Preliminary Phytochemical Screening, Antioxidant Activity & Cytotoxic Activity Evaluation of *Spondias pinnata* Barks

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Submitted By

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ID: 2010-1-70-040

Submitted to

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CERTIFICATE

This is to certify that, the research work on "Preliminary Phytochemical Screening, Antioxidant Activity & Cytotoxic Activity Evaluation of *Spondias pinnata* Barks" submitted to the department of Pharmacy, East West University, Aftabnagar, Dhaka**,** in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (B.Phrm) was carried out by Md. Al Hafiz, ID# 2010-1-70-040.

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CERTIFICATE BY THE SUPERVISOR

This is to certify that, the research work on "Preliminary Phytochemical Screening, Antioxidant Activity & Cytotoxic Activity Evaluation of *Spondias pinnata* Barks" submitted to the department of Pharmacy, East West University, Aftabnagar, Dhaka**,** in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (B.Phrm) was carried out by **Md. Al Hafiz**, ID# 2010-1-70-040, under my guidance and supervision and I further certify that all the resources of the information in this connection are duly acknowledged.

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled "Preliminary Phytochemical Screening, Antioxidant activity & Cytotoxic Activity Evaluation of *Spondias pinnata* Barks" is an authentic and genuine research work carried out by me under the guidance of **Mahbubul Hoque Shihan**, Senior Lecturer, Department of Pharmacy, East West University, Aftabnagar, Dhaka, Bangladesh.

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Dedication

This research paper is dedicated to

My beloved Father…..

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Abstract

Spondias pinnata is a flowering and glabrous tree upto 10.5 m high with straight trunk and smooth ash coloured bark having pleasant aromatic smell. The leaves and bark are aromatic, astringent and useful in preventing vomiting, dysentery and diarrhoea. The purpose of the study was to investigate the phytochemical compounds and cytotoxic compounds from ethyl acetate extract which will be pharmacologically effective. From phytochemical screening, the presence of some compounds was found such as alkaloids, flavonols, phenols, steroidal compounds and saponins. This indicates, it will be effective pharmacologically. Antioxidant properties are also found by DPPH test, Total Reducing power test and Total phenol content test. The Reducing power test showed percentage of reducing potential which is similar to standard (ascorbic acid). Sometimes it showed greater potential than the standard indicates the presence of phenol and polyphenolic compounds. For example, in the concentration of 1000 (µg/ml) the reducing potential of extract was 24.397% and of the standard was 13.941%. DPPH scavenging activity test also showed similar activity as ascorbic acid. Here, the extract showed greater activity than the standard. The extract showed the IC_{50} of 409(µg/ml) and the standard showed IC_{50} of 425(µg/ml). Estimation of total phenolic content also determined by Folin-Ciocalteu method and showed significant amount of phenolic compound's presence. The extract showed greater absorbance than the standard. These indicate that, it will be effective pharmacologically to treat free radical mediated diseases. Cytotoxic activity was also found by Brine Shrimp Lethality test. The LD_{50} was measured for both the standard and the extract. The extract showed greater activity than the standard. That means the ethyl acetate extract of ***Spondias pinnata*** has potential cytoxic effect.

Key words: *Spondias pinnata,* Antioxidant, Total phenol content, Reducing potential, DPPH, Cytotoxicity, Brine shrimp.

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1.1.1 Medicinal plants

Medicinal plants are important therapeutic aid for various ailments. Today there is widespread interest in drugs deriving from medicinal plants. This interest primarily stems from the belief that green medicine is safe and dependable, compared with costly synthetic drugs that have toxic side effects (Das J, *et al*, 2005). A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis. When a plant is designated as 'medicinal', it is implied that the said plant is useful as a drug or therapeutic agent or an active ingredient of a medicinal preparation. Medicinal plants may therefore be defined as a group of plants that possess some special properties or virtues that qualify them as articles of drugs and therapeutic agents, and are used for medicinal purposes. Since most medicinal plants occur naturally in a large number of countries, a plant of potential importance in one country may well have been studied by scientists elsewhere. Considerable time and effort could be saved if their findings could make available to all interested people. World Health Organization (WHO) has provided a definition of medicinal plants, that is "A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for synthesis of useful drug".

Some medicinal plants are wild crafted, meaning that they are harvested in the wild by people who are skilled at plant identification. Sometimes, plants cannot be cultivated, making wild crafting the only way to get them, and some people believe that wild plants have more medicinal properties. Wild crafting can also be done to gather herbs for home use, with people seeking them out to use in their own medicinal preparations. Other plants may be cultivated. One of the advantages of cultivation is that it allows for greater control over growing conditions, which can result in a more predictable and consistent crop. Cultivation also allows for mass production, which makes plants more commercially viable, as they can be processed in large numbers and priced low enough that people will be able to afford them.

The most important ingredients present in plant communities turn out to be alkaloids, terpenoids, steroids, phenols glycosides and tannins (Abayomi, 1993)

1.1.2 History of Medicinal Plants

1. The use of plants as medicines predates written human history. Many of the herbs and spices used by humans to season food also yield useful medicinal compounds. The use of herbs and spices in cuisine developed in part as a response to the threat of food-borne pathogens. Studies show that in tropical climates where pathogens are the most abundant, recipes are the most highly spiced. Further, the spices with the most potent antimicrobial activity tend to be selected. In all cultures vegetables are spiced less than meat, presumably because they are more resistant to spoilage. Many of the common weeds that populate human settlements such as nettle, dandelion and chickweed, also

2. A large amount of archaeological evidence exists which indicates that humans were using medicinal plants during the Paleolithic, approximately 60,000 years ago. Furthermore, other non-human primates are also known to ingest medicinal plants to treat illness in Ancient times. The Ebers Papyrus (ca. 1550 BCE) from Ancient has a prescription for Cannabis applied topically for inflammation. The essential oil of common thyme contains 20- 54%thymol. Thymol is a powerful antiseptic and antifungal that is used in a variety of products. Before the advent of modern antibiotics, oil of thyme was used to medicate bandages.

3. In the written record, the study of herbs dates back over 5,000 years to the Sumerians, who created clay tablets with lists of hundreds of medicinal plants such as myrrh and opium. In 1500 B.C., the Ancient Egyptians wrote the Ebers Papyrus, which contains information on over 850 plant medicines, including garlic, juniper, cannabis, castor bean, aloe, and mandrake. In India, Ayurveda medicine has used many herbs such as turmeric possibly as early as 1900 BC. Sanskrit writings from around 1500 B.C., such as the Rig Veda, are some of the earliest available documents detailing the medical knowledge that formed the basis of the Ayurveda system. Many other herbs and minerals used in Ayurveda were later described by ancient Indian herbal lists such as Charaka and Sushruta during the 1st millennium BC. The Sushruta Samhita attributed to Sushruta in the 6^{th} century BC describes 700 medicinal plants, 64 preparations from mineral sources, and 57 preparations based on animal sources. The Chinese emperor Shen Nung is said to have written the first Chinese herbal, the Pen Tsao. The Pen Tsao lists 365 medicinal plants and their uses - including Ephedra (the shrub that introduced the drug ephedrine to modern

medicine), hemp, and chaulmoogra is one of the first effective treatments (Levetin and Mahon M, 2003).

4. Middle Ages Benedictine monasteries were the primary source of medical knowledge in Europe and England during the Early Middle Ages. However, most of these monastic scholars' efforts were focused on translating and copying ancient Greco-Roman and Arabic works, rather than creating substantial new information and practices. Many Greek and Roman writings on medicine, as on other subjects, were preserved by hand copying of manuscripts in monasteries. The monasteries thus tended to become local centers of medical knowledge, and their herb gardens provided the raw materials for simple treatment of common disorders. At the same time, folk medicine in the home and village continued uninterrupted, supporting numerous wandering and settled herbalists. Among these were the "wise-women", who prescribed herbal remedies often along with spells and enchantments. It was not until the late middle Ages that women who were knowledgeable in herb lore became the targets of the witch hysteria.

5. Dioscorides' Materia Medica, c. 1334 copy in Arabic, describes medicinal features of cumin and dill. Baghdad was an important center for Arab herbalism, as was Al-Andalus between 800and 1400. Abulcasis of Cordoba authored The Book of Simples, an important source for later European herbals, while Ibn al-Baitar of Malaga authored the Corpus of Simples, the most complete Arab herbal which introduced 200 new healing herbs, including tamarind, Aconitum, and nux vomica. Avicenna's The Canon of Medicine lists 800 tested drugs, plants and minerals. Book Two is devoted to a discussion of the healing properties of herbs, including nutmeg, senna, sandalwood, rhubarb, myrrh, cinammon, and rosewater. The Canon of Medicine remained a medical authority, used at many European and Arab medical schools, until the early 19th century. Other pharmacopoeia books include that written by Abu-Rayhan Biruni in the 11th century and Ibn Zuhr in the12th century, Peter of Spains Commentary on Isaac, and John of St Amands Commentary on the Antedotary of Nicholas. In particular, the Canon introduced clinical trials, randomized controlled trials, and efficacy tests.

6. Early modern era the fifteenth, sixteenth, and seventeenth centuries were the great age of herbals, many of them available for the first time in English and other languages rather than Latin or Greek. The first herbal to be published in English was the anonymous Grete Herbal of 1526. The two best-known herbals in English were The Herbal or General History of Plants

(1597) by John Gerard and The English Physician Enlarged (1653) by Nicholas Culpeper. Gerard's text was basically a pirated translation of a book by the Belgian herbalist Dodoens and his illustrations came from a German botanical work. The original edition contained many errors due to faulty matching of the two parts. Culpeper's blend of traditional medicine with astrology, magic, and folklore was ridiculed by the physicians of his day yet his book - like Gerard's and other herbals - enjoyed phenomenal popularity. The Age of Exploration and the Columbian Exchange introduced new medicinal plants to Europe. The Badianus Manuscript was an illustrated Aztec herbal translated into Latin in the 16th century the second millennium, however, also saw the beginning of a slow erosion of the pre-eminent position held by plants as sources of therapeutic effects. This began with the Black Death, which the then dominant Four Element medical system proved powerless to stop. A century later, Paracelsus introduced the use of active chemical drugs (like arsenic, copper sulfate, iron, mercury, and sulfur). These were accepted even though they had toxic effects because of the urgent need to treat Syphilis (Levetin and Mahon M, 2003).

1.1.3 Traditional medicine

According to WHO, Traditional medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness.

Among the largest ethnic group, the Bangalees on the main land, there are two distinct forms of Traditional medicine practice:

1. One is the old and original form based on old knowledge, experience and belief of the older generations. This includes:

i) Folk medicine, which uses mainly plant and animal parts and their products as medicines for treating different diseases and also includes treatments like blood-letting, bone-setting, hot and cold baths, therapeutic fasting and cauterization.

ii) Religious medicine, which includes use of verses from religious books written on papers and given as amulets, religious verses recited and blown on the face or on water to drink or on food to eat, sacrifices and offerings in the name of God and gods, etc. and

iii) Spiritual medicine, which utilizes methods like communicating with the supernatural beings, spirits or ancestors through human media, torturous treatment of the patient along with incantations to drive away the imaginary evil spirits and other similar methods.

2. The other is the improved and modified form based on the following two main traditional systems:

i) The Unani-Tibb or Graeco-Arab system which has been developed by the Arab and Muslim scholars from the ancient Greek system, and

ii) The Ayurvedic system which is the old Indian system based on the Vedas, the oldest scriptures of the Hindu saints of the Aryan age (Ghani A, 1990).

1.1.4 History of Plant Based Traditional Medicine

Plants have formed the basis of sophisticated traditional medicine practices that have been used for thousands of years by people in China, India, and many other countries. Some of the earliest records of the usage of plants as drugs are found in the Artharvaveda, which is the basis for Ayurvedic medicine in India (dating back to 2000 BCE), the clay tablets in Mesopotamia (1700 BCE), and the Eber Papyrus in Egypt (1550 BCE). Other famous literature sources on medicinal plant include “De Materia Medica,” written by Dioscorides between CE 60 and 78, and “Pen Ts’ao Ching Classic of Materia Medica” (written around 200 CE).

Nowadays plants are still important sources of medicines, especially in developing countries that still use plant-based TM for their healthcare. In 1985, it was estimated in the Bulletin of the World Health Organization (WHO) that around 80 % of the world’s population relied on medicinal plants as their primary healthcare source. And it was presumed that a major part of traditional medicine involves the extracts or active principles (Kamboj, 2000). Even though a more recent figure is not available, the WHO has estimated that up to 80 % of the population in Africa and the majority of the populations in Asia and Latin America still use traditional

medicine for their primary healthcare needs. In industrialized countries, plant-based traditional medicines or phytotherapeuticals are often termed complementary or alternative medicine, and their use has increased steadily over the last 10 years. In the USA alone, the total estimated "herbal" sales for 2005 were $4.4 billion, a significant increase from $2.5 billion in 1995. However, such "botanical dietary supplements" are regulated as foods rather than drugs by the United States Food and Drug Administration (Herborn, 1998).

1.1.5 Examples of Some Modern Medicine Discovered from Plants

Plants can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and reduced toxicity. The small fraction of flowering plants that have so far been investigated have yielded about 120 therapeutic agents of known structure from about 90 species of plants. Some of the useful plant drugs include vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capscicine, allicin, curcumin, artemesinin and ephedrine among others. In some cases, the crude extract of medicinal plants may be used as medicaments. About 121 (45 tropical and 76 subtropical) major plant drugs have been identified for which no synthetic one is currently available. More than 64 plants have been found to possess significant antibacterial properties; and more than 24 plantshave been found to possess antidiabetic properties, antimicrobial studies of plants (Samy P *et al*, 2006), plant for antidotes activity-*Daboiarussellii* and *Najakaouthia* venom neutralization by lupeolacetate isolated from the root extract of Indian sarsaparilla *Hemidesmusindicus* (Chatterjee*et al.*, 2006). Which effectively neutralized *Daboiarussellii* venom induced pathophysiological changes (Alam *et al*, 1994).

It has been estimated that more than 400 traditional plants or plant-derived products have been used for the management of type 2 diabetes across geographically. Galegine, a substance produced by the herb Galega officinalis, provides an excellent example of such a discovery. Experimental and clinical evaluations of galegine, provided the pharmacological and chemical basis for the discovery of metformin which is the foundation therapy for type 2 diabetes. Plant derived agents are also being used for the treatment of cancer. Several anticancer agents including taxol, vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, and etoposide derived from epipodophyllotoxin are in clinical use all over the world. Vincristine

is recommended for acute lymphocytic leukemia in childhood advanced stages of hodgkins, lympho sarcoma, small cell lung, cervical and breast cancer (Farnsworth and Bingel, 1977).

In conclusion, plants have provided humans with many of their essential needs, including life-saving pharmaceutical agents. Recently the World Health Organization estimated that 80% people worldwide rely on herbal medicines for some aspect. Many developing countries have intensified their efforts in documenting the ethnomedical data and scientific research on medicinal plants. Natural products or natural product derivatives comprised 14 of the top 35 drugs in 2000 based on worldwide sales. There are more than 270,000 higher plants existing on this planet. But only a small portion has been explored phytochemically. So, it is anticipated that plants can provide potential bioactive compounds for the development of new 'leads' to combat various diseases. As a vast proportion of the available higher plant species have not yet been screened for biologically active compounds, drug discovery from plants should remain an essential component in the search for new medicines & the scientific study of traditional medicines, concerned medicinal plants are thus of great importance.

1.1.6 Medicinal plant part utilization

For medicinal preparations, people mostly use above ground plant parts (76%), followed by belowground parts (17%) and whole plants (7%). Of the above ground parts, leaves are used most frequently (25%), followed by roots and fruits (20% each), bark (16%), whole plants (9%), flowers (4%), latex (4%) and seed (2%). In most cases, the paste and juice made from leaves and barks are used in medicine, while fruits are eaten raw (Kitula, R.A., 2007).

1.2.1 Plant family

Spondias pinnata is a medicinal plant of Anacardiaceae family.

Anacardiaceae, the sumac family of flowering plants in the order Sapindales, with about 70 genera and 650 species of ever green or deciduous trees, shrubs, and woody vines. It is native to tropical and subtropical areas of the world, but a few species occur in temperate regions. Members of the family have resin ducts in the bark, leaves usually composed of leaflets in various arrangements, flowers often with only male or female parts, and usually fleshy fruits. The pistachio and cashew produce edible nuts, and mango, mombin, and wild plum, or Kaffir plum, has edible fruits. The mastic tree and the varnish tree contain useful oils, resins, and lacquers. The reddish brown wood of quebracho trees yields commercial tannin. The Peruvian pepper tree, *Cotinus* species, and several species of sumac are cultivated as ornamentals. Poison ivy, poison oak, and poison sumac are irritating to the skin (Tianlu M, Barfod A, 2004).

Table 1.2.1: Chief genera and species of Anacardiaceae family

Genera	**Species**
Spondias	S.pinnata
Mangifera	M. indica
Anacardium	A.occidentale
Rhus	R. ovate
Schinus	S.molle
Pistacia	P.mexicana
Cotinus	C.obovatus
Bouea	B.oppositifolia
Buchanania	B.arborescens
Harpephyllum	H.caffrum
Loxostylis	L. alata
Mangifera	M.casturi
Cotinus	C.coggygria
Toxicodendron	T.vernix
Rhus	R.typhina

1.2.2 Plant information

Spondias pinnata is a glabrous tree upto 10.5 m high with straight trunk and smooth ash coloured bark having pleasant aromatic smell. In Ayurveda, the unripe fruits are believed to destroy "vata", enrich the blood and cures rheumatism. Leafs are glossy, green in color and having a lovely foliage. Green leaf buds are sour in taste and smells similar to the fruit. Stalked compound leaflets of this plant are having 30-40cm long leafs oppositely arranged on it. The leaves and bark are aromatic, astringent and useful in preventing vomiting, dysentery and diarrhoea. The plant is reported to have anti-tubercular properties. The tribes of Orissa use the paste of the bark orally for treating diarrhoea in children. The paste is also used in adults for promoting diuresis in the adults (Hazra B *et al,* 2008). Flowers of this wild mango are very small in size and come around a long stalk in a group. Small flower are mainly white in color and stalkless. Fruits are rounding, ovate, elliptic in shaped. Pulp of the fruit is sour in taste. It is being cultivated commercially in our country for both local and international market.

Figure 1.2.1: *Spondias pinnata*

1.2.3 Other names

***Spondias pinn*ata** distributes around 20 countries all over the world and normally in India, Sri Lanka and South East Asian countries. In India it is commonly seen the deciduous to semi-evergreen forests of the Western Ghats. The genus spondias includes 17 described species, 7 of which are native to the neotropics and about 10 are native to Asia. In different countrys, it is

known differently. Sometimes various names are used based on the area of the country. Some names of the plant are listed bellow,

Table 1.2.2: Common names of the ***Spondias pinnata***

Assamese	Aamrata, Amora
Bengali	Aamada, Aamraata, Aamraataka, Amra
Chinese	Bin lang qing, Mu ge, Zhao wa wen po
German	Mangopflaume
Gujarati	Ambaada
Hindi	Ambara, Ambari, Amra, Amara, Bhringi-phal, Metula, Pashu-haritaki,Pitan
Japanese	Amura tamagonoki
Kannada	Amategayi mara, Ambatte mara, Marahunsie, Muthiga
Malayalam	Ambazham
Marathi	Amada, Ambada, Dholamba, Khatamba, Ranamba
Nepalese	Amaaro
Oriya	Ambaada
Portuguese	Cajamangueira, Cajá-manga, Imbú manga
Sanskrit	Aamraata, Amraatakah, Metula, Pitan
Spanish	Ciruela mango, Jobo de la India, Mango jobo
Tamil	Ambalam, Ambazham, Kincam, Pulima
Telegu	Adavimamidi, Adhvamu, Ambalamu
Thai	Makok, Má kok pa

1.2.4 Botanical Description

Spondias pinnata is a medium to tall tree reaching a height of about 25 m and a diameter of about 60 cm. It is wholly or partly deciduous with occasionally small buttress. Its bark surface is smooth, with irregular cracks, greyish to pale reddish brown, exceeding a clear, sticky sap with turpentine smell. Leaves are arranged spirally, leaflets are alternate to opposite. Flowers are bisexual. Fruit is yellow in color, fleshy, drupe with a finely flavored edible pulp; seed is hard, ridged and has a fibrous surface. *S. pinnata* is a light-demanding species.

1.2.5 Leaves

The leaves are aromatic, acidic and astringent. They are spirally arranged, pinnate, rarely bipinnate or simple. They are used for flavoring. The leaves are 20-45 cm long and hairy underneath. Leaves have a sour taste are edible. Young leaves are used as ingredient in meat and in fish soup, a Bicolano delicacy. Bicolanos also use dried young leaves in the preparation of "laing", a favorite and popular dish among the local people. Leaves are also used as feeds for cattle.

Figure 1.2.2: Leaves of *Spondias pinnata*

1.2.6 Flowers

Flowers white to cream, arranged in a many-flowered inflorescence, in the upper axils, bisexual and unisexual flowers on the same tree, pedicels up to 3 mm long (Watson, L, Dallwitz, M.J, 1992).

Figure 1.2.3: Flowers of Spondias pinnata

1.2.7 Fruits

This is a common fruit of Bangladesh called "Amra". The fruits have a sour taste. They are eaten raw and can be made into jams, jellies and juices. It is also given to pigs as feeds. The fruits are eaten as a vegetable when green and as a fruit when ripe. The unripe fruits contain some proteolytic enzymes apart from several terpenes, aldehydes and esters. Fruits are very nutritious and rich in vitamin A, minerals and iron content. They can be used to treat coughs. The fruit is also known for having anti inflammatory properties and also prevent free radicals, fight cancer and reduce inflammations caused by various conditions.

Figure 1.2.4: Fruit of Spondias pinnata

1.2.8 Seeds

Small heaps of stones are used for sowing and one stone of *S. pinnata* contains 1-3 viable seeds. There are 250 fresh stones/kg. Seed viability is up to one year. The seeds collected from such heaps germinate well. Germinative power decreases by 50% after a year of storage. Germination percent is 5-20% in 12-51 days. Seeds can be damage because of the consumption of fruit by birds, monkey and other animals.

1.2.9 Root

The tree has a large root system that stores quite a bit of water for drier seasons. The root is considered useful in regulating menstruation.

1.2.10 Stem

The stem barks are used in folk medicine in the treatment of antidiarrhoea, dysentery, rheumatism, gonorrhea and anti-tubercular.

1.2.11 Origin and Distribution

This is native from Melanesia through Polynesia and has been introduced into tropical areas of both the Old and New World. It is common in Malayan gardens and fairly frequent in India and

Ceylon. The fruits are sold in markets in Vietnam and elsewhere in former Indochina. It first fruited in the Philippines in 1915. It is cultivated in Queensland, Australia, and grown on a small scale in Gabon and Zanzibar.It was introduced into Jamaica in 1782 and again 10 years later by Captain Bligh, probably from Hawaii where it has been grown for many years. It is cultivated in Cuba, Haiti, the Dominican Republic, and from Puerto Rico to Trinidad; also in Central America, Venezuela, and Surinam; is rare in Brazil and other parts of tropical America. Popenoe said there were only a few trees in the Province of Guayas, Ecuador, in 1924.The United States Department of Agriculture received seeds from Liberia in 1909, though Wester reported at that time that the tree had already been fruiting for 4 years in Miami, Florida. In 1911, additional seeds reached Washington from Queensland, Australia. A number of specimens are scattered around the tip of Florida, from Palm Beach southward, but the tree has never become common here. Some that were planted in the past have disappeared.

1.2.12 Climate

The tree flourishes in humid tropical and subtropical areas, being only a trifle tenderer than its close relative, the mango. It succeeds up to an altitude of 2,300 ft (700 m). In Israel, the tree does not thrive, remaining small and bearing only a few, inferior fruits.

1.2.13 Soil

The ambarella grows on all types of soil, including oolitic limestone in Florida, as long as they are well-drained.

1.2.14 Propagation

The tree is easily propagated by seeds, which germinate in about 4 weeks, or by large hardwood cuttings, or air-layers. It can be grafted on its own rootstock, but Firminger says that in India it is usually grafted on the native *S. pinnata*. Wester advised: "Use non-petioled, slender, mature, but green and smooth budwood; cut large buds with ample wood-shield, 1 1/2 to 1 3/4 in (4-4.5 cm) long; insert the buds in the stock at a point of approximately the same age and appearance as the scion."

1.2.15 Culture

Seedlings may fruit when only 4 years old. Ochse recommends that the young trees be given light shade. Mature trees are somewhat brittle and apt to be damaged by strong winds; therefore, sheltered locations are preferred.

1.2.16 Season

In Hawaii, the fruit ripens from November to April; in Tahiti, from May to July. In Florida, a single tree provides a steady supply for a family from fall to midwinter, at a time when mangos and many other popular fruits are out of season.

1.2.17 Pests and Diseases

Ochse says that in Indonesia the leaves are severely attacked by the larvae of the kedongdong spring-beetle, *Podontia affinis.* In Costa Rica, the bark is eaten by a wasp ("Congo"), causing necrosis which leads to death. No particular insects or diseases have been reported in Florida. In Jamaica, the tree is subject to gummosis and is consequently short-lived.

1.2.18 Food Uses

The ambarella has suffered by comparison with the mango and by repetition in literature of its inferior quality. However, taken at the proper stage, while still firm, it is relished by many out-of-hands, and it yields a delicious juice for cold beverages. If the crisp sliced flesh is stewed with a little water and sugar and then strained through a wire sieve, it makes a most acceptable product, much like traditional applesauce but with a richer flavor. With the addition of cinnamon or any other spices desired, this sauce can be slowly cooked down to a thick consistency to make a preserve very similar to apple butter. Unripe fruits can be made into jelly, pickles or relishes, or used for flavoring sauces, soups and stews.

Young ambarella leaves are appealingly acid and consumed raw in Southeast Asia. In Indonesia, they are steamed and eaten as a vegetable with salted fish and rice, and also used as seasoning for various dishes. They are sometimes cooked with meat to tenderize it.

1.2.19 Using Information

The bark is astringent and refrigerant; infusion of the bark is given in dysentery, diarrhoea and to prevent vomiting. Paste of the bark is used as an embrocation for both articular and muscular rheumatism. Decoction of the bark is given in gonorrhoea. Gum of the bark is demulcent. Roots are useful in regulating menstruation. The leaves are appetizing and astringent. Fruit possesses antiscorbutic and astringent properties; used in bilious dyspepsia. The unripe fruit is good for rheumatism and sore throat. Ripe fruit is tonic, aphrodisiac and astringent to the bowels; cures burning sensation (Morton J, 1997).

1.2.20 Constituents

Phytochemical studies have yielded flavonoids, tannins, saponins and terpenoids. Essential oil from the pulp yielded carboxylic acids and esters, alcohols, aromatic hydrocarbons. The major compounds were 9, 12, 15-octadecatrien-1-ol (36.78%), hexadecanoic acid (25.27%) and furfural (19.77%). Study isolated 24-methylene cycloartanone, stigma-4en-3one, lignoceric acid, β-sitosterol and its β-D-glucoside. Fruits yield ß-amyrin, oeanolic acid, glycine, cystine, serine, alanine, and leucine. Aerial parts yield lignoceric acid, ß-sitosterol and its glucoside.

2.1 Phytochemical study of *Spondias pinnata*

2.1.1 Preliminary evaluation of nutraceutical and therapeutic potential of raw of fruit of *Spondias pinnata* K.

The underutilized, edible green raw fruits of ***Spondias pinnata*** K. from the eastern region of India were investigated for their nutraceutical and therapeutic potential. A thorough nutritional characterization of this fruit demonstrated it as a source of energy, phenolic compounds, natural antioxidants and minerals. It is also a moderate source of ascorbic acid, malic acid, calcium, phosphorus and other nutrients. The phytochemical screening revealed alkaloids followed by saponins and tannins. Total phenolic, flavonoid and flavonol contents were obtained. Antioxidant activity of different extracts also obtained. The presence of gallic acid, salicylic acid, chlorogenic acid, ellagic acid, p-coumaric acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, quercetin, catechin, myrecetin and rutin is also obtained. The antimicrobial activity and α-amylase inhibition capacity are also obtained. Analysis of volatile flavor showed isopropyl myristinate as a major compound followed by the other monoterpenes and sesquiterpenes. The current study explains the nutritional as well as medicinal utility of the fruit which is a rich source of minerals and antioxidants such as phenols and flavonoids (Satpathi, Tyagi and Gupta, 2011)**.**

2.1.2 Structural features of the acidic polysaccharide from gum exudate of *Spondias pinnata***.**

The purified, homogeneous, acidic polysaccharide isolated from the gum exudate of *Spondias pinnata*, and its degraded product prepared by controlled autohydrolysis, were found to contain d-galactose, l-arabinose, and d-galacturonic acid. Complete methylation followed by hydrolysis, both before and after reduction with lithium aluminum hydride, revealed the probability of a (1 →3)-linked, galactan backbone (Ghosal and Thkur, 1981).

2.1.3 Study on microscopic observation and TLC identification of Dai medicine from *spondias pinnata.*

Characteristic, microscopic observation and TLC identification were used to authenticate this crude drug. The characters of the cross section, powder and TLC of the drug were reported, and the relevant drawings of the tissue, powder and TLC of this ethno medicine were drawn. These results can supply evidences for the identification of the ethno medicine in its exploitation and utilization (Xu *et al*., 2009).

2.1.4 1D- and 2D-NMR spectroscopy studies of the polysaccharide gum from *Spondias purpurea* var.

Spondias purpurea var. *lutea* trees located in Venezuela, South America, produce a clear gum very soluble in water. The polysaccharide, from this gum, contains galactosyl, arabinosyl, xylosyl, rhamnosyl and uronic acid residues. Degraded gums A and B were prepared by mild acid hydrolysis and Smith degradation, respectively. Application of 1D- and 2D-NMR spectroscopy to the original gum and its degraded products, in combination with chemical data, led to confirm that the structure of the original polysaccharide contains 3-*O*- and 6-*O*-galactosyl residues, terminal and 3-*O*-α-l-arabinofuranosyl, terminal rhamnosyl residues and uronic acids, represented by β-d-glucuronic acid and its 4-*O*-methyl derivative. It was demonstrated that 2D-NMR spectroscopy is a good tool for structural elucidation of complex heteropolysaccharides (Omaira *et al*., 2005).

Table 2.1: Summaries of Phytochemical study on ***Spondias pinnata***

Part of the plant	**Findings**	**references**
Fruit	Energy, phenolic compounds, natural antioxidants and minerals, ascorbic acid, malic acid, calcium, phosphorus and other nutrients were found. The phytochemical screening revealed alkaloids followed by saponins and tannins	Satpathi, Tyagi and Gupta, 2011
Gum exudate	The purified, homogeneous, acidic polysaccharide was isolated	Ghosal and Thkur, 1981
Crude	ethno medicine were found by microscopic TLC observation	Xu *et al*., 2009
Gum	The polysaccharide, from this gum, contains galactosyl, arabinosyl, xylosyl, rhamnosyl and uronic acid residues	Omaira *et al*., 2005

2.2 Pharmacological study of *Spondias pinnata*

2.2.1 Studies on diuretic and laxative activity of bark extracts of *Spondias pinnata* (Linn. f) Kurz

The diuretic and laxative activity of different extracts of the barks of *Spondias pinnata* (Linn. f) Kurz were studied in Wistar albino rats. Furosemide and agar-agar were used as reference standards respectively for activity comparison. The chloroform and methanol extracts produced significant diuretic and laxative activity. On the other hand, the petroleum ether extract did not reveal significant activity. Urinary levels of sodium, potassium and chloride were estimated (Mondal, S. *et al.*, 2009).

2.2.2 Antioxidant and free radical scavenging activity of Spondias pinnata.

A 70% methanol extract of Spondias pinnata stem bark was studied in vitro for total antioxidant activity, for scavenging of hydroxyl radicals, superoxide anions, nitric oxide, hydrogen peroxide, peroxynitrite, singlet oxygen and hypochlorous acid, and for iron chelating capacity, reducing power, and phenolic and flavonoid contents. The extract showed total antioxidant activity with a trolox equivalent antioxidant concentration value of 0.78 +/- 0.02. The IC50 values for scavenging of free radicals were 112.18 +/- 3.27 microg/ml, 13.46 +/- 0.66 microg/ml and 24.48 +/- 2.31 microg/ml for hydroxyl, superoxide and nitric oxide, respectively. The IC50 for hydrogen peroxide scavenging was 44.74 +/- 25.61 mg/ml. For the peroxynitrite, singlet oxygen and hypochlorous acid scavenging activities the IC50 values were 716.32 +/- 32.25 microg/ml, 58.07 +/- 5.36 microg/ml and 127.99 +/- 6.26 microg/ml, respectively. The extract was found to be a potent iron chelator with IC50 = 66.54 +/- 0.84 microg/ml. The reducing power was increased with increasing amounts of extract. The plant extract (100 mg) yielded 91.47 +/- 0.004 mg/ml gallic acid-equivalent phenolic content and 350.5 +/- 0.004 mg/ml quercetin-equivalent flavonoid content (Hazra, B.; Biswas, S.; Mondal, N., 2008).

2.2.3 In vitro anticancer activity of *Spondias pinnata* on human lung and breast carcinoma

Spondias pinnata, a commonly distributed tree in India, previously proven for various pharmacological properties and also reported for efficient anti-oxidant, free radical scavenging

and iron chelating activity, continuing this, the present study is aimed to investigate the role of 70 % methanolic extract of *S. pinnata* bark in promoting apoptosis in human lung adenocarcinoma cell line and human breast adenocarcinoma cell line. These two malignant cell lines and a normal cell line were treated with increasing concentrations of the extract and cell viability is calculated. The extract showed significant cytotoxicity to both the carcinoma cells with an IC_{50} value of 147.84 ± 3.74 and 149.34 ± 13.30 µg/ml, respectively, whereas, comparatively no cytotoxicity was found in normal human lung fibroblast cell line with IC_{50} value of 932.38 ± 84.44 µg/ml (Ghate, B.N. *et al.*, 2013)

2.2.4 Antibacterial and cytotoxic activities of *Spondias pinnata* (Linn. f.) Kurz fruit extract

Attempt was undertaken to study the antibacterial potency and cytotoxic activity of 80% ethanol extract of the fruits of *spondias pinnata*. The antibacterial activity was performed by the disc diffusion method and cytotoxicity was observed by brine shrimp lethality bioassay. The fruit extract exhibited mild to potent antibacterial activity against some Gram-positive and Gram negative bacteria at a concentration of 500 µg/disc. Among them *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* shows promising result. The ethanolic extract revealed strong cytotoxicity having LC_{50} of 2.12 ± 0.09 µg/ml (Muhammad *et al.*, 2011).

2.2.5 Hypoglycemic activity of the bark of *Spondias pinnata* Linn. kurz.

The various extracts of the barks of *Spondias pinnata* was evaluated for hypoglycemic activity on adult Wistar albino rats at dose levels of 300 mg/kg p.o. each using normoglycaemic, glucose loaded and alloxan induced hyperglycaemic rats. Glibenclamide was used as reference standard for activity comparison. Among the tested extracts, the methanol extract was found to produce promising results that is comparable to that of the reference standard glibenclamide (Mondal, S.; Dash, G.K., 2009).

2.2.6 Analgesic activities of the stem bark extract of *Spondias pinata* (Linn.f) Kurz.

The ethanol extract of *Spondias pinnata* was obtained from the dried stem barks of *S. pinnata* and its analgesic properties investigated using acetic acid, formalin test and hot plate model. Ethanol extract of *S. pinnata* showed analgesic effects in a dose dependent manner in the acetic

acid test and in the second phase of formalin test which were comparable to the effects observed with acetylsalicylic acid. The results of this study lead credit to the traditional uses *S. pinnata*, especially as an analgesic (Panda, B.K., *et al.,* 2009).

2.2.7 Antibacterial, antidiarrhoeal and ulcer-protective activity of methanolic extract of *Spondias mangifera* bark.

The extracts of S. mangifera were tested for castor-oil induced diarrhea, and intestinal fluid accumulation and propulsion in rats using diphenoxylate hydrochloride and atropine as standard drug. The effect of the extracts on indomethacin-induced ulceration in rats was also evaluated. Cimetidine was used as positive control. In-vitro antibacterial activity of methanolic and aqueous extract was also evaluated against Escherichia coli, *Salmonella typhimurium* and *Vibrio cholerae* bacteria (Arif *et al.*, 2008).

2.2.8 *Spondias pinnata* stem bark extract lessens iron overloaded liver toxicity due to hemosiderosis in Swiss albino mice.

The study was designed to evaluate the ameliorating effect of 70% methanol extract of Spondias pinnata on iron overload induced liver injury. Iron overload was induced by intraperitoneal administration of iron-dextran into mice and resulting liver damage was manifested by significant rise in serum enzyme markers and reduction in liver antioxidants. Hepatic iron, serum ferritin, lipid peroxidation, protein carbonyl and hydroxyproline contents were measured in response to the oral administration of the extract of different doses. In order to determine the efficiency as iron chelating drug, the release of iron from ferritin by the extract was further studied. Enhanced levels of antioxidant enzymes were detected in the extract treated mice. The extract produced a dose dependent inhibition of lipid peroxidation, protein oxidation, liver fibrosis; and levels of serum enzyme markers and ferritin were also reduced dose dependently. The liver iron content was also found to be less in the extract treated group compared to control grou (Hazra, Sarkar and Mandal, 2013).

2.2.9 Anthelmintic activities of *Spondias pinnata.*

The stem heart wood and bark of *Spondias pinnata* when tested in vitro, showed potent anthelmintic activity on the earthworm, Pheretima posthuma. While stem heart wood methanolic extract of *S. pinnata* was also more potent than the bark extract (Panda *et al.*, 2011).

Table 2.2: Summaries of Pharmacological study on Spondias pinnata

Part of the plant	Findings	References
Barks	The chloroform and methanol extracts produced significant diuretic and laxative activity	Mondal, S. *et al.*, 2009
Stem barks	Total antioxidant activity, scavenging of hydroxyl radicals, superoxide anions, nitric oxide, hydrogen peroxide, iron chelating capacity, reducing power, and phenolic and flavonoid contents were found	Hazra, B.; BIswas, S.; Mondal, N., 2008
Bark	Methanolic extract promotes apoptosis in human lung adenocarcinoma cell line and human breast adenocarcinoma cell line	Ghate, B.N. *et al.*, 2013
Fruit	Ethanol extract shows antibacterial and cytotoxic activity	Muhammad *et al.*, 2011
Bark	Methanol extract shows hypoglycemic activity on adult Wistar albino rats	Mondal, S.; Dash, G.K., 2009
Bark	Ethanol extract shows analgesic activity	Panda, B.K. *et al.*, 2009
Bark	Antibacterial, antidiarrhoeal and ulcer-protective activity of methanolic extract were found	Arif *et al.*, 2008
Bark	Methanol extract lessens iron overloaded liver toxicity	Hazra, Sarkar and Mandal, 2013
Stem heart wood and bark	When tested in vitro, showed potent anthelmintic activity on the earthworm, Pheretima posthuma	Panda *et al.*, 2011

3.1 Extraction of leaves of *Spondias pinnata*

Extraction procedure: During extraction procedure of the experimental plat, following apparatus and solvents were used.

3.1.1 Materials:

Table 3.1: Apparatus and reagent used for extraction

Chemicals	Equipments	Glass apparatus
Methanol	Balance	Beaker
	Blender	Conical flask
	Rotary evaporator	Measuring cylinder
		Funnel

3.1.2 Collection of Plant and identification

The whole plant was collected from Belkuchy, Sirajgonj in January 2013. The plant was taxonomically identified by experts in Bangladesh National Herbarium, Mirpur, Dhaka, where a Voucher specimen (DACB Accession No. 351037) has been deposited for future reference.

3.1.3 Method

3.1.3.1 Drying of the barks

The collected Bark of the plant (around 1000 kilogram) was dried after cutting and slicing in the sun for about two weeks. In general the plant material should be dried at temperature bellow 30°C to avoid the decomposition of thermo labile compounds. The plant was dried in sun light thus chemical decomposition cannot take place.

3.1.3.2 Grinding of the dried barks

After drying, the barks were weighted in an electrical balance and the total weight was found to be 550 kilogram. The dried leaves were ground to course powder with a mechanical grinder. Before grinding of sample, the grinder was thoroughly cleaned to avoid contamination with any

remnant of previously ground material or other foreign matter deposited on the grinder. Grinding improves the efficiency of extraction by increasing surface area. After grinding, the weight of the grinded leaves was measured and the weight was about 500 gm. All grinded barks were stored in an air tight container.

Figure 3.1: Grinding machine

3.1.3.3 Procedure

After getting the sample as dried powder, the sample (500 gm) was then soaked in 1000ml of methanol for seven days. This process is termed as maceration. A glass made jar with plastic cover was taken and washed thoroughly with methanol and dried. Then the dried powder sample was taken in the jar. After that methanol (1000ml) was poured into the jar up to 1-inch height above the sample surface as it can sufficiently cover the sample surface. The plastic cover with aluminum foil was closed properly to resist the entrance of air into the jar. This process was performed for seven days. The jar was shaken in several times during the process for more interaction between the powdered particles and the solvent.

3.1.3.4 Filtration of the Extract

After the extraction process the plant extract was filtered with sterilized cotton filter. The cotton was rinsed with methanol and fitted in a funnel. The filtrate was collected in a beaker. Then again it was filtered and this time What man's filter paper was used for getting more clear extract which would be useful making the sample more concentrated in Rotary Evaporator Technique.

Then the filtrate was taken into a volumetric flask and covered with aluminum foil paper and was prepared for rotary evaporator.

3.1.3.5 Principle of a Rotary Evaporator

A rotary evaporator is a specially designed instrument for the evaporation of solvent (single-stage or straight distillation) under vacuum. The evaporator consists of a heating bath with a rotating flask, in which the liquid is distributed as a thin film over the hot wall surfaces and can evaporate easily. The evaporation rate is regulated by the heating bath materials and method temperature, the size of the flask, the pressure of distillation and the speed of rotation (Sepos E, 2012).

Figure 3.2: Rotary evapoprator

3.1.3.6 Procedure

After the filtration process two parts were obtained namely `residual part' and filtered part or filtrate'. The filtered part, which contains the substance soluble in methanol, was putted into a 1000ml round bottom flask and then the flask was place in a rotary evaporator. The evaporation was done at 50 temperatures. The number of rotation per minute was selected as 120 rpm. The pressure of the vacuum pumper machine was 6bar. The water flow through the distillation chamber was also provided in a satisfactory flow rate. When the evaporation seemed to be satisfactory, then the methanolic extract was collected in a 50mL beaker. The extraction was collected from the evaporating flask and the solvent is collected from the receiving flask. The

evaporator flask was rinsed by methanol. Then the beaker was covered with aluminum foil paper and kept for 60 minutes. Finally the concentrated methanolic plant extract was found and stored in the laboratory refrigerator from which the extract was used for many chemical investigations.

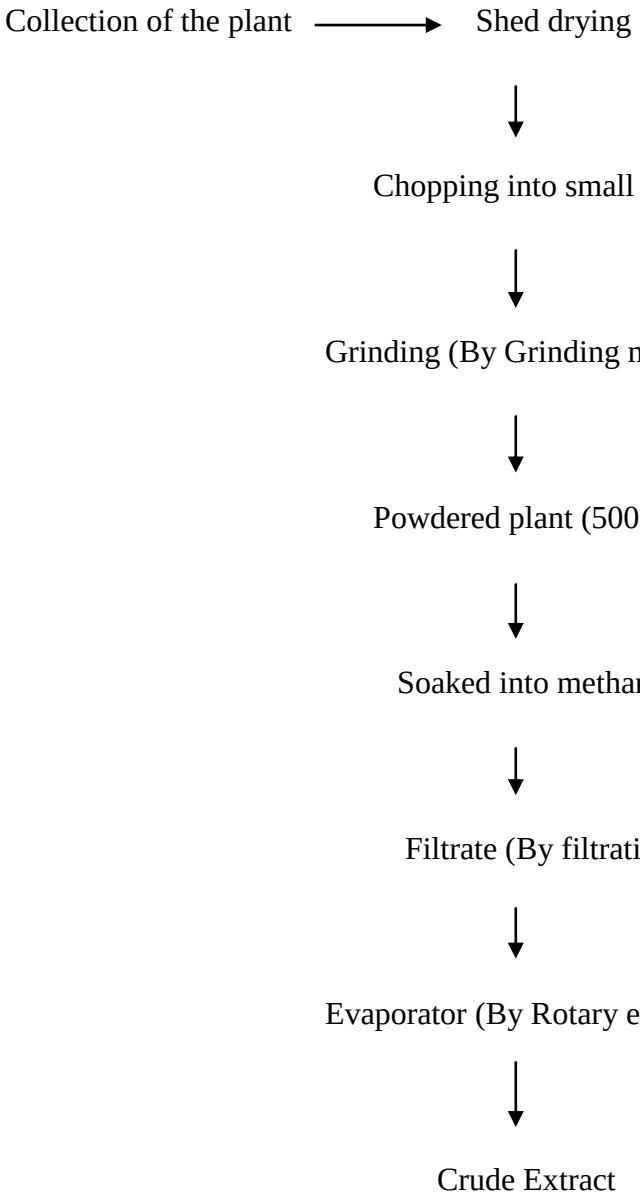

Figure 3.3: Schematic presentation of the crude preparation from the plant

3.1.3.7 Preparation of Mother Solution

5 gm of methanolic crude extract was again dissolved with 90 ml of methanol containing 10 ml of distilled water. The crude extract was dissolved completely. This is the mother solution, which was partitioned off successively by three solvents of different polarity. In subsequent stages each

of the fractions was analyzed separately for the detection and identification of compounds having antibacterial, cyto-toxic, antioxidant and other pharmacological properties.

3.1.3.8 Partition with Ethyl Acetate

The mother solution was taken in a separating funnel. 100 ml of the ethyl acetate was added to it and the funnel was shaken and then kept undisturbed. The extracted portion was collected. The process was repeated thrice (100 ml X 3). The ethyl acetate fraction was then air dried.

3.1.3.9 Partition with Chloroform

To the mother solution that left after partitioning with ethyl acetate, 16 ml of distilled water was added and mixed uniformly. The mother solution was then taken in a separating funnel and extracted with $CHCl_3$ (100 ml X 3). The $CHCl_3$ soluble fractions were collected together and air dried. The aqueous methanolic fraction was preserved as aqueous fraction.

3.2 Preliminary Phytochemical Screening

3.2.1 Materials

Table 3.2: Reagents and Apparatus used in Preliminary Phytochemical Screening Assay

10% Ferric Chloride Solution	Acetic Acid
1% Aqueous Hydrochloric Acid	Distilled Water
Acetic Anhydride	Bismuth Nitrate
Glacial Acetic Acid	Benzene
0.1% Ferric Chloride	Potassium Iodide
Concentrated Sulfuric Acid	Ethyl Acetate
Sodium Hydroxide Solution	10% Lead Acetate Solution
Dilute Sodium Hydroxide Solution	10% Ammonia Solution
Copper (II) Sulfate Crystal	10% Sulfuric Acid
Sodium Potassium Tartrate	Sodium Hydroxide
Glacial Acetic Acid	Screw Cap Test Tubes
Sonicator	Filter Papers

3.2.2 Test for Alkaloids

At first, 0.17 gm Bismuth nitrate in 2 mL Acetic Acid and 8 mL distilled water to prepare the Solution A. Then 4 gm Potassium Iodide was dissolved in 10 ml Acetic Acid and 8 ml Distilled Water to prepare the Solution B. Both solution A and B were mixed together in equal volume and distilled water added up to 100 ml to prepare Dragendorffs Reagent (Savithramma et al., 2011). A 100 mg of an extract was dissolved in dilute hydrochloric acid. Solution was clarified by filtration. Filtrate was tested with the prepared Dragendroff's reagents. The treated solutions were observed for any reddish brown precipitation (Kujur *et al*., 2010).

3.2.3 Test for Saponins

3.2.3.1 Froth test

0.5 g of extract was boiled with 5 ml of distilled water in a water bath for 10minutes. The mixture was filtered while hot and allowed to cool. 1 ml of filtrate was diluted to 5 ml with 4 ml distilled water and shaken vigorously for 2minutes. Appearance of frothing indicated the presence of saponin in the filtrate (Ajayi *et al*., 2011).

3.2.4 Test for Flavonoids

3.2.4.1 Test for free flavonoids

Five milliliters of ethyl acetate was added to a solution of 0.5 g of the extract in water. The mixture was shaken, allowed to settle, and inspected for the production of yellow color in the organic layer, which is taken as positive for free flavonoids (Kujur *et al*., 2010).

3.2.5 Test for Phenols

3.2.5.1 Ferric chloride Test:

Extract were treated with 3-4 drops of ferric chloride solution. Formulation of bluish black colour indicates the presence of phenols.

3.2.6 Test for Steroidal Compounds

3.2.6.1 Lieberman's test

0.5 g extracts were dissolved in 2 ml of acetic anhydride and cooled well in an ice-bath. Concentrated sulfuric acid was then carefully added. A color change from purple to blue to green indicated the presence of a steroid nucleus (Kujur *et al*., 2010).

3.3 Total Phenol content determination

Table 3.3: Apparatus and Reagents used in Estimation of Total Phenolic Content

Methanol	Test Tubes
Gallic Acid	Beaker
Folin	Pipette both 10 and 2 ml
Ciocalteu	Pumper
Na2CO3	Funnel
UV – Visible Spectrophotometer	Spatula
Measuring Cylinder	Volumetric Flask

3.3.1 Principle

The total phenolic concentration of the extract of was determined by the modified Folin-Ciocalteu method. The process of measuring total phenolic content of the crude extract of *Spondias pinnata* 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).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 and Thorpe, 1954).

3.3.2 Preparation of 7.5 % Sodium Carbonate Solution

7.5 g sodium carbonate was taken into a 100 ml of volumetric flask and the volume was adjusted by distilled water.

3.3.3 Preparation of 10% Folin- ciocalteu reagent

10 ml of Folin-ciocalteu reagent was taken in 100 ml volumetric flask and adjusted by distilled water.

3.3.4 Preparation of Standard Solution

The stock solution was prepared by taking .025 g of Gallic acid and dissolved into 5 ml of distilled water. The concentration of this solution was 5 µg/µl gallic acid. The experimental concentration from this stock solution was prepared by following manner.

Table 3.4: Preparation of standard solution

Concentration (µg/ ml)	Solution taken from stock solution	Adjust the volume by distilled water	Final volume
250	250µl	4.75ml	5ml
200	200µl	4.80ml	5ml
150	150µl	4.85ml	5ml
100	100µl	4.90ml	5ml
50	50µl	4.95	5ml

3.3.5 Preparation of extract solution

0.025gm of ethyl acetate extract was taken and dissolved into 5ml of distilled water. The concentration of this solution was 5 µg/µl of plant extract. The experimental concentration from this stock solution was prepared by following manner.

Table 3.5: Preparation of extract solution

Concentration (µg/ ml)	Solution taken from stock solution	Adjust the volume by distilled water	Final volume
250	250µl	4.75ml	5ml
200	200µl	4.80ml	5ml
150	150µl	4.85ml	5ml
100	100µl	4.90ml	5ml
50	50µl	4.95	5ml

3.3.6 Procedure

1.0ml of plant extract or standard of different concentration solution were taken in test tubes and 5ml of folin-ciocalteu (diluted 10 fold) reagent solution was added to the test tubes. 4 ml of sodium carbonate solution was added into the test tubes. The test tubes of standard solution were incubated for 30 minutes at 20^{o}c temperature. The test tubes of plant extracts solution were incubated for 1 hour at 20^{o}c to complete the reaction. The absorbances of the solutions were measured at 765 nm using a spectrophotometer against blank.

3.4 DPPH Free Radical Scavenging Assay

3.4.1 Materials

Table 3.6: Apparatus and Reagents used in DPPH Test

Methanol	Beaker
Distilled Water	Pipette both 10 and 2 ml
UV – Vis Spectrophotometer	Pumper
Test Tubes	Funnel
Micropipette	Spatula
Screw Cap Test Tubes	

3.4.2 Principle

The DPPH method measures electron-donating activity of other compounds in the mixture and hence provides an evaluation of antioxidant activity due to free radical scavenging. Any molecule that can donate an electron or hydrogen to a mixture will react with and bleach DPPH. DPPH is reduced from a purple compound to a light yellow compound by electrons from oxidant compounds. Reaction of DPPH with hydroxyl groups involves a hemolytic substitution of one of the phenyl rings of DPPH yielding 2-(4-hydroxyphenyl)-2-phenyl-1- picryl hydrazine as a major product whilst 2-(4nitrophenyl)-2phenyl-1-picrylhydrazine is also formed via a series of secondary processes. The concentration of DPPH at the end of a reaction will depend on the concentration and structure of the compound being scavenged.

3.4.3 Preparation of DPPH solution

4 mg of DPPH was taken and dissolved in 10ml of methanol. The solution was kept in dark place for 30 minutes.

3.4.4 Preparation of extract solution

4 mg of ethyl acetate extract were taken and dissolved in 40 ml of methanol. The concentration of the solution is 100µg/ml.

3.4.5 Preparation of standard solution

Ascorbic acid is taken as standard. 4mg of ascorbic acid is dissolved in 40ml of methanol and kept the concentration at solution is 100 µg/ml.

3.4.6 Procedure

1ml of extract or standard solution was taken from the stock in different test tubes and 4ml of methanol was added to make 5 ml solution. The concentration of the solution is 20µg/ml. Then 2 ml of stock solution was added to other test tubes and 3 ml of methanol was added to the test tubes. The concentration of the solution is 40µg/ml. then 3ml, 4ml, 5ml of stock solution was mixed with 2ml, 1ml and 0ml of methanol to make concentration of 60, 80, 100 100µg/ml. 5 ml of methanol was taken in a test tube as blank. Then 100µl of DPPH solution was added to each test tube. The test tubes were kept in dark place for 20 minute. After that, the absorbance was taken at 517 nm.

3.4.7 Calculation of % inhibition

The radical scavenging activity was expressed as the percentage inhibition which was calculated by using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Blank Absorbance} - \text{Sample Absorbance}) \times 100}{\text{Blank Absorbance}}$$

Here, Blank Absorbance = 0.373

3.5 Total Reducing Power

3.5.1 Materials

Table 3.7: Apparatus and Reagents used in Total Reducing Power

Phosphate Buffer (0.2 M, pH 6.6)	Screw Cap Test Tubes
1% Potassium Ferric cyanide (10 mg/ml)	Beaker
10% Trichloroacetic Acid	Pipette both 10 and 2 ml
Distilled Water	Pumper
Ferric chloride (0.5 ml, 0.1%)	Falcon Tube
Sonicator	Reagent Bottle
Ice bath	Filter Paper
Centrifuge Machine	Funnel
Double Beam UV – Vis Spectrophotometer	Spatula
Water Bath	Conical Flask

3.5.2 Introduction

The oxidation induced by Reactive Oxygen Species can result in cell membrane disintegration, membrane protein damage and DNA mutation which can further initiate or propagate the development of many diseases such as cancer, liver injury and cardiovascular disorders. Although our body has its own defense mechanism but continuous exposure to chemicals and contaminants may lead to an increased amount of free radicals in the body beyond its capacity to control them and cause irreversible oxidative damage.So therefore the antioxidants with free radical scavenging activity play an important role in case of this problem. The synthetic antioxidants produce much toxicity. So the main focus is on the natural antioxidants especially of plant origin (Jayaprakash and Rao, 2000).

3.5.3 Preparation of Reagent

1) Phosphate buffer (2.5 ml, 0.2 H, pH6.6)
    A. 27.8 gm monobasic sodium phosphate dissolved in 500 ml water.
    B. 53.65 gm of dibasic phosphate dissolved in 500 ml water.

    62.5 ml from solution A and 37.5 ml from solution B were taken and mixed to form buffer solution.

3.5.4 Preparation of potassium ferricyanide solution (1%)

1gm of potassium ferricyanide was taken into 100ml of volumetric flask and adjusted with distilled water.

Preparation of trichloro acetic acid (10%) solution

10gm of trichloroacetic acid was taken into 100ml volumetric flask and adjusted with distilled water.

3.5.5 Preparation of ferric chloride (.1%) solution

0.1 gm of ferric chloride was taken into 100 ml volumetric flask and adjusted with distilled water.

3.5.6 Preparation of sample

12 mg of extract dissolved in 10 ml of methanol. The coincentration of this solution is 1200 µg/ ml. Then serial dilution was applied to create.

3.6 Brine Shrimp Lethality Test

3.6.1 Objective of Brine Shrimp Lethality Bioassay

Bioactive compounds are always toxic to living body at some higher doses and it justifies the statement that 'Pharmacology is simply toxicology at some higher doses and toxicology is simply pharmacology at some lower doses'. Brine shrimp lethality bioassay is a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origin. By this method, natural product extracts, fractions as well as pure compounds can be tested for their bioactivity. In this method *In vivo* lethality in a simple zoological organism (Brine shrimp nauplii) is used as a favorable monitor for screening and fractionation in the discovery of new bioactive natural products.

This bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal and anti-tumor etc. of the compounds.

Brine shrimp lethality bioassay technique stands superior to other cytotoxity testing procedures because it is rapid in process, inexpensive and requires no special equipment or aseptic technique. It utilizes a large numbers of organisms for statistical validation and a relatively small amount of sample. Furthermore, unlike other methods, it does not require animal serum.

3.6.2 Principle

Brine shrimp eggs are hatched in simulated sea water to get nauplii. By the addition of calculated amount of Dimethyl sulfoxide (DMSO), desired concentrations of the test sample is prepared. The nauplii are counted by visual inspection and are taken in vials containing 5 ml of simulated sea water. Then samples of different concentrations are added to pre-marked vials using micropipettes. Then the vials are left for 24 hours. Survivors are counted after 24 hours.

3.6.3 Materials

Table 3.8: Materials for Brine shrimp lethality test

Artemia salina leach (Brine shrimp eggs)	Sea salt
Test samples of the experimental plants	Lamp to attract the shrimps
Small tank with perforated dividing damns	Pipettes
Micropipettes	Test tubes
Glass vials	Magnifying glass

Test samples (Bark extract of *Spondias pinnata*) for brine shrimp lethality bioassay

Code no.	Test sample	Amount (mg)
EAF	Ethyl Acetate fraction of methanol extract	4.0

3.6.4 Preparation of seawater

38 gm sea salt (pure NaCl) was weighed, dissolved in one litre of distilled water and filtered off to get a clear solution. 1-2 drops of NaOH solution of 1N was added with a dropper to obtain the pH 7.4 as sea water.

3.6.5 Hatching of Brine Shrimps

Brine shrimp eggs were collected from pet shops was used as the test organism. Sea water was taken in a small tank and shrimp eggs were added to the one side of the tank and then this side was covered.

One day was allowed to hatch the shrimps and to be matured as nauplii. Constant oxygen supply was carried through the hatching time. The hatched shrimps were attracted to the lamp through the perforated damn and they were taken for experiment. With the help of Pasteur pipette 10 living shrimps nauplii were added to each of the test tubes containing 5 ml of sea water.

3.6.6 Preparation of the Test sample of Experimental plant

All the test samples (ethyl acetate, chloroform extract & crude fraction) were taken in vials and dissolved in 100 µl of pure Dimethyl sulfoxide (DMSO) to get stock solution. Then 100 µl of this solution was taken in the first test tube containing 5 ml of sea water and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400 µg/ ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. In every case, 100 µl of the test samples were added to the test tube and fresh 100 µl DMSO was added to the vial. Thus different concentrations were found in the different test tubes.

Table 3.9: Test sample with concentration values after serial dilution

Test tube no.	Concentration (µg/ ml)
01	400.0
02	200.0
03	100.0
04	50.00
05	25.00
06	12.50
07	6.250
08	3.125
09	1.563
10	0.781

3.6.7 Preparation of the control group

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

i. Positive control group
ii. Negative control group

3.6.8 Preparation of the positive control group

Positive control in a cytotoxity study is a widely accepted cytotoxic agent and the result of the test sample was compared with the result of obtained for the positive control. In the present study Vincristine tamoxifen was used as the positive control. Measured amount of the tamoxifen was dissolved in DMSO then the positive control solution were added to the pre-marked vials containing 10 living shrimps nauplii in 5 ml simulated sea water to get positive control groups. The concentration is maintained 400 µg/ ml, 200 µg/ ml, 100 µg/ ml, 50 µg/ ml, 25 µg/ ml, 12.50

µg/ ml, 6.25 µg/ ml, 3.125 µg/ ml, 1.5625 µg/ ml and 0.78125 µg/ ml by serial dilution as the sample prepared.

3.6.9 Preparation of the negative control group

100 µl DMSO was added to each three pre-marked glass vials containing 10 living shrimps nauplii in 5 ml simulated sea water to used as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxity of the compounds.

3.6.10 Counting of nauplii

After 24 hours, the vials were using a magnifying glass and the numbers of survivors were counted. The percent (%) mortality was diluted for each dilution. The concentration- mortality data was analyzed statistically by using linear regression using a simple IBM-PC program. The effectiveness or the concentration- mortality relationship of plan product is usually expressed as a median lethal concentration (LC_{50}) value. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

4.1 Phytochemical Screening of ethyl acetate extract of *Spondias pinnata* bark

4.1.1 Result: Phytochemical screening is listed below:

Table 4.1: Results of Phytochemical Screening

Secondary Metabolites	**Screening Result**
Test for Alkaloids	- -
Test for Flavonoids	+ +
Detection of Phenols	- -
Test for Steroidal compund	-
Test for Saponins	+ + +

"+ + + +" = Highly present, "+ + +" = moderately present, "+ +" =slightly present and "-" = absent.

4.1.2 Discussion: From the experiment it was found that, the ethyl acetate extract of *Spondias pinnata* bark contains most of the phytoconstituents. It contains Alkaloids, flavonoids, phenols, Saponins etc. Saponins were present in greater amount, whereas flavonoids were present in moderate amount. Steroidal compound, aljkaloids and phenol were absent in the initial screening.

4.2 Total reducing power of Ethyl Acetate extract of *Spondias pinnata* bark

4.2.1 Result: The absorbance at 700nm was taken using UV-Visible spectrophotometer and the absorbance of sample and standard was recorded.

Table 4.2: Absorbance of sample at different concentrations

Concentration (µg/ml)	Absorbance (nm)	% Reducing Potential (Blank absorbance – Sample absorbance) ×100 / Absorbance of blank
1200	0.282	24.397
1000	0.281	24.665
800	0.353	5.362
600	0.391	-4.826
400	0.298	20.107
200	0.294	21.179

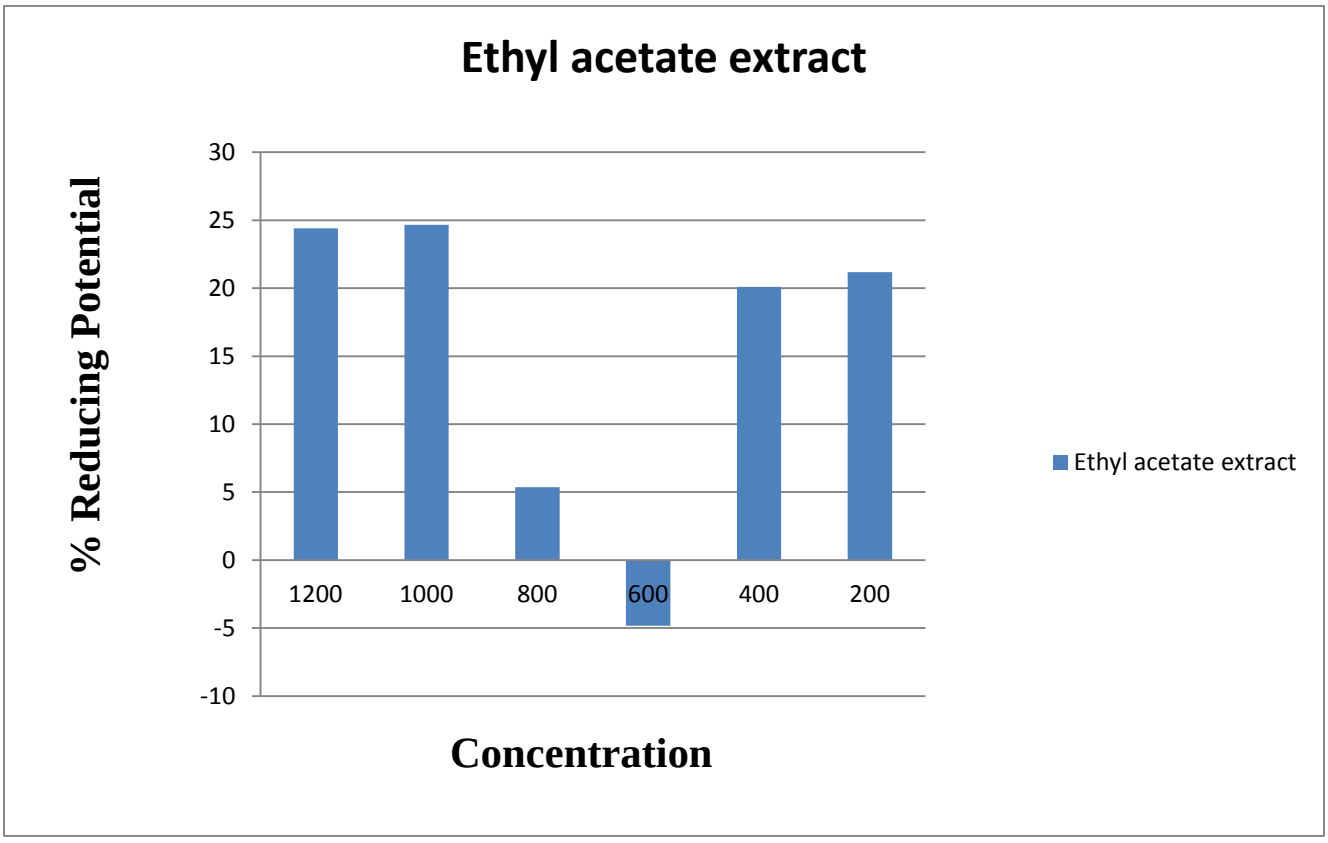

Figure 4.1: Absorbance of sample at different concentration

Table 4.3: Absorbance of standard at different concentration

Concentration (µg/ml)	Absorbance (nm)	% Reducing Potential $\frac{\text{(Blank absorbance – Sample absorbance)} \times 100}{\text{Absorbance of blank}}$
1200	0.321	13.941
1000	0.280	24.933
800	0.305	18.231
600	0.296	20.643
400	0.271	27.346
200	0.314	15.818

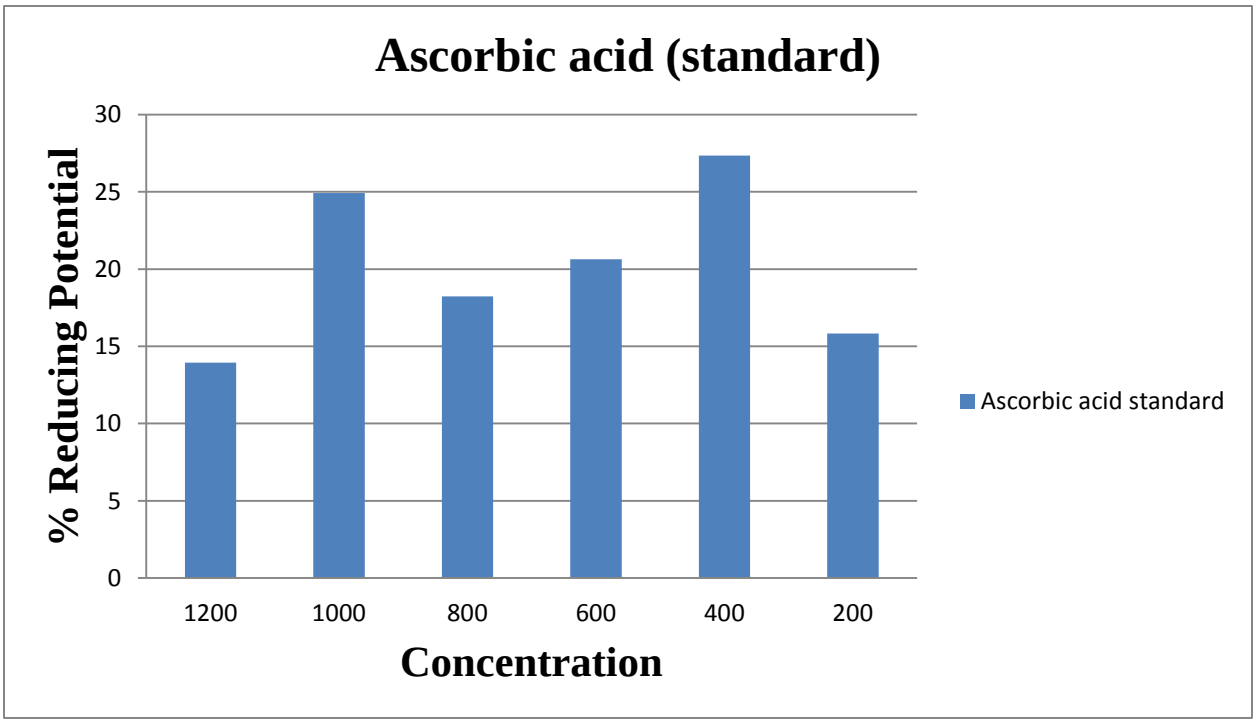

Figure 4.2: Absorbance of standard at different concentration

4.2.2 Discussion: From the curve, standard and plant extract showed almost similar pattern of absorbance. High absorbance indicates the high reducing power (Dharmendra, 2009).The reducing power of the plant extract increased as the amount of extract increases and the activity of the sample is higher than the standard. For example, in the concentration of 1000 (µg/ml) the reducing potential of extract was 24.397% and of the standard was 13.941%. Sharma *et al.* stated that phenolics or polyphenol compounds causes the greater reducing power and therefore, can act as antioxidants. Total phenolic content experiment, stated that plant extract contains significant amount phenolic compound. From the results, it was evident that the extracts possess significant reducing power as compared to standards.

4.3 Evaluation of antioxidant property of Ethyl Acetate extract of *Spondias pinnata* bark by DPPH free radical scavenging assay

4.3.1 Result: The absorbance at 517nm was taken using UV-Visible spectrophotometer and absorbance of DPPH for extract, extract blank, ascorbic acid was recorded. The absorbance and percent scavenging of different sample is in the following table.

Table 4.4: Absorbance and %inhibition by DPPH for Test Sample

Concentration (µg/ml)	Absorbance (nm)	% inhibition $\frac{\text{(Blank absorbance – Sample absorbance)} \times 100}{\text{Absorbance of blank}}$	X value (IC_{50})
20	0.11	70.509	425
40	0.125	66.488	
60	0.151	59.517	
80	0.172	53.887	
100	0.22	41.019	

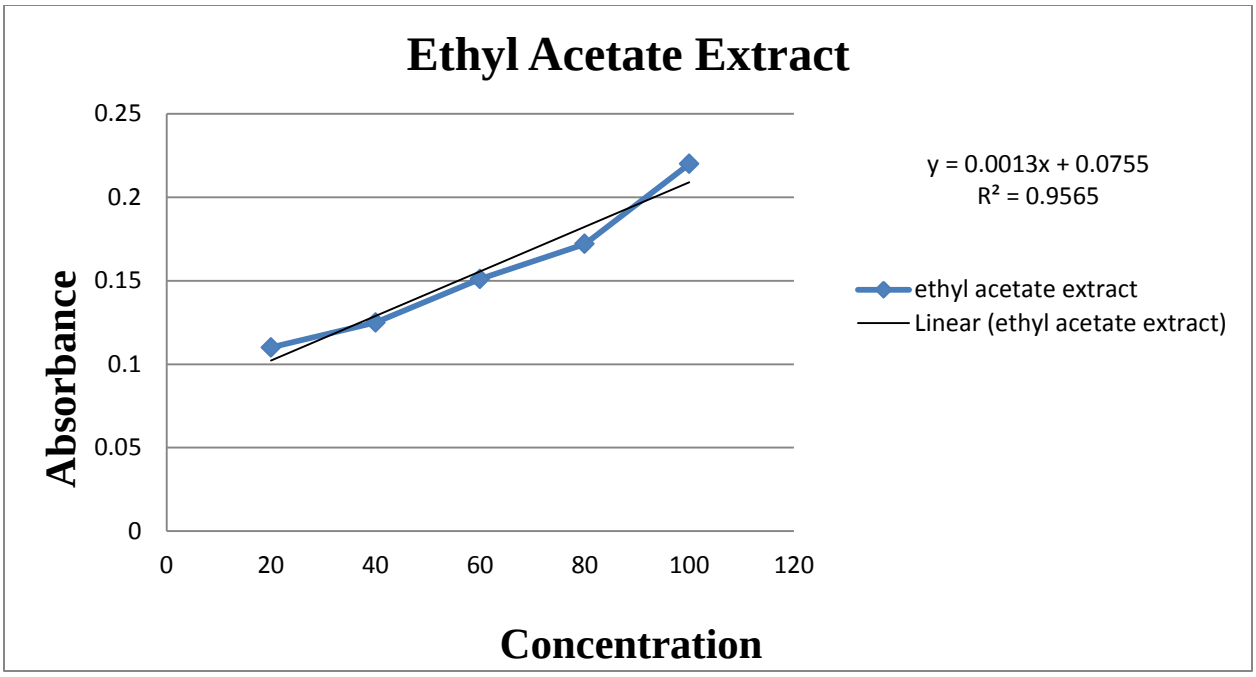

Figure 4.3: Graph of **c**oncentration versus absorbance for test sample

Table 4.4: Absorbance and %inhibition by DPPH for Ascorbic Acid

Concentration (µg/ml)	Absorbance (nm)	% inhibition $\frac{\text{(Blank absorbance – Sample absorbance)} \times 100}{\text{Absorbance of blank}}$	X value (IC_{50})
20	0.107	71.314	409
40	0.14	62.466	
60	0.168	54.959	
80	0.179	52.011	
100	0.199	46.649	

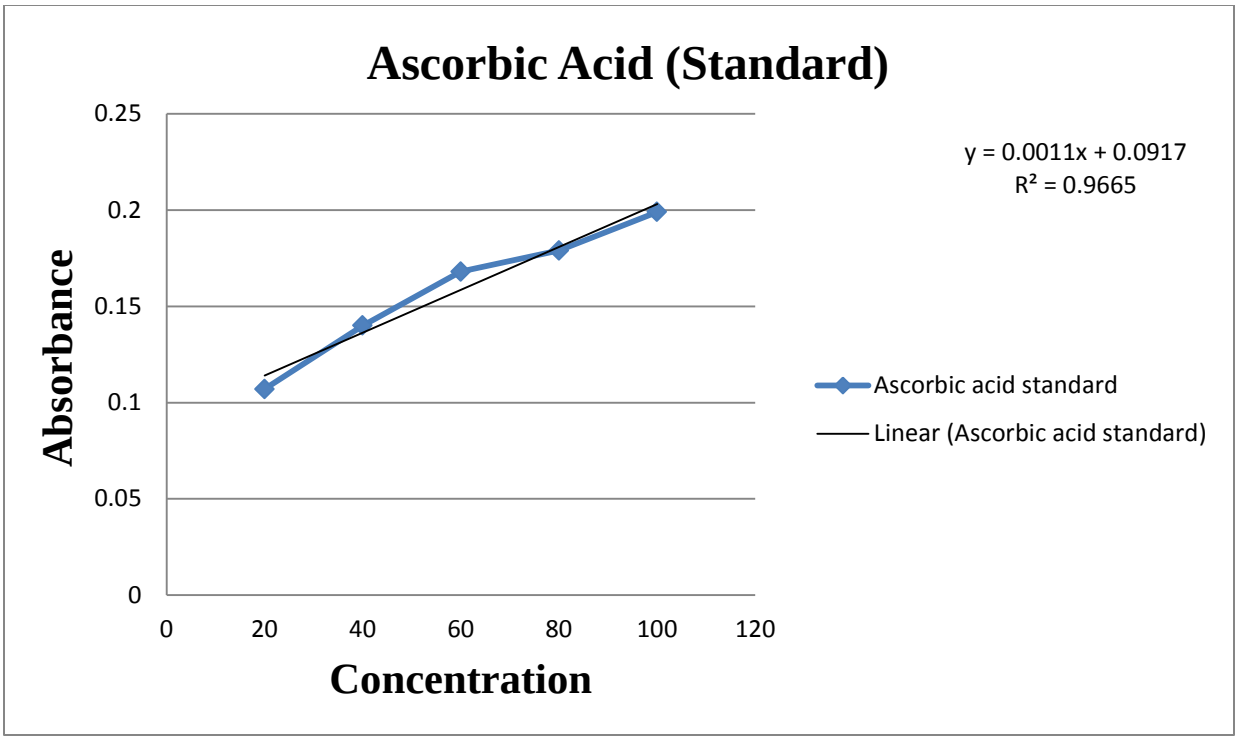

Figure 4.4: Graph of concentration versus absorbance for ascorbic acid

4.3.2 Discussion: From the curve, percentage scavenging activity of extract and the ascorbic acid at different concentration was observed almost similar. The IC_{50} of extract is 425(µg/ml) and the IC50 of standard is 409(µg/ml). This means the plant extract has antioxidant activity. It may be, due to the presence of polyphenolic compounds such as flavonoids and tannins in the extract of the plant (Paixao *et al*., 2007).

4.4 Brine Shrimp Lethality Test

The Ethyl Acetate extracts of bark were subjected to brine shrimp lethality bioassay following the procedure (Manik *et al.,* 2013). The lethality of the extractives to brine shrimps was determined and the results are given in Table below.

The lethal concentration (LC_{50}) of the test samples after 24 hours was obtained by a plot of percentage of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. Tamoxifen was used as standard and the LC_{50} was found. The result of ethyl acetate extract was compared with the standard tamoxifen.

Table 4.5: Effect of Ethyl Acetate extract on shrimp nauplii

Concentration µg/ml	Log C	No. of Nauplii dead	% Mortality	Best Fit Equation	LC_{50} (µg/ ml)
400	2.602	10	100	Y=17.51x + 63.14	0.178
200	2.301	10	100		
100	2.00	10	100		
50	1.699	10	100		
25	1.398	10	100		
12.5	1.097	10	100		
6.25	0.796	7	70		
3.125	0.495	5	50		
1.5625	0.194	5	50		
0.781	-0.107	8	80		

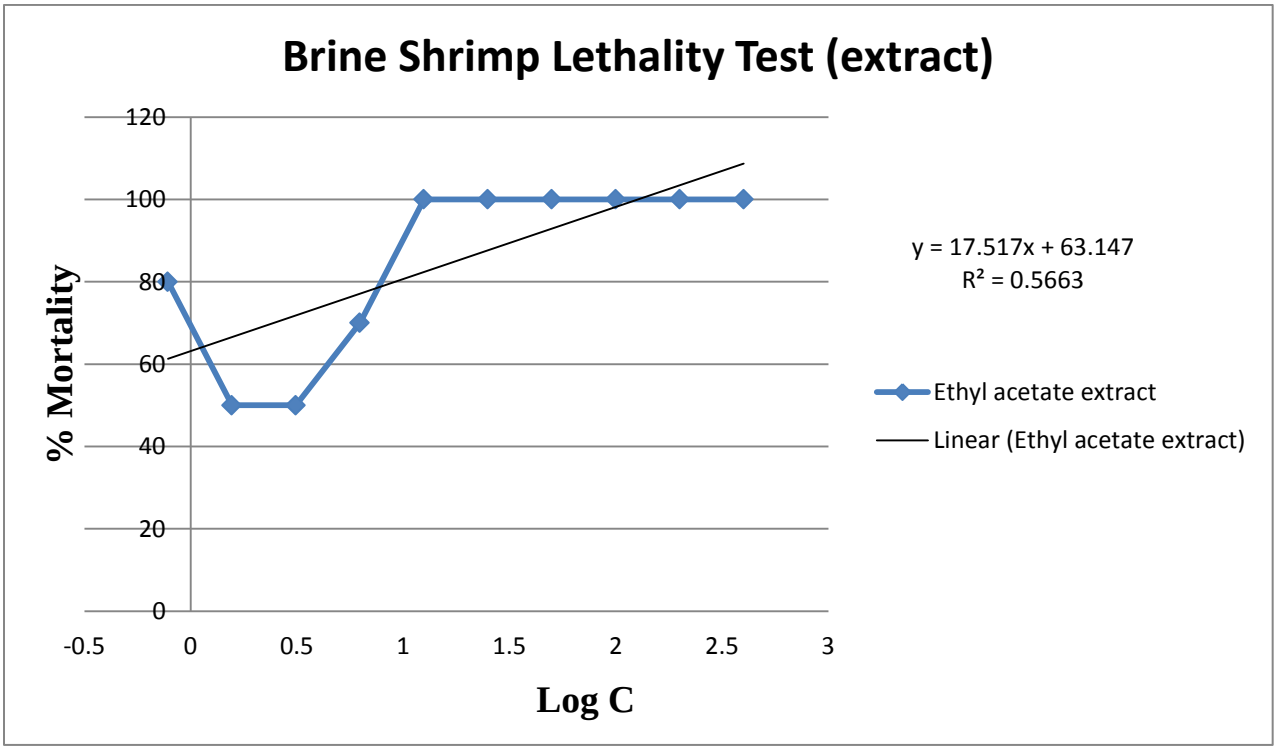

Figure 4.5: Graph of % of mortality of brine shrimp nauplii by tested sample

Table 4.6: Effect of tamoxifen on shrimp nauplii

Concentration µg/ml	Log C	No. of Nauplii dead	% Mortality	Best Fit Equation	R2 Value	LC_{50} (µg/ ml)
400	2.602	10	100	Y=19.73x +61.38	0.720	1.142
200	2.301	10	100			
100	2.00	10	100			
50	1.699	10	100			
25	1.398	10	100			
12.5	1.097	10	100			
6.25	0.796	9	90			
3.125	0.495	7	70			
1.5625	0.194	5	50			
0.781	-0.107	5	50			

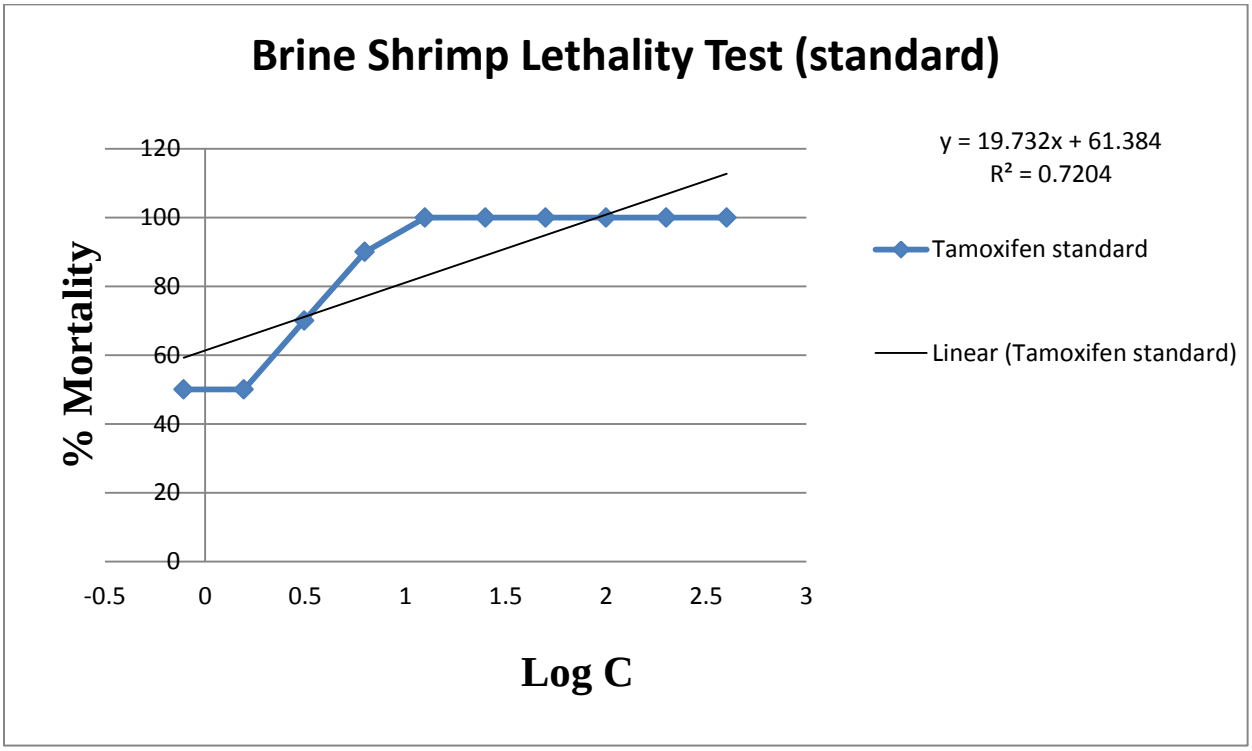

Figure 4.6: Graph of % of mortality of brine shrimp nauplii standard tamoxifen

4.4.2 Discussion: From the result it was found that the % of mortality of extract is greater than the standard. The extract showed the LD_{50} of 0.178 (µg/ ml) and the standard showed the LD_{50} of 1.142 (µg/ ml). This indicates that the ethyl acetate extract of Spondias pinnata bark has potential cytotoxic activity.

4.5 Estimation of total phenolic content of ethyl acetate extract of *Spondias pinnata* bark

4.5.1 Result: The methanol extract of *Spondias pinnata* (bark) 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 equivalents, Total phenolic content of the samples are expressed as mg of GAE (Gallic acid equivalent) per gm of dried extract. Absorbance Values are expressed as average ± SD (n=3). The absorbance of phenol content for Gallic acid at 760nm, equation for best fitted line and R^2 value is given in a table below using the standard curve also shown below. The absorbance of phenol content for gallic acid at 760nm, equation for best fitted line and R2value is given in a table below using the standard curve also shown below.

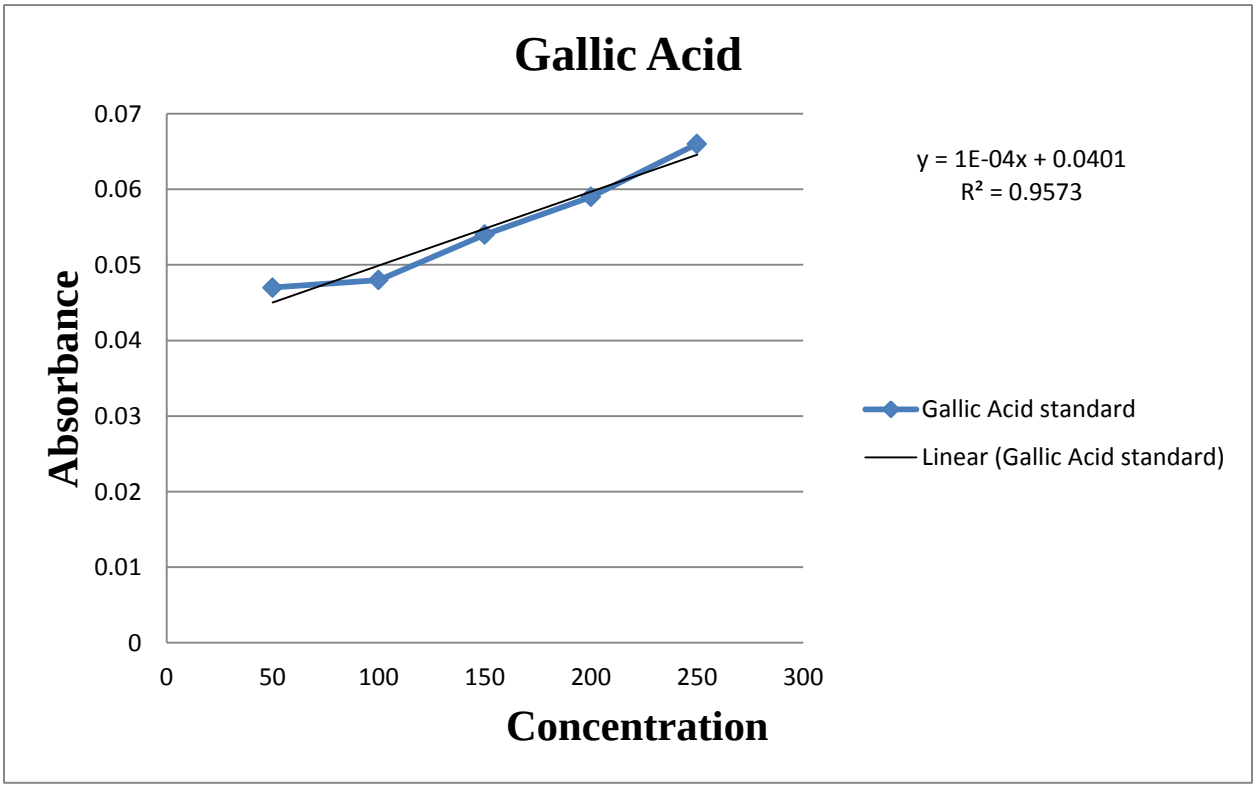

Figure 4.7: Standard curve of Gallic Acid

Table 4.7: Absorbance of phenol for gallic acid, best fit equation and R^2 value

Concentration	Absorbance	Best Fit Equation	R^2 Value	X value
250	0.066	Y= .0001 x + 0.040	0.957	260
200	0.059			190
150	0.054			140
100	0.048			80
50	0.047			70

Table 4.8: Absorbance of phenol for Ethyl acetate Extract, best fit equation and R^2 value

Concentration	Absorbance	X value
250	0.060	200
200	0.054	140
150	0.051	110
100	0.048	80
50	0.045	50

4.5.2 Discussion: From the standard curve, we found an equation Y= .0001 x + 0.040. By incorporating the absorbance of extract and standard on the Y value of this equation, we find the x value for different concentration of sample and standard. Then we found that the x value of sample (200, 140, 110, 80 and 50) mg of GAE/gm is comparatively lower than the x value of (260, 190, 140, 80 and 70) mg of GAE/gm. Here, GAE means Gallic acid equivalent. So, it is evident that the plant extract of *Spondious pinnata* possess significantly lower phenolic content as compared to the standards.

5.1 Conclusion

The phytochemical constituents of *Spondias pinnata barks* are responsible for medicinal value. The bark contains varieties of phytochemical compounds. The antioxidant and free radical scavenging activity is responsible for their usefulness in the treatment of many diseases. Oxidation is responsible for many diseases such as ageing, ischemia, anemia, cancer, arthritis, asthma, neurodegenaration, inflammation, Pakinson's disease etc. In reducing power test, the extract showed potential activity similar to standard. Potential scavenging activity was also found by DPPH scavenging activity test. Total phenolic content experiment also stated that the plant extract contains significant amount of phenolic compounds. Due to the antioxidant activity; it will be useful for the treatment of those diseases. The bark also has the cytotoxic activity. So, it can be used as cytotoxic drug in cancer treatment. Further studies and researches are required to see tge biological activity of the extract. The isolation and identification should be done and tested in higher animal to see the efficacy of the compound.

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