethylacetate.

In antimicrobial activity investigation, methanolic crude extract, dichloromethane fraction, ethylacetade fraction & n-hexane fraction of methanolic extract of ***Streblus asper*** (leaves) showed moderate activity against the tested organisms. They were appeared almost no zone of inhibition against gram positive & gram negative bacteria. The range of zone of inhibition was 8.5-10mm, 8-9.25mm, 6.00-7.50mm,7.0-8.0mm respectively dichloromethane, ethylacetade, n-hexane and methanolic extract. Which is compared to kanamycin (30 μg/disc) used as positive control in this study. Bioactivity guided isolation can be carried out to separate bioactive metabolites from these extracts.

Methanolic crude extract of ***Streblus asper*** (leaves) and its different fractions were screened for cytotoxic properties using brine shrimp lethality bioassay where Vincristine sulfate (LC_{50} value 3.23 μg/ml) was used as a positive control. From the results of the brine shrimp lethality bioassay, it can be predicted that the methanolic crude extract, dichloromethane fraction, ethylacetade fraction & n-hexane fraction of methanolic extract possess cytotoxic principles and have cytotoxic potency having LC_{50} values of 0.025 μg/ml, 3.67μg/ml, 21.96 μg/ml and 0.29μg/ml respectively. The degree of lethality of brine shrimp increases in a dose dependent manner.

In evacuation of total phenolic content of different extraction of ***Streblus asper.*** The total phenolic content of methanolic crude extract, dichloromethane fraction, ethylacetade fraction & n-hexane fraction of methanolic extract is respectively is 131.70mgGAE/g, 122.50mgGAE/g, 154.81mgGAE/g, 96.79mgGAE/g.

In evaluation of free radical scavenging activity it was found that crude methanolic extract of ***Streblus asper*** (leaves) exhibited remarkable antioxidant activity with much lower IC_{50} value. IC_{50} value of methanolic crude extract, dichloromethane fraction, ethylacetade fraction & n-hexane fraction of methanolic extract is respectively is 0.696 µg/ml, 0.427 µg/ml, 0.414µg/ml, 0576µg/ml.

Chapter-1
Introduction

1.1 Introduction

The study of disease and their treatment have been existing since the beginning of human civilization. Norman R. Farnsworth of the University of Illinois declared that, for every disease that affect mankind there is a treatment and cure occurring naturally on the earth. Plant kingdom is one of the major search areas for effective works of recent days. The importance of plants in search of new drugs is increasing with the advancements of medical sciences. For example, ricin, a toxin produced by the beans of *Ricinus communis*, has been found to be effectively couple to tumor targeted monoclonal antibiotics and has proved to be a very potent antitumor drug. Further have the HIV inhibitory activity has been observed in some novel coumarins (complex angular pyranocoumarins) isolated from *Calophyllum lanigerum* and glycerrhizin (from Glycerrhiza species). Hypericin from *Hypercium* species) is an anticancer agent. Taxol is another example of one of the most potent antitumor agent found from *Taxus bravifolia*.

Ayurvedic medicine is a system of healing that relies heavily on herbs and other plants—including oils and common spices. Currently, more than 600 herbal formulas and 250 single plant drugs are included in the “pharmacy” of Ayurvedic treatments. Historically, Ayurvedic medicine has grouped plant compounds into categories according to their effects (for example, healing, promoting vitality, or relieving pain).

Ayurvedic medicine (also called Ayurveda) is one of the world’s oldest medical systems. It originated in India and has evolved there over thousands of years. In the United States, Ayurvedic medicine is considered complementary and alternative medicine (CAM)—more specifically, a CAM whole_medical_system. Many therapies used in Ayurvedic medicine are also used on their own as CAM—for example, herbs, massage, and specialized diets.

Ayurvedic medicine aims to integrate and balance the body, mind, and spirit; thus, some view it as “holistic.” This balance is believed to lead to happiness and health, and to help prevent illness. Ayurvedic medicine also treats specific physical and mental health problems. A chief aim of Ayurvedic practices is to cleanse the body of substances that can cause disease, thus helping to reestablish harmony and balance.

1.2 History of ayurveda

Ayurvedic medicine, also called Ayurveda, originated in India several thousand years ago. The term “Ayurveda” combines the Sanskrit words *ayur* (life) and *veda* (science or knowledge). Thus, Ayurveda means “the science of life.”

There are two main re-organizers of Ayurveda whose works are still existing intact today - Charak and Sushrut. The third major treatise is called the Ashtanga Hridaya, which is a concise version of the works of Charak and Sushrut. Thus the three main Ayurvedic texts that are still used today are the Charak Samhita (compilation of the oldest book Atreya Samhita), Sushrut Samhita and the Ashtangha Hridaya Samhita. These books are believed to be over 1,200 years old. It is because these texts still contain the original and complete knowledge of this Ayurvedic world medicine, that Ayurveda is known today as the only complete medical system still in existence. Other forms of medicine from various cultures, although parallel are missing parts of the original information. In 16th Century Europe, Paracelsus, who is known as the father of modem Western medicine, practiced and propagated a system of medicine which borrowed heavily from Ayurveda.

People from numerous countries came to Indian Ayurvedic schools to learn about this world medicine and the religious scriptures it sprang from. Learned men from China, Tibet, the Greeks, Romans, Egyptians, Afghanistanis, Persians, and more traveled to learn the complete wisdom and bring it back to their own countries. Ayurvedic texts were translated in Arabic and under physicians such as Avicenna and Razi Sempion, both of whom quoted Indian Ayurvedic texts, established Islamic medicine. This style became popular in Europe, and helped to form the foundation of the European tradition in medicine. Thus ayurvedic system sread out throughout world.

1.3 Medicinal plants

Medicinal plants, plants used as natural medicines. This practice has existed since prehistoric times. There are three ways in which plants have been found useful in medicine. First, they may be used directly as teas or in other extracted forms for their natural chemical constituents. Second, they may be used as agents in the synthesis of drugs. Finally, the organic molecules found in plants may be used as models for synthetic drugs. Historically, the medicinal value of plants was tested by trial and error, as in the Doctrine of Signatures . Modern approaches to determining the medicinal properties of plants involve collaborative efforts that can include ethnobotanists, anthropologists, pharmaceutical chemists, and physicians. Many modern medicines had their origin in medicinal plants. Examples include aspirin from willow bark (*Salix spp.*), digitalis from foxglove (*Digitalis purpurea*), and vinblastine from Madagascar periwinkle (*Vinca rosea*) for the treatment of childhood leukemia .

a large group of plants used in medicine or veterinary practice for therapeutic or prophylactic purposes. The actual number of medicinal plants is not known, but there is no doubt that their number is very big at present. So a large number of plants with medicinal constituents have been described, the number of which presently stands at about one-sixth the number of the flowering plants so far pharmacologically evaluated.

In Bangladesh Unani and Ayurvedic medicines were being prepared from plants following the age-old traditional methods available in literature. These herbal medicines may thus be termed as upgraded herbal medicine or modern herbal medicines. The herbal medicine manufacturers of the modern herbal drugs are adopting scientific techniques to a great extent in order to keep the therapeutic value of the active constituents' intact following modern manufacturing process. In the manufacture of a particular medicine, several medicinal plants of similar efficacy are used with the consequent synergistic effect of the active constituents present in the plant materials.

Tab 1.1: Drugs of plant origin used in modern medicine

Common name	**Botanical name**	**use**
Amla	Emblica officinalis	Vitamin - C, Cough , Diabetes, cold, Laxativ, hyper acidity.
Ashok	Saraca Asoca	Menstrual Pain, uterine, disorder, Deiabetes.
Bael / Bilva	Aegle marmelous	Diarrrhoea, Dysentry, Constipation.
Chiraita	Swertia Chiraita	Skin Desease, Burning, censation, fever.
Kalmegh/ Bhui neem	Andrographis Paniculata	Fever, weekness, release of gas.
Long peeper / Pippali	Peeper longum	Appetizer, enlarged spleen , Bronchities, Cold, antidote.
Pashan Bheda / Pathar Chur	Coleus barbatus	Kidny stone, Calculus.
Sandal Wood	Santalum Album	Skin disorder, Burning, sensation, Jaundice, Cough.
Satavari	Asparagus Racemosus	Enhance lactation, general weekness, fatigue, cough.
. Senna	Casia augustifolia	General debility tonic, aphrodisiac.
. Tulsi	Ocimum sanclum	Cough, Cold, bronchitis,expectorand
. Pippermint	Mentha pipertia	Digestive, Pain killer
. Henna/Mehd	Lawsennia iermis	Burning, Steam, Anti Imflamatary
. Gritkumari	Aloe Verra	Laxative, Wound healing, Skin burns & care,Ulcer
. Sada Bahar	Vincea rosea	Leaukamia, Hypotensiv,
. Vringraj	Eclipta alba	Anti-inflamatory, Digestive, hairtonic

. Neem	Azardirchata - indica	Sdedative, analgesic, epilepsy.
. Anantamool/sariva	Hemibi smus Indicus	Appetiser, Carminative, aphrodisiac, Astringent
. Kantakari	Solanum Xanthocarpum	Diuretic, Antiinflamatory, Appetiser, Stomachic
. Shankhamul	Geodorum denciflorum	Antidiabetic

At present, thousands of plant metabolites are being successfully used in the treatment of variety of diseases. A few striking examples of plant metabolites include taxol from *Taxus brevifalia*, vincristine and vinblastine from *Vinca rosea*, of which are important anticancer agents being used clinically. In the current popular field of chemotherapy, cepharanthine, isolated is being used as a prophylactic in the management of tuberculosis.

In China, about 15,000 factories are involved in producing herbal drugs; Herbal medicines have been developed to a remarkable standard by applying modern scientific technology in many countries, such as China, India, Bangladesh, Sri Lanka, Thailand and United Kingdom. In these countries, the dependence of allopathic drugs has been described to greater extent.

"Modern medicine still has much to learn from the collector of herbs" said Dr. Hafdan Mohler, director general of World Health Organization. Many of the plants, familiar to the witch doctor really do have the healing power that tradition attaches to them. The age old art of the herbalist must be tapped.

Chapter-2
Plant Details

2.1 A brief about Mulberry family

Moraceae often called the mulberry family or fig family. These are a family of flowering plants comprising about 40 genera and over 1000 species. Plants of the family contain a milky latex and have alternate or opposite leaves and small, petal less male or female flowers. The fruits of many species are multiple because fruits from different flowers become joined together.

Some genera produce edible fruits, such as the mulberry (*Morus*), fig (*Ficus carica*), breadfruit and jackfruit (*Artocarpus*), and affon, or African breadfruit (*Treculia*). Others, such as *Antiaris, Ficus,* and *Castilla,* are important for their timber and latex. The latex of the upas tree (*Antiaris toxicaria*) of Java is used as an arrow poison; the latex of the cow tree (*Brosimum utile*) of tropical America is sweet and nutritious. *Ficus*, the largest genus in the mulberry family, contains the banyan and the India rubber tree.

The bark of the paper mulberry (*Broussonetia*) has been used for the manufacture of cloth and paper products. Among the ornamentals in the family are paper mulberry and Osage orange.

2.2 Different Plant of Mulberry family

Tab 2.1: Different Plant of Mulberry family

Name of Plant	Figure of plant
1. *Streblus asper, Shaora*	
2. *Artocarpus breadfruit*	

3. *Artocarpus heterophyllus*, jack fruit	
4. *Broussonetia papyrifera*, paper mulberry	
5. *Ficus pumila*, climbing fig	

6. *Ficus lyrata*, fiddle	
7. *Ficus microcarpa*, Chinese banyan	
8. *Ficus religiosa*, Bo tree, Buddha tree	

2.3 Introduction to *Streblus asper*

Tab 2.2: A brief introduction to *Streblus asper*

Family	Moracea
Tribe	Moreae
Genus	Streblus
Species	Streblus asper
Synonyms	In different Countries ***Streblus asper*** has different name, these are • Bangladesh=Shaora • Malaysia=Kesinai, Serinai • English=Sandpaper tree, Siamese Rough brush, Tooth brush tree • Indonesia=Peleh, Serut • Thailand=Khoi, Kak maifol • Myanmer=Okhne • Philippines=Kalios

2.4 Taxonomy of *Streblus asper* Lour

Kingdom- Plantae

Subkingdom- Tracheobionta

Superdivision- Spermatophyta

Order- Rosales

Family- Moracea

Tribe-Moreae

Genus-Streblus

Species- ***Streblus asper***

2.5 Botanical description of *Streblus asper*

Streblus asper is a rigid and densely branched tree growing from 4-10 m in height. The leaves are oblong-obovate to sub-rhomboid, 4-12 cm long, very rough on both sides, with finely toothed margin, the tip blunt or tapering to a point and the base narrowed.

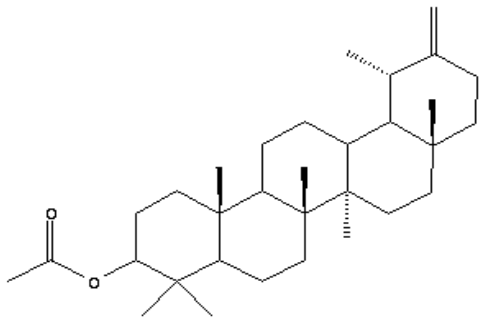
The male flowers are in rounded heads, 4-7 mm in diameter, short peduncled, greenish-yellow, or nearly white. The female flowers are stalked, usually in pairs, green; the sepals become larger after flowering, and nearly enclose the fruit. The fruit is ovoid, 8-10 mm long, pale yellow, and the pericarp soft and fleshy.

The seed is ovoid, and 5-6 mm long. Thrue to its name, the leaves of Sand Paper Tree are rough and are utilized for cleaning cooking utensils and as a substitute for sandpaper.

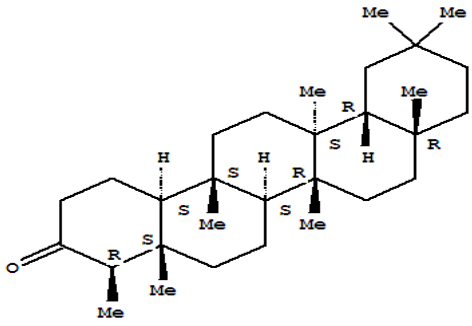
Fig 2.1: Leave of *Streblus asper*

2.6 Chemical Constituents

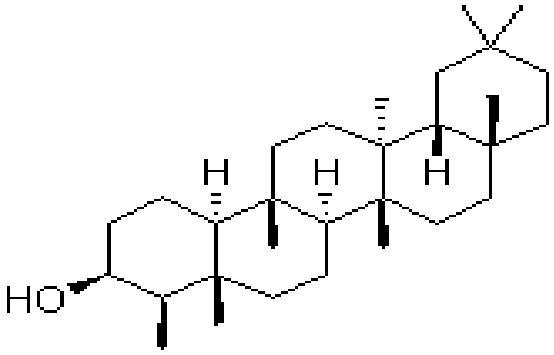
Plant contains triterpenoids - friedelin, epifriedelinol, taraxasteryl acetate. Root bark contains cardiac glycoside vijaloside, cardenolide, kamaloside, asperoside, indroside, lucknoside, glycosides G, G and H, cannodimethoside, strophalloside, glucogitodimethoside, strophanolloside, glucokamaloside, sarmethoside and glucostrebloside. Stem bark contains cardiac glycoside stebloside, mansonin



taraxasteryl acetate



triterpenoids – friedelin



epifriedelinol

Fig 2.2: Different chemical compound of *Streblus asper*

2.7 Uses

Leaves used as a galactogogue; their poultice applied to swellings and buboes. Roots given in dysentery. Decoction of bark used in fevers, diarrhoea and dysentery.

Latex astringent and antiseptic applied to sore heals, chapped hands and glandular swellings; also applied to temples as a sedative in neuralgia. Seeds used in epistaxis, piles and diarrhoea; externally, paste applied to leucoderma.

2.8 Collection

The plant ***Streblus asper*** was collected from Munshigong, Dhaka.
And the leave of ***Streblus asper*** is identified from Bangladesh National Herbarium (BNH) and the accession number is 37567.

Chapter-3
Literature Review

3.1 *Streblus asper* Lour. (Shakhotaka): A Review of its Chemical, Pharmacological and Ethnomedicinal Properties

Source:

Subha Rastogi, Dinesh K. Kulshreshtha and Ajay Kumar Singh Rawat
Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute and
2Medicinal Chemistry Division, Central Drug Research Institute, Lucknow 226001, India

- Streblus asper Lour is a small tree found in tropical countries, such as India, Sri Lanka, Malaysia, The Philippines and Thailand.
- Various parts of this plant are used in Ayurveda and other folk Medicines for the treatment of different ailments such as filariasis, leprosy, toothache, diarrhea, dysentery and cancer.
- Research carried out using different in vitro and in vivo techniques of biological evaluation support most of these claims.
- This review presents the botany, chemistry, traditional uses and pharmacology of this medicinal plant.

3.2 In-vivo and In-vitro antioxidant properties of methanol extraction of *Streblus asper* Lour

Source:

Bibhuti B Kakoti , V. Thamil Selvan, Prerona Saha, M. Gupta, U.K Mazumder

- The *in vivo* and *in vitro* antioxidant properties of methanol extract of *Streblus asper* Lour
- (Family: Moraceae) (MESA) was evaluated.
- The *in vitro* antioxidant potential determined by performing various assays such as DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, lipid peroxidation inhibition assay, hydroxyl radical scavenging assay, nitric oxide scavenging assay, and reducing ability. 3, 5-Ditert- butyl-4-hydroxytoluene (BHT) was used as a standard.

- The IC50 values of MESA and BHT in DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging, lipid peroxidation.
- The obtained *in vitro* and *in vivo* results suggested that MESA possesses a significant antioxidant and hepatoprotective property.

3.3 Evaluation of antihyperglycemic and antioxidant properties of *Streblus asper* Lour against streptozotocin-induced diabetes in rats

Source:

RB Suresh Kumar, Biswakanth Kar, Narayan Dolai1, Asis Bala, Pallab Kanti Haldar
Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India-700032
Himalayan Pharmacy Institute, Sikkim, India-737102

- To evaluate antidiabetic and antioxidant role of methanol extract of Streblus asper (S. asper) root bark in wister rats.

3.4 Effect of *Streblus asper* Leaf extract on selected anaerobic bacteria

Source:

S. Taweechaisupapong, S. Singhara, T. Choopan

- The purpose of this in vitro study was to determine the antibacterial effects of leaf extract of koi (*Streblus asper*) against six anaerobic bacteria: *Porphyromonas gingivalis* W50, *Prevotella intermedia, Actinomyces naeslundii* (T14V), *Peptostreptococcus micros, Actinobacillus actinomycetemcomitans* ATCC 43717 and ATCC 43718.
- The dried-pulverized leaves were extracted using 50% (v/v) redistilled ethanol. Employing the disc diffusion method, it was demonstrated that 15 µL of the leaf extract at 250 and 500 mg/mL had inhibitory effects towards all bacterial strains tested except *A. actinomycetemcomitans* ATCC 43717

Chapter-4
Pharmacological Review

4.1 Traditional use of *Streblus asper*

Traditional or folk medicines have been widely employed for centuries, and they remain one important source for the discovery of new bio-active compounds. Ayurveda, an ancient traditional system of medicine that has been practiced in India since 200 B.C. employs a large number of medicinal plants used in the prevention and treatment of a wide number of diseases. *Streblus asper* is a well known ethnomedicinal plant which is also used in Ayurveda. Its use in the Indian traditional folk medicine is also well documented.

Tab 4.1: List of traditional medicinal plants

Plant part	Traditional uses
Root	As an application to unhealthy ulcers and sinuses and as antidote to snake bite, in epilepsy.
Stem	Toothache
Stem bark	Given in fever, dysentery and diarrhea stomachache and urinary complaints, useful in piles, edema and wounds decoction effective against lymphadema, chylurea and other effects of filariasis
Leaves	Eye complaints
Milky juice/latex	Antiseptic, astringent, applied to chapped hands and sore feet, in pneumonia and swells of cheek
Fruit	Eye complaints
Seeds	Epistaxis and diarrhe
Part not specified	Cancer, cholera, colic, diarrhea dysentery and menorrhagia, epilepsy.

4.2 Review of Pharmacological Report

Streblus asper a plant from mulberry family this likely use for treatment of cholera, piles, wounds and as a mild pain reliever or analgesic.

The bark of the tree can be boiled in water and the resultant liquid is used as a disinfectant for wounds and skin problems. A decoction of the bark is used for fever, diarrhea and dysentery, while the bark itself is chewed as an antidote to snake bites. In the Philippines a tea is made from the leaves and drunk as a beverage.

The powdered root is used for diarrhea and dysentery and can be made into a poultice for ulcers on the skin. The root is used for epilepsy, inflammation and boils, and the root extract is astringent and antiseptic. The bark exudes latex when cut which is used in India for chapped hands and feet. It is also used for swollen glands, being put on them externally. The seeds are used for nosebleeds, piles and diarrhea too.

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Chapter-5
Material Requirement

5.1 Materials for chemical investigation

Tab 5.1: lists of Glass wares

Materials	Source
Conical flasks	Laboratory Equipments
Beakers	Laboratory Equipments
Test tubes	Laboratory Equipments
Funnels	Laboratory Equipments
Measuring cylinders	Laboratory Equipments
Pipettes etc	Laboratory Equipments
Pasteur pipettes	Laboratory Equipments

Tab 5.2: lists of Solvents

Materials	Source
Methanol	Merck, Germany and Sigma, USA
Di-Chloro-Methane	Merck, Germany and Sigma, USA
N-Hexane	Merck, Germany and Sigma, USA
Ethyl acetate	Merck, Germany and Sigma, USA

Tab 5.3: Lists of Equipments

Equipments	Source
Rotary vacuum evaporator	University Instruments Lab
Electronic balance	Denver Instruments M-220
Grinding machine	University Instruments Lab
Oven (0°C-210°C)	Gallen Kamp Hotbox
Solvent distillation plant	University Instruments Lab
Distilled water plant	University Instruments Lab

5.2 Material for brine shrimp lethality bioassay

- *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

5.3 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

Chapter-6
Study Protocol

6.1 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 *Streblus asper* (Family: Moracea). The study protocol consisted of the following steps:

- Cold extraction of the powdered stem bark 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, dicholoromethane 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.

6.2 Phytochemical Investigation

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

- Collection and proper identification of the plant material
- Preparation of plant sample
- Extraction of the plant material

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 Pharmacological Research Laboratory, Faculty of Pharmacy, and 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:

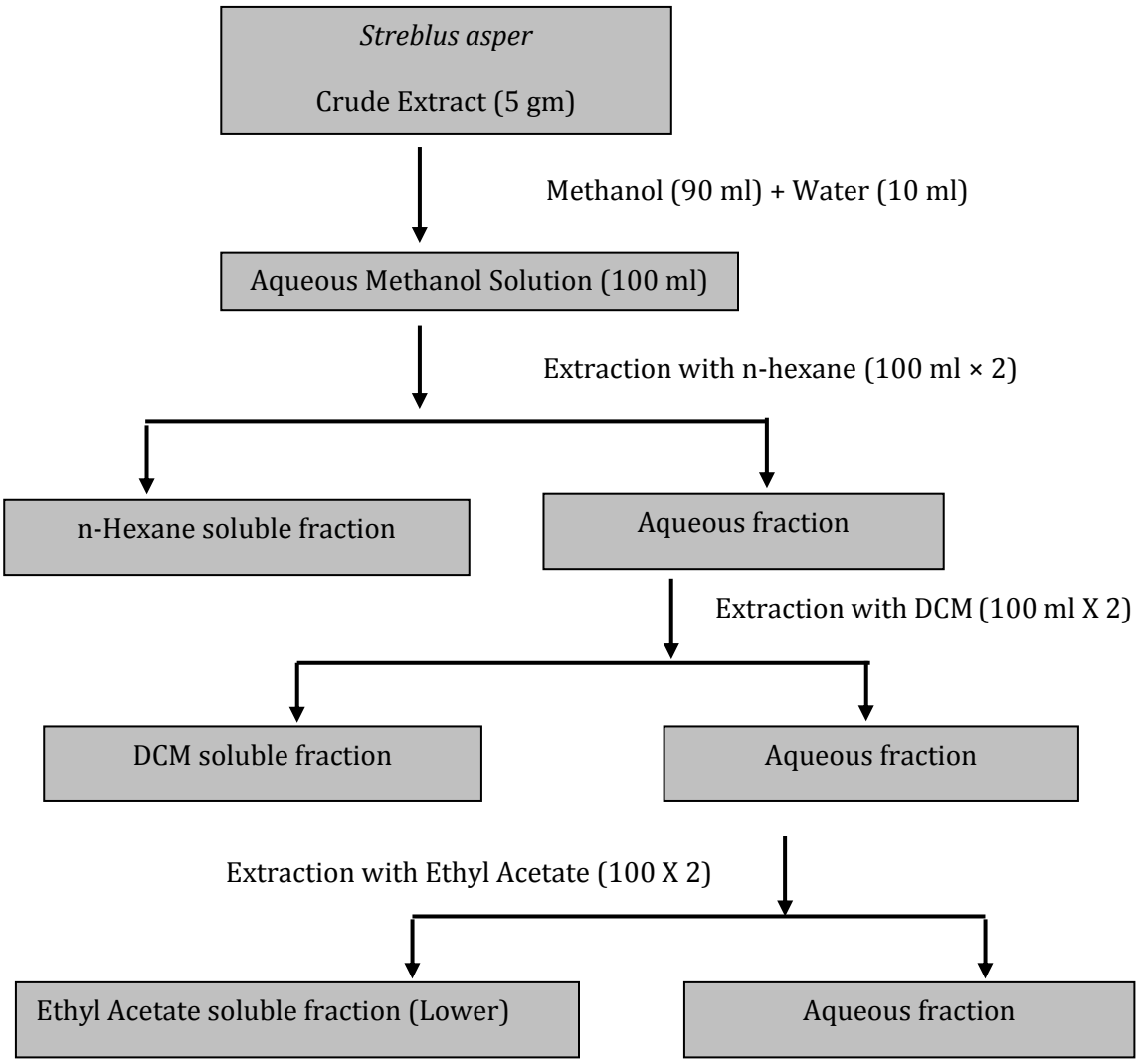


Fig 6.1: Schematic representation of the modified kupchan partitioning of methanolic crude extract of *Streblus asper*

Tab 6.1: After evaporation the weight of the different fractions obtained are as follows

Plant	Part	Extract and Fraction	Weight
Streblus asper	Leave	Crude of methanol extract	35.0 g
Streblus asper	Leave	n-Hexane fraction	6.57 g
		Ethyl acetate fraction	4.567 g
		Methanol fraction	2.94g
		Dichloro methane	5.77 g

Chapter-7
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 (Pinner *et al*., 1996).

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

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).

Fig 7.1: Disc diffusion method

7.3 Apparatus, reagent and test sample

Tab 7.1: Apparatus and reagents

Apparatus and reagents	
Filter paper discs	Autoclave
Nutrient Agar Medium	Laminar air flow hood
Petri dishes	Spirit burner
Sterile cotton	Refrigerator
Sterile forceps	Incubator and Micropipette
Inoculating loop	Nose mask and Hand gloves

7.3.1 Test materials of *Streblus Asper*

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

7.3.2 Test organisms

The bacterial and fungal strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka.Test organisms are given below.

Tab 7.2: List of test bacteria

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

7.4 Culture medium and their composition

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

Tab 7.3: Composition of nutrient agar medium

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)

7.5 Experimental procedure

7.5.1 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.

7.5.2 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. Petri dishes 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.

7.5.3 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.

7.5.4 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.

7.6 Preparation of discs

Three types of discs were used for antimicrobial screening.

7.6.1 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.

7.6.2 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.

7.7 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)

Tab 7.4: Amount of test sample taken

Sample	Dose (μg/disc)	Required amount for disc (mg)
Dichloromethane	200	4
N-Hexane	200	4
Ethylacetade	200	4
Methanol	200	4

7.8 Application of the test samples

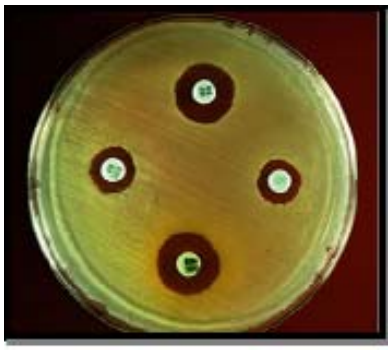
Standard Kanamycin (30 mg/disc) discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. Blank discs were used as negative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

7.8.1 Diffusion and incubation

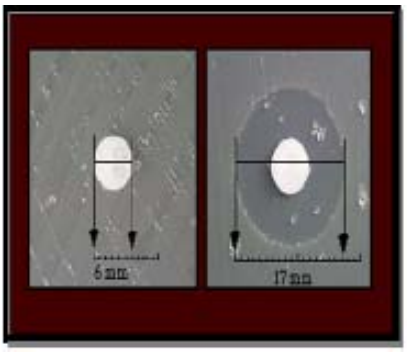
The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4 ^{0}C for about 24 hours upside down to allow sufficient diffusion of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

7.8.2 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)

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

Tab 7.5: Antimicrobial activity of test samples of *Streblus asper* (leaves)

Test microorganisms	Diameter of zone of inhibition (mm)				
	DCM	**EAE**	**NHE**	**MEL**	Kanamycin
Gram positive bacteria					
Bacillus sereus	-	-	6.5	-	-
Staphylococcus aureus	-	-	-	8.5	34

Test microorganisms	Diameter of zone of inhibition (mm)				
	DCM	**EAE**	**NHE**	**MEL**	Kanamycin
Gram negative bacteria					
Escherichia coli	8.5	9.25	6.5	-	37
Shigella boydii	10	8.5	6.25	7.75	37
Vibrio mimicus	9	-	7.25	8.75	37

Chapter-8
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.

Tab 8.1: Test samples of experimental plant (*Streblus asper*)

Plant Part	**Test samples**	**Measured amount (mg)**
Leaves	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

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.

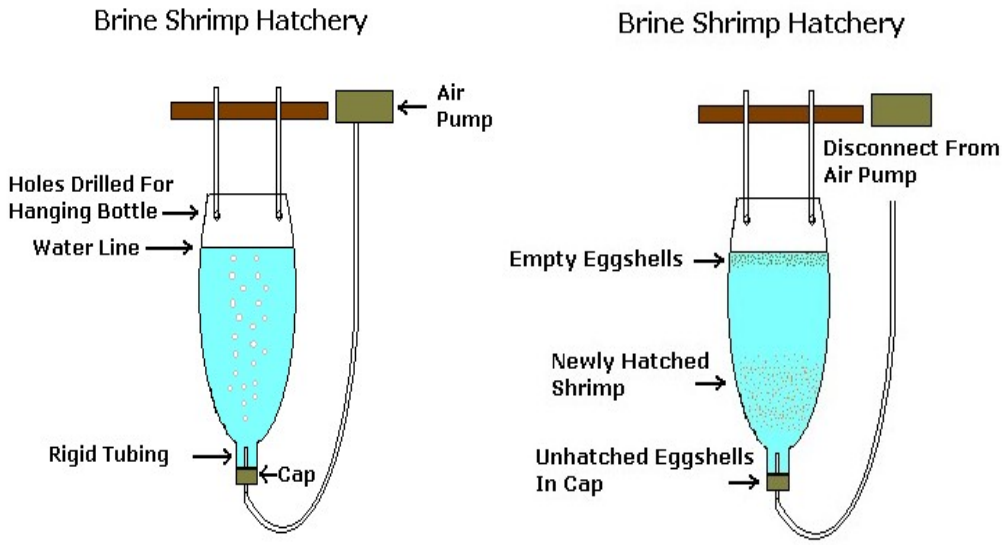


Fig 8.1: Brine shrimp Hatchery

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.

8.5 Preparation of control group

Control groups are used in cytotoxicity study to validate the test method and ensure that the results obtained are only due to the activity of the test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used

i) Positive control

ii) Negative control

8.5.1 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.

8.5.2 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.

8.6 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.

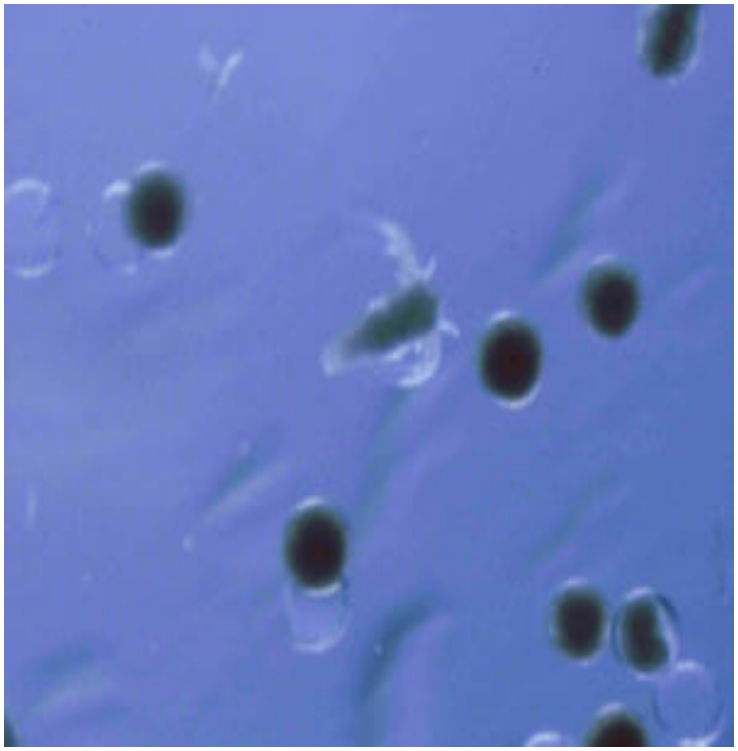


Fig 8.2: Brine shrimp nauplii

8.7 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.

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

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	

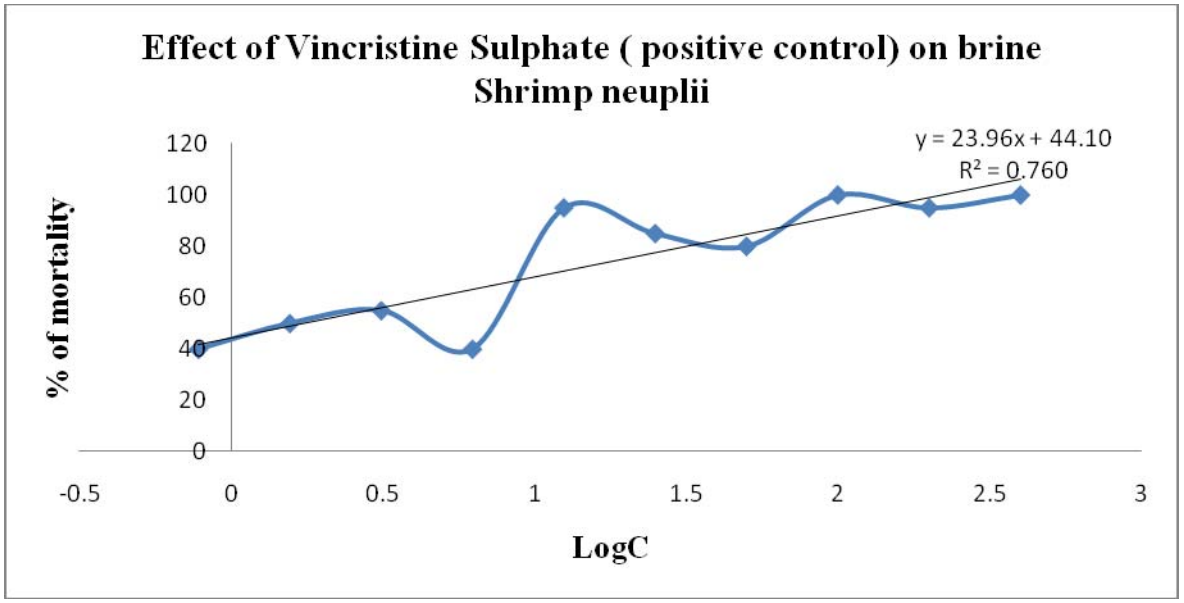


Fig 8.3: Effect of Vincristine Sulphate (positive control) on brine shrimp nauplii

8.8 Graphical representation of different extract of *Streblus asper*

Tab 8.3: Effect of Dichloromethane fraction of crud extraction of *Streblus asper* on brine shrimp nauplii

Test tube No.	Concentration (C) (µg/ml)	LogC	% Mortality	LC_{50} (µg/ml)
1	400	2.602	90	3.67
2	200	2.301	50	
3	100	2.000	80	
4	50	1.699	70	
5	25	1.398	85	
6	12.5	1.097	95	
7	6.250	0.796	00	
8	3.125	0.495	50	
9	1.563	0.194	50	
10	0.781	-0.107	40	

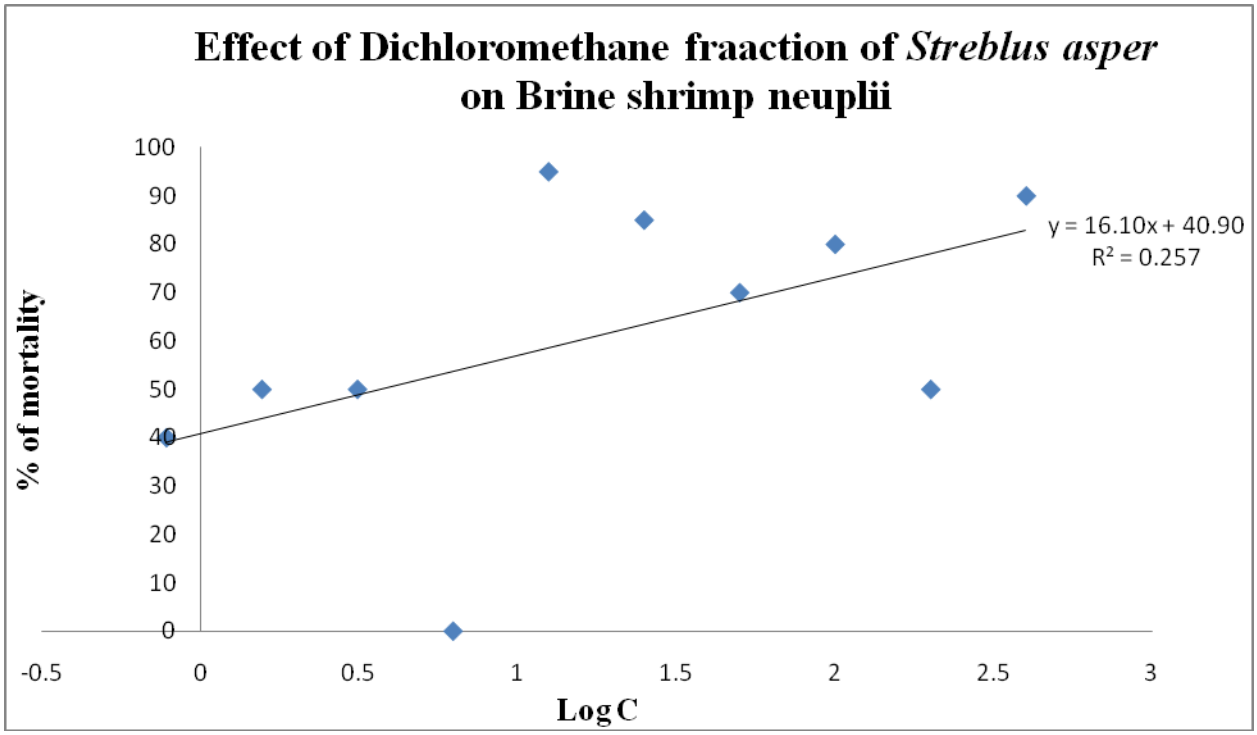


Fig 8.4: Effect of Dichloromethane fraction of crud extraction on brine shrimp nauplii

Tab 8.4: Effect of N-Hexane fraction of crud extraction of *Streblus asper* on brine shrimp nauplii

Test tube No.	Concentration (C) (µg/ml)	LogC	% Mortality	LC_{50} (µg/ml)
1	400	2.602	50	0.29
2	200	2.301	35	
3	100	2.000	80	
4	50	1.699	80	
5	25	1.398	55	
6	12.5	1.097	45	
7	6.250	0.796	40	
8	3.125	0.495	50	
9	1.563	0.194	70	
10	0.781	-0.107	40	

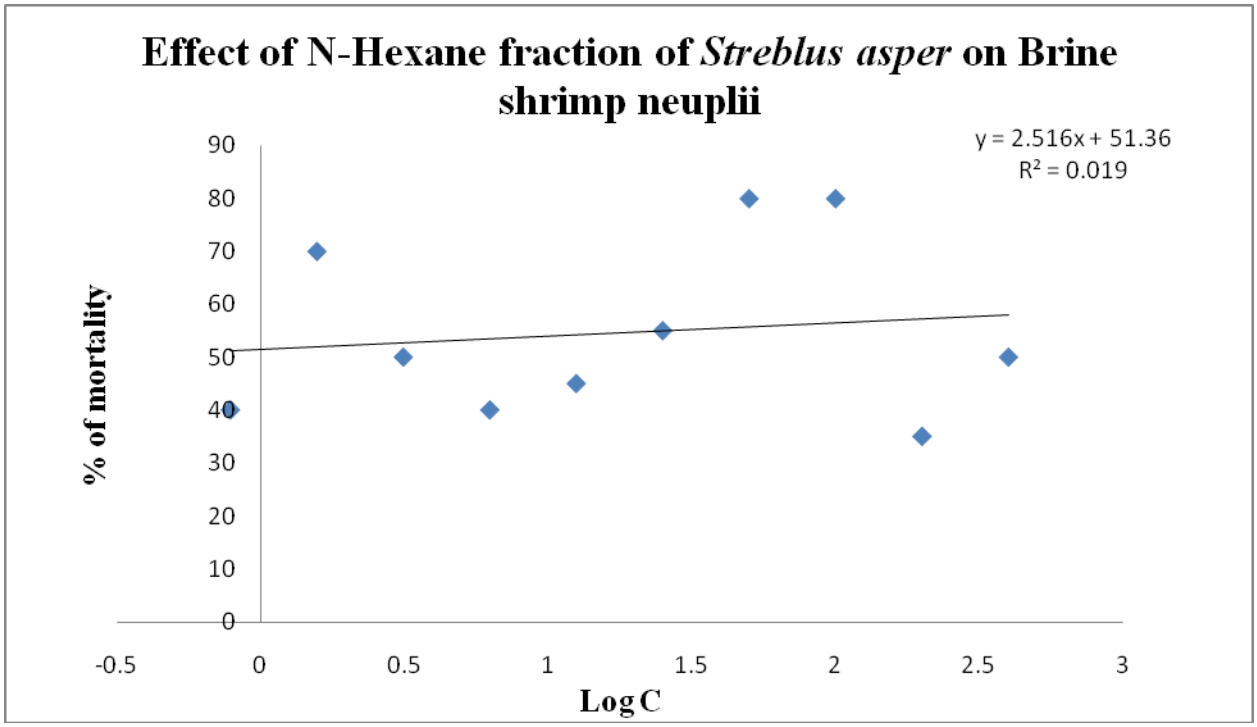


Fig 8.5: Effect of N-Hexane fraction of crud extraction on brine shrimp naupli

Tab 8.5: Effect of Ethylacetade fraction of crud extraction of *Streblus asper* on brine shrimp nauplii

Test tube No.	Concentration (C) (µg/ml)	LogC	% Mortality	LC_{50} (µg/ml)
1	400	2.602	80	21.96
2	200	2.301	50	
3	100	2.000	70	
4	50	1.699	60	
5	25	1.398	50	
6	12.5	1.097	65	
7	6.250	0.796	40	
8	3.125	0.495	35	
9	1.563	0.194	20	
10	0.781	-0.107	10	

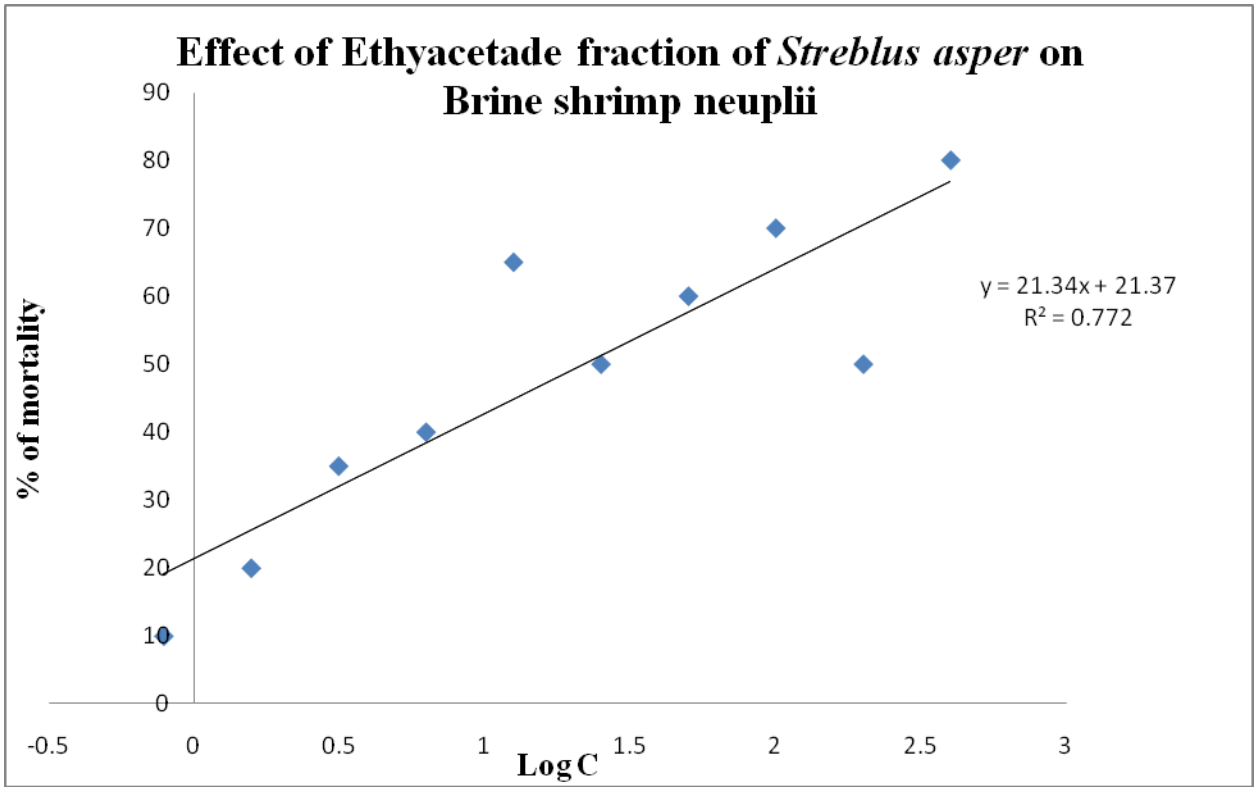


Fig 8.6: Effect of Ethylacetade fraction of crud extraction on brine shrimp nauplii

Tab 8.6: Effect of Methanol fraction of crud extraction of *Streblus asper* on brine shrimp nauplii

Test tube No.	Concentration (C) (µg/ml)	LogC	% Mortality	LC_{50} (µg/ml)
1	400	2.602	100	0.025
2	200	2.301	100	
3	100	2.000	80	
4	50	1.699	90	
5	25	1.398	70	
6	12.5	1.097	75	
7	6.250	0.796	80	
8	3.125	0.495	70	
9	1.563	0.194	60	
10	0.781	-0.107	80	

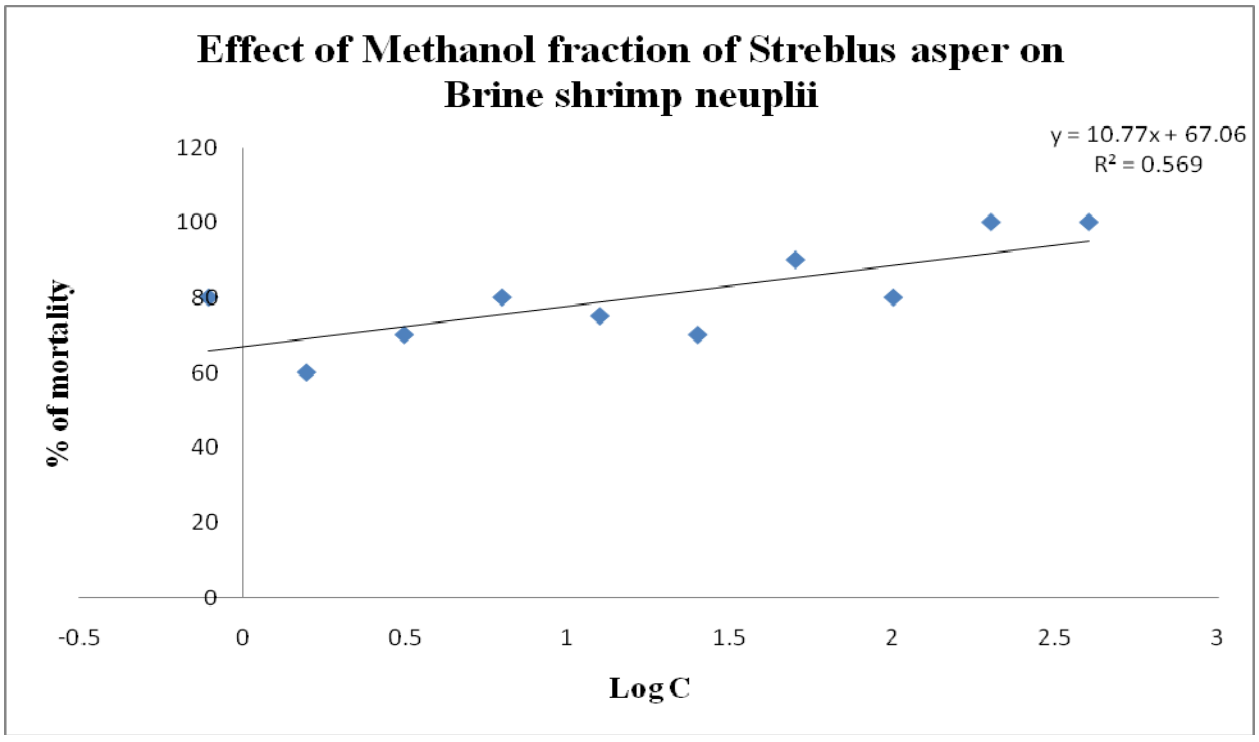


Fig 8.7: Effect of Methanol fraction of crud extraction on brine shrimp nauplii

Chapter-9
Total Phenolic 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 *Streblus Asper* -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 *Streblus Asper* 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).

Tab 9.1: Composition of Folin-Ciocalteu Reagent

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

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

Tab 9.2: Test samples for total phenolic content determination

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

9.6 Total phenolic compound analysis

Flow diagram for Total Phenolic Content Assay:

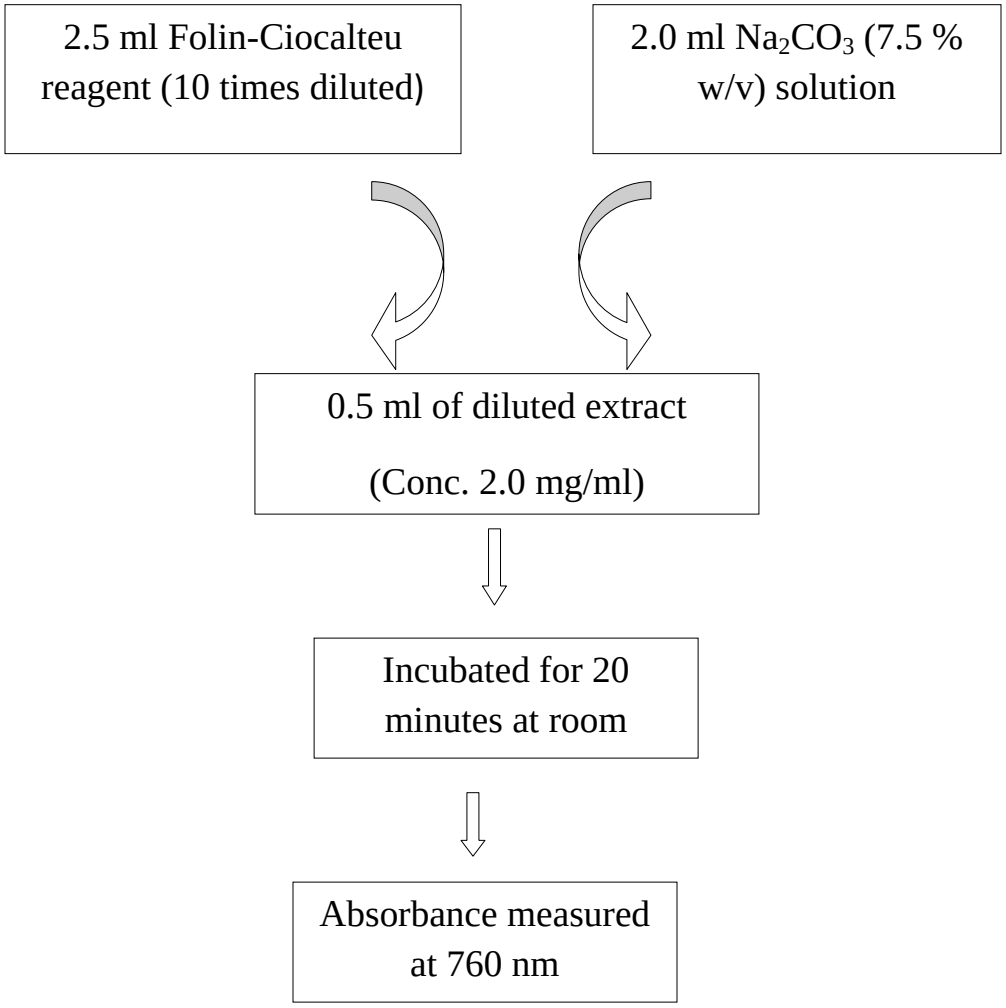


Fig 9.1: Schematic representation of the total phenolic content determination

9.7 Result of Total Phenolic Content

Amount of total phenolic content in *Streblus Asper.*

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

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		

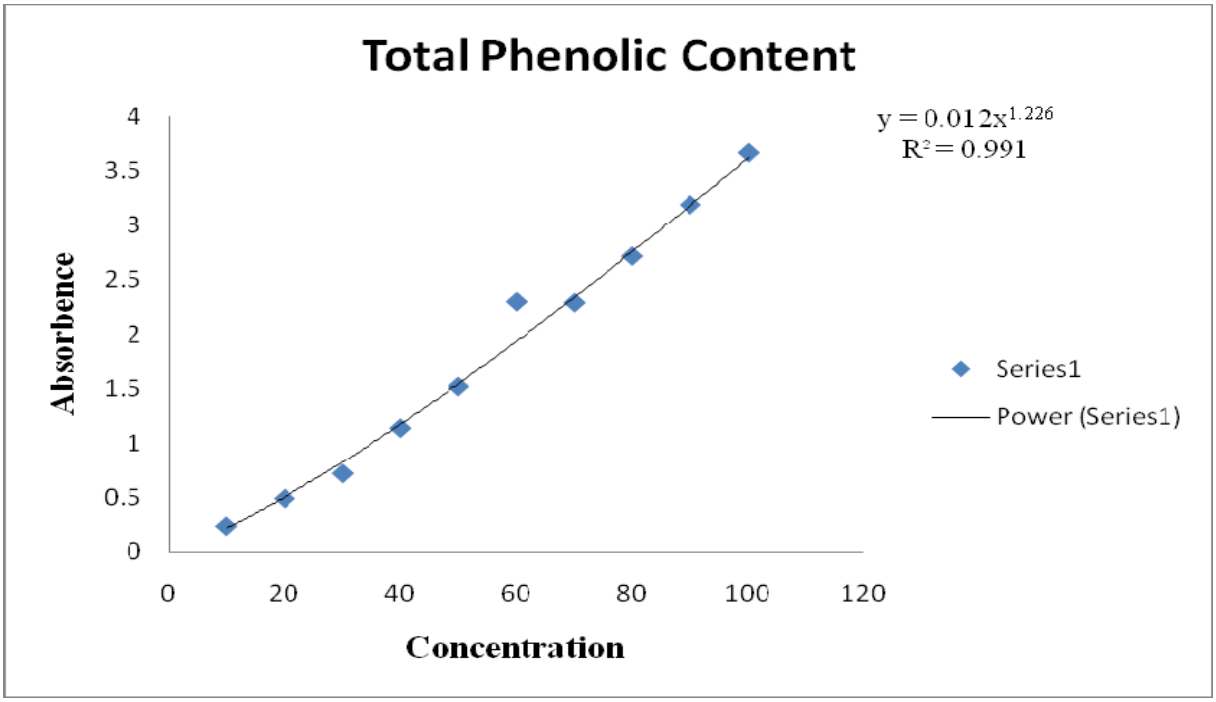


Fig 9.2: Graphical representation of assay of Phenolic compound in different extract of *Streblus asper*.

Tab 9.4: Total phenolic content of fraction of whole plant of *Streblus asper*

Name of fraction	Absorbance	mgGAE/g
N-Hexane (NHE)	0.194	96.79
Dichloromethane (DCM)	0.259	122.50
Ethylacetade (EAE)	0.385	154.81
Methanol (MEL)	0.283	131.70

9.8 Total phenolic content of different fraction of *Streblus asper*

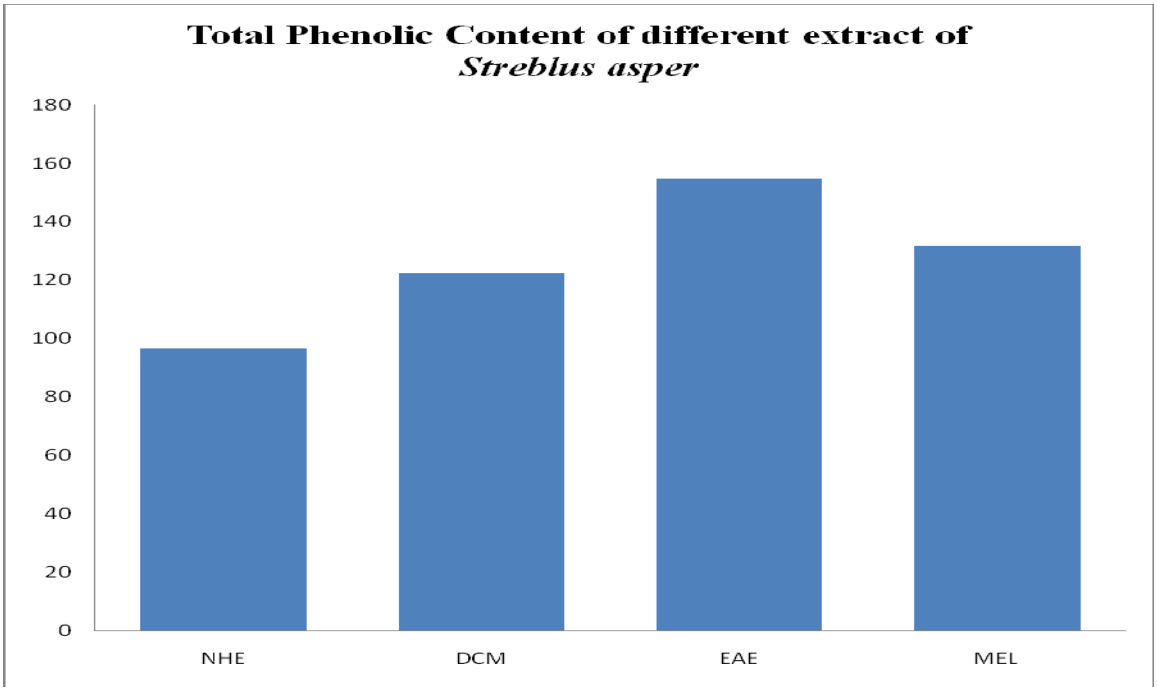


Fig 9.3: Total phenolic content of different fraction of *Streblus asper*

9.9 Discussion

The Total Phenolic content of *Streblus asper* was found. The plant extract contain phenolic content. The total phenolic content of methanolic crude extract, dichloromethane fraction, ethylacetade fraction & n-hexane fraction of methanolic extract is respectively is 131.70mgGAE/g, 122.50mgGAE/g, 154.81mgGAE/g, 96.79mgGAE/g. Further research is needed on the determination of the correlation between the antioxidant capacity and the chemical components of the plant.

Chapter-10
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).

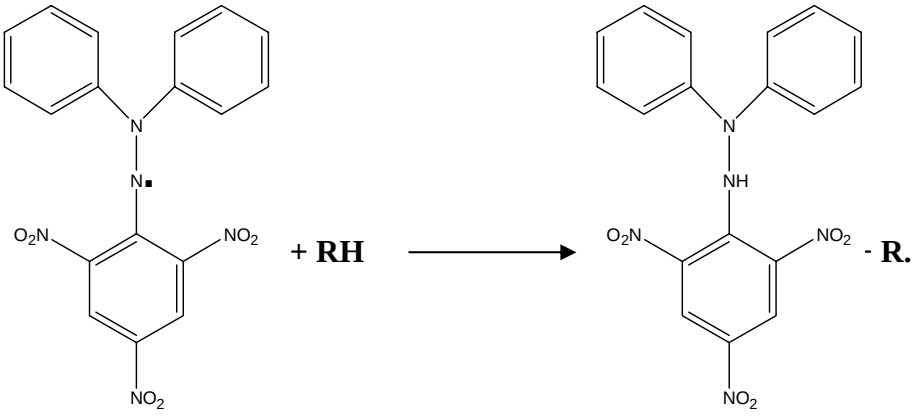
Dietary food contains a wide variety of free radical-scavenging antioxidants; for example, flavonoids and antioxidant vitamins such as ascorbic acid and ∞-tocopherol. These compounds are particularly rich in vegetables, fruits, tea, and wine. Epidemiological studies have shown that higher intake of fresh 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 *Streblus asper* (leaves) 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**

Fig 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.

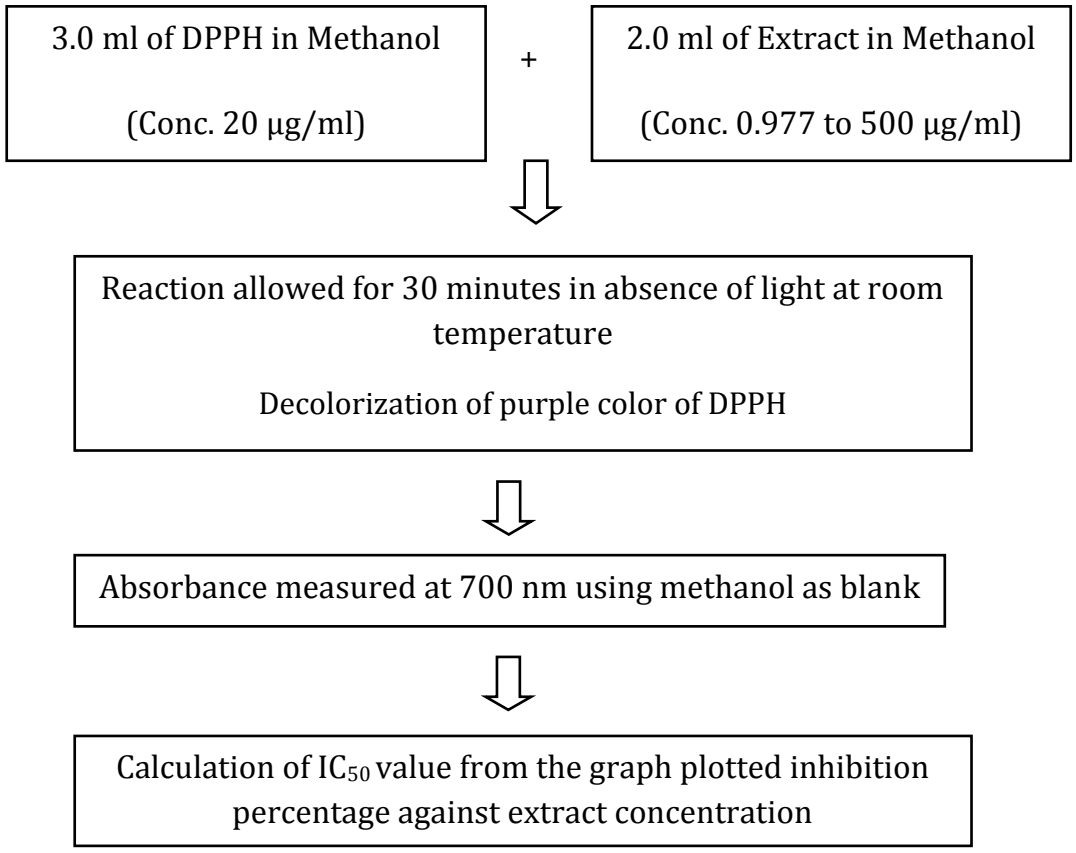


Fig 10.2: Schematic representation of assaying free radical scavenging activity

10.4 Results and Discussion

Different partitionates of methanolic extract of *Streblus asper* (leaves) 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.

Tab 10.1: IC_{50} values of standard and different fractions of *Streblus asper* (leaves)

Code	Sample	IC_{50} (µg/ml)
BTH	*tert*-butyl-1-hydroxytoluene	18.21
NHE	N-Hexane Crude Extract	0.576
DCM	Dichloromethane Crude Extract	0.427
EAE	Ethylacetade Crude Extract	0.414
MEL	Methanol Crude Extract	0.696

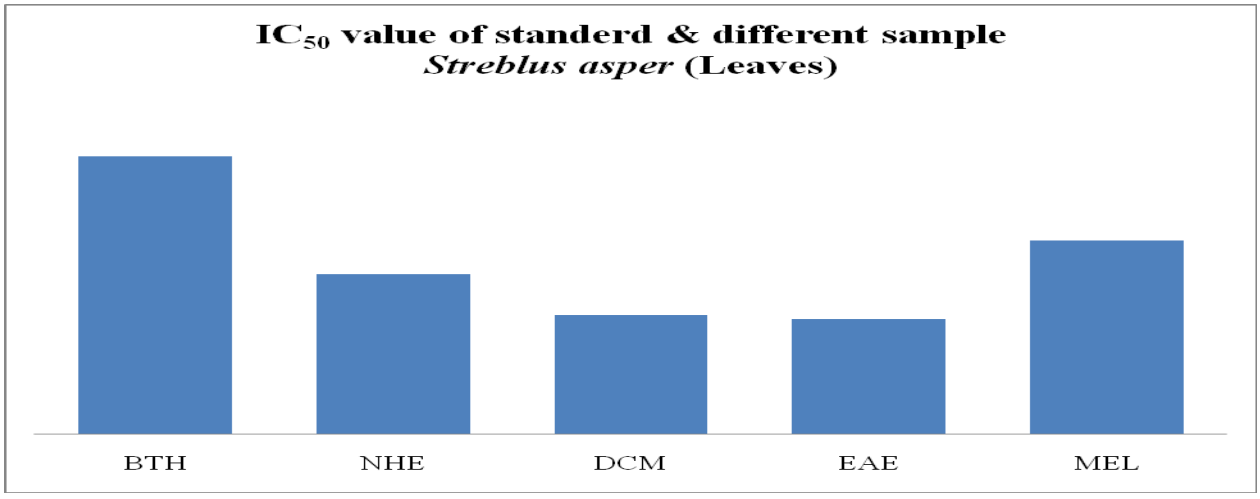


Fig 10.3: IC_{50} values of standard and different partitionates of *Streblus asper* (leaves)

Tab 10.2: IC_{50} value of Dichloromethane (DCM)

Test tube No.	Absorbance of Blank	Concentration (µg/ml)	Absorbance of sample	% Inhibition	IC_{50} (µg/ml)
1	0.674	500	0.032	95.25	0.427
2		250	0.050	95.58	
3		125	0.078	88.43	
4		62.5	0.098	85.46	
5		31.25	0.129	80.86	
6		15.625	0.153	77.30	
7		7.813	0.202	70.03	
8		3.906	0.240	64.39	
9		1.953	0.274	59.34	
10		0.977	0.304	54.90	

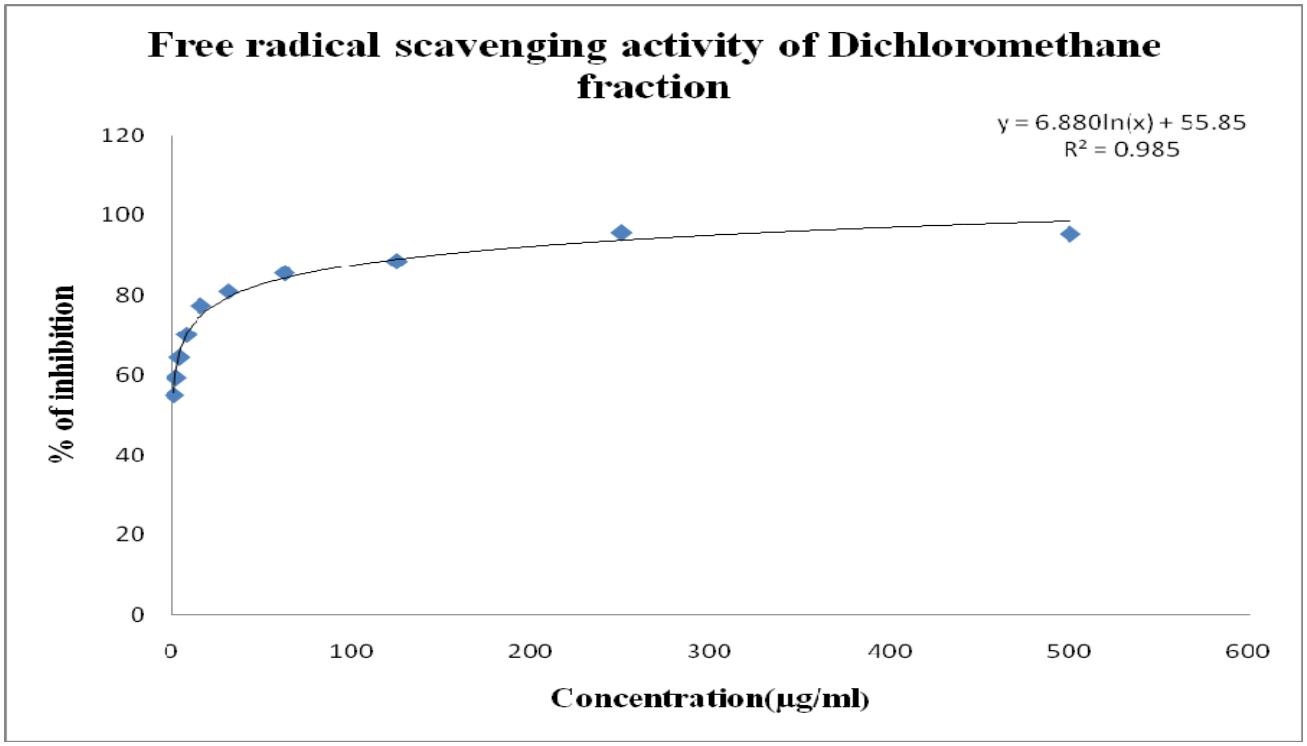


Fig 10.4: Free radical scavenging activity of Dichloromethane (DCM)

Tab 10.3: IC_{50} value of Ethylacetade (EAE)

Test tube No.	Absorbance of Blank	Concentration (µg/ml)	Absorbance of sample	% Inhibition	IC_{50} (µg/ml)
1	0.674	500	0.028	95.856	0.414
2		250	0.015	97.774	
3		125	0.088	86.945	
4		62.5	0.086	87.389	
5		31.25	0.161	76.113	
6		15.625	0.214	68.249	
7		7.813	0.233	65.430	
8		3.906	0.235	65.133	
9		1.953	0.257	61.869	
10		0.977	0.272	59.644	

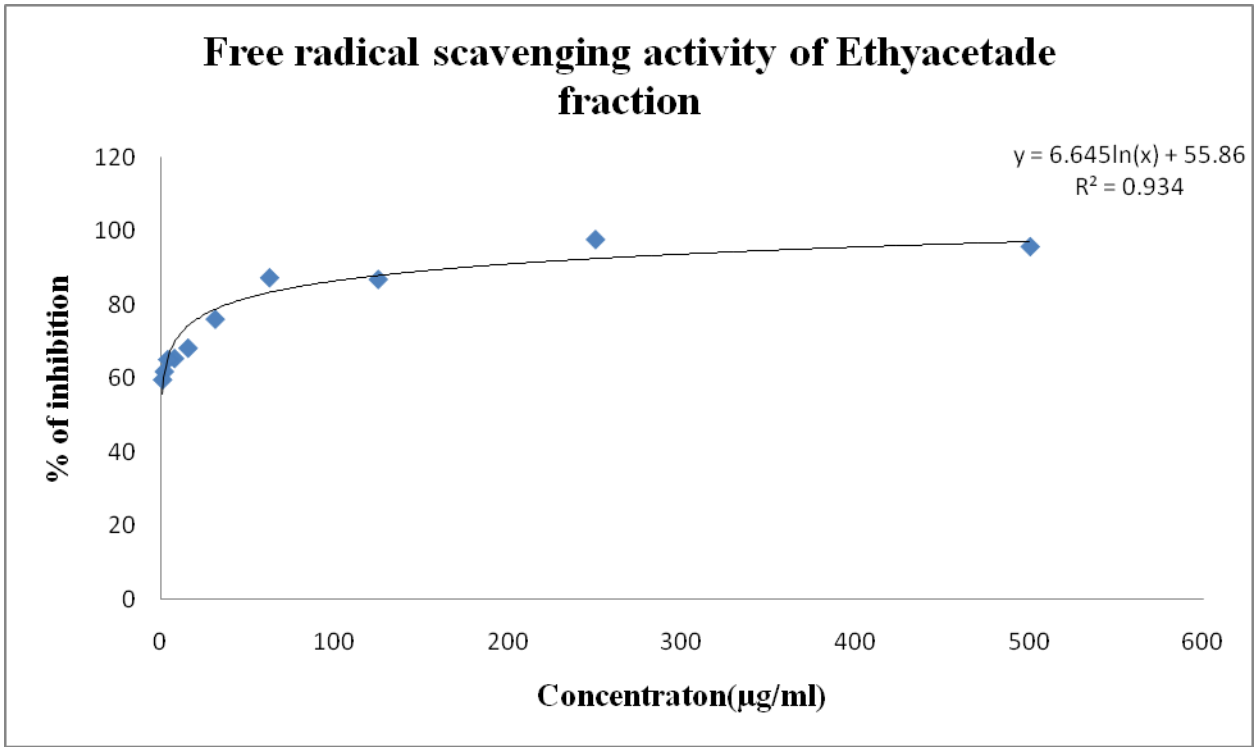


Fig 10.5: Free radical scavenging activity of Ethylacetade

Table 10.4: IC_{50} value of N-Hexane

Test tube No.	Absorbance of Blank	Concentration (µg/ml)	Absorbance of sample	% Inhibition	IC_{50} (µg/ml)
1	0.674	500	0.017	97.48	0.576
2		250	0.026	96.61	
3		125	0.051	92.43	
4		62.5	0.081	87.98	
5		31.25	0.113	83.23	
6		15.625	0.158	76.56	
7		7.813	0.212	68.55	
8		3.906	0.257	61.87	
9		1.953	0.292	56.68	
10		0.977	0.296	56.08	

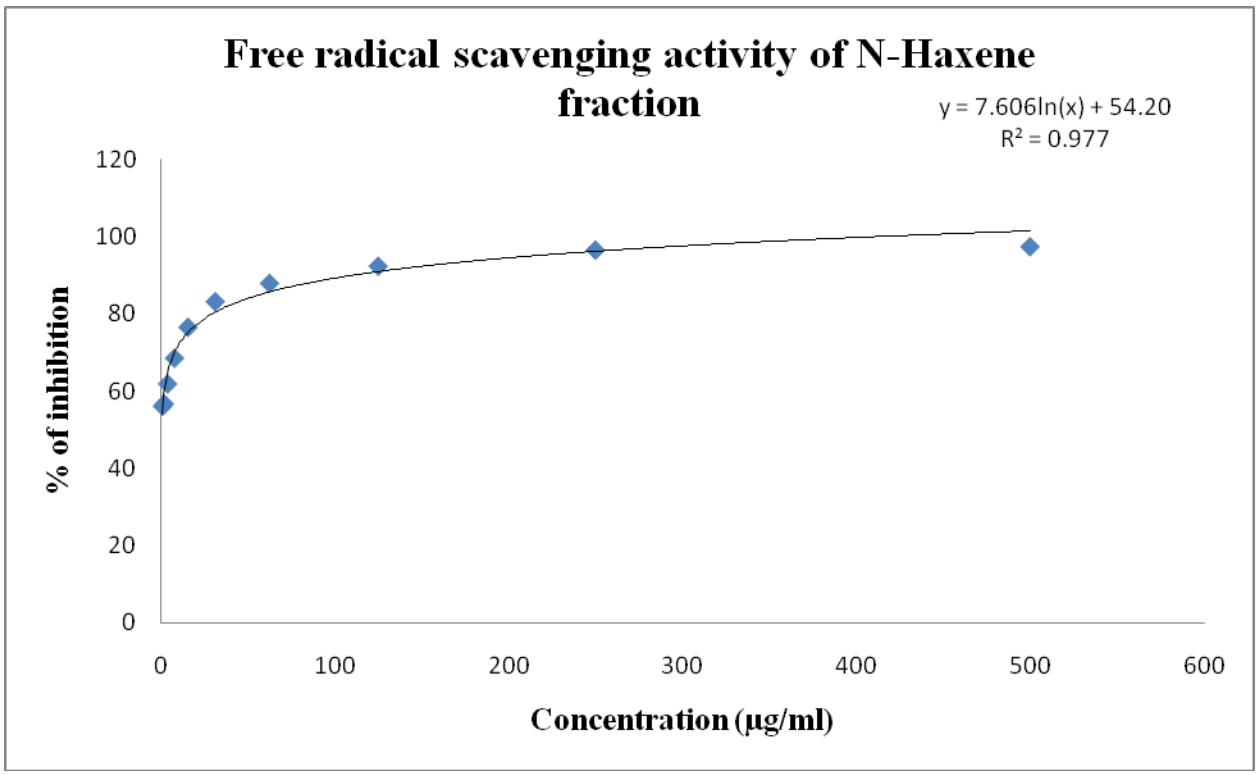


Fig 10.6: Free radical scavenging activity of N-Hexane

Table 10.5: IC_{50} value of Methanol

Test tube No.	Absorbance of Blank	Concentration (µg/ml)	Absorbance of sample	% Inhibition	IC_{50} (µg/ml)
1	0.674	500	0.027	95.99	0.696
2		250	0.036	94.67	
3		125	0.061	90.95	
4		62.5	0.091	86.50	
5		31.25	0.123	81.75	
6		15.625	0.168	75.07	
7		7.813	0.222	67.06	
8		3.906	0.267	60.39	
9		1.953	0.302	55.19	
10		0.977	0.306	54.60	

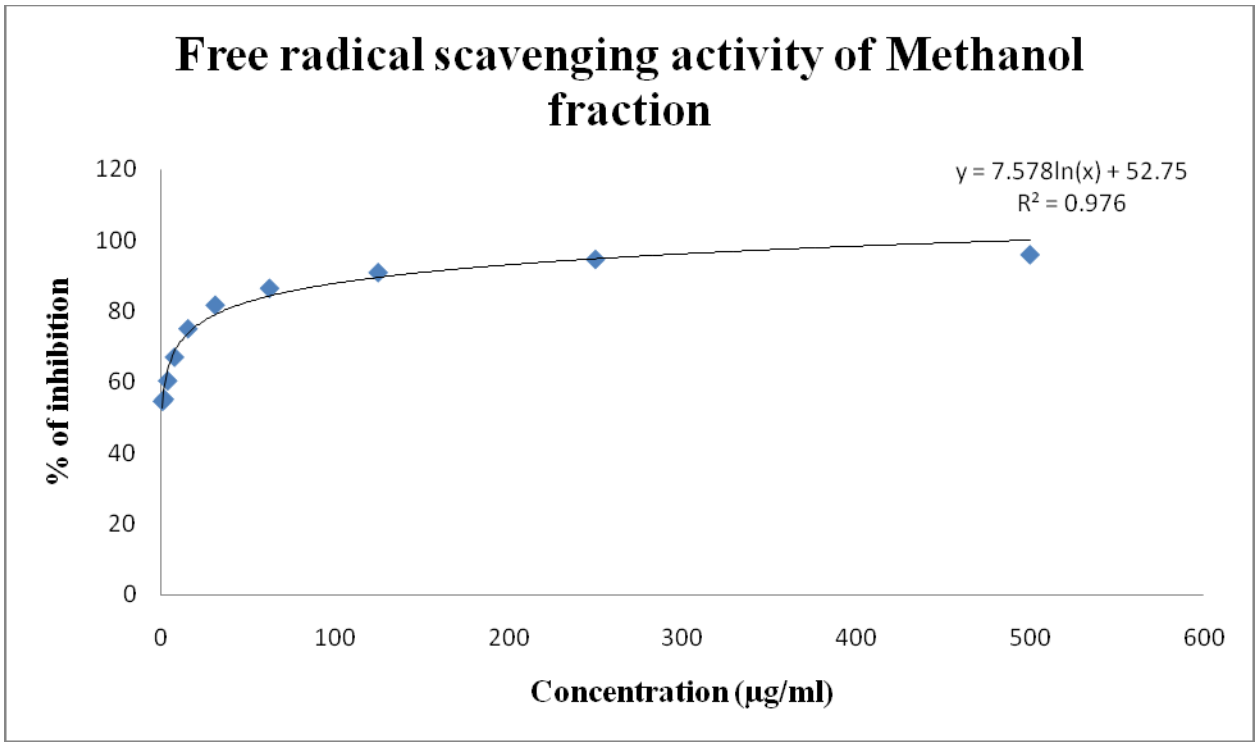


Fig 10.7: Free radical scavenging activity of Methanol

Table 10.6: IC_{50} value of *tert*-butyl-1-bydroxy toluene (BHT)

12	Absorbance of Blank	Concentration (µg/ml)	Absorbance of sample	% Inhibition	IC_{50} (µg/ml)
1	0.326	500	0.016	95.12	18.21
2		250	0.067	79.57	
3		125	0.088	73.01	
4		62.5	0.134	59.15	
5		31.25	0.160	51.22	
6		15.625	0.174	46.95	
7		7.813	0.207	36.89	
8		3.906	0.224	31.71	
9		1.953	0.239	27.13	
10		0.977	0.257	21.65	

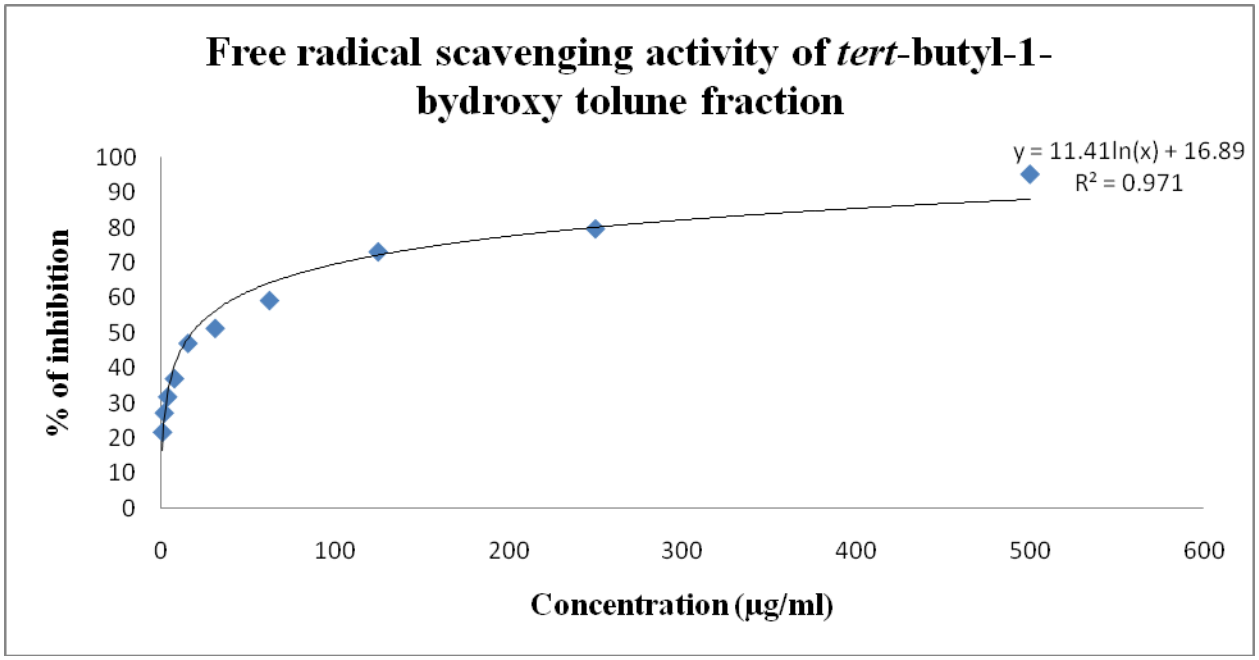


Fig 10.6: Free radical scavenging activity of tert-butyl-1-bydroxy toluene fraction.

Chapter-11
Result

Pharmacological Investigations of *Streblus asper* (Leaves)

11.1 Screening of in-vitro antimicrobial activity

The antimicrobial activities of different fractions of ***Streblus asper*** leaves 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)

Tab 11.1: 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	-	-	6.5	-	-
Staphylococcus aureus	-	-	-	8.5	34

Test microorganisms	Diameter of zone of inhibition (mm)				
	DCM	**EAE**	**NHE**	**MEL**	Kanamycin
Gram negative bacteria					
Escherichia coli	8.5	9.25	6.5	-	37
Shigella boydii	10	8.5	6.25	7.75	37
Vibrio mimicus	9	-	7.25	8.75	37

11.1.1 Result and Discussion of Antimicrobial Activity

All fractions are showing the low Antimicrobial activity. And the growth of Gram (+ve) bacteria *Bacillus sereus* & *Staphylococcus aureus* are not inhibited properly.

The Gram (-ve) bacteria *Escherichia coli*, *Shigella boydii* & *Staphylococcus aureus* are also not inhibited by the fractions.

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 leaves of ***Streblus asper*** 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:

Tab 11.2: Test samples of experimental plant (*Streblus asper*)

Plant Part	**Test samples**	**Measured amount (mg)**
Leaves	Dichloromethane fraction	0.008
	N-Hexane fraction	0.008
	Ethylacetade fraction	0.008
	Methanol fraction	0.008

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 ***Streblus asper*** 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, where as 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.

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

Tab 11.3: Results of the test samples of *Streblus asper*

Sample	**LC_{50} (µg/ml)**	**Regression equation**	**R^2**
Vincristine sulphate **(VIS)**	3.23	y = 23.96x + 34.82	0.760
Dichloromethane fraction of crud extraction **(DCM)**	3.67	y = 16.10x + 40.90	0.257
N-Hexane fraction of crud extraction **(NHE)**	0.29	y = 2.516x + 51.56	0.019
Ethylacetade fraction of crud extraction **(EAE)**	21.96	y = 21.34x + 21.37	0.772
Methanol fraction of crud extraction **(MEL)**	0.025	y = 10.77x + 67.06	0.569

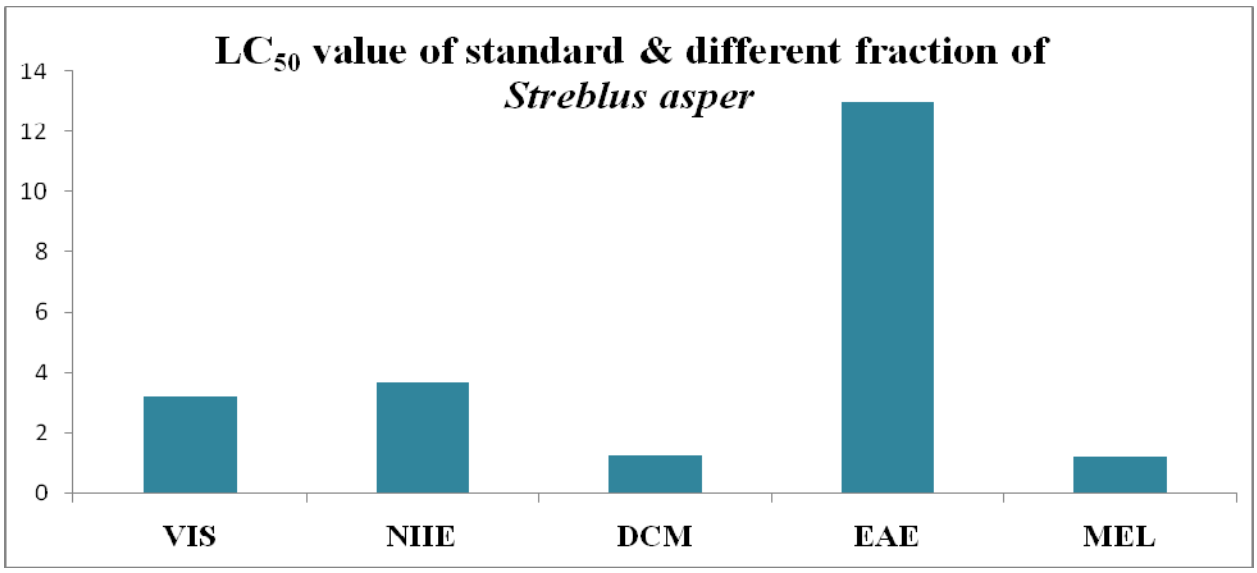


Fig 11.1: LC_{50} values of standard and different fraction of *Streblus asper* (leaves)

11.3 Free Radical Scavenging Activity

Different fraction of *Streblus asper* 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.

Tab 11.4: Results of free Radical Scavenging Activity he test samples of *Streblus asper*

Code	**Sample**	**IC_{50} (µg/ml)**
BTH	*tert*-butyl-1-hydroxytoluene	18.21
NHE	N-Hexane Crude Extract	0.576
DCM	Dichloromethane Crude Extract	0.427
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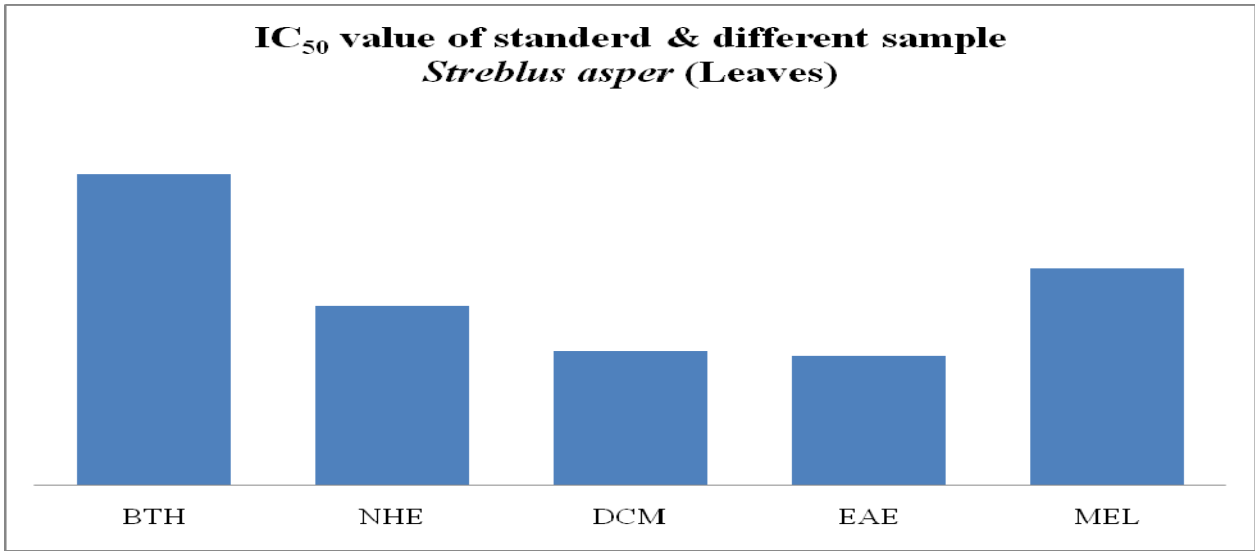

Fig 11.2: IC_{50} values of standard and different fractions of *Streblus asper* (leaves)

11.4 Total phenolic compound analysis

The amount of total phenolic content in *Streblus asper.*

Tab 11.5: **Standard curve preparation by using gallic acid**

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		

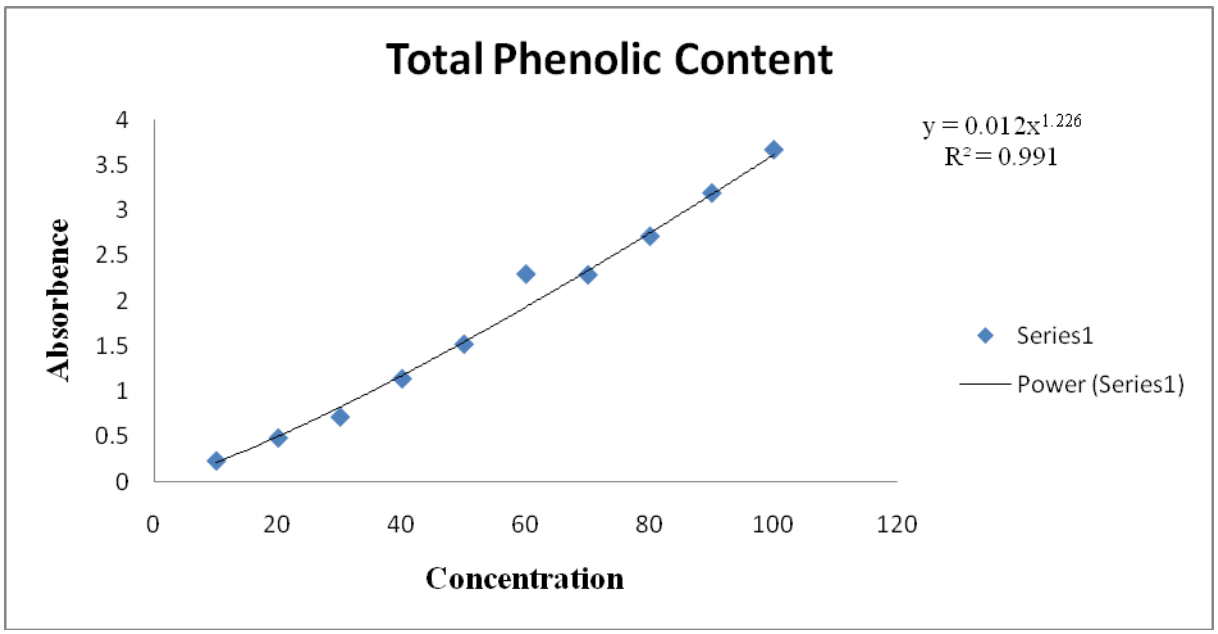

Fig 11.3: Graphical representation of assay of Phenolic compound in different extract of *Streblus asper*.

Tab 11.6: Total phenolic content of fraction of whole plant of *Streblus asper*

Name of fraction	Absorbance	mgGAE/g
NHE	0.194	96.79
DCM	0.259	122.50
EAE	0.385	154.81
MEL	0.283	131.70

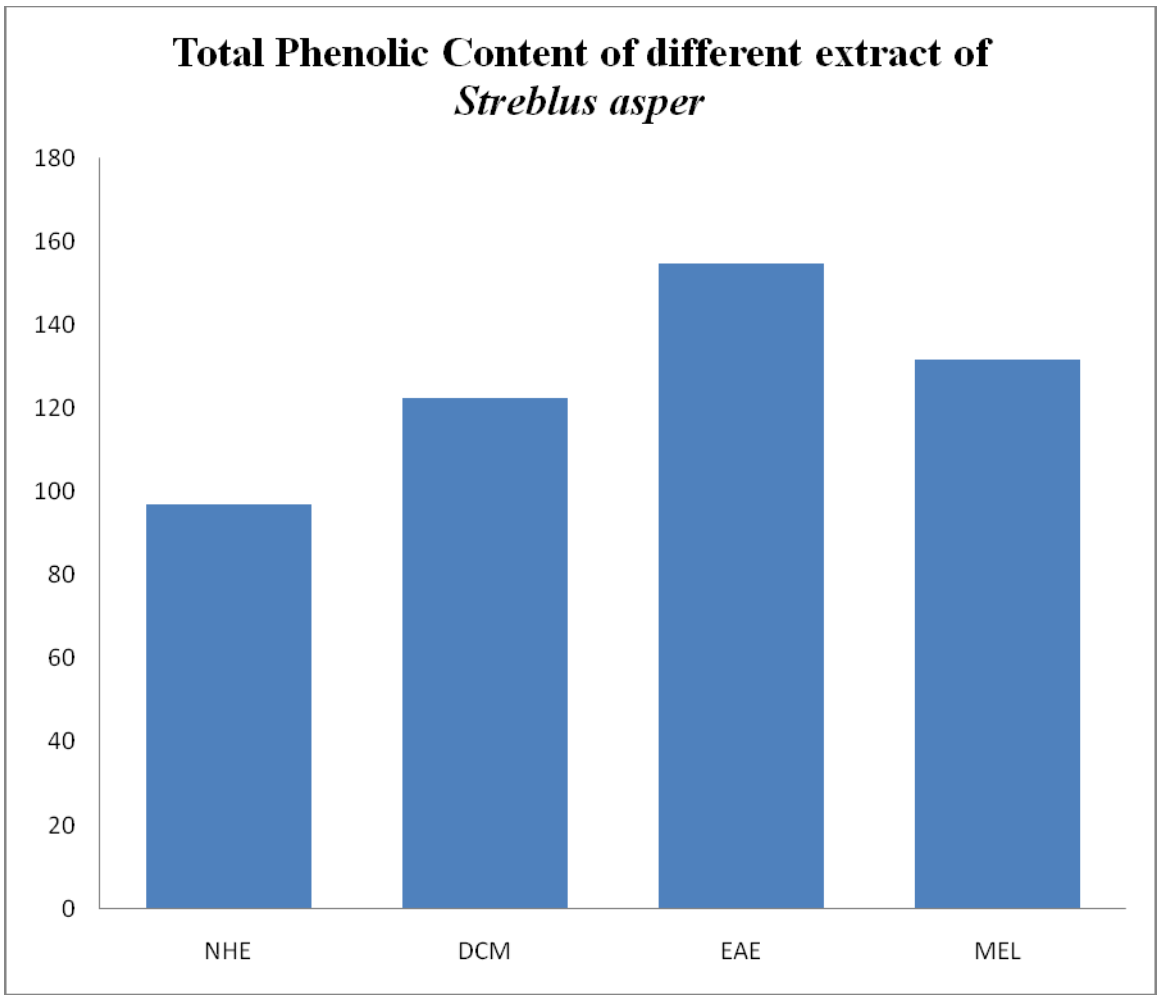

Fig 11.4: Total phenolic content of different fraction of *Streblus asper*

The Total Phenolic content of *Streblus asper* was found. The plant extract contain phenolic content. The total phenolic content of methanolic crude extract, dichloromethane fraction, ethylacetade fraction & n-hexane fraction of methanolic extract is respectively is 131.70mgGAE/g, 122.50mgGAE/g, 154.81mgGAE/g, 96.79mgGAE/g. Further research is needed on the determination of the correlation between the antioxidant capacity and the chemical components of the plant.

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