Biological Investigation of *Crataeva religiosa*

A project submitted

by

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Dedicated to my parents to whom I owe my achievements.

Certification statement

This is to certify that, this project titled 'Biological Investigation of *Crataeva religiosa*' submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Ridwan Islam, Senior Lecturer, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

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Countersigned by the Supervisor

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Acknowledgement

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Abstract

Current study strives to establish the scientific basis of the use of *Crataeva religiosa* as a healing and medical agent in traditional medicine for the first time. The leaves of the plant were extracted using methanol and different partitionates were obtained using different organic solvents, viz., petroleum ether, chloroform and ethyl acetate. All these fractions were subjected to in vitro biological screening. Crude methanolic extract showed maximum phenolic content in total phenolic content determination. On the other hand, Petroleum ether soluble fraction showed the highest activity in the DPPH free radical scavenging assay; corroborating the antioxidant and potential of the plant. Antimicrobial activity was estimated by agar disc diffusion method and was not found to be high. Methanolic fraction showed highest thrombolytic activity and membrane stabilizing effect, establishing the potential of using this plant as a possible source of discovering anti-coagulants and anti-inflammatory agents. Thus, this investigation might lead to the development of new drugs to treat a wide variety of diseases.

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Abbreviations

DPPH - 1, 1-diphenyl-2-picrylhydrazyl

ASA - Ascorbic Acid

DMSO – Dimethylsulphoxide

GAE - Gallic Acid Extract

VS – Vincristine Sulphate

TPC -Total Phenol Content

WHO – World Health Organization

ROS - Reactive Oxygen Species

ME – Methanolic extract

PESF – Petroleum ether soluble fraction

EASF – Ethyl acetate soluble fraction

CSF – Chloroform soluble fraction

AQSF – Aqueous soluble fraction

SK - Streptokinase

Chapter One

Introduction

Chapter One: Introduction

1.1 Introduction

Traditional medicines are being regarded as an alternative form of health care to treat human diseases by researchers and physicians for centuries. One of the most important reasons behind this is that medicinal plants contain a wide variety of chemical components of therapeutic value (Nostro et al, 2000). According to the World Health Organization (WHO) in 2008, more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (Pierangeli and Windell, 2009). Many of the plants could be used as stimulants, poisons, hallucinogens or as medicine because of the presence of unique or rich biological-active plant chemicals i.e. Chemical compounds that have a biological effect on another organism (Sharmin, 2004).

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

(i) Pharmaceutical industries

(ii) Traditional healthcare systems

(iii) Individual traditional health practitioners

(iv) Women in family health care" (Lambert et al., 1997).

The estimated amount of higher plants mainly angiosperms and gymnosperms on earth is 250,000 (Ayensu and De Filipps, 1978), with an upper level as high as high as 500,000 (Tippo et al., 1977 and Schultes, 1972). More than 6% of the medicinal plants have been screened for biological activity. Whereas, phytochemical investigations have been run on 15% plants. (Verpoorte, 2000). In reality, obtaining medicinal plants are much easier than pharmaceutical products by breeder (Latifou et al., 2011). Furthermore, utilization of indigenous systems of medicines based on the locally available medicinal plants and raw materials has always been emphasized by the World Health Organization. Because, such act can be exceptionally beneficial in lessening consumption of synthetic drugs and antibiotics. Moreover, the use of expensive intermediary chemicals will also fell down (Ali, 2010).

A variety of compounds are created by plants which are biologically active to both the plant itself, as well as, in other living organisms. A portion of these chemical compounds are used to prevent growth of competing plant (e.g. salicylic acid produced by willows), whereas, some are utilized for plants' biological activities. (United States forest service). Quite of a lot of compounds isolated from numerous plants have been reported to show multiple biological effects, like anti-inflammatory, anti-oxidant, free radical scavenging ability, thrombolytic, antimicrobial as well as, cytotoxic activities. Several active plant ingredients have been proved to exert medicinal uses for humans.

(i) Alkaloids

(ii) Bitters

(iii) Cardiac Glycosides

(iv) Cyanogenic Glycosides

(v) Flavonoids

(vi) Minerals

(vii) Phenols

(viii) Polysaccharides

(ix) Proanthocyanins

(x) Saponins

(xi) Tannins

(xii) Vitamins

(xiii) Volatile oils (United States forest service).

Nowadays the medicinal plants are not only being used by pharmaceutical industry but also by makers of cosmetics, detergents, dyes, insecticides, foods and paints etc. (Sharmin, 2004).

Treatment of diseases from medicinal plant dates back an extended period of time. Our ancestors were compelled to use medicinal plants to ease the sufferings from illnesses of chronic and acute origin, physical discomforts or injuries like wounds and in some cases terminal diseases. Still this tradition is kind of unchanged. In a variety of developing countries, ancient medicines are still used as a lifeline of health care. Within the developed countries, more and more individuals are taking seasoned (herbal) and unani remedies these days and at an equivalent time most of the factory-made essential medications are extracted from medicinal plants (Motaleb, 2011). WHO claims that approximately one-third of all drugs are plant based and if bacteria and fungi are also included, nearly 60% of pharmaceuticals are of plant origin (Ali, 2010).

It's a well-known fact that many plants especially plants which are being used by traditional healers, do produces pharmaceutically active compounds that poses antimicrobial, anti-helminthic, antifungal, antiviral, anti-inflammatory and antioxidant activity (Latifou et al., 2011). Bangladesh has enriched herself with a wide variety of plants. More than 5,700 angiosperm species, 1,700 species of pteridophytes and 3 species of gymnosperms prevails in Bangladesh, among which a total of 24 plants are in various degrees of threat of extinction. (Ali Reza, 2002). Dhaka, Rajshahi, Sylhet and Chittagong division contains a rich amount of medicinal plants (Ghani, 1998). So far, the number of medicinal plant enlisted as growing in Bangladesh is more than 500 of species (Motaleb, 2011). Costly imported drugs as well as inaccessibility to western health care facility

convey that the traditional mode of health care is the only affordable and available form of health care.

The work described in this dissertation is an attempt to investigate the biological activities of medicinal plat *Crataeva religiosa* which includes:

- Extraction of the dried powder of *Crataeva religiosa* with methanol by mesceration extraction method. And also fractionation of methanolic extract and separation of methanol, PET ether, chloroform, ethyl acetate and aqueous fraction.
- Determination of total phenolic content of all the five fractions by Folin-Ciocalteu reagent.
- *In vitro* cytotoxicity investigation using Brine shrimp with all the five fractions.
- Antioxidant activity investigation using DPPH radical scavanging assay method.
- Anti-microbial and thrombolytic activity investigation.
- Anti-inflammatory activity investigation using membrane stabilizing activity test method.

1.2 The Plant Family: Capparaceae (Caper Family)

Crataeva religiosa is a small spreading tree from the family Capparaceae and native to Asia-temperate - Eastern Asia: Japan, Asia-tropical - Indian subcontinent, Malaysia, Indonesia and Australia (Encyclopedia of Medicinal Plants, 2014 and U.S. National Plant Germplasm System).

The family Capparaceae is also known as the Caper family consisting of 210 plants and so far accepted genera of 49 (U.S. National Plant Germplasm System). Capparaceae family are mesophytes with a propensity to grow in semi-dry regions. This familyusually consists of herbs and shrubs, although there are some exceptions. For example, *Crataeva* genus plants are mostly trees, *Capparis aphylla* is a leafless shrub (xerophytic). Few species of *Capparis* and *Maerua*are climbing shrubs and they may be armed with curver stipular spines.Plants are annuals or prennials (Sambamurty, 2010).

Leaf is kind of alternate, simple, 3-9 foliolate leaves are seen. And also, incompletely developed and spiny stipules are observed. Three to four sepals and usually 4-6 petals are seen in flowers (actinomorphic or zygomorphic) under this family. In between sepals and the point of attachment to the stamens, a stalk is often observed. Different shapes exist in case of fruits. However, capsule and berry shape is common (Flora of Zimbabwe).

Pedicellate, bracteates, complete tetramerous, regular, bisexual and hypogynous types flowers exist in this family. The thalamus in *Capparis* and *Crataeva*is elongated into a gynophore above the stamens (Sambamurty, 2010).

1.3 The plant genus: Crataeva

The flowering genus *Crataeva* comes from the Capparacea family and also falls under the group Angiosperms (The Plant List, 2013). The genus name *Crataeva* emerges from Crataevus, name of a Greek botanist. Crataevus indicates growth near places of worship (Panda, 2002). *Crataeva* is a large genus group consisting of evergreen non-marine flowering plants (The Plant List, 2013) or medium-sized deciduous trees used in various medicinal systems (Kher et al, 2016). They are widely distributed to tropical countries, for example, Asia: Japan, India, Myanmar, Malaysia, Bangladesh, China and Sri Lanka; Africa and Madagascar; South America: Brazil, Colombia and Argentina; Mesoamerica: Honduras, Nicaragua; Australia. Though, so far, 14 scientific names of species have been identified under the genus, at least 53 scientific names have been included in the plant list. And also 12 infraspecific ranked plant names have been uploaded (The Plant List, 2013).

The enticing flowers of *Crataeva* genus plants build themselves as completely unique decorative trees for agriculture and landscaping. *In vitro* propagation methods are helpful for large-scale propagation and standardization of plant material. It can be hoped that once an efficient tissue culture system has been established, long preservation of necessary germplasm through cryopreservation will be done (Kher et al, 2016).

1.4 Distribution / Habitat

Native distribution of *Crataeva* has been documented in India, Bangladesh, Sri Lanka, Malaysia, Papua New Guinea; Philippines, Myanmar and Indonesia. Some species are also been found in Australia and South America.

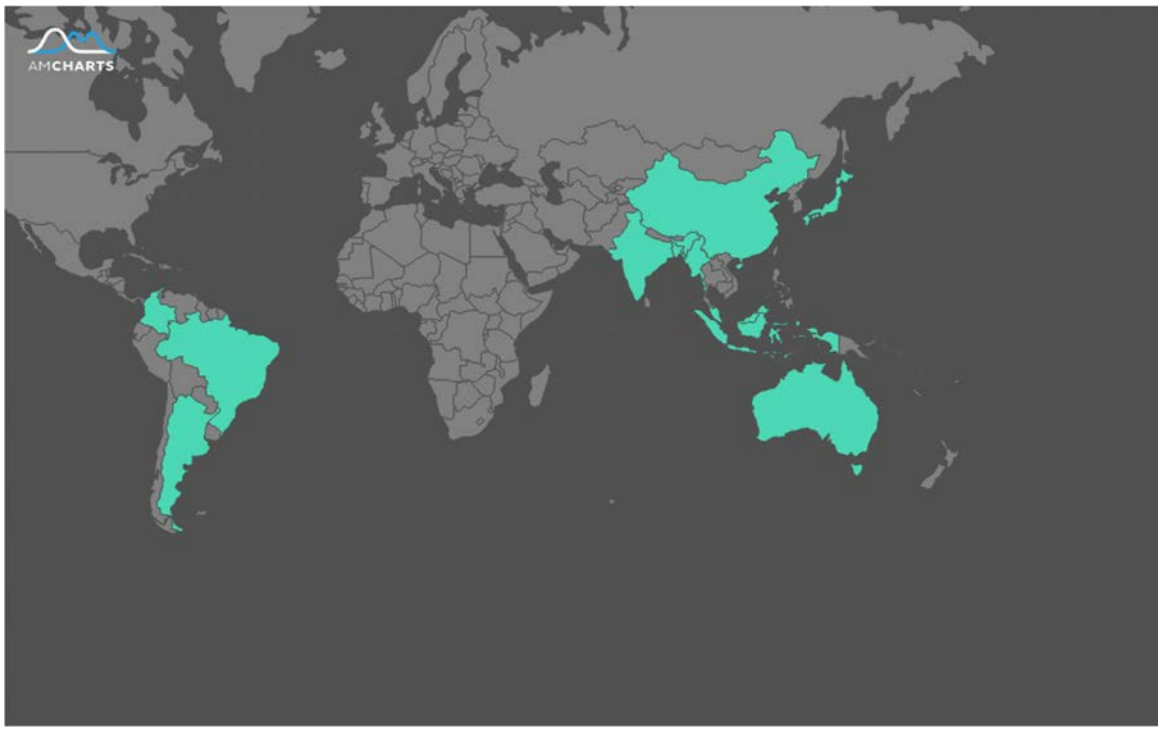


Figure 1.1: Countries which contains Crataeva species (drawn by AMCHARTS)

The map shows countries wherever the species has been planted. It neither recommends that the species can be planted in every ecological zone within that country, nor that the species cannot be planted in other countries than that represented.

1.4.1 Ecology

Crataeva religiosa is a habitat of island and lowland forest, usually grow up in places surrounded with fresh water. This plant is usually found near to temple side and along the bank of the river (Patil and Gaiwad, 2011). This non-marine plant is evergreen or semi evergreen in nature, having fleshy types fruit and 510 mm^3 seed volume (Encyclopedia of Life, 2016).

1.5 Some reported species

- *Crateva adansonii* DC.
- *Crateva excelsa* Bojer
- *Crateva greveana* Baill.
- *Crateva humblotii* (Baill.) Hadj-Moust.
- *Crateva hygrophila* Kurz
- *Crateva nurvala* Buch.–Ham
- *Crateva obovata* Vahl
- *Crateva palmeri* Rose
- *Crateva religiosa* G.Forst
- *Crateva simplicifolia* J.S.Mill.
- *Crateva suaresensis* Baill
- *Crateva tapia* L.
- *Crateva unilocularis* Buch.–Ham.
- *Crateva urbaniana* R. Rankin

1.6 Description of *Crataeva religiosa*

Crataeva religiosa is a species in the family Capparaceae and also known as sacred garlic pear (Kanakam et al., 2016). The word *Crataeva* comes as a form of honor to the Greek botanist Crataevas, who lived the era of Hippocrates. *Religiosa* in Latin means pertaining to religion (Parker, 1999). Three leaved capper is the trade name given to this plant (Kanakam et al., 2016). This plant is widely distributed in Australia, China, Indonesia, Myanmar, Malaysia, Sri Lanka, India and Bangladesh. Also, Sikkim, Andaman and Nicobar islands are enriched with *Crataeva religiosa* (Patil and Gaiwad, 2011). It is found in many parts of Africa, especially in the forest galleries of African Sudanese area

(Latifou et al., 2011). In India, it is found in the Peninsular India, Western and Eastern India, Gangetic plains, up to Tripura and Monipur (Patil and Gaiwad, 2011). In Bangladesh, *Crataeva religiosa* is found in areas like Munshigonj, Manikgonj, Mymensing and Dhaka.

1.6.1 Taxonomic hierarchy

According to the Integrated Taxonomic Information System (ITIS) Data Portal Classification:

Kingdom: Plantae

Subkingdom: Virdiplantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Brassicales

Family: Capparaceae

Genus: *Crataeva*

Species: *Crataeva religiosa*

Scientific name: *Crataeva religiosa* (G.Forst)

Local names: Bangali (Borun, Barna, Boinna)

English (Temple plant, Sacred garlic pear, Spider tree, Three-leaved caper)

Chinese (Yin tu chih, Yu mu)

Cambodia (Tonliem)

Indonesia (Jaranan, Barunday, Sibaluak)

Laos (Kumz)

Tamil (Navala, Mavilankai)

Thailand (Kum-bok, Kum nam)

Sanskrit (Varuna)

Hindi (Barna, Barni)

Manipuri (Loiyumba lei)

Kannada (Nirvala)

Telegu (Voolemara)

Malayalam (Nir mathalam)

Benin (Goriguiberou, Wontonzonzwen, Tanilabia, Tcharouwenwe)

Bombay state (Vayarana)

German (Abiyuch)

1.6.2 General Botanical Data

Crataeva religiosa is a small or medium sized deciduous tree up to 6-15 meter tall, glabrous in nature and trunk diameter of 40 cm (Encyclopedia of Life, 2016 and Parker, 1999).The plant is self-incompatible and obligate out-crosser (Sharma, 2006). According to World Heritage Encyclopedia, the pierid butterfly (*Hebomoia glaucippe*) is a frequent visitor to this plant (World eBook Library, 2016)

Table 1.1: Description of different plant parts of *Crataeva religiosa*

Part of plant	Description
Bark	• Outer surface of the bark is wrinkled • Pale grey or grey-white in color • Bark is covered with a large number of lenticels
Leaves	• Leaves contains 3 leaflets which is 2 to 6 inches long on a common stalk 1.5 to 4 inches long

	• Leaves are trifoliate, glabrous and ovate
Flowers	• Normally 2 to 3 inches long • White, pale yellow and reddish yellow colored • In loose clusters each on a stalk 1 to 2 inches long • 4 deciduous sepals • Normally 4 petals are seen and each petal is 1 to 1.5 inches long • Terminal dense corymbs • Stamens purple, indefinite, adnate to base of gynophore • Ovary stays on a slender stalk
Fruit	• Berry, globose or sometimes oblong with woody rind • Embed seeds in the yellow pulp • Tree flowers and fruits in the month of December to May
Seeds	• Smooth or crested with hard points • Many seeded fruits and the seeds are found in pulp inside fruit • Seeds ripen by July
Root	• Root-suckers are widely produced by *Crataeva religiosa* plant • These can be dug up and used for planting as a form of propagation (Parker, 1999).

1.7 Chemistry of *Crataeva religiosa*

1.7.1 Chemical constituents

1. Fruits

- Dodecanoic anhydride
- Methyl pentacosanoate
- Kaemferol-3-O-α-D glucoside
- Querscitin 3-O- α-D glucoside
- Glucocaparin
- Pentadecane
- Octanamide
- 12-tricosanonoe
- Friedelin (Kanakam Vijayabhaskar and Naresh, 2016)

2. Leaves

- Isovitexin
- Proanthocyanidins
- Myricetin
- Phenolic acid
- p-hydroxyl benzoic acid
- Vanilic acid
- Ferulic acid
- Sinapic acid (Kanakam Vijayabhaskar and Naresh, 2016)

3. Root bark

- Lupeol
- Lupeol acetate
- Varunaol
- Spinasterol acetate
- Taraxasterol

- 3-epilupeol (Udaysing Hari Patil, 2011)

4. Root

- A triterpene
- Two alkaloids – Cadabicine and Cadabcine deacetate
- Diosgenin (Udaysing Hari Patil, 2011)

5. Stem bark

- Two triterpenoids (Phragmalin triacetate and Lupeol)
- Epiafzemechin
- 5-glucoside (Udaysing Hari Patil, 2011)

1.8 Uses of *Crataeva religiosa*

1.8.1 Medicinal Uses

Various plant parts as well as, whole plant of *Crataeva religiosa* are used as traditional medicines.

Whole plant

- Ethnopharmacological uses include diuretic, laxative, lithonotriptic, antirehumatic, antiperiodic, bitter tonic, rubifacient and counterirritant. Other applications include antipyretic, antilithitic, antihelminthic, demulcent, in blood and chest diseases treatment.
- In Ayurvadic medication, Crataeva religiosa is utilized in the treatment of
  1. Vata (blood flow, waste elimination, breathing)
  2. Pitta (fever and metabolic disorder)
  3. Kapha (joint lubrication, skin moisture, wound healing, strength and vigour, memory loss, heart and lung weakness, and weak immune system)
- An ayurvedic preparation 'Varunal' is a combination product of Crataeva, *Eclipts, Picrorrhiza, Achillea, Cichotium, Solanum, Arjuna* and *Cassia* seeds. This

preparation shows activity against hepatitis, edema, ascites, urinary stones and arthritis.

- Traditionally *Crataeva religiosa* is used as an oxitosic and tonic. Also, this plant is used to treat kidney stones in rheumatic fever and also to treat bladder stone.
- Two polyherbal formulations called NR-AG-I (containing *Crataeva religiosa, Dollichosbiflorus, Tribulus terrestris* and *Shilajit*) and NRAG-II (combination of *Crataeva religiosa, Boerrhavia diffusa, Sacchaarum officinarum.* and *Butea frondosa*) are used as diuretic.
- The juice of fruit, leaves and bark is applied internally and tropically to cure snakebite, infected wounds and cuts. It increases appetite and controls other skin diseases.

Table 1.2: Uses of different plant parts of *Crataeva religiosa*

Plant Part	Uses
Leaf	• External use as rubifacient, vasicant and used inrheumatism • Internal use as febrifuge and tonic • Philippine communities use the leaves in treating stomachic and irregular menstruation • Crushed leaves are applied in the form of paste for swelling of feet and also for a burning -sensation in the soles of feet • Dwellers of Kango and Yurubas use leaf paste in water as counter irritant • Dried leaves are smoked in caries of nasal bones, the smoke being exhaled through the nose in neurologic pains.
Stem bark	• Decease the secretion of the bile in unani medicines and promote the appetite
Bark	• Used in treating urinary disorders including kidney and bladder stones, antiemetic, and calculous affections and also used as an antidote in snakebite

	• Contraceptive, diuretic and cytotoxic and useful in kidney bladder stones, fever vomiting and gastric irritation • *Tephrosia purpurea* leaves (10%) and *Crataeva religiosa* bark (25%) was prepared and 4gm of mixture given to patient twice daily with water in urinary disorder • In Philippines, bark is used to cure convulsions and tympanites
Fruit	• Astringent • Rind of the fruit is used as mordant in dying • Fruits are edible and used as spice because of its garlictaste
Roots and bark combination	• Laxative rubifacient and vesicant • Lithontipic and increase biliary secretion and appetite
Bark and leaves combination	• Bark and the leaves are pounded and applied in the form of a poultice in rheumatism.
Bark and leaves combination	• In Pallaypatty village of Tamil Nadu, people use the leaves and bark of this tree to cure jaundice, eczema, rabies.

1.8.2 Commercial Uses

- Rind of *Crataeva religiosa* plant's fruit is used as a mordant in dyeing.
- Wood is not durable although is used for making drums, combs and turnery articles.
- Since *Crataeva religiosa* looks very beautiful in flowering time (April and May), it is very often planted as an ornament.

1.8.3 Rationale of the study

According to world health organization (WHO) a huge proportion of world's population depend on medicinal plants to satisfy their needs of primary health care. Apart from that, only six percent of the medicinal plants have been screened so far for finding out the biological activities. No biological screening has been done on *Crataeva religiosa* so far. That's the reason which makes it a unique plant to run on biological screening for the first time.

1.8.4 Aim and objectives of the study

- Aim of this study is establishing the biological properties of *Crataeva religiosa* medicinal plant.
- Objectives of this is to find out the followings
    1. Cytotoxicity activity using brine shrimp method.
    2. Thrombolytic activity.
    3. Antioxidant property using DPPH radical scavenging method.
    4. Antimicrobial activities.
    5. Membrane stabilizing property.

Chapter Two

Methodology

Chapter Two: Methodology

2.1 Chemical investigation of the experimental plants

Crataeva religiosa, belonging to the family Capparaceae, was investigated for its chemical constituents.

2.1.1. Collection and preparation of the plant material

The leaves of *Crataeva religiosa* were collected from Dhaka in March, 2016.After proper washing the leaves were sun dried for several days. The dried leaves were then ground to a coarse powder using high capacity grinding machine. The coarse powder was then stored in an air tight container and kept in cool and dry place.

2.1.2 Extraction of the plant material

Seven hundred grams of the powdered material was taken in two clean, round bottomed flasks (5 L) and soaked in 3.0 liter of methanol. The container with its content was sealed and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through a fresh cotton plug and finally with a Whatman No.1 filter paper. The volume of the filtrate was then reduced using a Rotary evaporator at low temperature and pressure. The weight of the crude extract was 40gm.

2.1.3 Solvent-Solvent partition of crude extract

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wagenen et al. (1993). The crude extract (5gm) was dissolved in 10% aqueous methanol. It was extracted with Petroleum ether, then with Chloroform and finally with Ethyl acetate. The whole partitioning process is schematically shown in Figure 2.1.

All the four fractions were evaporated to dryness Table 2.1 and were used for further analysis.

2.1.3.1 Partitioning with petroleum ether

The mother solution was taken in a separating funnel. 100 mL of the petroleum ether was added to it and the funnel was shaken and then kept undisturbed. The organic portion was collected. The process was repeated thrice and the fractions collected were evaporated together in Rotary evaporator.

2.1.3.2 Partitioning with chloroform

To the mother solution that left after washing with petroleum ether, 16 mL of distilled water was added and mixed uniformly. The mother solution was then taken in a separating funnel and extracted with trichloromethane ($CHCl_3$) (100 mL × 3). The $CHCl_3$ soluble fractions were collected together and evaporated. The aqueous methanolic fraction was preserved as aqueous fraction.

2.1.3.3 Partitioning with ethyl acetate

To the mother solution that left after washing with petroleum ether and trichloromethane, 20mL of distilled water was added and mixed uniformly. The mother solution was then taken in a separating funnel and extracted with ethyl acetate ($CH_3COOC_2H_5$) (100 mL × 3). The $CH_3COOC_2H_5$ soluble fractions were collected together and evaporated. The aqueous methanolic fraction was preserved as aqueous fraction.

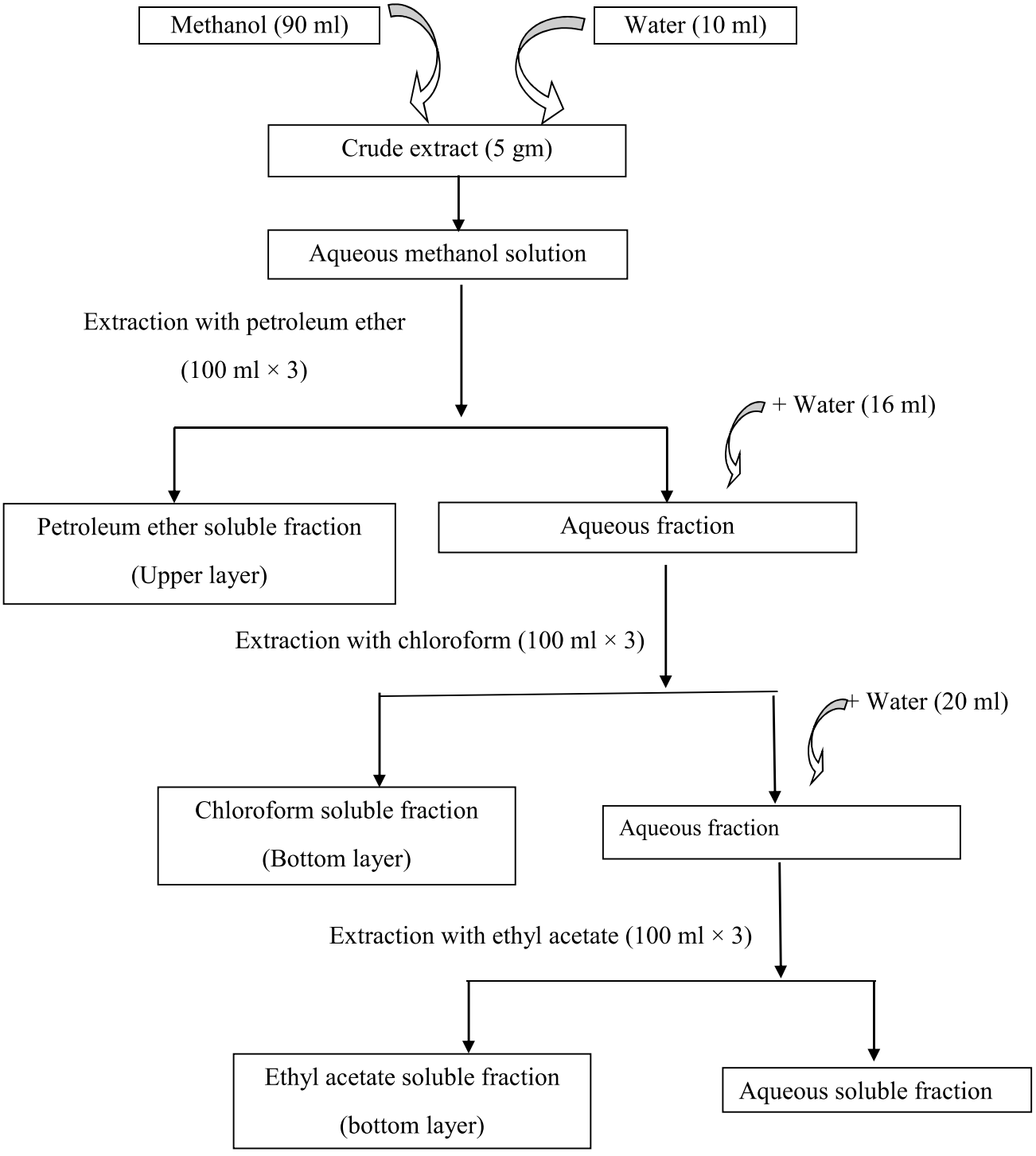


Figure 2.1: Schematic representation of the Kupchan partitioning of methanolic crude extract of leaves of *Crataeva religiosa*

This process was repeated for several times and after evaporation the weight of the different fractions obtained are mentioned in Table 2.1

Table 2.1: Amount of partitionates obtained from (60 gm) methanolic extract

Plant part	Sample code	Fraction	Weight (gm)
Leaves	PESF	Petroleum ether soluble fraction	14
	CSF	Chloroform soluble fraction	12
	EASF	Ethyl acetate soluble fraction	12
	AQSF	Aqueous soluble fraction	10

2.2 Evaluation of Antioxidant activity

The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability.

The antioxidant activity assayed in terms of

- Determination of total phenolic content.
- Determination of antioxidant properties: DPPH assay

2.2.1 Determination of total phenolic content

Neutralization and absorption of free radicals, reduction of triplet as well singlet compounds and lastly, decomposition of peroxides of human body are vital roles played by antioxidants (Osawa, 1994). Generally the antioxidative action are shown by phenolics, phenolic acid, phenolic diterpenes and flavonoids. Chemical properties of the phenolic compounds show that they exert their antioxidative properties by redox reaction (Pietta, 1998; Shahidi, Janitha and Wanasundara, 1992). Researches show that many of the phytochemicals possess antioxidant capacities which might be linked with lower incidence and lower mortality rates in human cancer (Velioglu et al, 1998).

2.2.2 Principle

Phenols get ionized in alkaline condition which is why the Folin-Ciocalteu reagent is used which readily gets ionized in phenolic solution. Oxidized reagent turns blue from yellow. Color change intensity is measured as absorbance at 760 nm by UV spectrophotometer. Absorbance indicates the TPC (Total Phenolic Content) of particular test compound (Harbertson and Spayed, 2006).

$$\text{Phenols} + Na_2CO_3 \xrightarrow{\text{Complete ionization}} \text{Ionized phenols}$$

$$\text{Ionized phenols} + \underset{\text{(Yellow)}}{\text{Folin-Ciocalteu}} \xrightarrow{\text{Oxidation}} \underset{\text{complex (Blue)}}{\text{Folin-Ciocalteu reagent}}$$

2.2.3 Materials and methods

Total phenolic content of leaves of *Crataeva religiosa* extractives 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).

2.2.4 Materials

- Folin-Ciocalteu reagent (10 fold diluted)
- Na_2CO_3 solution (7.5%)
- Distilled water
- Ascorbic acid
- Methanol
- Chloroform
- Ethyl acetate
- Pet ether
- UV-spectrophotometer
- Vial
- Beaker (100 and 200 mL)
- Test tube
- Pipette (1 mL)
- Pipette (5 mL)
- Micropipette (50-200 µL)

2.2.5 Composition of Folin-Ciocalteu reagent

Water	57.5%
Lithium sulfate	15.0%
Sodium tungstate dihydrate	10.0%
Hydrochloric acid ≥ 25%	10.0%
Phosphoric acid 85% solution in water	5.0%
Molybdic acid sodium dihydrate	2.5%

2.2.6 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 0 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 is used as a standard curve for the determination of the total phenolic content of the test samples.

2.2.7 Sample preparation

2 mg of the extractives was taken and dissolved in the distilled water to get a sample concentration of 2 mg/mL in every case. The samples along with their concentration for the total phenolic content measurement are given in the Table 2.2.

Table 2.2: Test samples for total phenolic content determination

Plant part	Sample code	Test Sample	Conc. (mg/mL)
Leaves of *Crataeva religiosa*	ME	Methanolic crude extract	2.0
	PESF	Petroleum ether soluble fraction	2.0
	EASF	Ethyl acetate soluble fraction	2.0
	CSF	Chloroform soluble fraction	2.0
	AQSF	Aqueous soluble fraction	2.0

2.2.8 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.

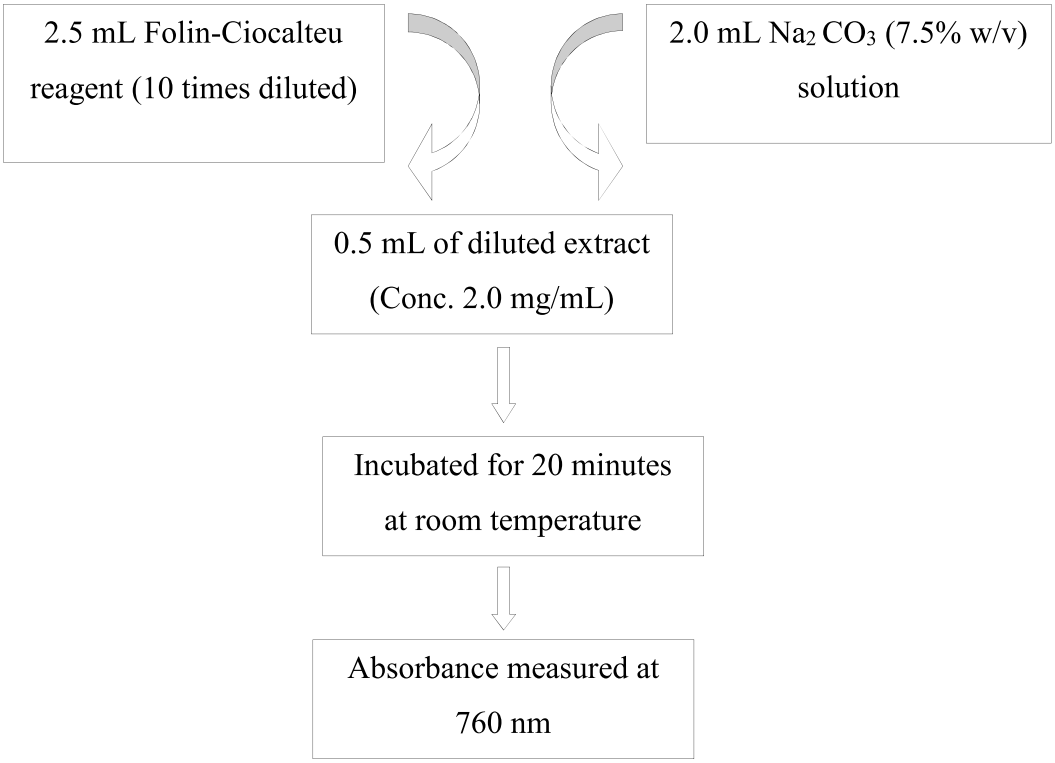
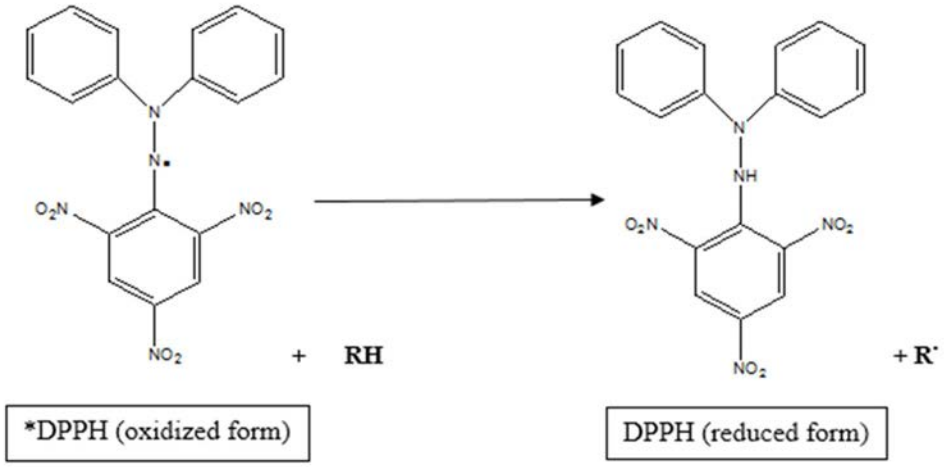


Figure 2.2: Schematic representation of the total phenolic content determination

2.2.9 DPPH assay: Principle

According to the method described by (Brand-Williams et al., 1995) the free radical scavenging activities or antioxidant property of plant extracts are measured using DPPH (1, 1-diphenyl-2-picrylhydrazyl) reagent. Above mentioned method follows addition of extract's methanol solution (2 mL) with DPPH methanol solution (3 mL, conc. 20µg/mL).

Decoloration of purple colored DPPH methanol solution by the test plant extract is compared with a standard ascorbic acid.



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

2.2.10 Materials and Methods

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

1,1-diphenyl-2-picrylhydrazyl	UV-spectrophotometer
Micropipette (50-200 µl)	Beaker (100 and 200mL)
Ascorbic acid	Amber reagent bottle
Distilled water	Test tube
Methanol	Light-proof box
Chloroform	Pipette (5mL)
Ethyl acetate	

2.2.11 Control preparation for antioxidant activity measurement

Ascorbic acid was used as positive control. Calculated amount of ASA were dissolved in methanol to get a mother solution having a concentration 1000 µg/mL. Serial dilution was made using the mother solution to get different concentration ranging from 500.0 to 0.977 µg/mL.

2.2.12 Test sample preparation

Calculated amount of different extractives were measured and dissolved in methanol to get the mother solution (Conc. 1000 μg/mL). Serial dilution of the mother solution gave different concentration ranging from 500.0 to 0.977 μg/mL which were kept in the marked flasks.

2.2.13 DPPH solution preparation

Twenty milligram of DPPH powder was weighed and dissolved in methanol to get a DPPH solution having a concentration 20 μg/mL. The solution was prepared in the amber reagent bottle and kept in the light proof box.

2.2.14 Assay of free radical scavenging activity

Two milligram of a methanol solution of the sample (extractives/control) at different concentration (500 μg/mL to 0.977 μg/mL) were mixed with 3.0 mL of a DPPH methanol solution (20 μg/mL). After 30 min reaction period at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by UV spetrophotometer.

Inhibition of free radical DPPH in percent (I%) was calculated as follows:

$$(I\%) = (1 - A_{sample}/A_{blank}) \times 100$$

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.

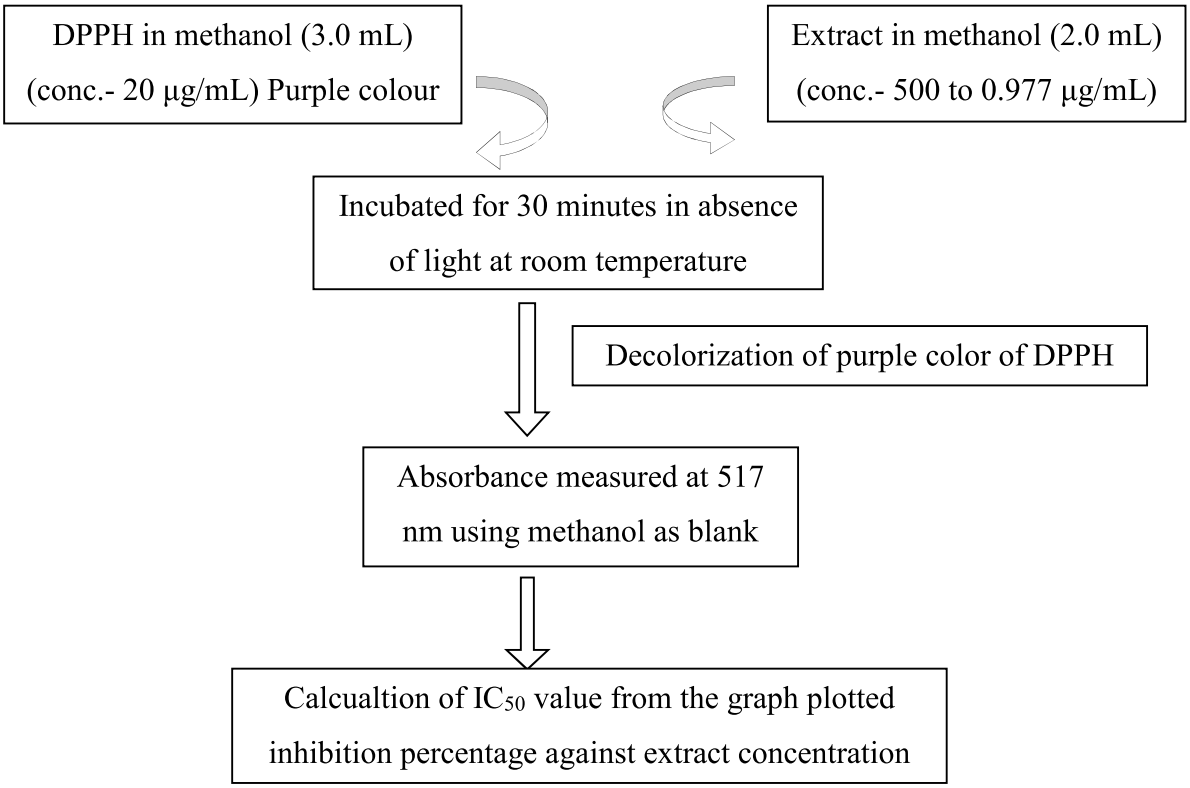


Figure 2.3: Schematic representation of the method of assaying free radical scavenging activity

2.3 Thrombolytic Activity Investigation

Human blood circulation gets hindered by thrombus or embolus formation as these kind of development can easily block the blood vessels. Blood vessel blocking can result in deprivation of blood flow and low oxygen supply to tissue leading to cell necrosis (Ramjan A, 2014). A common thrombolytic disorder named CVST (cerebral venous sinus thrombosis) shows notable morbidity and mortality (Watson et al., 2002) and treated with heparin, an anticoagulant agent regarded as first line of treatment because of its efficacy, safety and feasibility (Biousse and Newman, 2004). In management of patients with CVST, tissue plasminogen activator (t-PA), urokinase, streptokinase perform a very vital role. That's why this study will study the thrombolytic activity of the crude methanolic extract as well as other fractions of *Crataeva religiosa.*

2.3.1 Materials and methods

2.3.1.1 Preparation of sample

The thrombolytic activity of all extractives was evaluated by a method using streptokinase (SK) as standard substance. The dry crude extract (100 mg) was suspended in 10 mL of distilled water and it was kept overnight. Then the soluble supernatant was decanted and filtered.

2.3.1.2 Streptokinase

Commercially available lyophilized Altepase (Streptokinase) vial (Beacon pharmaceutical Ltd) of 15,00,000 I.U., was collected and 5 mL sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30,000 I.U) was used for *in vitro* thrombolysis.

2.3.1.3 Blood sample

Whole blood (n=10) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1mL of blood was transferred to the previously weighed micro centrifuge tubes and was allowed to form clots.

2.3.1.4 Thrombolytic activity

Aliquots (5 mL) of venous blood were drawn from healthy volunteers which were distributed in five different pre weighed sterile micro centrifuge tube (1 mL/tube) and incubated at 37°C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone).

To each micro centrifuge tube containing pre-weighed clot, 100µL aqueous solutions of different partitionates along with the crude extract was added separately. As a positive control, 100µL of streptokinase (SK) and as a negative non thrombolytic control, 100µL of distilled water were separately added to the control tubes. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, the

released of fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

$$\% \text{ of clot lysis} = (\text{wt of released clot} / \text{clot wt}) \times 100$$

2.4 Antimicrobial screening

Infectious diseases play a vital role in most of the deaths (approximately on-half of all deaths) in tropical countries, which is not a surprising scenario in case of lower developed and developing countries. Whereas, in developed countries the mortality rate due to infectious diseases are rising alarmingly. Death from infectious disease, ranked 5th in 1981, has become the 3rd leading cause of death in 1992, an increase of 58% .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 millenium. 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 (Ayafor, 1972).

- Disc diffusion method
- Serial dilution method
- Bioautographic method

Among the above mentioned techniques the disc diffusion (Bayer et al., 1966) is a widely accepted *in vitro* investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method (Roland R, 1982).

2.4.1 Principle of disc diffusion method

In this classical method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Dried and sterilized filter paper discs (6 mm diameter) containing the test samples of known amounts are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs and blank discs are used as positive and negative control. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion of the test materials to the surrounding media (Barry, 1976). The plates are then inverted and incubated at 37°C for 24 hours for optimum growth of the organisms. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimeter (Barry, 1976; Bayer et al., 1966.)

In the present study the crude extracts as well as fractions were tested for antimicrobial activity by disc diffusion method. The experiment is carried out more than once and the mean of the readings is required (Bayer et al., 1966).

2.4.2 Experimental

2.4.2.1 Apparatus and reagents

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

2.4.2.2 Test organisms

The bacterial and fungal strains used for the experiment were collected as pure cultures from department of Pharmacy, State University of Bangladesh (SUB). Both gram positive and gram-negative organisms were taken for the test and they are listed in the Table 2.5

Table 2.3 Different strains used in antimicrobial screening

Gram positive Bacteria	Gram negative Bacteria	Fungi
Bacillus cereus	*Escherichia coli*	*Aspergillus niger*
Bacillus megaterium	*Salmonella paratyphi*	*Candida albicans*
Bacillus subtilis	*Salmonella typhi*	*Sacharomyces*
Staphylococcus aureus	*Vibrio parahemolyticus*	*cerevacae*
Sarcina lutea	*Shigella boydii*	
	Shigella dysenteriae	
	Pseudomonas aeruginosa	
	Vibrio mimicus	

Table 2.4: List of test materials

Plant part	Sample code	Test Sample
Leaves of *Crataeva religiosa*	ME	Methanolic crude extract of *Crataeva religiosa*
	PESF	Petroleum ether soluble fraction
	EASF	Ethyl acetate soluble partitionate
	CSF	Chloroform soluble partitionate
	AQSF	Aqueous soluble partitionate

2.4.3 Composition of culture medium

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

a) Nutrient agar medium

Ingredients	Amount
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. to 100 mL
pH	7.2 + 0.1 at 25^0C

b) Nutrient broth medium

Ingredients	Amount
Bacto beef extract	0.3 gm
Bacto peptone	0.5 gm
Distilled water	q.s. to 100 mL
pH	7.2 + 0.1 at 25^0C

c) Muller – Hunton medium

Ingredients	Amount
Beef infusion	30 gm
Casamino acid	1.75 gm
Starch	0.15 gm
Bacto agar	1.70 gm
Distilled water	q.s. to 100 mL
pH	7.3 + 0.2 at 25^0C

d) Tryptic soya broth medium (TSB)

Ingredients	Amount
Bacto tryptone	1.70 gm
Bacto soytone	0.30 gm
Bacto dextrose	0.25 gm
Sodium chloride	0.50 gm
Di potassium hydrogen Phosphate	0.25 gm
Distilled water	q.s. to 100 mL
pH	7.3 + 0.2 at 25^0 C

Nutrient agar medium is the most frequently used and also used in the present study for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures.

2.4.4 Preparation of 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 25^0C) 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 at 121^0C for 20

minutes. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

2.4.5 Sterilization procedure

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

2.4.6 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 37^0C for their optimum growth. These fresh cultures were used for the sensitivity test.

2.4.7 Preparation of test plate

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.

2.4.8 Preparation of discs

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

Table 2.5: Preparation of sample discs

Plant part	Test Sample	Dose (µg/disc)	Required amount for 20 disc (mg)
Leaf of *Crataeva religiosa*	Methanolic extract of Bark	400	8.0
	Petroleum ether partitionate	400	8.0
	Ethyl acetate soluble partitionate	400	8.0
	Chloroform soluble partitionate	400	8.0
	Aqueous soluble partitionate	400	8.0

Standard Ciprofloxacin (30 µg/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.

2.4.9 Diffusion and incubation

The sample discs, the standard antibiotic disc0s 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 4^0C 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 37^0C for 24 hours.

2.4.10 Determination of the zone of inhibition

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

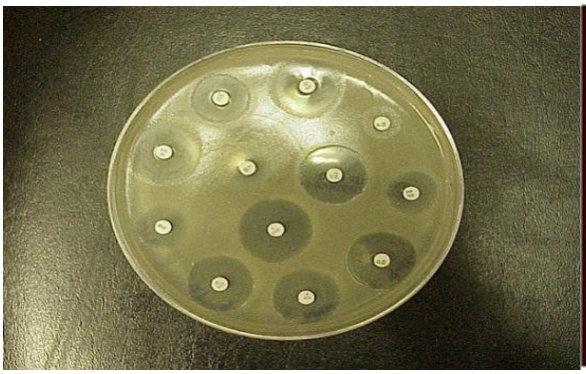


Figure 2.4: Clear zone of inhibition

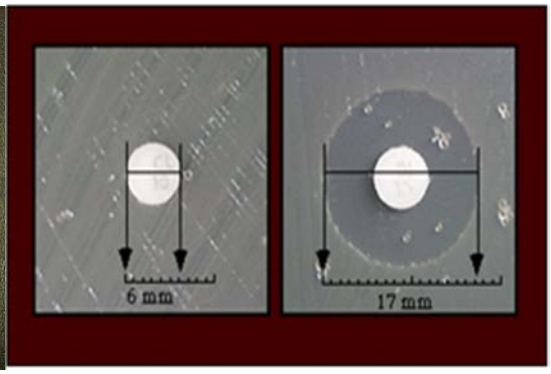


Figure 2.5: Determination of clear zone

2.5 Membrane stabilizing activity investigation

Compounds possessing scavenging ability may exert therapeutic potential against inflammatory diseases. The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane (Omale, 2008). Therefore, as membrane stabilizes that interfere in the release and or action of mediators like histamine, serotonin, prostaglandins, leukotrienes etc. (Shinde et al., 1999). Thus, the aim of the present study was to investigate the anti-inflammatory activity of methanolic extract and its different fraction of leaves of *Crataeva religiosa.*

2.5.1 Materials and methods

2.5.2 Preparation of the extract

Table 2.6: Preparation of different extracts

Plant part	Sample code	Test Sample	Concentration
Leaves of *Crataeva religiosa*	Hypotonic medium	------	50 mM
	ME	Methanolic extract	1 mg/mL
	PESF	Petroleum ether soluble fraction	1 mg/mL
	EASF	Ethyl acetate soluble fraction	1 mg/mL

	CSF	Chloroform soluble fraction	1 mg/mL
	AQSF	Aqueous soluble fraction	1 mg/mL
	ASA	Acetyl salicylic acid	0.10 mg/mL

Solvent used: Methanol analytical grade

2.5.3 Drug

Standard acetyl salicylic acid (ASA) or Aspirin was used as standard drug for comparison with different methanolic extracts of leaves of *Crataeva religiosa.*

2.5.4 Red blood cells (RBC) collection

Human RBCs were collected for the study. RBCs collected from the human was male, 70 kg, fare complexion and free from diseases. The collected RBCs were kept in a test tube with an anticoagulant EDTA under standard conditions of temperature 23 ± 2°C and relative humidity 55 ± 10%.

2.5.5 Preparation of phosphate buffer solution

A buffer is an aqueous solution that has a highly stable pH. The buffer was prepared at pH 7 using monosodium phosphate and its conjugate base, disodium phosphate.

2.5.5.1 Phosphate buffer materials

- Monosodium phosphate
- Disodium phosphate
- Water
- pH meter
- Glassware
- Stirring bar

2.5.5.2 Calculation of Phosphate buffer

A pH of about 7.4 with buffer strength of 10 mM was obtained using 0.0352% monosodium phosphate dehydrate and 0.1099% disodium phosphate anhydrate. The buffer was made by adding 0.352 gm monosodium phosphate dehydrate and 1.099 gm disodium phosphate anhydrate to 1000 mL water.

- pH 7.4
- Buffer strength: 10.00 mM
- Monosodium phosphate, dehydrate: 0.0352%
- Disodium phosphate, anhydrate: 0.1099%

2.5.6 Preparation of isotonic solution

A solution that has a concentration of electrolytes, nonelectrolytes or a combination of the two that will exert equivalent osmotic pressure as that solution with which it is being compared.
Either 0.16 M sodium chloride (NaCl) solution (approximately 0.95% salt in water) or 0.3 M nonelectrolyte solution is approximately isotonic with human red blood cells.
For the preparation of 500 mL isotonic solution of 154 mM strength, 4.5 gm NaCl was added and mixed.

2.5.6.1 Material for isotonic solution

- Sodium chloride (NaCl)
- Water
- Glassware
- Stirring bar

2.5.6.2 Calculation for isotonic solution

1000mL solution of strength 1 M contain = 58.5 gm NaCl
500mL solution of strength 1 M contain = 29.25 gm NaCl

500mL solution of strength 1000 mM contain = 29.25 gm NaCl

500mL solution of strength 154 mM contain = 58.5 × (154 ÷ 2) × 1000 gm NaCl

= 4.5 gm NaCl

2.5.7 Preparation of hypotonic solution

A solution of lower osmotic pressure than that of a reference solution or of an isotonic solution is called hypotonic solution. For the preparation of 500 mL hypotonic solution, having strength of 50 mM, 1.5 gm NaCl was added and mixed.

2.5.7.1 Materials for hypotonic solution

- Sodium chloride (NaCl)
- Water
- Glassware
- Stirring bar

2.5.7.2 Calculation for hypotonic solution

1000 mL solution of strength 1 M contain = 58.5 gm NaCl

500 mL solution of strength 1 M contain = 29.25 gm NaCl

500 mL solution of strength 1000 mM contain = 29.25 gm NaCl

500 mL solution of strength 50 mM contain = 58.5 × 50/2 × 1000 gm NaCl

= 1.5 gm NaCl

2.5.8 Effect on haemolysis

2.5.8.1 Erythrocyte suspension

Whole blood was collected from male human under standard condition. EDTA was used to prevent clotting. The blood was washed three times with isotonic solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) through centrifuge action for 10 min at 3000 g. Thus the suspension finally collected was the stock erythrocyte (RBC) suspension.

2.5.8.2 Hypotonic solution- induced haemolysis

The experiments were carried out with hypotonic solution. The test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffer saline (pH 7.4) containing the different methanolic extract (1.0 mg/mL). Acontrol was used as a reference standard. The mixtures were incubated for 10 min at room temperature, centrifuged for 10 min at 3000 g and the absorbance (O.D.) of the supernated was measured at 540 nm using Shimadzu UV spectrophotometer.

The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

$$\% \text{ inhibition of haemolysis} = 100 \times \{(OD_1 - OD_2)/ OD_1\}$$

Where,

OD_1 = Optical density of hypotonic-buffered saline solution alone (control) and

OD_2 = Optical density of test sample in hypotonic solution.

2.5.8.3 Heat induced haemolysis

Aliquots (5 mL) of the isotonic buffer containing 1.0 mg/mL of different extractives of plant was put into two duplicate sets of centrifuge tubes (Shinde *et al.,* 1999). The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30 µL) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54^0 C for 20 min in a water bath. The other pair was maintained at 0-5^0 C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm.

The percentage inhibition or, acceleration of haemolysis in tests was calculated according to the following equation:

$$\% \text{ Inhibition of haemolysis} = 100 \times [1 - (OD_2 - OD_1/ OD_3 - OD_1)]$$

Where,

OD_1 = test sample unheated, OD_2 = test sample heated, OD_3 = control sample heated

Chapter Three

Result and Discussion

Chapter Three: Result and Discussion

3.1 Determination of total phenolic content

The methanolic extract (ME) of leaves of *Crataeva religiosa* and its different partitionates i.e. petroleum ether (PESF), chloroform (CSF), ethyl acetate (EASF) and aqueous soluble fractions (AQSF) were tested for total phenolic content. Folin-Ciocalteu reagent was used for the test. Based on the absorbance values of the various extract solutions the colorimetric analysis of the total phenolics of different extracts were determined and compared with the standard solutions of gallic acid (table 3.1) equivalents. Total phenolic content of the samples are expressed as mg of GAE (gallic acid equivalent) per gm of extractives and are given in table 3.2.

The amount of total phenolic content differed in different extractives and ranged from 75.89 mg of GAE per gm of extractives to 328.36 mg of GAE per gm of extractives of leaves of *Crataeva religiosa*. Among all extractives of leaves of *Crataeva religiosa* the highest phenolic content was found in methanolic crude extract (ME) 328.36 mg of GAE per gm of extractives which follows by 230.21 (PESF), 143.67 (AQSF), 140.09 (CSF), 75.89 (EASF) mg of GAE per gm of extractives.

Table 3.1: Standard curve prepration by using gallic acid

Sl. No.	Conc. of the standard (µg/mL)	Absorbance	Regression line	R^2
1	100	0.800	y = 0.0081x - 0.0007	0.9975
2	50	0.423		
3	25	0.215		
4	12.5	0.123		
5	6.25	0.047		
6	3.125	0.007		
7	1.5625	0.003		
8	0.78125	0.000		
9	0.3906	0.000		

Sl. No.	Conc. of the standard (µg/mL)	Absorbance		
10	0	0.000		

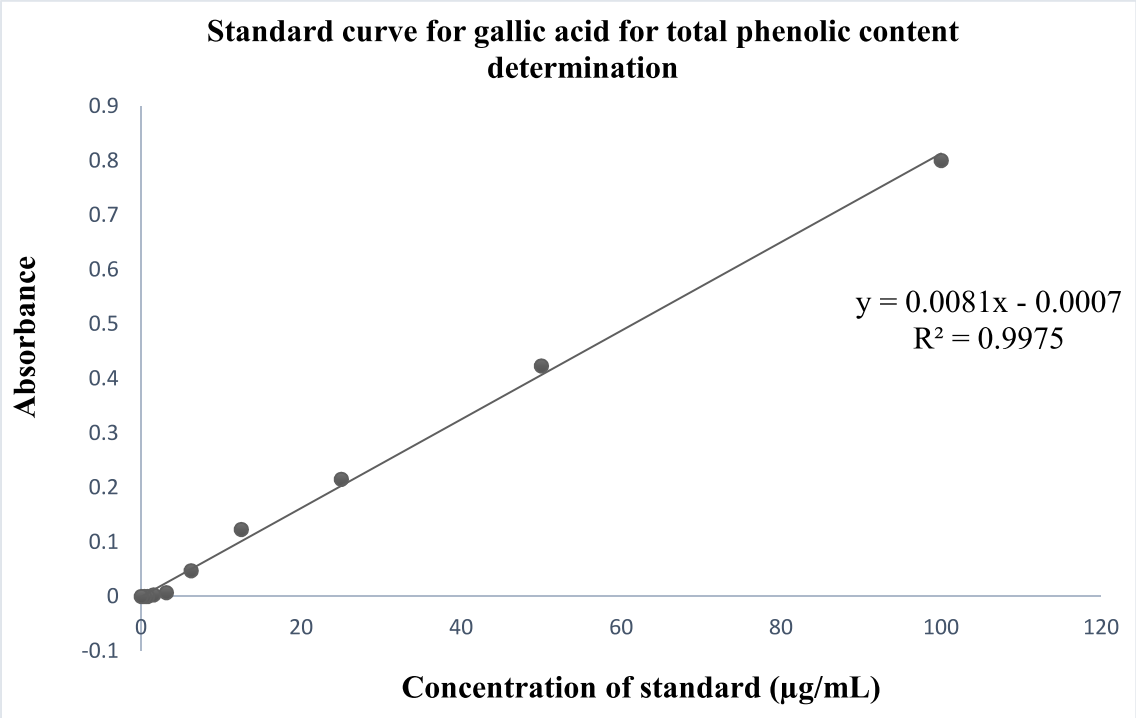


Figure 3.1: Standard curve of gallic acid for total phenolic determination

Table 3.2: Test samples for total phenolic content determination

Plant part	Sample code	Test Sample	Total phenolic content (mg of GAE per gm of extractives
Leaves of *Crataeva religiosa*	ME	Methanolic extract	328.36
	PESF	Petroleum ether soluble fraction	230.21
	EASF	Ethyl acetate soluble fraction	75.89
	CSF	Chloroform soluble fraction	140.09
	AQSF	Aqueous soluble fraction	143.67

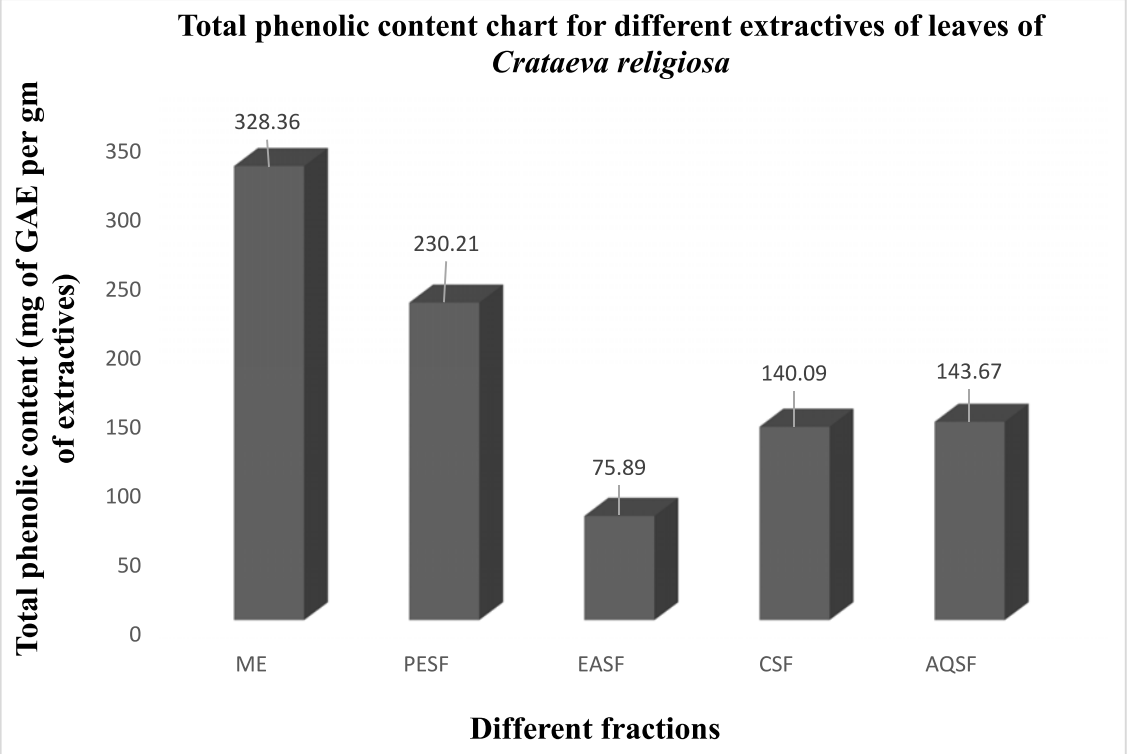


Figure 3.2: Graphical representation of total phenolic content (mg of GAE per gm of extractives) of different extractives of leaves of *Crataeva religiosa*

3.2 DPPH Assay

The methanolic crude extract of leaves of *Crataeva religiosa* (ME) and its different partitionates i.e. pet-ether (PESF), chloroform (CSF), ethyl acetate (EASF) soluble fractions of the methanolic extract of leaves of *Crataeva religiosa* were subjected to free radical scavenging activity using the method of Brand-Williams et al., 1995. Here, ascorbic acid (ASA) was used as reference standards.

In this investigation, aqueous soluble fraction PESF showed the notable free radical scavenging activity with IC_{50} value of 124.78 µg/mL for leaves of *Crataeva religiosa*. The other partitionates like MESF, CSF and EASF exhibited moderate scavenging activity having IC_{50} values 277.80 µg/mL, 1388.41 µg/mL and 272.11 µg/mL, respectively.

Table 3.3: IC_{50} values of the standard and partitionates of leaves of *Crataeva religiosa*

Plant part	Sample code	Test Sample	IC_{50} (µg/mL)
Leaves of *Crataeva religiosa*	ME	Methanolic extract	277.80
	PESF	Petroleum ether soluble fraction	124.78
	CSF	Chloroform soluble fraction	1388.41
	EASF	Ethyl acetate soluble fraction	272.11
ASA (Ascorbic acid) (standard)			5.80

Table 3.4: IC_{50} value of ascorbic acid

Absorbance of the blank	Conc. (µg/mL)	Absorbance of the extract	% Inhibition	IC_{50} (µg/mL)
0.325	500	0.005	98.46	5.80
	250	0.006	98.15	
	125	0.015	95.38	
	62.5	0.024	92.61	
	31.25	0.068	79.07	
	15.625	0.098	69.84	
	7.813	0.139	57.23	
	3.906	0.186	42.76	
	1.953	0.175	46.15	
	0.977	0.193	40.61	

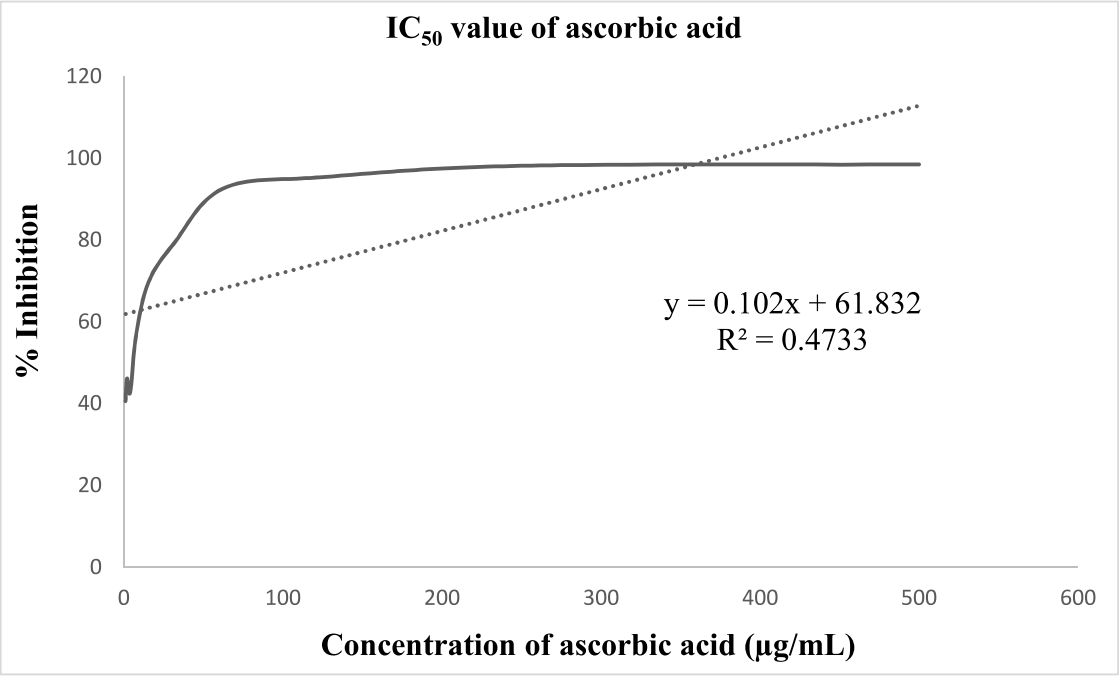


Figure 3.3: Graphical representation of IC_{50} value of ascorbic acid

Table 3.5: IC_{50} value of methanolic crude extract (ME)

Absorbance of the blank	Conc. (µg/mL)	Absorbance of the extract	% Inhibition	IC_{50} (µg/mL)
0.399	500	0.067	83.20	277.80
	250	0.18	54.88	
	125	0.275	34.83	
	62.5	0.351	12.03	
	31.25	0.36	9.77	
	15.625	0.366	8.27	
	7.813	0.367	8.02	
	3.906	0.386	3.25	
	1.953	0.328	1.77	
	0.977	0.391	2.01	

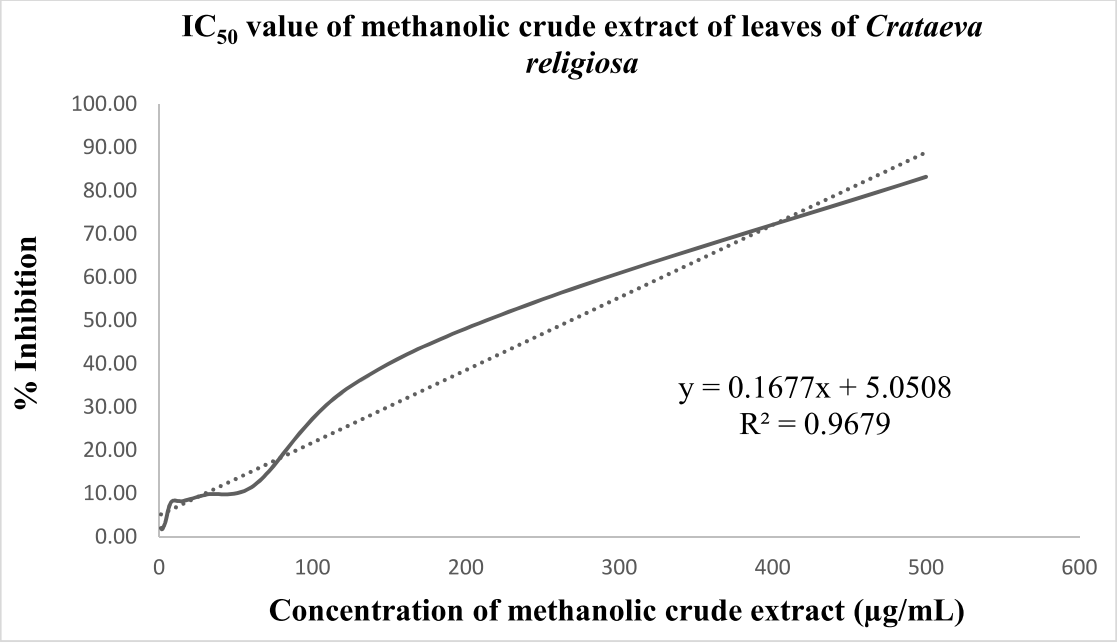


Figure 3.4: Graphical representation of IC_{50} value of methanolic crude extract of leaves of *Crataeva religiosa*

Table 3.6: IC_{50} value of petroleum ether soluble fraction (PESF)

Absorbance of the blank	Conc. (µg/mL)	Absorbance of the extract	% Inhibition	IC_{50} (µg/mL)
0.399	500	0.101	74.68	124.78
	250	0.091	77.19	
	125	0.265	33.58	
	62.5	0.272	31.82	
	31.25	0.275	31.07	
	15.625	0.327	18.04	
	7.813	0.352	11.77	
	3.906	0.363	9.02	
	1.953	0.375	6.01	
	0.977	0.396	0.01	

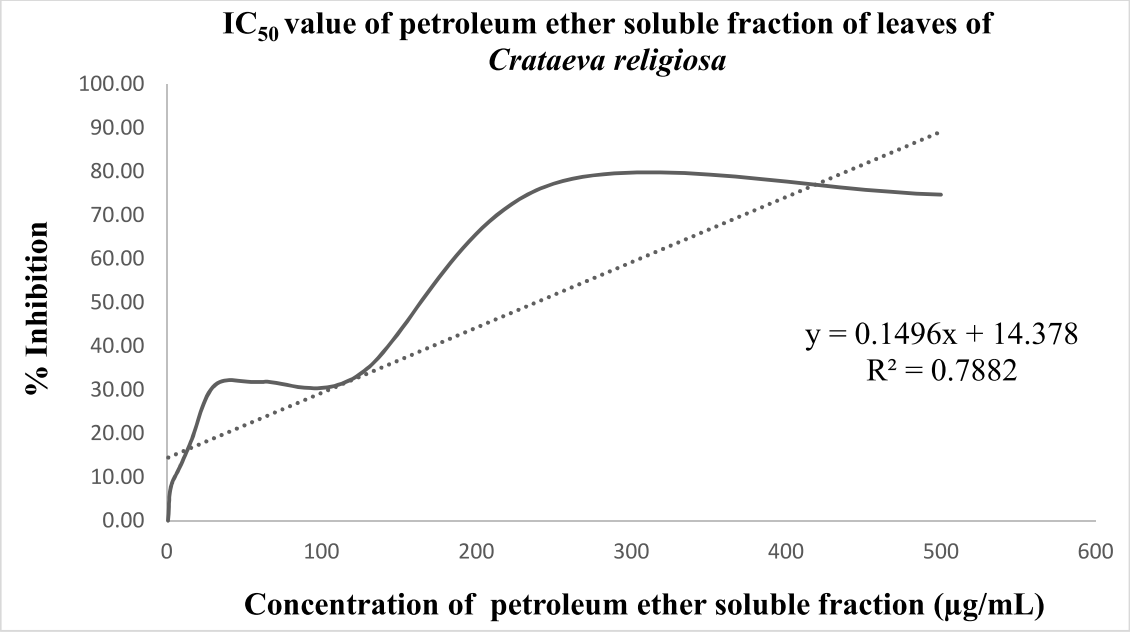


Figure 3.5: Graphical representation of IC_{50} value of petroleum ether soluble fraction of leaves of *Crataeva religiosa*

Table 3.7: IC_{50} value of chloroform soluble fraction (CSF)

Absorbance of the blank	Conc. (µgm/mL)	Absorbance of the extract	% Inhibition	IC_{50} (µg/mL)
0.399	500	0.214	46.36	1388.41
	250	0.238	40.35	
	125	0.298	25.31	
	62.5	0.25	37.34	
	31.25	0.338	15.28	
	15.625	0.371	7.01	
	7.813	0.355	11.02	
	3.906	0.384	3.75	
	1.953	0.35	1.22	
	0.977	0.384	3.75	

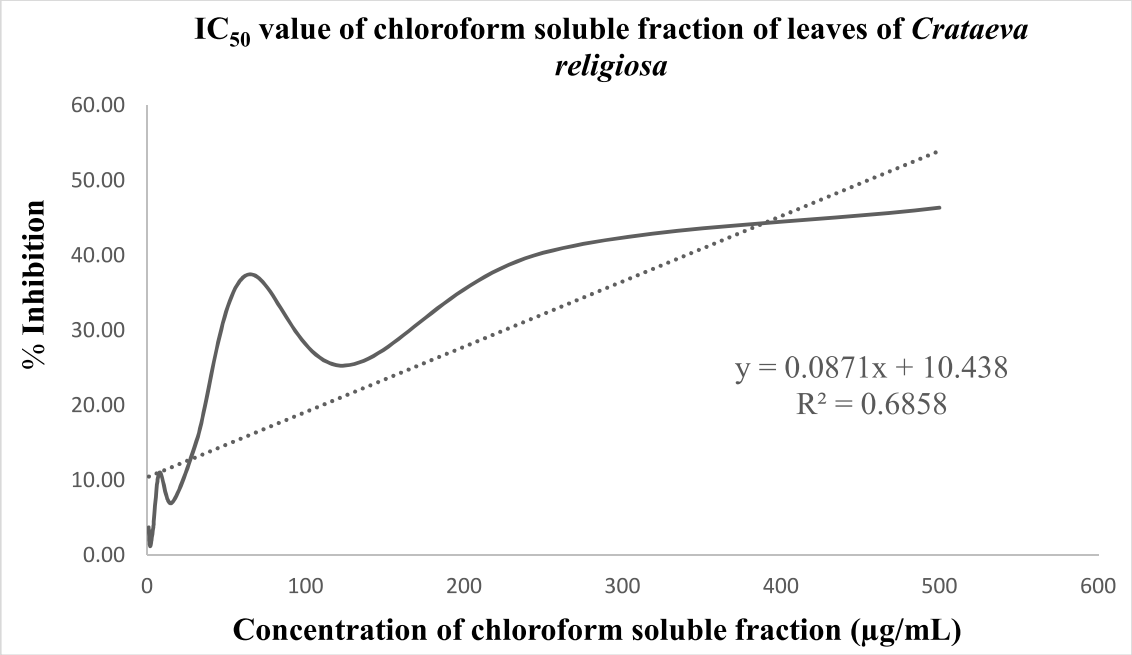


Figure 3.6: Graphical representation of IC_{50} value of chloroform soluble fraction of leaves of *Crataeva religiosa*

Table 3.8: IC_{50} value of ethyl acetate soluble fraction (EASF)

Absorbance of the blank	Conc. (µg/mL)	Absorbance of the extract	% Inhibition	IC_{50} (µg/mL)
0.399	500	0.057	85.71	272.11
	250	0.177	55.63	
	125	0.292	26.81	
	62.5	0.303	24.06	
	31.25	0.366	8.27	
	15.625	0.336	1.58	
	7.813	0.349	1.25	
	3.906	0.376	5.76	
	1.953	0.391	2.01	
	0.977	0.389	2.51	

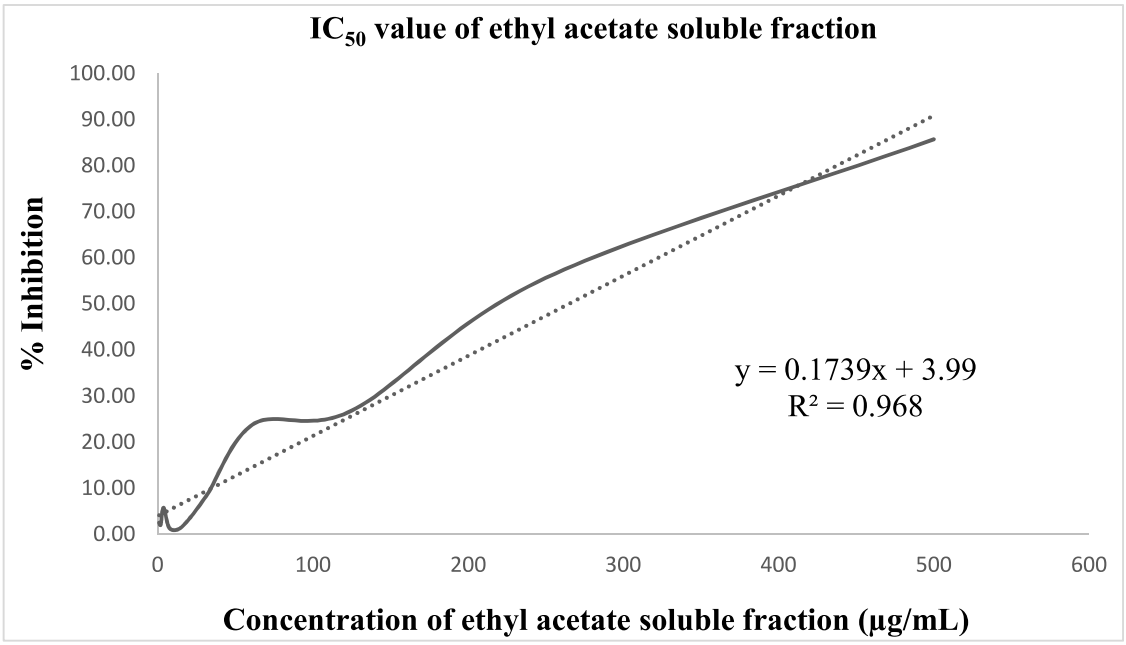


Figure 3.7: Graphical representation of IC_{50} value of ethyl acetate soluble fraction of leaves of *Crataeva religiosa*

3.3 Thrombolytic activity investigation

To find out cardio protective drugs from natural sources the extractives of *Crataeva religiosa* were assessed for thrombolytic activity and the results are presented in Table 3.9. Addition of 100µl SK, a positive control (30,000 I.U.), to the clots and subsequent incubation for 90 minutes at 37°C, showed 66.77% lysis of clot. On the other hand, distilled water was treated as negative control which exhibited a negligible percentage of lysis of clot (3.09%). The mean difference in clot lysis percentage between positive and negative control was found very significant. In this study, the methanolic soluble fraction showed highest thrombolytic activity (99.09%). However, the other partitionates of *Crataeva religiosa* i.e. petroleum ether soluble fraction (PESF), chloroform soluble fraction (CSF), ethyl acetate soluble fraction (EASF) and aqueous soluble fraction (AQSF) showed mild to moderate thrombolytic activity.

Table 3.9: Thrombolytic activity (in terms of % of clot lysis) of leaves of *Crataeva religiosa*

Fractions	Weight of empty eppendorf tube (W_1)	Weight of clot containing eppendorf tube before clot disruption (W_2)	Weight of clot containing eppendorf tube after clot disruption (W_3)	% of clot lysis
MESF	4738.1	54627	5191	99.09
PESF	4831	5563.1	5306	35.11
CSF	4768.3	5434.9	5320	17.24
EASF	4724.1	5306.2	5147.8	27.21
AQSF	4874	5617.4	5343	36.91
Blank	0.919	1.476	1.275	36.09
SK	0.905	1.913	1.24	66.77

W_1 = Weight of empty eppendorf tube; W_2 = Weight of clot containing eppendorf tube before clot disruption; W_3 = Weight of clot containing eppendorf tube after clot disruption; SK =Streptokinase

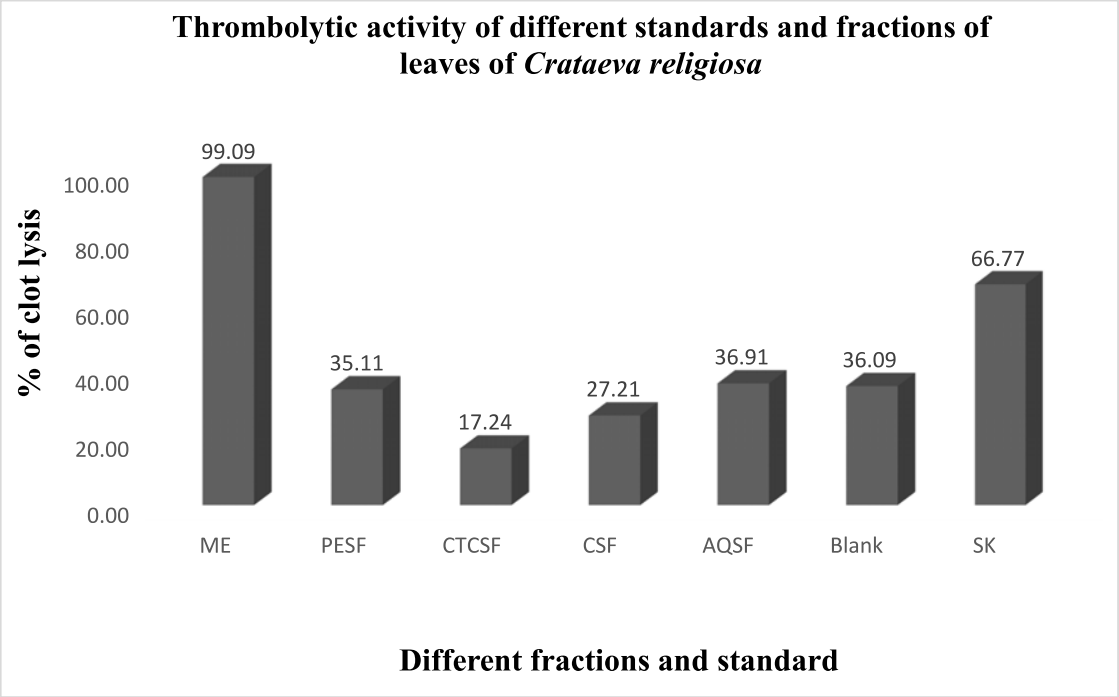


Figure 3.8: Graphical representation of thrombolytic activity of different standards and fractions of leaves of *Crataeva religiosa*

From this experiment, it can be concluded that few of the extractives of *Crataeva religiosa* showed mild to moderate clot lysis activity. Once found these herbal preparations may be incorporated as a thrombolytic agent for the improvement of the patients suffering from Atherothrombotic diseases. This is only a preliminary study and to make final comment the extract should thoroughly investigated phytochemically and pharmacologically to exploit their medicinal and pharmaceutical potentialities.

3.4 Antimicrobial Screening

The methanolic crude extract (ME) of leaves of *Crataeva religiosa* and its different partitionates i.e. petroleum ether (PESF), chloroform (CSF), ethyl acetate (EASF) and aqueous (AQSF) soluble fractions of leaves of *Crataeva religiosa* were subjected to antimicrobial screening with a concentration of 400 μg/disc in every case. Among the extractives, PESF and EASF exhibited mild to moderate activity against different tested microorganisms. Whereas, other partitionates have not shown any antimicrobial activity. The results are given in the Table 3.10.

The petroleum ether soluble fraction as well as the ethyl acetate soluble fraction exhibited the highest inhibition against microbial growth having zone of inhibition ranged from 7.0 mm to 9.0 mm. The maximum zone of inhibition produced by PESF was found to be 9.0 mm against *Shigella dysenteriae*. This partitionate also showed mild antifungal activity against *Aspergillus niger* (having zone of inhibition of 7.0 mm) and *Sacharomyces cerevacae* (having zone of inhibition of 7.0 mm) and no antibacterial activity against *S. typh*i (0.0 mm), mild activity against *Salmonella Paratyphi* (7.0 mm) and *Staphylococcus aureus* (8.0 mm). Ethyl acetate fraction showed highest activity against *Salmonellatyphi* (zone of inhibition of 9.0 mm). And also showed activity against *Vibrio parahemolyticus* (zone of inhibition of 8.0 mm). No antibacterial activity was observed against *Escherichia coli*.

Table 3.10: Antimicrobial activity of test samples of leaves of *Crataeva religiosa*

Test microorganisms	Diameter of zone of inhibition (mm)					
	ME	**PEP**	**CSF**	**EASF**	**AQP**	**Ciprofloxacin**
Gram positive bacteria						
Bacillus cereus	0	7	0	7	0	40
B. megaterium	0	7	0	7	0	42
B. subtilis	0	7	0	8	0	47

Test microorganisms	Diameter of zone of inhibition (mm)					
	ME	PEP	CSF	EASF	AQP	Ciprofloxacin
Gram positive bacteria						
Staphylococcus aureus	0	8	0	8	0	43
Sarcina lutea	0	0	0	7	0	48
Gram negative bacteria						
Escherichia coli	0	8	0	0	0	48
Pseudomonas aeruginosa	0	7	0	7	0	41
Salmonella paratyphi	0	7	0	7	0	49
S. typhi	0	0	0	9	0	40
Shigella boydii	0	7	0	7	0	40
Sh. dysenteriae	0	9	0	7	0	39
Vibrio mimicus	0	8	0	0	0	44
V. parahemolyticus	0	8	0	8	0	45
Fungi						
Candida albicans	0	0	0	7	0	41
Aspergillus niger	0	7	0	7	0	45
Sacharomyces cerevacae	0	7	0	0	0	40

3.5 Membrane stabilizing activity investigation

3.5.1 Results and discussion of hypotonic solution-induced haemolysis

The various extractives of leaves *Crataeva religiosa* at concentration 1.0 mg/mL were tested to evaluate the activity against lysis of human erythrocyte membrane induced by hypotonic solution, as compared to the standard acetyl salicylic acid (0.10 mg/mL) In hypotonic solution induced condition the methanolic fraction (MESF) inhibited highest percentage of haemolysis of RBCs (34.12%). The other partitionates i.e. petroleum ether (PESF) and chloroform (CSF) soluble fractions showed good inhibition of haemolysis of RBCs as compared to 42.20% inhibited by acetyl salicylic acid (0.10 mg/mL).

Table 3.11: Effect of different extractives of leaves of *Crataeva religiosa* on hypotonic solution-induced hemolysis of erythrocyte membrane.

Sample code	Concentration	% inhibition of haemolysis hypotonic solution induced
ASA	0.10 mg/mL	71.91
MESF	1 mg/mL	34.120
PESF	1 mg/mL	33.730
CSF	1 mg/mL	27.760

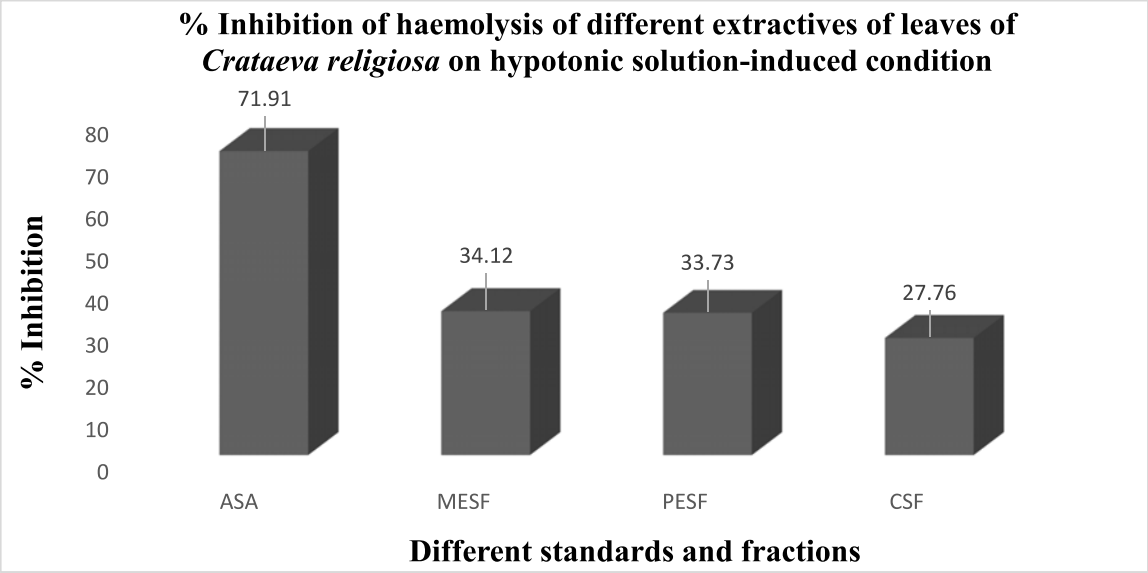


Figure 3.9: Graphical representation of % inhibition of haemolysis of different extractives of leaves of *Crataeva religiosa* on hypotonic solution-induced condition.

3.5.2 Results and discussion of heat induced haemolysis

The various extracts of leaves of *Crataeva religiosa* at concentration 1.0 mg/mL were tested to evaluate the activity against lysis of human erythrocyte membrane induced by heat, as compared to the standard acetyl salicylic acid (0.10 mg/mL) (Table- 3.19). At a concentration of 1.0 mg/mL and in heat induced condition the methanolic fraction inhibited highest percentage of haemolysis of RBC which is about 1728.16% as compared to the standard. Other partitionates i.e. pet ether soluble fraction (PESF) and chloroform soluble fraction (CSF) showed good inhibition of haemolysis of RBCs as compared to 95.60% inhibited by acetyl salicylic acid (0.10 mg/mL).

Table 3.12: Effect of different extractives of leaves of *Crataeva religiosa* on heat induced haemolysis of erythrocyte membrane.

Sample code	Concentration	% inhibition of haemolysis (Heat induced)
ASA	0.10 mg/mL	42.20
MESF	1 mg/mL	1728.16
PESF	1 mg/mL	591.67
CSF	1 mg/mL	652.91

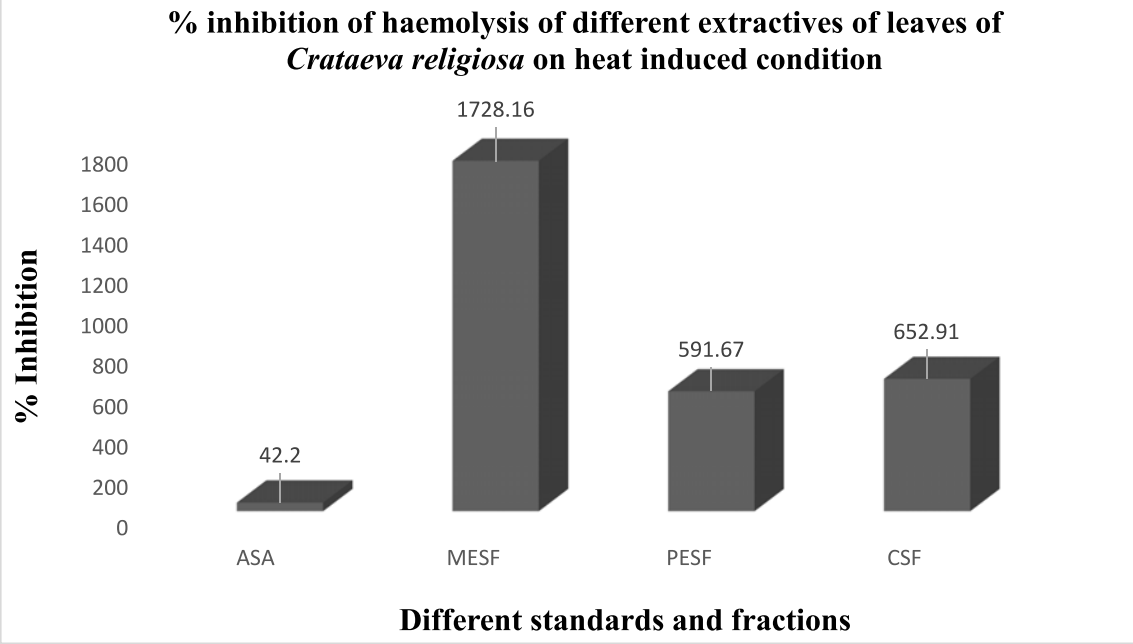


Figure 3.10: Graphical representation of % inhibition of haemolysis of different extractives of leaves of *Crataeva religiosa* on heat induced condition.

Chapter Four

Chapter Four: Conclusion

The findings of the biological investigations on five different fractions of *Crataeva religiosa* leaves on biological screening delivers effective information that can be further employed in isolation of a number of compounds with therapeutic potentials and also for different traditional purpose enormously. Moreover, further studies should be carried on different parts of the plant other than leaf using various solvents and on other research areas like pharmacological and chemical screenings.

The crude methanolic extract of the plant exhibited the highest amount of total phenolic content, and the petroleum ether soluble fraction showed prominent DPPH free radical scavenging assay, making *Crataeva religiosa* a potent source of antioxidant compounds.

Other *in-vitro* biological investigations, viz. thrombolytic and membrane stabilizing activity testes, have presented strong evidence of the potential of *Crataeva religiosa* plant's leaf extracts in the treatment of relevant diseases.

It can be stated that, the different extracts of *Crataeva religiosa* plant's leaves can act as an important contributor in drug discovery. However, extensive *in-vivo* investigations should be carried out to validate the existing herbal and traditional uses and to explore any other therapeutic activity along with their possible side effects. These will ultimately lead to gathering of substantive understanding about the safety and efficacy of *Crataeva religiosa*. Also, further phytochemical analysis of different extracts will be helpful in characterization and isolation of its active compounds.

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