Investigation of Phytochemical and Cytotoxic Activity of Methanol Extract of *Spondias pinnata* Bark

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ENDORSEMENT BY THE CHAIRPERSON

This is to certify that the dissertation entitled “Investigation of Phytochemical and Cytotoxic Activity of Methanol Extract of *Spondias pinnata* Bark” is a genuine research work carried out byEkhlas Uddin Khan, under the supervision of Mahbubul Hoque Shihan (Senior Lecturer, Department of Pharmacy, East West University, Dhaka). I further certify that no part of the thesis has been submitted for any other degree and all the resources of the information in this connection are duly acknowledged.

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CERTIFICATE BY THE SUPERVISOR

This is to certify that the dissertation entitled "Investigation of Phytochemical and Cytotoxic Activity of Methanol Extract of *Spondias pinnata* Bark", submitted to the Department of Pharmacy, East West University, Dhaka, in partial fulfillment of the requirements for the Degree of Bachelor of Pharmacy, was carried out by Ekhlas Uddin Khan, ID No. 2010-1-70-037 under my supervision and no part of this dissertation has been or is being submitted elsewhere for the award of any Degree/Diploma.

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DECLARATION BY THE CANDIDATE

I, Ekhlas Uddin Khan, hereby declare that the dissertation entitled "Investigation of Different Pharmacological Activity of the Methanol Extract of *Spondias pinnata* Barks", submitted by me to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Honors) is a confide record of original research work carried out by me under the supervision and guidance of Mahbubul Hoque Shihan, Senior Lecturer, Dept. of Pharmacy, East West University.

_____

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Dedication

This research paper is dedicated to
My beloved Mother…

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ABSTRACT

*Spondias pinnata*is a medicinal herb belonging to the family Family- Anacardiaceae also known as wild mango, hog-plum, which has been used to dysentery, diarrhea, stomach ache, rheumatism properties according to indigenous systems of medicine in this sub continent. But its antioxidant, antimicrobial, cytotoxic potential and phytochemical screening is still unknown. Therefore, the present study was designed to investigate antioxidant, antimicrobial and cytotoxic properties and to investigate the phytochemical compounds of *spondias pinnata*. For phytochemical screening, we found the presence of alkaloids, flavonols, phenols and saponins. In order to investigate the antioxidant property, we are experiencing DPPH test, Total Reducing power test and Total phenol content test. In DPPH scavenging IC_{50} value of extract was 397 whereas the IC_{50} value of standard was 409. The Reducing power test showed lower reducing potential of extract compared to standards which indicates the slightly presence of phenol and polyphenolic compounds. For example, in the concentration of 1000 (µg/ml) the reducing potential of extract was 8.58% and of the standard was 24.93 %. In Brine shrimp lethality test the LC_{50} of the extract was 0.362 and the LC_{50} of the standard was 1.412 which showed lower cytotoxic activity compare to standards.

Key Words: *Spondias pinnata*, Anacardiaceae, Cytotoxicity, DPPH free scavenging, Reducing potential and Brine shrimp lethality.

Chapter One

INTRODUCTION

Chapter Two

LITERATURE REVIEW

Chapter Three

METHODS & MATERIALS

Chapter Four

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1.1 Introduction of Medicinal Plants

1.1.1. Medicinal Plants

Plants form the main ingredients of medicines in traditional systems of healing and have been the source of inspiration for several major pharmaceutical drugs. Roughly 50,000 species of higher plants have been used medicinally. This represents by far the biggest use of the natural world in terms of number of species. 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. Nearly, twenty-five per cent of all of our prescription drugs are derived from plants, many of them from tropical rainforests; and as many as 70% of our pharmaceuticals are modeled after constituents found in plants. Even today, 80% of the world's population relies on botanical medicines as their primary means of health care (Herborn, 1998).

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" (Sofowora, 1982). Almost every family in rural Amazonian villages has their own family medicinal plants garden. 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 (Latha *et al.*, 2010).

The most important ingredients present in plant communities turn out to be alkaloids, terpenoids, steroids, phenols glycosides and tannins (Stelling, 1992). The information obtained from extracts of medicinal plants makes pharmacological studies possible. If the active ingredients are

characterized, then the mode of action of plants producing therapeutic effects can also be better investigated. Infectious diseases are the leading cause of death worldwide. The clinical efficiency of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogens (Bandow *et al*., 2003). Bacterial pathogens have involved numerous defense mechanisms against antimicrobial agents and resistance to old and newly produced drug is on the rise. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity (Colombo and Bosisio, 1996). There are several reports in the literature regarding the antimicrobial activity of crude extracts prepared from plants. Plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines. Higher plants as sources of medicinal compounds have continued to play a dominant role in the maintenance of human health care since ancient times. Over 50% of all modern clinical drugs are of natural product origin and natural products play a vital role in modern drug development in the pharmaceutical industry (El-seedi *et al.*, 2002).

1.1.2. History of Plants in Medicine

- Plants have been used from ancient times to attempt cures for diseases and to relive physical suffering. Ancient peoples all had acquired some knowledge of medicinal plants. Oftentimes these primitive attempts at medicine were based on superstition and speculation. Evil spirits in the body were thought to be the cause of medical problems. They could be driven out of the body through the use of poisonous or disagreeable plant substances that rendered the body a disagreeable habitat. Medicine men or women of a tribe were usually charged with knowledge of such plants. The progress of medicine has often been guided by the earlier observations and beliefs. (Levetin and Mahon M, 2003).
- Drug plants were always of especial interest. As early as 5,000 B.C. many drugs were in use in China. Sanskrit writings testify to methods of gathering and preparing drugs in these early times. The Babylonians, ancient Hebrews and Assyrians were all familiar with medicinal plants. From Egypt there are records dating to 1,600 B.C. naming many of the medicinal plants used by physicians of that period, among which myrr, opium, cannabis, aloes, cassia and hemlock are prominent. The Greeks were familiar with many of the drugs of today, evidenced by the works of Hippocrates, Theophrastus, Aristotle and

Pythagoras. The supernatural element continued to remain prominent in their culture, however. Only a few individuals were thought able because of some special power to distinguish harmful from valuable plants. This "rhizotomoi" or root diggers were an important caste in ancient Greece. In Rome there was less interest in plants that had healing powers. But by 77 BC Dioscorides wrote in his treatise, "De Materia Medica," dealing with the nature and properties of all the medicinal substances known at that time. This work was highly esteemed for 15 centuries and to this day is valued in parts of Turkey and North Africa. Pliny and Galen also described the nature of some drug plants(Halberstain, 2005) .

- Following the Dark Ages there began a period of the encyclopedists and herbalists. The monasteries of Northern Europe produced large compendiums of information regarding plants, much of which was false. They stressed the medicinal value and folklore of plants. About the same time there appeared a "Doctrine of Signatures." This superstitious doctrine suggested that all plants possessed some sign, given by the Creator, which indicated the use for which they were intended. A plant with heart-shaped leaves was good for heart ailments; the liverleaf with its 3-lobed leaves was good for liver problems, etc. Many of the common names of plants owe their origin to this superstition. Names such as heartease, dogtooth violet, Solomon's seal and liverwort are examples (Petrovska, 2012).
- 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. 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).

- The Arabs introduced numerous new plants in pharmacotherapy, mostly from India, a country they used to have trade relations with, whereas the majority of the plants were with real medicinal value, and they have persisted in all pharmacopoeias in the world till today. The Arabs used aloe, deadly nightshade, henbane, coffee, ginger, strychnos, saffron, curcuma, pepper, cinnamon, rheum, senna, and so forth. Certain drugs with strong action were replaced by drugs with mild action, for instance, *Sennae folium* was used as a mild laxative, compared to the purgatives *Heleborus odorus* and *Euphorbium* used until then (Petrovska, 2012).
- Pharmacology and pharmacognosy owe their beginnings to the earlier beliefs and knowledge about medicinal plants. The interest in medicinal plants was especially pronounced among the early botanists who were often physicians (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 (Chopra, Nayar and Chopra, 1956)

Although the use of traditional medicine is so deeply rooted in the cultural heritage of Bangladesh the concept, practice, type and method of application of traditional medicine vary widely among the different ethnic groups. Traditional medical practice among the tribal people is guided by their culture and life style and is mainly based on the use of plant and animal parts and their various products as items of medicine. But the method of treatment and application of the medicament are greatly influenced by the religious beliefs of the different tribes and their concept of natural and supernatural causes of diseases (Kamboj, 2000).

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

- One is the old and original form based on old knowledge, experience and belief of the older generations. This includes:
  - ✓ *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 cauterisation.
  - ✓ *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.
  - ✓ *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.
- The other is the improved and modified form based on the following two main traditional systems:
  - ✓ The *Unani-Tibb or Graeco-Arab system* which has been developed by the Arab and Muslim scholars from the ancient Greek system, and
  - ✓ The *Ayurvedic system* which is the old Indian system based on the *Vedas*, the oldest scriptures of the Hindu saints of the Aryan age (Chopra,Nayar and Chopra, 1956)

1.1.4. Official Status of Tradition Medicinal in Bangladesh

Unani and Ayurvedic systems of medicine were officially recognised by the Government of Bangladesh immediately after independence and at the same time a Board of Unani and Ayurvedic systems of medicine was constituted. After the introduction of a National Drug Policy in 1982, Unani and Ayurvedic drugs have been brought under the control of the Drugs Administration Department of the Ministry of Health and Family Welfare by legislation to control and regulate the commercial manufacturing and marketing of quality Unani and

Ayurvedic drugs. The Board of Unani and Ayurvedic systems of medicine performs the following specific functions: registration of the traditional medicine practitioners, recognition of the relevant teaching institutions, holding of qualifying examinations, publication of text books, standardisation of Unani and Ayurvedic drugs, preparation and publication of Pharmacopoeias/Formularies and undertaking research and development programmes. The Board has by this time published two National Formularies:- one for Unani and the other for Ayurvedic drugs, which have already been approved by the Government. They are now in use as official guides for the manufacture of all recognised Unani and Ayurvedic medicinal preparations (Labu, 2013).

1.1.5: Role of Plants in Human History

Plants have also been used in the production of stimulant beverages (e.g. tea, coffee, cocoa, and cola) and inebriants or intoxicants (e.g., wine, beer, kava) in many cultures since ancient times, and this trend continues till today. Tea (*Camellia sinensis* Kuntze) was first consumed in ancient China (the earliest reference is around CE 350), while coffee (*Coffea arabica* L.) was initially cultivated in Yemen for commercial purposes in the 9th century. The Aztec nobility used to consume bitter beverages containing raw cocoa beans (*Theobroma cacao* L.), red peppers, and various herbs. Nowadays, tea, coffee, and cocoa are important commodities and their consumption has spread worldwide. The active components of these stimulants are methylated xanthine derivatives, namely caffeine, theophylline, and theobromine, which are the main constituents of coffee, tea, and cocoa, respectively (Ahmed, 2011)

The most popular inebriants in society today are wine, beer, and liquor made from the fermentation of fruits and cereals. Wine was first fermented about 6000–8000 years ago in the Middle East, while the first beer was brewed around 5000–6000 BCE by the Babylonians. The intoxicating ingredient of these drinks is ethanol, a by-product of bacterial fermentation, rather than secondary plant metabolites. Recent studies have shown that a low to moderate consumption of red wine is associated with reduction of mortality due to cardiovascular disease and cancer (Ahmed, 2011).

1.1.6. Drugs discovered from plants

That branch of medical science dealing with the drug plants themselves is known as Pharmacognosy. It is concerned with the history, commerce, collection, selection, identification and preservation of crude drugs and raw materials. The action of drugs is Pharmacology. Worldwide there are several thousand plants that have been and are still being used for medical purposes. Many of these are restricted in use by native people who have long resided in any given area (Harbon, 1998).

The Pure Food and Drug Act of 1906 in the United States have standardized most of the truly valuable drug plants. Such drugs are referred to as "official." Details about these plants may be found at the United States Pharmacopoeia, the Homeopathic Pharmacopoeia and the National Formulary, and various other sources in the United States and Europe. Some examples of drugs which are discovered from plants are given below:

Table 1.1: Example of some drugs discovered from plants

Plant Roots	Bark of plants, stems and woods	Plant leaves	Flowers	Fruits and seeds	Lower Plants
Aconite Colchicum Gentian Goldenseal Ginseng Ipecac Jalap Podophyllum Rhubarb Valerian	Cascara Curare Quinine Ephedrine Guiacum Oleoresins	Aloe Belladonna Cocaine Buchu Digitalis Lobelia Stramonium Wormeood	Chamomile Hops Santonin	Colocynth Croton Oil Nux Vomica Opium Psyllium	Penicillin Streptomycin Chloromycetin Aureomycin Neomycin Terramycin

1.1.7 Population Using Traditional Medicine

In some Asian and African countries, 80% of the population depends on traditional medicine for primary health care. In many developed countries, 70% to 80% of the population has used some form of alternative or complementary medicine (e.g. acupuncture). Herbal treatments are the most popular form of traditional medicine, and are highly lucrative in the international marketplace. Annual revenues in Western Europe reached US$ 5 billion in 2003-2004. In China, sales of products totaled US$ 14 billion in 2005. Herbal medicine revenue in Brazil was US$ 160 million in 2007 (Chaudhary*et al*., 2010).

1.1.8 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, 2007).

1.2.1 Plant Family

Spondias Pinnata is a traditional medicinal plant of the anacardiaceae family.Anacardiaceae, the Cashew family, includes approximately 800 species in 82 genera. It is native to tropical and subtropical areas of the world, but a few species occur in temperate regions. Many members have economic importance as sources of timber, lacquer, oil, wax, dye, and for their often edible fruit or nuts. The family is characterized by shrubs, small trees, or woody vines, with resin ducts in the bark and/or the foliage. The leaves are simple or odd-pinnately compound, trifoliate, alternate, and deciduous or evergreen. The fruit of these plants is a drupe. (Pell, 2009)

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 medicinal herb belonging to the family Anacardiaceae, also known as wild mango, hog plum, which has been reported to dysentery, diarrhea, stomach ache, rheumatism etc. Genus *Spondias* includes 17 described species, 7 of which are native to the neotropics and about 10 are native to tropical Asia. It is found wild or cultivated throughout the Indian subcontinent and in the Andamans up to an altitude of 1500 m in Himalayas. However, *S. pinnata* is widely available in Bangladesh especially wild in the forests of Chittagong, Cox's Bazar, Tangail, Sylhet and Dinajpur (Manik *et al.,* 2013)

Figure 1.2.1:*Spondias pinnata*

1.2.3: Common name

Spondias pinnata is distributed around 20 different countries all over the world.In different countries, it is known as different name. Sometimes various names are used based on the area of the country. *Spondias pinaata* is a fast growing equatorial tree with edible fruits which is popular in Bangladesh in the name of amra while its English name is hog plum or golden apple. (Panda *et al* 2011)

Table 1.2.2: Common name of *Spondias Pinnata*

Location	Name
Bangladesh	Aamada
	Aamraata
	Aamraataka
	Amra.
China	Bin lang qing
	Mu ge
	Zhao wa wen po
Japan	Amura tamagonoki
Portugal	Cajamangueira
	Cajá-manga
	Imbú manga.
Canada	Amategayi mara
	Marahunsie
	Muthiga.
India	Amratakah
	Pulima
	Amora
	Bhringi-phal
	Ranamba
German	Mangopflaume
English	Hog plum
	Golden Apple

1.2.4: Taxonomical Classification:

Kingdom: Plantae

Phyllum: Magnoliophyta

Class: Magnoliopsida

Superorder: Rosanae

Order: Sapindales

Family: Anacardiaceae

Subfamily: Spondioideae

Genus: Spondias

Species:*Spondias Pinnata*

(National Herbarium, 2013).

1.2.5: 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, grayish 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 (Muhammad *et al.,* 2011).

1.2.6: 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 (Nebedum *et al.*, 2009)

Figure 1.2.2: Leaves of *Spondias Pinnata*

1.2.7: Flowers

The flowers are sour and used in curry as a flavoring and also eaten raw. They are also used in herbal medicine. They are typically prepared in an infusion and used for eye infections and cataracts, as a soothing tea for sore throat, laryngitis and mouth sores, as well as a heart tonic. There are usually separate male and female flowers, although bisexual flowers are produced infrequently (Badonmi and Bisht, 2009).

Figure 1.2.3: Flowers of *Spondias pinnata*

1.2.8: 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 (Rahman, 2013).

Figure 1.2.4: Fruits of *Spondias pinnata*

1.2.9: 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 (Ajayi, Ajibade and Oderinde, 2011).

Figure 1.2.5: Fruit section and seeds

1.2.10: 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 (Nebedum *et al.*, 2009).

1.2.11: Stem

The stem barks are used in folk medicine in the treatment of antidiarrhoea, dysentery, rheumatism, gonorrhea and anti-tubercular (Panda *et al.*, 2009).

Figure 1.2.6 Stem of *Spondias pinnata*

1.2.12: Chemical Composition:

Aerial parts have been found to contain 24-methylene cycloartenone, stigmast-4-en-3-one, β -sitosterol, glycoside of β-sitosterol and lignoceric acid. Fruits contain water-soluble polysaccharides, composed of mainly L-arabinose, D-galactose and galacturonic acid8. Presence of β-amyrin and oleanolic acid, glycine, cystine, serine, alanine and leucine has also been detected in the fruits of this plant (Tandon and Rastogi, 1976).

1.2.13: Wood and Bark

The bark is light grey-brown and smooth. The light brown wood is buoyant and has been used to manufacture canoes on the Society Islands. (Panda *et al.*, 2009)

1.2.14: Growth

In their natural habitat these fast-growing trees grow to a height of up to 18 metres. As a potted plant they usually reach three to four metres. They have a tight upright habit with arching branches. Especially fruiting branches droop under the weigth and may even break (Ismail, Marjan and Foong, 2004).

1.2.15: Distribution

Spondias pinnata is native to Polynesia and Melanesia. It has been introduced into a large part of the tropics and is today very common e.g. in Malaysia, India, Sri Lanka, many of the Carribean Islands, and Central America (Csurhes MS, 1999).

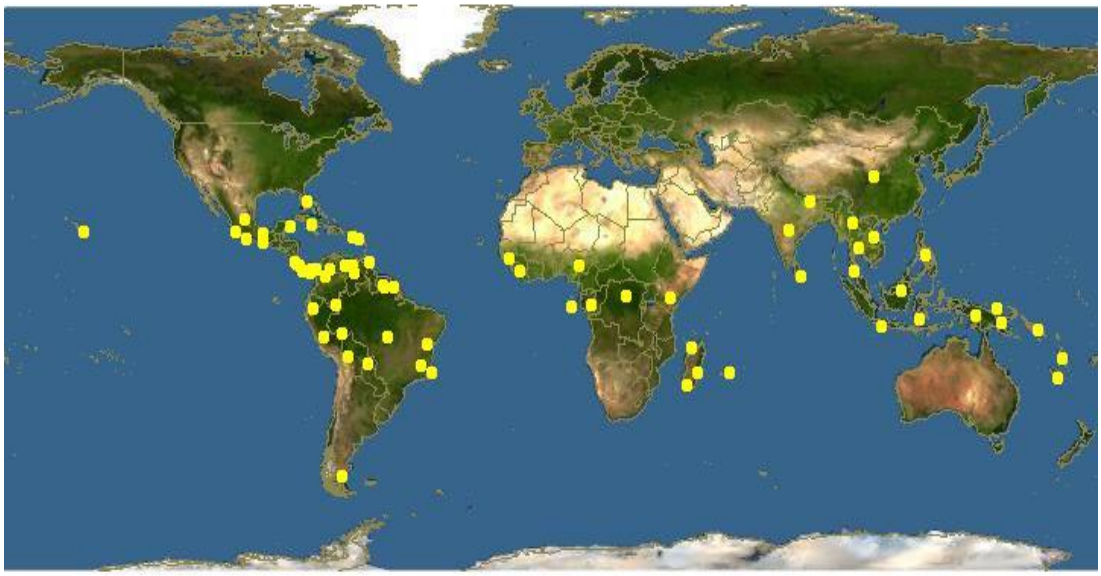


Figure 1.2.7: Geographical Distribution of *Spondias pinnata*

1.2.16: Cultivation

The Polynesian plum can withstand temperatures only above 1, 2º C and prefers a sunny site. It grows on any cultivated soil as long as it is well drained. In pots use a mix of loamy potting soil and sand or perlite. Young specimens should have a little shade. Since the branches get brittle with age the trees should be protected from strong winds (Manik *et al.*, 2013).

1.2.17: Maintenance and Propagation

Water freely during growth and apply a liquid balanced fertilizer every two weeks. In winter keep the plants in a bright place at around 10°C while keeping the soil just moist. Propagate by sowing or by hardwood cuttings. In the tropics the trees are often graftes using *Spondias pinnata as a rootstock. Since the seeds do not stock well they should be extracted from the puld and sown as soon as ripe. Let them soak in lukewarm* water for a day or two and then put them into a mix of potting soil and sand or perlite. They germinate at temperatures between 25 and 28°C. The plants usually bear fruits for the first time after three to four years(Panda *et al* 2011).

2.1 Pharmacological study of *Spondias pinnata*

2.1.1 Studies on Hypoglycaemic Activity of the Different Extracts of *Spondias pinnata*

Diabetes, the most prevailing metabolic disorder is attracting present research attention towards it. In the present study, the various extracts of the roots of *Spondias mangifera* (Family: Anacardiaceae) was evaluated for hypoglycemic activity on adult Wistar albino rats at dose levels of 100, 200 and 400 mg/kg p.o. respectively each using normoglycaemic, glucose loaded and alloxan induced hyperglycaemic rats. Glibenclamide (2.5 mg/kg) 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. The preliminary phytochemical examination of the methanol extract revealed presence of flavonoids, tannins, saponins and terpenoids. The present work justifies the use of the roots in the folklore treatment in diabetes (Acharyya, 2010).

2.1.2 Anthelmentic activity of *Spondias pinnata*

Present study reports anthelmintic activity of various extracts of the bark of Spondias Pinnata against Indian earthworms Pheritima posthuma. The results revealed that all the tested extracts of Spondias Pinnata bark possess anthelmintic activity in a dose dependant manner. Potency of the test samples was found to be inversely proportional to the time taken for paralysis/death of the worms. The activities were comparable with the reference drug piperazine citrate. Among the tested extracts, the chloroform extract was found to possess promising anthelmintic activity in comparison to other extracts. The present study therefore justifies its use in the folklore remedies as an anthelmintic drug of natural origin (Panda *et al.*, 2011).

2.1.3 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 (Family: Rubiaceae) were studied in Wistar albino rats. Furosemide (10 mg/kg, p.o.) and agar-agar (300 mg/kg, p.o.) 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 (by flame photometry) and chloride (by titrimetry) were estimated (Mondol *et al.*, 2009).

2.1.4 Anthelmintic activities of Glycosmis pentaphylla and Spondias pinnata

Extracts from Glycosmis pentaphylla roots, and stem heart wood and bark of *Spondias pinnata* when tested in vitro, showed potent Anthelmintic activity on the earthworm, Pheretima posthuma. Methanolic extract of *G. pentaphylla* was more active than its chloroform extract ($p<0.001$), while stem heart wood Methanolic extract of *S. pinnata* was also more potent than the bark extract (Gangarao and Jayaraju, 2009).

2.1.5 Chloroform and Ethanol extracts of *Spondias pinnata* and its different pharmacologic activity

SPONDIAS PINNATA. Is a medicinal herb belonging to the family Family- Anacardiaceae also known as wild mango, hog-plum, which has been reported to dysentery, diarrhea, stomach ache, rheumatism properties according to indigenous systems of medicine in this sub continent. But its antioxidant,antimicrobial and cytotoxic potential is still unknown. Therefore, the present study was designed to investigate antioxidant, antimicrobial and cytotoxic properties of *spondias pinnata*. The extract was examined for its antioxidant action using 1, 1-diphenyl-2 picrylhydrazyl (DPPH) radical scavenging and reducing power scavenging assays,total phenol and flavonoid also determined. The extract displayed an excellent scavenging of DPPH radical and reducing power. In DPPH scavenging IC_{50} value of ethanolic extract was 44.335 whereas IC_{50} value of chloroform extract was 61.91. In case of cytotoxicity of ethanolic extract LC_{50} was 65 and LC_{90} was 160.while chloroform extract exhibited LC_{50} value 170 and LC_{90} value 325.In case of antibacterial screening crude methanolic extract showed moderate antibacterial activity ranged from 17.3-23.5 against eight microorganisms and good activity is observed by the chloroform extract where ranged was 17.1- 23.5.When phytochemical property was tested Flavonoid, glycoside, alkaloid, carbohydrate, saponin, steroids were found in ethanol extract glycoside, alkaloid, carbohydrate, resin and steroid was observed in chloroform extract(Das *et al*., 2011)

2.1.6 Antioxidant and free radical scavenging activity of *Spondias pinnata*.

The extract showed total antioxidant activity with a trolox equivalent antioxidant concentration (TEAC) value of 0.78 +/- 0.02. The IC_{50} 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 IC_{50} 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 IC_{50} = 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. The present study provides evidence that a 70% methanol extract of *Spondias pinnata* stem bark is a potential source of natural antioxidants (Hazra, Biswas and Modol, 2008).

2.1.7 A comparative study of the antioxidant, antimicrobial, cytotoxic and thrombolytic potential of the fruits and leaves of *Spondias dulcis*

The methanolic fruit extract exhibited the highest phenolic content, flavonoid content and antioxidant capacity, among the other extracts, with the highest DPPH radical scavenging activity at a concentration of 10 µg/mL (IC_{50}: 1.91 µg/mL) and maximum reducing power at a concentration of 100 µg/mL (EC_{50}: 3.58 µg/mL). Though all extract showed moderate antimicrobial activity against the bacterial strains, weak or no activity against fungus. The range of LC_{50} value of all extracts was 1.335-14.057 µg/mL which was far lower than the cut off index for cytotoxicity. All extracts exhibited statistically significant (P<0.001) thrombolytic activity (Manik *et al.,* 2013).

2.1.8 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)

2.1.9 In vitro anticancer activity of *Spondias pinnata* bark 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 et al., 2013).

2.1.10 Investigation of *In Vitro* Antioxidant, Antimicrobial and Thrombolytic Activity of the Exocarp of *Spondias pinnata* (Anacardiaceae)

The aim of the current study was to evaluate the antioxidant, antimicrobial and thrombolytic potentials of the exocarp of *Spondias pinnata* fruits. The crude ethanolic extract of the exocarp of *S. pinnata* fruit was partitioned successively by solvents of different polarity. All fractions were then investigated for qualitative preliminary phytochemical screening by specific standard procedure. The antioxidant potential of all fractions were then evaluated in terms of total phenolic content, total flavonoid content, DPPH free radical scavenging potential, reducing potential and total antioxidant capacity by specific standard procedure. The disc diffusion method was incorporated to evaluate the *in vitro* antimicrobial activity on nutrient agar medium. The highest total phenolic content was found in aqueous fraction (570.20±0.48 mg GAE/g of dried extract) while the lowest in the n-Hexane fraction (337.51±0.21 mg GAE/g of dried extract). However, ethyl acetate fraction exhibited the highest flavonoid content which amounted to 132.27±0.25 mg quercetin equivalents/g of dried extract. Likewise, ethyl acetate fraction

showed the highest antioxidant capacity (21.61±0.11 g of L-ascorbic acid equivalents/g of dried extract) along with the lowest IC_{50} (1.72±0.39 μg/ml) & EC_{50} (2.25±0.75 μg/ml) value. However, DPPH free radical scavenging activity and reducing power of all fractions were found to be concentration dependent. Nonetheless, comparatively more polar fractions (ethyl acetate and aqueous fraction) were found to be ineffective against all the microbial strains except *S. dysentery* and *P. aeruginosa* while non-polar fractions (n-hexane and dichloromethane fraction) showed variable antimicrobial activity. In addition, all fractions produced statistically significant ($P<0.05$ for ethyl acetate and aqueous fraction, $P<0.001$ for others) thrombolytic activity. To conclude, our present study suggested that exocarp of *S. pinnata* fruit exhibits antimicrobial activity against a wide variety of strains while it produces noteworthy antioxidant and significant thrombolytic activity (Islam *et al*, 2013).

2.1.11*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 group (Hazra, Sarkar and Mandal, 2013)

Table 2.1: 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	Mondol *et al.*, 2009
Stem barks	Methanolic extract show antibacterial, antidiarrhoeal and ulcer-protective activity	Arif *et al.*, 2008
Bark	Methanolic extract promotes apoptosis in human lung adenocarcinoma cell line and human breast adenocarcinoma cell line	Ghate et al., 2013
Fruit	Ethanol extract shows anmicrobial and thrombolytic activity	Islam *et al*, 2013
Bark	Methanol extract shows hypoglycemic activity on adult Wistar albino rats	Acharyya, 2010
Stem heart wood	Methanol extract show anthelmentic activity	Gangarao and Jayaraju, 2009
Bark	Methanol extract lessens iron overloaded liver toxicity	Hazra, Sarkar and Mandal, 2013
Bark	Chloroform and methanol extract show antioxidant, antimicrobial and cytotoxic	Das *et al*., 2011
Fruits and leaves	Methanolic fruit extratc show antioxidant, antimicrobial, cytotoxic and thrombolytic	Manik *et al.*, 2013
Bark	Various extract show anthelmentic activity of *Spondias pinnata*	Panda *et al.*, 2011
Stembark	Methanol extract show antioxidant and free redical scavenging activity	Hazra, Biswas and Modol, 2008

2.2 Phytochemical study of Spondias pinnata

2.2.1 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)

2.2.2 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)

2.2.3 Composition of the Leaf Oils of Four *Spondias* Species from Brazil

The chemical compositions of the leaf oils of *Spondias mombim* L., S. *purpurea* L., S. *tuberosa* Arr. and *S. cytherea* Sonn. were analyzed by GC/MS. *S. mombim* oil was found to be rich in 3-hexenol (38.07%) and β-caryophyllene (13.73%), *S. purpurea* oil contained β-caryophyllene

(11.16%), δ-cadinene (10.29%), torreyol (11.63%) and T-muurolol (10.09%), *S. tuberosa* oil contained 3-hexenol (13.56%), α-copaene (13.25%), β-caryophyllene (50.01%) and δ-cadinene, while *S. cytherea* oil was found to be rich in 3-hexenol (12.03%), α-terpineol (14.02%), β-caryophyllene (27.69%), α-selinene (10.53%) and hexadcanoic acid (11.17%). (Lemos *et al.*, 1995)

2.2.4 1D- and 2D-NMR spectroscopy studies of the polysaccharide gum from *Spondias purpurea* var. *lutea*

Spondias purpurea var. *lutea* (Anacardiaceae) 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)

2.2.5 Structural investigation of the polysaccharide of *Spondias pinnata* gum

The polysaccharide, isolated from *Spondias pinnatan* gum exudate, was subjected to acid hydrolysis and Smith degradation processes in order to investigate its relevant structural features. Chemical and spectral evidence suggested the existence of small blocks of (1→3)-linked β-d-galactosyl residues, interspersed with 6-*O* substituted d-galactosyl residues, in the backbone of the investigated structure; these residues are also in the side-chains. In addition, there was arabinose, up to three units long, predominantly in the form of furanosyl residues (terminal, 2-*O*- and 3-*O*-linked). Arabinopyranosyl residues (terminal and 2-*O*-linked) as well as rhamnose and mannose are also present in minor amounts. Uronic acids, β-d-glucuronic acid and its 4-*O*-methyl analogue, are attached at the C-6 and C-4 positions of the galactose moieties in the backbone. These sugar acid residues were difficult to remove from the core. ^{13}C-NMR spectroscopy confirmed the results obtained by chemical methods, and showed the presence of reducing terminal sugar residues and internal acetyl groups (Ghosal and Thkur, 1981)

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

Part of the plant	**Findings**	**References**
Fruit	The phytochemical screening revealed alkaloids followed by saponins and tannins	Satpathi, Tyagi and Gupta, 2011
Leaf oil	Four *Spondias* species analyze the chemical composition	Lemos *et al.,* 1995
Gum exudate	The purified, homogeneous, acidic polysaccharide was isolated	Ghosal and Thkur, 1981
Crude	ethno medicine were found bymicroscopic 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

3.1 Extraction of leaves of *spondias pinnata*

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

3.1.1 Materials:

Table 3.1: Apparatus and Reagent used for Extraction

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

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, and Dhaka, where a Voucher specimen (DACB Accession No. 35937) has been deposited for future reference.

3.1.3 Method

3.1.3.1 Drying of the bark

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 degree 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 bark

After drying, the barks were weighted in an electrical balance and the total weight was found to be 550 kilogram. The dried barks 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.

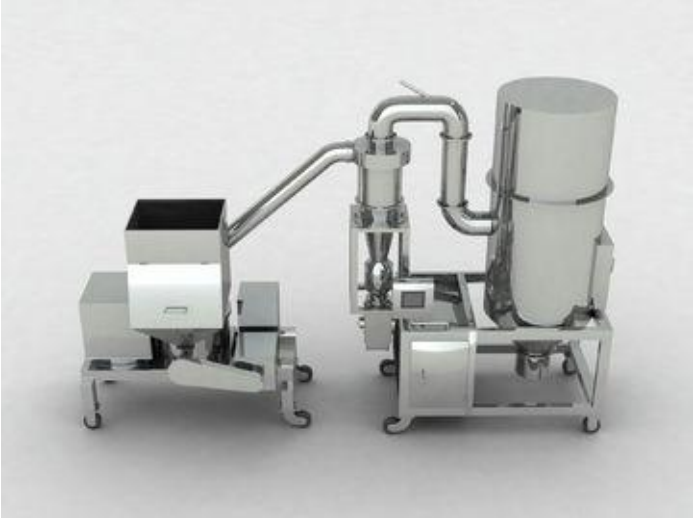


Figure 3.1: Grinding Machine

After grinding, the weight of the grinded bark was measured and the weight was about 500 gm. All grinded barks were stored in an air tight container.

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.

Figure 3.2: 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 (Elliot, 2012).

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.

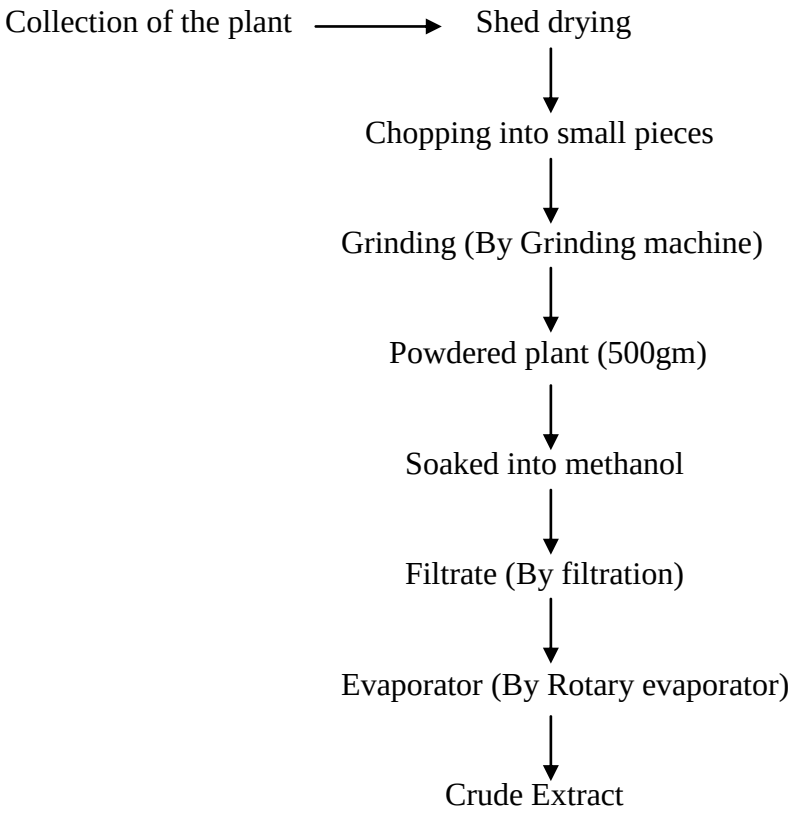


Figure 3.3: Schematic presentation of the crude preparation from the plant

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 was 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 Phenolic Content

3.3.1 Materials

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
Na_2CO_3	Funnel
UV – Visible Spectrophotometer	Spatula
Measuring Cylinder	Volumetric Flask

3.3.2 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, Rudolf and Rosa, 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).

3.3.3 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.

3.3.4 Preparation of 10% Folin- ciocalteu reagent

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

3.3.5 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

3.3.6 Preparation of extract solution

0.025gm of methanol 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

3.3.7 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°c temperature. The test tubes of plant extracts solution were incubated for 1 hour at 20°c to complete the reaction. The absorbance of the solution was 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 methanol 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 100µg/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 (Das *et al*., 2011). 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 (Hazra, Biswas and Modol, 2008).

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.

3.5.5 Preparation of trichloro acetic acid (10%) solution

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

3.5.6 Preparation of ferric chloride (.1%) solution

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

3.5.7 Preparation of sample

12 mg of extract dissolved in 10 ml of methanol. The concentration 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)**
ME	Methanol (crude) 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.

Figure 3.4: Brine shrimp

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

Controls 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 methanolic extracts of *Spondias pinnata* bark

4.1.1 Result:

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 methanolic extract of *Spondias pinnata* bark contains most of the phytoconstituents. It contains Alkaloids, flavonoids, phenols, Saponins etc. Alkaloids were present in greater amount, whereas flavonoids and phenols were present in moderate amount. On the other way, saponins were present in slightly amount. Steroidal compound were absent in the initial screening.

4.2 Total reducing power of Methanol 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 concentration

Concentration	**Absorbance**	**% Reducing Potential** **(Blank absorbance – Sample absorbance) ×100** **Absorbance of blank**
1200	0.345	7.51
1000	0.341	8.58
800	0.311	16.62
600	0.294	21.18
400	0.301	19.30
200	0.303	18.77

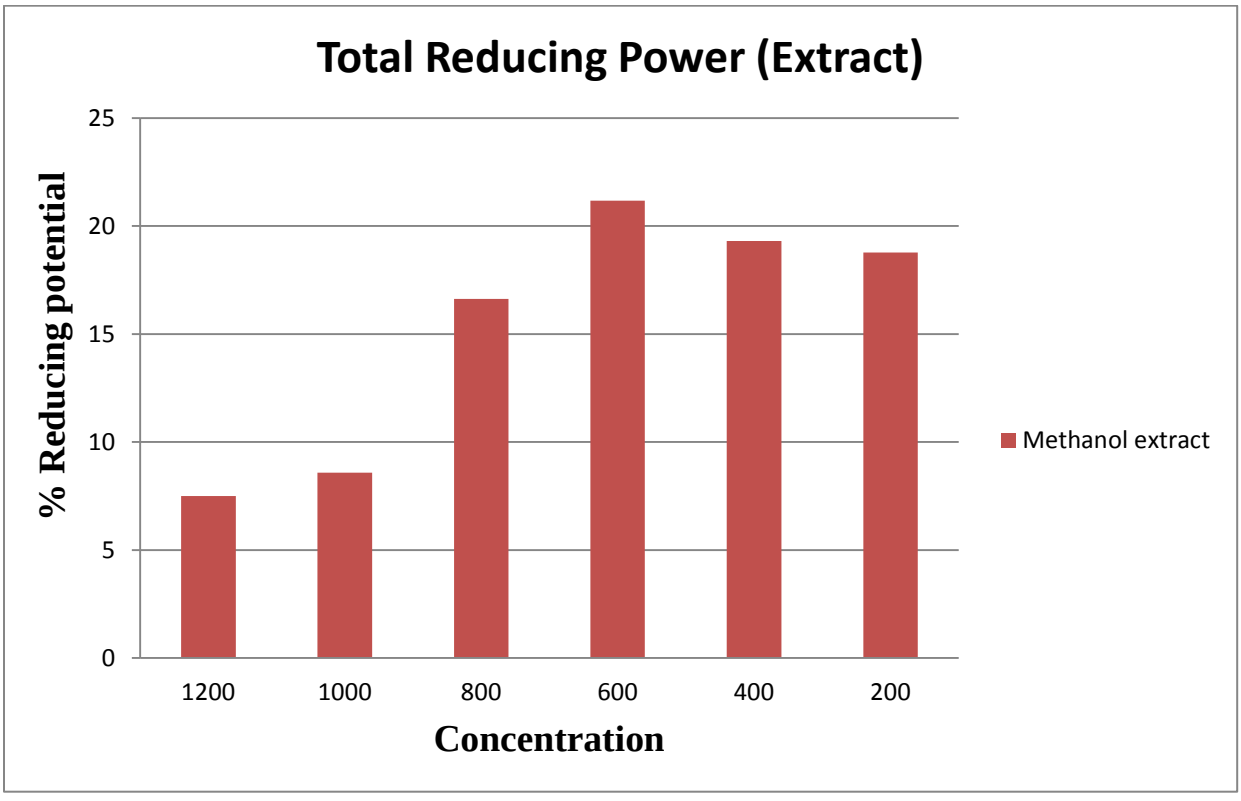


Figure 4.1: Graph of the reducing power of *Spondias pinnata* bark

Table 4.3: Absorbance of standard at different concentration

Concentration	**Absorbance**	**% Reducing Potential** **(Blank absorbance – Sample absorbance) ×100** **Absorbance of blank**
1200	0.321	13.94
1000	0.280	24.93
800	0.305	18.23
600	0.296	20.64
400	0.271	27.35
200	0.314	15.82

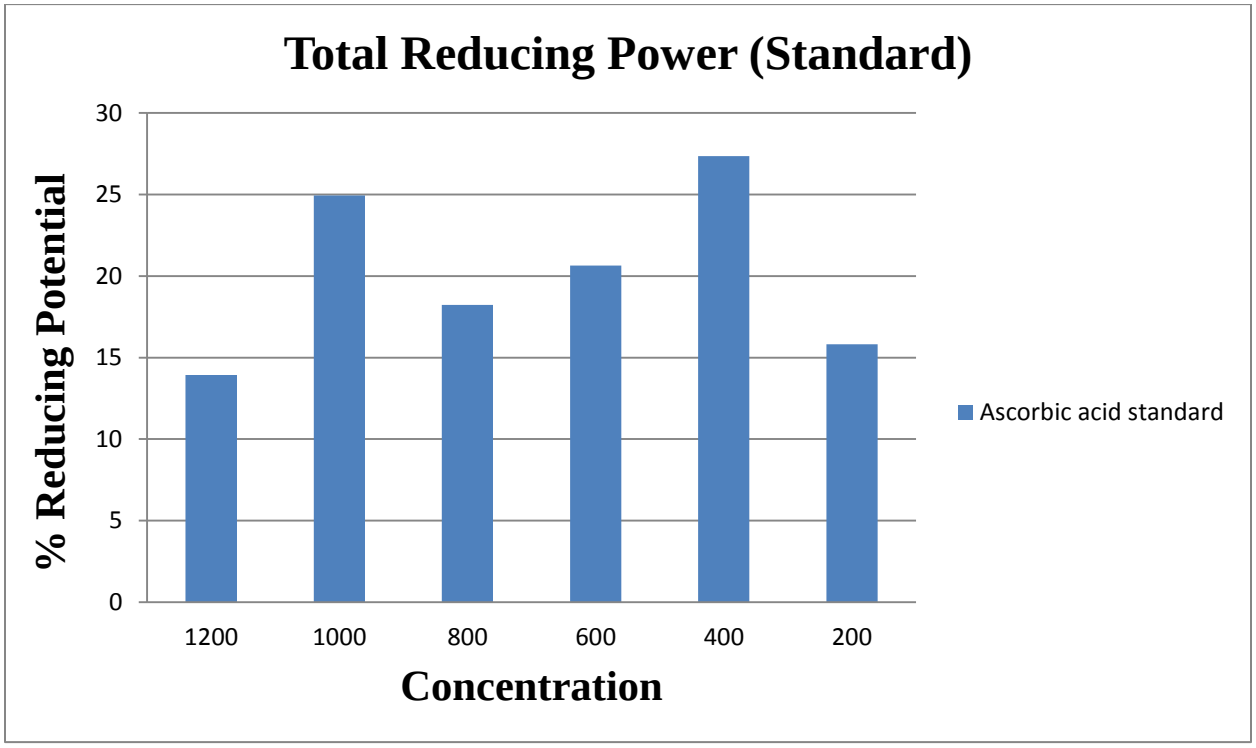


Figure 4.2: Graph of the reducing power of Ascorbic acid standard.

4.2.2 Discussion: From the bar diagram, standard and plant extract showed almost similar pattern of percent reducing potential. High percent reducing potential indicates the high reducing power (Das *et al*., 2009).In this experiment, we found the % reducing potential of methanol extracts are

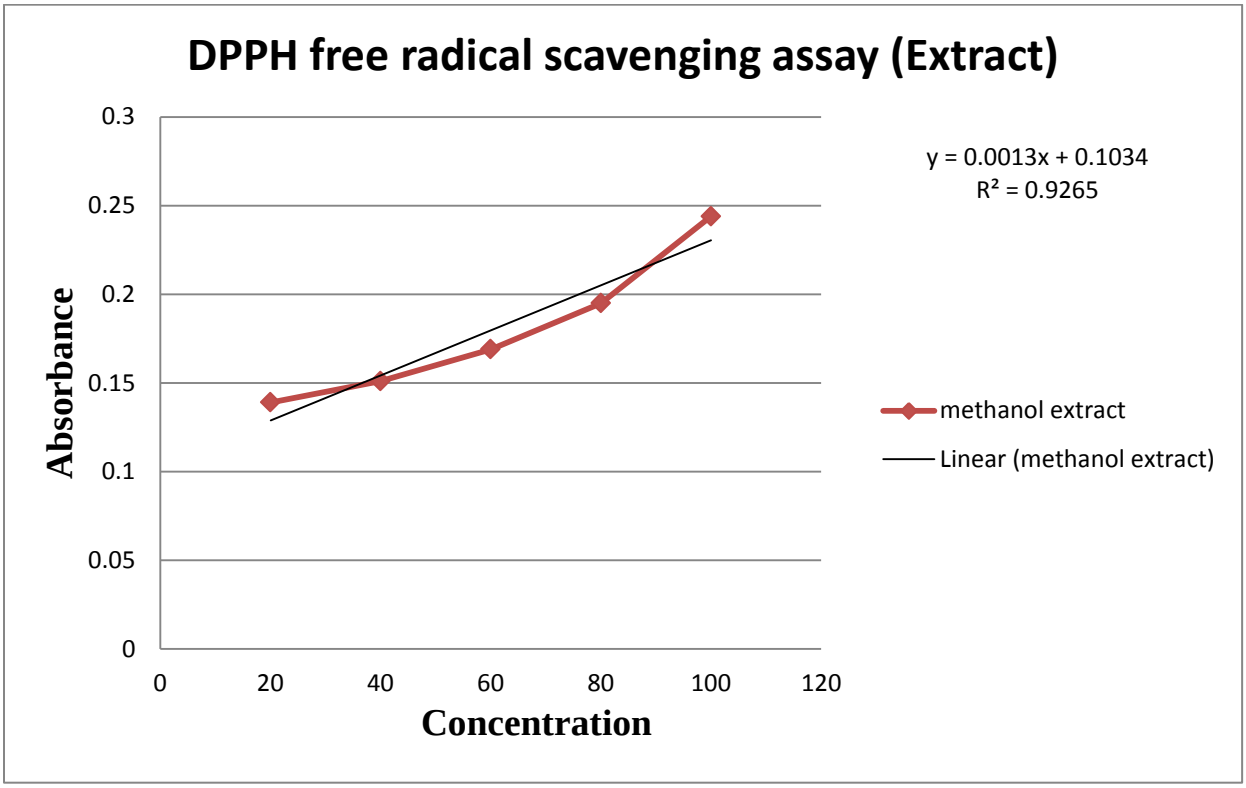
7.51, 8.58, 16.62, 21.18, 19.30 and 18.77; whereas the % reducing potential of standard are 13.94, 24.93, 18.23, 20.64, 27.35 and 15.82. So, we can say that our sample extract possess lower reducing potential than the ascorbic acid standard.

4.3 Evaluation of antioxidant property of Methanol 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 of sample for DPPH free radical scavenging assay

Concentration (µg/ml)	**Absorbance**	**% inhibition** $\frac{\text{(Blank absorbance – Sample absorbance)} \times 100}{\text{Absorbance of blank}}$	**IC_{50} (µg/ml)**
20	0.139	62.734	397
40	0.151	59.517	
60	0.169	54.692	
80	0.195	47.721	
100	0.244	34.584	



Frigur 4.4: Graph of DPPH free radical scavenging assayof different test sample

Table 4.5: Absorbance and % inhibition of standard for DPPH free radical scavenging assay

Concentration (µg/ml)	**Absorbance**	**% inhibition** **(Blank absorbance – Sample absorbance) ×100** / **Absorbance of blank**	**IC_{50} (µg/ml)**
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	

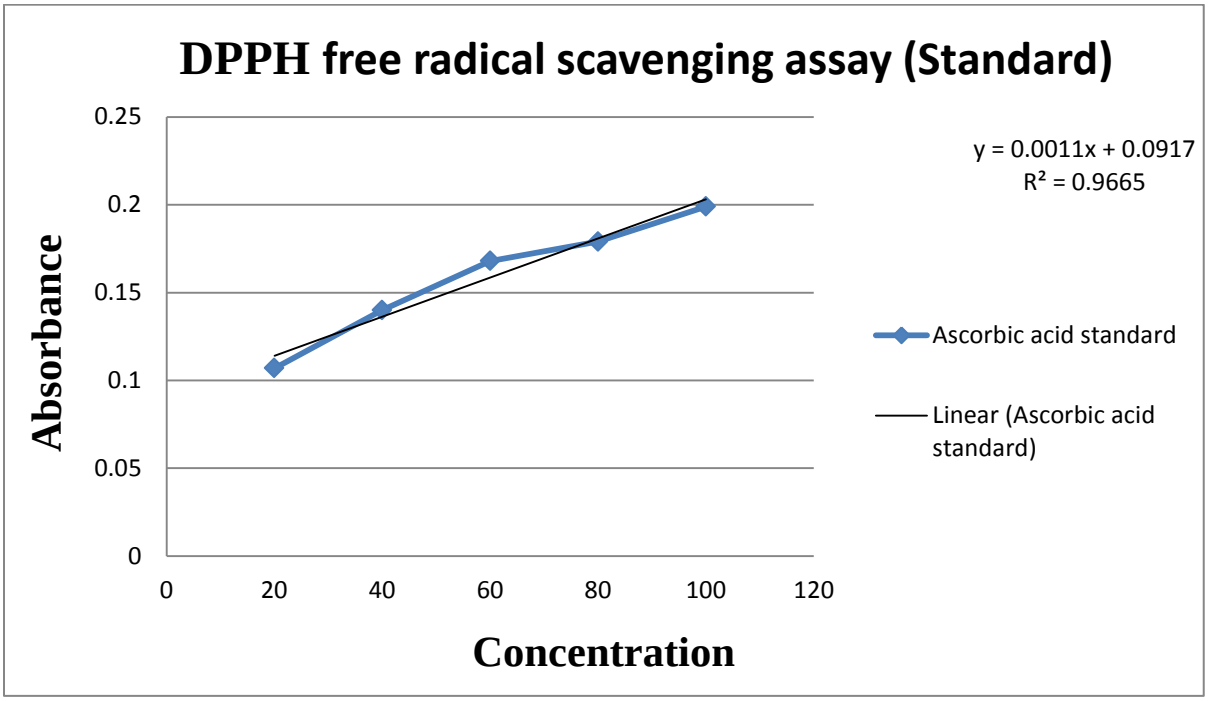


Figure 4.4: Graph of DPPHfree radical scavenging assay of Ascorbic Acid standard

4.3.2 Discussion: From the curve, % inhibiton of the ascorbic acid at different concentration was observed almost similar. On the other hand, IC_{50} value of methanol extract is 397, where the IC_{50}value of the standard 409. Compare to each other, the results are quite similar and we can say that our plant extract has significant antioxidant activity. (Das*et al.*, 2011).

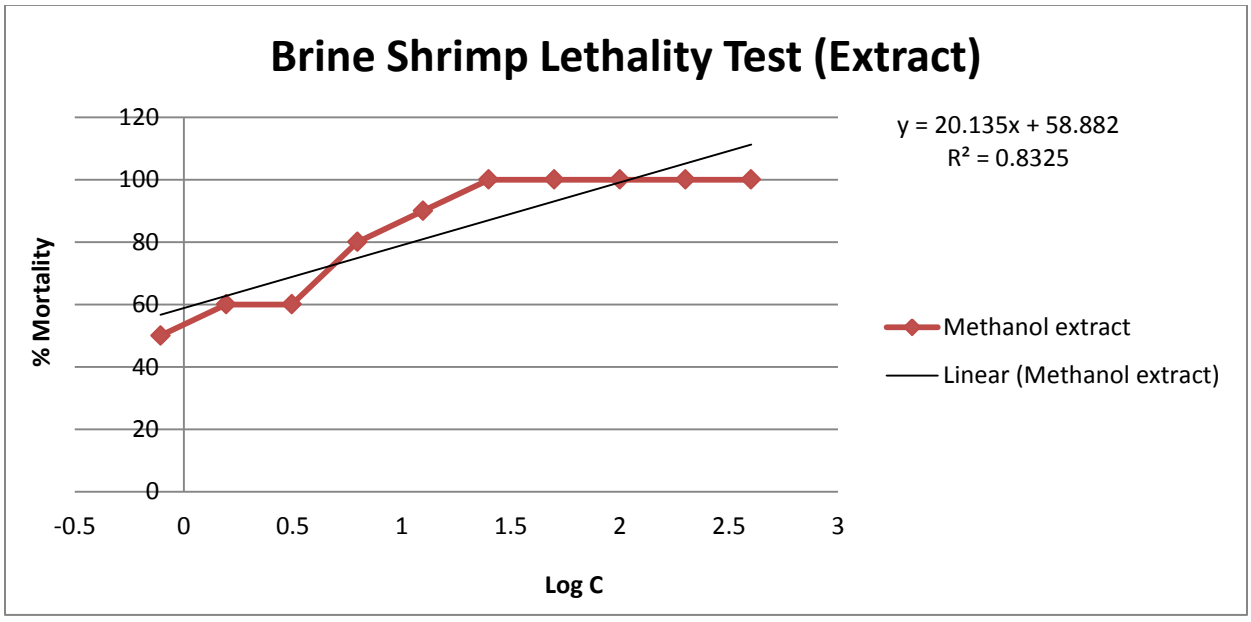
4.4 Brine Shrimp Lethality Test

4.4.1 Result: The Methanol 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 as. Compared with the negative control VS (positive control) gave significant mortality and the LC_{50} values of the different extractives were compared with negative control.

Table 4.6 Effect of Methanol extract on shrimp nauplii

Concentration µg/ml	Log C	No. of Nauplii dead	% Mortality	Best Fit Equation	R^2 Value	LC_{50} (µg/ml)
400	2.602	10	100	Y=20.13x + 58.88	0.832	0.362
200	2.301	10	100			
100	2.00	10	100			
50	1.699	10	100			
25	1.398	10	100			
12.5	1.097	9	90			
6.25	0.796	8	80			
3.125	0.495	6	60			
1.5625	0.194	6	60			
0.781	-0.107	5	50			



Figur 4.5: Graph of the plant extract for cytotoxicity

Table 4.7Effect of Tamoxifen (standard) on shrimp nauplii

Concentration µg/ml	Log C	No. of Nauplii dead	% Mortality	Best Fit Equation	R^2 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			

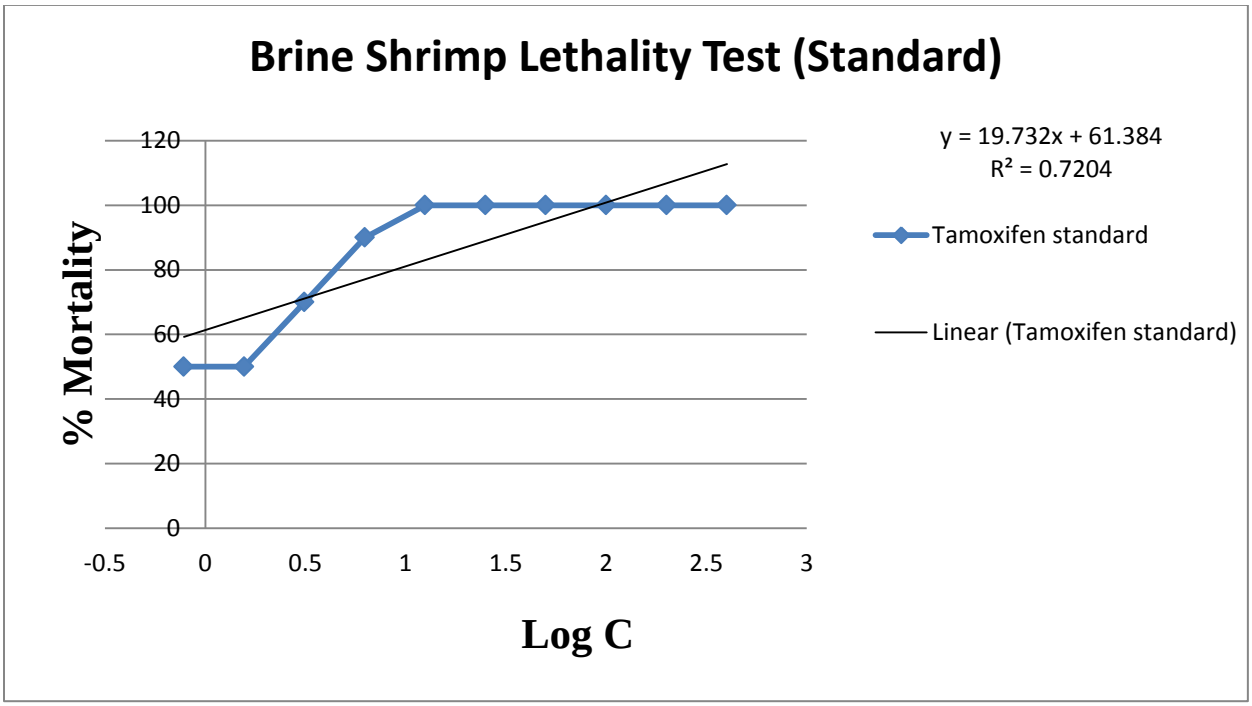


Figure 4.6: Graph of Tamoxifen (standard) for cytotoxicity

4.4.2 Discussion: The lethal concentration LC_{50} of the test samples after 24 hr. was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration (toxicant concentration) and the result is 0.362 µg/ ml, where our standard concentration is 1.142 µg/ ml. so it show significant level of toxic component. Best-fit line was obtained from the curve data by means of regression analysis**.**

4.5 Estimation of total phenolic content of Methanol 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^2value is given in a table below using the standard curve also shown below.

Table 4.8: Effect of Gallic acid standard on Total phenolic content

Concentration	**Absorbance**	**Best Fit Equation**	**R^2 Value**	**X value (Mg of GAE/gm)**
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

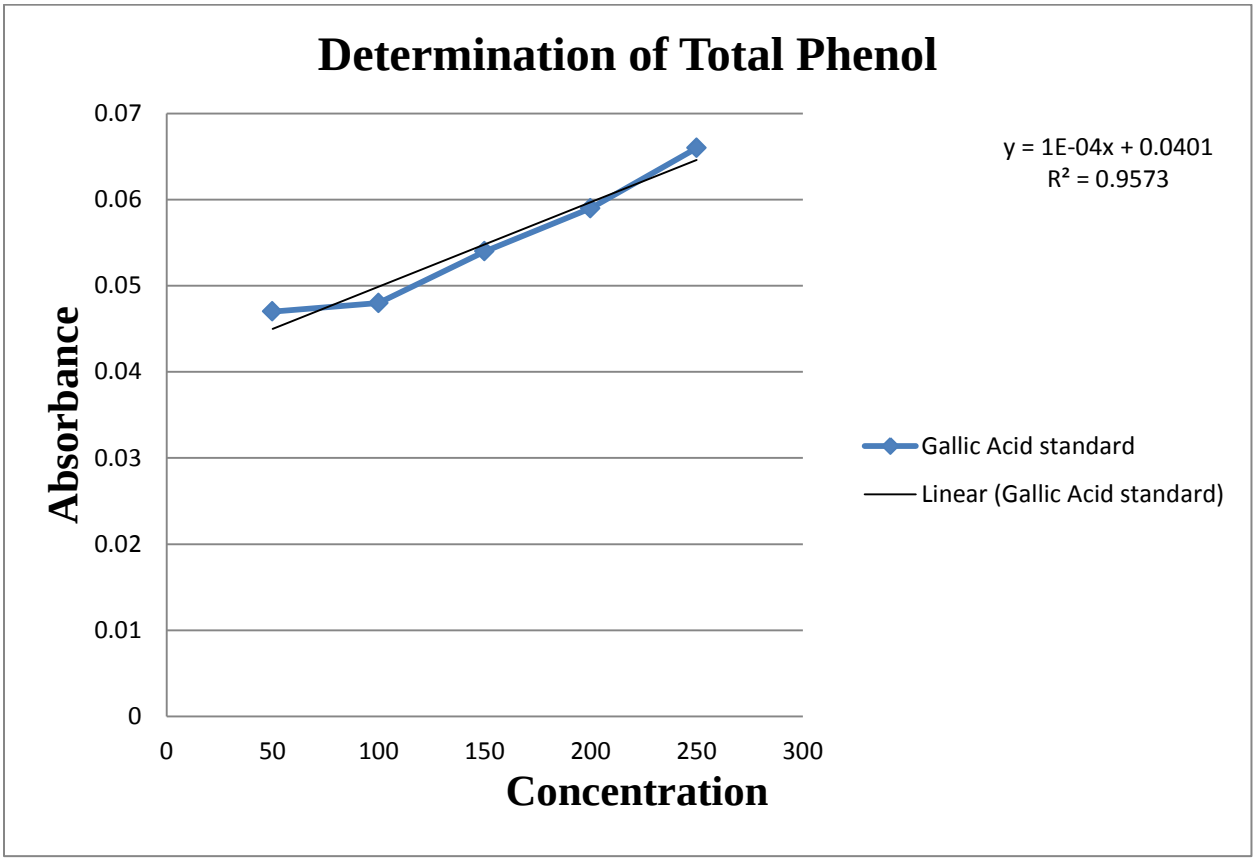


Figure 4.7: Graph of Gallic Acid on Total phenolic content

Table 4.9: Effect of Methanol extract on Total phenolic content

Concentration	**Absorbance**	**X value (Mg of GAE/gm)**
250	0.059	190
200	0.055	150
150	0.052	120
100	0.049	90
50	0.046	60

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 (190, 150, 120, 90 and 60) 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

To conclude, the present study demonstrates that the methanol extract of *Spondias pinnata* barkcan be considered as a valuable source of therapeutic agents for human health, as an antioxidant, antimicrobial and cytotoxic agent. The medicinal values of the *Spondias pinnata* bark may be related to their constituent phytochemicals. Their antioxidant activity may be responsible for their usefulness in the management and treatment of various diseases. Due to the antioxidant activity; it will be useful for the treatment of the diseases which are caused by oxidation such as, Parkinson's disease, neurodegenatation, asthma, anemia, cancer, ischemia etc. By this study, we can also conclude that the bark can be used as a cytotoxic drug. However further research and studies are required for identification and isolation of the biological activityof the extract on higher animal to know about the efficacy of the compound.

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