Biological Investigation of Fruit *of Spondias pinnata* (Anacardiaceae)

A dissertation submitted to the Department of Pharmacy, East West University in the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B. Pharm.)

Submitted by : **Shawkat Md. Aminul Islam**
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DEDICATED

TO

MY PARENTS

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Shawkat Md. Aminul Islam
Author

Certificate

Certificate

This is to certify that the research work on "Biological Investigation of *Spondias pinnata* fruit" submitted to the Department of Pharmacy, East West University in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy was carried out by Shawkat Md. Aminul Islam (ID: 2008-3-70-085) under or guidance and supervision and that no part of the thesis has been submitted for any other degree. We further certify that all the sources of information and facilities availed of this connection are duly acknowledged.

……..

Kh. Tanvir Ahmed
Lecturer
Department of Pharmacy
East West University

…….

Sufia Islam, PhD
Chairperson
Department of Pharmcay
East West University

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Abstract

Abstract

Spondias pinnata belonging to the Anacardiaceae family has been investigated for the evaluation of biological activities of the crude extract with especial emphasis to the antimicrobial activity, antioxidant activity and thrombolytic activity.

The powdered fruit of *S. pinnata* was extracted with 96% ethanol. The concentrated crude ethanolic extract of *S. pinnata* was then evaluated for antioxidant activity. The crude ethanolic extract of *S. pinnata* exhibited significant antioxidant activity with the IC_{50} value 1.91µg/ml. The mean phenolic content found in the crude ethanolic extract of *S. pinnata* (fruit) was 659.74±3.58 mg of gallic acid (GAE) per gm of dried extract. Reducing power assay of the crude ethanolic extract of *S. pinnata* (fruit) revealed that the EC_{50} value of the crude extract was 6.39 µg/ml. The mean flavonoids content found in the crude ethanolic extract of *S. pinnata* (fruit) was 225.60±1.20 mg of quercetin per gm of dried extract. The mean antioxidant capacity found in crude ethanolic extract of *S. pinnata* (fruit) was 25.52±0.17 gm of Ascorbic acid per gram of dried extract.

The concentrated crude ethanolic extract of *S. pinnata* (fruit) was also subjected to microbiological investigation by the disc diffusion method. The crude extract of *S. pinnata* showed mild to moderate antimicrobial activity. The chloroform soluble fraction is moderately active against *Sarcina lutea*, *Staphylococcus aureus*, *Shigella boydii*, *Salmonella paratyphi* and *Salmonella typhi* with the diameter of zone of inhibition of (12 mm for 400 µg/disc, 16 mm for 800 µg/disc) , (10 mm for 400 µg/disc, 15 mm for 800 µg/disc), (12 mm for 400 µg/disc, 15 mm for 800 µg/disc), (10 mm for 400 µg/disc, 15 mm for 800 µg/disc), and (9 mm for 400 µg/disc, 13 mm for 800 µg/disc) respectively. Besides the MIC for *S. aureus*, *S.typhi*, *S.paratyphi*, *B.subtilis*, and *S.boydii* was 0.078125 mg/ml and the lowest MBC was found against *S. typhi* (0.3125mg/ml).

In the in vitro thrombolytic assay, the average percentage of clot lysis by crude ethanolic extract of *Spondias pinnata* (fruit) were 25.23 ± 0.51 for volunteer 1 and 25.37 ± 0.03 for volunteer 2. In contrast, the average clot lysis by streptokinase in volunteer 1 and volunteer 2 was 50.21 and 49.80 respectively.

CHAPTER

1

Introduction

Chapter

1

Introduction

1.1 Rationale and Objective of the Work

Terrestrial plants, especially higher plants, have a long history of use in the treatment of human diseases (Balunas MJ *et al.,* 2006). Fossil records date human use of plants as medicines at least to the Middle Paleolithic age some 60,000 years ago (Solecki & Shanidar, 1975). Several well known species, including licorice (*Glycyrrhiza glabra*), myrrh (*Commiphora* species), and poppy capsule latex (*Papaver somniferum*), were referred to by the first known written record on clay tablets from Mesopotamia in 2600 BC, and these plants are still in use today for the treatment of various diseases as ingredients of official drugs or herbal preparations used in systems of traditional medicine. Furthermore, morphine, codeine, noscapine (narcotine), and papaverine isolated from *P. somniferum* were developed as single chemical drugs and are still clinically used. Hemisuccinate carbenoxolone sodium, a semi-synthetic derivative of glycyrrhetic acid found in licorice, is prescribed for the treatment of gastric and duodenal ulcers in various countries (Balunas MJ *et al.,* 2006).

Success in natural products research is conditioned by a careful plant selection, based on various criteria such as chemotaxonomic data, ethnomedical information, field observations or even random collection (Kurt Hostettmann and Christian Terreaux, 2000). Historical experiences with plants as therapeutic tools have helped to introduce single chemical entities in modern medicine. Plants, especially those with ethnopharmacological uses, have been the primary sources of medicines for early drug discovery. In fact, a recent analysis by Fabricant and Farnsworth showed that the uses of 80% of 122 plant-derived drugs were related to their original ethnopharmacological purposes. Current drug discovery from terrestrial plants has mainly relied on bioactivity-guided isolation methods, which, for example, have led to discoveries of the important anticancer agents, paclitaxel from *Taxus brevifolia* and camptothecin from *Camptotheca acuminata* (Balunas MJ *et al.,* 2006).

The goals of using plants as sources of therapeutic agents are (Fabricant DS and Farnsworth NR, 2001)

a) to isolate bioactive compounds for direct use as drugs, e.g. digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine;
b) to produce bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon (and other narcotic analgesics), taxotere, teniposide, verapamil, and miodarone, which are based, respectively, on galegine, Δ9-tetrahydrocannabinol, morphine, taxol, podophyllotoxin, and khellin;
c) to use agents as pharmacologic tools, e.g., lysergic acid diethylamide (LSD), mescaline, yohimbine; and
d) to use the whole plant or part of it as a herbal remedy, e.g., cranberry, echinacea, feverfew, garlic, etc.

The number of higher plant species (angiosperms and gymnosperms) on this planet is estimated at 250,000 (Ayensu & DeFilipps, 1978) with a lower level at 215,000 (Cronquist, 1981; Cronquist, 1988) and an upper level as high as 500,000 (Tippo & Stern, 1977; Schultes, 1972). Of these, only about 6% have been screened for biologic activity, and a reported 15% have been evaluated phytochemically (Verpoorte, 2000). It was estimated that in 1991 in the United States, for every 10,000 pure compounds (most likely those based on synthesis) that are biologically evaluated (primarily in vitro), 20 would be tested in animal models, and 10 of these would be clinically evaluated, and only one would reach U.S. Food and Drug Administration approval for marketing. The time required for this process was estimated as 10 years at a cost of $231 million (U.S.) (Vagelos, 1991).

The major drawback of this strategy is the frequent isolation of known metabolites. Therefore, hyphenated techniques (LC-UV, LC-MS, LC-NMR) have been developed, in order to detect as early as possible potential original structures. These compounds can then be tested in various bioassays (Kurt Hostettmann and Christian Terreaux, 2000). More recently combinatorial chemistry and high throughput robotic screening techniques have been employed as viable strategies for drug discovery programs (Berhanu M. *et al.,* 1999).

Chemical diversity of secondary plant metabolites that results from plant evolution is superior to that found in synthetic combinatorial chemical libraries (Vagelos, 1991). Medicinal plants have

played an essential role in the development of human culture, for example religions and different ceremonies. (E.g. *Dutura* has long been associated with the worship of Shiva, the Indian god). Plants are directly used as medicines by a majority of cultures around the world, for example Chinese medicine and Indian medicine. Many food crops have medicinal effects, for example garlic. Medicinal plants are resources of new drugs. Studying medicinal plants helps to understand the plant toxicity and protect human and animals from natural poisons. Cultivation and preservation of medicinal plants protect biological diversity, for example metabolic engineering of plants (Naik S.N and Panda V.S, 2008). The medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry. With onset of scientific research in herbals, it is becoming clearer that the medicinal herbs have a potential in today's synthetic era, as numbers of medicines are becoming resistant. According to one estimate only 20% of the plant flora has been studied and 60%of synthetic medicines owe their origin to plants. Ancient knowledge coupled with scientific principles can come to the forefront and provide us with powerful remedies to eradicate the diseases. In real sense, coupling of ancient knowledge and scientific principle is essential-

(1) To identify alternative and complementary medicine.

(2) To reduce the toxicity of drug therapy especially toxicity reduction of synthetic and semi synthetic drugs.

(3) To find the lead compound diversification to treat various diseases (Ayuvedaherbs, 2005).

1.2 History of traditional herbal medicine in Bangladesh

Traditional Medicine is the medicine or treatment based on traditional uses of plants, animals or their products, other natural substances (including some inorganic chemicals), religious verses, cultural practices, and physical manipulations including torture. As this system of medicine has been in use almost unchanged generation after generation throughout the ages for the treatment of various physical and psychological diseases, it is called traditional. Most of the times, the type, preparation, and uses of traditional medicines are largely influenced by folklore customs and the cultural habits, social practices, religious beliefs and, in many cases, superstitions of the people who prescribe or use them (Ghani A., and Pasha M.K, 2006).

The earliest mention of traditional medicine is found in ''*Rigveda'',* the oldest repository of knowledge in this subcontinent. Later ''*Ayurveda'',* developed from the *Vedic* concept of life,

became the important source of all systems of medical sciences. In course of time it became a part of culture and heritage of the people of the Indian subcontinent.

Traditional medicine involves the use of both material and non-material components. The material components invariably comprise parts or organs of plants and their products. They also consist of animal organs, minerals and other natural substances. The non-material components, which constitute important items of religious and spiritual medicines, include torture, charms, magic, incantations, religious verses, amulets and rituals like sacrifices, appeasement of evil spirits, etc (Ghani A., and Pasha M.K, 2006).

Treatments in traditional medicine are carried out by internal and external application of medicaments, physical manipulation of various parts of the body, performing rituals, psychological treatment, and also by minor surgery. *Ayurvedic* medicinal preparations consist mainly of plant materials in the form of powders, semi-solid preparations, decoctions, elixirs and distillates. Many of them also contain inorganic chemical substances, minerals and animal products. Alcoholic extracts and alcoholic solutions of the ingredients, tinctures and elixirs are also frequently used in *Ayurvedi*c medicine (Ghani A., and Pasha M.K, 2006).

Whole plants or their powders or pastes or products and their extracts, infusions, decoctions and distillates constitute the major constituents of *Unani* medicine. Minerals, inorganic chemicals and animal products are also frequently used in preparing these medicines.

For hundreds of years, the medical knowledge of the Indian subcontinent is termed as Ayurveda. Ayurveda remains an important system of medicine and drug therapy in India and Bangladesh. Plant alkaloids are the primary active ingredients of Ayurvedic drugs. Today the pharmacologically active ingredients of many Ayurvedic medicines are being identified and their usefulness in drug therapy being determined. As only a certain percentage of plants are used in traditional medicines, it is roughly estimated that of the discovered 17,000 species, nearly 3,000 species are used in medicinal field (Samy RP *et al.,* 2008). Some crude drugs used as medicine in Bangladesh are reported in following table (Ghani, 2003).

Table 1.1 Some crude drugs used as medicine in Bangladesh

Bangali Name	**Scientific Name**	**Used part**	**Uses**
Nayantara	*Catharanthus roseus*	Leaves, flowers	Cancer, insomnia, blood pressure and diabetes.
Sarpagandha	*Rauvolfia serpentian*	Root	Blood pressure and dysentery

Ghritokumari	*Aloe indica*	Leaves	Constipation, anthelemintic, fistula, piles, leucorrhoea, burns and jaundice
Lajjabati	*Mimosa pudica*	Whole plant	Blood purification, toothache, convulsion fistula and piles
Assamlata	*Makania cordata*	Leaves	Dysentery.
Ulatkambal	*Abroma augsta*	Bark, leaves	Gonorrhea, diarrhea, urethritis and irregular menstruation
Jogyadumur	*Ficus hispida*	Bark, latex, root	Insects bites, boils, asthma, piles, cough, bronchitis, and diarrhea.
Shatamuli	*Asparagus racemosus*	Roots	Cancer, bacteria and fungal disease, tonic, appetizer, jaundice and diabetes.
Anatamul	*Tylophora indica*	Root, leaves	Asthma, cough, bronchitis, diarrhea, dysentery and stimulant
Mahedi	*Lawsonia inermis*	Leaves, flower	Skin disease, pox, burns, dandruff and insomnia.
Bohera	*Terminalia belerica*	Fruit, bark	Constipation, diarrhea, dysentery, leprosy, rheumatisms and piles.
Bherenda	*Ricinus communis*	Roots, seeds	Constipation and rheumatisms.
Ghandabadal	*Paederia foetida*	Leaves	Diarrhea, uriticaria, paralysis, piles and toothache
Haritaki	*Terminalia chebula*	Fruit, Bark	Indigestions, jaundice, piles, skin disease and ulceration of gum.
Thankuni	*Cliotoria ternatea*	Whole plant	Weakness, dermatitis, jaundice and stomach disorder
Neem	*Azadirachta indica*	Leaves	Anathematic, fever dermatitis, stomach disorder, jaundice, nausea, and ruminates
Tulshi	*Ocimum sanctum*	Leaves, flower, seeds	Stomach disorder, malaria, common cold, and hypertension.

Nishinda	*Vitex negunda*	Leaves, barks	Weakness, cough, headache, malaria, and kalazar
Basak	*Adhatoda vasica*	Root, leaves, flowers	Cough, asthma, arthritis, dysentery, and malaria
Arahar	*Cajanus cajan*	Leaves, seeds	Jaundice, mouth sore and leprosy.
Arjun	*Terminalia arjuna*	Bark	Heart disease, tonic
Kalojira	*Nigella sativa*	Seeds	Common cold, rheumatisms, galactagogue and carminative
Hatishur	*Heliotropoum indicum*	Root, leaves	Fever, rheumatisms, wound, skin disease, and diuretic
Amloki	*Phyllanthus emblica*	Bark, flower, fruit	Hair tonic, cough, diuretic, stomach ache, dysentery, jaundice, dermatitis.
Halud	*Curcuma longa*	Rhizomes	Blood purification, skin disease, eye disease, tonic, and stomachache
Methi	*Trigonella foenum-graceum*	Seeds	Hypertension and diabetes.

(Ghani, 2003)

1.3 Research of traditional drugs in Bangladesh

In rural areas medicinal plants have been being used as remedy for disease for a long time. They not only cure the disease but also provide an important role in the economy. Medicinal plants are cheap and easy to get to those people who knew it very well. Bioactive compounds are deposited in medicinal plants; it can serve as important raw materials for pharmaceutical manufacturing. They comprise a precious asset of a country and donate to its health care system. Well-judged and scientific investigation of this wealth can significantly contribute to the public health. Besides being available commodity of commerce, a country can also earn a good amount of foreign currency by exporting this natural wealth to other countries. More than 500 of such medicinal plants have so far been established as growing in Bangladesh (Ghani, 2003). Almost all of these indigenous medicinal plants are extensively used in the preparation of unani, ayurvedic and homeopathic medicines in Bangladesh.

A survey conducted in 1990 in different villages of Bangladesh shows that on average of 14% of people suffering from illness approach qualified allopathic doctors, 29% contact unqualified village doctors, 10% contact mullahs, 29% contact quack and 19% contact homeopaths. The survey indicates an extensive use of medicinal plants, most of which are served in crude and substandard form, by our people (Haque MM *et al.,* 1990). Traditional medicines are still manufactured in our country by following the age-old unscientific, traditional methods. Hundreds of indigenous medicinal plants are employed in different Ayurvedic and Unani commercial preparations without proper standardization, quality control, evaluation and determination of the chemical nature, pharmacological and toxicological studies of the active components which are essential to utilize their therapeutic potential fully. Toxicity of the plants or plant extracts is coming to light with the advancement of science. Since Bangladesh is a country of low economic growth, a proper health care system can be established by supplying low cost medicines to its population. This may be possible only by developing standard drugs from our natural resources of medicinal plants. In order to achieve this goal research and development of traditional medicines should be given the due priority (Ghani, 2003).

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

1.4 General Approaches to Drug Discovery from Natural Sources

In general, three different approaches have been, and continue to be used in the drug discovery process from natural sources. These approaches are: traditional, empirical and molecular (Harvey A.L, 2008). During the vedic period the ‘‘Susruta samhita’’ and the ‘‘charaka samhita’’ were influential works on traditional medicine. Hundreds of medicinal plant were identified and have been traditionally used since then. Over the following centuries, Ayurvedic practitioners developed a number of medicinal preparations and surgical procedures for the treatment of various ailments and diseases. WHO (World Health Organization) estimates that 80% of the populations living in the developing countries rely exclusively on traditional medicine for their primary health care needs. In almost all the traditional medicine, the traditional plants play a major role and constitute the backbone of the traditional medicine. Indian Materia medica includes about 1600 drugs of vegetable origin almost all of which are derived from different

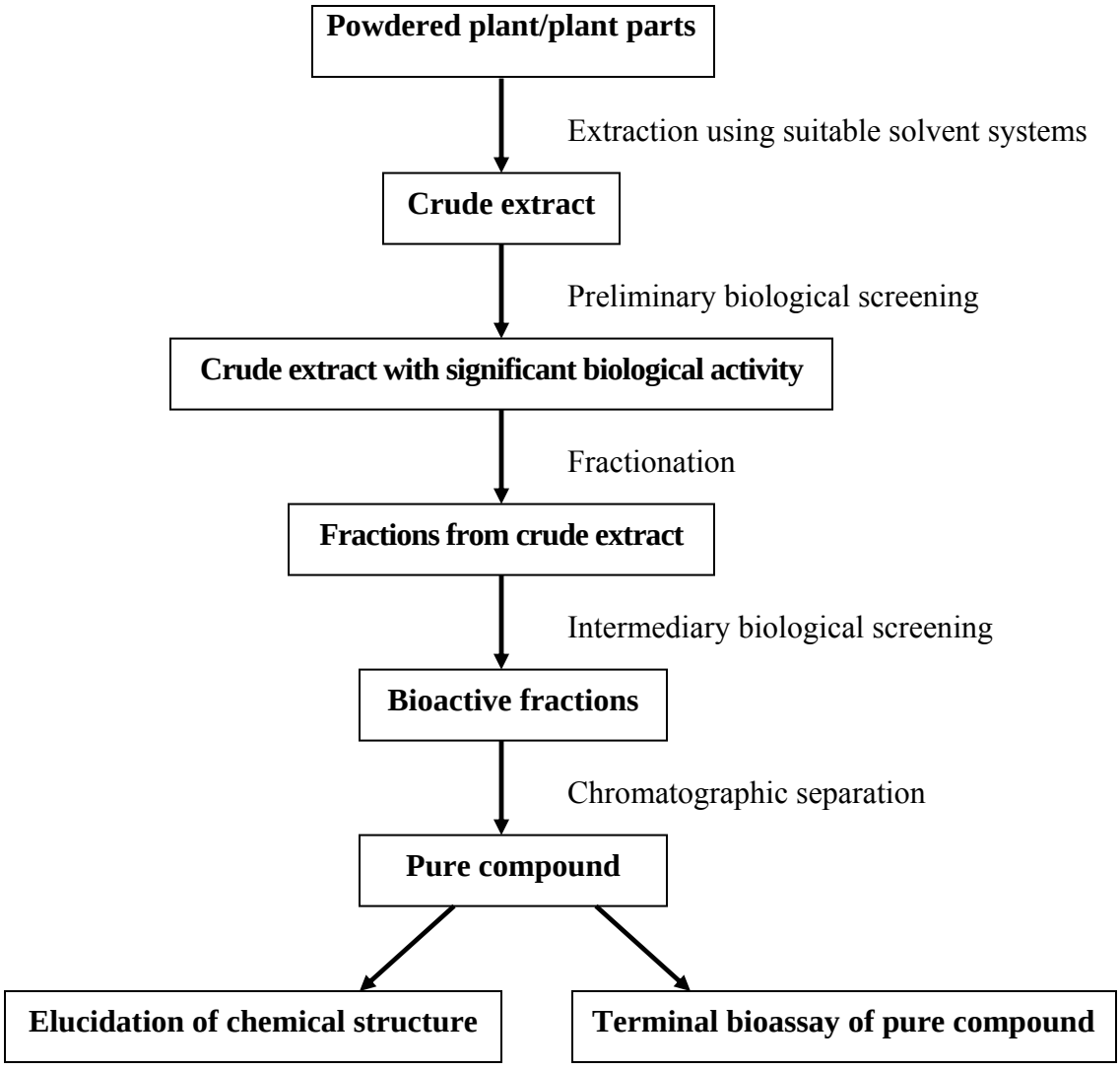
traditional system and folklore practices (Sashidhara K.V, 2005). Examples include drugs like morphine, quinine and ephedrine that have been in widespread use for a long time, and more recently adopted compounds such as the antimalarial artemisinin. The empirical approach builds on an understanding of a relevant physiological process and often develops a therapeutic agent from a naturally occurring lead molecule. Examples include tubocurarine and other muscle relaxants, propranolol and other β-adrenoceptor antagonists, and cimetidine and other H_2 receptor blockers. Development of molecular biological techniques and the advances in genomics lead to molecular approach. The molecular approach to drug discovery can be further subdivided into three general categories. The first is rational drug design using computer aided techniques. A second area is the antisense approach, which is based on manipulation of genetic targets. The third technique, which currently dominates drug discovery activity, is the pragmatic approach of random screening. With recent technological developments in molecular biology, instrumentation and information technology, screening of compounds can be conducted by high throughput screening method. High throughput screening is an automated testing process of large number of compounds versus a large number of targets which is particularly effective in identifying potential lead compounds. Robotics and miniaturization of in vitro tests on genetically modified cells has lead to high throughput screening (Harvey A.L, 2008).

The major advantage of natural products for random screening is the structural diversity. Since Bioactive natural products often occur as a part of a family of related molecules, it is therefore possible to isolate a number of homologues compounds and obtain structure-activity relationship. Of course, lead compounds found from screening of natural products can be optimised by traditional medicinal chemistry or by application of combinatorial approaches. Overall, when faced with molecular targets in screening assays for which there is no information about low molecular weight leads, use of a natural products library seems more likely to provide the chemical diversity to yield a hit than a library of similar numbers of compounds made by combinatorial synthesis. Since only a small fraction of the world's biodiversity has been tested for biological activity, it can be assumed that natural products will continue to offer novel leads for novel therapeutic agents (Harvey A.L, 2008).

In earlier times, all drugs and medicinal agents were derived from natural substances, and most of these remedies were obtained from higher plants. Today, many new chemotherapeutic agents are synthetically derived, based on "rational" drug design. The study of natural products has

advantages over synthetic drug design in that it leads optimally to materials having new structural features with novel biological activity. Not only do plants continue to serve as important sources of new drugs, but phytochemicals derived from them are also extremely useful as lead structures for synthetic modification and optimization of bioactivity. The starting materials for about one-half of the medicines we use today come from natural sources. Virtually every pharmacological class of drugs includes a natural product prototype. The future of plants as sources of medicinal agents for use in investigation, prevention, and treatment of diseases is very promising (Setzer W.N, 1999).

1.5 Flow Chart of Bioactivity Guided Phytochemical Approach



The objective of this dissertation is to identify the biological activity of the fruit of an indigenous medicinal plant, viz., *Spondias pinnata* (L.f.) Kurz (Family: Anacardiaceae) and to evaluate the possible pharmacological and microbiological profiles of the crude extracts. So far some chemical and biological investigations have been carried out on this plant mainly focusing on the bark and root of the plant. That's why the goal of this framework is to explore the potential possibilities of developing new drug candidates from the fruit of this plant which could be crucial for the treatment of various ailments.

1.6 The plant family: Anacardiaceae

The plant under investigation- *Spondias pinnata* (L.f.) Kurz belongs to the family Anacardiaceae. Anacardiaceae, the cashew family, includes approximately 800 species in 82 genera. The genera are given in table 1.2 (Pell S.K, 2009).

Table 1.2 Various genera of Anacardiaceae family

Abrahamia	*Actinocheita*	*Allospondias*	*Amphipterygium*	*Anacardium*
Androtium	*Antrocaryon*	*Apterokarpos*	*Astronium*	*Baronia*
Blepharocarya	*Bonetiella*	*Bouea*	*Buchanania*	*Campnosperma*
Campylopetalum	*Cardenasiodendron*	*Choerospondias*	*Comocladia*	*Cotinus*
Cyrtocarpa	*Dobinea*	*Dracontomelon*	*Drimycarpus*	*Euroschinus*
Fegimanra	*Faguetia*	*Gluta*	*Haematostaphis*	*Haplospondias*
Haplorhus	*Harpephyllum*	*Heeria*	*Hermogenodendron*	*Holigarna*
Koordersiodendron	*Lannea*	*Laurophyllus*	*Lithrea*	*Loxopterygium*
Loxostylis	*Malosma*	*Mangifera*	*Mauria*	*Melanochyla*
Melanococca	*Metopium*	*Micronychia*	*Mosquitoxylum*	*Myracrodruon*
Nothopegia	*Ochoterenaea*	*Operculicarya*	*Orthopterygium*	*Ozoroa*
Pachycormus	*Parishia*	*Pegia*	*Pentaspadon*	*Pistacia*
Pleiogynium	*Poupartia*	*Poupartiopsis*	*Protorhus*	*Pseudosmodingium*
Pseudospondias	*Rhodosphaera*	*Rhus*	*Schinopsis*	*Schinus*
Sclerocarya	*Searsia*	*Semecarpus*	*Smodingium*	*Solenocarpus*
Sorindeia	*Spondias*	*Swintonia*	*Tapirira*	*Thyrsodium*
Toxicodendron	*Trichoscypha*			(Pell S.K, 2009)

Members of the family are cultivated throughout the world for their edible fruits and seeds, medicinal compounds, valuable timber, and landscape appeal. Some of the products of Anacardiaceae, including mango (*Mangifera indica*), pistachio (*Pistacia vera*), cashew (*Anacardium occidentale*), and pink peppercorn (*Schinus terebinthifolia*), are enjoyed worldwide while other notables such as the pantropical *Spondias* fruits, the marula of Africa (*Sclerocarya birrea*), and the Neotropical fruits of Antrocaryon are restricted to localized cultivation and consumption and are not generally transported far distances to larger markets (Pell S.K, 2009).

1.6.1 Taxonomy

The exact definition of taxonomy varies slightly from source to source, but the core of the discipline remains: the identification, naming, and classifying of organisms. As points of reference, three recent textbook definitions are presented below:

1. Theory and practice of grouping individuals into species, arranging species into larger groups, and giving those groups names, thus producing a classification (Judd *et al.,* 2007)

2. A field of science (and major component of systematics) that encompasses description, identification, nomenclature, and classification (Simpson, 2010).

3. The science of classification, in biology the arrangement of organisms into a classification (Kirk *et al.,* 2008).

In 1759, Bernard de Jussieu arranged the plants in the royal garden of the Trianon at Versailles, according to his own scheme. That classification included a description of an order called Terebintaceae which contained a suborder that included Cassuvium (Anacardium), Anacardium (Semecarpus), Mangifera, Connarus, Rhus and Rourea. In 1789, Antoine Laurent de Jussieu, nephew of Bernard de Jussieu, published that classification scheme (Bernard J, 1789).
Robert Brown described a subset of Terebintaceae called Cassuvlae or Anacardeae in 1818, using the herbarium that was collected by Christen Smith during a fated expedition headed by James Kingston Tuckey to explore the River Congo. The name and genera were based on the order with the same name that had been described by Bernard de Jussieu in 1759. The herbarium from that expedition contained only one genus from the family, Rhus (Brown R, 1818).

Augustin Pyramus de Candolle in 1824, used Robert Browns name Cassuvlae or Anacardeae, wrote another description of the group and filled it with the genera Anacardium, Semecarpus, Holigarna, Mangifera, Buchanania, Pistacia, Astronium, Comocladia and Picramnia (Candolle AP, 1825).

John Lindley described the "Essential character" of Anacardiaceae, the "Cashew Tribe" in 1831, adopting the order that was described by Jussieu but abandoning the name Terebintaceae. He includes the genera which were found in de Candolle's Anacardieae and Sumachineae: *Anacardium, Holigarna, Mangifera, Rhus* and *Mauria* (Lindley F.R.S., 1831).

The genus Pistacia has sometimes been separated into its own family, Pistaciaceae, based on the reduced flower structure, differences in pollen, and the feathery style of the flowers. However, the nature of the ovary does suggest it belongs in the Anacardiaceae, a position which is supported by morphological and molecular studies, and recent classifications have included Pistacia in the Anacardiaceae (Tingshuang Y, 2008).

1.6.2 Botanical features of Anacardiaceae family

Anacardiaceae, the sumac family of flowering plants includes primarily trees and shrubs (rarely lianas or subshrubs), with resin canals and clear to milky exudates (Pell S.K, 2009).

Habit and leaf form: Members of Anacardiaceae family have resin ducts in the bark, leaves usually composed of leaflets in various arrangements, inconspicuous flowers often with only male or female parts, and usually fleshy fruits (Encyclopaedia britannica, 2012). Resin-canals located in the inner fibrous bark of plants fibrovascular system found in the stems, roots and leaves is characteristic of all members of this family; resin-canals located in the pith is a characteristic of many of the cashew family species and several species have them located in the primary cortex or the regular bark. Tannin sacs are also widespread among the family. The wood of Anacardiaceae has the frequent occurrence of simple small holes in the vessels, occasionally in some species side by side with scalariform holes (in *Campnosperma*, *Micronychia* and *Anaphrenium argenteum*). The simple pits are located along the vessel wall and in contact with the parenchyma (Solereder H, 1908).

The sumacs have either simple or pinnately compound leaves, depending on the species. It is common for members of Anacardiaceae to have pinnately compound leaves (e.g. *Toxicodendron,*

Schinus). Many members of Anacardiaceae (eg - *Rhus, Toxicondendron*) produce compounds on their leaves that cause rash (Hickman J.C, 1993).

Leaf anatomy: The leaves are duciduous or evergreen, estipulate & usually alternate (opposite in *Bouea*, *Blepharocarya*). Most taxa have imparipinnate leaves (rarely paripinnate, bipinnate in *Spondias bipinnata*), usually with opposite leaflets (rarely alternate in e.g. *Pseudospondias, Sorindeia, Thyrsodium*), while others have trifoliolate leaves (e.g. *Rhus, Searsia, Toxicodendron*) or unifoliolate leaves (e.g. *Anacardium, Lithrea*). The unifoliolate leaves are rarely palmate (*Campylopetalum*). Leaf margins can be entire, dentate, serrate or crenate, prominently revolute (e.g. *Anacardium*) or rarely spinose (e.g. *Comocladia*). Primary leaf venation is pinnate, rarely palmate (e.g. *Campylopetalum*). Secondary venation is most commonly eucamptodromous, brochidodromous, craspedodromous, semi craspedodromous or cladodromous and rarely exmedially reticulodromous (e.g. *Rhus thouarsii*). An intramarginal vein is rarely present (e.g. *Spondias*). Resin canals are found in Anacardiaceae leaves and reproductive structures. Resin canals run parallel to the phloem in leaf petioles and in major lamina veins, and are absent only in the most minor veins. They are also associated with the phloem of every vascular bundle in the reproductive structures of most genera (Pell S.K *et al.,* 2011).

Reproductive type, pollination: Anacardiaceae are primarily entomophilous but some exceptions are found. *Anacardium* species are pollinated by butterflies and moths, and secondarily by bats. Some *Mangifera* are pollinated by flies and secondarily by flying foxes. A few genera are wind pollinated (*Amphipterygium, Dobinea, Orthopterygium*) (Pell S.K *et al.,* 2011).

Inflorescence, floral, fruit and seed morphology: Inflorescence structure is quite diverse in Anacardiaceae but basically appears axillary and more branched. Inflorescences are often pseudoterminal (e.g. *Spondias, Tapirira*) but can be truly terminal (e.g. *Apterokarpos, Cotinus, Rhus*) or rarely cauliflorous (e.g. *Sorindeia, Semecarpus*). Most are thyrso-paniculate but some are racemes and spikes. Rarely female inflorescences are reduced to solitary flowers (*e.g. Sclerocarya, Choerospondias*) (Pell S.K *et al.,* 2011).

Flowers grow at the end of a branch or stem or at an angle from where the leaf joins the stem and have bracts. Often with this family bisexual and male flowers on some plants, and bisexual and female flowers on others or flowers having both stamens and pistils (perfect). Calyx with 3 to 7 cleft sepals and the same number of petals, occasionally no petals, overlapping each other in the bud. Stamens twice as many or equal to the number of petals, inserted at the base of the fleshy ring or cup-shaped disk, and inserted below the pistil(s). Stamen stalks separate, anthers able to move. Flowers have the ovary free, but the petals and stamen are borne on the calyx. In the stamenate flowers, ovaries are 1-celled. In the pistillate flowers, ovaries are 1-celled or sometimes 4-5-celled. 1-3 styles and 1 ovule in each cavity (Britton N.L and Brown A, 1897).

Fruit: The fruits are drupes or samaras and all appear to be derived from a fundamentally drupaceous fruit type. They are most often 1-locular, but incompletely 2- ,3- ,4- and 5- locular fruits are not rare. The pericarp is multilayered and well differentiated within the family. The exocarp varies in thickness and can have a lignified outer epidermis (Some *Anacardioideae*) or subepidermal sclereids (*Pentaspadon*). In some taxa (e.g. *Lithrea, Toxicodendron*), the exocarp is brittle and chartaceous and separates from the mesocarp at maturity. The mesocarp is usually fleshy and resinous, can be waxy or oily, and is often pulpy and edible (e.g. *Mangifera, Spondias*), dry (e.g. *Schinopsis, Pachycormus*) and sometimes contains dermatitis causing liquid in usually black resin canals of varying thickness (e.g. *Anacardium, Mangifera, Gluta*). In *Melanochyla* both mesocarp and endocarp contains black resin (Pell S.K *et al.,* 2011).

Seeds: Seeds are generally ellipsoid, ovoid, falcate, lenticular or reniform. They vary in size from 2mm to more than 10cm. Species of Mangifera have labyrinth seeds in which the seed coat deeply encroaches on the endosperm and embryo. The seed coat is usually undifferentiated and/or reduced, while the chalaza is well developed and forms the greater part of the seed coats. The embryo of Anacardiaceae is oily, curved or straight with two expanded cotyledons (Pell S.K *et al.,* 2011).

Seedling: Both epigeal and hypogeal germination are found in the family (sometimes within one genus). Epigeal and phanerocotylar seedlings are found in *Anacardium, Lannea, Rhus, Spondias,* and *Toxicodendron.* Hypogeal and cryptocotylar seedlings are found in *Gluta, Lannea,*

Mangifera, Melanochyla and *Semecarpus*. Epigeal and cryptocotylar seedlings are found in *Swintonia, Astronium graveolens* (Pell S.K *et al.,* 2011).

1.6.3 Distribution of Anacardiaceae family

Anacardiaceae are found worldwide in dry to moist, mostly lowland habitats, primarily in the tropics and subtropics but extending into the temperate zone. The family is native to the western hemisphere (from southern Canada south to Patagonia), Africa, southern Europe, temperate and tropical Asia, tropical and subtropical Australia, and most of the Pacific Islands. Anacardiaceae are absent from northern Europe, temperate and arid Australia, New Zealand, the Galapagos Islands, and extreme desert and high elevation habitats (although they can reach elevations as high as 3,500 m) (Pell S.K, 2009).

1.6.4 Anacardiaceae Species Available in Bangladesh

Scientific name	**Genus**	**Local name**	**Medicinal uses**	**Location**
Lannea coromandelica (Houtt.) Merr	*Lannea*	Jiga, Jika, Jeol, Bhadi	The water extract of stem bark soaked overnight is used for liver pain. (Anisuzzaman, 2007) Green fruit is used in chicken pox. (Pavel, 2007) Its bark along with the bark of Aegle mermelos, Artocarpus heterophyllus and Sygygium cumini is useful in impotency. (Pavel, 2007) The lotion of the bark is used in leprous, heart diseases, and obstinate ulcers. (Pavel, 2007)	Throughout Bangladesh. Utrail (Netrokona), Bheduria and Thanarbaid (Tangail).

Mangifera laurina Blume	*Mangifera*	Milam, Jangli Aam	Fruits are used in dog bite. Two teaspoonful of the decoction of it's bark along with bark of *Syzygium cumini* and fruits of *Phyllanthus emblica* and *Terminalia chebula* is taken 4 times daily for 14 days for loss of sensitivity in skin, continuous sneezing, small pustules on the skin of children (Chowdhury JU *et al.*, 2010)	Barisal, forests of Chittagong, Chittagong Hill Tracts and Cox's Bazar.
Semecarpus anacardium L.f	*Semecarpus*	Bhela, Beula, Beda	Ripe fruits are used as carminative, aphrodisiac and stimulant; good for piles (Anisuzzaman, 2007). Juice of the pericarp is powerful escharotics. Nut oil is a powerful antiseptic and anthelmintic; used externally in acute rheumatism, leprosy and psoriasis. (Pavel, 2007) Paste of the bark is used both articular and muscular rheumatism. Decoction of the bark is used in gonorrhoea. Root is used in menstruation(Pavel, 2007).	Pirgacha, Tangail, Forests of Chittagong, Chittagong Hill Tracts, Sylhet and Sal forests of Modhupur.

Buchanania lanzan	*Buchanania*	Pial, Piyar, Nala amsi	Fruits are laxative and aphrodisiac which is used to cure fever and ulcers. Seeds are aphrodisiac, expectorant, astringent to bowels, stomachic, cardiac and brain tonic. Gum exudes from bark is administered in diarrhoea. The roots and leaves, pounded and mixed with buttermilk cures diarrhea. (Longman O, 2005)	Forest of Chittagong Hill Tracts and Cox's Bazar.
Anacardium occidentale L	*Anacardium*	Kaju badam	Bark tea is used for diarrhoea and dysentery. Root is purgative. The fruit is anti-diarrhoeal. Liquor made from cashew apple is valued as diuretic. Leaf, bark and fruit are used as Anthelmintic and antiasthmatic. Leaf tea is used for diarrhoea, and stomachache (Uddin, 2010)	Cultivated in Chittagong Hill Tracts in limited scale.
Schinus polygama	*Schinus*	Morich gach	It has anti inflammatory and analgesic property. The folk medicine employs it to treat arthritic pain and cleansing of wounds (Rahmatullah M *et al.*, 2010).	Cultivated throughout Bangladesh

Mangifera indica L	*Mangifera*	Aam	Juice from 4-5 leaves is taken to cure sudden stomach ache (Chowdhury JU *et al.*, 2010). Fresh stem-bark juice is mixed with sugar and is used for dysentery (Pavel, 2007). Decoction of the leaves is used in fever, diarrhoea and toothache. The unripe fruit is astringent to the bowels and cures dysentery and urinary discharges. The seeds are used in chronic diarrhoea and asthma. The kernel is also anthelmintic; useful in menorrhagia. (Anisuzzaman, 2007)	Cultivated throughout Bangladesh including Barisal.
Toxicodendron acuminatum	*Toxicodendron*	Bichuti	The fluid extract prepared from fresh leaves is used as rubefacient and vesicant. It is also recommended in cases of incontinence of urine. It is also used in the form of tincture as a sedative (Rahmatullah M *et al.*, 2010).	Found in Bangladesh including Khulna.

Mangifera longipes Griff	*Mangifera*	Milam, Jangli Aam, Uri Aam	Fruits are used in dog bite in the country. Leaves are used to brush teeth in toothache (Pavel, 2007).	Cox's Bazar, Chittagong (Forest and Hill tracts).
Lannea grandis	*Lannea*	Jikkha	Leaf is taken as diuretic and tonic for debility. It is used in various gastrointestinal disorders including dysentery, diarrhea, indigestion, constipation, and bloating. (Mollik, A.H., *et al.,* 2010)	Throughout Bangladesh including bagarhat and bagha.

1.7 Introduction to *Spondias pinnata*

1.7.1 Taxonomic hierarchy of the investigated plant (Ethnobotanybd, 2010)

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Sapindales
Family	Anacardiaceae
Genus	*Spondias* L.
Species	*Spondias pinnata* (L.f.) Kurz

1.7.2 Common names (Longman O, 2005; Ayurvedic pharmacopoeia of India, 2001)

Language	Vernacular name
Bengali	Amra, Piala, Pial
Tribal	Thoura (Mogh); Ambi-thong (Garo)
English	Wild Mango, Hog-plum
Hindi	Ambara, Amra, Bhringi-phal, Pashu-haritaki
Sanskrit	Aamraata
Manipuri	Eikhoi
Telugu	Adavimamidi
Nepali	Amaro
Malayalam	Ambazham, Ambalam, Mambuli
Tamil	Pulicha kaai

1.7.3 Botanical features (Gardner S *et al.,* 2000; Longman O, 2005; Ethnobotanybd, 2010)

Category: Ornamental tree

Diagnostic characters: Deciduous trees with a pleasant smell. Bark is smooth. Sap white, rapidly turning black. Leaves compound with the leaflets base often asymmetric. Inflorescence axillary, flowers white to cream, polygamous. Fruit a drupe with a large stone.

Height: Deciduous tree up to 27 m tall.

Girth: Usually 2.5 m

Trunk & bark: Bole straight, bark pale grey, smooth with rounded knobs.

Branches and branchlets or twigs : Twigs terete, hairy.

Exudates: Exudate white turning to black.

Leaves: Leaves compound, imparipinnate, alternate and spiral, leaflets elliptic, and apex acuminate, base acute, often asymmetrical, with distinct marginal vein, margin entire. Midrib flat above, secondary veins obtuse, widely parallel, tertiary veins reticulate. Stipules are absent. Leaves are 30-40 cm long, compound with 5-11 opposite leaflets. Leaflets are stalked, ovate-oblong to elliptic-oblong, 7-12 cm long, 4-5 cm wide, papery. Leaf base is wedge- shaped to rounded, often oblique, margin toothed or entire, with a tapering tip.

Inflorescences or flowers: Polygamous tiny white to cream flowers are borne in panicles at the end of branches, 25-35 cm long. Flowers are stalkless, white. Sepals are triangular, about 0.5 mm. Petals are ovate-oblong, about 2.5 × 1.5 mm, pointed.

Fruits: Fruit is ellipsoid to elliptic-ovoid, yellowish orange at maturity, 3.5-5 × 2.5-3.5 cm. Inner part of endocarp is woody and grooved, outer part is fibrous. Ripen fruits have pleasant fragrance.

Propagation: By seeds.

Seeds: Usually stony. Mature fruit is usually with 2 or 3 seeds.

Flowering time: May-July.

Fruiting time: November-December.

Stem: Soft Wooded

Roots: Deep roots, Tap roots

1.7.4 Images of various parts of *Spondias pinnata*

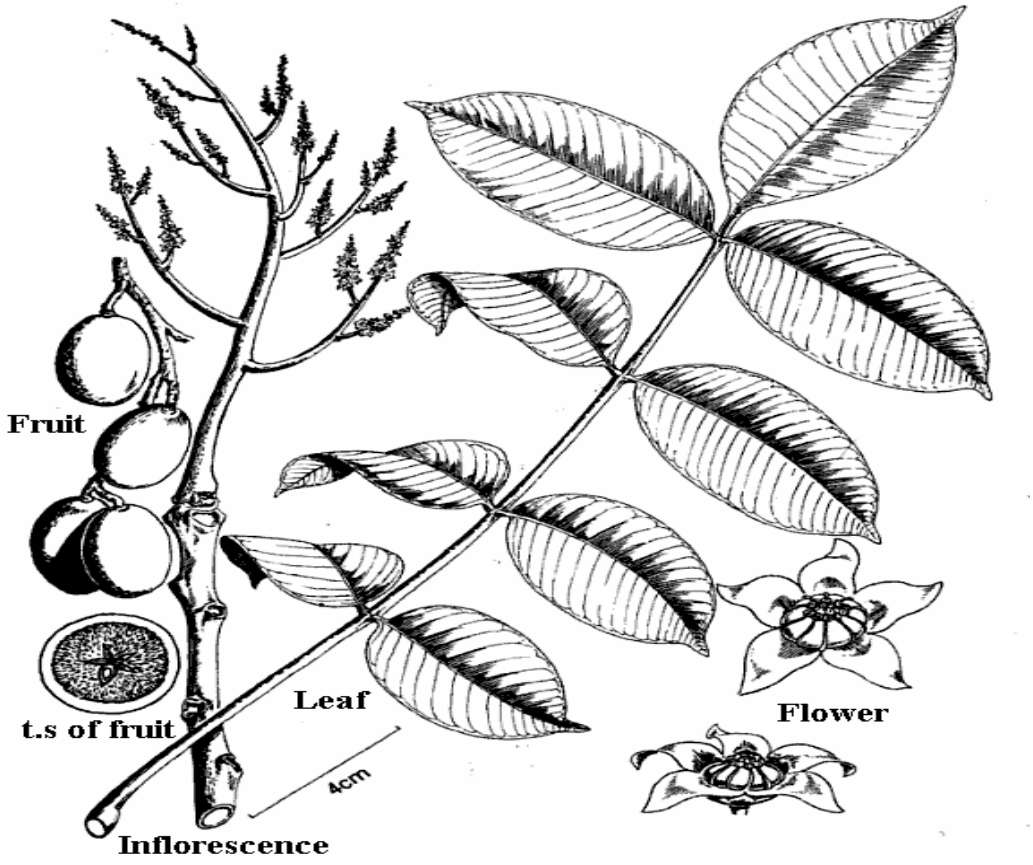


Figure 1.1 *Spondias pinnata* Plant

Figure 1.2 *Spondias pinnata* flower

Figure 1.3 *Spondias pinnata* bark

Figure 1.4 *Spondias pinnata* leaflet

Figure 1.5 *Spondias pinnata* fruit

Figure 1.6 *Spondias pinnata* leaf

Figure 1.7 *Spondias pinnata* whole plant

1.7.5 Distribution of *Spondias pinnata*

Native to Southeast Asia, this slow growing fruit tree is seen everywhere in Bangladesh for it's fruits including wild forests of Chittagong, Chittagong Hill Tract, Cox's Bazar, Tangail, Sylhet and Dinajpur. It is also found in village shrubberies throughout the country (Ethnobotanybd, 2010). Besides it is found in India, Srilanka, Assam, Myanmar, China, Malaysia and Thailand (Badoni, A. and Bisht, C, 2009).

1.7.6 Growing conditions

The plants are easily grown with adequate moisture preferring humus rich, moist but well drained soil. Like the Mango, the tree thrives in humid tropical and subtropical areas growing up to 2 metres in a single growing season. It grows on all types of soil, as long as they are well drained. It has been noted that some trees can suffer from some nutritional disorders if the soil is too alkaline. Trees are cold sensitive when small and should be protected from serious frost and strong wind. Trees do best in full sun, but will produce some fruit in light shade. As a large and vigorous tree, they prefer not be planted underneath other large trees and unlike some mango varieties they are not too fussed on salt spray (Morton J, 1987).

1.7.7 Chemical constituents of *Spondias pinnata*

Plant Part	Chemical Constituent
Aerial parts	24-methylene cycloartenone, stigmast-4-en-3-one, β -sitosterol, glycoside of β-sitosterol and lignoceric acid (Ghani, 2003)
Fruits	Water soluble polysaccharides, composed of mainly L-arabinose, D-galactose and galacturonic acid (Ghani, 2003)
	β-amyrin and oleanolic acid, glycine, cystine, serine, alanine and leucine (Rastogi & Mehrotra, 1993)

1.7.8 Ethnobotany of *Spondias pinnata*

Timber is used for interior furniture. This plant is used to treat diarrhea, ear aches, and to make a cancer home remedy. The fruit contains 40-70 mg Vitamin C per 100 g and is domesticated for food production. Unripe fruits are used to make pickles. The young leaves and flowers are edible too (Ethnobotanybd, 2010).

1.7.9 Nutritive and mineral potential of ripe fruits of *Spondias pinnata* (Purohit, V.K *et al.*, 2010)

Energy	189–203 kcal/g
Crude fat	12.23–12.54%
Crude fiber	3.13–4.03%
Total carbohydrate	16.30–23.54%
Sodium	0.96–1.38%
Calcium	0.15–0.93%
Iron	1.3–1.5%
Copper	0.9–1.23%
Protein	0.50-0.80%
Acid	0.47%

1.7.10 Medicinal Potentials of *Spondias pinnata*

Plant Part	Medicinal properties
Leaf	It is aromatic, acidic, appetizing and astringent, and is used in dysentery. The juice of the leaves is recommended for local application in otalgia (Ethnobotanybd, 2010). In Nigeria, a decoction of the mashed leaves is used for washing a swollen face. A leaf infusion is a common cough remedy & used as a laxative for fever with constipation. A leaf decoction is used for gonorrhoea. All these leaves are used for leprosy. Crushed with lemon they are effective for worms in children. A decoction of pounded leaves is used as an eye lotion and the juice pressed from young, warm leaves is given to children for stomach troubles. The young leaves are used as an infusion taken internally or as a warm astringent lotion by women in confinement in Sierra Leone. In Suriname's traditional medicine, the infusion of the leaves is used as a treatment of eye inflammation, diarrhoea and venereal diseases (Faiz M, 2011).
Fruit (Unripe)	It is astringent, sour, thermogenic, appetizer and aphrodisiac, and is good for rheumatism and sore throat (Ethnobotanybd, 2010).
Root	Regulation of menstruation. It is used in fever in Thailand (Faiz M, 2011).

Fruit (Ripe)	It is sweet, astringent, cooling, emollient, tonic, constipating and antiscorbutic, and is administered in bilious dyspepsia, diarrhea, and vitiated conditions of tridosa. Ripe fruit is aphrodisiac & cures burning sensation. The fruit-juice is used as a febrifuge and diuretic (Ethnobotanybd, 2010).
Bark	It is aromatic, astringent and refrigerant, and infusion of the bark is administered in dysentery, diarrhea, vomiting. Paste of the bark is used as an embrocation for both articular and muscular rheumatism. Decoction of the bark is given in gonorrhoea & severe cough. Gum of the bark is demulcent. Bark is used as purgative and in local applications for leprosy (Faiz M, 2011).

1.7.11 Reported Biological Works on *Spondias pinnata*

Plant part	Investigation & Result	Reference
Whole plant	The ethanolic extract was investigated for total phenolic activity, total flavonoid & free radical scavenging activity.	Maisuthisakul, P. *et al* (2007)
Fruit	The methanolic extract was investigated for total phenolic activity, total flavonoid & DPPH radical scavenging activity.	Wetwitayaklung, P. *et al* (2012)
Stem bark	The 70% methanolic extract was investigated for hydroxyl radical scavenging, superoxide radical scavenging, NO radical scavenging, Hydrogen peroxide radical scavenging, Peroxynitrite radical scavenging, Singlet oxygen scavenging, Hypochlorous acid scavenging, reducing power, ferrous chelation, total phenolic activity & total flavonoid activity.	Hazra, B. *et al* (2008)
Fruit	The extract was investigated for total phenolic activity, total flavonoid & DPPH radical scavenging & ferric reducing activity.	Kubola, J. *et al* (2011)

Bark	The chloroform & ethanolic extract was investigated for cytotoxic, antioxidant, antibacterial, & phytochemical screening.	Das, J. *et al.*(2011)
Fruit	The 80% ethanolic extract was investigated for cytotoxic and antibacterial activity	Muhammad, A. *et al.*(2011)
Fruit	The extract was investigated for the correlation analysis between total acid, total phenolic and ascorbic acid contents and their antioxidant activities	Samee, W. *et al*. (2006)
Stem heart wood	The methanolic and ethyl acetate extract was investigated for hepatoprotective activity.	Rao, BG. *et al* (2010).
Fruit	The hexane extract was investigated for Anti HIV-1 reverse transcriptase activity.	Silprasit, K. *et al.* (2011)
Bark	Hypoglycemic activity of the bark of *Spondias pinnata* Linn. kurz	Mondal, S. *et al.* (2009)
Leaf	Aqueous & methanolic extracts was investigated for anti diarrheal activity	Panda, SK. *et al.* (2012)
Bark	Petroleum ether, chloroform and methanolic extracts was investigated for diuretic and laxative activity	Mondal, S. *et al.* (2009)
Stem heart wood and bark	Methanolic extracts was investigated for antihelmintic activity	Jayaraju, N. *et al.* (2009)
Stem bark	Ethanolic extracts was investigated for analgesic activity	Panda, BK. *et al.* (2009)
Fruits pulp	Polysaccharide has been identified from the fruits pulp which has eliciting activity on peritoneal macrophages	Iacomini *et al.* (2005)
Whole plant	Isolation of 24-methylelle cydoartanone, stigma-4en-3one, lignoceric acid, β-sitosterol and its β- D-glucoside	Rastogi, R.P. *et al* (1976)

Fruit	Isolation and characterization of active compound an oleanolic acid (3α hydroxyolea-12-en-28-oic acid)	Kandali, R. *et al.* (2011)
Pulp	Isolation and characterization of 5-hydroxymethylfurfural, 1,4-pentadiene, 3,5-dihydroxy-2-methyl-5,6-dihydropyran-4-one and furfural from the methanolic extract	Pholsongkram, K. *et al* (2009)
Pulp	Isolation and characterization of 9,12,15-octadecatrien-1-ol, hexadecanoic acid and furfural from the essential oil of the pulp	Liawruangrath, B. *et al* (2009)
Stem bark	Methanolic extracts was investigated for antibacterial activity	Chetia, B., and Gogoi, S. (2011)

CHAPTER

2

Study protocol

Chapter

2 Study Protocol

2.1 Present Study Protocol

Our present study was designed to observe pharmacological activities of the crude ethanolic extract of the plant *Spondias pinnata (L.f.) Kurz* (Family: Anacardiaceae). The study protocol consisted of the following steps:

- Extraction at room temperature of the fruit with 96% ethanol for 10 days.
- Filtration of the crude ethanolic extract by using cotton and subsequently through the Double rings filter paper (9.0 cm) and solvent evaporation.
- Phytochemical screening of the crude ethanolic extract of *Spondias pinnata* fruit.
- Screening of in vitro antioxidant activity of crude ethanolic extract.
- Investigation of in vitro antimicrobial activity of crude ethanolic extract and determination of Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) of crude ethanolic extract.
- Investigation of *in vitro* thrombolytic activity of crude ethanolic extract.

2.1.1 Phytochemical Screening

Freshly prepared crude extracts of *S. pinnata* were qualitatively tested for the presence of various chemical constituents including alkaloids, flavonoids, steroids, terpenoids, reducing sugars, tannins, anthraquinone, cardiac glycoside, and saponins by following standard procedures.

2.1.2 Evaluation of Antioxidant activity

Antioxidant test was done to determine the antioxidant capacity of the crude ethanolic extract of the *S .pinnata* fruit. Antioxidant effects play an important role in many human diseases, including cancer, diabetic, complications, heart disease, liver damage, autism and Alzheimer's disease, *etc*. Recently, free radicals (FR) and reactive oxygen species (ROS) have been considered as one of the main causes of these diseases. The protective effects of antioxidants on cell membrane lipid bilayers attacked by free radicals are attracting more interest (Lai SC *et al.,* 2010). Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease (Miller, H.E. *et al.,* 2000). Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. They exert their effects by scavenging reactive oxygen species, activating a battery of detoxifying proteins, or preventing the generation of reactive oxygen species (Halliwell, 1992). It has been reported that many compounds such as phenol acids, flavonoids, saponins, tannins, alkaloids and polysaccharides have antioxidant activity in vitro or in vivo. These compounds are abundant in herbs and food additives. A number of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been extensively added to foodstuffs, although their use has begun to be questioned because of their toxicity (Ito N *et al.,* 1985). Therefore more recently, interest in the use of natural antioxidants obtained from botanical sources, especially herbal plants for the prevention and treatment of cancer has increased greatly, and oxidative stress was shown to influence treatment efficacy and survival of non-small cell lung cancer patients (Gupta A *et al.,* 2010)

Antioxidant property of the various fraction of the plant was determined by following methods-

1. Determination of total phenolic content
2. Determination of DPPH radical scavenging assay (Qualitative and Quantitative analysis)
3. Determination of reducing power ability
4. Determination of total antioxidant capacity by phosphomolybdenum method
5. Determination of total flavonoids content

2.1.3 Antimicrobial Screening

The *in vitro* antimicrobial study was designed to investigate the antibacterial as well as antifungal spectrum of the crude ethanolic extract by observing the growth response. The rationale for these experiments is based on the fact that bacteria and fungi are responsible for many infectious diseases, and if the test material inhibits bacterial or fungal growth then it may be used in those particular diseases. However, a number of factors viz. the extraction method (Nadir *et al.*, 1986), inocula volume, culture medium composition (Bauer *et al.*, 1966), pH (Levan *et al.*, 1979), and incubation temperature (Lorian, 1991) can influence the results.

Antimicrobial activity was observed by using two methods. The methods are-

a. Kirby- Bauer disk diffusion method.

b. Determination of Minimum Inhibitory Conentration (MIC) & Minimum Bactericidal Concentration (MBC).

2.1.4 Evaluation of Thrombolytic Activity

One of the major causes of blood circulation problem is the formation of blood clots. Thrombi or emboli can lodge in a blood vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. This can result in damage, destruction (infarction), or even death of the tissues (necrosis) in that area (Vorvick LJ, 2010). A blood clot (thrombus) is formed from fibrinogen by thrombin and is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator (tPA). Fribrinolytic drugs has been used to dissolve thrombi in acutely occluded coronary arteries there by to restore blood supply to ischaemic myocardium, to limit necrosis and to improve prognosis (Laurence DR, 1992). Streptokinase is an antigenic thrombolytic agent used for the treatment of acute myocardial infarction. It reduces mortality as effectively as the nonantigenic altreplase in most infarct patients while having the advantages of being much less expensive. Tissue Plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators. All available thrombolytic agents still have significant shortcomings, including the need for large doses to be maximally effective, limited fibrin specificity and a significant associated bleeding tendency.

Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs (Nicolini FA *et al.,* 1992; Adams DS *et al.,* 1991; Lijnen HR *et al.,* 1991).The plant kingdom represents an enormous reservoir of biologically active compounds with various chemical structures and disease preventive properties. Nearly 50% of drugs used in medicine are of plant origin, and only a small fraction of plants with medicinal activity has been assayed. Therefore much current research devoted to the phytochemical investigation of higher plants which have ethnobotanical information associated with them. The phytochemicals isolated are then screened for different types of biological activity like thrombolytic potentials (Harborne JB, 1998). Herbal preparations are used potential source of medicine since ancient times to maintain health and regain healthy state of mind. Herbs showing thrombolytic activity have been studied and some significant observations have been reported (Basta G *et al.,* 2004)

CHAPTER

3

Collection, Preparation and Phytochemical Screening

Chapter

3

Collection, Preparation and Phytochemical Screening

3.1 Collection and preparation of the plant material

Spondias pinnata fruit was collected from Mirzapur (Tangail) in August 2011. The plant was identified by Bangladesh National Herbarium. One voucher specimen has been deposited in Bangladesh National Herbarium (DACB accession no. 36703) and another one to East West University. After removing the peel or rind & seed, the fruit was then cut into small pieces & was air dried for several days. After drying, the net weight of the fruit was reduced to about 500 gm from 18 kg. The dried fruit pieces were then ground in coarse powder in Holy Chemical Lab (Tongi.) using high capacity grinding machine.

3.2 Extraction of the Plant material

The air-dried and pulverized plant material (about 500gm) was taken in a separate clean container (2.5 L) and soaked in 2 liters of ethanol. The container with its content was then sealed by cotton plug and aluminum foil and kept for a period of 10 days. Within this period of time, the container was shaken occasionally. The whole mixture was then filtered through cotton followed by Double rings filter paper (9.0 cm) and the filtrate thus obtained was concentrated at 55°C with a rotary evaporator (IKA HB10 Basic, Biometra). The concentrated extract was then air dried to solid residue. The weight of the crude ethanolic extract obtained was 13.4 gm.

Figure 3.1 Rotary Evaporator (IKA HB10 Basic, Biometra)

3.3 Preliminary phytochemical investigations of crude extract

Freshly prepared crude extracts of *Spondias pinnata* were qualitatively tested to detect the presence of various secondary metabolites of the plant including alkaloids, flavonoids, steroids, terpenoids, reducing sugars, tannins, anthraquinone, cardiac glycoside, and saponins by following standard procedures.

3.3.1 Apparatus

Test tube	Filter paper
Conical flask	Bunsen burner
Beaker	Pipette
Electronic balance	Dropper
Test tube holder	Vortex mixer

3.3.2 Reagents

Concentrated Sulphuric acid (H_2SO_4)	Methanol
Dragandroff reagent (Solution A & Solution B) Solution A: Bismuth Nitrate (0.17g) in AcOH (2ml) & H_2O (8ml) Solution B: KI (4g) in AcOH (2ml) & H_2O (8ml)	Dilute (10%) ammonia
1% HCl	Chloroform
Acetic anhydride	Distilled water
Fehling's solution (Solution A & Solution B)	0.1 % Ferric Chloride
Dilute Sulphuric acid (H_2SO_4)	Glacial acetic acid
Olive oil	

3.3.3 Methods & results of preliminary phytochemical investigations of crude extract

Preliminary qualitative chemical tests were carried out on the ethanolic extract of *S. pinnata* (fruit) using standard procedures to identify the constituents. (Sofowara A, 1993; Harborne JB, 1998; Kokate CK, 2001; Trease and Evans, 1989)

Secondary metabolite	Testing procedure	Result
Alkaloids (Dragandroff's test)	10 ml methanol was added to 20 mg plant crude extract. Then it was placed in sonic bath to dissolve. Then it was filtered & 2 ml filtrate was mixed with 1% HCl. 6 drops of Dragandroff reagent was added to 1 ml of the liquid. An orange precipitate indicates the presence of alkaloid.	Alkaloids are present in the crude ethanolic extract of *Spondias pinnata* fruit.
Flavonoids	5 ml dilute (10%) ammonia solution was added to a 4 ml of the filtrate of crude extract. Then 1 ml conc. H_2SO_4 was added. A yellow color indicates the presence of flavonoids.	Flavonoids are present in the crude ethanolic extract of *Spondias pinnata* fruit.

Steroids	1 ml methanol was added to 20 mg plant crude extract. Then it was filtered. Then 1 ml chloroform & 1 ml conc. H_2SO_4 was added to the filtrate. The upper layer turns red and sulphuric acid layer showed a yellow green fluorescence indicates the presence of steroids.	Steroids are present in the crude ethanolic extract of *Spondias pinnata* fruit.
Terpenoids (Salkowski test)	4 mg extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. 3 ml conc. H_2SO_4 was added slowly. A red violet color indicates the presence of terpenoids.	Terpenoids are present in the crude ethanolic extract of *Spondias pinnata* fruit.
Reducing Sugars (Fehling test)	1 ml distilled water and 5-8 drops of Fehling's solutin (both A & B are added in equal amount) was added to 0.5 ml of extract solution. Then it was heated. A brick red precipitate indicates the presence of reducing sugars.	Reducing sugars are absent in the crude ethanolic extract of *Spondias pinnata* fruit.
Tannins	0.5 ml of dried crude extract was boiled in 20 ml of distilled water in a test tube. Then it was filtered. Then 1-2 drops of 0.1% ferric chloride was added. A brownish or blue-black color indicates the presence of tannins.	Tannins are present in the crude ethanolic extract of *Spondias pinnata* fruit.
Anthraquinones	0.5 ml of dried crude extract was boiled with 10 ml dilute H_2SO_4. Then it was filtered while hot. The filtrate	Anthraquinones are absent in the crude ethanolic extract of *Spondias pinnata* fruit.

	was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube. 1 ml dilute ammonia was then added to it. A change in color of the resulting solution indicates the presence of anthraquinones.	
Cardiac glycosides (Keller-Killani test)	5 ml crude extract was mixed with 2 ml glacial acetic acid & 1 drop of ferric chloride. 1 ml conc. H_2SO_4 was then added. A brown ring at the interface indicates the deoxysugar characteristics of cardenolides and the presence of cardiac glycosides.	Cardiac glycosides are present in the crude ethanolic extract of *Spondias pinnata* fruit.
Saponins	5 ml distilled water was added to a test tube containing 0.5 g of crude extract. Then the solution was shaken vigorously & observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil & shaken vigorously. Formation of an emulsion indicates the presence of saponins.	Saponins are present in the crude ethanolic extract of *Spondias pinnata* fruit.

CHAPTER

4

Antimicrobial Screening

Chapter

4

Antimicrobial Screening

4.1 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. 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. 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.

i) Disc diffusion method

ii) Serial dilution method

iii) Bioautographic method

But there is no standardized method for expressing the results of antimicrobial screening (Ayafor *et al*., 1982). Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods, inoculum volume, culture medium composition (Bayer *et al*., 1966), p^H, moisture and incubation temperature can influence the results.

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. That's why the antimicrobial ability of the crude ethanolic extract of *Spondias pinnata* is estimated by Disc diffusion method. 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).

4.2 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, fractions as well as some pure compounds 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).

4.3 Experimental Design

4.3.1 Apparatus and Reagents

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

4.3.2 Test Organisms

The bacterial and fungal strains used for the experiment were collected as pure cultures from the East West University microbiology lab. Both gram positive and gram-negative organisms were taken for the test and they are listed in the Table 4.1.

Table 4.1 List of test microorganisms used for antimicrobial screening

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

4.3.3 Test Materials of *Spondias pinnata*

Crude ethanolic extract

4.3.4 Culture Medium and its Composition

Nutrient agar medium is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms. The agar medium was directly brought from the market. The composition of the culture medium is stated below:

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.	100 ml
p^H	7.2 ± 0.1 at 25^0C

4.3.5 Preparation of the Medium

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

4.3.6 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.

4.3.7 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.

4.3.8 Preparation of the Test Plate

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

4.3.9 Preparation of Discs

Three types of discs were used for antimicrobial screening.

4.3.10 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, Cephradin (30µg/disc/disc) standard disc was used as the reference.

4.3.11 Blank Discs

These 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.

4.3.12 Preparation of Sample Discs with Test Sample

Measured amount of each test sample (specified in table) was dissolved in specific volume of solvent (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 4.2 Preparation of Sample Discs

Test Sample	Dose (μg/disc)	Required amount for 20 disc (mg)
Crude ethanolic extract of *Spondias pinnata* fruit	400	8.0
Crude ethanolic extract of *Spondias pinnata* fruit	800	16

4.3.13 Application of the Test Samples

Standard Cephradin (30 μg/disc/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.

4.3.14 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 4^0C 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.

Figure 4.1 Laminar air flow

Figure 4.2 Incubation of petri dish

4.3.15 Determination of Antimicrobial Activity by Measuring the Zone of Inhibition

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

Figure 4.3: Zone of inhibition

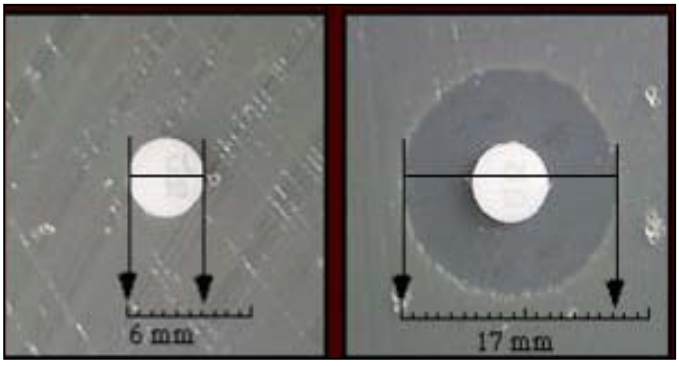


Figure 4.4: Determination of clear zone of inhibition

4.4 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The aim of this study was to determine the minimum inhibitory concentration (MIC) of the crude ethanolic extract of *Spondias pinnata* (fruit) required to kill *Staphylococcus aureus, Salmonella*

paratyphi, Salmonella typhi and Shigella boydii. In the experiment, medicaments were added to bacterial species into eppendrof tube, in 10 different concentrations. The MIC is the lowest concentration of the crude drug at which bacterial growth could not be observed.

4.4.1 Principle of MIC

The disc diffusion method is a semi quantitative method. Determination of MIC values provides a quantitative measure for the level of resistance expressed by the test organism. Next to the agar dilution method, the broth dilution method is one of the most frequently used methods to determine minimum inhibitory concentration (MIC) of the crude extract. The method is based on the inoculation of a standardized liquid culture of the test strain in a dilution series of the extract for which the MIC is determined. The first concentration of the dilution series at which no visual growth of microorganism can be determined is considered as the MIC (Huys G, 2002).

4.5 Experimental Design for MIC & MBC

4.5.1 Apparatus and Reagents

Nutrient Agar Medium	Autoclave
Nutrient Broth Medium	Eppendrof tube
Petridishes	Laminar air flow hood
Sterile cotton	Spirit burner
Micropipette	Refrigerator
Inoculating loop	Incubator
Sterile forceps	Ethanol
Screw cap test tubes	Nosemask and Hand gloves

4.5.2 Test Organisms

The bacterial strains used for the experiment were collected as pure cultures from the East West University microbiology lab. Both gram positive and gram-negative organisms were taken for the test and they are listed in the Table 4.3.

Table 4.3 List of microorganisms used for MIC & MBC

Gram positive bacteria	**Gram negative bacteria**
Staphylococcus aureus	*Salmonella paratyphi*
Bacillus subtilis	*Salmonella typhi*
	Shigella boydii

4.5.3 Test Materials of *Spondias pinnata*

Crude ethanolic extract

4.5.4 Culture Medium and their Composition

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

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. 100 ml
P^H	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. 100 ml
P^H	7.2 ± 0.1 at 25^0C

4.5.5 Method of determination of MIC & MBC (Huys G, 2002)

I. Bacterial cultivation and material preparations (Day 1-2)

• The organism to be tested should be sub cultured using a suitable medium under optimal incubation conditions to obtain a fresh overnight grown culture. As standard conditions, growth in Agar and Broth at 28 °C under aerobic atmosphere is recommended. For sub culturing,

remove one bead from the cryovial and streak out onto a nutrient agar plate. Incubate at 28°C overnight (or longer until clear visible growth is observed).

• After overnight incubation, the streak cultures are checked for purity. A number of pure colonies (app. 5 or more if the isolate is a suspected fastidious organism) are introduced into a glass culture tube containing 10 ml broth and incubated at 28°C overnight (or longer until clear visible growth is observed). Ideally, the culture tubes should fit into a portable spectrophotometer. In this way, the overnight grown culture can be easily adjusted to a standardized cell density by dilution with sterile broth

• Control cultures should be included during each series of MIC determinations.

• A bottle with the necessary volume of double-distilled water should be prepared.

II. Preparation and inoculation of the dilution series (Day 3)

• The optical density of the overnight culture of the strain is determined spectrophotometrically at 590 nm and is standardized at 0.1 ± 0.02 (i.e. app. 10E8 CFU/ml) by diluting with sterile broth.

• For each batch of 4 strains, two 6 ml sterile stock solutions of extract should be prepared in the suitable solvent. However, subsequent dilutions of these stock solutions can be made up in sterile water. The use of two stock solutions is recommended to minimize quantitative errors in the low concentration range of the serial dilution series.

• Extract concentrations 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, and 0.078125 mg/ml are obtained by making serial dilutions from stock solution. It is absolutely crucial to thoroughly mix every freshly prepared antibiotic dilution prior to using it to prepare the next dilution. It should be noted that each antibiotic dilution undergoes a final 1:2 dilution when the broth culture is added. If required, the tested MIC range can be extended with additional concentrations.

• Following the preparation of the serial dilutions of extract, 2 ml of freshly standardized broth culture of the strain is inoculated in each tube of the dilution series. In this regard, it is important to note that the standardized cultures should be processed within the hour after preparation. Dilutions and broth cultures should be well homogenized prior to mixing.

• For each batch of MIC determinations, a blank tube (i.e. 2 ml non-inoculated broth mixed with 2 ml water) should be included. In addition, a positive control should be included for each strain.

The positive control is made up by mixing 2 ml adjusted broth culture with 2 ml sterile double distilled water.

• All MIC tubes and control tubes of the test isolates and the control strain as well as the blank are incubated aerobically at 28°C for 24h.

• Each isolate should also be checked for purity by plating a drop of the adjusted culture onto agar medium, and this plate should be incubated under the same conditions as the MIC test itself.

III. Reading of the MIC (Day 4)

• The purity of the broth culture is checked on agar on the basis of uniform colonial morphology. If contamination is noted than all data generated from the involved strain should be rejected.

• Following a 24 hour incubation (or longer until clear visual growth can be determined in the positive control tubes), growth is determined visually among the different tubes of the serial dilution by comparing with the positive control and with the blank. Any series where discontinuity in growth is observed (e.g. growth in tubes 5 and 7 but not in tube 6) should be discarded. The end-point is defined as the lowest antibiotic concentration for which there is no visual growth. This concentration should be reported as the MIC of that antibiotic for that particular strain. If trailing end-points are observed, this should be reported as a remark and a 80% reduction in growth should be reported as end-point.

Then a sterilized cotton bud is dipped into the clear solution obtained then applied to an agar plate.

IV. Reading of the MBC (Day 5)

The agar plates then checked for the growth.

CHAPTER

5

Evaluation of Antioxidant Activity

Chapter

5

Evaluation of Antioxidant Activity

5.1 Rational and objective

There is considerable recent evidence that free radical induce oxidative damage to biomolecules. This damage causes cancer, aging, neurodegenerative diseases, atherosclerosis, malaria 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 diseases (Steinmetz and Potter, 1996; Aruoma, 1998; Bandoniene *et al*., 2000; Pieroni *et al.,* 2002; Couladis *et al.,* 2003). A number of reports on the isolation and testing of plant 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; Pietta *et al.,* 1998)

Lipid peroxidation is one of the main reasons for deterioration of food products during processing and storage. Synthetic antioxidant such as *tert*-butyl-1-hydroxytoluene (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 *et al*., 1983). Therefore, the interest in natural antioxidant, especially of plant origin, has greatly increased in recent years (Jayaprakasha & Jaganmohan Rao, 2000)

5.2 Mechanism of Antioxidant

Antioxidant may be defined as 'any substance that when present at low concentrations, compared with those of the oxidizable substrate, significantly delays or inhibits oxidation of that substrate' (Gutteridge, J.M.C, 1994). For convenience, antioxidants have been traditionally divided into two classes, primary or chain breaking antioxidants and secondary or preventative antioxidants. Secondary or preventative antioxidants are compounds that retard the rate of oxidation. This may be achieved in a number of ways including removal of substrate or singlet oxygen quenching. Primary antioxidants, AH, when present in trace amounts, may either delay or inhibit the initiation step by reacting with a lipid radical ($L^{\bullet}$) or inhibit the propagation step by reacting with peroxyl or alkoxyl radicals:

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet}$$
$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$$
$$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet}$$

The antioxidant free radical may further interfere with chain propagation reactions by forming peroxy antioxidant compounds: (Jadhav SJ *et al.,* 1996)

$$A^{\bullet} + LOO^{\bullet} \rightarrow LOOA$$
$$A^{\bullet} + LO^{\bullet} \rightarrow LOA$$

Chain breaking antioxidants may occur naturally or they may be produced synthetically as in the case of Butylated hydroxyanisole, Butylated hydroxytoluene, *tert*-Butylhydroquinone and the gallates. The use of naturally occurring antioxidants has been promoted because of concerns regarding the safety of synthetic antioxidants (Williams GM *et al.,* 1999), with natural alternatives (*e.g.*, plant biophenols) possessing antioxidant activity similar to or even higher than that of synthetic antioxidants (Velioglu YS *et al.,* 1998)

5.3 Methods of evaluating antioxidant activity

Antioxidant property of the various fraction of the plant was determined by following methods-

1. Determination of DPPH radical scavenging assay (Quantitative analysis)
2. Determination of total phenolic content
3. Determination of reducing power ability
4. Determination of total flavonoids content
5. Determination of total antioxidant capacity by phosphomolybdenum method

5.3.1 Determination of DPPH radical scavenging assay

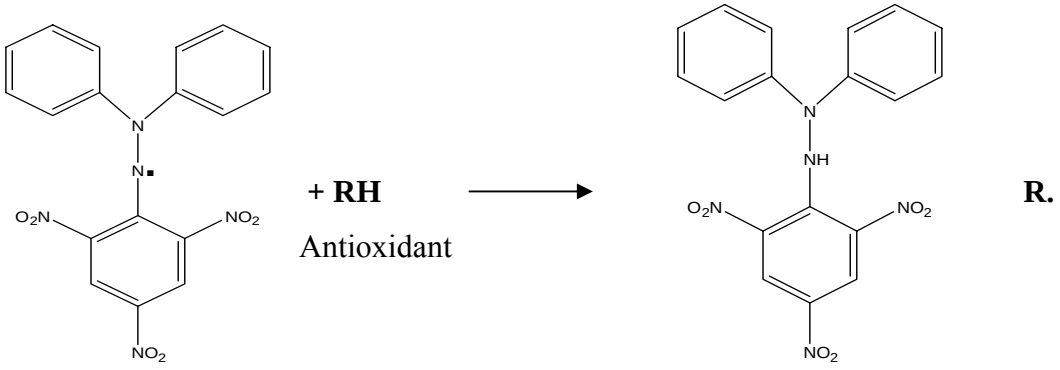
Antioxidant activities of the crude ethanolic extract of *Spondias pinnata* (fruit) was determined on the basis of their scavenging potential of the stable DPPH free radical in both qualitative and quantitative assay.

5.3.1 (1) Qualitative assay: A suitably diluted stock solutions were spotted on pre-coated silica gel TLC plates and the plates were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extracts. The plates were dried at room temperature and were sprayed with 0.02% DPPH in ethanol. Bleaching of DPPH by the resolved band was observed for 10 minutes and the color changes (yellow on purple background) were noted (Sadhu *et al.,* 2003).

5.3.1 (2) Quantitative assay

5.3.1.1 Principle of Quantitative Assay (Miller E *et al.,* 2000)

A rapid, simple and convenient method to measure free radical scavenging capacity of antioxidants involves the use of the free radical, 1,1-Diphenyl-2-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity. DPPH is a stable nitrogen centered free radical with purple color and the odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm. When the odd electron of DPPH radical becomes paired with a hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H, then the color turns from purple to yellow as the molar absorptivity of the DPPH radical reduces from 9660 to 1640 at 517 nm. Scavenging of DPPH free radicals by antioxidants decreases the absorbance. The lower the absorbance at 517 nm, the greater the free radical scavenging capacity of the crude extracts.



DPPH (oxidized form)
Diphenyl picrylhydrazyl (λ_{max} 517 nm)

DPPH (reduced form)

5.3.1.2 Materials & Reagents

1,1-diphenyl-2-picrylhydrazyl	UV- visible spectrophotometer
L-Ascorbic acid	Beaker (100 & 200ml)
Distilled water	Test tube
Methanol	Aluminium foil
Pipette (5ml)	Spatula
Analytical balance	

5.3.1.3 Methods (Choi *et al.,* 2000; Desmarchelier *et al.,* 1997)

- 2.0 ml of a methanol solution of the extract at different concentration (2, 4, 6, 8, 10µg/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 using a UV- visible spetrophotometer.
- 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). L-Ascorbic acid was used as positive control.

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

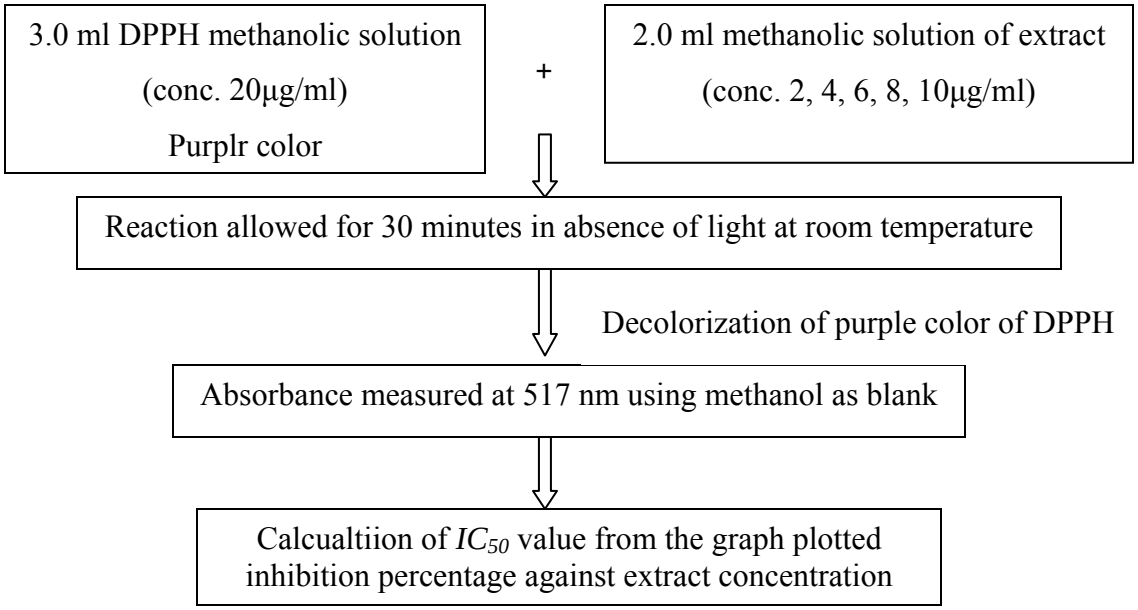


Figure 5.1 Schematic representation of the method of assaying free radical scavenging activity

5.3.2. Determination of total phenolic content

The antioxidative effect is mainly due to phenolic components, such as flavonoids (Pietta, 1998), phenolic acids, and phenolic diterpenes (Shahidi *et al*., 1992). The phenolic compounds exert their antioxidant properties by redox reaction, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994). Many phytochemicals possess significant antioxidant capacities that may be associated with lower incidence and lower mortality rates of cancer in several human populations (Velioglu *et al*., 1998).

5.3.2.1 Principle

The total phenolic concentration of the extract of *Spondias pinnata* fruit was determined by the modified Folin-Ciocalteu method (Wolfe *et al.,* 2003). The process of measuring total phenolic content of the crude extract of *Spondias pinnata* fruit involves the use of Folin-Ciocalteu reagent. The Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidants. It measures the amount of substance being tested needed to inhibit the oxidation of the Folin-Ciocalteu reagent (Singleton VL *et al.,* 1999; Vinson JA *et al.,* 2005). The reagent does not contain phenol. Rather, the reagent will react with phenols and nonphenolic reducing substances to form chromogens that can be detected spectrophotometrically. The color development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic-phosphotungstic acid complexes to form chromogens in which the metals have lower valence. The generated chromogens give a strong absorption maximum at 760 nm (Bray HG and Thorpe WV, 1954)

$$\text{Phenols} \xrightarrow[\text{Complete ionization}]{\text{Alkaline condition}} \text{Ionized Phenols}$$

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

5.3.2.2 Materials & Reagents

Test tube	Analytical balance
Pipette	UV- visible spectrophotometer
Spatula	Vortex mixer

Folin-Ciocalteu reagent	Distilled water
Sodium carbonate (Na_2CO_3)	Methanol
Gallic acid	Aluminium foil

5.3.2.3 Composition of Folin-Ciocalteu reagent

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

5.3.2.4 Methods (Wolfe *et al.*, 2003)

- 0.5 ml of a methanol solution of the crude extract of concentration of 1 mg/ml was mixed with 5 ml Folin ciocalteu reagent (1:10 v/v distilled water) and 4 ml (75g/L) of Sodium carbonate.
- The mixture was vortexed for 15 second and allowed to stand for 30min at room temperature in dark place for color development and the absorbance was measured at 760 nm against methanol as blank by using a UV- visible spetrophotometer.
- The total phenolics was expressed as mg of GAE (gallic acid equivalent) per gm of the dried extract using the following equation obtained from a standard Gallic acid calibration curve: $y = 0.0162x + 0.0215$, $R^2=0.9985$.

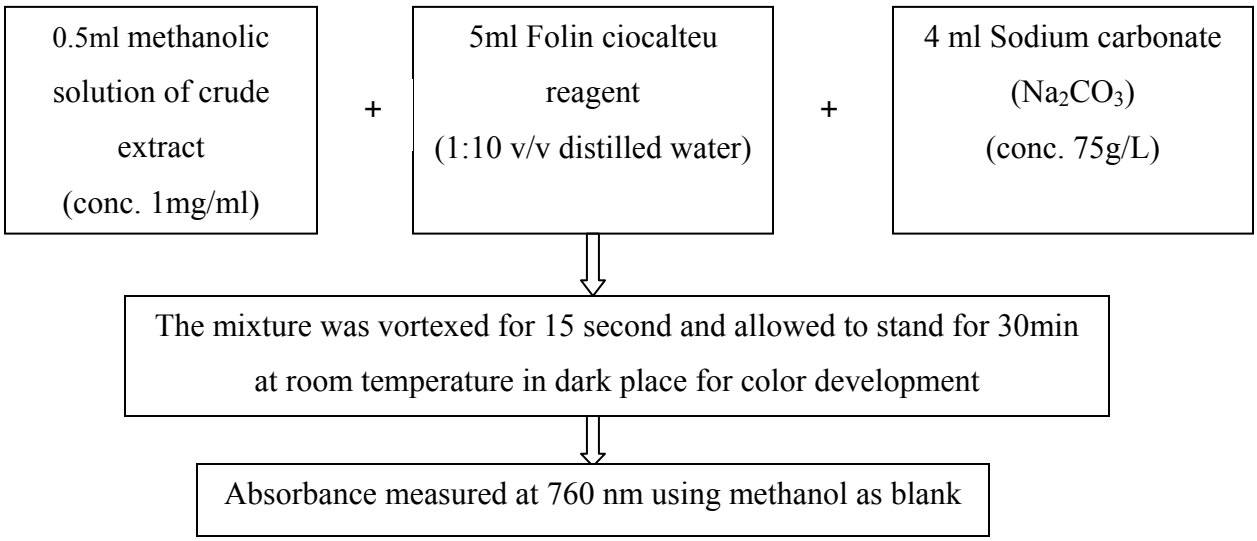


Figure 5.2 Schematic representation of the method of assaying total phenolic content

5.3.3 Determination of reducing power assay

5.3.3.1 Principle

The reducing power assay of the extract of *Spondias pinnata* fruit was determined according to the method previously described by (Oyaizu *et.al.,* 1986). Reducing power assay is based on the principle that substances which have reduction potential react with potassium ferricyanide [$K_3Fe(CN)_6$] to form potassium ferrocyanide [$K_4Fe(CN)_6$], which then reacts with ferric chloride to form ferrous complex that has an absorption maximum at 700nm (Hemalatha S *et al.,* 2010). The reducing capacity of a compound may serve as a significant indicator of it's potential antioxidant activity (Vishal DJ *et al.,* 2009). The presence of reducing agent such as antioxidant substances in the crude extract causes the reduction of the ferricyanide (Fe^{3+}) complex to the ferrous form (Fe^{2+}). The resulting ferrous complex (Fe^{2+}) can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. A blank was prepared without adding extract. Ascorbic acid at various concentrations was used as standard. Increased absorbance of the reaction mixture indicated the increased reducing power of the crude extract. The following reaction occurs in reducing power assay:

$$\mathbf{K_3Fe(CN)_6 + FeCl_3} \xrightarrow{\text{Antioxidant}} \mathbf{K_4Fe(CN)_6 + FeCl_2}$$ (Hemalatha S *et al.,* 2010)

5.3.3.2 Materials & Reagents

Test tube	Analytical balance
Pipette	UV- visible spectrophotometer
Spatula	Hot air oven
Vortex mixer	Centrifuge machine
0.2 M Phosphate buffer	1 % Potassium ferricyanide [$\mathbf{K_3Fe(CN)_6}$]
10 % Trichloroacetic acid (TCA)	Distilled water
Ferric chloride **($\mathbf{FeCl_3}$)**	L-ascorbic acid

5.3.3.3 Methods (Oyaizu *et.al.,* 1986)

- 1 ml of the methanol solution of the crude extract of different concentrations (1, 5, 10, 50, 100 µg/ml) was mixed with 2.5 ml phosphate buffer and 2.5 ml potassium ferricyanide [$K_3Fe(CN)_6$] (1%).
- The mixture was incubated at 50°C for 20min.

- 2.5 ml of trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged at 3000rpm for 10min.
- The upper layer of the solution was separated and mixed with 2.5 ml distilled water and 0.5ml $FeCl_3$.
- The absorbance was measured against a blank at 700nm.
- All the tests were carried out in triplicate and average absorption was noted for each time.
- L-Ascorbic acid was used as positive control.
- Percentage (%) increase in reducing power was calculated as follows:

  % Increase in reducing power = (A_{Test} / A_{Blank}) -1 X 100, where A_{Blank} is absorbance of blank (containing all reagents except the test material) and A_{Test} is absorbance of test solution.

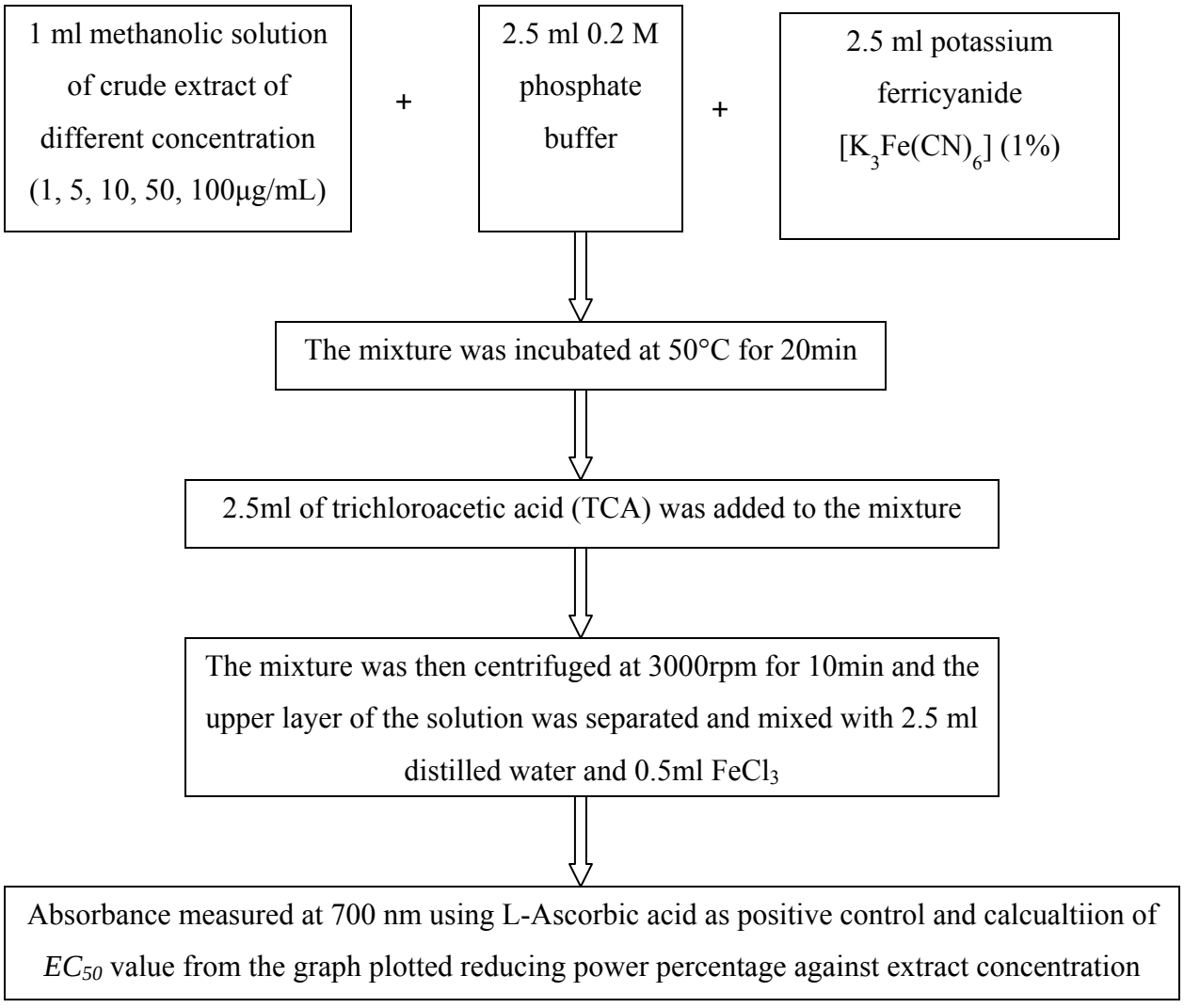


Figure 5.3 Schematic representation of the method of reducing power assay

5.3.4 Total Flavonoids Concentration

5.3.4.1 Principle

Aluminium chloride ($AlCl_3$) (Chang C *et al.,* 2002) colorimetric method is incorporated to determine the total flavonoid contents of the crude plant extract. The basic principle of the assay method is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols of the crude extract. In addition aluminium chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids. The formed flavonoid-aluminium complex between flavonoid of the crude extract and aluminium chloride has an absorptivity maximum at 415 nm (Chang C *et al.,* 2002). Therefore, the amount of flavonoid in the crude extract can be quantified by measuring the absorbance of reaction mixture at 415 nm using a UV-visible spectrophotometer against a blank containing all reagents except the extracts. Quercetin at various concentrations was used as standard.

Flavonoid (Extract) + $AlCl_3$ (reagent) = Formation of flavonoid-aluminium complex (λ_{max} 415 nm)

5.3.4.2 Materials & Reagents

Methanol	UV- visible spectrophotometer
10% aluminum chloride ($AlCl_3$)	Test tube
1M potassium acetate (CH_3COOK)	Aluminium foil
Distilled water	Spatula
Pipette (5ml)	Analytical balance

5.3.4.3 Methods (Chang C *et al.,* 2002)

- 0.5 ml of a methanol solution of the extract of concentration of 10 mg/ml was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. Sample blank was prepared in similar way by replacing aluminium chloride with distilled water.
- Both sample and blank solution were filtered through double rings filter paper before measuring the absorbance
- Both sample and blank solution were allowed to stand for 30 min at room temperature.
- After 30 minute incubation period, the absorbance of the reaction mixture was measured at 415 nm against a blank by using a UV- visible spectrophotometer.

- All the tests were carried out in triplicate and average absorption was noted for each time.
- The total flavonoids was expressed as mg of Quercetin equivalent per gram of dried extract by using the equation obtained from a standard Quercetin calibration curve $y = 0.002x + 0.0318$; $R^2 = 0.9989$.

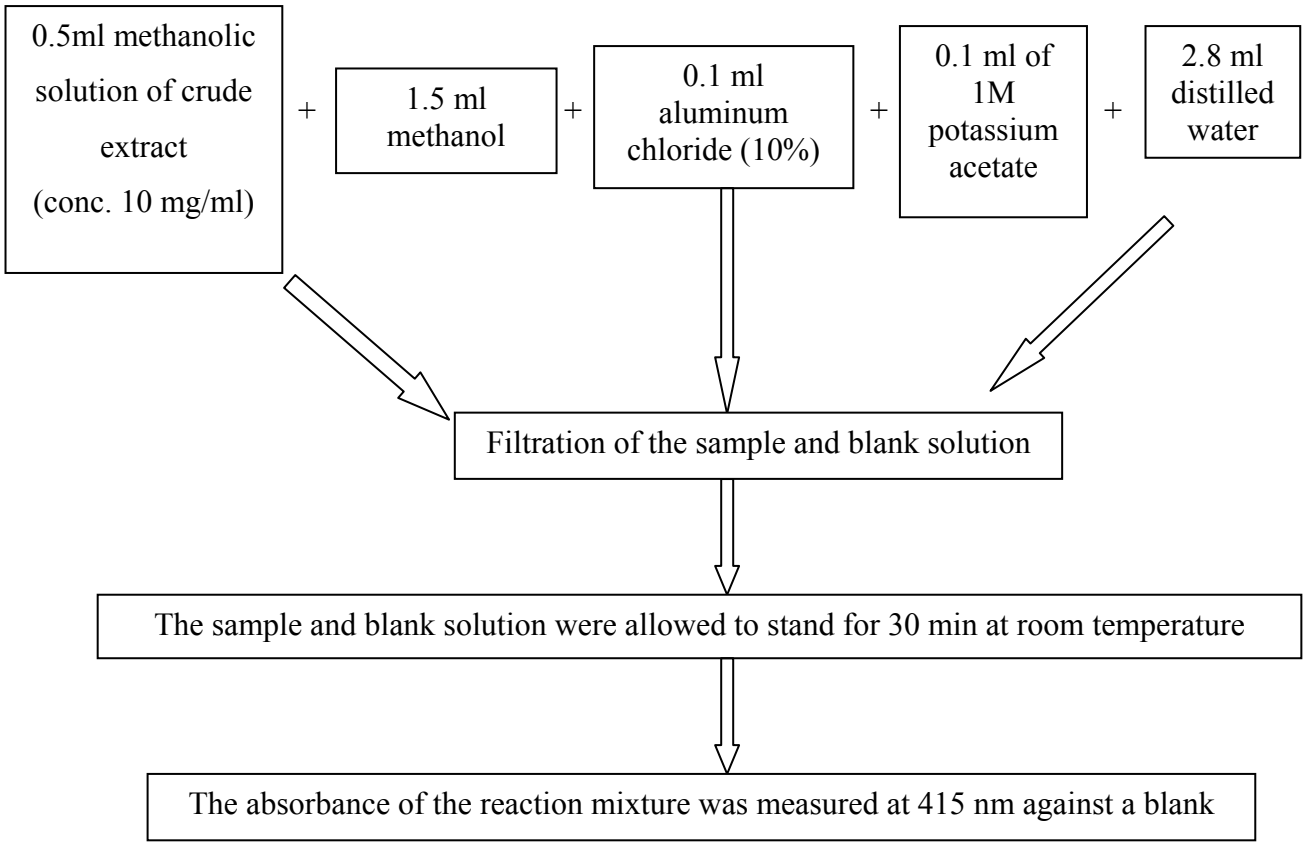


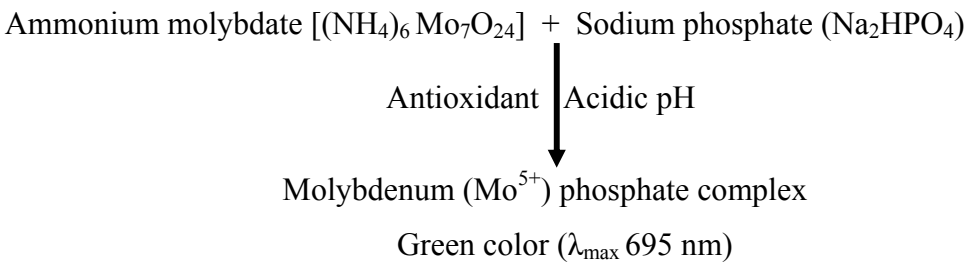
Figure 5.4 Schematic representation of the method of total flavonoids content assay

5.3.5 Determination of total antioxidant capacity by phosphomolybdenum method

5.3.5.1 Principle

Phosphomolybdenum assay method is a spectroscopic method for the quantitative determination of antioxidant capacity of crude plant extract. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte which has reduction potential and the subsequent formation of a stable green Mo (V) phosphate complex at acidic pH with the chromogenic reagent. The formed Mo (V) phosphate complex has an absorptivity maximum at 695 nm (Prieto *et al*., 1999). Therefore, the antioxidant capacity of the crude extract can be quantified by measuring the absorbance of reaction mixture at 695 nm after cooling to room temperature by using a UV-

visible spectrophotometer against a blank containing all reagents except the extracts. Ascorbic acid at various concentrations was used as standard. The higher absorbance value indicated higher antioxidant activity.



5.3.5.2 Materials & Reagents

Ethanol	UV- visible spectrophotometer
0.6 M Sulfuric acid (H_2SO_4)	Test tube
28mM Sodium Phosphate (Na_2HPO_4)	Aluminium foil
4mM Ammonium molybdate [$(NH_4)_6 Mo_7O_{24}$]	Spatula
Pipette (5ml)	Analytical balance
Hot air oven	L-Ascorbic acid

5.3.5.3 Methods (Prieto *et al*., 1999)

- 0.3 ml of ethanolic solution of the extract of concentration of 2 mg/ml was mixed with 3 ml of reagent solution (0.6 M Sulfuric acid, 28mM Sodium Phosphate and 4mM Ammonium molybdate) (all of them were taken in equal volume). Sample blank was prepared in similar way by replacing sample extract with ethanol.
- The reaction mixture was incubated at 95°C for 90 min.
- After that, the samples were allowed to cool to room temperature.
- The absorbance of the reaction mixture was measured at 695 nm against a blank by using a UV- visible spectrophotometer.
- All the tests were carried out in triplicate and average absorption was noted for each time.
- The total antioxidant activity of the crude extract was expressed as the number of grams of ascorbic acid equivalents per gram of dried extract by using the equation obtained from a standard Ascorbic acid calibration curve

  $y = 0.113x - 0.146$; $R^2 = 0.9916$.

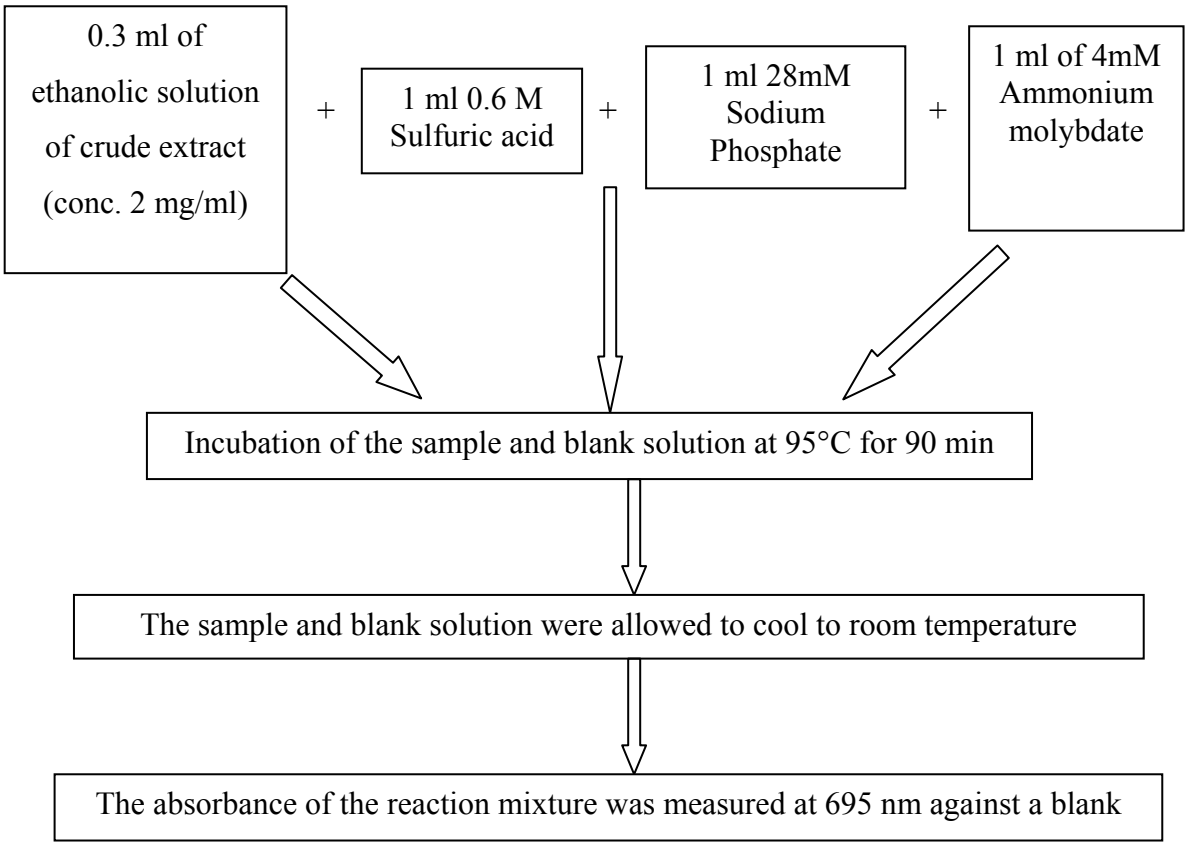


Figure 5.5 Schematic representation of the method of total antioxidant capacity assay

CHAPTER

6

Evaluation of Thrombolytic Activity

Chapter

6

Evaluation of Thrombolytic Activity

6.1 Introduction

Formation of blood clot (thrombus), one of the major causes of blood circulation problem can lodge in a blood vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. This may result in damage, destruction (infarction), or even death of the localized tissues (necrosis) in that area (Vorvick LJ, 2010). Thrombus is formed from fibrinogen by thrombin and is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator (tPA). All thrombolytic agents activate the enzyme plasminogen that clears the cross linked fibrin mesh. Fribrinolytic drugs can dissolve thrombi in acutely occluded coronary arteries thereby can restore blood supply to ischaemic myocardium and can limit necrosis (Laurence DR, 1992).

Streptokinase is an antigenic thrombolytic agent used for the treatment of acute myocardial infarction. It reduces mortality as effectively as the nonantigenic altreplase in most infarct patients. Tissue-type Plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators. All available thrombolytic agents still have significant shortcomings: (Nicolini FA *et al.,* 1992; Adams DS *et al.,* 1991; Lijnen HR *et al.*, 1991)

1. Large doses are required to be maximally effective.
2. Limited fibrin specificity.
3. Significant bleeding tendency.

The plant kingdom represents an enormous reservoir of biologically active compounds with diversified structures having disease preventive properties. Nearly 50% of drugs used in medicine are of plant origin, and only a small fraction of plants with medicinal activity has been assayed. Therefore much current research devoted to the phytochemical investigation of higher plants which have ethnobotanical information associated with them. The phytochemicals isolated

are then screened for different types of biological activity like thrombolytic potentials which could overcome the shortcomings of the current thrombolytic agents (Harborne JB, 1998).

6.2 Principle

The in vitro thrombolytic activity of the crude ethanolic extract *Spondias pinnata* (fruit) was determined according to the method reported earlier by Prasad, S. *et al.* (2006). Whole blood drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy was incubated for 45 minutes at 37°C to form clot. After clot formation, the serum was completely removed without disturbing the clot and the weight of the formed clot was taken. Addition of crude extracts having thrombolytic potential result in the lysis of some of the clot. For clot lysis, the tubes were incubated for 90 minutes at 37°C. Thereby the weight of the clot will decrease. Weight loss of clot after application of crude extract solution was considered as the functional indication of thrombolytic activity. The study was implemented on two volunteers and the average value of weight loss (in %) was calculated to examine the variation of two volunteer. Difference obtained in weight taken before and after clot lysis was expressed as percentage (%) of clot lysis as shown below: **(**Prasad, S. *et al.* 2006)

% clot lysis = (Weight of the released clot / Weight of clot before lysis) × 100

The thrombolytic potential of the crude extract is compared with streptokinase (standard thrombolytic agent).

Clot + Extract (thrombolytic potential) ⟹ Lysis of clot ⟹ Weight of clot decreases

6.3 Materials & Reagents

Eppendorf tube (500 µl/tube)	Syringe (5 ml)
Analytical balance	Distilled water
Micropipette (100 µl)	Test tube
Streptokinase	Vortex mixer
Filter paper (Double ring)	Blood Sample

6.4 Preparation of test sample **(**Prasad, S. *et al.* 2006)

100 mg of ethanolic extract of *Spondias pinnata* (fruit) was suspended in 10 ml distilled water and shaken vigorously on a vortex mixer. Then the suspension was kept overnight and decanted

to remove the soluble supernatant, which was filtered through filter paper. The solution was then ready for in vitro evaluation of clot lysis activity.

6.5 Methods (Prasad, S. *et al.* 2006)

- 10 sterile eppendorf tubes were taken and weights (W_1) were measured.
- 10 ml venous blood samples were drawn from two healthy human volunteers (5 ml from each) without a history of oral contraceptive or anticoagulant therapy.
- The blood samples were then equally distributed into 10 separate pre weighed (W_1) eppendorf tubes (each contains 1 ml) and incubated at 37°C for 45 minutes for clot formation.
- After clot formation, serum was completely decanted and removed without disturbing the clot formed.
- Each eppendorf tube having clot was again weighed to determine the clot weight.
  Clot weight (ΔW) = (weight of clot containing tube, W_2 – weight of tube alone, W_1)
- Each eppendorf tube containing clot was properly labeled and 100 µl of plant extract was added to six eppendorf tubes.
- Streptokinase (100 µl) was added to two eppendorf tubes. (Positive control)
- Distilled water (100 µl) was added to two eppendorf tubes. (Negative control)
- All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis.
- After incubation, fluid obtained was removed and eppendorf tubes were again weighed (W_3)
- The weight of released clot was then calculated.
  Weight of released clot = (weight of clot containing tube, W_2 – weight of tube after after clot disruption, W_3)
- Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

  % clot lysis = (Weight of the released clot / Weight of clot before lysis) × 100

  $$= [(W_2 - W_3) / (W_2 - W_1)] \times 100$$

- The study was implemented on two volunteers and the average value of weight loss (in %) was calculated to examine the variation of two volunteer.

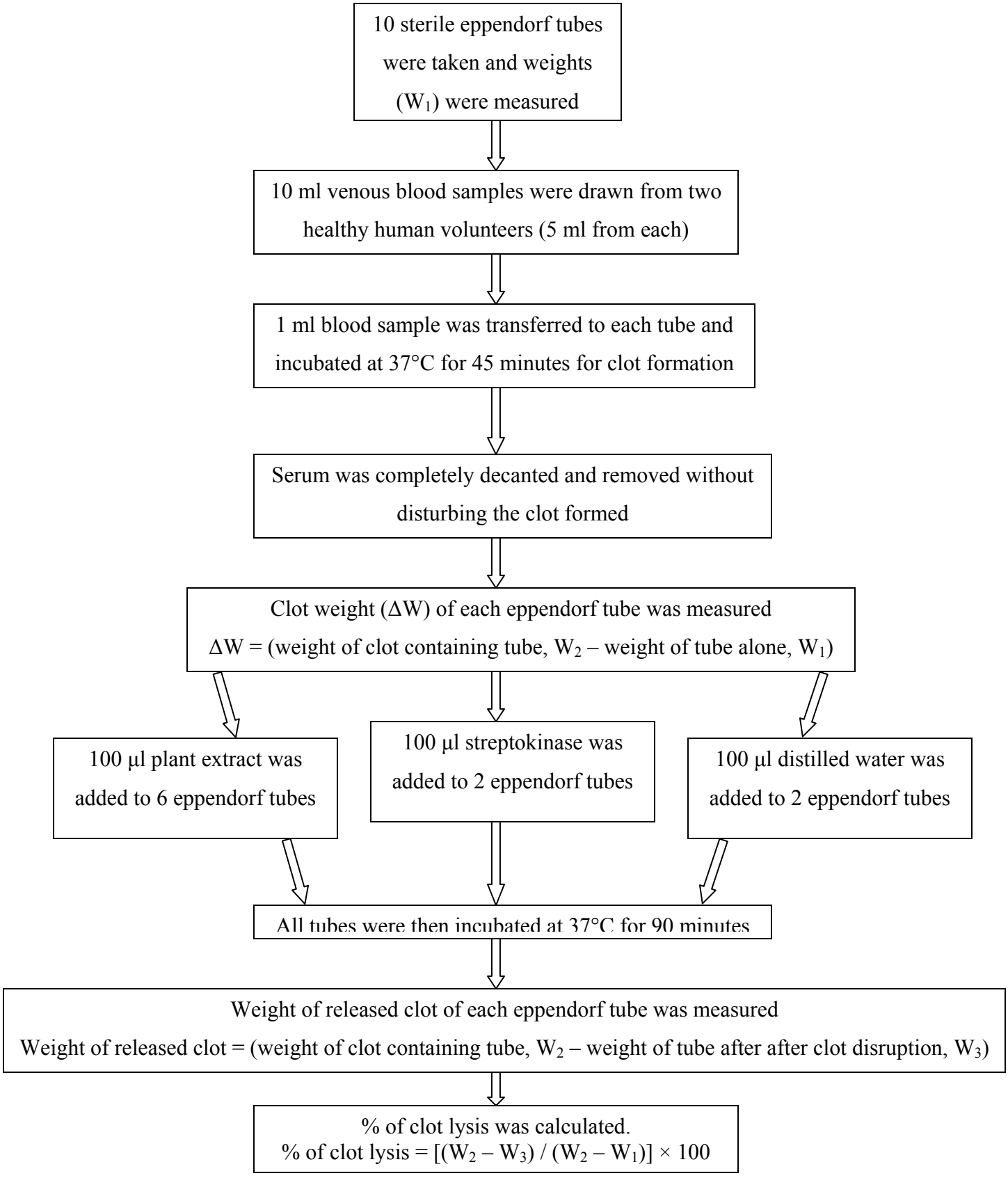


Figure 6.1 Schematic representation of the method of thrombolytic activity study

CHAPTER

7

Results and Discussion

Chapter

7

Results & Discussion

7.1 Results and discussion of the test samples of *Spondias pinnata* (fruit)

The aim of this chapter is to illustrate the results and discussions of crude ethanolic extract of *Spondias pinnata* (fruit). This chapter will include the following results and discussions of the test samples of *Spondias pinnata* (fruit):

1. In vitro antimicrobial screening
2. Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC)
3. In vitro antioxidant activity
    - 3.1. Total phenolic content
    - 3.2. DPPH radical scavenging assay (Quantitative analysis) and IC_{50}
    - 3.3. Reducing power assay
    - 3.4. Total antioxidant capacity by phosphomolybdenum method
    - 3.5. Total flavonoids content
4. In vitro thrombolytic activity

Besides the results of various tests are represented through suitable graphical representation methods. All the data are expressed as mean ± standard deviation (n=3).

7.1.1 In vitro antimicrobial screening

The antimicrobial activities of extracts were examined in the present study. The crude extract produced strong activity against a number of the test organisms. The results are given in table 7.1. The zones of inhibitions produced by the crude ethanolic extract of the fruit of *Spondias pinnata* were ranged from 0-12 mm at a concentration of 400µg/disc. While the zones of inhibition produced by by the crude ethanolic extract of the fruit of *Spondias pinnata* were ranged from 0-16 mm at a concentration of 800µg/disc.

The ethanolic extract showed greatest activity against *Sarcina lutea* having the zone of inhibition of 12 mm (400 µg/disc) and 16 mm (800 µg/disc). Besides the extract showed strong activity against *Staphylococcus aureus* (15mm for 800µg/disc and 10 mm for 400µg/disc), *Shigella boydii* (15 mm for 800µg/disc and 12 mm for 400µg/disc), *Salmonella paratyphi* (15 mm for 800µg/disc and 10 mm for 400µg/disc). Moderate activity was found against *Salmonella typhi* (13 mm for 800µg/disc and 9 mm for 400µg/disc), *Shigella dysentery* (13 mm for 800µg/disc and 7 mm for 400µg/disc), *Pseudomonas aeruginosa* (13 mm for 800µg/disc and 7 mm for 400µg/disc), *Candida albicans* (13 mm for 800µg/disc and 7 mm for 400µg/disc), *Bacillus subtilis* (12 mm for 800µg/disc and 7 mm for 400µg/disc), *Escherichia coli* (11 mm for 800µg/disc and 7 mm for 400µg/disc), *Vibrio mimicus* (10 mm for 800µg/disc and 6 mm for 400µg/disc), *Bacillus cereus* (9 mm for 800µg/disc and 5 mm for 400µg/disc). The crude ethanolic extract of *Spondias pinnata* fruit showed no activity against *Sacharomyces cerevacae*.

Among the tested fungi, the extract showed greatest activity against *Candida albicans* (13 mm for 800µg/disc and 7 mm for 400µg/disc). The crude extract failed to inhibit the growth of *Sacharomyces cerevacae* having zone of inhibition of 0 mm for both 400µg/disc and 800µg/disc respectively.

Among the tested Gram positive bacteria, the extract showed greatest activity against *Sarcina lutea* having the zone of inhibition of 12 mm (400 µg/disc) and 16 mm (800 µg/disc). Besides, the extract showed significant inhibitory activity against *Staphylococcus aureus* (15mm for 800µg/disc and 10 mm for 400µg/disc).

Among the tested Gram negative bacteria, the extract showed strong activity against *Shigella boydii* (15 mm for 800µg/disc and 12 mm for 400µg/disc) and *Salmonella paratyphi* (15 mm for 800µg/disc and 10 mm for 400µg/disc). Besides the extract showed a similar significant extent of inhibitory activity against *Shigella dysentery* and *Pseudomonas aeruginosa* having zone of inhibition of 13 mm for 800µg/disc and 7 mm for 400µg/disc against both of them. The extract showed a more potent inhibitory activity against *Salmonella typhi* (13 mm for 800µg/disc and 9 mm for 400µg/disc) than *Shigella dysentery* and *Pseudomonas aeruginosa*.

In brief, the ehanolic extract of *Spondias pinnata* fruit showed a potent inhibitory activity against *Sarcina lutea* and *Shigella boydii*.

Table 7.1 Antimicrobial activity of *Spondias pinnata* (Fruit)

Test microorganisms	Diameter of zone of inhibition (mm)		
	Crude extract (Dose 800 μg/disc)	Crude extract (Dose 400 μg/disc)	Control Cephradin (30 μg/disc)
Gram positive bacteria			
Bacillus cereus	9	5	25
Bacillus subtilis	12	7	26
Sarcina lutea	16	12	21
Staphylococcus aureus	15	10	15
Gram negative bacteria			
Escherichia coli	11	7	27
Salmonella paratyphi	15	10	25
Salmonella typhi	13	9	20
Shigella boydii	15	12	23
Shigella dysentery	13	7	22
Pseudomonas aeruginosa	13	7	25
Vibrio mimicus	10	6	22
Fungi			
Candida albicans	13	7	27
Sacharomyces cerevacae	0	0	20

The following bar diagram (Figure 7.1) illustrates the in vitro antimicrobial activity of the crude ethanolic extract of *Spondias pinnata* (fruit) in compared to the positive control cephradin. Besides some figures (Figure 7.2-7.8) show the zone of inhibition of crude plant extract for particular microorganism.

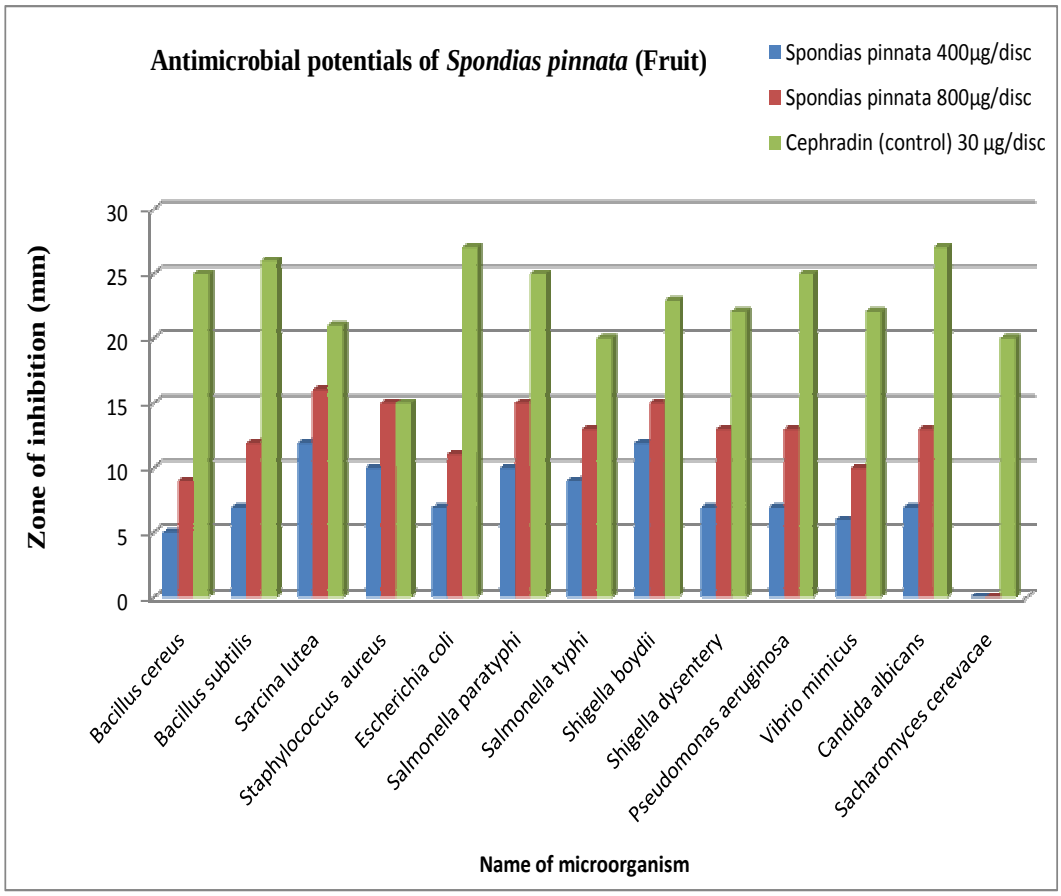


Figure 7.1 Comparison between the antimicrobial activities (zone of inhibition) of the crude ethanolic extract of *Spondias pinnata* (fruit) and positive control cephradin

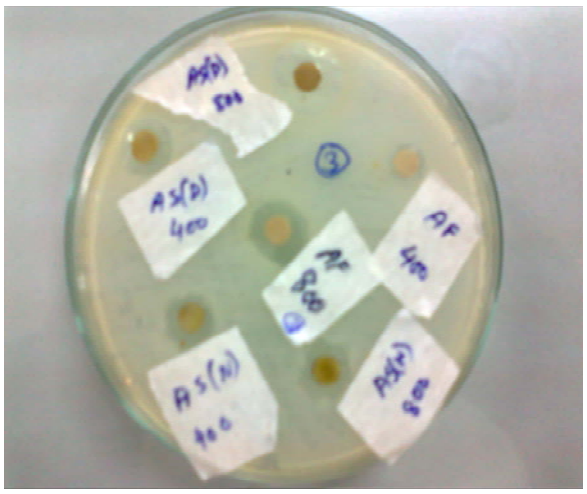


Figure 7.2: Zone of inhibition for *C. albicans*

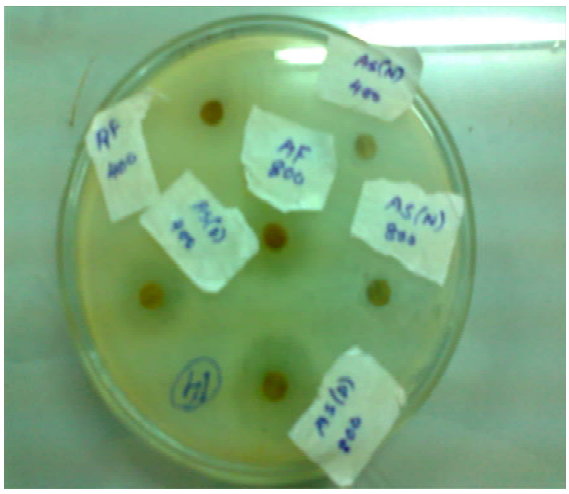


Figure 7.3: Zone of inhibition for *S. aureus*

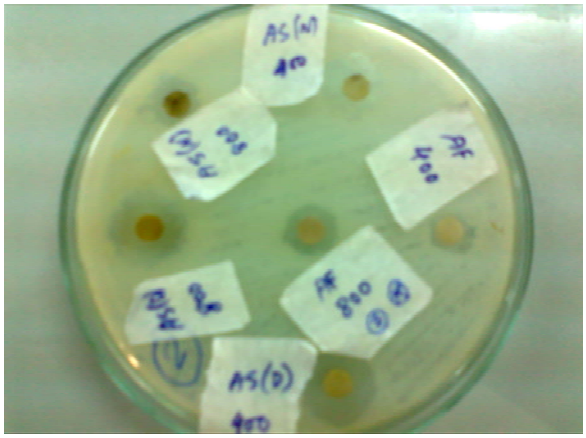


Figure 7.4: Zone of inhibition for *B. subtilis*

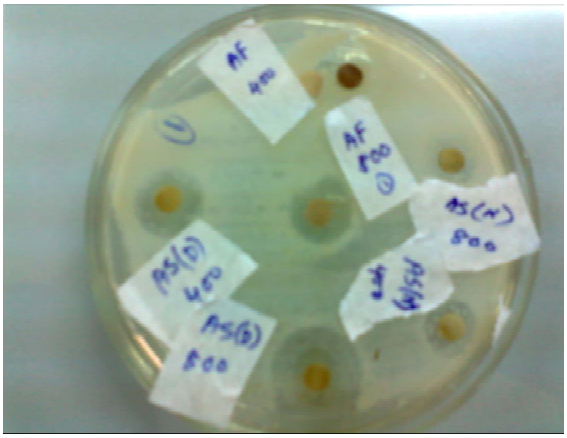


Figure 7.5: Zone of inhibition for *S. boydii*

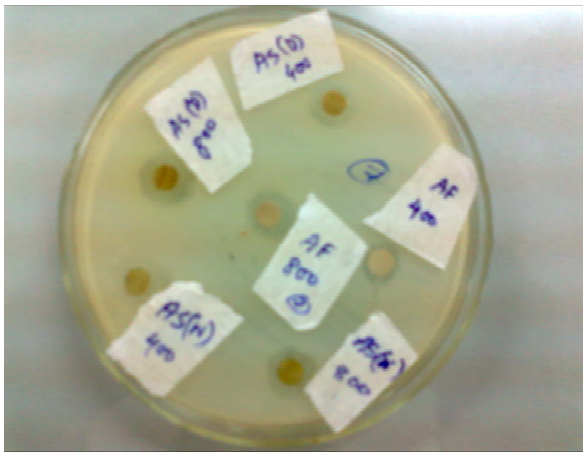


Figure 7.6: Zone of inhibition for *S. dysentery*

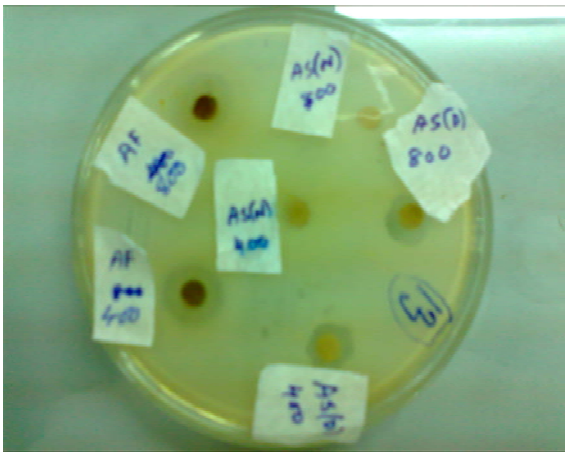


Figure 7.7: Zone of inhibition for *S. lutea*

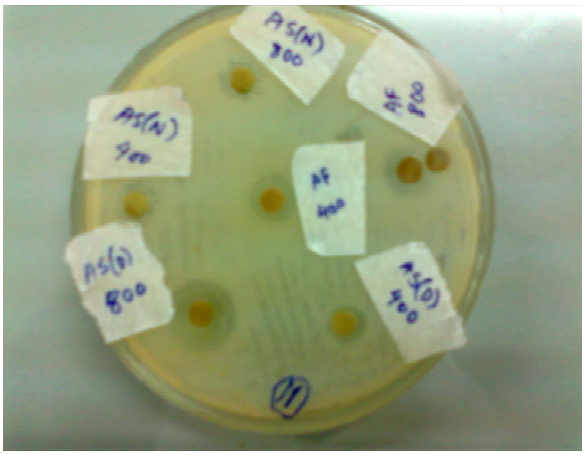


Figure 7.8: Zone of inhibition for *S. paratyphi*

7.1.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the crude extract was determined for five microorganisms (*S. aureus, S.typhi, S.paratyphi, B.subtilis,* and *S.boydii*) against which the crude ethanolic extract of *Spondias pinnata* (fruit) showed promising and potent antimicrobial activity in the preliminary in vitro antimicrobial screening test. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of crude ethanolic extract of *Spondias pinnata* (fruit) for the test microorganisms is expressed in table 7.2 and is shown by figures (Figure 7.9-7.11). The MIC for all five test organisms was 0.078125 mg/ml but the lowest MBC was found against *S. typhi* (0.3125mg/ml).

Table 7.2 Result of MIC and MBC

Extract	Bacteria									
	(1)*S. aureus*		(2)*S.paratyphi*		(3) *S. typhi*		(4)*B.subtilis*		(5)*S.boydii*	
	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml
S. pinnata fruit EtOH extract	0.078125	5	0.078125	1.25	0.078125	0.3125	0.078125	2.5	0.078125	0.625

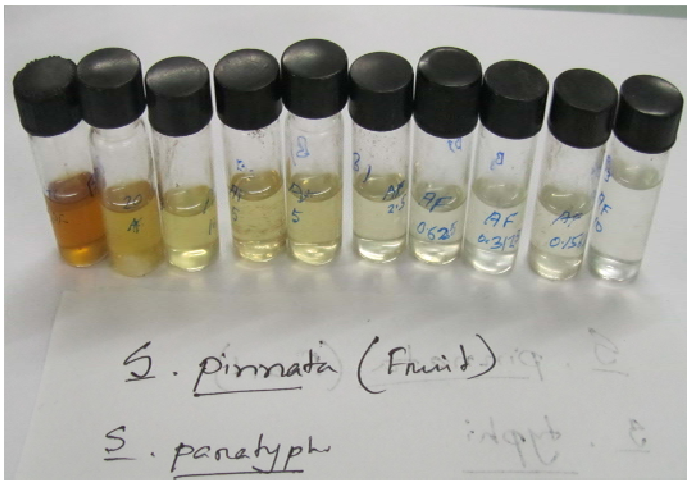


Figure 7.9 MIC for *S. paratyphi*

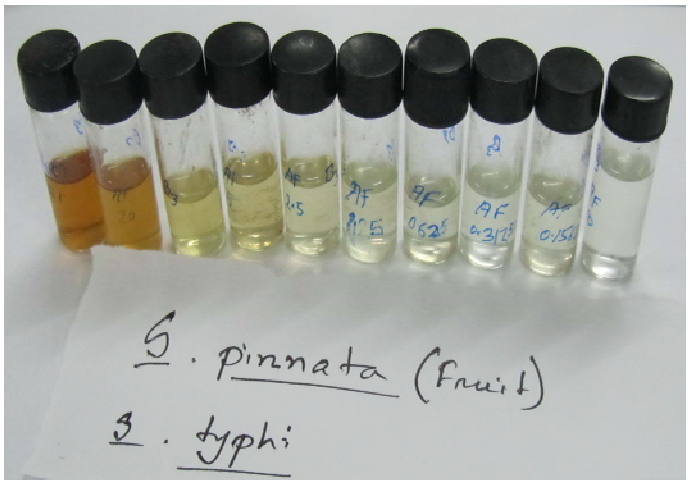


Figure 7.10 MIC for *S. typhi*

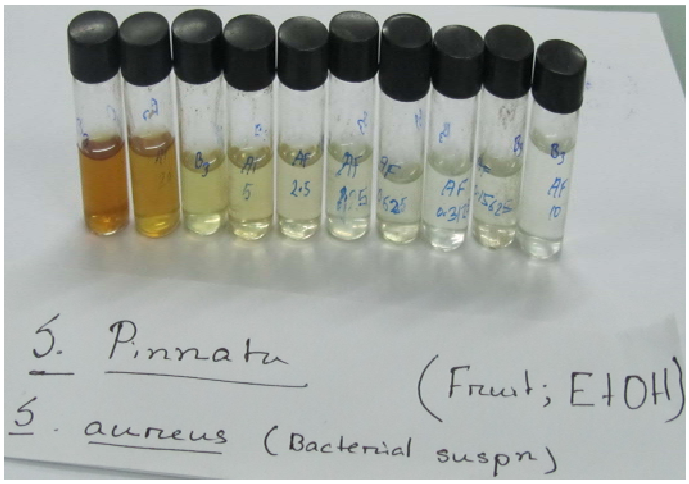


Figure 7.11 MIC for *S. aureus*

7.1.3.1 Total phenolic content

The crude ethanolic extract of *Spondias pinnata* (fruit) was subjected to total phenolic content determination. Based on the absorbance values of the extract solution, reacted with Folin-Ciocalteu reagent and compared with the standard solutions of gallic acid (table 7.3) equivalents, result of the colorimetric analysis of the total phenolics are given in table 7.4. Total phenolic content of the samples are expressed as mg of GAE (gallic acid equivalent) per gm of dried extract. The phenolic content found in the crude ethanolic extract of *Spondias pinnata* (fruit) was 659.74±3.58 mg of gallic acid (GAE) per gm of dried extract. Absorbance Values are expressed as average ± SD (n=3).

Table 7.3 Standard curve preparation by using gallic acid

SL. No.	Concentration (µg /ml)	Absorbance	Regression line	R^2
1	100	1.620	y = 0.0162x + 0.0215	0.9985
2	50	0.866		
3	25	0.450		
4	12.5	0.253		
5	6.25	0.120		
6	3.125	0.059		
7	1.5625	0.034		
8	0.78125	0.022		
9	0.3906	0.020		
10	0	0.011		

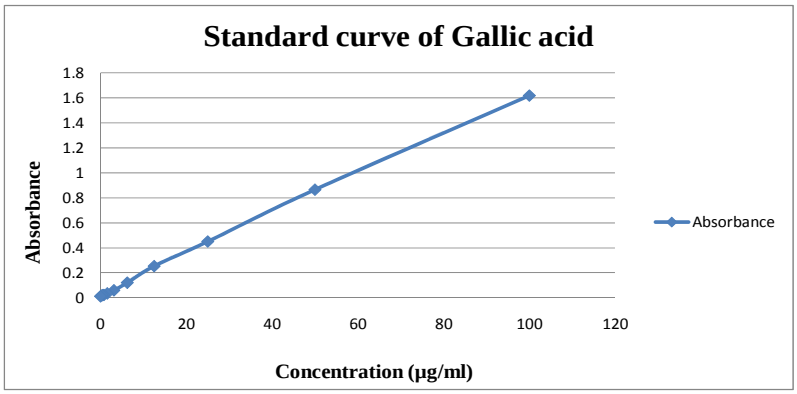


Figure 7.12 Standard curve for gallic acid

Table 7.4 Total phenolic content assay

Sample	Conc (mg/ml)	Absorbance	mg of Gallic acid equivalent (GAE) per gm of dried extract)
Crude ethanolic extract of Spondias pinnata (fruit)	1mg/ml	0.584±0.0031	659.74±3.58

7.1.3.2 DPPH radical scavenging assay (Quantitative analysis) and IC_{50}

The crude ethanolic extract of *Spondias pinnata* (fruit) was subjected to free radical scavenging activity and it showed significant free radical scavenging activity with an IC_{50} value of 1.91µg/ml. The percentage inhibition of free radical DPPH and the IC_{50} value of crude ethanolic extract of *Spondias pinnata* (fruit) are given in table 7.5 and figure 7.13. Absorbance Values are expressed as average ± SD (n=3).

Table 7.5 IC_{50} value of crude ethanolic extract of *Spondias pinnata* (fruit)

SL no	A_{Blank}	Concentration (µg/ml)	A_{Sample}	% inhibition of free radical DPPH = $(1 - A_{Sample}/A_{Blank}) \times 100$	IC_{50} µg/ml
1	0.116	2	0.059	49.14	1.91
2		4	0.032	72.41	
3		6	0.017	85.344	
4		8	0.012	89.655	
5		10	0.008	93.1	

Here, A_{Blank} and A_{Sample} are the absorbance of blank and sample respectively.

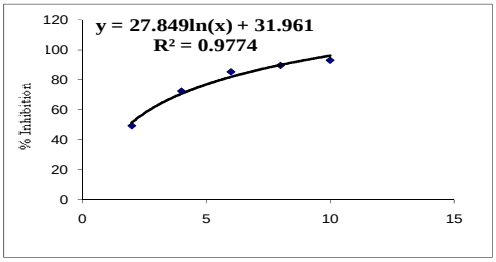


Figure 7.13 DPPH scavenging potential & IC_{50} value of crude ethanolic extract of *Spondias pinnata* (fruit)

7.1.3.3 Reducing power assay

The reducing properties are generally associated with the presence of reductanes which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Duh *et al.,* 1999). The reducing power ability of crude ethanolic extract of *Spondias pinnata* (fruit) was determined using L-ascorbic acid as positive control. In case of reducing power, the higher the concentration of the test samples, the higher the absorbance. The higher the absorbance, the higher the inhibition. The reducing power of various concentrations of crude ethanolic extract of *Spondias pinnata* (fruit) are given in table 7.6 & figure 7.15. The percentage increase in reducing power of various concentrations of test material and EC_{50} value of the extract is represented in figure 7.14. The highest % increase in reducing power was observed for 100 µg/ml concentration of crude ethanolic extract and it was 89.89%. The EC_{50} value of the extract was found to be 6.39 µg/ml for crude ethanolic extract of *Spondias pinnata* (fruit).

Table 7.6 EC_{50} value of crude ethanolic extract of *Spondias pinnata* (fruit)

SL no	Absorbance of blank, A_{Blank}	Concentration (µg/ml)	Absorbance of test, A_{Test}	% increase in reducing power = (A_{Test} / A_{Blank}) -1 X 100	EC_{50} µg/ml
1	0.089	1	0.109	22.47	6.39
2		5	0.127	42.70	
3		10	0.144	61.80	
4		50	0.160	79.70	
5		100	0.169	89.89	

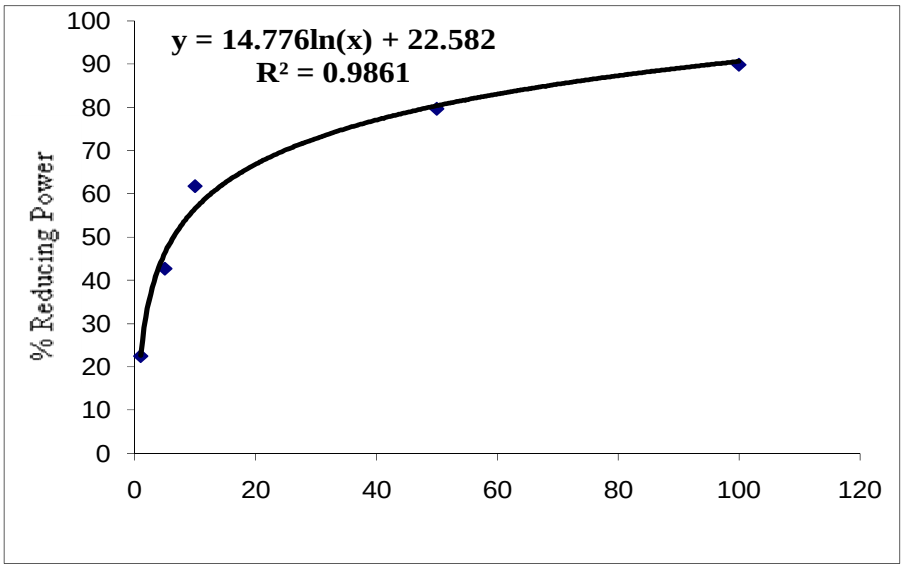


Figure 7.14 % reducing power & EC_{50} value of crude ethanolic extract of *Spondias pinnata*

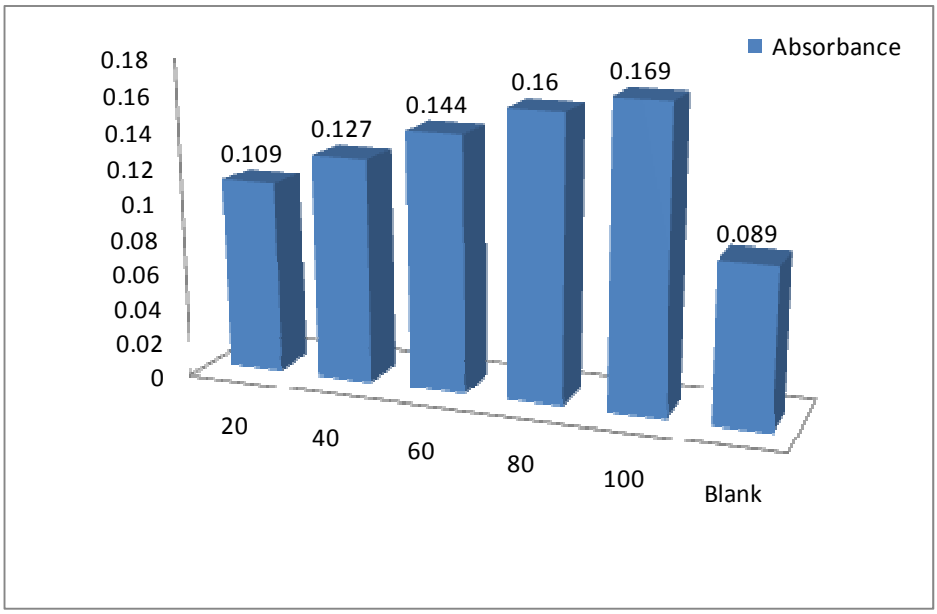


Figure 7.15 Bar diagram of Reducing power assay of various concentration of crude extract of *Spondias pinnata* (fruit)

7.1.3.4 Total antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of crude ethanolic plant extract was determined by phosphomolybdenum method. Sometimes a correlation analysis is performed between the total phenolic content and total antioxidant capacity to reveal the correlation. It is obvious that the plant phenolic compounds contribute to the the major antioxidant activity. Based on the

absorbance values of the extract solution, reacted with reagent solution (0.6 M Sulfuric acid, 28mM Sodium Phosphate and 4mM Ammonium molybdate) and compared with the standard solutions of L-ascorbic acid (table 7.7) equivalents, result of the colorimetric analysis of the total antioxidant capacity is given in table 7.8. Total antioxidant capacity of the sample is expressed as mg of L-ascorbic acid per gm of dried extract. The total antioxidant capacity found in the crude ethanolic extract of *Spondias pinnata* (fruit) was 25.52±0.17 gm of L-ascorbic acid per gm of dried extract. Absorbance Values are expressed as average ± SD (n=3).

Table 7.7 Standard curve preparation by using L-ascorbic acid

SL. No.	Concentration (mg /ml)	Absorbance	Regression line	R^2
1	2	0.12	y = 0.113x - 0.146	0.9916
2	4	0.27		
3	6	0.5		
4	8	0.77		
5	10	1.0		

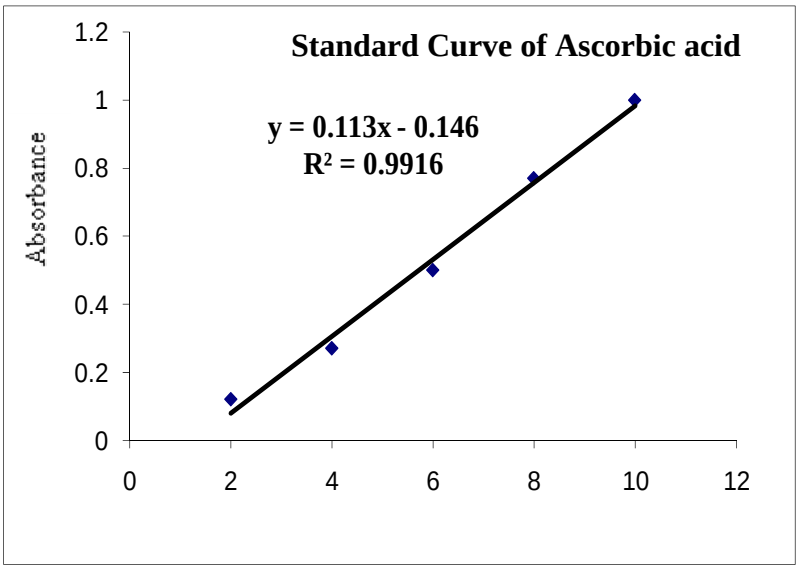


Figure 7.16 Standard curve for L-ascorbic acid

Table 7.8 Total antioxidant capacity by phosphomolybdenum method

Sample	Conc (mg/ml)	Absorbance	gm of L-ascorbic acid equivalent per g of dried extract)
Crude ethanolic extract of Spondias pinnata (fruit)	2 mg/ml	0.373±0.003	25.52±0.17

7.1.3.5 Total flavonoids content

To determine the total flavonoids content of crude ethanolic extract of *Spondias pinnat*a (fruit) using Chang et al method, a standard curve is needed which is obtained from a series of different quercetin concentrations (table 7.9). The total flavonoids content of the sample is expressed as mg of quercetin per gm of dried extract in table 7.10 by using the standard curve equation of quercetin ($y = 0.002x + 0.0318$, $R^2 = 0.9989$). Where y is absorbance at 415 nm and x is flavonoid content of crude plant extract. The total flavonoids content found in the crude ethanolic extract of *Spondias pinnata* (fruit) was 225.60±1.20 mg of quercetin per gm of dried extract. Absorbance Values are expressed as average ± SD (n=3).

Table 7.9 Standard curve preparation by using quercetin

SL. No.	Concentration (µg /ml)	Absorbance	Regression line	R^2
1	2.5	0.0365	y = 0.002x + 0.0318	0.9989
2	5	0.0417		
3	10	0.0521		
4	20	0.0735		
5	30	0.0906		
6	40	0.1127		

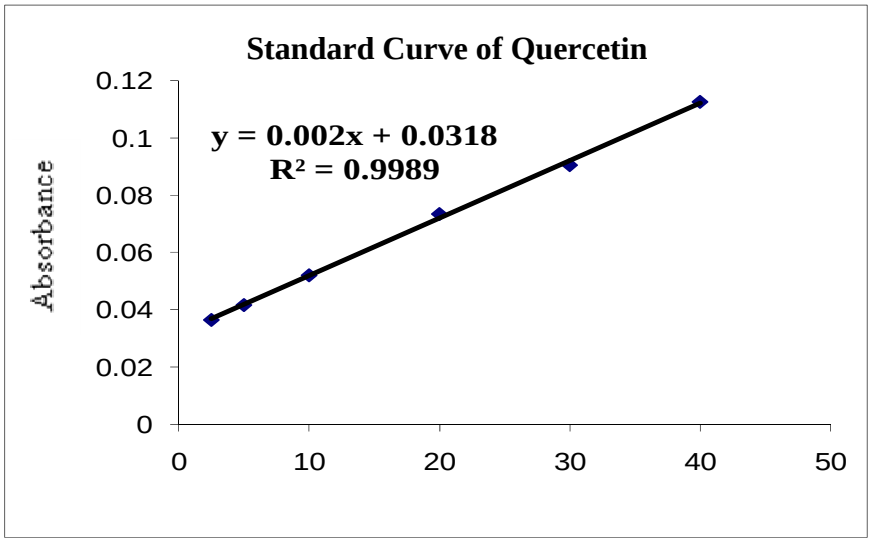


Figure 7.17 Standard curve for quercetin

Table 7.10 Total flavonoids content assay

Sample	Conc (mg/ml)	Absorbance	mg of quercetin equivalent per gm of dried extract)
Crude ethanolic extract of Spondias pinnata (fruit)	10 mg/ml	0.483±0.002	225.60±1.20

7.1.4 In vitro thrombolytic activity

The percentage of weight loss of clot after the application of crude ethanolic *Spondias pinnata* extract solution was taken as the functuional indication of thrombolytic activity. The study was implemented on two volunteer with 5 blood sample (for each) and average value of weight loss (in %) was calculated to examine the variation of two volunteer with the following formula:

% clot lysis = (Weight of the released clot / Weight of clot before lysis) × 100

$= [(W_2 – W_3) / (W_2 – W_1)] \times 100$

Here, W_1, W_2, W_3 are the Weight of empty Eppendrof, Wt of Eppendrof with clot, and Wt of Eppendrof after clot lysis respectively.

The percentage clot lysis for both volunteer is represented by using a bar diagram (figure 7.18) and the overall result of thrombolytic activity of crude plant material in compared with standard and blank is given in table 7.11.

Table 7.11 Thrombolytic activity of *Spondias pinnata* (fruit)

No of sample	Volunteer 1				Volunteer 2			
	Wt of empty Eppendrof (W_1) gm	Wt of Eppendrof with clot (W_2) gm	Wt of Eppendrof after clot lysis (W_3) gm	% of Clot lysis = [(W_2 – W_3) / (W_2 – W_1)] × 100	Wt of empty Eppendr of (W_1) gm	Wt of Eppendr of with clot (W_2) gm	Wt of Eppendr of after clot lysis (W_3) gm	% of Clot lysis = [(W_2 – W_3) / (W_2 – W_1)] × 100
SPC 1	0.871	1.116	1.053	25.71	0.887	1.064	1.018	25.99
SPC 2	0.882	1.121	1.062	24.69	0.872	1.143	1.076	24.72
SPC 3	0.871	1.120	1.057	25.30	0.873	1.117	1.055	25.41
STD	0.882	1.123	1.002	50.21	0.875	1.128	1.002	49.80
Blank	0.881	1.167	1.162	1.75	0.886	1.113	1.109	1.76

Here, SPC 1, 2, 3 are the samples of crude ethanolic extract of *Spondias pinnata* (fruit)

STD is the standard (streptokinase).

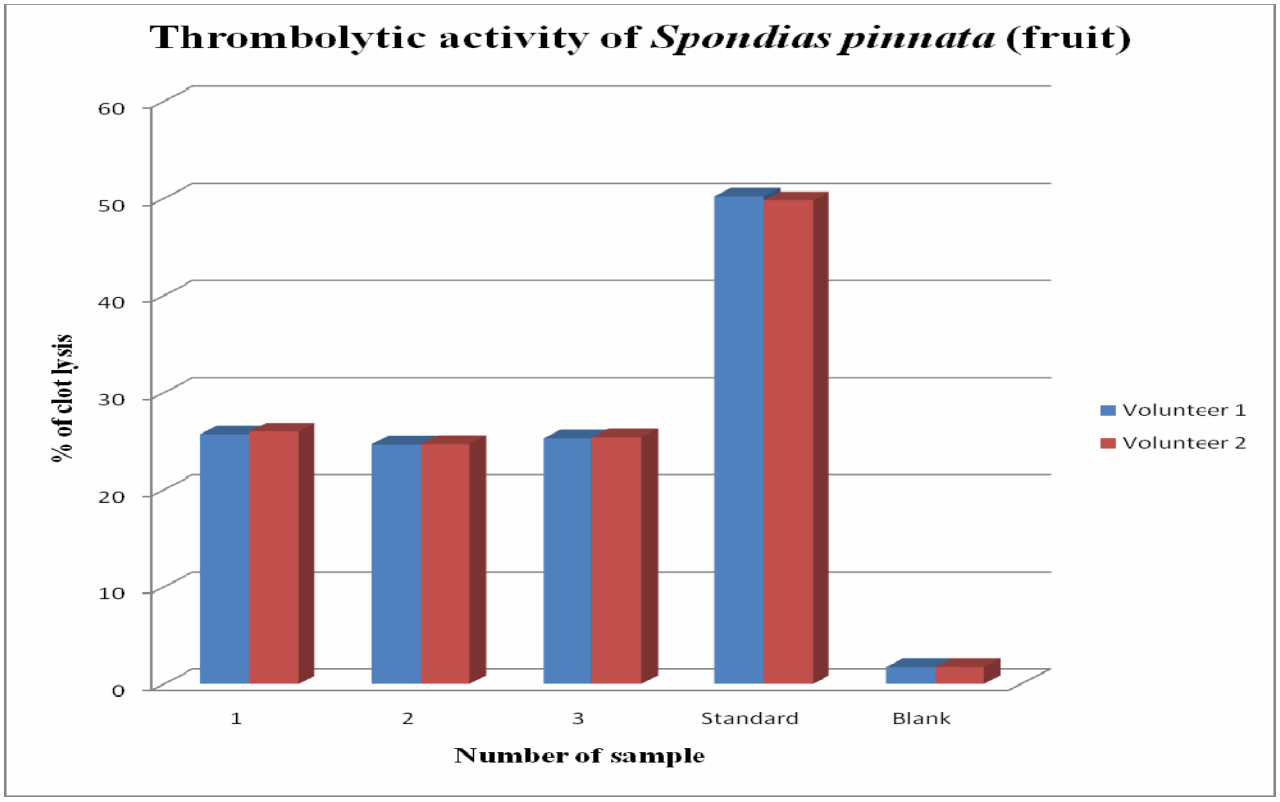


Figure 7.18 Bar diagram of thrombolytic activity

The average clot lysis by streptokinase in volunteer 1 and volunteer 2 was 50.21 and 49.80 respectively. It was found that average % of clot lysis by crude ethanolic extract of *Spondias pinnata* (fruit) were 25.23 ± 0.51 for volunteer 1 and 25.37 ± 0.03 for volunteer 2. The difference in the thrombolytic action between two volunteer in response to average activity was due to body physiological differences (age, sex, body weight, % of clotting factor and food habit etc.)

Conclusion

Conclusion

The crude ethanolic extract of *Spondias pinnata* showed significant antioxidant, antimicrobial and thrombolytic activities, some of which supports the traditional use of this plant in various diseases.

The plant can be further screened against various diseases in order to find out its unexplored efficacy and can be a potential source of chemically interesting and biologically important drug candidates. Very few compounds are isolated from the *S. pinnata*. Therefore there is huge potential to find active principles which could be beneficial for mankind for targeting various diseases.

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