CHEMICAL AND BIOLOGICAL INVESTIGATIONS ON CHLOROFORM EXTRACT OF *Spondias pinnata* BARK

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF PHARMACY, EAST WEST UNIVERSITY IN THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF PHARMACY

SUBMITTED BY

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January 2014

CERTIFICATE BY THE SUPERVISOR

This is to certify that, the research work on "Chemical and biological investigations on chloroform extract of *Spondias pinnata* bark" 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 **Rumana Akhter**, ID# 2009-3-70-011, 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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Date: 17/1/2014

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled "Chemical amd biological investigations on chloroform extract of *Spondias pinnata* bark" submitted by me to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the award of the degree of Bachelor of Pharmacy (B.PHARM) is a complete record of original research work carried out by me during the period 2013 under the supervision and guidance of **Mehreen Rahman**, Lecturer, Department of Pharmacy, East West University and co-supervisor **Mahbubul Hoque Shihan**, Senior Lecturer, Department of Pharmacy, East West University and it has not formed the basis for the award of any other Degree/Diploma/Fellowship or other similar title to any candidate of any University.

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Thesis Certificate

This is to certify that the thesis entitled "Chemical and biological investigations on chloroform extract of *Spondias pinnata* bark", submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the award of the degree of Bachelor of Pharmacy (B.PHARM) is a complete record of original research work carried out by **Rumana Akhter**, ID# 2009-3-70-011, during the period 2013 of her research in the Department of Pharmacy at East West University, under my supervision and guidance and the thesis has not formed the basis for the award of any other Degree/Diploma/Fellowship or other similar title to any candidate of any University. We further certify that all the sources of information and facilities availed of this connection are duly acknowledged.

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Date: 17/1/2014

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January 2014 Rumana Akhter

Dedication:

This research paper is dedicated to

My respected parents

Abstract

The purpose of this study was to understand and investigate the phytochemical contents, cytotoxic effect of the traditional medicinal plant *Spondias pinnata.* It is classified under the family Anacardiaceae. In this paper the study was conveyed on the chloroform extract of the bark of *Spondias pinnata*. Many researches were done on this plant species by many scholars at different time period but the intention of the study was to emphasize on the possibility of presence of its antioxidant, cytotoxic property. To prove the cytotoxic property and detect its intensity, the Brine Shrimp Lethality Bio-assay test was done and the result is very significant. Sample LC_{50} value 4.102 µg/ ml where the standards Tamoxifen show LC_{50} value 1.142 µg/ ml. To detect the presence of antioxidant property, total phenolic contents, DPPH free radical scavenging activity, was done. DPPH test show IC_{50} value of Sample is 319.08, where standard Ascorbic acid has IC_{50} value of 371.18. Total phenolic content test for 250, 200, 150, 100, 50 µg/ml concentration of sample it has X value of 255, 190, 129, 95, 59, where standard Gallic acid contains value 260, 190, 140, 80, 70 mg of GAE (Gallic acid equivalent) per gm of dried extract, respectively. So chloroform extract of *Spondias pinnata* (bark) have significant amount of phenol.

After collection, identification and drying the bark sample, extraction was performed by using chloroform solvent system.

Keywords: *Spondias pinnata*, Anacardiaceae, Cytotoxicity, Brine Shrimp Lethality Bio-assay, Antioxidant activity

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INTRODUCTION

1 INTRODUCTION

"A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs". (Sofowora, 1982, Medicinal Plant and Traditional Medicine in Africa)…This definition of Medicinal Plant has been formulated by WHO (World Health Organization).

Medicinal plants have been used by mankind for millennia; their use is as old as humanity itself. The range of species used and their scope for healing is vast. Cures as yet undiscovered may exist in plants as yet undescribed. Currently, it is estimated that the number of higher plant species used worldwide for medicinal purposes is more than 50,000 (Schippmann et al., 2002). This equates to approximately 20% of the world's vascular flora and constitutes the biggest spectrum of biodiversity used by people for a specific purpose (Hamilton et al., 2006).

1.1 History of medicinal plant:

Medicinal plants continue to be an important therapeutic aid for alleviating ailments of humankind. Search for eternal health and longevity and to seek remedy to relieve pain and discomfort prompted the early man to explore his immediate natural surrounding and tried many plants, animal products and minerals and developed a variety of therapeutic agents. Over millenia that followed the effective agents amongst them were selected by the process of trial, error, empirical reasoning and even by experimentation. These efforts have gone in history by the name discovery of 'medicine'. In many eastern cultures such as those of India, China and the Arab/Persian world this experience was systematically recorded and incorporated into regular system of medicine that refined and developed and became a part of the Materia Medica of these countries. The ancient civilization of India, China, Greece, Arab and other countries of the world developed their systems of medicine independent of each other but all of them were predominantly plant based. But the theoretical foundation and the insights and in depth understanding on the practice of medicine that we find in Ayurveda is much superior among organized ancient systems of medicine (Rahman, 2007).

Natural product research and drug discovery

Nature appears to be a therapeutic cornucopia to treat superfluity of diseases ranging from common cold to multifarious type of illness since the dawn of civilization. Overwhelming evidence has accumulated showing that natural products from plants, microorganisms and marine organisms comprise major portion of the total repertoire of the available therapeutic drugs. Products of natural origins are often called __natural products.'' Natural products include: an entire organism (e.g., a plant, an animal, or a microorganism) that has not

undergone any kind of processing or treatment other than a simple process of preservation (e.g., drying), part of an organism (e.g., leaves or flowers of a plant, an isolated animal organ), an extract of an organism or part of an organism, and exudates, and pure compounds (e.g., alkaloids, glycosides, sugars, flavonoids, coumarins, lignans, steroids, terpenoids, etc.) isolated from plants, animals, or microorganisms (Samuelsson, 1999). However, in most cases the term natural products refer to secondary metabolites, small molecules (mol wt <2000 amu) produced by an organism that are not strictly necessary for the survival of the organism (Cannell, 1998).

Natural products have played a key role in drug discovery research, as many medicines are either natural products or derivatives thereof. Indeed, it is estimated that about 40% of all medicines is either natural products or their semi-synthetic derivatives. This may not be surprising as herbal medicine is a tradition of healthcare since ancient times and natural extracts screening has been one of the roots of drug discovery research, where erythromycin and rifampicin (bacterial infections), statins (hyperlipidemia), quinines and artimesinin (malaria), paclitaxel, vinblastine and vincristine (cancer), are a few well-known natural products-based medicines. For bacterial infections, over 80% of all medicines in clinical use is either natural products or their derivatives, while for anticancer agents over 60% of all drugs is either natural products or derivatives thereof; examples of several potential lead molecules are vincristine, vinblastine, taxol, camptothecin, podophyllotoxin, combretastatins, etc which have been isolated from plants for successful use in cancer treatment (Newman and Cragg, 2007). In earlier times, all drugs and medicinal agents were derived from natural substances, and most of these remedies

1.2 The Plants Role in Human:

A modern dictionary defines health as soundness of physical, mental or moral condition especially freedom from pain or diseases. But true health is more than that- it includes the joy of living, the power and ability to lead a satisfying and purposeful life.

Modern drugs or conventional medicine is often viewed as impersonal, emphasizing crisis intervention. It is not only expensive also many of them bring about side effect, which are sometimes more dangerous than the disease itself. Plants contain natural substances that can promote health and alleviate illness. They are source of medicines directly. The origins of medicine are tied up with plants. Alcohol was produced from starch early in civilization for use as a beverage preservative, but also as a surface sterilizing agent. Quinine from plant bark prevents malaria. Morphine, codeine, and cocaine are useful as local anesthetics and serve

other purposes through side effects too. Digitoxin from foxglove has been used to regulate heartbeat. Caffeine from plants is an important daily stimulant for many humans. Nicotine and A-9-THC are important recreational drugs. There are probably many more important medicines to be found in the tropical forests of the world where countless species remain unknown. Medicinal drugs now used in the developed world come from only about 95 of the 250,000 known species of flowering plants on earth. The study of new plants can and has led to the creation of medicines that can save lives and cure illnesses. As the tropical rainforest shrinks day by day, the potential to discover new plants shrinks as well, yet few researchers are actively seeking new plant species in the jungles of South America. (Verun hey wood hug-1991)

1.3 Approaches to natural product research and drug discovery :

Different approaches to drug discovery from plants can be enumerated as: random selection followed by chemical screening, random selection followed by one or more biological assays, follow-up of biological activity reports, follow-up of ethnomedical (traditional medicine) use of plants, use of appropriate plant parts as such in powdered form or preparation of enriched / standardised extracts (herbal product development), use of a plant product, biologically potent but beset with other issues, as a lead for further chemistry, and single new compounds as drugs. The objective of the later approach is the targeted isolation of new bioactive plant products, i.e. lead substances with novel structures and novel mechanisms of action. This approach has provided a few classical examples, but the problem most often encountered here is not enough availability. The problem of availability can be overcome by semi-synthesis/synthesis or using tissue culture techniques (by genetically modifying the biosynthetic pathway of the compound of interest).

Drug discovery from plants involves a multidisciplinary approach combining botanical, ethnobotanical, phytochemical and biological techniques. The search for bioactive chemicals from the unstudied part of the plant kingdom can be conducted essentially with three methods (Cotton, 1996): the random method involves the collection of all plants found in a given area of study, phylogenetic targeting means the collection of all members of those plant families which are known to be rich in bioactive compounds, and the ethnobotanical approach is based on the traditional knowledge of medicinal plant use. It has been suggested that the ethno-directed sampling is most likely to succeed in identifying drugs for use in the treatment of gastrointestinal, inflammatory and dermatological complaints. Strategies for research in the area of natural products have evolved quite significantly over the last couple of decades. These can be broadly divided into two categories:

Older approach

- Focused on chemistry of compounds from natural sources, but not on activity.
- Straightforward isolation and identification of compounds from natural sources followed by testing of biological activity in animal model.
- Chemotaxonomic investigation.
- Selection of organisms primarily based on ethnopharmacological information, folkloric reputations, or traditional uses.

Modern approach

- Bioassay-directed (mainly *in vitro*) isolation and identification of active lead compounds from natural sources.
- Production of natural products libraries.
- Production of active compounds by cell or tissue culture, genetic manipulation, natural combinatorial chemistry and so on.
- More focused on bioactivity.
- Introduction of the concepts of dereplication, chemical fingerprinting, and metabolomics.
- Selection of organisms based on ethnopharmacological information, folkloric reputations, or traditional uses, and also those randomly selected.

1.4 Source of Modern Medicine

Allopathic or 'modern'medicine owes a great deal to medicinal plants.

Table 1.1: Major plant drugs for which no synthetic one is currently available (Kumar *et al*, 1997).

Drug	Plant sources	Use
Vinblastine	*Catharanthus roseus*	Anticancer
Ajmalacine	*Catharanthus roseus*	Anticancer, hypotensive
Rescinnamine	*Rauvolfia serpentina*	Tranquilizer
Quinine	*Cinchona sp.*	Antimalarial, amoebic dysentery
Pilocarpine	*Pilocarpus jaborandi*	Antiglucoma
Cocaine Topical	*Erythroxylum coca*	Anaesthetic
Morphine	*Papaver somniferum*	Painkiller
Codeine	*Papaver somniferum*	Anticough
Atropine	*Atropa belladonna*	Spasmolytic, cold
Atropine	*Hyoscyamus niger*	Spasmolytic, cold
Cardiac glycosides	*Digitalis sp.*	For congestive heart failure
Artemisinin	*Artemesia annua*	Antimalarial
Taxol	*Taxus baccata* T. brevifolia	Breast and ovary cancer Antitumor
Berberine	*Berberis*	For leishmaniasis
Pristimerin	*Celastrus paniculata*	Antimalarial
Quassinoids	*Ailanthus*	Antiprotozoal
Plumbagin	*Plumbago indica*	Antibacterial, antifungal
Gossypol	*Gossypium sp.*	Antispermatogenic
Allicin	*Allium sativum*	Antifungal, amoebiasis
Emetine	*Cephaelis ipecacuanha*	Amoebiasis
Glycyrrhizin	*Glycyrrhizia glabra*	Antiulcer
Nimbidin	*Azadirachta indica*	Antiulcer
Catechin	*Acacia catechu*	Antiulcer
Sophoradin	*Sophora subprostrata*	Antiulcer

Magnolol	*Magnolia bark*	Peptic ulcer
Forskolin	*Coleus forskohlii*	Hypotensive, cardiotonic
Digitoxin, Digoxin	*Digitalis, Thevetia*	Cardio tonic
Thevenerin	*Thevetia*	Cardio tonic
Nerrifolin	*Thevetia*	Cardio tonic
Podophyllin	*Podophyllum emodi*	Anticancer
Indicine N-oxide	*Heliotropium indicum*	Anticancer

1.5 Drug Discovery from Natural sources:

Drug discovery from medicinal plants involves a multifaceted approach combining botanical, phytochemical, biological, and molecular techniques. Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, and pain. Several natural product drugs of plant origin have either recently been introduced to the United States market, including arteether, galantamine, nitisinone, and tiotropium, or are currently involved in late-phase clinical trials. As part of our National Cooperative Drug Discovery Group (NCDDG) research project, numerous compounds from tropical rainforest plant species with potential anticancer activity have been identified. Our group has also isolated several compounds, mainly from edible plant species or plants used as dietary supplements that may act as chemo preventive agents. Although drug discovery from medicinal plants continues to provide an important source of new drug leads, numerous challenges are encountered including the procurement of plant materials, the selection and implementation of appropriate high-throughput screening bioassays, and the scale-up of active compound (Hill, A., 1972)

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

traditional plants play a major role and constitute the backbone of the traditional medicine. Indian Materia medica includes about 1600 drugs of vegetable origin almost all of which are derived from different traditional system and folklore practices (Sashidhara K.V, 2005). Examples include drugs like morphine, quinine and ephedrine that have been in widespread use for a long time, and more recently adopted compounds such as the antimalarial artemisinin. The empirical approach builds on an understanding of a relevant physiological process and often develops a therapeutic agent from a naturally occurring lead molecule. Examples include tubocurarine and other muscle relaxants, propranolol and other β-adrenoceptor antagonists, and cimetidine and other H_2 receptor blockers. Development of molecular biological techniques and the advances in genomics lead to molecular approach. The molecular approach to drug discovery can be further subdivided into three general categories. The first is rational drug design using computer aided techniques. A second area is the antisense approach, which is based on manipulation of genetic targets. The third technique, which currently dominates drug discovery activity, is the pragmatic approach of random screening. With recent technological developments in molecular biology, instrumentation and information technology, screening of compounds can be conducted by high throughput screening method. High throughput screening is an automated testing process of large number of compounds versus a large number of targets which is particularly effective in identifying potential lead compounds. Robotics and miniaturization of in vitro tests on genetically modified cells has lead to high throughput screening (Harvey A.L, 2008).

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

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

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

Table 1.2: Some crude drugs used as medicine in Bangladesh (Ghani, 2003)

Common name	Botanical name	Uses
Amla	*Emblicaofficinalis*	Vitamin - C, Cough, Diabetes, cold, Laxative, hyper acidity.
Ashok	*Saracaasoca*	Menstrual Pain, uterine, disorder, Deiabetes.
Bael / Bilva	*Aeglemarmelous*	Diarrrhoea, Dysentry, Constipation.
Chiraita	*Swertiachiraita*	Skin Desease, Burning, censation, fever.
Kalmegh/ Bhuineem	*Andrographispaniculata*	Fever, weekness, release of gas.
Long peeper / Pippali	*Peeper longum*	Appetizer, enlarged spleen,Bronchities, Cold, antidote.
PashanBheda / PatharChur	*Coleus barbatus*	Kidny stone, Calculus.
Sandal Wood	*Santalum album*	Skin disorder, Burning, sensation, Jaundice, Cough.
Satavari	*Asparagus racemosus*	Enhance lactation, general weekness, fatigue, and cough.
Senna	*Casiaaugustifolia*	General debility tonic, aphrodisiac.

Tulsi	*Ocimumsanclum*	Cough, Cold, bronchitis,expectorand
Pippermint	*Menthapipertia*	Digestive, Pain killer
Henna/Mehd	*Lawsenniaiermis*	Burning, Steam, Anti Imflamatary
Gritkumari	*Aloe verra*	Laxative, Wound healing, Skin burns &care,Ulcer
SadaBahar	*Vincearosea*	Leaukamia, Hypotensiv, Antispasmodic , Atidot
Vringraj	*Eclipta alba*	Anti-inflamatory, Digestive, hairtonic
Neem	*Azardirchataindica*	Sdedative, analgesic, epilepsy, hypertensive
Anantamool/sariva	*Hemibismusindicus*	Appetiser, Carminative, aphrodisiac, Astringent
Kantakari	*Solanumxanthocarpum*	Diuretic, Antiinflamatory, Appetiser, Stomachic
Shankhamul	*Geodorumdenciflorum*	Antidiabetic

1.6 Opportunities in drug discovery from medicinal plants:

Bio prospecting demands a number of requirements which should be co-coordinated, such as team of scientific experts (from all the relevant interdisciplinary fields) along with expertise in a wide range of human endeavors, including international laws and legal understanding, social sciences, politics and anthropology. In our context, Ayurveda and other traditional systems of medicine, rich genetic resources and associated ethno medical knowledge are key components for sustainable bio prospecting and value-addition processes. For drug-targeted bio prospecting an industrial partner is needed, which will be instrumental in converting the discovery into a commercial product. Important in any bio prospecting is the drafting and signing of an agreement or Memorandum of Understanding that should cover issues on access to the genetic resources (biodiversity), on intellectual property related to discovery, on the sharing of benefits as part of the process (short term), and in the event of discovery and

commercialization of a product (long term), as well as on the conservation of the biological resources for the future generations. When ethnobotanical or ethnopharmacological approach is utilised, additional specific requirements that relate to prior informed consent, recognition of Indigenous Intellectual Property and Indigenous Intellectual Property Rights as well as short- and long-term benefit sharing need to be taken into account (Patwardhan, 2005).

In order to screen thousands of plant species at one go for as many bioassays as possible, we must have a collection of a large number of extracts. Globally, there is a need to build natural products extract libraries. The extract libraries offer various advantages, such as reduction in cost and time for repeat collection of plants and availability of properly encoded and preserved extracts in large numbers for biological screening in terms of high-throughput screenings and obtaining hits within a short period. Such libraries could serve as a powerful tool and source of extracts to be screened for biological activities using high-throughput assays.

1.7 Medicinal plants of Bangladesh:

Being naturally gifted by a suitable tropical climate and fertile soil, Bangladesh possesses a rich flora of tropical plants. Around 5000 species of phanerogams and pteridophytes grow in its forests, jungles, wastelands and roadsides as indigenous, naturalised and cultivated plants. Out of them, more than a thousand have been claimed to possess medicinal and / or poisonous properties, of which 546 have recently been enumerated with their medicinal properties and therapeutic uses (Ghani, 2003).

In addition to possessing various other medicinal properties, 257 of these medicinal plants have been identified as efficacious remedies for diarrhoeal diseases and 47 for diabetes. Medicinal plants are an accessible, affordable and culturally appropriate source of primary health care system in Bangladesh. Marginalised, rural and indigenous people, who cannot afford or access formal health care systems, are especially dependent on these culturally familiar, technically simple, financially affordable and generally effective traditional medicines. As such, there is widespread interest in promoting traditional health systems to meet primary health care needs. This is especially true in this country, as prices of modern medicines spiral and governments find it increasingly difficult to meet the cost of pharmaceutical-based health care.

However, it has been observed that many other medicinal plants growing in the country have not been identified taxonomically and that there are many of them, which have not been chemically examined and no attention has yet been paid to characterize them from the pharmacognostic viewpoint. Thus, it is expected that the number of medicinal plants growing

or available in Bangladesh may be more than what has so far been enumerated. It has further been observed that the countless herbs found in Bangladesh should be used for promotion of health and for fighting many diseases. Thus medicinal plants of Bangladesh hold good promises as potential resources for drug development.

However, in order to develop these medicinal plants as drugs, attempts should be first made to certainly identify them and preclinical studies on them should be carried out to establish their claimed therapeutic properties. Since the chemical constituents and pharmacological actions of most of these plants are known and as they are in current use in traditional medicines, their clinical evaluation can be undertaken.

Table 1.3: Primary health care plants used in Bangladesh (From Thompson *et al*, 2005)

Local Name	Botanical Name
Amloki	*Emblica officinalis*
Anantamul	*Hemidesmus indica*
Apang	*Achyranthes aspera*
Arohor	*Cajanus cajan*
Ashok	*Saraca asoca*
Bahera	*Terminalia belerica*
Basok	*Adhatoda vasica*
Bael	*Aegle marmelos*
Chatim	*Alastonia scholaris*
Dalim	*Punica granatum*
Dondokolosh	*Leucas linifolia*
Durba	*Cynodon dactylon*
Gandhavadule	*Paederia foetida*
Ghritokumari	*Aloe indica*
Gulancha	*Tinospora cordifolia*
Horitoki	*Terminalia chebula*
Jute	*Corchorus capsularis*
Kalomegh	*Andrographis paniculata*
Sugarcane	*Saccharum officinalis*
Turmeric	*Curcuma longa*

1.8 Objective of the research study:

The objective of this study was to assess biological activity of *Spondius pinnata* using selected bench top bioassays, including total reducing capacity, antioxidant activity, toxicity assays--brine shrimp,chemical tests. Increased knowledge about phytomedicines can:

- Serve as alternative solutions where orthodox medicines have limitations, for example antibiotics (in case of antibacterial-drug resistance), anticancer drugs from plants, like tubulin polymerization inhibitors (which is less toxic than current anti-cancer drugs such as Actinomycin D).
- Provide man with necessary knowledge to avoid or minimize unwanted side effects from toxicities resulting from use of herbal medicines.
- To determine plants' extraordinary ability to synthesize secondary metabolites. Plants' defence mechanisms are sophisticated which allow them to survive. They have to be able to defend themselves against all the hazards to be able to survive. They do this with an enormous variety of secondary metabolites that they synthesize. Several types of thousands of secondary metabolites have already been isolated and their structure elucidated.

The main roles of secondary metabolites have been identified to be:

- Defence against herbivores (insects, vertebrates),
- Defence against fungi and bacteria,
- Defence against viruses,
- Defence against other plants competing for light, water and nutrients,
- Signal compounds to attract pollinating and seed dispersing animals,
- Signals for communication between plants and symbiotic microorganism (N-fixing Rhizobia or mycorrhizal fungi) and Protection against UV-light or physical stress. (Wink, 1999).

Medicinal plants have played an essential role in the development of human culture, for example religions and different ceremonies. (E.g. Dutura has long been associated with the worship of Shiva, the Indian god). Plants are directly used as medicines by a majority of cultures around the world, for example Chinese medicine and Indian medicine. Many food crops have medicinal effects, for example garlic.

With onset of scientific research in herbals, it is becoming clearer that the medicinal herbs have a potential in today's synthetic era, as numbers of medicines are becoming resistant.

1.9 The plant family: Anacardiaceae

Anacardiaceae, the cashew family, includes approximately 800 species in 82 genera. Members of the family are cultivated throughout the world for their edible fruits and seeds, medicinal compounds, valuable timber, and landscape appeal. Some of the products of Anacardiaceae, including mango (*Mangifera indica*), pistachio (*Pistacia vera*), cashew (*Anacardium occidentale*), and pink peppercorn (*Schinus terebinthifolia*), are enjoyed worldwide while other notables such as the pantropical Spondias fruits, the marula of Africa (*Sclerocarya birrea*), and the Neotropical fruits of Antrocaryon are restricted to localized cultivation and consumption and are not generally transported far distances to larger markets.

1.10 distribution of Anacardiaceae family:

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

1.11 Plant description:

1.11.1 Local names:

Bengali	Aamada, Aamraata, Aamraataka, Amra.
England	Hog plum
Chinese	Bin lang qing, Mu ge, Zhao wa wen po.
German	Mangopflaume
Gujarati	Ambaada
Hindi	Ambara,Ambari,Amra, Amara, Bhringi-phal, Metula, Pashuharitaki.
Japanese	Amura tamagonoki
Malayalam	Ambazham
Marathi	Amada,Ambada,Dholamba, Khatamba, Ranamba.
Nepalese	Amaaro

1.11.2 Taxonomic Hierarchy of investigated plant:

Kingdom	Plantae
Subkingdom	Tracheobionta
Super division	Spermatophyta
Subclass	Rosids
Order	Sapindales
Family	Anacardiaceae
Genus	*Spondias*
Species	*Spondias pinnata*

1.11.3 Physical Description:

Spondias pinnata is a medium to tall tree reaching a height of about 25 m and a diameter of about 60cm. It is wholly or partly deciduous with occasionally small buttress.

Figure 1.1: *Spondias pinnata* plant

Barks

Its bark surface is smooth, with irregular cracks, greyish to pale reddish brown, exceeding a clear, sticky sap with turpentine smell.

Figure 1.2: Bark of *Spondias pinnata*

Leaves

Leaves are arranged spirally, leaflets are alternate to opposite. Flowers are bisexual.

Figure 1.3: Leaves of *Spondias pinnata*

Fruit

Fruit is yellow in color, fleshy, drupe with a finely flavored edible pulp.

Figure 1. 4: Immature fruit and mature fruit of *Spondias pinnata*

Seed

Seed is hard, ridged and has a fibrous surface. *S. pinnata* is a light-demanding species. (Linn.f.) Kurz:

Figure 1. 5: Seed of *Spondias pinnata*

1.11.4 Distribution:

S. pinnata is native to India, Burma (Myanmar), Indonesia, Southern China, Thailand and throughout Malesia to the Solomon Islands. It is widely distributed in the Philippines.

1.11.5 Environmental requirements:

The suitable elevation for *S. pinnata* is from lowland up to 500 m asl. It requires areas with welldrained soil and limestone sites.

1.11.6 Phenology:

Flowers appear before the leaves or accompanied by very young ones. Flowering and fruiting is from June to October.

1.11.7 Propagation:

Through seeds, cuttings and air layering.

1.11.8 Growth:

The mean annual diameter increment of a 22-year-old S*pondias pinnata* tree in India was 1.1 cm. (Ghani, 1998)

1.12 Uses of *Spondias pinnata*:

1.12.1Whole plant:

1. The tree is used as shade and living fence posts.

2. Wood is utilized in temporary construction, mouldings, interior finish, drawers, turnery articles, carvings, core stock of plywood and pulp. Because of its lightness and softness, the wood is more suitable in the manufacture of matchsticks, matchboxes, boxes and crates.

3. The plant is reported to have anti-tubercular properties.

1.12.2 Leaves:

1. Leaves have a sour taste are edible. When young leaves are used as ingredient in meat, stew and filling for fish sinanglay, a bicolano delicacy. Bicolanos also use dried young leaves in the preparation of "laing", a favorite and popular dish among the local people.

2. Leaves are also used as feeds for cattle.

1.12.3 Fruits:

1. The fruits are eaten as a vegetable when green and as a fruit when ripe. Fruits are very nutritious and rich in vitamin A, minerals and iron content.

2. The fruits have a sour taste. They are eaten raw and can be made into jams, jellies and juices.

3. Through value addition of this wild edible fruit tree plant the local people make chutney, jam and pickle. By production and marketing of these products, the local people may increase their socio-economic status (Florido and Cortiguerra, 2003).

1.12.4 Flowers:

1. The flowers are sour and used in curry as a flavoring and also eaten raw.

1.12.5 Bark and Root:

1. The bark is useful in dysentery and diarrhea, and is also given to prevent vomiting.

2. The root is considered useful in regulating menstruation.

3. The bark leaves and fruits have medicinal properties and is used in the treatment of wounds, sores and burns.

Chapter 2

LITERATURE REVIEW

2. Literature review:

2.1. The phytochemistry of *Spondias pinnata (S. pinnata*) had been studied and it was found that the plant contained sterols, flavonoids and gums (Tandon and Rastogi, 1976).

2.2. The phytochemical studies isolated β amyrin, oleanolic acid and amino acid (leucine and alanine) compounds from *Spondias pinnata* (Rastogi and Mehrotra, 1993).

2.3. Analgesic activities of the stem bark extract of *Spondiaspinata* (Linn.f) Kurz.
Spondias pinnata is a strugging; scandent shrub belongs to family Anacardeaceae. The stem barks are used in folk medicine in the treatment of antidiarrhoea, dysentery, rheumatism, gonorrhea and anti-tubercular. The ethanol extract of *Spondiaspinnata* 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 (50-100 mg/kg; p.o) manner in the acetic acid test and in the second phase of formalin test which were comparable to the effects observed with acetylsalicylic acid (20 mg/kg). The results of this study lead credit to the traditional uses *S. pinnata*, especially as an analgesic. Phytochemical studies on this plant reveal the presence of terpenoids, flavonoids, tannins and these might be responsible for the analgesic activity of this plant. (Panda, B. K. *et al* 2009).

2.4. Structural features of the acidic polysaccharide of *Spondias pinnata* gum exudate:

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. The linkages of the interior parts were confirmed by methylation studies, and by the results of periodate oxidation and Smith degradation. (Ghoshal, P.K. *et al* 2008)

2.5. Qualitative phytochemical tests of *S. pinnata* resin extracts were carried out for detecting the presence of saponins (by foaming test), carbohydrates (by Fehling's test), proteins and free

amino acids (by Biuret method), fixed oils (by spot test). Tannins, phytosterols, flavonoids were detected by colour development tests (Gupta *et al*., 2010).

2.6. The qualitative screening of the phytochemical components in the stem bark of *S. pinnata* revealed the presence of alkaloids, flavonoids and polyphenols (Chetia and Gogoi, 2011).

2.7. The phytochemical screening of *S. pinnata* revealed the presence of alkaloids, saponins and tannins. LC– MS/MS analysis of *S. pinnata* revealed 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. GC/MS screening showed the presence of vitamin E, furfural, phytosterol, campesterol and fatty acids. Analysis of volatile flavor showed isopropyl Study of bark of *Spondias pinnata* 26
myristinate as a major compound followed by the other monoterpenes and sesquiterpenes (Satpathy, 2011).

Table 2.1: Summary of the phytochemical studies on *Spondias pinnata*

Parts of the plant	Findings References	References
Whole plant	Sterols, flavonoids and gums	Tandon and Rastogi, 1976.
Whole plant	β amyrin, oleanolic acid and amino acid (leucine and alanine)	Rastogi and Mehrotra, 1993.
Ethanol extract of stem bark	Terpenoids and tannins	Panda *et al*., 2009.
Resin extracts	Saponins, carbohydrates, proteins and free amino acids, fixed oils, Tannins, phytosterols, flavonoids.	Gupta *et al*., 2010.
Stem bark	Alkaloids, flavonoids and	Chetia and Gogoi, 2011.

	polyphenols	
Whole plant	Alkaloids, saponins, tannins, gallic acid, salicylic acid,	Satpathy, 2011.

2.8. A crude extract of *S. pinnata* has been reported to show antibacterial activity (Bibitha*et al*., 2002).

2.9. A 70% methanol extract of *Spondia spinnata* stem bark was studied *in vitro* for total antioxidant activity. The study provided evidence that a 70% methanol extract of *Spondias pinnata* stem bark is a potential source of natural antioxidants (Hazra*et al*., 2008).

2.10. Stem heart wood and bark of *Spondias pinnata* when tested in vitro, showed potent Anthelmintic activity on the. Stem heart wood methanolic extract of *S. pinnata* was also more potent than the bark extract (Gangarao and Jayaraju, 2009).

2.11. Earlier studies have shown that the ethanolic extracts of pulp of *S. pinnata* Kurz. have antibacterial activity against *Staphylococcus aureus, E. coli* and *Pseudomonas aeruginosa*(bacteria) and antifungal activity against yeast *Candida albicans*and fungus *Aspergillus flavus* (Keawsa-ard and Liawruangrath, 2009).

2.12. A comparative study of anthelmintic study between actone and ethanolic extract of bark stem of *Spondias pinnata* was done. Ethanol extract showed better anthelmintic activity than acetone extract (Panda *et al*., 2011).

2.13. To evaluate the antimicrobial activity of methanolic extract of fruit of *Spondias pinnata in vivo* study was carried out. Methanolic extract of fruit of *Spondias pinnata* was found to be more effective against *P. aeroginosa* (Borkotoky*et al*., 2013).

Table 2.2: Summary of the pharmacological studies on *Spondias pinnata*

Parts of the plant	Findings	References
Crude extract	Antibacterial activity	Bibitha*et al*., 2002. .
70% methanol extract of stem bark	Antioxidant activity	Hazra*et al*., 2008.
Ethanolic extracts of pulp	Antibacterial and antifungal Activity	Keawsa-ard and Liawruangrath, 2009.
Actone and ethanolic extract of bark stem	Anthelmintic activity	Panda et al., 2011.
Methanolic extract of fruit	Antimicrobial activity	Borkotoky*et al*., 2013

Chapter: 3

MATERIALS AND METHODS

3. Materials and methods

Materials:

Table3.1: Apparatus and Reagent used for Extraction

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

Method:

3.1. 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. 35937) has been deposited for future reference.

3.2 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.

3.3 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.

3.3.1 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.

3.4 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.

3.4.1 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.

3.4.2 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.

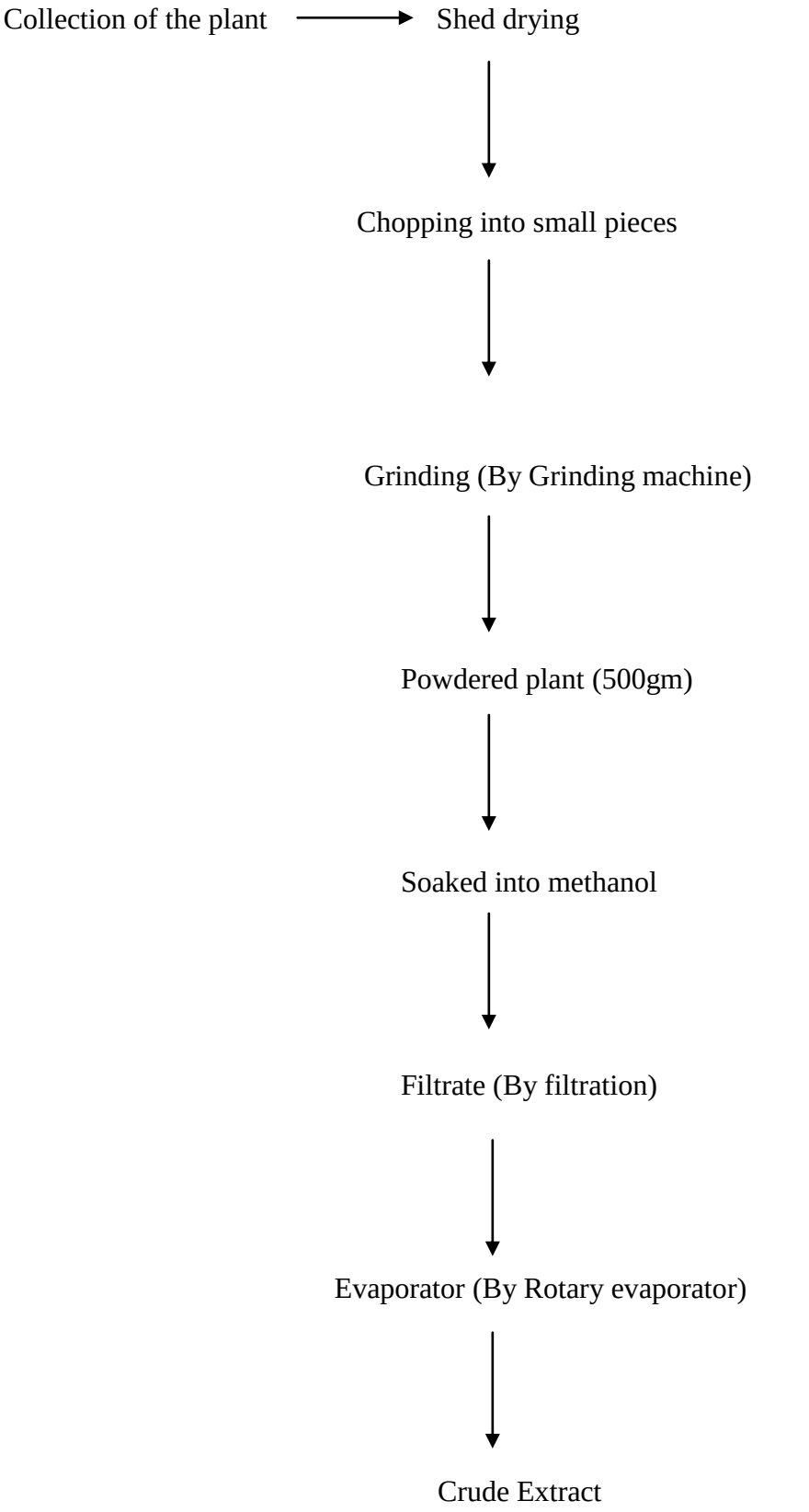


Figure 3.1: Schematic presentation of the crude preparation from the plant

Extraction of bark of *spondias pinnata:*

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

3.5.1 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.

3.5.2 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.

3.6 Preliminary Phytochemical Screening

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

3.6.1 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).

3.6.2 Test for Saponins

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).

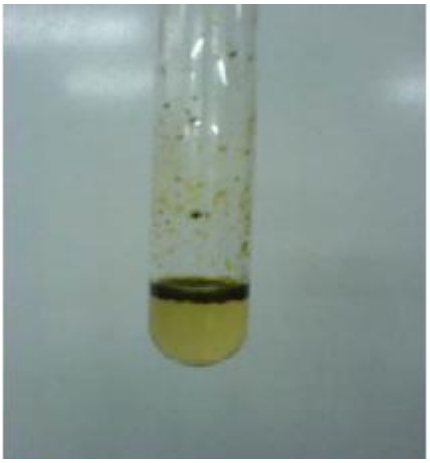


Figure 3.2: Froth test for Saponons

3.6.3 Test for 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).

3.6.4 Test for Phenols

Ferric chloride Test:

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

3.6.5 Test for Steroidal Compounds

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).

3.7 Total Phenol content determination

Table3.3: 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

3.7.1 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).

3.7.2 Preeparation 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.

3.7.3 Preparation of 10% Folin- ciocalteu reagent:-

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

3.7.4 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.

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

3.7.5 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.

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

3.7.6 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 solution were measured at 765 nm using a spectrophotometer against blank.

3.8 DPPH Free Radical Scavenging Assay

Table3.4: 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	

3.8.1 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.

3.8.2 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.

3.8.3 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.

3.8.4 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.

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.

Calculation of % inhibition

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

$$\% \text{ Inhibition} = \frac{(\text{Control Absorbance} - \text{Test Absorbance}) \times 100}{\text{Control Absorbance}}$$

3.9 Total Reducing Power

Materials

Table3.5: 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

3.9.1 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 (Liao and Yin, 2000). 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).

3.9.2 Preparation of Reagent:

- **Phosphate buffer (2.5 ml, 0.2 H, pH6.6)**

  27.8 gm monobasic sodium phosphate dissolved in 500 ml water.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.

- **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.

- **Preparation of ferric chloride (.1%) solution:**

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

3.9.3 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.

3.10 Brine Shrimp Lethality Test

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 (McLaughlin, 1990; Persoone, 1980) 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, pesticide and anti-tumor etc. of the compounds (Meyer, 1982; McLaughlin, 1988).

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.

3.10.1 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.

3.10.2 Materials

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
EAF	Ethyl Acetate fraction of methanol extract	4.0
CF	Chloroform fraction of methanol extract	4.0

3.10.3 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.

3.10.4 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.

3.10.5 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.

3.10.6 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

3.10.7 Preparation of the control group

Controls groups are used in the cytotoxicity 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

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.

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.

Figure 3.3: Brine shrimp

3.10.8 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.

Chapter: 4

RESULTS AND DISCUSSIONS

4. Results and Discussions

4.1 Result of Phytochemical Screening

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 From the experiment it was found that, the chloroform extracts of *Spondias pinnata* leaves contains most of the phytoconstituents.

Discussion:
Chloroform fraction has shown the presence of the, flavonoid, steroid, alkaloids and saponin in all tests. This fraction has not shown the presence of the phenols. Also there is no evidence of the presence of cardiac glycoside.

4.2 Result of Brine Shrimp Lethality Bioassay

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 best-fit line were obtained from the curve data by means of regression analysis.

Table 4.2: Result of lethality of Tamoxifen (standard) on brine shrimp

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			

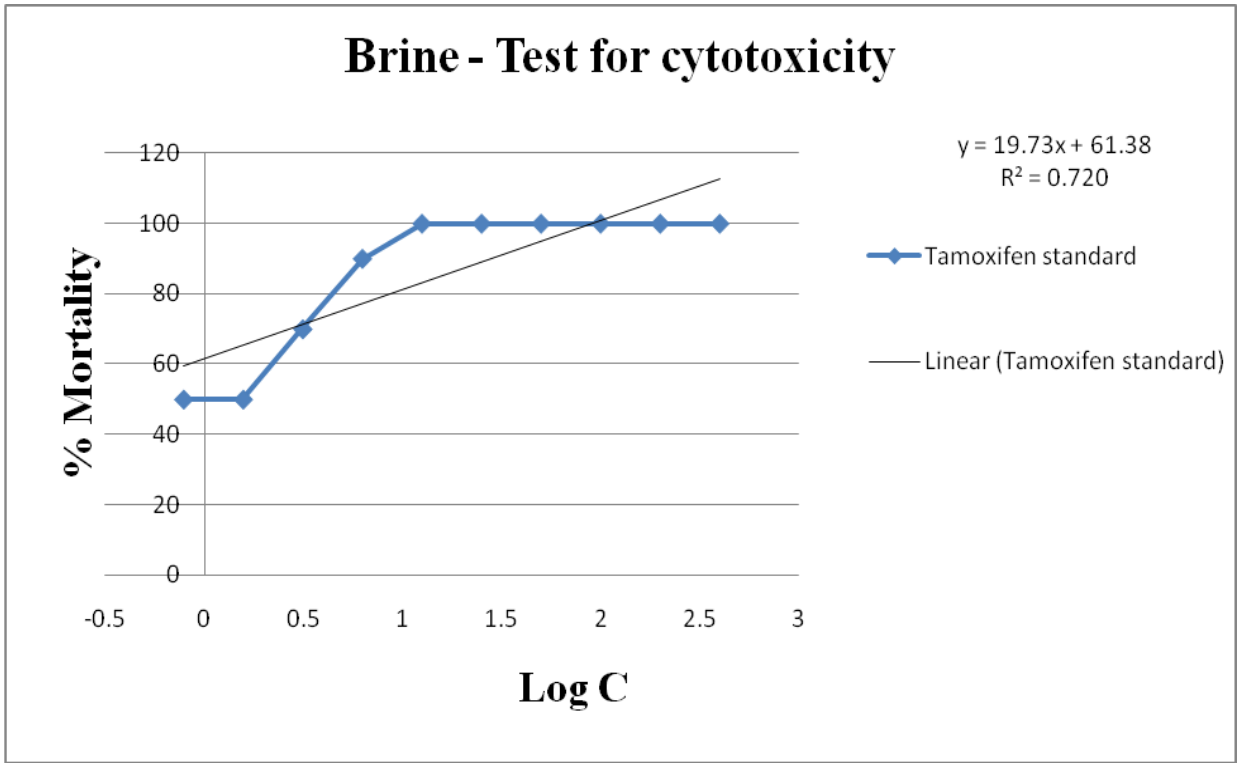


Figure 4.1: Standard curve of Tamoxifen (standard)

TABLE 4.3: Effects of Chloroform extract of *Spondias pinnata* on Brine 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=26.77x +33.59	4.102
200	2.301	10	100		
100	2.00	10	100		
50	1.699	8	80		
25	1.398	6	60		
12.5	1.097	5	50		
6.25	0.796	6	60		
3.125	0.495	4	40		
1.5625	0.194	4	40		
0.781	-0.107	4	40		

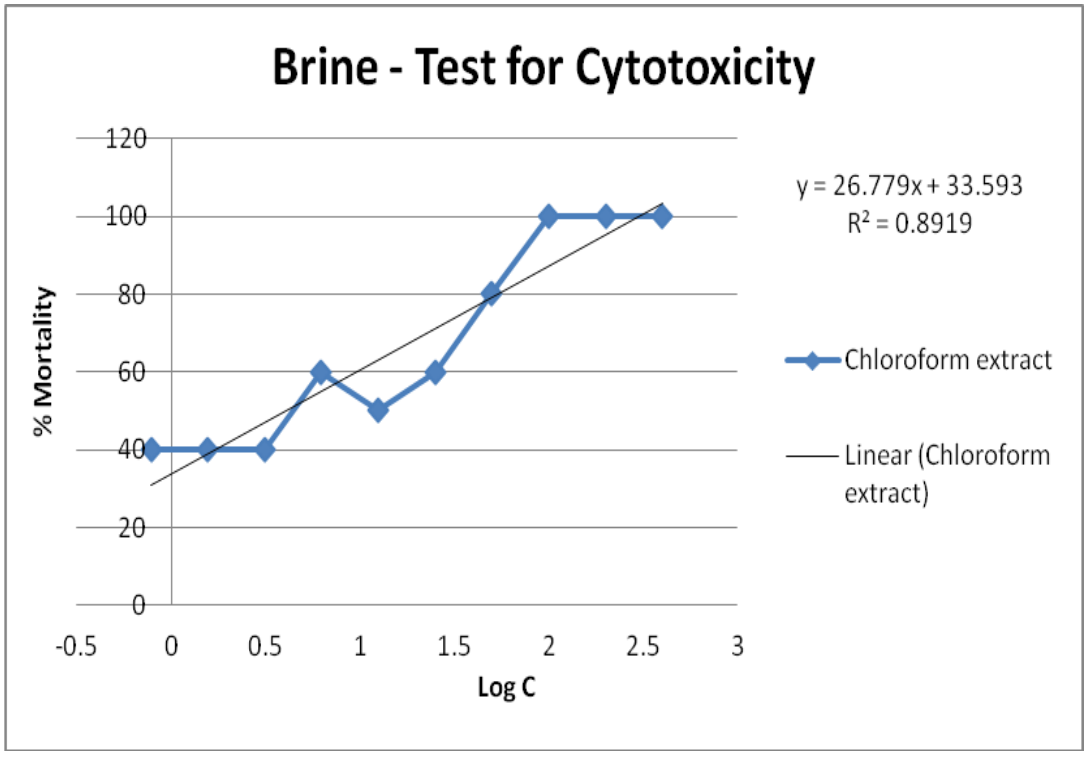


Figure 4.2: Graphical presentation of effect of chloroform extract of *Spondias pinnata* bark on brine shrimp nauplii.

Discussion:

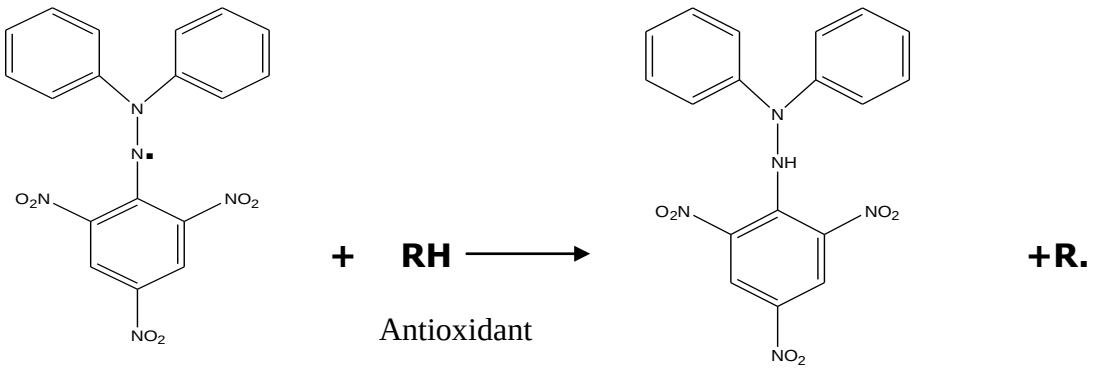
The lethal concentration LC50 of the chloroform extract after 24 hr. was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration (toxicant concentration) and the result is 4.102 µg/ ml, where our standard concentration is 1.142 µg/ ml. so it show significant level of toxic component. Best-fit line was obtained from the curve data by means of regression analysis**.**

4.3 Result of antioxidant property:

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

(At normal temperature)

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



DPPH (oxidized form)

Diphenyl picrylhydrazyl (λ_{max} 517 nm)

DPPH (reduced form)

Table 4.4: IC_{50} value of Ascorbic acid (standard) (at room temperature)

Concentration (µg/ml)	Absorbance	% inhibition $\frac{\text{(Blank absorbance – Sample absorbance)} \times 100}{\text{Absorbance of blank}}$	X value (IC_{50})
20	0.107	71.314	371.18
40	0.14	62.466	
60	0.168	54.959	
80	0.179	52.011	
100	0.199	46.649	

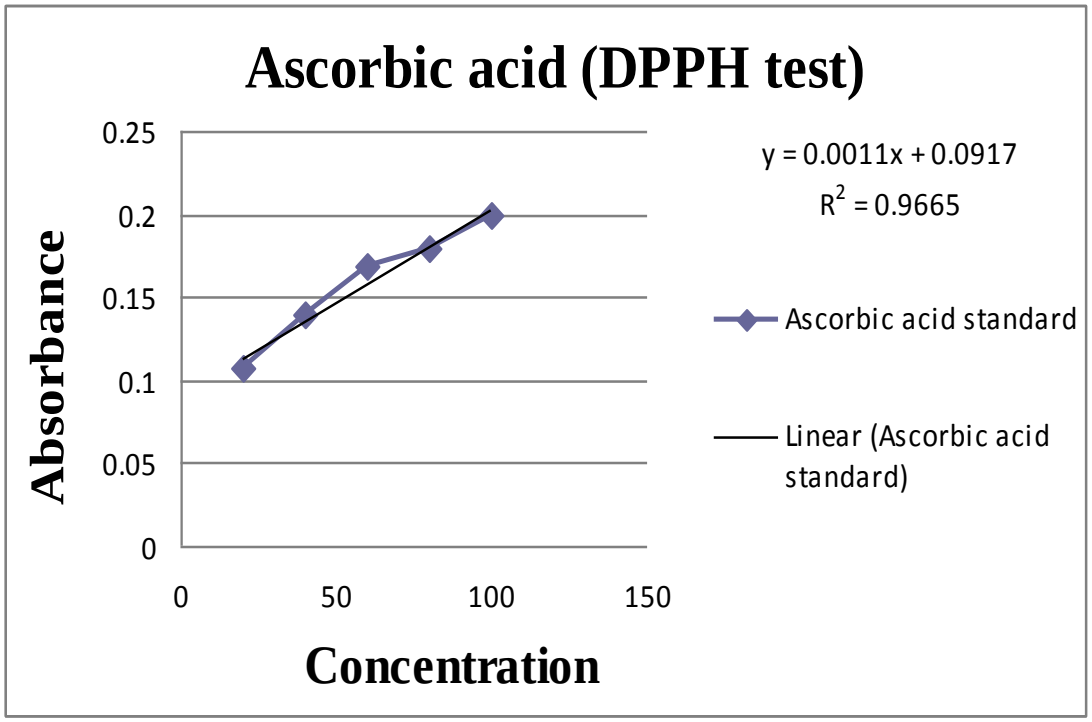


Figure4.3: Standard curve (Ascorbic acid)

Table 4.5: IC_{50} value of chloroform extract of *Spondias pinnata* (bark) (at room temperature)

Concentration (µg/ml)	Absorbance	% inhibition (Blank absorbance – Sample absorbance) ×100 / Absorbance of blank	X value (IC_{50})
20	0.155	58.445	319.083
40	0.162	56.568	
60	0.17	54.424	
80	0.199	46.648	
100	0.255	31.635	

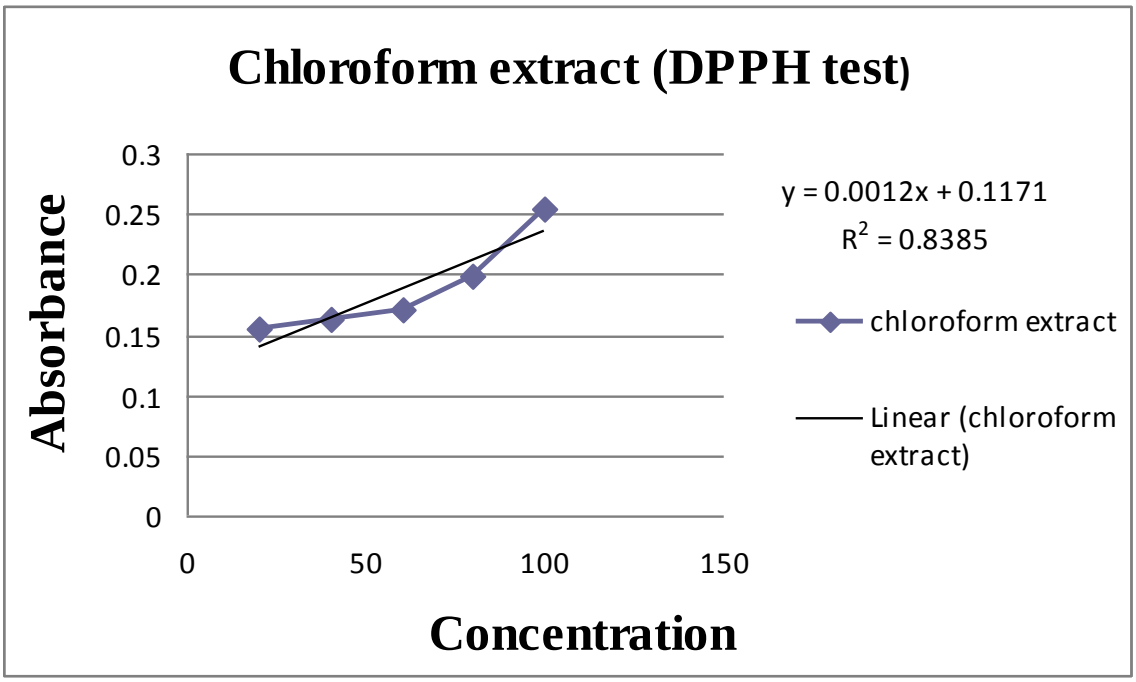


Figure 4.4: Standard curve for DPPH scavenging potential & IC_{50} value of chloroform extract of *Spondias pinnata* (bark)

Discussion:

The chloroform extract of *Spondias pinnata* (bark) was subjected to free radical scavenging activity and it showed significant free radical scavenging activity with an IC_{50} value of 319.08

µg/ml at normal temperature, where standard ascorbic acid show a value of 371.18 µg/ml. So we can say that our chloroform show free radical scavenging activity.

4.3.2 Result of Total phenolic content

The crude chloroform 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).

Table 4.6: Total phenolic content of Gallic acid (standard)

Concentration	Absorbance	Best Fit Equation	R2 Value	X value
250	0.066	Y= 0.0001x + 0.040	0.957	260
200	0.059			190
150	0.054			140
100	0.048			80
50	0.047			70

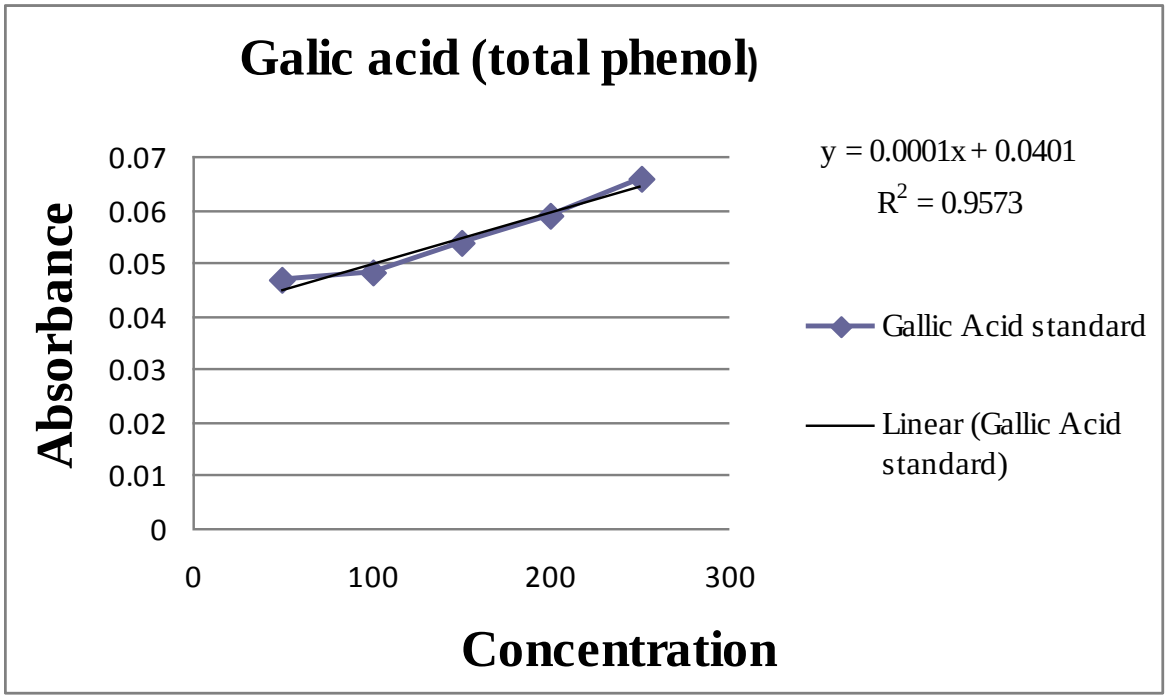


Figure 4.5: Standard curve for Gallic acid

Table 4.7: Total phenolic content of chloroform extract of *Spondias pinnata* (bark)

Concentration	Absorbance	X value
250	0.231	255
200	0.166	190
150	0.105	129
100	0.071	95
50	0.035	59

Discussion:

In test sample for 250, 200, 150, 100, 50 µg/ml concentration it has X value of 255, 190, 129, 95, 59, where standard Gallic acid contains 260, 190, 140, 80, 70 mg of GAE (Gallic acid equivalent) per gm of dried extract, respectively. So chloroform extract of *Spondias pinnata* (bark) have significant amount of phenol.

Chapter: 5

CONCLUSION

5. Conclusion

Phytochemical screenings of the extract showed that the *Spondias pinnata* bark possess alkaloids, saponins, and steroids. The presence of the identified phytochemicals makes the pharmacologically active. Brine Shrimp Lethality Bio-assay test was done and the result is very significant LC_{50} value 4.102 µg/ ml where the standards Tamoxifen show LC_{50} value 1.142 µg/ ml. The pharmacological profiles of the present investigation of the chloroform fractionation of the *Spondias pinnata* barks indicate that it posses significant level of cytotoxic compound. DPPH test show IC_{50} value of Sample is 319.08, where standard Ascorbic acid has IC_{50} value of 371.18. So it possesses some free radical scavenging activity. Total phenolic content test for 250, 200, 150, 100, 50 µg/ml concentration of sample it has X value of 255, 190, 129, 95, 59, where standard galic acid contains value 260, 190, 140, 80, 70 mg of GAE (gallic acid equivalent) per gm of dried extract, respectively. So chloroform extract of *Spondias pinnata* (bark) have significant amount of phenol.

The extract possesses free radical scavenging activity, strong cytotoxic properties and might, in part, be due to the presence of such compounds. The results also suggest a rationale for the traditional uses of this plant. However, studies are required on higher animal model and subsequently on human subjects to prove efficacy as an antioxidant, and cytotoxic agent.

Chapter: 6

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