STRUCTURAL ELUCIDATION OF MAJOR BIOACTIVE COMPOUNDS AND GENOME ANALYSIS OF *SPONDIAS PINNATA* KURZ. (AMARA) AND *STREBLUS ASPER* LOUR. (SOURA).

A thesis submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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Dedicated
to
'Rit' and 'Lu'

ABSTRACT

Spondias pinnata Kurz. and *Streblus asper* Lour. are two important medicinal plants of Assam and the North Eastern region. *Spondias pinnata* plants on maturity attains height of 27.4±2.302 m and girth of 2.42±0.449 m. The bark is thick and greyish brown in colour bearing longitudinal cracks. The leaf size varies from 16.20±2.084 x 6.98±0.923 cm to 18.70±2.350 x 7.92±0.526 cm. The leaf area ranges from 95.63±4.725 - 108.19±3.375 cm^2. Flowering starts from February and lasts till end of June. The inflorescence is a terminal panicle with a length 29.80±4.508 - 39.38±2.334 cm. A mature plant of *Streblus asper* with thick foliage attains the height of 7.96±1.533 m and the girth 0.88±0.216 m at maturity. The bark is warty, wrinkled and greyish white in colour. A sticky milky white juice is exuded by the bark on injury. Leaves are alternate, entire, obovate and acute. Leaf size ranges from 4.70±1.445 x 2.89±0.387 cm - 6.10±1.937 x cm 3.46±0.421 cm. The leaf area ranges from 11.40±1.733 - 15.84±2.683 cm^2. The dioecious plant flowers during February to May.

The per cent moisture content of *Spondias pinnata* fruit was 76.62± 0.785. A comparatively high percentage of crude protein 3.336±0.195 is available in the fruits. The mature fruits contains a high amount of reducing sugar, 69.56±1.060 mg g^{-1} and crude fibre 23.07±0.780 mg g^{-1}. The composition of minerals like phosphorous, iron, calcium and potassium are 0.483±0.032, 0.043±0.005, 5.967±0.472 and 83.60±1.520 mg g^{-1}, respectively. The leaf moisture content of *Streblus asper* is 75.64±1.820 per cent where as crude protein and fat content 16.73±0.079 and 1.029±0.029 per cent, respectively. The starch, reducing sugar, crude fibre and ash content in leaves of the plant are 12.05±0.710, 1.15±0.060, 17.08±0.120 and 8.113±0.256 mg g^{-1}, respectively. The composition of

minerals like phosphorus, iron, calcium and potassium are 0.236±0.015, 0.040±0.010, 14.33±0.577 and 33.46±0.611 mg g^{-1}, respectively.

The methanolic extract of fruits (500 g powder) on partitioning with chloroform could produce a fraction which possessed antimicrobial activity against *Bacillus subtilis, Escherichia coli, Staphyllococcus aureus* and *Candida albicans*, but not against *Klebsiella pneumoniae.* The antimicrobial chloroform extract following chromatography on a silica gel column with chloroform-methanol (9:1 to 1:9) could afford two fractions, fraction 1 (2.0 g) and 2 (1.4 g). On thin layer chromatography the fraction 1 on plates prepared with silica using chloroform-methanol (60:40) as the solvent could afford 3 pure compounds viz. 1a, 1b and 1c and the fraction 2 also different 3 compounds viz. 2a, 2b and 2c. Out of six compounds 1c (SP1) showed antimicrobial activity. The highest activity was against *Staphyllococcus aureus* (19.0±0.2 mm) followed by *Bacillus subtilis* (18.0±0.40 mm) and *Escherichia coli* (15.0±0.40 mm). The IR, ^{1}H-NMR, ^{13}C-NMR and HR-FAB mass analysis revealed the molecular formula of the compound SP1 (283 mg; yield 0.0566% and R_f-0.45) to be $C_{30}H_{48}O_3$ with molecular mass of 456 and is a pentacyclic triterpene with a double bond C12-C13. On the basis of IR, HRFABMS, ^{1}H NMR, ^{13}C NMR spectroscopic data and available reference data, the structure of the compound was determined to be '3 β-hydroxyolea-12-en-28-oic acid' commonly known as 'oleanolic acid'.

The methanolic extract of the powdered stem bark of *Streblus asper* on partitioning with petroleum ether produced a fraction, which exhibited antimicrobial activity. The petroleum ether extract showed antimicrobial activity against all the tested organisms except *Klebsiella pneumoniae.* The petroleum ether extract on silica gel-based

chromatography afforded fractions 1 and 2. The thin layer chromatography revealed 3 pure compounds viz. 1a, 1b and 1c from that of fraction 1 and 2 viz. 2a and 2b from fraction 2. The compound 2b (SA2) showed antimicrobial activity against the test organisms. The highest activity was recorded against *Bacillus subtilis* (17.0±0.5 mm) followed by *Staphyllococcus aureus* (14.0±0.60 mm) and *Candida albicans* (12.0±0.90 mm). The IR, ^{1}H-NMR, ^{13}C-NMR and HR-FAB mass analysis revealed the molecular formula of the compound to be $C_{30}H_{50}O$ with molecular mass 426 and the compound was a pentacyclic triterpene with an isopropenyl group. On the basis of all these data and references available the structure of the compound was determined to be 'Lup-20(29)-en-3 β-ol' commonly known as 'lupeol'.

The 2C nuclear DNA content of the extracted nuclei of *Spondias pinnata* and *Streblus asper* was determined by flow cytometry using *Pisum sativum* as the external reference standard. The C-value of *Spondias pinnata* was estimated to be 2.36 pg or $2.30x10^9$ bp and *Streblus asper* 3.93 pg or $3.84x10^9$ bp.

Genome size of the plants was also determined using the simple and cost effective method developed by Konwar *et al* (2007). The yield of genomic DNA per gram of fresh leaf tissue was 43.1 µg in *Spondias pinnata* and 48.5 µg in *Streblus asper.* The purity as judged from A260 : A280 ratio was 1.77 in *Spondias pinnata* and 1.89 *Streblus asper.* Following the method, genome size or C-value of *Spondias pinnata* and *Streblus asper* was determined to be 2.36 pg or $2.25x10^9$ bp and 3.93 pg or $3.72x10^9$ bp. The C-value determined by this method possessed a minor variation of 0.04 pg in the case of *Spondias pinnata* and 0.12 pg in the case of *Streblus asper* from that determined by flow cytometry.

DECLARATION

I hereby declare that the thesis entitled 'STRUCTURAL ELUCIDATION OF MAJOR BIOACTIVE COMPOUNDS AND GENOME ANALYSIS OF *SPONDIAS PINNATA* KURZ. (AMARA) AND *STREBLUS ASPER* LOUR. (SOURA)' being submitted to the department of Molecular Biology and Biotechnology, Tezpur University, is a record of original research work carried out by me. Any text, figures, methods or results that are not of own devising are appropriately referenced in order to give credit to the original author(s). All sources of assistance have assigned due acknowledgement. I also declare that neither this work as a whole nor a part of it has been submitted to any other university or Institute for any other degree, diploma or award.

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CERTIFICATE BY THE PRINCIPAL SUPERVISOR

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All help received by him from various sources have been duly acknowledged.

No part of this thesis has been reproduced elsewhere for award of any other degree.

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CERTIFICATE OF THE EXTERNAL EXAMINER

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ABBREVIATION AND SYMBOL USED

m	: meter	cm	: centimeter
cm^2	: square centimeter	mm	: milimeter
μm	: micrometer	μm^3	: cubic micrometer
nm	: nanometer	g	: gram
g^{-1}	: per gram	mg	: miligram
μg	: microgram	pg	: picogram
ng	: nanogram	l	: litre
ml	:mililitre	μl	:microlitre
SD	: standard deviation	R_f	: retention factor
$CDCl_3$	: deuterated chloroform	IR	: infrared
NMR	: nuclear magnetic resonance		
HRFABMS	: High resolution fast atom bombardment mass spectroscopy	KBr	: potassium bromide
%	: per cent	DNA	: deoxyribonucleic acid
PR	: peak ratio	bp	: base pair
OD	: optical density	MeOH	:methanol
$CHCl_3$	: chloroform	TLC	: thin layer chromatography
TMS	: tetramethylsilane	NBA	: m-nitrobenzyl alcohol
mA	: miliampere	kV	: kilovolt
V	: volt	^0C	: degree centigrade
h	: hour	HCl	: hydrochloric acid
ppm	: parts per million	NA	: Nutrient Agar

MHA : Mullar Hinton Agar

PCR : Polymerase chain reaction

Tris : Tris(hydroxymethyl)aminomethane

RAPD : Random amplified polymorphic DNA

EDTA : Etylene diamine tetra acetic acid

mM : milimole

CTAB : Cetyltrimethylamoniumbromide

TAE : Tris Acetate EDTA

rpm : revolution per minute

UV : ultraviolet

DMSO : Dimethyl sulfoxide

dH_2O : distilled water

NaOH : Sodium hydroxide

NaCl : Sodium chloride

M : mole

Na_2 EDTA: Sodium EDTA

PVP : Polyvinylpyrolidone

TE : Tris EDTA

Rnase : ribonuclease

Vis : visible

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Chapter I

Introduction

CHAPTER I

INTRODUCTION

1.1. General

The history of the use of medicinal plants for the alleviation of diseases by human beings has its origin in primitive times. The plant kingdom contributed immensely to human health when no synthetic medicines were available, and when no concept of surgery existed. Primitive men lived in harmony with their surrounding environment. This entailed the use of herbal medicines as healthcare and also as medicinal treatment to various diseases. Illness, physical discomfort, wounds, and fear of death forced primitive men to use the natural substances around them. During antiquity, although guided by different cultural contexts, health in the Western and Eastern systems was based on holism, that is, treatment should act on the organism as a whole, integrated with the Universe. According to past records, Babylonians (about 3,000 B.C.) were aware of a large number of medicinal plants and their properties. Some of the plants are still used today in the same way and for the same purposes. The earliest mention of the medicinal use of plants in the Indian subcontinent is found in the *Rig Veda* (4,500–1,600 B.C.), which noted that the Indo-Aryans used the Soma plant (*Amanita muscaria*), a narcotic and hallucinogenic mushroom, as a medicinal agent.

1.2. World trend

Even with the progress of civilization men have not been able to dissociate themselves from plants rather the dependence is on a upward trend. The World Health Organization (WHO) has listed 20,000 medicinal plants globally (Gupta and Chadha, 1995). According to the WHO estimate, about 80 percent of the population in the

developing countries depends directly on plants for medicines (Pareek, 1996). There is a growing demand today for plant-based medicines, health products, pharmaceuticals, food supplements etc in the international market. The international market of medicinal plants is over 60 billion US dollar per year, which is growing at a rate of 7 percent per annum (Chatterjee, 2002). Medicinal, aromatic and dye plants (MADPs) also have potential to present as commodities with competitive advantages for the poor regions of Asia, Africa and Latin America. It is observed that some of the poorest regions of the world such as the Western and Eastern regions of the Himalayas, Borneo and Sumatra regions in Asia; Congo basin in Africa and Amazon catchments in South America are also rich in biological diversity where medicinal plants and other non-timber forest product (NTFP) species grow in abundance. If a balanced conservation and cultivation of medicinal, aromatic and dye plants can be promoted, the effort will protect human health, help to treat domestic animals to alleviate rural poverty, to address gender imbalances and to improve local economy.

1.3. Danger of extinction

Plant resources are depleting at an alarming rate and a number of economically and medicinally important plant species are likely to be extinct soon. An estimated 4,000-10,000 species of medicinal plants face potential local, regional or global extinction, with subsequent serious consequences for livelihoods, economies and healthcare systems (Hamilton, 2004). There are many potential causes of rarity in medicinal plant species, such as habitat specificity, narrow range of distribution, land use disturbances, introduction of non-natives, habitat alteration, climatic changes, heavy livestock grazing, explosion of human population, fragmentation and degradation of population, population

bottleneck and genetic drift. Additionally, natural enemies such as pathogens, herbivores and seed predators could substantially limit the abundance of rare medicinal plant species in any given area (Kala *et al.*, 2006). Medicinal plants are now under great pressure due to their excessive collection or exploitation.

1.4. Indian scenario

India (80-30^{0}N and 68-97.5^{0}E) exhibits a wide range in topography and climate, which has a bearing on its vegetation and floristic composition. This subcontinent is one of the world's 12 leading biodiversity centres, encompassing 16 different agro-climatic zones, 10 vegetation zones, 25 biotic provinces and about 426 habitats of specific species. In India, more than 80 percent of medicinal and aromatic plants are collected from 17 million hectares of Indian forestland. There are about 17,000 species of higher plants, and 7,500 of them are known for medicinal uses (Kala *et al.*, 2006). About 2,000 drugs used in the country are of plant origin (Dikshit, 1999).

With the increased collection, the degree of threat to natural populations of medicinal plants has increased because more than 90 percent of medicinal plant-raw materials for herbal industries in India and also for export is drawn from natural habitats (Dhar *et al.*, 2002). At present, India exports herbal raw materials and medicines of about 100-114 million US dollar per year (Chatterjee, 2002). India and China are two of the largest countries in Asia, which have the richest arrays of registered and relatively well known medicinal plants (Raven, 1998). China is estimated to have 12,807 species of plants, out of which 11,146 are classified as medicinal plants used in traditional Chinese medicine (TCM). It is estimated that up to 492 species are currently under cultivation and the remaining 10,654 species are harvested from wild habitats (SEPA, 1997). According

to Wang *et al.* (2002), the total production of medicinal plants from the wild sources is 8.5 million tons and the production of cultivated medicinal plants was estimated to be 0.3 million tons in 2001-02. The plant based medicines not only contribute to the health of Chinese people but also add approximately 2 billion USD to the national economy annually.

Since, Indian subcontinent is well known for its diversity of forest products and the age-old healthcare traditions, there is an urgent need to establish these traditional values in both national and international perspectives realizing the ongoing developmental trends in traditional knowledge. A great deal of traditional knowledge on the use of various plant species is still intact with the indigenous people living in less accessible mountainous areas. The Planning Commission and the National Medicinal Plants Board (NMPB) of the Government of India have prepared a policy document on the promotional and commercial aspects of the medicinal plant sector. The Biodiversity Act 2002 framed many rules for the sustainable utilization of medicinal plants (Puspangadan, 2005).

1.5. Cultural preference

Apart from human use, many plant species are used in animal husbandry as the primary source of healthcare in India. The reliance on medicinal plants is also due to the cultural preference of different communities. Medicinal plants have strong acceptance in religious activities of the native communities of India, who worship plants in the form of gods, goddesses and minor deities. *Origanum vulgare* (Oregano), *Saussurea obvallata* (snow lotus or Brahma Kamal), *Ocimum sanctum* (Holy Basil or Tulsi), *Cedrus deodara* (Cedarwood or Deodar cedar), *Cynodon dactylon* (Bermudagrass or Dubori), *Aegle*

marmelos (Bael fruit), *Juniperus communis* (Common juniper), *Nardostachys grandiflora* (Jatamansi or Indian Spikenard), *Zanthoxylum armatum* (Winged prickly-ash or Darmar), *Ficus benghalensis* (Indian Banyan tree) and *Ficus religiosa* (Bodhi tree) are some of the examples of plants highly used for medicinal as well as for religious purposes by the Hindus in Northern India (Kala *et al.*, 2006). The Buddhist community in Northern India regards *Terminalia chebula* (Myrobalan) as an important medicinal as well as a sacred fruit tree.

1.6. Alternative medicine

Already institutionalised to a large extent globally, alternative medicine forms a link between folk medicine and modern Western medicine. Many of the healing concepts date back to well before the advent of modern medicine. With the rapid and enormous progress of scientifically based modern Western medicines, they were however largely repressed. The last couple of decades have, notwithstanding, seen a global resurgence of these alternative medicinal systems, some of the best known being Homeopathy, Ayurvedic, Unani and traditional Chinese medicine.

There are two ancient systems of medicine in India, the Siddha that flourished in the South and the Ayurveda prevalent in the North. Ayurveda has alone reported approximately 2,000 medicinal plant species whereas more than 1,100 medicinal plant species are reported by Siddha (Kala *et al*, 2006). The 'Charak Samhita', an age-old written document on herbal therapy, reports on the production of 340 herbal drugs and their indigenous uses (Prajapati *et al.*, 2003). The traditional medical systems of India comprise of a major part of time-tested culture and are honoured by people till today.

These traditions have successfully set an example of natural resource utilization in curing many complex diseases for more than 3,000 years.

1.7. Merits of herbal therapy

Many advantages of such eco-friendly traditions exist. The plants used for various therapies are readily available, are easy to transport and have a relatively long shelf life. The most important advantage of herbal medicine is the minimal side effect and relatively low cost as compared to synthetic medicines. Synthetic drugs gained popularity against green remedies for their fast-acting effects; on the other hand, the healing process using medicinal plants is slow. But, people have begun to realize the benefits associated with natural remedies again. Chemically prepared drugs may act quickly but they have various side effects, which affect our body negatively in the long run, whereas medicinal plants work in an *integrated* or *pro-biotic approach* and with little adverse effects. For example, a regular intake of garlic can control cholesterol and high blood pressure within a moderate period of time, but taking synthetic drugs make the person's body completely dependent on that particular medicine (Babu and Madhavi, 2001). Allopathic medicines may cure a wide range of diseases; however, its high prices and side effects are causing many people to return to herbal medicines.

1.8. Limitations of herbal medications

Apart from the slow healing process by the medicinal plants, another limitation of the herbal medication is that the actual working principles of most of the medications are still not known. Of late, a number of screening programmes, aimed at finding out new compounds of pharmacological activity or new sources of known compounds are being conducted. The instant increasing demand of plant-based drugs is unfortunately creating

heavy pressure on some selected high-value medicinal plant species in the wild due to over-harvesting. Several of these medicinal plant species possess slow growth, low population density and narrow geographic ranges; therefore, they are more prone to extinction (Kala *et al.*, 2006).

1.9. North East India- a store house of potential medicinal plants

The North-East India is located between $87^0 32'E$ - $95^0 52'E$ and $21^0 34'N$ - $29^0 50'N$ and is a genetic treasure house of plant, animal and microbial resources. The region forms a distinctive part of the Indo-Burma hotspot which ranks the sixth among the 25 biodiversity hotspots of the world and is the prime one among the two identified in the Indian subcontinent (Sing, 2006). The region also falls in the bio-geographic tri-junction of Indian, the Himalayan and the oriental landmasses. The flora of the North Eastern Region is among the richest in the world and naturally a sizeable number of medicinal plants of tropical and subtropical nature grow here. The North Eastern Region of India that comprises of seven sister states viz. Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, and Tripura along with Sikkim harbors more than 130 major tribal communities of the total of 427 tribal communities found in India (Sajem and Gosai, 2006). The North East India is blessed with nature's bounty in regard to the wealth of industrially potential plants. The people from the region traditionally use various plant species as a home remedy for various ailments. But, proper phytochemical analysis of these plants is yet to be carried out. Modernization of the traditional knowledge and sustainable use of the medicinal plants through biochemical and biotehnological interventions will ensure the economic prosperity of the region. Some

important plant based medications prevalent among the different communities of North Eastern states are given below:

Alternanthera sessilis R. Br. (Matikanduri, family- Amaranthaceae) is useful in diarrhoea and dysentery

Amaranthus spinosus Linn (Hati khutura, family- Amaranthaceae) is used in leprosy, bronchitis and fever.

Andrographis paniculata Burm. f Wall ex. Nees (Kalmegh, family- Acanthaceae) is useful in fever, inflammations, cough and intestinal worms.

Bacopa monieri (L) Wettst (Brahmi, family- Scrophulariaceae) is used as a tonic for nerves.

Centella asiatica (L) Urban (Bormanimuni, family- Umbelliferae) is useful in dysentery, diarrhoea and skin diseases.

Clerodendron colebrookianum Walp (Nephaphu, family- Verbenaceae) leaf is used in high blood pressure

Cajanus cajan (L) Mill (Rohar mah, family- Leguminosae) leaf is consumed to cure jaundice.

Drymaria cordata Willd (Lai jabori, family- Caryophyllaceae) is useful in sinus problems.

Euphorbia hirta Linn. (Dudh bon, family- Euphorbiaceae) is used to cure bronchitis.

Flemingia strobilifera R.Br (Makhioti, family- Fabaceae) is used against dental bleeding.

Jatropa curcas Linn. (Bongali era or bhut era, family- Euphorbiaceae) is used to cure toothache.

Meyna laxiflora Robyns. (Khutura, family- Rubiaceae) is useful for piles and dysentery.

Rauvolfia serpentina (L) Benth ex. Kurg (Sarpagandha, family- Apocynaceae) roots are advocated for high blood pressure.

Stellaria media Linn. (Morolia sak, family- Caryophyllaceae) is useful against enlargement of spleen and ulcers.

Withania somnifera Dunal (Aswagandha, family- Solanaceae) is useful for rheumatism and nervous disorder

1.10. Medicinal flora of Assam

Assam is specially renowned for its herbal treasure. Dependence on traditional herbs has a long history in Assam. The flora of the state is among the richest in the world and a number of classical medicinal plants and other high value plants of tropical and sub-tropical nature grow here. It has been estimated that there are about 3,000 species of plants having medicinal properties to cure and prevent different diseases (Kalita, 2006). The rural areas of the state inhabited by different communities particularly by the tribal people and socio-economically backward communities are the major sources of traditional knowledge about the ethnomedicinal use of the medicinal plants. The village elders as well as 'Kabiraj', 'Ozas' or 'Bezs' have traditionally used these medicinal plants to cure different ailments. They prepare medicines from different parts of medicinal plants. Various communities of Assam have their own traditional plant-based medications for the treatment.

Assam has a geographical area of 78,563 sq. km. with an average annual rainfall of 2,500 mm. The state is divided into six agroclimatic zones viz. North Bank Plains, Upper Brahmaputra Valley, Lower Brahmaputra Valley, Central Brahmaputra Valley,

Barak Valley and Hill Zone. The state has a sizable forest area. This includes reserved forests including national parks, biosphere reserves and sanctuaries, unclassed forests, village forests, private forests and botanical gardens. They harbor very rich species diversity. Although the state is very rich in medicinal plant diversity, the exploration is not up to the mark. The continuous increase in the demand and expanding trade on medicinal and aromatic plants worldwide, have jeopardized the survival of several plant species due to indiscriminate harvesting of natural flora including those in forests. It was estimated that about 90% collection of medicinal plants is from wild source and 70% of collections involve destructive harvesting. As a result of that, many species become extinct and some are endangered. For the sustainable growth in this sector, some necessary steps have to be initiated. Firstly, necessary legislative, legal and administrative legislations have to be made to prohibit the collection of those wild plant species which are rare, threatened, endangered and vulnerable. Secondly, the indigenous knowledge system has to be well documented. The traditional medicinal (herbal) practitioners should be given due recognition, their efforts and activities supported by helping them to tie up with research institutions. Thirdly, biochemistry and biotechnology of wild economic plants be pursued for future need-base commercial access and a chain of botanical gardens be established at different altitudes to conserve and multiply the germplasm of rare and endangered species.

Some of the important medicinal plants of the state are *Andrographis paniculata, Cinchona* spp., *Phyllanthus emblica, Bacopa monnieri, Terminalia arjuna, Ficus religiosa, Saraca asoca, Croton tiglium, Cissus quadrangularis, Colocasia* spp., *Spondias pinnata, Streblus asper, Ocimum sanctum, Cynodon dactylon, Aegle marmelos,*

Xanthyxylum oxifolium, Rubus alceifolius, Meyna spinosa, Ficus benghalensis, Withania somnifera etc. All these plants need scientific validation as well as detailed documentation including molecular parameters.

Spondias pinnata Kurz (Syn. *Spondias mangifera* ; Assamese name - amara) is an important medicinal plant belonging to anacardiaceae family, commonly found in Assam and the adjoining states which has many ethno-medicinal uses. Roots are used in regulating menstruation in womanfolk. The aromatic astringent and refrigerant bark is used in dysentery, diarrhoea, vomiting and muscular rheumatism. Leaves are also aromatic, astringent and used in dysentery; juice extracted from leaves is recommended for local application in otalgia. The ripe fruits are sweet, astringent, cooling, emollient, tonic, constipating and antiscorbutic as well as useful in bilious dyspepsia, diarrhoea, general debility and vitiated conditions of tridosa (Warrier *et al.*, 1996).

Streblus asper Lour (Assamese name – Soura or Siora) is a small evergreen tree of moraceae family, abundantly found throughout the state and adjoining states. This plant is known for its medicinal properties. The decoction of its bark is consumed in fever, dysentery and diarrhoea whereas the root is known to possess antiseptic and astringent properties. The decoction of the leaves of the plant is prescribed in the treatment of high blood pressure, and also for easy painless and quick delivery during labour. The boiled juicy extract of the stem is used against common intestinal worms for large animals. The ability of the tree foliage to remain green during the dry season makes them a potential source of protein and energy supplement to both domestic and wild animals. To avoid the problem of gastrointestinal worms and poor nutrition in grazing animals, fresh leaves of *Streblus asper* is fed at least for seven days, along with other

fodder species. Leaves of the plant are used by traditional fisherman of the state as an antidote against 'flesh decaying disease' of cultivated fishes. Moreover, branch segments of the plant are used as tooth brush and are believed to contain compounds effective against tooth decay. It is reported to be a favoured food of rhinoceros. The plant is distributed in the entire North Eastern region; however, the population is dwindling rapidly with the possibility of being extinct in near future.

1.11. Documentation and identification of medicinal plants

Plants have been used in traditional medicine for several thousand years. The knowledge of medicinal plants has been accumulated in the course of many centuries based on different medicinal systems such as Ayurveda, Unani and Siddha. In India, it is reported that traditional healers use about 2,500 plant species and 100 of them serve as regular sources of medicine (Mutthu *et al.*, 2006). During the last few decades, there has been an increasing interest in the study of medicinal plants and their traditional use in different parts of the world. Documentation of the indigenous knowledge through ethnobotanical studies and molecular characterization is important for the conservation and utilization of biological resources. There are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plants for the treatment of various diseases. Due to poor communication, poverty, ignorance and unavailability of modern health care facilities; most of the people especially tribal and rural mass in the North Eastern Region are still forced to practice traditional medicines for their common ailments. Most of these people form the poorest link in the trade of medicinal plants. A vast knowledge on the use of plants against different illnesses might have accumulated in areas where the use of plants is still of great importance. Traditional knowledge on

medicinal plants and their use by indigenous cultures are not only useful in the conservation of traditions and biodiversity but also for the community healthcare and drug development in the present and future. Apart from the tribal groups, many other forest dwellers and rural people also possess unique knowledge about plants. Due to lack of interest among the younger generation as well as their tendency to migrate to cities for lucrative jobs, this great wealth of knowledge is declining. Right from its beginning, the documentation of traditional knowledge especially on the medicinal uses of plants, has provided many important drugs of modern day. But, information on the use of plants for medicine and molecular parameters present in them from this area of the country is rare. Thus, many important leads to drug discovery may be lost in the absence of proper documentation and identification of the medicinal plants.

1.12. Active compounds in medicinal plants

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. They are usually secondary metabolites, of which at least 12,000 have been isolated, which is less than 10% of the total (Schultes, 1978). In many cases, aromatic substances serve as the component of plant defense mechanism against microorganisms, insects and herbivores. Some, such compounds like terpenoids, give plants their aroma; others (quinones and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavor, such as the terpenoid capsaicin from chili, pepper, and some other herbs and spices used by human beings to season food, yield useful medicinal compounds (Cowan, 1999). Medicinal plants have attracted considerable research attention due to their prospect to use as antimicrobial agents. The natural products derived from medicinal plants have

proven to be the abundant source of biologically active compounds, many of which have been the basis for the development of new pharmaceutical molecules. With respect to infectious diseases, the increasing resistance in many common pathogens to currently used therapeutic agents, such as antibiotics, has led to the renewed interest in the discovery of novel antiinfective compounds from plants and such other sources. As there are approximately 5,00,000 plant species occurring worldwide, of which only 1.0% (5,000) has been phytochemically investigated; thus there is great potential for discovering novel bioactive compounds (Palombo, 2006). However, according to the World Conservation Monitoring Centre under the United Nations Environment Programme, the current extinction rates of plants and animals mean that the world is losing one major drug every two years (Groombridge and Jenkins, 2002). Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as micro-organisms, animals and plants. One of such resources is the folk medicine. Systematic screening of them may result in the discovery of novel effective compounds. The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection- fighting strategies. Contrary to the synthetic drugs, antimicrobial compounds of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases (Janovská *et al.*, 2003).

1.13. Genome size

Genome size of an organism is the amount of nuclear DNA in its unreplicated gametic nucleus, irrespective of the ploidy level or taxon (Singh, 2003). It is measured by

weight or number of base pairs where 1 picogram (pg) equals to 978 megabases (Mb). Hinegardner (1976) described genome size as an important biodiversity character with fundamental significance and wide range of modern biological uses. Till date, genome size of only a fraction of plant species are known (Dolezel and Bartos, 2005). Any breeding program requires information on nuclear DNA content or genome size of the particular plant species. Furthermore, geo-botanical studies may use genome size as an additional parameter for the interpretation of species-specific phenology and the composition of plant communities (Lysak *et al.*, 2000). Interest in the origin, extent and significance of genome size variation has increased greatly in the last decade. Nuclear DNA amount and genome size (C-value) are important biodiversity characters, whose study provides a strong unifying element in biology with practical and predictive uses. Variation in DNA amount between species begins with changes within species, yet intraspecific variation remains one of the most controversial topics in the study of plant genome size. Dolezel and Bartos (2005) observed that previous estimations of DNA peak ratios obtained in four different laboratories for otherwise identical species pairs differed between 1.8 and 15.6%. They opined that absolute DNA amounts of a given plant species determined in different laboratories should be compared with caution and in no way should the differences be interpreted in terms of intraspecific variation in genome size. It is clear that genome size impacts on other areas of research and its knowledge can be important when framing questions or planning research. DNA fingerprinting and quantitative genetics are two such examples where knowledge of genome size may be important. Microsatellites are used widely for DNA fingerprinting in population genetic studies analysing population structure, gene flow, genetic diversity etc and yet their

successful analysis has been shown in part to be determined by genome size. There exists a highly significant positive correlation between genome size and the successful amplification of microsatellites (Garner, 2002). The use of a related DNA fingerprinting technique, amplified fragment length polypmorphisms (AFLPs), is similarly affected by the genome size. The knowledge of genome size and ploidy level generated on the basis of AFLP analyses on plant species ranging in C-value from 0.2 to 32.25 pg are important for determining what protocol is most likely to yield informative data for population genetic analyses (Fay *et al.*, 2005). Genome size has now been recognized to be potentially important in the field of quantitative genetics, which aims to analyse and understand the genetic basis of characters showing continuous variation. A simple and less expensive method on genome size determination was developed by Konwar *et al.* (2007)

Objectives

Spondias pinnata Kurz. and *Streblus asper* Lour are two of the important medicinal plants of the entire North East India including the state of Assam. Proper phytochemical analysis and documentation of knowledge on these two plant species may lead to their effective use, conservation and improvement.

On the basis of the above facts, the present investigation has been proposed with the following objectives:

1. To extract and identify major bioactive compounds present in *Spondias pinnata* Kurz. (*Amara*) and *Streblus asper* Lour. (*Soura*).
2. To assess the antimicrobial activity of the bioactive compounds under *in vitro* condition and their structural elucidation.
3. To determine the genome size of the plants.

Chapter II

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

2.1. General

Looking for healing powers by human beings in plants is an ancient idea. According to Thomson (1978) the Neanderthals living 60,000 years ago in present-day Iraq used plants such as hollyhock; these plants are still widely used in ethnomedicine around the world. Schultes (1978) while studying the works of Hippocrates (in the late fifth century B.C.) reported that he mentioned the use of 300 to 400 medicinal plants for the maintenance of good health. In the first century A.D., Dioscorides wrote *De Materia Medica*, a medicinal plant catalog, which became the prototype for modern pharmacopoeias. The Bible offers description of approximately 30 healing plants. In the Indian sub-continent, the oldest medical system 'Ayurveda' has reported 2000 medicinal plant species. According to Prajapati (2003) the Charak Samhita, an-age-old written document on herbal therapy, reports production and indigenous uses of 340 herbal drugs.

2.2. Studies on medicinal plants in N. E. India

The North Eastern Region harbours more than 130 tribal communities out of 427 found in India (Sajem and Gosai, 2006). In general, the tribes of North East India have been categorized into two broad groups: Khasi and Jaintia tribes of Meghalaya, who belong to 'Monkhemar' culture of Austric dialect and the rest of the tribal groups are basically Mongoloid, who belongs to Tibeto-Burman subfamily of Tibeto-Chinese group.

Sajem and Gosai (2006) studied the traditional use of medicinal plants by the Jaintia tribes in North Cachar Hills district of Assam. Altogether, 30 types of ailments have been reported to be cured by using 39 medicinal plant species. For curing diverse

form of ailments, the use of aboveground plant parts was higher (76.59%) than the underground parts (23.41%). Of the aboveground parts, leaf was used in majority of cases (23 species), followed by fruit (4). Different underground parts such as root, tuber, rhizome, bulb and pseudo-bulb were also found to be in use by the Jaintia tribe as medicine. Begum and Nath (2000) studied the medicinal plants used for skin diseases and related problems in North Eastern India. According to them, out of 275 plant species, 224 are used for treatment of specific human ailments such as allergies, burns, cuts and wounds, inflammation, leprosy, leucoderma, scabies, smallpox and sexually transmitted diseases. Some of the plant species, including *Artemisia nilagirica* (Cl) Pamp., *Calotropis gigantea* (L) R. Br., *Cannabis sativa* L., *Cassia alata* L., *C. fistula* L., *Centella asiatica* L., *Cyclea peltata* Hk., *Datura metal* L., *Drymaria cordata* (L.) Willd. ex Roam & Schult., *Jatropha curcus* L., *Litsea cubeba* Pers., *Mimosa pudica* L., *Plantago major* L. and *Plumeria acutifolia* Ait are used for disease treatments by different ethnic groups. Temjenmongla and Yadav (2005) studied the anticestodal efficacy of folklore-based medicinal plants of Naga tribes in North East India. They found that the leaves of *Psidium guajava*, *Houttuynia cordata* and stalk of *Lasia spinosa* possess a profound anticestodal efficacy as evident by the mean mortality time of *R. echinobothrida* which ranged from 1 to 3.66 h, following exposure to 5 - 40 mg/ml concentration of these plant extracts. Moderate activity was recorded for the leaves of *Clerodendrum colebrookianum*, *Lasia spinosa* and *Centella asiatica*, while *Curcuma longa*, *Cinnamomum cassia*, *Gynura angulosa, Lasia spinosa* (stem) and *Aloe vera* revealed a negligible degree of anticestodal activity. Das *et al.* (2006) studied the medicinal plants of North Kamrup district of Assam used in primary healthcare system. They found that out of 31 medicinal plants, 8 are used

in stomach disorder, 4 in body pain, 3 in piles, 2 in skin disease, 2 in ulcer and remaining in dysurea, boils, nervous affection, spermatorrhoea, jaundice, toothache, hydrophobia, sinusitis, asthmatic trouble and obstetrics problem. Borah *et al.* (2006) studied the traditional medicine in the treatment of gastrointestinal diseases in the Upper Assam.The results revealed use of 38 plant species represented by 36 genera and 29 families for the treatment of various gastrointestinal diseases. Das *et al.* (2005) reported the use of *Jatropha curcus* Lin. for the treatment of dysmenorrhoea by the Koch-Rajbongshi tribe in Nalbari district of Assam. Borah *et al.* (2001) highlighted the use of 25 medicinal plants by the rural and tribal communities of Darrang district of Assam. They reported that *Spondias pinnata* (syn. *S. mangifera*) fruit and bark was used to cure dysentery.

Works on important medicinal and aromatic plants of North East India has been initiated in the department of Molecular biology and Biotechnology, Tezpur University, Assam. Chowdhury and Konwar (2006) isolated a flavoury compound '1-methoxy-4-(1-propenyl)-benzene' from 'Karphul', an important medicinal plant of Assam. Chowdhury and Konwar (2006) carried out the morphophenological and karyotype study of *Schumannianthus dichotmus* (Roxb) Gagnep, synonym *Clinogyne dichotoma* Salisb (Patidoi), a traditional plant of Assam. They reported that the plant is a perennial shrub with petiolated leaves which flower during May-June. They further revealed that the species is diploid with 2n=20 (where n=x=10). Buragohain and Konwar (2006) studied the antimicrobial potentiality of two fractions obtained from crude methanolic extracts of fruits of *Meyna spinosa* Roxb. Ex Link, a potential medicinal plant of North East India. They reported antimicrobial activity of both fractions against *Staphylococcus aureus, Escherichia coli* and *Candida albicans.*

2.3. Medicinal plants and antimicrobial activity

In the past few decades, the search for new anti-infection agents has been a major thrust area of research in the field of ethnopharmacology. Various compounds belonging to various chemical groups viz. phenolic compounds, quinones, flavones, tannin, terpenes and alkaloids have been identified as antimicrobial agents. Some of these compounds were isolated by bio-guided isolation after detecting their antimicrobial activity.

Phenolic compounds are important for their wide range of antimicrobial properties. Caffeic acid, catechol and pyrogallol are examples of antimicrobial agents belonging to this group. Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive. In addition to providing a source of stable free radicals, quinones are known to form complexes irreversibly with nucleophilic amino acids in proteins (Stern *et al.*, 1996), often leading to inactivation of the protein and the loss of function. Flavones are phenolic structures containing one carbonyl group. Since they are known to be synthesized by plants in response to microbial infection (Dixon *et al.*, 1983), it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. The activity is probably due to their ability to form complexes with extracellular, soluble proteins and bacterial cell wall, as described above for quinones. Catechins, a flavonoid group compounds, deserve special mention. Tea was reported to have antimicrobial activity (Toda *et al.*, 1989) and that they contain a mixture of catechin compounds. "Tannin" is a general descriptive name for a group of polymeric phenolic substances and they are found in almost every plant part: bark, wood, leaves, fruits, and roots. They are divided into two groups, hydrolyzable and condensed tannins. Scalbert (1991) reviewed the antimicrobial

properties of tannins and observed that tannins can be toxic to filamentous fungi, yeasts, and bacteria. Terpenes occur as diterpenes, triterpenes, and tetraterpenes, as well as hemiterpenes and sesquiterpenes. When the compounds contain additional elements, usually oxygen, they are termed terpenoids. Terpenenes or terpenoids are active against bacteria (Ahmed *et al.,* 1993), fungi (Harrigan *et al.,* 1993), viruses (Fujioka and Kashiwada.,1994), and protozoa (Ghoshal *et al.,* 1996). The triterpenoid betulinic acid is just one of several terpenoids reported to inhibit HIV. Heterocyclic nitrogen compounds are called alkaloids. While alkaloids have been found to have microbiocidal effects, the major antidiarrheal effect is probably due to their effects on transit time in the small intestine. Berberine is an important representative of the alkaloid. It is potentially effective against trypanosomes (Freiburghaus *et al.,* 1996) and plasmodia (Omulokoli *et al.,* 1997).

Scientific analysis of plant components follows a logical pathway. Plants are collected either randomly or by following leads supplied by local healers in geographical areas where the plants are found. Initial screening of plants for possible antimicrobial activities typically begin by using crude aqueous or alcohol extractions and can be followed by various organic extraction methods. Almost all the identified aromatic or saturated organic components from plants active against microorganisms are usually obtained through initial ethanol or methanol extraction. Two most commonly used screening methods to determine antimicrobial susceptibility are dilution assay (Ayafor *et al.* 1994) and disc or agar well diffusion assay (Navarro, *et al.* 1996).

2.4. Morphophenological study of *Spondias pinnata*

Warrier *et al.*, (1996) reported that *Spondias pinnata* was a medium sized deciduous tree of 27 m height and 2.5 m girth, with compound leaves crowded at branches

and the bark was thick, greyish brown which cracked longitudinally. Kanjilal and Bor (1997) reported that *Spondias pinnata* was a middle size tree with brown or grey bark and with horizental wrinkles, leaflets were 22-33 cm long and 3-10 cm broad, entire and petioles were 0.5 cm long. They further observed that flowers were polygamous, scented, subsessile in spreading terminal panicles.

2.5. Phytochemistry of *Spondias pinnata*

Tandon and Rastogi (1976) studied the chemical constituents of *Spondias pinnata.* They isolated several compounds from aerial parts of *Spondias pinnata.* The ethanolic extract of the plant material was fractionated into hexane, chloroform and aquous phases over alumina and silica gel respectively and subsequent chromatographic purification yielded 24-methylenecycloartanone, stigma-4-en-3-one, β-sitosterol, lignoceric acid and β-sitosterol-β-D-glucoside. Sing and Saxena (1976) isolated beta-amyrin and oleonolic acid from its fruits.

2.6. Antimicrobial activity of *Spondias pinnata*

Babu *et al.* (2002) studied the antibacterial activity of fifty eight plants mentioned in the traditional systems of medicine as sources of aseptic agents. Fifteen of them were found to possess antibacterial activity, out of which nine showed broad-spectrum activity, by disc diffussion method. Both crude and acetone extracts of *Spondias pinnata* were active against all the test organisms. The crude extract was found to be more active than the acetone extract.

2.7. Morphophenological study of *Streblus asper*

Warrier *et al.,* (1996) reported *Streblus asper* to be a small rigid evergreen tree up to 15 m height with milky white latex, light grey bark, leaves simple, alternate, flower

diocious, male heads globose, female flowers solitary or 2-4 together and fruits one seeded berry. Kanjilal and Bor (1997) reported that *Streblus asper* Lour. was a small rigid evergreen tree, with milky juice, bark greyish white, warty and wrinkled, 0.16 inch thick, leaves alternate, acute, moreor less serrate, lateral nerves 4-6 on either half. They also reported male flowers to be in globose pedunculate heads and female flowers in solitary, pedunculate and the perianth embracing ovary. The fruit was berry, one seeded globose and yellow when ripe.

2.8. Phytochemistry of *Streblus asper*

Fernandes *et al.* (1961) reported an α β-unsaurated lactone from the root bark of *Streblus asper.* This plant is a rich source of cardiac glycosides. Khare *et al.* (1962 a and b) and Manzetti and Reichstein (1964 a and b) isolated more than twenty glycosides from the root bark of *Streblus asper* and fifteen of them were structurally characterized mainly applying degradative techniques. Some of them are kamloside, asperoside, strebloside, indroside, cannodimemoside, strophalloside, strophanolloside, 16-O-acetyl glucogitomethoside, glucogitomethoside, glucokamloside, sarmethoside and glucostrebloside. Barua *et al.* (1968) isolated α-amyrin acetate, lupeol acetate, β-sitosterol, α-amyrin, lupeol and diol from the stem bark of this plant. Mukherjee and Roy (1983) isolated β-sitosterol, amyrin and lupeol from the root bark and leaves. Chaturvedi and Saxena (1984) reported the presence of other glycosides in the root viz. β-sitosterol-3-O- β-D-arabinofuranosyl-O- α-L-rhamnopyranosyl-O- β-D glucopyranoside.

Chaturvedi and Saxena (1985) isolated a new saponin from the plant. The methanol soluble part of the rectified spirit extract of roots yielded a saponin which was homogenous on TLC and identified as lupanol-3-O-β-D-glucopyranosyl-(1-5)-O- β-D-

xylofuranoside. Saxena and Chaturvedi (1985) reported the isolation of vijaloside from its roots. Fiebig *et al.* (1985) isolated some other compounds from the stem bark including strebloside and mansonin. Chawla *et al.* (1990) on the other hand isolated N-tricontane, β-sitosterol, stigmasterol, betulin and oleanolic acid from the aerial parts. Prakash *et al.* (1992) reported the presence of a pregnane glycoside named as sioraside in the roots of the plant. Column chromatography of a chloroform-ethanol (2:1) extract of dried roots of *Streblus asper* gave a novel substance named sioraside.

Phutdhawong *et al.* (2004) obtained 0.005% yield of volatile oil as a brown liquid from the fresh leaves of *Streblus asper.* The major constituents of the volatile oil were phytol (45.1%), caryophyllene (4.9%) and trans-trans-α-farnesin (2.0%). The other constituents were α-copaene, β-elemene, geranyl acetone, germacrene, δ-cadinene, caryophyllene oxide and 8-heptadecane.

Yazid *et al* (1999) studied the proteolysis of milk and casein fractions by *Streblus asper* leaf extract with the objective of using enzymes from higher plants in cheese making. Whole milk (12.5 per cent total solids) and casein fractions (1 per cent w/v) were reacted with *Streblus asper* leaf extract, maxiren and rennilase, separately. Their electrophoretic profiles were determined and compared. Close similarity in electrophoretic profile of milk coagulum and whey were observed for the milk treated with *Streblus asper* extract and maxiren. Casein-loss studies showed that casein protein retained in coagulum after proteolysis of 1.21, 2.26 and 3.15 % for rennilase, *Streblus asper* extract and maxiren, respectively. Results of the proteolytic and casein-loss studies showed that *Streblus asper* extract was comparable to rennilase which was used

commercially in cheese making. They concluded that *Streblus asper* extract was therefore potentially useful in cheese making.

2.9. Cardiotonic activity of *Streblus asper*

Fernandes *et al.* (1961) reported that the total ethanolic extract of the root bark of *Streblus asper* indicated interesting activity on blood pressure, isolated frog heart, isolated rabbit intestine and guinea pig uterus. Intravenous administereing of α-β-unsaturated lactone isolated from the plant gave an LD_{50} dose of 4.8 mg kg^{-1} in the case of white mice. Studies on isolated frog heart showed that it induces a positive ionotropic effect in 10^{-5} dilution and a systolic response in 10^{-4} dilution. *In vitro* pronounced and spasmodic effect of the compound was observed on the smooth muscles of the rabbit intestine and guinea pig uterus in those high dilutions. Pharmacological studies carried out have indicated that the drug has got definite action on myocardium.

2.10. Antifilarial activity of *Streblus asper*

A preliminary study of *Streblus asper* as an antilymphoedematous agent was carried out by Baranwal *et al.* (1978). The effect of aqueous and alcoholic extracts of *Streblus asper* was studied by Nazneen *et al.* (1989) on the spontaneous movements of the whole worm and nerve-muscle of *Setaria cervi*, the bovine filarial parasite, and also on the survival of microfilariae *in vitro*. *Setaria cervi* is a cosmopolitan nematode of cattle, used to assess the efficacy of potential antifilarial agents, and it bears close similarity in response to drug to that of human filarial worms. Aqueous as well as alcoholic extracts caused inhibition of the spontaneous motility of the whole worm and the nerve muscle preparation of *Setaria cervi* characterized by decreased tone, amplitude and rate of contractions. The concentration required to inhibit movements of the nerve-

muscle preparation was 1/25 for the aqueous and 1/160 for the alcoholic extracts suggesting a cuticular permeability barrrier. The stimulatory response of acetylcholine was blocked by the alcoholic and not by the aqueous extract of *Streblus asper*. Both alcoholic and aqueous extracts caused death of microfilariae *in vitro*, LC_{50} and LC_{90} being 90 and 33.5 ng ml^{-1}, respectively.

Chatterjee *et al.* (1992) reported that the crude aqueous extract of the stem bark of *Streblus asper* showed macrofilaricidal activity against *Litomosoides carinii* and *Brugia malayi* in rodents. The study revealed two cardiac glycosides, asperoside and strebloside, of the extract to be responsible for antifilarial activity. Of the two glycosides, the more effective macrofilaricide was asperoside which was active at 50 mg kg^{-1} orally against *L. carinii* in cotton rats (>90%), *B. malayi* in mastomys (>70%) and *Acanthocheilonema viteae* in mastomys natalensis (>70%). The glycosides were also active *in vitro* against all three filarial species. Significantly weak activity was detected in glycon and aglycon portions of the parent glycosides (asperoside and strebloside). Several cardiac glycosides of other origins did not show any comparable antifilarial efficacy. The aglycosidic portion of the extract, however, showed poor adulticidal activity (44.5% activity at 1 g kg^{-1} against *L. carinii*). *Streblus asper* has been used in the preparation of a few formulations also.

Pandey and Das (1990) reported that Shakhotaka Ghana Vati prepared from its stem bark was found to be useful in filariasis. The *in vitro* effects of asperoside and strebloside on *Setaria cervi* females were also studied by Singh *et al.* (1994). They found that both asperoside and strebloside caused death of the worms within 2–3 h at concentrations of 10 g ml^{-1} (1.7 pmol) and were found to inhibit motility and glucose

uptake of the parasites at lower concentrations (0.1 g ml^{-1}; 0.17 pmol). These glycosides also inhibited the incorporation of [U-14] C-glucose into macromolecules of *Setaria cervi* females. Parasites pre-incubated with either asperoside or strebloside had lowered profiles of glucokinase (EC 2.7.1.2), malate dehydrogenase (EC 1.1.1.37) and succinate dehydrogenase (EC 1.3.99.1) activities, suggesting the lethal effects of the glycosides were due to the glucose metabolism.

Singh *et al.* (1998) found that asperoside and strebloside interfere with the glutathione metabolism of the adult *Setaria cervi*, which caused disturbances in various vital activities of the parasites that ultimately result in the death of the parasites.

Hashmi and Singh (2002) reported a safe and effective filaricide from the stem bark of *Streblus asper* named 'Filacid'. A series of extraneous investigations involving hundreds of patients infested with filarial parasites have also established its efficacy against filariasis.

2.11. Anticancer Activity of *Streblus asper*

Fiebig *et al.* (1985) reported that two cytotoxic cardiac glycosides, strebloside and mansonin, isolated from *Streblus asper* displayed significant activity in KB cell culture system with ED_{50} values of 0.035 and 0.042 μg ml^{-1}, respectively. An isolate is considered to be active in a system if it shows an ED_{50} of 4μg ml^{-1}.

Rastogi and Dhawan (1990) also showed that *Streblus asper* possess anticancer activity. KB cytotoxicity was found to be concentrated sequentially in the methanol and dichloromethane extracts of *S. asper* stem bark.

Phutdhawong *et al.* (2004) reported that the volatile oil from fresh leaves of *Streblus asper* showed significant anticancer activity (ED_{50} <<30 μg ml^{-1}) on cytotoxicity

primary screening tests with P388 (mouse lymphocytic leukemia) cells but no significant antioxidant activity (IC_{50} values >>100 μg ml^{-1}) in a DPPH radical scavenging assay.

2.12. Antimicrobial Activity of *Streblus asper*

Different studies were carried out to determine the antimicrobial potential of leaf extracts of *Streblus asper*.

2.12.1. For oral hygiene

Studies conducted by Triratana and Thaweboon (1987) demonstrated antimicrobial activity of *Streblus asper* leaf extracts on various microorganisms involving oral and nasopharyngeal infections, especially *Streptococcus mutans*. The ethanol extract from the sticks and leaves of *Streblus asper* inhibited the growth of *Streptococcus mutans*.

Taweechaisupapong *et al.* (2000) carried out an *in vivo* one group time series design and single blind study to determine the antimicrobial effectiveness of a mouth-rinse containing *Streblus asper* leaf extract on *Streptococcus mutans* and total salivary bacteria following a single sixty second rinse. Change in salivary pH and buffer capacity during the experimental period was also measured. Thirty human subjects participated in the study. At each experimental session, a pre-test saliva sample was taken. After giving the pre-test samples, the subjects rinsed with *Streblus asper* leaf extract or distilled water control for sixty seconds, then the post-test saliva samples were collected at 0, 0.5, 1, 3, 5 and 6 h. The samples were used to determine the numbers of *Streptococcus mutans* and total salivary bacteria. The results indicated that *Streblus asper*-leaf extract significantly reduced *Streptococcus mutans* count as compared to distilled water rinse. However, the mean difference from the baseline of total salivary bacterial count was not significantly

different between rinsing with *Streblus asper* leaf extract and distilled water. Moreover, *Streblus asper* leaf extract showed no effect in modifying the salivary pH and buffer capacity. From the study, it was concluded that the mouth-rinse containing *Streblus asper* leaf extract could reduce *Streptococcus mutans* without changing an oral ecology.

Wongkham *et al.* (2001) reported that bactericidal activity was found in the 50% ethanol (v/v) extract of *Streblus asper* leaves. The extract possessed a selective bactericidal activity towards *Streptococcus*, especially to *Streptococcus mutans*, which is strongly associated with the dental caries. The extract had no effect on cultures of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus coagulase* positive, *Staphylococcus coagulase negative, Serratia marcescens, Klebsiella pneumoniae, Enterobacter, P. aeruginosa, Burkholderia pseudomeallei* and *Candida albicans*. The minimum growth inhibitory concentration and the minimum bactericidal concentration of *Streblus asper* extract against 10^8 CFU per ml of *Streptococcus mutans* was 2 mg ml^{-1}.

Taweechaisupapong *et al.* (2002) carried out a single blind and crossover design study to determine the effect of the mouth-rinse containing *Streblus asper* leaf extract on gingivitis and plaque formation. The results revealed that when used in mouth-rinse, the *Streblus asper* leaf extract significantly effected the gingival health only. It reduced the gingival index but no significant effect on the plaque growth.

Limsong *et al.* (2004) investigated the inhibitory effect of *Streblus asper* extract solution at 0.5% concentration (w/v) on adherence of *Streptococcus mutans* on the glass surface. However, it did not show significant inhibitory effect on bacterial adherence to glass surfaces.

Taweechaisupapong *et al.* (2005a) carried out *in vitro* study to determine the effects of a sublethal concentration of *Streblus asper* leaf ethanolic extract on adherence of *Candida albicans* to human buccal epithelial cells (HBEC). The minimum concentration of *Streblus asper* leaf ethanolic extract (SAE) that significantly reduced adherence ($P<0.05$) after a 'one hour exposure' was 15.6 mg/ml. However, there was a significant reduction ($P<0.05$) of candidal adhesion to HBEC after one-minute exposure to 125 mg/ml of SAE. Pre-treatment of either *Candida* or HBEC, or both, with 125 mg/ml of SAE for one hour resulted in reduced adherence. SAE at concentrations of 125 and 25 mg/ml also showed 41 and 61 per cent inhibition of germ tube formation, respectively, which might affect adherence. These findings indicated that the sublethal concentration of this extract might modulate candidal colonization of the oral mucosa thereby suppressing the invasive potential of the pathogen.

2.12.2. Against anaerobic bacteria

In vitro study was also carried out by Taweechaisupapong *et al.* (2005b) to determine the antibacterial effect of the leaf extract of *Streblus asper* against the following six anaerobic bacteria: *Porphyromonas gingivalis* W50, *Prevotella intermedia, Actinomyces naeslundii* (T14V), *Peptostreptococcus micros, Actinobacillus actinomycetemcomitans* ATCC 43717 and ATCC 43718. It was demonstrated that 15 µl of the leaf extract at 250 and 500 mg ml^{-1} had inhibitory effect towards all bacterial strains tested except *Actinobacillus actinomycetemcomitans* ATCC 43717. The extract had no bactericidal activity against *Prevotella intermedia* and *Actinomyces naeslundii* (T14V). Although the extract did not show inhibitory effect towards *Actinobacillus actinomycetemcomitans* ATCC 43717 in the disc diffusion method, but it did inhibit

growth of *Actinobacillus actinomycetemcomitans* ATCC 43717 in the broth microdilution method.

2.13. Anti-allergic activity of *Streblus asper*

Amarnath *et al.* (2002) reported that the leaf extract of *Streblus asper* showed promising anti-allergic activity in experimental models. Anti-PCA (passive cutaneous anaphylaxis) and mast cell stabilizing activity of *Streblus asper* were investigated in mice and rats. Disodium cromoglycate (DSCG) was used as the standard anti-allergic drug. *Streblus asper* (50–100 mg kg^{-1}, p.o.) in mice showed 60–74% anti-PCA activity. In rats it showed dose-dependent (50–200 mg kg^{-1}, p.o.) anti-PCA activity (56–85%). The mast cell stabilizing activity in rats (10 mg kg^{-1}, p.o. x 4 days) showed 62% protection against compound 48/80 induced degranulation. In egg albumin induced degranulation sensitized rats there was a 67% protection with *Streblus asper*. These results were comparable with that of DSCG (50 mg kg^{-1}; i.p.).

2.14. Insecticidal activity of *Streblus asper*

Atal (1969) demonstrated the presence of the insecticidal effect in the stem extract of *Streblus asper*. Hashim and Devi (2003) reported that the extract from the stem bark of *Streblus asper* possesed insecticidal activity against the fifth instar of *Dysdercus cingulatus*. The methanolic extract showed an LC50 value of 5.56 µg per insect. Partition with chloroform increased the insecticidal activity (LC_{50} 2.01 µg per insect). Three polyphenolic rich fractions were obtained from silica gel column chromatography of the chloroform fraction and found to have noteworthy insecticidal activity (LC_{50}: 1.82, 2.70 and 2.26 µg per insect) by topical application. This might provide a useful beginning for the development of biopesticides.

2.15. Antiparasitic activity of *Streblus asper*

Das and Beuria (1991) studied the antimalarial property of the stem bark extract of *Streblus asper* on murine malaria. The stem bark extract of *Streblus asper* intra-peritoneally stimulated a host immune response against *Plasmodium berghei* in mice.

2.16. DNA isolation

Medicinal and aromatic plants contain exceptionally high amounts of secondary metabolites that interfere with DNA isolation. To address this problem, many methods were developed over the years. Peterson *et al.* (1997) developed a protocol for the isolation of milligram quantities of highly purified DNA from tomato nuclei. The protocol utilized fresh seedlings or leaves without freezing and used Triton X-100 as the detergent.

Khanuja *et al.* (1999) reported a rapid DNA isolation protocol that could be used for diverse medicinal and aromatic plants, which produced essential oils and secondary metabolites such as alkaloids, flavonoids, phenols, gummy polysaccharides, terpenes and quinones. Their protocol was essentially a CTAB based protocol, which produced fairly good yields of DNA and was equally effective in the isolation of DNA from dry as well as fresh plant tissues. The isolated DNA proved amenable to PCR amplification and restriction digestion. Pirttila *et al.* (2001) developed two DNA isolation methods for *Drosera rotundifolia* and *Artemisia dracunculus* that produced DNA suitable for molecular biological applications. Michiels *et al.* (2003) developed a CTAB protocol for the isolation of genomic DNA from latex containing plants. They used etiolated leaf tissue for the extraction and carried out isopropanol precipitation at 25^0C overnight.

Baranwal *et al.* (2003) found that the addition of sodium sulphite to the extraction buffer improved the yield, quality and stability of plant DNA.

2.17 Genome size of plants

The term C-value was coined by Swift (1950), referring to the DNA content of an unreplicated haploid chromosome complement (n). Hinegardner (1976) described genome size as an important biodiversity character with fundamental significance and wide range of modern biological uses.

Bennett and Smith (1976) reported that the genome size varies among the species at least up to about 800-fold (*Fritillaria assyriaca*, 1C = 127.4 pg *vs. Arabidopsis thaliana* 1C = 0.165 pg). The genome size varies greatly between and even among species. The intra-specific variation in genome size is a particularly interesting field of research and is currently receiving much attention.

According to Bennett and Leitch (1995), the genome size of only about 1.7% of the global angiosperm flora has been investigated. Gregory and Hebert (1999) interpreted genome size and C-value of an organism as equivalent and defined it as the content of DNA (measured by weight or number of base pairs) in a single copy of the entire sequence of DNA found within a nucleus of that organism.

Singh (2003) defined genome size of an organism as the amount of nuclear DNA in its unreplicated gametic nucleus, irrespective of the ploidy level or taxon. It is measured by weight or number of base pairs where 1 picogram (pg) equals to 978 megabases (Mb). According to Bennett and Leitch (2005) nuclear DNA amount and genome size (C-value) are important biodiversity characters, whose study rovides a strong unifying element in biology with practical and predictive uses.

Dolezel and Bartos (2005) reported that as the sample preparation and analysis is convenient and rapid, DNA flow cytometry has become a popular method for ploidy screening, cell cycle analysis and estimation of absolute DNA amount or genome size. To estimate nuclear DNA content, nuclei are stained with a DNA specific fluorochrome and the amount of light emitted by each nucleus is quantified. The result of the analysis is displayed in the form of a histogram of relative fluorescence intensity, representing relative DNA content. As flow cytometry analyses relative fluorescence intensity, and hence relative DNA content, the genome size of an unknown sample may be determined only after a comparison with nuclei of a reference standard, whose genome size is known. They further observed that previous estimations of DNA peak ratios obtained in four different laboratories for otherwise identical species differed between 1.8 and 15.6%. They opined that absolute DNA amounts of a given plant species determined in different laboratories should be compared with caution and in no way should the difference be interpreted in terms of intraspecific variation in genome size.

Dolezel *et al.* (1998) estimated the 2C DNA content of pea to be 9.09 pg and it was most popularly used as the reference standard. Srisawat *et al.* (2005) estimated the nuclear DNA content of oil palm (*Elaeis guineensis* Jacq) by flow cytometry using different external reference plant species. They found the 2C nuclear DNA content of oil palm to be 4.72 pg while using *Zea mays* as the external reference standard.

Konwar *et al.* (2007) determined the genome size of medicinal plants viz. *Rubus alceifolius* Poir, *Meyna spinosa* Roxb. Ex. Link, *Zanthoxylum oxyphyllum* Edgew, *Spondias pinnata* Kurz and *Streblus asper* Lour by using a microscope based simple and less expensive technique.

Chapter III

Materials and Methods

CHAPTER III

MATERIAL AND METHODS

3.1. Mophophenological study

3.1.1. Mophophenological study of *Spondias pinnata* Kurz. and *Streblus asper* Lour.

Morphophenological study is a prerequisite for proper identification and systematic analysis of plants. Five fully grown (mature) plants of each of *Spondias pinnata* and *Streblus asper* were selected randomly for studying their morphological, flowering and fruit characters. The plants were under constant observation for studying morphological as well as phenological characters. The important morphophenological characters such as plant height, girth of the plant; leaf length, breath, area; petiole length; flower color, shape, size and bark thickness-colour etc were studied and recorded in the field as well as in the laboratory. The phenological data such as time of flowering and fruiting were studied and recorded at weekly intervals. The observations were repeated in all selected five plants and recorded.

Morphophenological characters of the selected plants were measured and recorded as per the procedure described below:

Morphological characters

Plant height – Height of the plant was measured in centimeter (cm) from the ground level to the base of the petiole of the top most leaf. For this a long rope was stretched from the top leaf of the top most branch to the base of the stem and the length measured.

Plant girth – It was measured in cm at 1m height of the stem from the ground level.

Leaf length– It is the length of the leaf blade from the base to the tip of the leaf. It was measured in cm with a Portable Laser area meter (Model-CI-203, make CID, Inc, USA).

Leaf breadth– It is the width of a leaf at the middle position of the leaf blade in cm. It was measured with a Portable Laser area meter (Model-CI-203, make CID, Inc, USA).

Leaf area- It is the area of the leaf in cm^2 and was measured with a Portable Laser area meter (Model-CI-203, make CID, Inc, USA).

Petiole length- It was measured from the stem to the base of leaf blade in cm.

Phenological characters

The phenological characters studied includes, inflorescence type and flower characters. The selected plants were monitored throughout the flowering season to study these characters. The inflorescence and flowers were collected and studied in the laboratory.

Flowering time- The flowering time was recorded from the initiation to end of flowering.

Flowers per inflorescence-The number of flowers per panicle as well as per male inflorescence (globose head) was counted and recorded.

Length of inflorescence- The length of panicle and also of the male inflorescence (globose head) was measured in cm.

Peduncle length – It is the stalk of a flower cluster or of a solitary flower. The length was measured in cm from the attachment point with stem to the top of the peduncle on which the flowers are aggregated.

Calyx- The number of sepals in each calyx was recorded.

Corolla- The number of petals in each corolla was recorded.

Flower length- The length of flower was measured in cm from the base of the flower to the top.

Flower diameter- The diameter of a flower was measured in cm.

Fruit characters

Period of fruiting- Period of fruiting was recorded from the onset of fruiting to end.

Fruit length- Fruit length was measured by making longitudinal cross section of fruits and recorded in cm.

Fruit diameter- Fruit diameter of both *S. pinnata* and *S. asper* was measured by making horizontal cross section of fruits and recorded in cm.

Fruit colour- Fruit colour was recorded at the ripening stage.

Seed weight- Individual seed weight was recorded in gram with an electronic balance (Sartorius, Model-BP121S).

From the recorded data for each character mean and standard deviation were calculated.

3.1.2. Herbarium preparation

Stem specimens with leaves, inflorescence and flowers were collected, dried and mounted neatly using narrow strips of gummed paper on a herbarium sheet of 25 x 43 cm size. The herbarium sheet was covered with a polythene sheet to avoid damages. The herbaria were prepared for both the specimen plants. A label was glued at the bottom right corner of each sheet. The herbaria *Spondias pinnata* Kurz. (MBBT/0205) and *Streblus asper* Lour. (MBBT/0206) were deposited in the Department of Molecular Biology and Biotechnology, Tezpur University, Napaam.

3.2. Biochemical analysis

3.2.1. Extraction and isolation of active compound from *Spondias pinnata* fruits

3.2.1.1. Plant material

Mature fruits of the plant were collected from plants growing in Tezpur University Campus, Napaam, Tezpur, Assam and authenticated. The collected fruits were washed thoroughly with tap water to remove the dirt and then shade dried. The edible fleshy portion of the fruits was peeled off, air-dried and ground into a fine powder using a mixer grinder (REMI, Auto-Mix-Blender).

3.2.1.2. Extraction of the compound

The protocol described by You *et al.* (2003) was used for the extraction of active compound from the fruit powder. The powdered fruit weighing 500 g was extracted twice with 2.5 litre of methanol for 3 days twice with occasional stirring. The methanolic extracts were combined and concentrated in vacuum to give a syrup which was suspended in water and partitioned in petroleum ether, ethyl acetate, chloroform and butanol successively in the increasing order of polarity. The solvent extracts obtained were concentrated to dryness and weighed. Each extract was tested for antimicrobial activity. The detailed extraction protocol from fruit powder to fractionation by column and thin layer chromatography is presented in Fig. 1.

3.2.1.3. Column chromatography

The chloroform extract having antimicrobial activity was chromatographed on a silica gel (60-120 mesh, E. Merk) column (40 mm internal diameter) using chloroform-

methanol (9:1 to 1:9) as eluents to afford two fractions viz. fraction 1 (2.0 g) and fraction 2 (1.4 g).

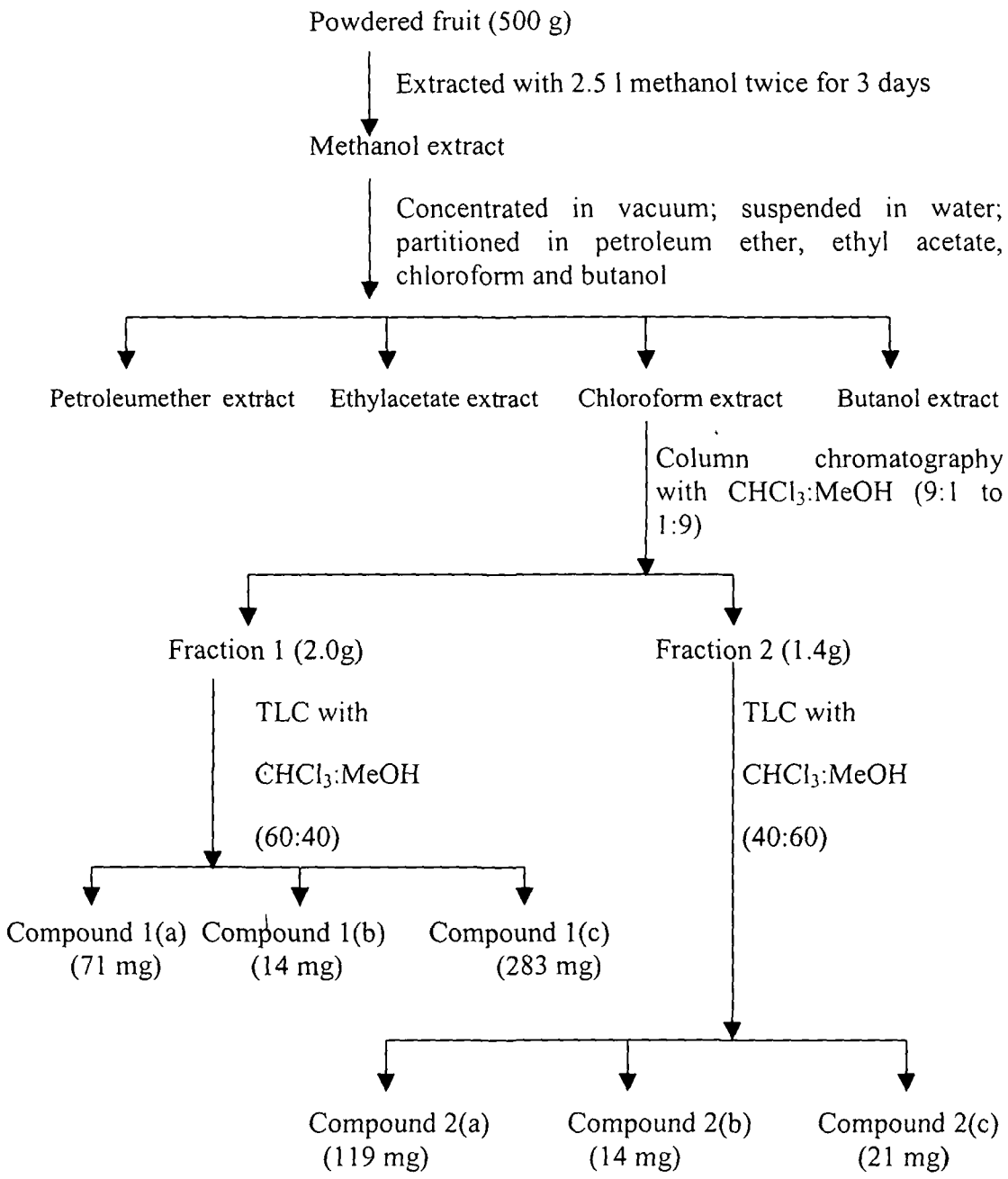


Fig.1 Schematic presentation of the extraction procedure of active compounds from *Spondias pinnata* Kurz.

3.2.1.4. Thin layer chromatography

The fraction 1 was subjected to thin layer chromatography in small plates (size: 5 X 20 cm) prepared with silica (Silica gel G, E. Merck) using chloroform-methanol (60:40) as the solvent system. This fractionation afforded three pure compounds viz. 1(a), 1(b) and 1(c). Then the fraction 2 was subjected to thin layer chromatography using chloroform-methanol (40:60) as the solvent system. This afforded three pure compounds viz. 2(a), 2(b) and 2(c). Spots on TLC plates were detected under the iodine vapour. Based on the TLC experiment, the preparative TLC was performed using the same solvent system in big TLC plates (size: 20 X 20 cm). The separated pure compounds by the TLC were assessed for their antimicrobial activity. Compounds having antimicrobial activity were subjected to IR spectroscopy, ^{1}HNMR spectroscopy, ^{13}CNMR spectroscopy and HR-FAB mass spectroscopy for the determination of molecular structure.

3.2.2. Extraction and identification of compounds from *Streblus asper* stem bark

3.2.2.1. Plant material

The bark of the stem was collected from the plants growing in Tezpur University Campus, Napaam, Tezpur, Assam and authenticated. The bark was cut into small pieces, washed thoroughly with tap water, air dried and ground in to a fine powder using a Willy Mill.

3.2.2.2. Extraction of the compound

The organic extracts were prepared from the stem bark-powder using the protocol of You *et al.* (2003). The powdered stem bark weighing 500 g was extracted twice with 2.5 litre of methanol for 3 days with occasional stirring. The two extracts were combined and concentrated in vacuum to give a syrup which was suspended in water and

partitioned in petroleum ether, ethyl acetate, chloroform and butanol successively in the increasing order of polarity. The different solvent extracts were concentrated to dryness and weighed. Each extract was assayed for antimicrobial activity. The detailed extraction protocol from the stem bark powder to the fraction by column and thin layer chromatography (TLC) is presented in Fig. 2.

3.2.2.3. Column chromatography

The petroleum ether extract having antimicrobial activity was chromatographed on a silica gel (60-120 mesh, E. Merck) column (40 mm internal diameter) using petroleum ether-ethyl acetate (9:1 to 1:9) as the eluent to afford two fractions (Fraction1 and 2).

3.2.2.4. Thin layer chromatography

The fraction 1 (1.0 g) was subjected to thin layer chromatography in small plates (size: 5 X 20 cm) prepared with silica (Silica gel G, E. Merck) using petroleum ether-ethyl acetate (60:40) as the solvent system to afford three pure compounds viz. 1(a), 1(b) and 1(c). The second fraction yielding 1.4 g was subjected to thin layer chromatography using petroleum ether:ethyl acetate (60:40) as the solvent system to afford two pure compounds viz. 2(a) and 2(b). Compound specific spots were detected under the iodine vapour. Based on the TLC experiment, the preparative TLC was performed with the same solvent system of petroleum ether-ethyl acetate (60:40) in big TLC plates (size: 20 X 20 cm). The compounds separated were then assessed for their antimicrobial activity and those possessing antimicrobial activity were subjected to IR spectroscopy, ^{1}HNMR spectroscopy, ^{13}CNMR spectroscopy and HR-FAB mass spectroscopy for the determination of molecular structure.

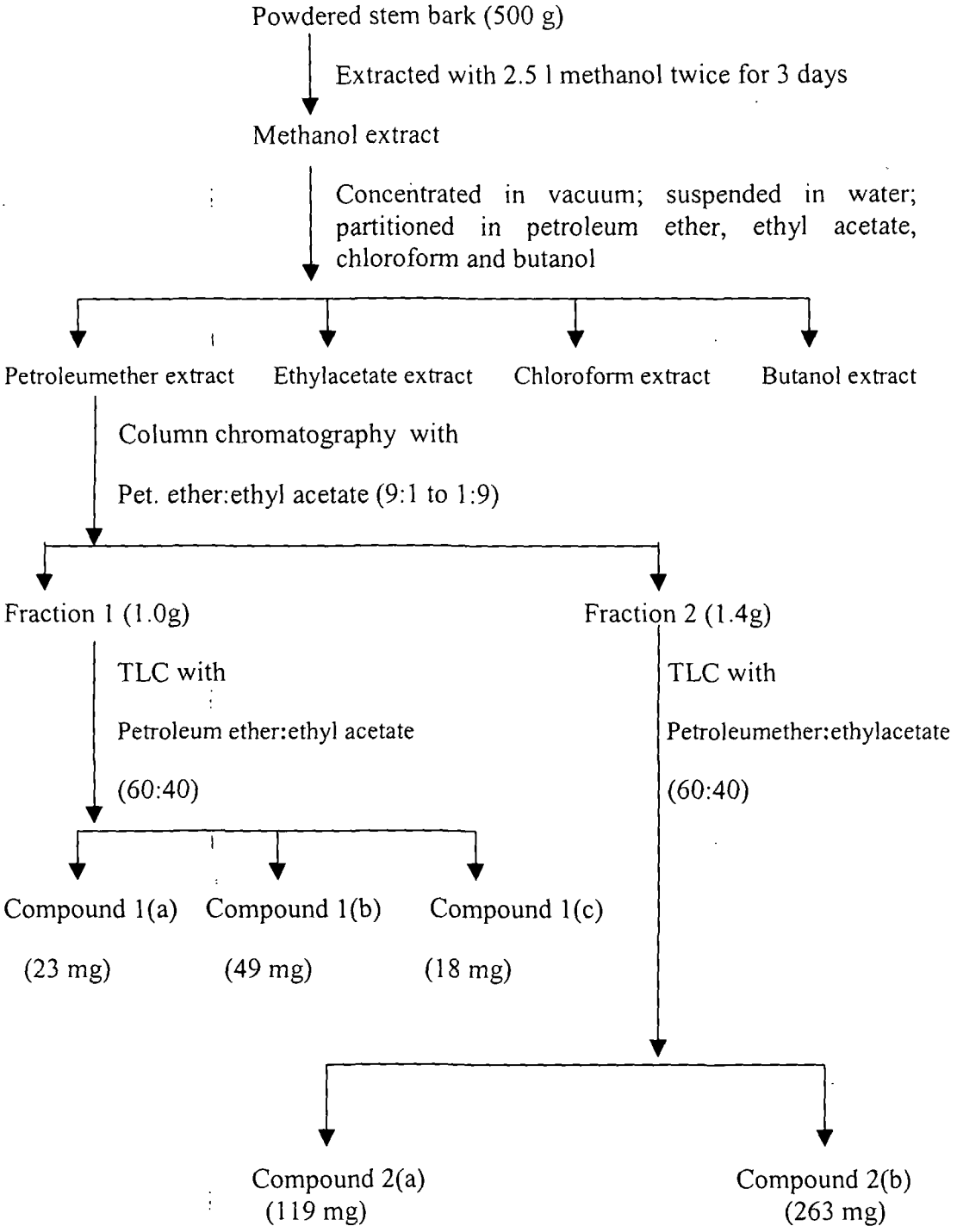


Fig.2 Schematic presentation of the extraction procedure of active compounds from *Streblus asper* Lour.

3.2.3. Structural elucidation of active compounds

For the identification and elucidation of molecular structure of the active compounds, different spectroscopic analysis of the compounds were carried out.

3.2.3.1. IR spectroscopy

The IR spectroscopy of the compounds was carried out using the following procedure.

1. Weighed amounts of sample (about 5 mg) and KBr powder (about 300mg) were transferred into an agate mortar. The KBr powder was of spectroscopic grade purity and spectroscopically dry.
2. Both sample and KBr powder were ground together with the agate mortar and pestle until the sample is well dispersed and the mixture possessing the consistency of a fine flour.
3. The KBr disk was transferred to a spectrometer disk holder. Enough care was taken to keep the disk translucent with the homogenous distribution of the sample in the disk.
4. The disk holder was mounted in the spectrometer. The IR spectrometer was a Nicolet Model Impact 410 FTIR spectrometer.
5. The IR spectra of the samples were collected using 500-4000 wave number (cm^{-1}).

3.2.3.2. ^{1}H-NMR spectroscopy

Sample preparation is a very important part of NMR experiment. If the sample is not prepared properly, the resulting NMR spectrum may be of poor quality. It is important to make sure that the NMR tubes are not scratched, cracked or chipped, and of

sufficiently good quality. The ^{1}H-NMR spectroscopy of the compounds was done with the following procedure.

1. About 8 mg of the sample was dissolved properly in a small amount of $CDCl_3$ solvent.
2. The NMR tube was filled up to a height of 5 cm with the sample and deuterated chloroform. Tetramethylsilane (TMS) was used as the internal standard.
3. The tube was then fitted with the spinner, the tube and the spinner were placed into the depth gauge to get the correct height. The spectrometer was Varian Mercury Plus-400 NMR spectrometer with a ^{1}H NMR frequency of 400 MHz.
4. After log on to the computer with VNMR software, the sample was loaded, locked and shimmed on. The purpose of the lock was to maintain the magnetic field at a constant value throughout the experiment. The purpose of shimming was to make the magnetic field homogeneous over the entire volume of the sample so that the lines in the NMR spectrum would be as narrow as possible.
5. Then the ^{1}H NMR spectrum was acquired.

3.2.3.3. ^{13}C-NMR spectroscopy

The ^{13}C-NMR spectroscopy of the compounds was carried out using the following procedure:

1. Good quality NMR tubes were selected.
2. About 25 mg of the sample was dissolved properly in a small amount of $CDCl_3$ solvent.
3. The NMR tube was filled up to a height of 5 cm with the sample and a deuterated chloroform. Tetramethylsilane (TMS) was used as the internal standard.

4. The tube was then fitted with the spinner, tube and spinner were placed into the depth gauge to obtain the correct height. The spectrometer was Varian Mercury Plus-400 NMR spectrometer with a ^{13}C NMR frequency of 100 MHz.
5. After log on to the computer with VNMR software, the sample was loaded, locked and shimmed on. The purpose of the lock was to maintain the magnetic field at a constant value throughout the experiment. The purpose of shimming was to make the magnetic field homogeneous over the entire volume of the sample so that lines in the NMR spectrum would be as narrow as possible.
6. Then the ^{13}C NMR spectrum was acquired.

3.2.3.4. HR-FAB mass spectroscopy

Fast-atom bombardment is a soft ionization method. Ionization of the sample is effected by bombarding a liquid solution of the analyte with fast atoms of argon. Small amount (8 mg) of the sample was dissolved in a liquid matrix of m-nitrobenzyl alcohol (NBA). The role of the FAB matrix was to absorb the energy of the impacting primary particles, to act as a solvent for the analyte, to refresh the surface of the drop and to assist ionization, e.g. by acting as a proton donor/acceptor or electron donor/acceptor. A drop of the sample/matrix mixture was placed at the end of an insertion probe and introduced to the source region. The fast atom beam was focused on the droplet to produce the analyte ions. FABMS was performed on a JEOL SX 102/D-6000 mass spectrometer, operated at 10 kV accelerating voltage, equipped with a Jeol MSFAB 10 D FAB gun operated at a 10 mA emission current, producing a beam of 6 kV argon atoms. The samples were measured over a mass range of *m:z* 10–1000. The spectra were recorded at the room temperature.

3.2.4. Nutritional analysis

Mature fruits of *Spondias pinnata* were collected from plants, the edible fleshy portion was peeled off, air-dried and ground into a fine powder and used for nutritional analysis. The nutritional parameters were also studied in the leaf of *Streblus asper*. Leaves of the plant were collected, air-dried and ground into a fine powder and used for nutritional analysis. The different nutritional parameters investigated were moisture content, crude protein, fat, starch, reducing sugar, crude fibre, ash, phosphorus, iron, calcium and potassium.

3.2.4.1. Moisture

The moisture content was determined by following AOAC (1970) method. For this 10 g of the powdered sample was accurately weighed in aluminum moisture boxes and dried in an oven at 100^0C ($\pm$ 2^0C) for 16 h, cooled in a dessicator and weighed. The experiment was conducted three times and the mean was recorded. The moisture content was determined using the following equation:

$$MC\ (\%) = \frac{Iw - Fw}{Sw} \times 100$$

Where,

MC- moisture content

Iw- Initial weight

Fw- Final weight

Sw- Weight of the sample

3.2.4.2. Crude protein

Crude protein was determined by micro-kjaldahl method (AOAC, 1965). The total nitrogen content was estimated and multiplied by 6.25 to arrive at the value of the crude protein. Estimations were repeated thrice and the average was recorded with standard deviation.

3.2.4.3. Fat

The fat content was estimated by the method of Sadasivam and Manickam (1996). The fat was extracted in soxhlet apparatus with petroleum ether as the solvent, then dried, weighed and the per cent fat was calculated. The estimation was repeated thrice and the average was recorded with the standard deviation.

3.2.4.4. Starch

Starch content was determined by the method of Chopra and Kanwar (1976). The sample was treated with 80 % ethanol to remove sugars. The starch content was extracted with perchloric acid and the estimation was done by measuring the intensity of the green colour when reacted with anthrone reagent. The glucose content was multiplied by a factor of 0.9 to arrive at the starch content. The estimation was repeated thrice and the average was recorded with the standard deviation.

3.2.4.5. Reducing sugar

Reducing sugar content was determined by using the method of Sadasivam and Manickam (1996). It was estimated by the dinitrosalicylic acid method. The reducing sugar present in the sample was calculated by using the standard graph. Estimation was repeated thrice and the average was recorded with the standard deviation.

3.2.4.6. Crude fibre

Crude fibre was estimated by using the method of Sadasivam and Manickam (1996). Crude fibre consists largely of cellulose and lignin along with some mineral components. In acid and subsequent alkali treatments, oxidative hydrolytic degradation of the native cellulose occurs which further leads to the degradation of lignins. The residue obtained after final filtration was weighed, incinerated, cooled and weighed. The loss in weight gave the crude fibre content. Estimation was repeated thrice and the average was recorded with the standard deviation.

3.2.4.7. Ash

Ash content was determined by following the method of AOAC (1970). For this analysis, powdered sample 5 g was taken in a silica crucible, charred in low Bunsen flame and finally ignited at 600^0C for 6 h in a muffle furnace.

$$\text{Ash content (\%)} = \frac{Aw}{Sw} \times 100$$

Where,

AC- Ash content

Aw- Weight of ash

Sw- Weight of the sample

Estimation was repeated thrice and the average was recorded with the standard deviation.

3.2.4.8. Preparation of mineral solution

The mineral solution was prepared according to the method of AOAC (1970). For this, ash was dissolved in HCl (1:1) and the solution was evaporated to dryness by putting in a water bath at 100^0C. Subsequently, 4 ml HCl with 2 ml distilled water was added,

warmed, the acid soluble portion separated with filtration, and the volume was made up to 100 ml by adding distilled water in a volumetric flask.

3.2.4.9. Phosphorus

Phosphorous was estimated colorimetrically by the method of Fiske and Subharow (1925). Orthophosphate and phosphate ion present in the sample react with ammonium molybdate in acidic solution to give phosphomolybdic acid, which upon reduction with hydroquinone produces a blue coloured complex exhibiting absorption at 660 nm. The phosphorus content was calculated from the standard curve prepared with the known concentrations of phosphorus. Estimations was repeated thrice and the average was recorded with the standard deviation.

3.2.4.10. Iron

Iron content was estimated by spectrophotometric method of Wong (1925). The reagent used was potassium thiocyanate. The ferric ion when reacted with potassium thiocyanate produced a blood red colour, which was measured at 540 nm. The iron content was calculated from the standard curve prepared with known concentrations of iron. Estimation was repeated thrice and the average was recorded with the standard deviation.

3.2.4.11. Calcium

Calcium was determined using a flame-photometer (Systronics ; model -MK III). Solutions with known concentrations of calcium viz. 50, 100 and 200 ppm were prepared and placed under the Nebuliser and the intensity of emitted light was measured. The readings were taken in a digital read out and a standard curve prepared. The sample solution was similarly placed under the Nebuliser and the reading was taken. The calcium

content in the sample was calculated from the standard curve. Estimation was repeated thrice and the average was recorded with the standard deviation.

3.2.4.12. Potassium

Potassium was determined using a flame-photometer (Systronics ; model -MK III). Solutions with known concentrations of potassium viz. 5, 10 and 20 ppm were prepared and placed under the Nebuliser and the intensity of emitted lights was measured. The data were collected in a digital mode and a standard curve was prepared. The sample solution was placed under the Nebuliser and reading was taken. The potassium content in the sample was calculated from the standard curve. Estimation was repeated thrice and the average was recorded with the standard deviation.

3.3. Determination of antimicrobial activity

The extracts obtained from each of the plant species were subjected to antimicrobial test. The active solvent extract was used for the isolation of compounds and each compound was tested for its ability to limit the microbial growth in culture medium.

3.3.1 Test microorganisms

The test organisms used in the study included two 'gram positive' bacteria viz. *Bacillus subtilis* (MTCC 619), *Staphylococcus aureus* (MTCC 737); two 'gram-negative' bacteria viz. *Escherichia coli* (MTCC 739), *Klebsiella pneumoniae* (MTCC 109) and one fungal pathogen *Candida albicans* (MTCC 3017). All were obtained from the MTCC, IMTECH, Chandigarh, India.

3.3.2 Media used

The bacterial test pathogens were maintained on Nutrient Agar (NA) medium. The composition of the medium is given below:

Composition of the Nutrient Agar Medium

Component	Concentration (g/l)
Peptone	10.0
Beef extract	10.0
Sodium chloride	5.0
Agar	12.0

pH = 7.3 ± 0.1

The fungal pathogen *C. albicans* was grown on M2 Agar Base medium. The composition of the M2 Agar Base Medium is given below:

Composition of the M2 Agar Base Medium

Component	Concentration (g/l)
Sodium chloride	10.0
Yeast extract	5.0
Monopotassium phosphate	0.1
Magnesium sulphate	0.05
Dextrose	10.0
Agar	15.0

pH = 7.1 ± 0.2

For antimicrobial susceptibility test, Mullar Hinton Agar (MHA) Medium was used. The composition of Mullar Hinton Agar Medium is given below:

Composition of the Mullar Hinton Agar Medium

Component	Concentration (g/l)
Beef infusion	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0

pH = 7.3 ± 0.1

All chemicals were procured from Hi-Media Private Limited, Mumbai, India.

3.3.3 Evaluation of antimicrobial activity

The antimicrobial activity of the extracts and each of the separated compounds was determined by the agar well diffusion method (Grammer, 1976). Spore or cell suspensions of the pathogens were prepared in Ringer's solution (sterile saline water) and adjusted to a final inoculum size of 3×10^5 cells/ml. One ml of the inoculum was added to 500 ml media at 45°C for each of bacterial and fungal strains. The suspension was mixed thoroughly and 20 ml of it was poured in to a sterile petriplate. After solidification of media, wells of 6 mm in diameter were cut in the agar plates. The compound weighing 20 mg was dissolved in 20 ml of dimethyl sulfoxide (DMSO) and then 200 µl of it was loaded into each well on the respective petriplate having bacterial and fungal test organisms. DMSO (200 µl) as solvent blank was added to each petriplate. After loading the samples into respective wells, the petriplates were kept for 60 minutes under the laminar hood to allow the test solution to diffuse in to the agar medium prior to their incubation. The plates were incubated at 37°C in the dark for 24 h for the growth of bacterial and 48 h for fungal pathogens. After the period, the diameter of the inhibition

zones around the wells was measured. Each experiment was repeated thrice for the confirmation.

3.4 Molecular characterization

3.4.1 Isolation of the genomic DNA

Isolation of genomic DNA, which is pure and suitable for restriction digestion and PCR amplification, is important for the application of molecular marker technologies to medicinal plants for their improvement. Several methods of genomic DNA isolation from medicinal plants were reported. Due to the presence of different secondary metabolites in differential quantities in different medicinal plants, these methods need to be modified to suit each medicinal plant species. Therefore, standardization of DNA isolation method is necessary for each plant species.

3.4.1.1. The plant material

For the isolation of genomic DNA of *Spondias pinnata* Kurz. and *Streblus asper* Lour, tender leaves were collected from the plants, washed thoroughly with distilled water. The excess water adhering to the leaves was soaked off using tissue paper. The leaves were then packed in polythene bags and stored in darkness at room temperature for the subsequent DNA extraction work.

3.4.1.2. Equipment used

Autoclave	High speed refrigerated centrifuge
Pestle and Mortar	Tabletop centrifuge
Electronic weighing balance	Magnetic stirrer
Micropipettes	Thermostatic water bath
Deep freezer,	Ultra violet (UV) light box

Refrigerator	Freeze-drier
Polypropelene tube	Gel Doc system
Oven	Incubator (37°C)
Gel apparatus	UV- spectrophotometer

3.4.1.3. Reagents, chemicals and solutions

Stock solutions

(a) 1M Tris-HCl (pH 8.0) –Tris base 121.1 g was dissolved in 800 ml of dH_2O. The pH was adjusted to 8.0 by adding concentrated HCl. The solution was allowed to cool to room temperature. The volume was adjusted to 1 liter and sterilized by autoclaving. The solution was stored at room temperature.

(b) 0.5 M EDTA (pH 8.0) –$Na_2EDTA.2H_2O$ 186.1 g was dissolved in 700 ml of dH_2O. The pH was adjusted to 8.0 with 10 M NaOH (~ 50 ml). The volume was adjusted to 1.0 litre and sterilized by autoclaving. The solution was stored at the room temperature.

(c) 5.0 M NaCl - NaCl 292 g was added to 900 ml of dH_2O. the volume was adjusted to 1.0 litre.

(d) CTAB (20%) –CTAB 20.0 g was dissolved in 100 ml of dH_2O.

Other reagents

β-mercaptoethanol

Chloroform: Isoamyl alcohol (24:1 v/v) mixture

Polyvinylpyrolidone (PVP)

Liquid Nitrogen

Loading dye (Bromophenol blue)

Ethidium bromide

Isopropanol

RNase

Agarose

Hind III digested λ DNA molecular weight marker

3.4.1.4. Preparation of CTAB extraction buffer

For 27.0 ml CTAB extraction buffer

Required concentration of chemicals	Amount of Stock solution/chemicals added
100 mM Tris-HCl (pH 8.0)	2.7 ml of 1 M Tris-HCl (pH 8.0)
25 mM EDTA	1.35 ml of 0.5 M EDTA (pH 8.0)
1.5 M NaCl	8.1 ml of 5.0 M NaCl
2.5% CTAB	3.375 ml of 20% CTAB
0.2% B-mercaptoethanol (v/v)	54 μl added immediately before use
PVP	300 mg of PVP (100 mg/g leaf sample) added immediately before use

The volume was adjusted to 27.0 ml by adding sterile dH_2O and stored at the room temperature.

3.4.1.5. Preparation of High salt TE buffer

For 100 ml High salt TE buffer

Required concentration of chemicals	Amount of Stock solution added
1 M NaCl	20.0 ml of 5 M NaCl
10 mM Tris-HCl (pH 8.0)	1.0 ml of 1M Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)	200 μ l of 0.5 M EDTA (pH 8.0)

The volume was adjusted to 100 ml by adding sterile dH_2O and stored at the room temperature.

3.4.1.6. Preparation of 50 X TAE electrophoresis buffer (Tris Acetate EDTA)

For 100 ml 50 X TAE stock solution

Required concentration of chemicals	Amount of chemicals added
0.5 M EDTA (pH 8.0)	3.7 g ($Na_2EDTA.2H_2O$)
Tris base	24.2 g
Glacial acetic acid	5.71 ml

The volume was adjusted to 100 ml by adding sterile dH_2O and stored at the room temperature.

3.4.1.7. Preparation of loading dye (Bromophenol blue)

6 X Loading dye (for 4.0 ml):

Chemicals	Amount
Bromophenol blue	10 mg
Xylene cyanol	10 mg
Glycerol (autoclaved)	1.2 ml

The volume was adjusted to 4 ml by adding sterile dH_2O and stored at 4^0C.

3.4.1.8. Preparation of ethidium bromide (10 mg/ml)

Ethidium bromide (100) mg was dissolved in 10 ml sterile dH_2O, mixed properly and then stored at 4^0C in darkness.

3.4.1.9. Preparation of 0.8% agarose gel

For 400 ml stock solution of 1 x TAE

An aliquot of 8.0 ml of 50 X TAE buffer was diluted to 400 ml by adding 392 ml sterile dH_2O to have 400 ml of 1 x TAE

Agarose 400 mg was dissolved in 50 ml of 1 X TAE completely by boiling for a few minutes. Ethidium bromide (10 mg/ml) 2 µl was added in to the gel.

3.4.1.10. DNA extraction protocol

A modified CTAB-based DNA isolation protocol was standardized for both plant species on the basis of the method described by Khanuja *et al.* (1999). The modifications incorporated in the procedure are overnight isopropanol precipitation, two washings with chloroform: isoamyl alcohol (24:1) and 2 h RNase treatment. The modified protocol is given below.

1. Fresh tender leaves weighing 1.0g were taken.

2. The leaves were surface sterilized with 70% ethyl alcohol and then cut in to smaller pieces.
3. The leaf material was ground in to a fine powder in liquid nitrogen using a pair of pre-chilled pestle and mortar.
4. The powdered leaf material was transferred to a pre-chilled polypropylene tube and added 3 ml of freshly prepared pre-warmed (60°C) extraction buffer and mixed gently by several inversions.
5. The sample was incubated at 60°C in a shaking waterbath for 2 h with occasional mixing to avoid reaggregation of the homogenate.
6. To the homogenate, 3 ml of chloroform: isoamyl alcohol (24:1) was added and mixed gently by inversion for 15 minutes.
7. The extract was centrifuged at 10,000 rpm for 10 minutes at 25°C and the clear upper aqueous layer was collected.
8. Added another 3 ml of chloroform: isoamyl alcohol (24:1) to the aqueous phase and mixed gently by inversion for 15 minutes and centrifuged at 10,000 rpm for 10 minutes at 25°C.
9. The upper clear the aqueous phase was taken in a clean polypropelene tube, 1.5 ml of 5 M NaCl added to the aqueous phase and mixed gently.
10. Then added 0.6 volume of isopropanol to the mixture and mixed by inversion. The mixture was allowed to stand at room temperature overnight for the precipitation of DNA.
11. The sample was centrifuged at 10,000 rpm for 10 minutes at 25°C. The supernatant was discarded and the pellet was carefully transferred to a clean microfuge tube and

washed with 80% ethanol.

12. The pellet was dried in a vacuum in a Maxi dry plus, Hoefer Pharmacia Biotech Inc., USA for 15 minutes and dissolved in 0.5 ml of high salt TE buffer.
13. RNase (5 µl) was added to the solution and incubated at 37°C for 2 h.
14. After incubation, the sample was extracted with equal volume of chloroform: isoamyl alcohol (24:1). The aqueous layer was transferred to a fresh 1.5 ml microfuge tube and was added 2 volumes of cold ethanol.
15. The sample was then centrifuged at 10,000 rpm for 10 minutes at 25°C.
16. The pellet was rinsed with 80% ethanol and then dried in vacuum. The pellet was dissolved in 100 µl of TE buffer and then stored at -20°C.

3.4.1.11. DNA quantification and purity test by UV spectroscopy

Quantification of DNA is an important step. Restriction digestion of DNA along with subsequent techniques such as polymerase chain reaction (PCR) and random amplified polymorphic DNA (RAPD) technique, require pure and clean DNA. The purity and the yield of DNA extracted was ascertained by UV-Spectroscopy and also by gel elctrophoresis.

3.4.1.11.1. UV-spectroscopic determination of yield and quality of DNA

The UV absorption of the extracted DNA was measured at the wave length of 260 and 280 nm in a Beckman DU® 530 Life Science UV/Vis Spectrophotometer. Amount of DNA was calculated by using the relationship of soluble standard DNA being O.D.1.0 at 260 nm = 50 µg/ml. The $A_{260\ 280}$ ratio was calculated to check the purity of the isolated DNA. A good DNA preparation exhibits a value in between 1.8 - 2.0.

3.4.1.11.2. Agarose gel electrophoresis

1. An agarose gel of 0.8% was prepared in 1XTAE.
2. Ethidium bromide (10 mg/ml) 2.0 µl was added to the agarose gel.
3. DNA sample (10 µl) was mixed with 2 µl of Bromophenol blue dye. The mixture was then loaded into the wells of the agarose gel along with the standard *Hind* III digested λ DNA molecular weight marker.
4. Electrophoresis was carried out at 60 – 70 V for 90 min.
5. The electrophoresed gel was exposed to UV light using a UV transilluminator for visualization of the DNA. DNA bands seen in the gel were documented by taking photographs with a Gel Doc system (BIO RAD Gel Doc 1000).

3.4.1.12. Restriction digestion of isolated DNA to check the quality of DNA

Purity of the extracted DNA can also be judged from the proper restriction digestion of the DNA by the restriction endonuclease enzymes. To check the purity, the extracted DNA samples were single digested with *Eco*R I, *Hind* III and double digested with *Eco*R I and *Hind* III combined restriction endonuclease enzymes. For the purpose, first 1.5 ml Eppendorf tubes were labelled appropriately for the restriction enzymes and the extracted DNA to be digested. The DNA samples, each measuring 5 µl (3 µg) were aliquoted in to the labelled tubes. The final volume of each of the samples was made to 20 ml and 25 ml for single and double digestions, respectively. The following were the reaction mixtures:

3.4.1.12.1. Digestion with *Eco*R I

Components	Amount
10X buffer for *Eco* RI	2.0 µl
Eco RI enzyme (20 units/µl)	2.0 µl
DNA (0.6µg/µl)	5.0 µl
Sterile distilled water	11.0 µl
Total volume	20.0 µl

3.4.1.12.2. Digestion with *Hind* III

Components	Amount
10X buffer for *Hind* III	2.0 µl
Hind III enzyme (20 units/µl)	2.0 µl
DNA (0.6 µg/µl)	5.0 µl
Sterile distilled water	11.0 µl
Total volume	20.0 µl

3.4.1.12.3. Double digestion with *Eco*R I and *Hind* III

Components	Amount
Buffer B (10X)	4.0 µl
Eco RI enzyme (20 units/µl)	2.0 µl
Hind III enzyme (20 units/µl)	2.0 µl
DNA (0.6 µg/µl)	5.0 µl
Sterile distilled water	12.0 µl
Total volume	25.0 µl

The reaction mixtures were incubated at 37°C for 4 h. After the incubation, 2.5 µl of the loading dye was added and reincubated for another 15 min. After the digestion, the

reaction mixtures were electrophoresed through ethidium bromide stained 0.8% agarose gel along with standard *Hind* III digested λ DNA molecular weight marker. The digested DNA was visualized under the UV-transilluminated gel and then photographed in the Gel Doc system.

3.4.2. Genome size determination of the plant species

3.4.2.1. Genome size determination by flow cytometry

3.4.2.1.1. Plant material

Seeds of the two plant species *Spondias pinnata* Kurz. and *Streblus asper* Lour were collected and seedlings were raised. Leaf tissues of about 2-3 cm^2 were taken for the isolation of nuclei from well defined adult leaves of 60 days old seedlings.

3.4.2.1.2. Preparation of Otto I buffer

Component	Amount
Citric acid monohydrate (0.1 M)	4.2 g
Tween 20 (0.5 %, v/v)	1.0 ml

The volume was adjusted to 200 ml by adding sterile dH_2O, filtered through a 0.22 µm filter and stored at 4^0C.

3.4.2.1.3. Preparation of Otto II buffer

Component	Amount
$Na_2HPO_4.12H_2O$ (0.4 M)	28.65 g

The volume was adjusted to 200 ml by adding sterile dH_2O, filtered through a 0.22 µm filter paper and stored at room temperature.

3.4.2.1.4. Extraction of nuclei

The method described by Otto (1990) was used with slight modification. The modified method is described below.

1. A small mass (20 mg) of the leaf tissue was chopped with a new razor blade in 0.5 ml ice-cold Otto I buffer in a Petri dish.
2. After chopping the mass of leaf tissues another 0.5 ml of ice-cold Otto I buffer was added and mixed well with a pipette.
3. The suspension was filtered through a nylon mesh of 42 μm.
4. It was then incubated for 15 minutes with occasional shaking.
5. Added 2 ml of Otto II buffer followed by propidium iodide (50 μg/ml) and RNase (50 μg/ml) treatment.
6. The relative DNA content of the isolated nuclei was analysed in a Flow Cytometer within 15 minutes of the preparation.

3.4.2.1.5. Flow cytometric analysis

The flow cytometer was a FAC Scan working with a software Cell Quest (Becton Dickinson, San Jose, CA). From laser excitation of propidium iodide at 488 nm, the reflection at 585 nm was measured to read 2C nuclear DNA content of 5,000 nuclei per sample. *Pisum sativum* was used as the external reference standard. During the analysis, after every three samples, the reference plant was used to check the calibration of the flow cytometer by adjusting the gain of *Pisum sativum* to the channel 200. All experiments were carried out at least 3 times.

3.4.2.2. Genome size determination by microscopy method

3.4.2.2.1. Determination of cell volume in leaf sections

Genome size of the plants was also determined by the microscopy method of Konwar *et al.* (2007). Fine transverse and longitudinal cross sections of the pre-weighed leaf tissue of 1 cm^2 size were obtained with a sharp razor blade and observed under a microscope (Leica ATC 2000) at 10 x 40*x* magnification. The volume of the whole tissue section (1 cm^2) was determined by the formulae: length x breath x thickness. The length, breath and thickness of rectangular cells, length and radius of cylindrical cells and radius of spherical cells were measured with a micro scale having 400x magnification. Data generated from five randomly selected cells of five random sections as well as cell volumes were recorded using mathematical calculations. The intercellular space of the leaf tissue of the plant was measured in five small leaf sections of known dimensions by measuring with a microscale at 10 X 40x magnification.

3.4.2.2.2. Genome size determination

The genome size of the plant species was determined with the following formulae:

Average volume of a single cell (l x b x t) = $x\ \mu^3$ where, l= length
b=breath
t=thickness

Volume of the tissue (l x b x t) = $t\ \mu^3$

Volume of the intercellular space = $s\ \mu^3$

Actual cell mass = $(t - s) = v\ \mu^3$

Total number of cells in the cell mass = $v\ \mu^3 / x\ \mu^3 = y$

The weight of the tissue section	= w g
w g tissue contains	= y cells
So, 1 g tissue will contain cells	= y/w
DNA yield per gram of leaf tissue	= d µg
	= d x 10^6 pg
So, one cell contains genomic DNA	= (d x 10^6) / (y/w) pg
	= (d x 10^6) / (y/w) x 978 Mbp

Chapter IV

Results

CHAPTER IV

RESULTS

4.1. Morphophenological characters

4.1.1. *Spondias pinnata*

Morphophenological characters like plant height, plant girth, leaf length, leaf breath, leaf area, petiole length, flowering time, flowers per inflorescence, length of inflorescence, peduncle length, flower length, flower diameter, number of sepals in each calyx, number of petals in each corolla, period of fruiting, fruit length, fruit diameter, fruit colour and seed weight were recorded on five randomly selected plants. The data on morphological characters are presented in Table 1. From the table, it was observed that a plant on maturity attains an average height of 27.4±2.302 m and girth of 2.42±0.449 m. The bark was found to be thick and greyish brown in colour bearing longitudinal cracks. Leaflets were imparipinnate and entire whereas, the apex was pointed. The length of leaf ranged from 16.20±2.084 to 18.70±2.350 and the breath varied in between 6.98±0.923 and 7.92±0.526 cm. The average leaf area ranged from 95.63±4.725 to 108.19±3.375 cm^2. The petiole length varied from 0.72±0.083 to 0.98±0.491 cm.

The phenological data are presented in Table 2. It was observed that flowering started in the month of February and extended till the end of June. The inflorescence was found to be a terminal panicle (Plate 1) with a length variation from 29.80±4.508 to 39.38±2.334 cm. The number of flowers per panicle ranged from 109.0±10.63 to 122.6±5.07. The length of flower ranged from 0.16±0.02 to 0.19±0.02 cm while the diameter of flowers varied from 0.16±0.02 to 0.20±0.05 cm. Flowers were bisexual, self

fertile, scented, inconspicuous and greyish white in colour (plate 1). Each calyx and corolla was found to contain five sepals and five petals, respectively.

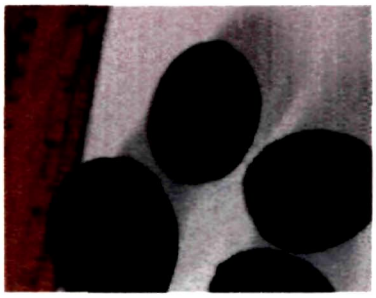
The fruit characters recorded on five randomly selected plants are presented in Table 3. The fruit was observed to be ovoid with length variation from 3.82±0.311 to 4.08±0.277 cm (plate 1) and diameter from 2.28±0.192 to 2.50±0.412 cm. The green fruits turn yellowish at ripening. Fruiting started from the month of July and lasted till the end of December. The fruits were born in terminal clusters of 10-15. The seed weight ranged from 4.225±0.271 to 4.622±0.588 g.

Table 1. Morphological characters of *Spondias pinnata* (mean of 3 replications ± SD)

Plant no	Plant height (m)	Girth of plant (m)	Leaf length (cm)	Leaf breath (cm)	Leaf area (sq. cm)	Petiole length (cm)
1	25.0	2.0	18.70±2.350	7.92±0.526	108.19±3.375	0.92±0.294
2	28.0	2.8	16.20±2.084	6.98±0.923	95.83±4.451	0.84±0.207
3	27.0	2.1	18.00±1.311	7.34±0.646	99.03±4.094	0.78±0.258
4	30.0	2.2	18.10±1.367	7.41±0.746	100.07±4.828	0.72±0.083
5	27.0	3.0	17.70±1.181	7.51±0.630	95.63±4.725	0.98±0.491

Inflorescence

Flowers



Fruits

Plate 1. *Spondias pinnata* inflorescence, flower and fruit.

Table 2. Phenological characters of *Spondias pinnata* (mean of 3 replications ± SD)

Plant no	Length of inflorescence (cm)	Flowers per Inflorescence	Flower length (cm)	Flower diameter (cm)
1	29.80±4.508	119.2±18.74	0.18±0.04	0.19±0.04
2	36.12±4.385	109.0±10.63	0.17±0.02	0.17±0.02
3	38.10±4.526	122.6±5 07	0.16±0.02	0.20±0.05
4	39.38±2.334	115.2±10.84	0.17±0.02	0.16±0.02
5	39.14±1.529	112.8±10.13	0.19±0.02	0.20±0.03

Table 3. Fruit characters of *Spondias pinnata* (mean of 3 replications ± SD)

Plant no	Fruit length (cm)	Fruit diameter (cm)	Seed weight (g)
1	3.86±0.260	2.50±0.412	4.225±0.271
2	4.06±0.270	2.30±0.200	4.230±0.574
3	3.82±0.311	2.28±0.192	4.622±0.588
4	4.08±0.277	2.32±0.216	4.250±0.528
5	3.98±0.178	2.34±0.130	4.388±0.391

4.1.2. *Streblus asper*

Morphophenological characters like plant height, plant girth, leaf length, leaf breath, leaf area, petiole length, flowering time, flowers per inflorescence, length of

inflorescence, diameter of inflorescence, peduncle length, flower length, flower diameter, number of sepals in each calyx, number of petals in each corolla, period of fruiting, fruit diameter, fruit colour and seed weight were recorded on five randomly selected plants. The data on morphological characters are presented in Table 4. From the table, it was observed that a mature plant attains an average height of 7.96±1.533 m and girth of about 0.88±0.216 m with thick foliage. The bark was found to be warty, wrinkled and greyish white in colour. A sticky milky white juice was found to be exuded by the bark on injury. Leaves were alternate, entire, obovate and acute (Plate 2). The length of leaf ranged from 4.70±1.445 to 6.10±1.937 cm and the breath varied in between 2.89±0.387 and 3.46±0.421 cm. The average leaf area ranged from 11.40±1.733 to 15.84±2.683 cm^2. The petiole length varied between 0.34±0.041 to 0.38±0.083 cm.

The phenological data are presented in Table 5. The plant was observed to be dioecious and to flower during the period from February to May. Male flowers were small and tightly aggregated into the axillary inflorescence. The calyx was found to contain four sepals with valvate aestivation. Androcium contained four stamens, oppositesepalous and free of the parianth. Female flowers were solitary or sometimes in clusters of 2-4 and pedunculate (Plate 2). The average peduncle length ranged from 0.50±0.070 to 0.60±0.122 cm. The gynocium was found to be stylate. Young shoots were generally found to bear female flowers. The length of female flowers ranged from 0.68±0.130 to 0.86±0.054 cm and the diameter between 0.42±0.083 and 0.50±0.070 cm. On the other hand, the length of the male inflorescence ranged from 0.96±0.114 to 1.06±0.240 cm and the diameter from 1.08±0.130 to 1.30±0.158 cm.

The fruit characters recorded on five randomly selected plants are presented in Table 6. The fruit was observed to be spherical with diameter varying from 0.21±0.054 to 0.25±0.050 cm. Plants started bearing fruits from the month of May and lasted till September. The green fruits on ripening were observed to turn orange in colour. The seed weight ranged from 0.0344±0.007 to 0.0418±0.005 g.

Table 4. Morphological characters of *Streblus asper* (mean of 3 replications ± SD)

Plant no	Plant height (m)	Plant girth (m)	Leaf length (cm)	Leaf breath (cm)	Leaf area (sq. cm)	Petiole length (cm)
1	6.0	0.7	4.70±1.445	3.02±0.465	11.44±1.022	0.38±0.083
2	7.5	0.7	5.50±0.969	3.05±0.680	12.68±1.030	0.38±0.044
3	8.9	1.0	5.40±0.707	3.32±0.454	13.53±1.532	0.36±0.054
4	7.4	0.8	6.10±1.937	3.46±0.421	15.84±2.683	0.37±0.054
5	10.0	1.2	5.00±1.708	2.89±0.387	11.40±1.733	0.34±0.041

Table 5. Phenological characters of *Streblus asper* (mean of 3 replications ± SD)

Plant no	Peduncle length (cm)	Length of female flower (cm)	Diameter of female flower (cm)	Length of male inflorescence (cm)	Diameter of male inflorescence (cm)
1	0.60±0.070	0.68±0.130	0.42±0.083	1.0±0.158	1.30±0.158
2	0.56±0134	0.78±0.130	0.44±0.114	1.02±0.178	1.08±0.130
3	0.60±0.122	0.68±0.148	0.48±0.083	1.00±0.158	1.22±0.148
4	0.55±0.130	0.86±0.054	0.46±0.089	1.06±0.240	1.26±0.114
5	0.50±0.070	0.74±0.089	0.50±0.070	0.96±0.114	1.16±0.114

A branch with the inflorescence of male flowers

A branch with individual female flowers

Plate 2. *Streblus asper* branch with male and female flower.

Table 6. Fruit characters of *Streblus asper* (mean of 3 replications ± SD)

Plant no	Fruit diameter (cm)	Seed weight (g)
1	0.22±0.057	0.0374±0.008
2	0.23±0.044	0.0388±0.003
3	0.25±0.050	0.0418±0.005
4	0.24±0.065	0.0344±0.007
5	0.21±0.054	0.0364±0.009

4.2. Nutritional analysis

4.2.1. Nutraceutical characteristics of *Spondias pinnata* fruit

Data recorded on the fruit characters including the moisture content are presented in Table 7.

Table 7. Neutraceutical constituents of *Spondias pinnata* fruit (mean of 3 replications ± SD)

Cohstituents	Content (on dry weight basis)
Moisture (%)	76.62±1.785
Crude protein (%)	3.336±0.195
Fat content (%)	1.027±0.009
Starch (mg g^{-1})	54.09±0.850
Reducing sugar (mg g^{-1})	69.56±1.060
Ash content (mg g^{-1})	6.239±0.186
Crude fibre (mg g^{-1})	23.07±0.780
Phosphorus (mg. g^{-1})	0.483±0.032
Iron (mg g^{-1})	0.043±0.005
Calcium (mg g^{-1})	5.967±0.472
Potassium (mg g^{-1})	83.60±1.520

4.2.2. Nutraceutical characters of *Streblus asper* leaf

The nutritional composition of leaves of the plant has been presented in Table 8.

Table 8. Nutritional constituents of *Streblus asper* leaf (mean of 3 replications± SD)

Constituents	Content (on dry weight basis)
Moisture (%)	75.64±1.820
Crude protein (%)	16.73±0.079
Fat content (%)	1.029±0.029
Starch (mg. g^{-1})	12.05±0.710
Reducing sugar (mg. g^{-1})	1.15±0.060
Ash content (mg. g^{-1})	8.113±0.256
Crude fibre (mg. g^{-1})	17.08±0.120
Phosphorus (mg. g^{-1})	0.236±0.015
Iron (mg. g^{-1})	0.040±0.010
Calcium (mg. g^{-1})	14.33±0.577
Potassium (mg. g^{-1})	33.46±0.611

4.3. Isolation and characterization of active compounds

4.3.1. Isolation of active compounds from *Spondias pinnata*

4.3.1.1. Crude extraction

The methanolic extract of fruits of *Spondias pinnata* being concentrated in vacuum to give a syrup, was suspended in water and partitioned in petroleum ether, ethyl acetate, chloroform and butanol successively. The solvent extracts obtained were concentrated into dryness and weighed (Table 9). The fractions with their yield are presented in Table 9.

Table 9. Yield of different solvent extracts from fruits of *Spondias pinnata*

Extract	Yield (g)
Petroleum ether	7.0
Ethyl acetate	10.0
Chloroform	17.0
Butanol	8.0

4.3.1.2. Determination of antimicrobial activity of the crude extracts isolated from *Spondias pinnata* fruits

All four extracts (Table 9) were subjected to antimicrobial activity test and data obtained are presented in Table 10. The chloroform-extract showed antimicrobial activity against all the test organisms except *Klebsiella pneumoniae.* The other extracts did not show any activity.

Table 10. Antimicrobial activity of the crude extracts of *Spondias pinnata* fruits

Extracts	Microorganisms				
	B.subtilis	*E. coli*	*S. aureus*	*K. pneumoniae*	*C. albicans*
Petroleum ether	-	-	-	-	-
Ethylacetate	-	-	-	-	-
Chloroform	+	+	+	-	+
Butanol	-	-	-	-	-

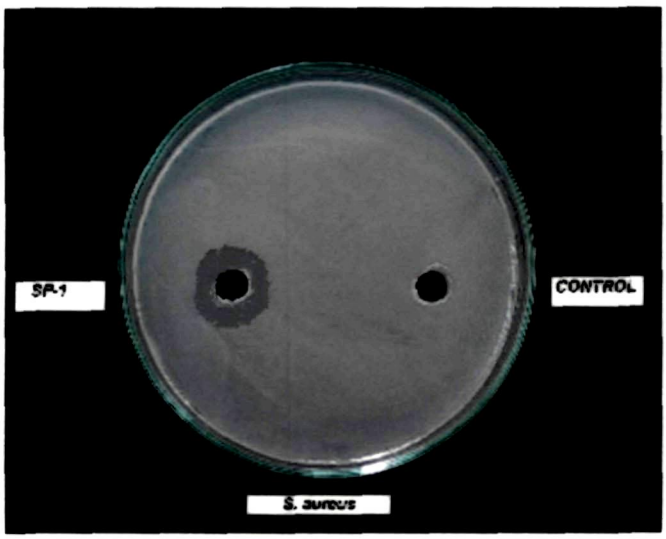
'+' activity present and '–' activity abscent

4.3.1.3. Purification of active compounds from the crude extract

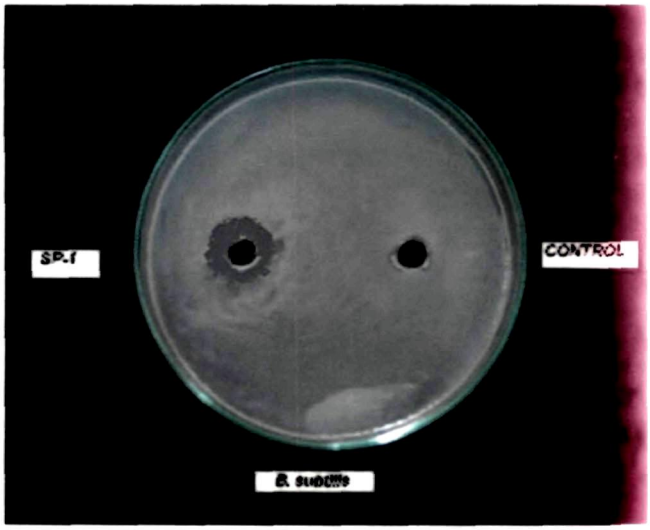
The chloroform-extract having antimicrobial activity on subsequent chromatography using chloroform-methanol (9:1 to 1:9) solvent system afforded two fractions, viz. fraction 1 (2.0 g) and fraction 2 (1.4 g). Both fractions on thin layer chromatography using chloroform-methanol (60:40) solvent system afforded three pure compounds 1a, 1b and 1c from fraction 1 and three pure compounds 2a, 2b and 2c from fraction 2.

4.3.1.4. Antimicrobial activity of the pure compounds isolated from *Spondias pinnata* fruits

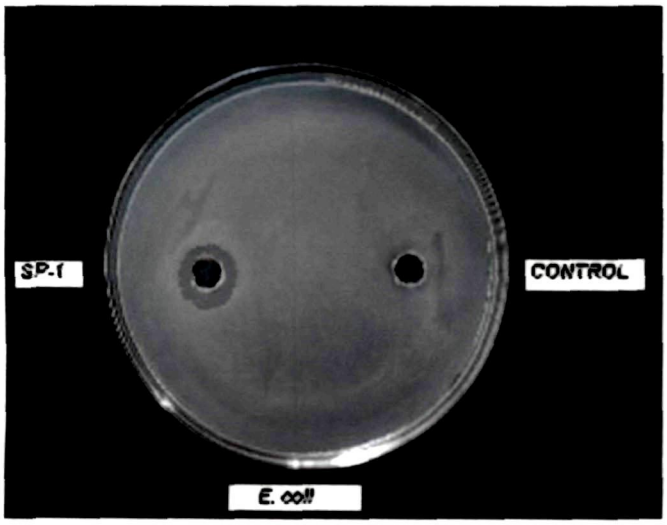
All six isolated and purified compounds were subjected to antimicrobial activity test and data thus obtained are presented in Table 11. It was observed that, the compound SP1 showed antimicrobial activity against all the test organisms except *Klebsiella pneumoniae*. The highest activity was recorded against *Staphyllococcus aureus* (19.0±0.2 mm) followed by *Bacillus subtilis, Escherichia coli* and *Candida albicans* (Plate 3). However, the other compounds did not show activity.



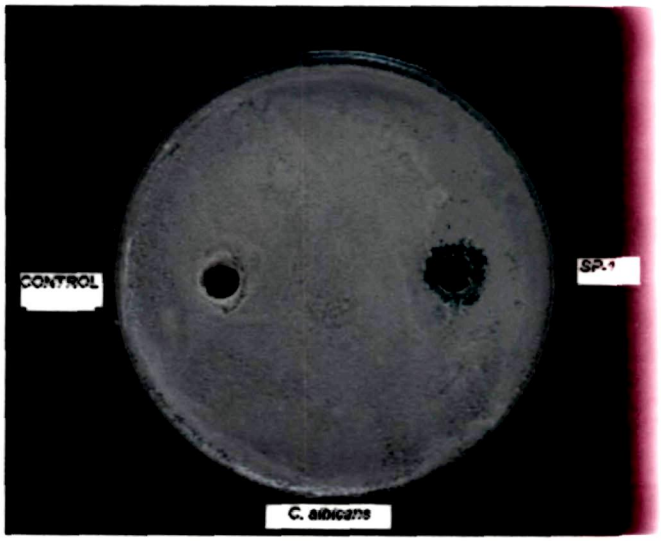
Staphyllococcus aureus



Bacillus subtilis



Escherichia coli



Candida albicans

Plate 3. Antimicrobial activity of SP1.

Table 11. Antimicrobial activity of the extracted compounds of *Spondias pinnata* fruit (zone of inhibition in mm, mean of 3 replications± SD)

Compound	Microorganisms with zone of inhibition in mm				
	B.subtilis	*E. coli*	*S. aureus*	*K. pneumoniae*	*C. albicans*
1a	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
1b	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
1c (SP1)	**18.0±0.40**	**15.0±0.40**	**19.0±0.20**	**0.0±0.0**	**13.0±0.40**
2a	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
2b	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
2c	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Control	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

The major active compound SP1 (283 mg; yield 0.0566% and R_f-0.45) was subjected to various spectroscopic analysis for elucidation of its structure.

4.3.2. Characterization of active compounds from *Spondias pinnata*

4.3.2.1. Spectral characteristics

Infrared spectroscopy: The active compound SP1 was subjected to infrared spectroscopy which revealed the following spectral characteristics:

IR (KBr) ν_{max}: 3459.01, 2944.54, 1694.95, 1464.34, 1387.12, 1362.98, 1275.78, 1032.10, 996.19 and 885.68 cm^{-1} (Fig. 1).

High resolution fast atom mombardment mass spectroscopy: The compound was also subjected to high resolution fast atom mombardment mass spectroscopy which revealed the following peaks:

HRFABMS m/z: 456.00(M^+), 455 (M^+-H) and 439 (M^+-OH) (Fig.2).

^{1}H Nuclear Magnetic Resonance spectroscopy : The active compound was then subjected to ^{1}H Nuclear magnetic resonance spectroscopy and the following peaks obtained:

^{1}H NMR ($CDCl_3$, 400MHz): δ 0.750, 0.772, 0.902, 0.912, 0.925, 0.984, 1.130 (each 3H, s, 7xCH_3), 5.264 (1H, m, C-12) and 3.202 (1H, m, C-11, 2.810 (1H, dd, J=4.0, 9.6 Hz, C-11) (Fig. 3).

^{13}C Nuclear Magnetic Resonance spectroscopy: The compound was hen finally subjected to ^{13}C Nuclear Magnetic Resonance spectroscopy and following characteristics were obtained:

^{13}C NMR ($CDCl_3$, 100MHz): δ 38.501(C-1), 27.290(C-2), 79.029(C-3), 38.837(C-4), 55.287(C-5), 18.435(C-6), 32.728(C-7), 41.071(C-8), 47.706(C-9), 37.189(C-10), 23.530(C-11), 122.508(C-12), 143.435(C-13), 41.681(C-14), 27.809(C-15), 23.050(C-16), 45.960(C-17), 41.681(C-18), 46.600(C-19), 30.768(C-20), 33.190(C-21), 32.545(C-22), 28.213(C-23), 15.675(C-24), 15.5(C-25), 17.284(C-26), 26.054(C-27), 182.103(C-28), 33.155(C- 29) and 23.698(C-30) (Fig. 4).

4.3.2.2. Structure elucidation

The HR-FAB mass spectrum of SP1 showed the presence of the molecular ion peaks at m/z 456, 439(M^+-OH) and 455(M^+-H) corresponding to the molecular formula $C_{30}H_{48}O_3$. The peak at m/z 248 was the characteristic for pentacyclic triterpene with a

double bond C12-C13. The IR spectrum showed absorptions at 3459.01 and 1694.95 cm^{-1} indicating the presence of hydroxyl and carbonyl function of carboxylic acid, respectively. The ^{1}H NMR spectrum of the compound showed the presence of an olefinic proton resonating at δ 5.264 (1H, m, J= 3.2 Hz) corresponding to H-12. The ^{1}H NMR spectrum also showed signal for seven methyl groups at δ 0.750, 0.772, 0.902, 0.912, 0.925, 0.984 and at 1.130 and the sharp singlet nature of signals indicated the tertiary nature of the methyl groups.

The ^{13}C NMR spectrum of the compound showed signals, chemical shifts of which were found to be identical to those of oleanolic acid.

On the basis of IR, HRFABMS, ^{1}H NMR and ^{13}C NMR spectroscopic data as well as all available reference data in various publications, the structure of the compound was determined to be '3 β-hydroxyolea-12-en-28-oic acid' commonly called as 'oleanolic acid'. The elucidated structure of the compound is presented in Fig. 5.

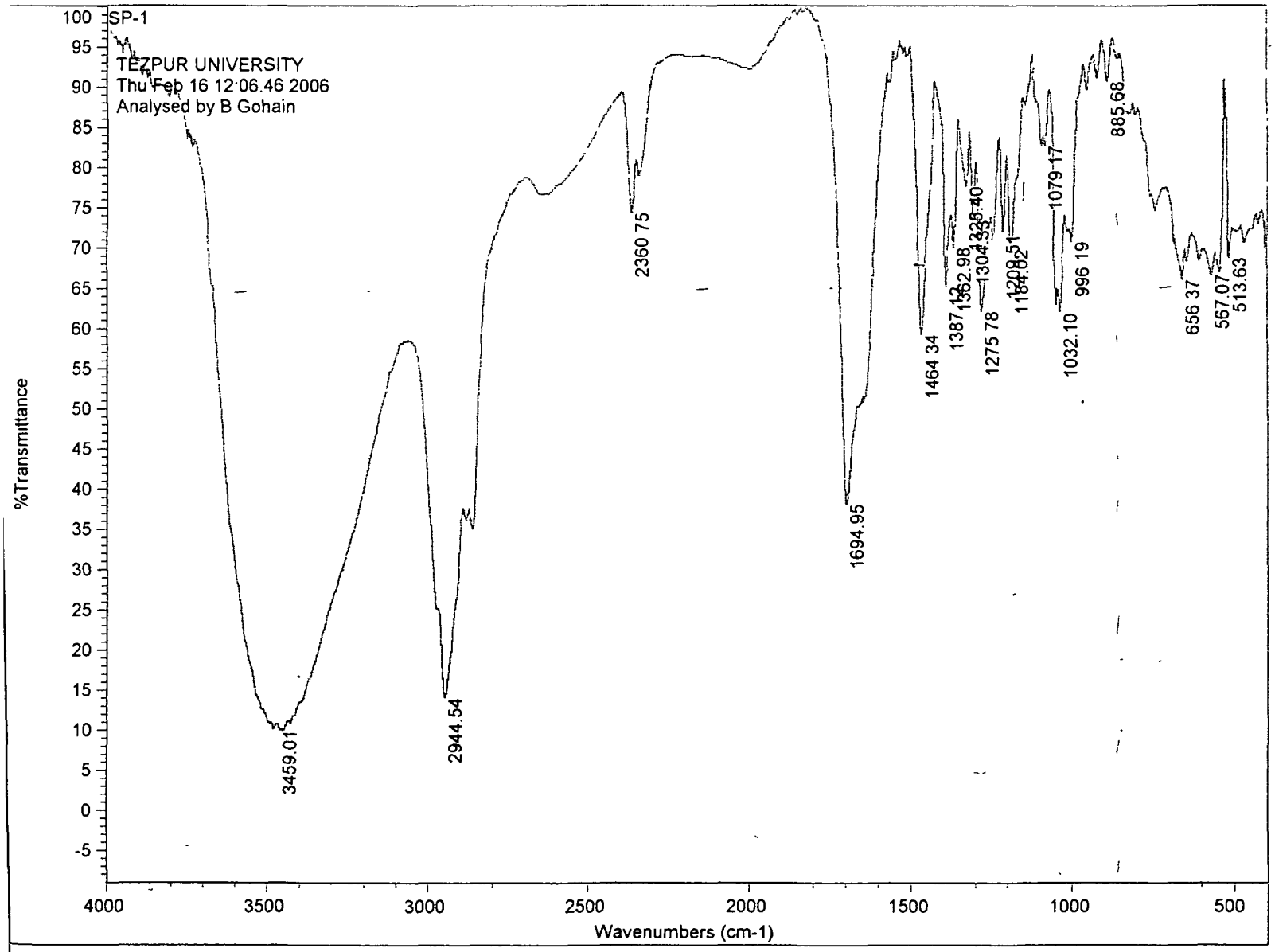


Fig. 1. IR spectra of SP1

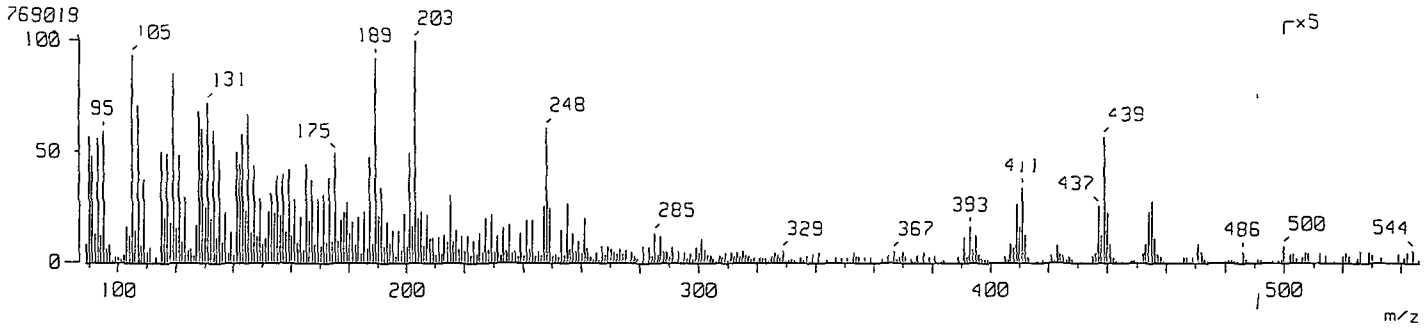


Fig 2. HRFAB mass spectra of SP 1

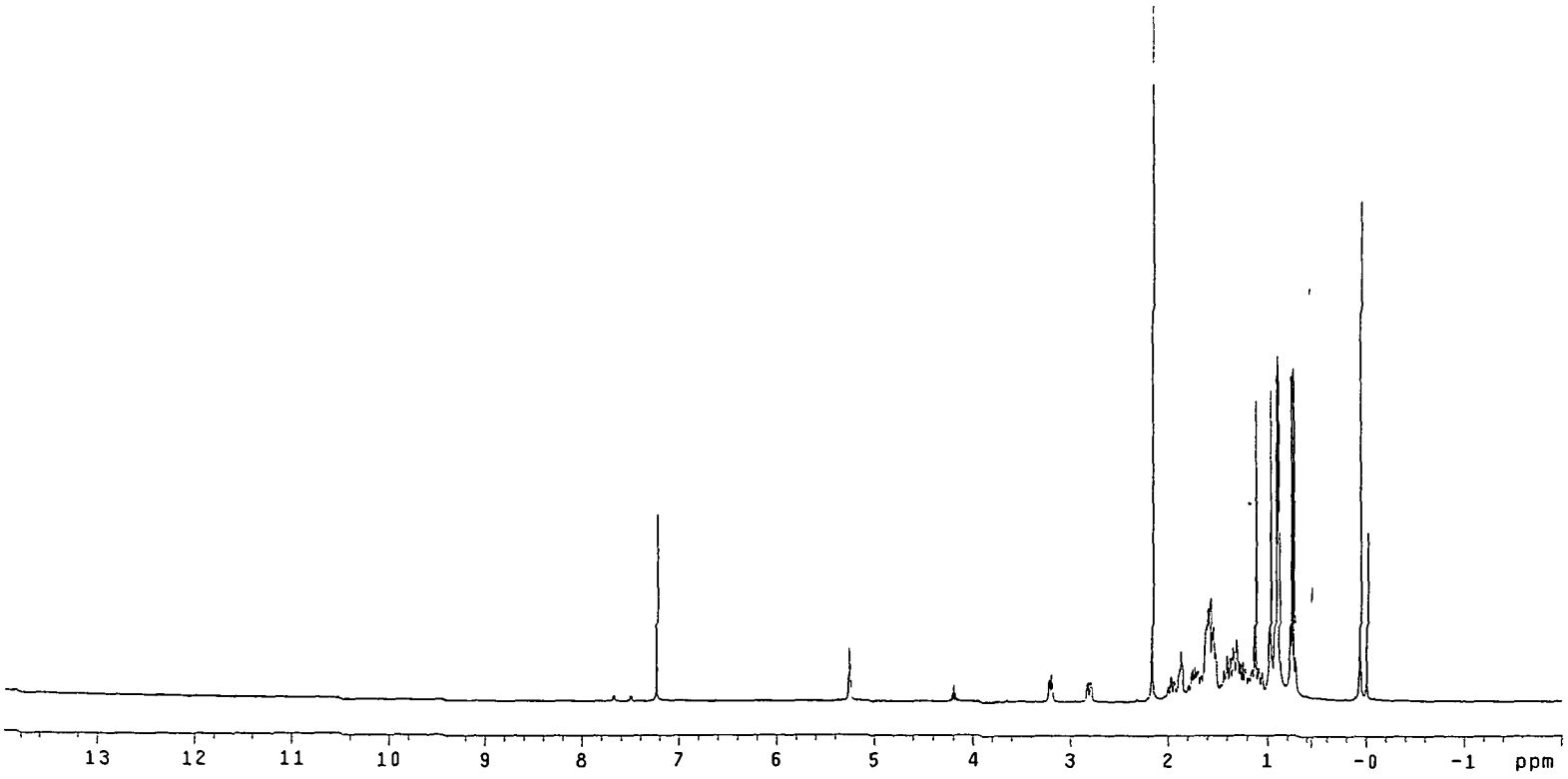


Fig 3 ^{1}H NMR spectra of SP1

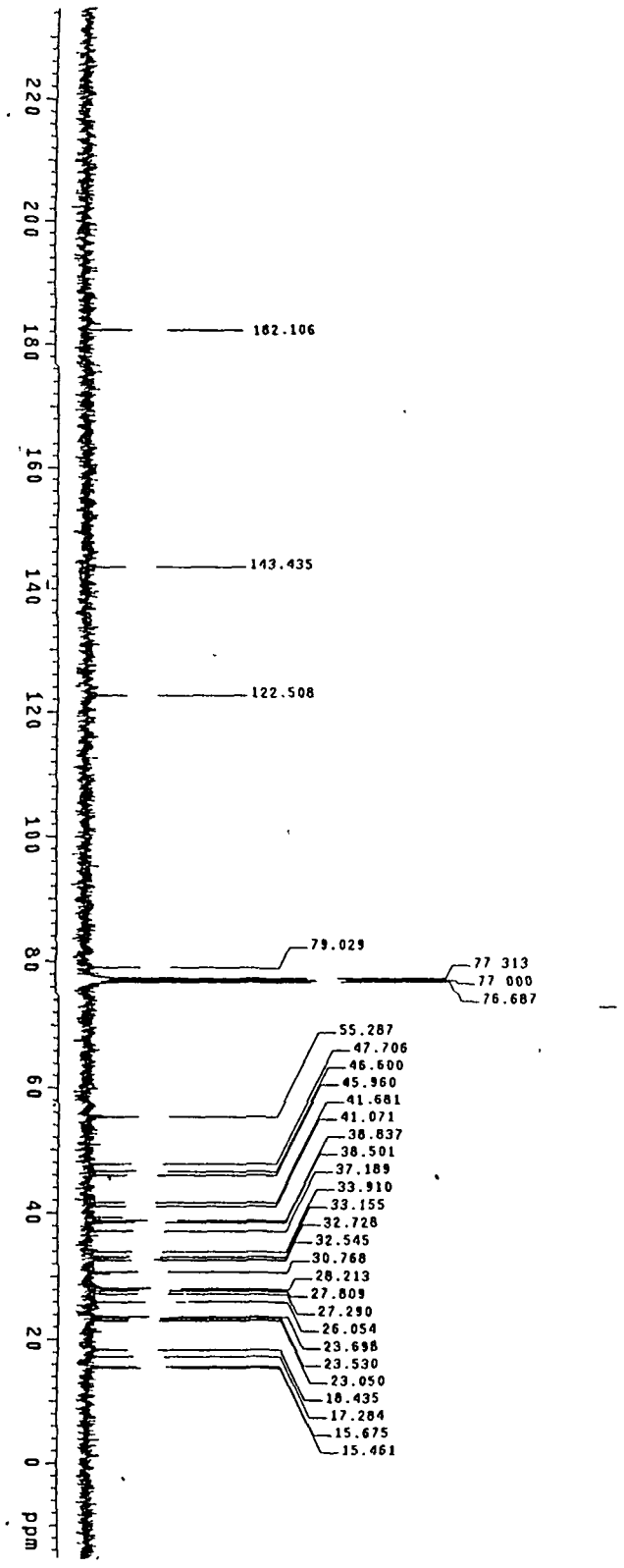


Fig 4. ^{13}C NMR spectra of SP1

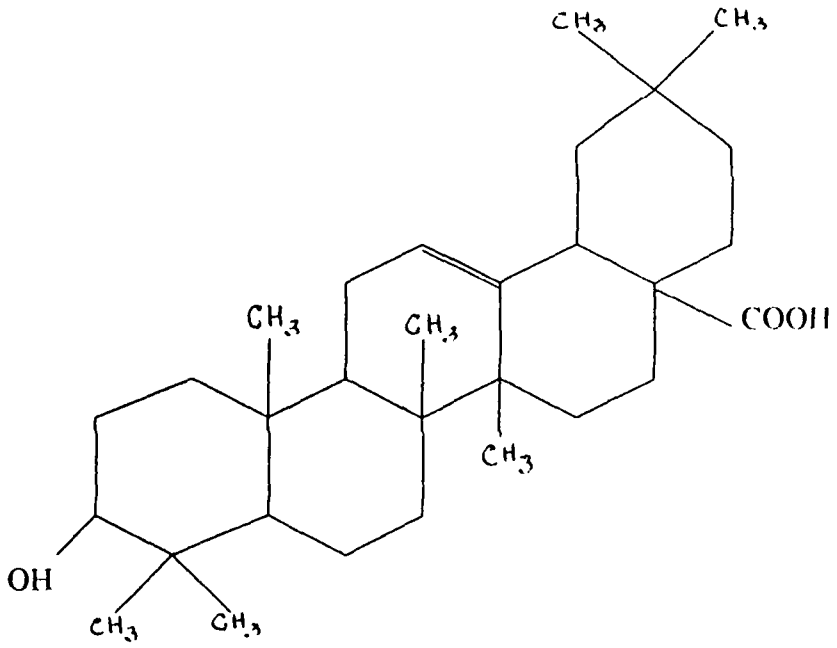


Fig 5. Structure ot oleanolic acid

4.3.3. Isolation of active compounds from *Streblus asper*

4.3.3.1. Crude extraction

The methanolic extracts of the stem bark of *Streblus* was being concentrated in vacuum to give a syrup, was suspended in water and partitioned sequentially with petroleum ether, ethyl acetate, chloroform and butanol. The solvent extracts were concentrated into dryness and weighed. The extracts with their yield are presented in Table 12.

Table 12. Yield of different solvent extracts from *Streblus asper* stem bark

Extracts	Yield (g)
Petroleum ether extract	8.5
Ethyl acetate extract	6.8
Chloroform extract	12.0
Butanol	9.0

4.3.3.2. Antimicrobial activity of the crude extract of the stem bark of *Streblus asper*

All four extracts (Table 12) were subjected to antimicrobial activity test and data obtained are presented in Table 13. The petroleum ether-extract showed antimicrobial activity against all the test organisms except *Klebsiella pneumoniae.* The other extracts did not show activity.

Table 13. Antimicrobial activity of the crude extracts of the stem bark of *Streblus asper*

Extracts	Microorganisms				
	B.subtilis	*E. coli*	*S. aureus*	*K. pneumoniae*	*C. albicans*
Petroleum ether	+	+	+	-	+
Ethylacetate	-	-	-	-	-
Chloroform	-	-	-	-	-
Butanol	-	-	-	-	-

'+' activity present and '–' activity abscent

4.3.3.3. Purification of the active compounds separated from the crude extract

Chromatography of the antimicrobial petroleum ether-extract with petroleum ether-ethyl acetate (9:1 to 1:9) solvent afforded two fractions, fraction 1 and 2. Thin layer chromatography using petroleum ether-ethyl acetate (60:40) as the solvent system afforded three pure compounds 1a, 1b and 1c from fraction 1 (1.0 g) and two pure compounds 2a and 2b from fraction 2 (1.4 g).

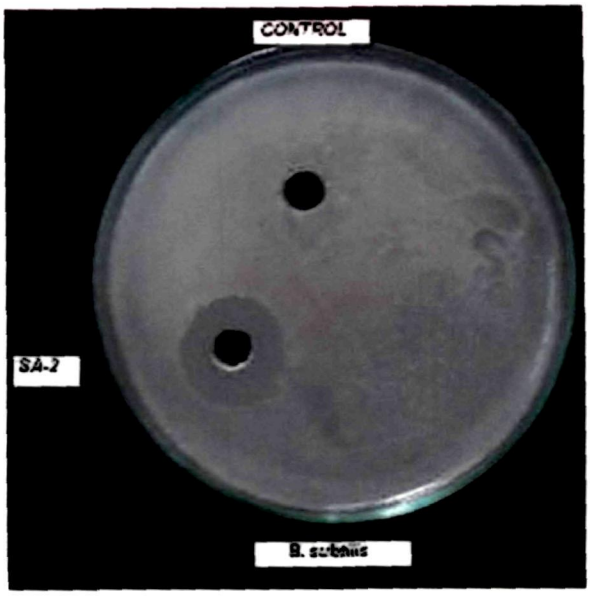
4.3.3.4. Antimicrobial activity of the pure compounds isolated from the stem bark of *Streblus asper*

All five compounds were subjected to antimicrobial activity test and data thus obtained are presented in Table 14. From the table, it was observed that, the compound SA2 showed antimicrobial activity against all the test organisms except *Klebsiella pneumoniae.* The highest activity was recorded against *Bacillus subtilis* (17.0±0.5 mm) followed by *Staphyllococcus aureus, Candida albicans* and *Escherichia coli* (Plate 4). The other compounds did not show any activity.

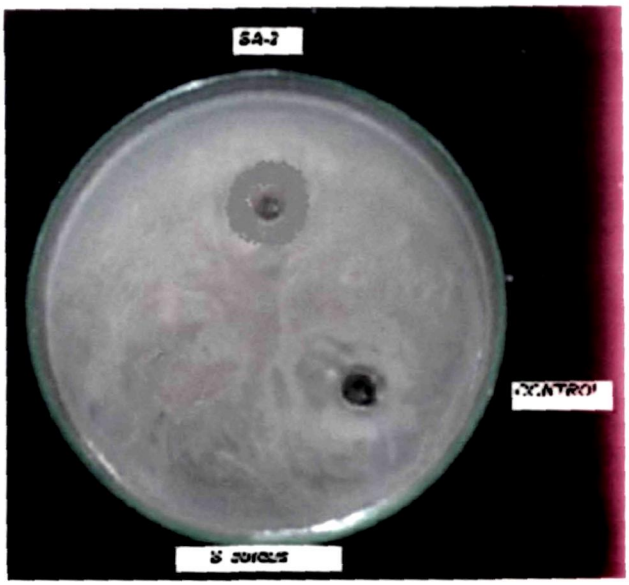
Table 14. Antimicrobial activity of the compounds extracted from the stem bark of *Streblus asper* (zone of inhibition in mm, mean of 3 replications± SD)

Compounds	Microorganisms				
	B.subtilis	*E. coli*	*S. aureus*	*K. pneumoniae*	*C. albicans*
1a	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
1b	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
1c	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
2a	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
2b (SA2)	**17.0±0.50**	**12.0±0.30**	**14.0±0.60**	**0.0±0.0**	**12.0±0.90**
Control	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

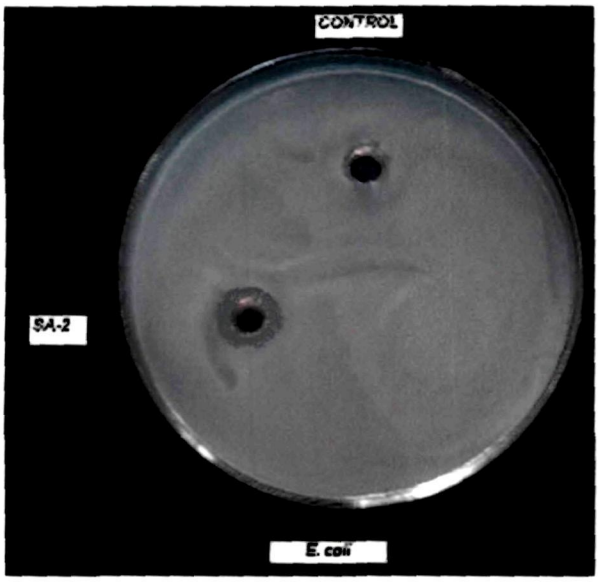
The major bioactive compound 2b or SA2 (263 mg or 0.0526 % w/w; R_f-0.35) was subjected to various spectroscopic analysis for the elucidation of its structure.



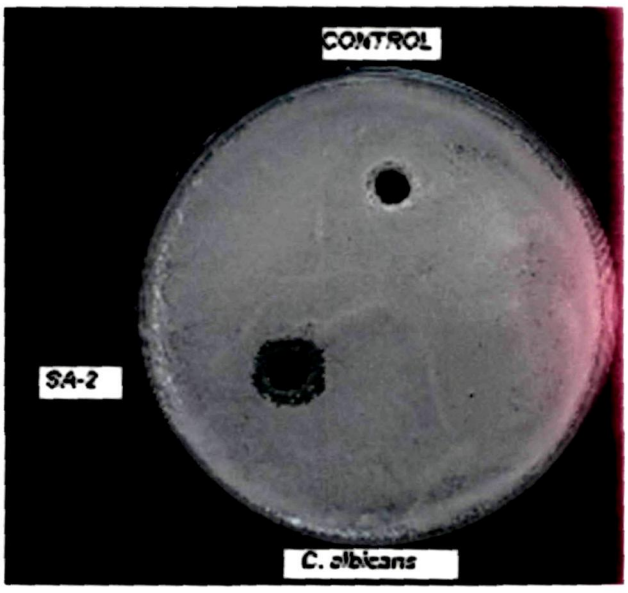
Bacillus subtilis



Staphyllococcus aureus



Escherichia coli



Candida albicans

Plate 4. Antimicrobial activity of SA2.

4.3.4. Characterization of active compound from *Streblus asper*

4.3.4.1. Spectral characteristics

Infrared spectroscopy: The active compound SA2 was subjected to infrared spectroscopy which revealed the following spectral characteristics:

IR (KBr) υ_{max}: 3319.19, 2938.36, 1643.85, 1450.11, 1382.92 and 1040.96 cm^{-1} (Fig 6).

High resolution fast atom mombardment mass spectroscopy: The compound was also subjected to High resolution fast atom mombardment mass spectroscopy which revealed the following peaks:

HRFABMS m/z : 426 (M^+), 425 (M^+-H) and 409 (M^+-OH) (Fig 7).

^{1}H Nuclear Magnetic Resonance spectroscopy : The active compound was then subjected to ^{1}H Nuclear magnetic resonance spectroscopy and the following peaks obtained:

^{1}HNMR ($CDCl_3$, 400MHz): δ 0.965 (3H, s, H-23), 0.758 (3H, s, H-24), 0.827 (3H, s, H-25), 1.027 (3H, s, H-26), 0.941 (3H, s, H-27), 0.786 (3H, s, H-28), 1.55 (3H, s, H-30), 4.551 (1H, s, H-29), 4.669 (1H, s, H-29), 2.377 (1H, ddd, J= 6.0, 5.6 & 5.6 Hz, H-19), 1.368 (1H, m, H-21), 1.918 (1H, m, H-21) and 3.19 (1H, dd, J= 5.6 & 10.8 Hz, H-3) (Fig 8).

^{13}C Nuclear Magnetic Resonance spectroscopy: The compound was hen finally subjected to ^{13}C Nuclear Magnetic Resonance spectroscopy and following characteristics were obtained:

^{13}CNMR($CDCl_3$, 100MHz): δ 38.814 (C-1), 27.557 (C-2), 78.998 (C-3), 38.959 (C-4), 55.371 (C-5), 18.466 (C-6), 34.406 (C-7), 40.934 (C-8), 50.513 (C-9), 37.273 (C-10), 21.074 (C-11), 25.292 (C-12), 38.158 (C-13), 42.924 (C-14), 27.557 (C-15), 35.694

(C-16), 43.085 (C-17), 48.393 (C-18), 48.057 (C-19), 150.795 (C-20), 29.974 (C-21), 40.095 (C-22), 28.106 (C-23), 15.514 (C-24), 16.132 (C-25), 16.262 (C-26), 14.706 (C-27), 18.146 (C-28), 109.230 (C-29) and 19.450 (C-30) (Fig 9).

4.3.4.2. Structure elucidation

The HR-FABMS of the compound established the molecular ion peaks at m/z 425 (M^+-H) and 409 (M^+-OH) corresponding to the molecular formula $C_{30}H_{50}O$. The diagnostic fragmented ion peaks to reveal pentacyclic triterpene with an isopropenyl group were also obtained at m/z 218 $(M-C_{15}H_{28})^+$ and 207 $(M-C_{16}H_{27})^+$. The IR spectrum of the compound showed absorption bands for hydroxyl group (3319.19 cm^{-1}) and terminal methylene group (3084.11, 1643.85 and 891.97 cm^{-1}). The ^{1}HNMR spectrum of the compound showed the presence of seven tertiary methyl groups at δ 0.965, 0.758, 0.827, 1.027, 0.941, 0.786 and 1.55 (3H each). All appeared as sharp singlets and hence the tertiary nature of methyl groups could be ascertained. A pair of multiplets at δ 4.551 and 4.669 (1H each) was an indicative of a terminal isopropenyl group which in turn indicated that the compound belonged to the lupane group of terpenoids. The double doublet at δ 3.19 (1H, dd, J= 5.6 and 10.8 Hz) was due to the proton attached to the carbon bearing hydroxyl group which is in beta orientation at C-3. In the ^{13}CNMR spectrum of the compound, the presence of two distinguished peaks at δ 150.79 and 109.23 revealed the presence of an isopropenyl group.

On the basis of IR, HRFABMS, ^{1}H NMR and ^{13}C NMR spectroscopic data as well as all available reference data in various publications, the structure of the compound was determined to be 'Lup-20 (29)-en-3 β-ol' commonly called as 'lupeol'. The elucidated structure of the compound is presented in Fig. 10.

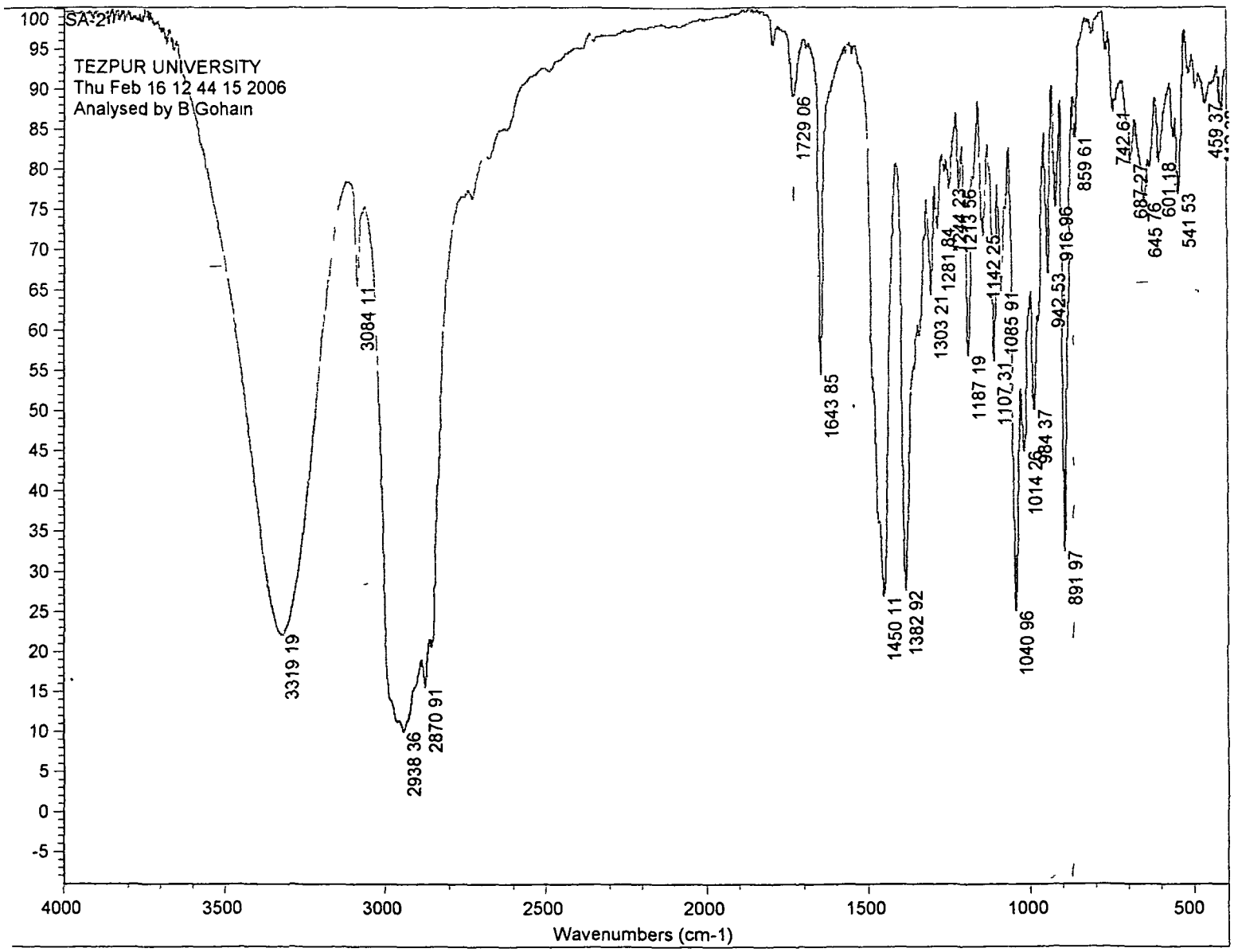


Fig 6 IR spectra of SA2

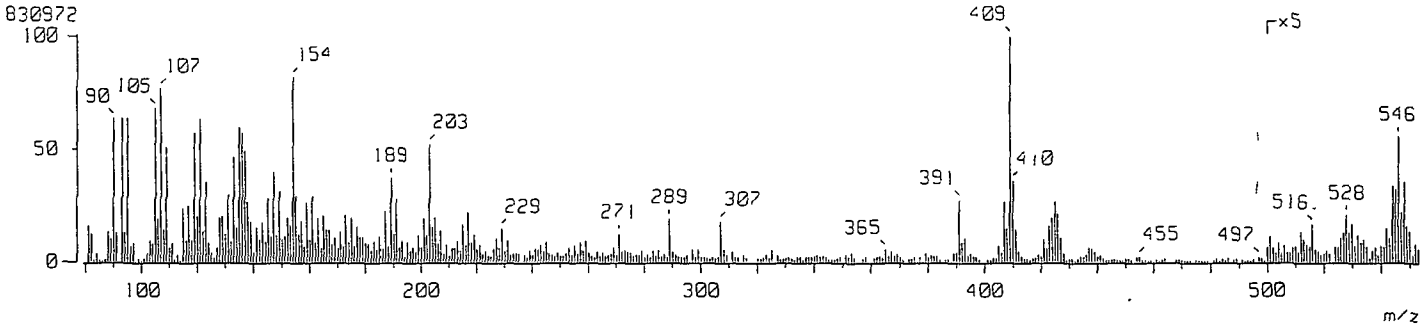


Fig 7. FAB mass spectra of SA2

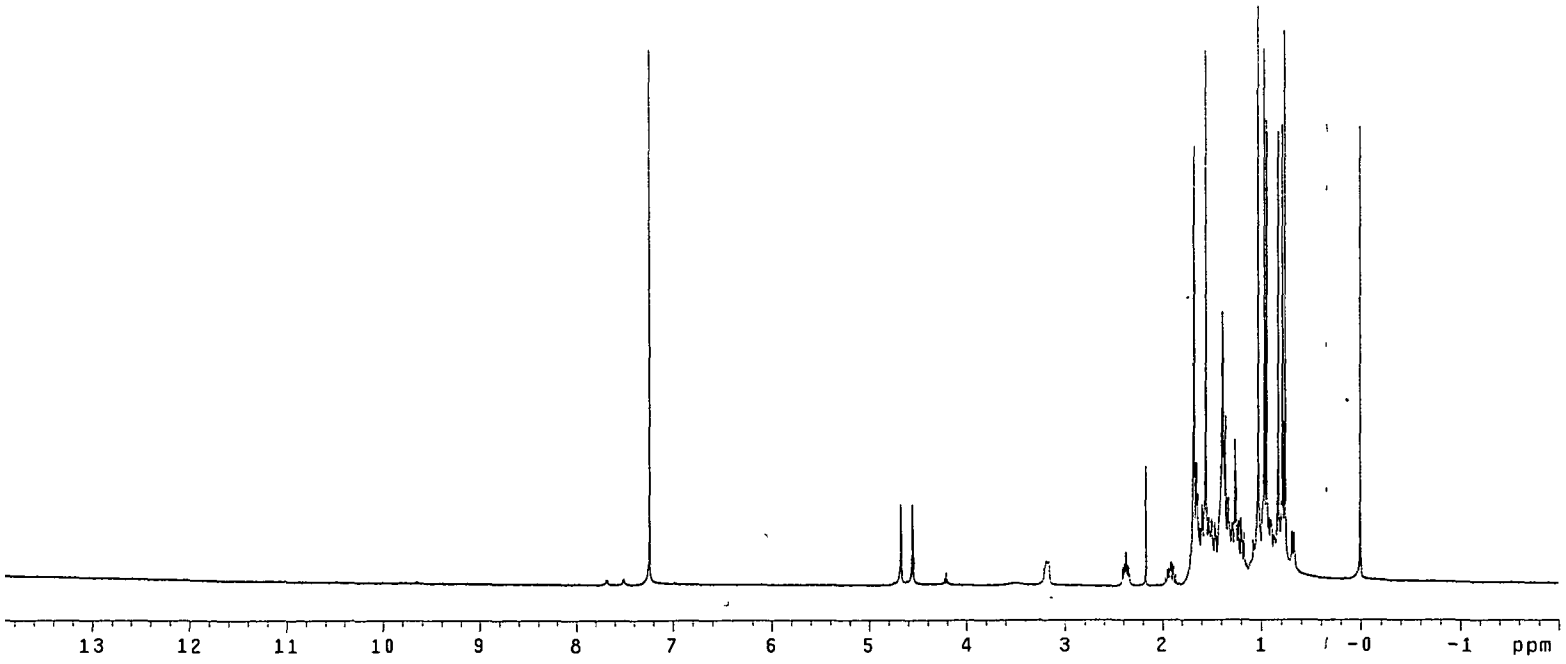


Fig 8. ^{1}H NMR spectra of SA2

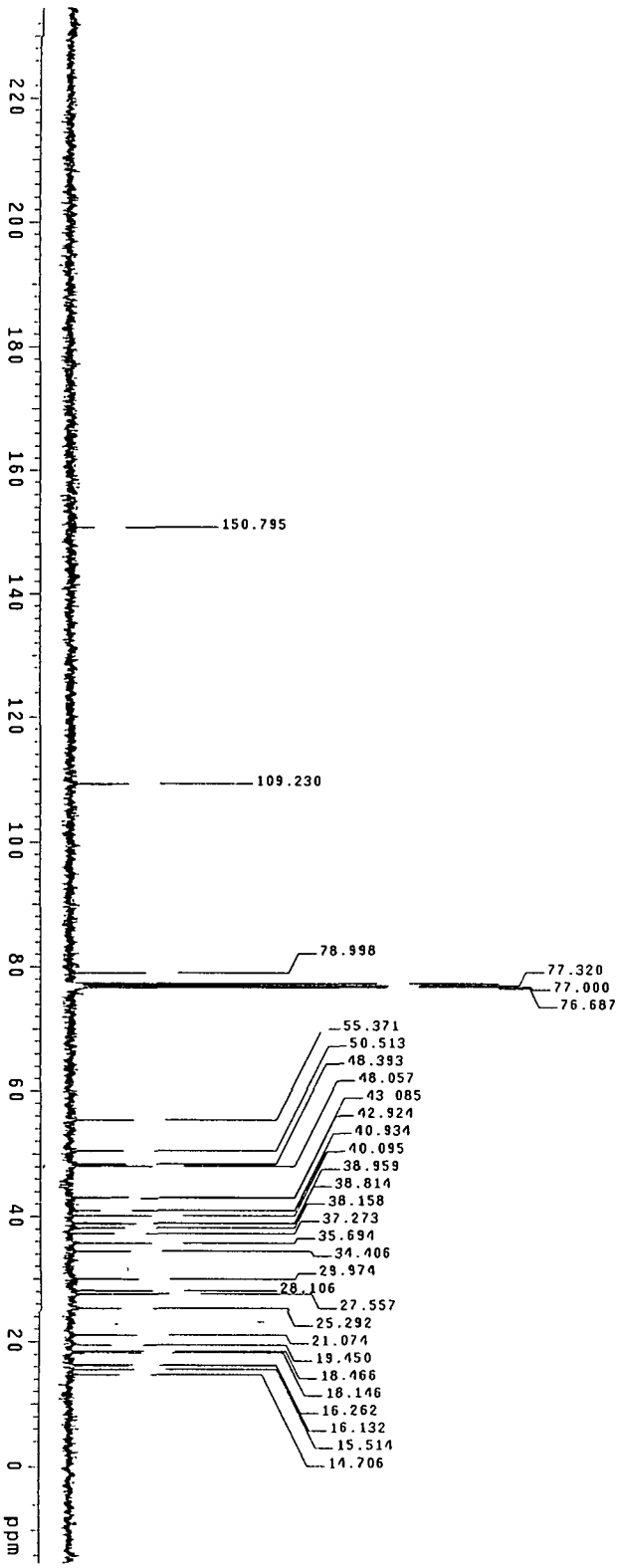


Fig 9. ^{13}C NMR spectra of SA2

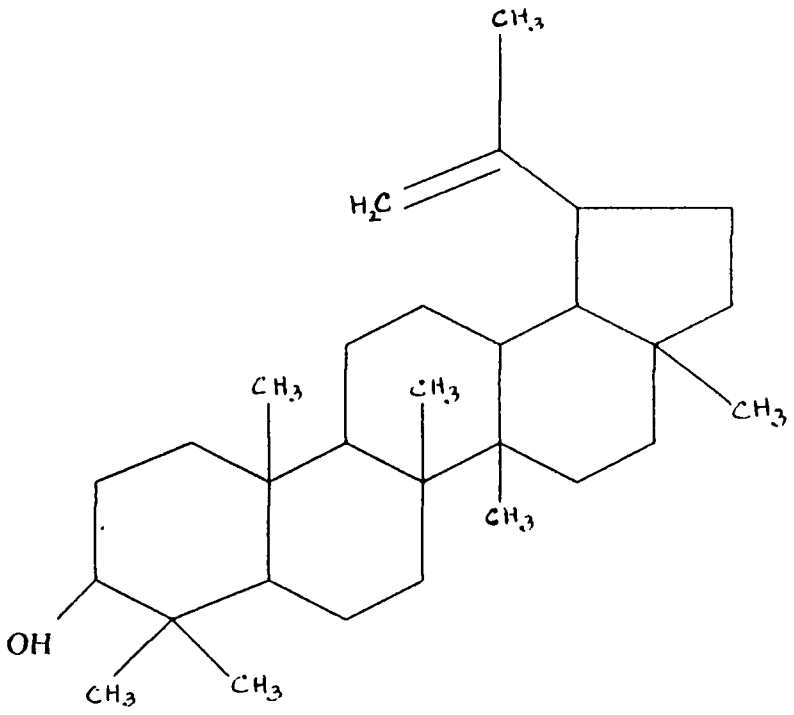


Fig 10 Structure of lupeol

4.4. Genome size determination

4.4.1. Determination of genome size of *Spondias pinnata* and *Streblus asper* by flow cytometry

Flow cytometric analysis of the isolated nuclei resulted in histograms of DNA content of both the standard and the test plants. The gain in the instrument was set so that the fluorescence peak of the external reference standard *Pisum sativum* could be placed in channel 200 of the 1023-channel scale (Figure 11a). The fluorescence peak of nuclei DNA of *Spondias pinnata* was recorded at channel 104 (Figure 11b) and that of *Streblus asper* at channel 173 (Figure 11c).

The peak ratio (PR) between positions of fluorescence peaks of a sample and standard was calculated as follows:

$$PR = \frac{\text{mean channel number of a sample}}{\text{mean channel number of the standard}}$$

The peak ratio of *Spondias pinnata* was 0.52 and *Streblus asper* 0.865. The absolute 2C nuclear DNA content of *Spondias pinnata* and *Streblus asper* was calculated to be 4.726 and 7.862, respectively (Table 15). The C-value of *Spondias pinnata* was estimated to be 2.36 pg or 2.30×10^9 bp and *Streblus asper* 3.93 pg or 3.84×10^9 bp.

Table 15. Fluorescence peak, peak ratio and 2C DNA content of *Pisum sativum*, *Spondias pinnata* and *Streblus asper*

Plant species	Fluorescence peaks	Peak ratio	2C DNA content (pg)	C-value (pg)	C-value (bp)
Pisum sativum	200	-	9.09	4.54	4.40×10^9
Spondias pinnata	104	0.520	4.726	2.36	2.30×10^9
Streblus asper	173	0.865	7.862	3.93	3.84×10^9

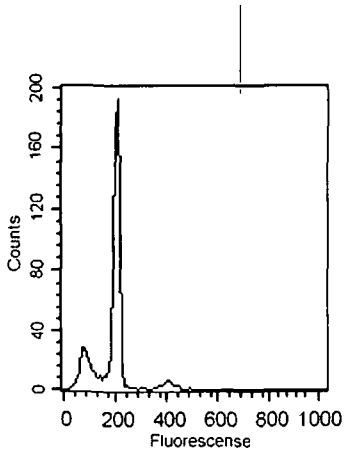


Fig 11a

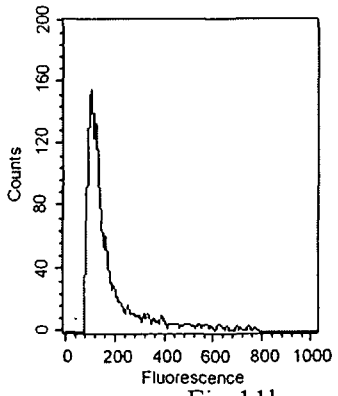


Fig 11b

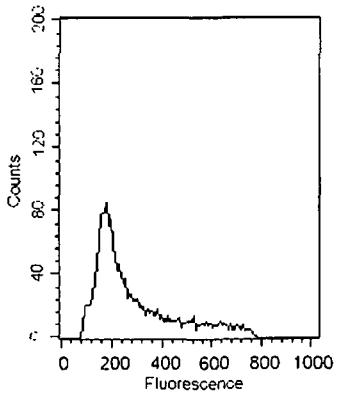


Fig 11c

Fig. 11. Histograms of relative fluorescence intensity obtained by flow cytometric analysis of propidium iodide stained nuclei of (a) *Pisum sativum* used as external reference standard, (b) *Spondias pinnata,* and (c) *Streblus asper.*

4.4.2. Genome size determination by microscopy

4.4.2.1. Genomic DNA

Genomic DNA of *Spondias pinnata* and *Streblus asper* was isolated with the help of a CTAB based isolation protocol developed by modifying the method of Khanuja *et al.* (1999). For quantification of DNA, optical density at 260 nm of the isolated genomic DNA was measured and quantified by the formula 1 O.D. (at 260 nm)= 50 µg/ml.

The yield and purity of the genomic DNA have been presented in Table 16. The yield of genomic DNA per gram of fresh leaf tissue of *Spondias pinnata* was 43.1 µg and *Streblus asper* 48.5 µg. The purity as judged from A260 : A280 ratio was 1.77 in *Spondias pinnata* and 1.89 in *Streblus asper.*

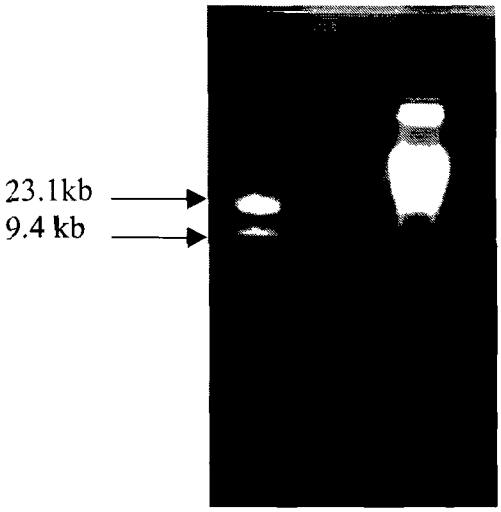
Table 16. Yield and quality of the isolated genomic DNA of the plant species

Plant species	Family	DNA yield (µg)/ g leaf tissue	DNA quality A260 : A280
Spondias pinnata Kurz	Anacardiaceae	43.1	1.77
Streblus asper Lour	Moraceae	48.5	1.89

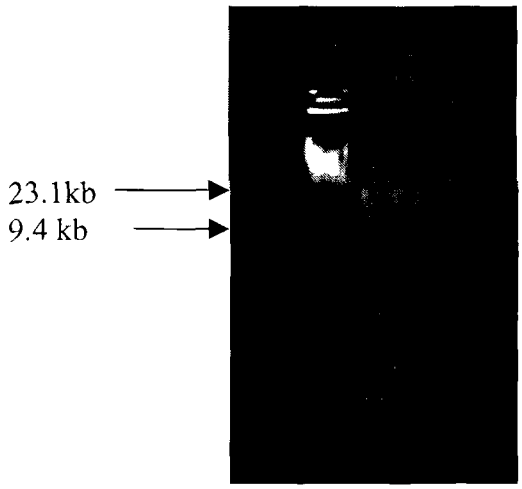
4.4.2.2. Electrophoretic data of restriction digested DNA

The isolated pure DNA, *Eco*R I, *Hind* III single digested and *Eco*R I-*Hind* III double digested DNA on electrophoresis alongside the *Hind* III-digested λ DNA revealed the banding pattern as shown in Plate 5 and 6.

The banding pattern revealed high yield of quality DNA.

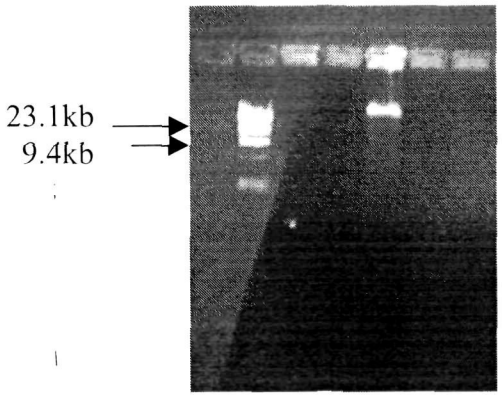


Genomic DNA of *Spondias pinnata* Kurz.
Lane 1- *Hind* III digested λ DNA (kb ladder);
Lane 2- *Spondias pinnata* genomic DNA

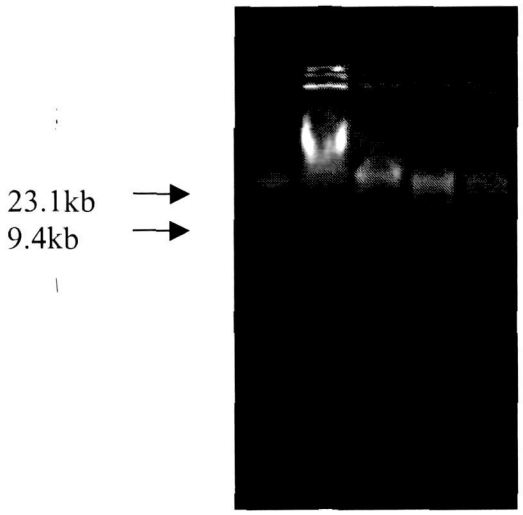


Restriction digestion of *Spondias pinnata* genomic DNA
Lane 1- *Hind* III digested λ DNA (kb ladder),
Lane 2-Control DNA (undigested),
Lane 3-DNA digested with *Eco* RI,
Lane 4-DNA digested with *Hind* III,
Lane 5- DNA digested with both *Eco* RI and *Hind* III

Plate 5. Genomic DNA of *Spondias pinnata* and its restriction digestion



Genomic DNA of *Streblus asper* Lour
Lane 1- *Hind* III digested λ DNA (kb ladder),
Lane 2- *S. asper* genomic DNA



Restriction digestion of *Streblus asper* genomic DNA
Lane 1- *Hind* III digested λ DNA (kb ladder),
Lane 2-Control DNA (undigested),
Lane 3-DNA digested with *Eco* RI,
Lane 4-DNA digested with *Hind* III,
Lane 5- DNA digested with both *Eco* RI and *Hind* III

Plate 6. Genomic DNA of *Streblus asper* and its restriction digestion

4.4.2.4. Genome size

Cell and tissue parameters measured microscopically are presented in Table 17. The volume of tissue section of the plant species *Spondias pinnata* and *Streblus asper* was $1.75x10^{10}$ μm^3 and $1.6x10^{10}$ μm^3, respectively. . The intercellular space in tissue sections was determined to be $6.1x10^9$ μm^3 in *Spondias pinnata* and $5.1x10^9$ μm^3 in *Streblus asper*. On the other hand, the average volume of a single cell was determined to be 61,383 μm^3 in *Spondias pinnata* and 74,490 μm^3 in *Streblus asper*. The weight of a tissue section of *Spondias pinnata* was determined to be 0.02 g and *Streblus asper* 0.23 g.

Table 17. Cell and tissue measurements of *Spondias pinnata* and *Streblus asper*

Plant species	Volume of the tissue section in μm^3 (t)	Volume of the intercellular space in μm^3 (s)	Average volume of single cell in μm^3 (x)	Weight of the tissue section in g (w)
S. pinnata	$1.75x10^{10}$	$6.1x10^9$	61,383	0.020
S. asper	$1.6x10^{10}$	$5.1x10^9$	74,490	0.023

The genome size of the plants determined by the microscopy method is presented in Table 18. The C-value of *Spondias pinnata* and *Streblus asper* was determined to be $2.25x10^9$ bp and $3.72x10^9$ bp, respectively.

Table 18. Genome size of *Spondias pinnata* and *Streblus asper*

Plant species	Genome size		
	2C-value (pg)	C-value (pg)	C-value (bp)
Spondias pinnata	4.64	2.32	$2.25x10^9$
Streblus asper	7.62	3.81	$3.72x10^9$

The genome size or C value of the two plants determined by flow cytometry and by microscopy method was found to be almost similar having a minor variation of 0.04 pg in *Spondias pinnata* and 0.12 pg in *Streblus asper* (Table 19).

Table 19. Comparison of C-value determined by flow cytometry and by microscopy

Plant species	C-value from flow cytometry (pg)	C-value from microscopy (pg)	Difference (pg)
Spondias pinnata	2.36	2.32	0.04
Streblus asper	3.93	3.81	0.12

Chapter V

Discussion

CHAPTER V

DISCUSSION

5.1. Morphophenological characters

5.1.1. Morphophenological characters of *Spondias pinnata*

Morphophenological characters recorded on five randomly selected plants revealed that the plant attains an average height of 27.4±2.302 m and girth of 2.42±0.449 m at maturity. The bark was found to be thick and greyish brown in colour and cracked longitudinally. Warrier *et al.* (1996) reported that *Spondias pinnata* was a medium sized deciduous tree of 27 m height and 2.5 m girth, with compound leaves crowded at branches and the bark was thick, greyish brown which cracked longitudinally. In the present investigation, it was observed that leaves were compound and crowded at the top end of branches. Leaflets were found to be imparipinnate, entire and having pointed apex. Leaf length ranged from 16.20±2.084 to 18.70±2.350 cm and leaf breath between 6.98±0.923 and 7.92±0.526 cm. The average leaf area ranged from 95.63±4.725 to 108.19±3.375 cm^2. Similar observations were reported by Kanjilal and Bor (1997) who reported that *Spondias pinnata* was a medium sized tree with brown or grey bark having horizental wrinkles, leaflets were 22-33 cm long and 3-10 cm broad, entire and petioles were 0.5 cm long. They further observed that flowers were polygamous, scented, subsessile in spreading terminal panicles. These observations were in conformity with our observations. In the present investigation, it was further observed that fruits were ovoid with 4-5 cm in length and were in terminal clusters of 10-15. Flowering started in the month of February and extended to June. The inflorescence was found to be a terminal panicle (plate 1) with a length variation from 30 to 40 cm. Flowers were bisexual, self

fertile, scented, inconspicuous and greyish white in colour. Each of sepals and petals were found to be five in numbers.

5.1.2. Morphophenological characters of *Streblus asper*

Data collected revealed *Streblus asper* Lour to be a small evergreen dioecious tree with the average height of 7.96±1.533 m and girth 0.88±0.216 m having thick foliage in a mature plant. The plant possessed a warty, wrinkled and greyish white bark and alternate, entire, obovate and acute leaves. The axillary inflorescence produced small aggregated male flowers. Female flowers could be single or in clusters of 2-4 and pedunculate. These observations were in conformity with the findings of Warrier *et al.* (1996). They reported *Streblus asper* to be a small rigid evergreen tree up to 15 m height having milky white latex, light grey bark, with simple and alternate leaves, diocious flower, globose male heads, solitary female flowers or 2-4 together and one seeded fruits berry.

5.2. Nutritional analysis

5.2.1. Nutraceutical characters of *Spondias pinnata* fruits

Data recorded on the fruit characters including the moisture content showed that the per cent moisture content was 76.62± 0.785. A comparatively high percentage of crude protein with 3.336±0.195 was recorded in the fruit. The mature fruits possessed a high amount of reducing sugar (69.56±1.060 mg g^{-1}). Fruits were found to contain crude fibre with a value of 23.07±0.780 mg g^{-1}. The composition of minerals like phosphorous, iron, calcium and potassium were recorded to be 0.483±0.032, 0.043±0.005, 5.967±0.472 and 83.60±1.520 mg g^{-1}, respectively. The iron content was observed to be high in comparison to many other common fruits like apple, amla, banana etc. Narasinga Rao *et al.* (1996) earlier reported that *Spondias pinnata* fruits contained 0.039mg g^{-1} iron and it

was higher than apple (0.0066 mg g^{-1}), amla (0.012 mg g^{-1}) and banana (0.0036 mg g^{-1}). This observation was in conformity with our results.

5.2.2. Nutraceutical characters of *Streblus asper* leaves

The leaves were found to be rich in protein and fat with 16.73±0.079 and 1.029±0.029 per cent, respectively. The ash content was found to be high in comparison to similar plants with the value of 8.113±0.256 mg g^{-1}. Similar results were reported by Kamalak *et al.* (2004) who studied the chemical composition of leaves of some tree species used for small ruminant animals in Turkey and reported that the crude protein content ranged from 5.62 to 14.1% and the ash content ranged from 4.99 to 15.8% in these plant species. Ping *et al.* (2002) determined the nutrient concentration in forage samples and reported that the crude protein in the forage was 15.22% on the dry weight basis at the maximum growth stage. These observations were in conformity with our observations. The starch and reducing sugar content were found to be 12.05±0.710 and 1.15±0.060 mg g^{-1}, respectively. The crude fibre content was high with an average value of 17.08±0.120 mg g^{-1} which indicated a high nutritive quality of leaves as fodder. The content of minerals like phosphorus, iron, calcium and potassium was found to be optimum. Similar observations were reported by Salazar-García and Becerra-Bernal (1998) by working on leaf mineral content of *Spondias purpurea* L., a tropical tree belonging to anacardiaceae family and used as fodder. They obtained P, K and Ca content of 1.0, 7.0 and 14.8 mg g^{-1}, respectively from leaves of the plant on dry weight basis.

5.3. Isolation and characterization of active compounds

5.3.1. Isolation and characterization of active compounds from *Spondias pinnata*

The methanolic extract of fruits partitioned in chloroform revealed antimicrobial activity. The extract having antimicrobial activity was subjected to column chromatography to afford fraction 1 and 2. Thin layer chromatography of fraction 1 afforded pure compounds 1a, 1b and 1c; the fraction 2 afforded pure compounds 2a, 2b and 2c. The separated compound 1c (SP1) exhibited antimicrobial activity. The compound was subjected to structure elucidation.

Structure elucidation of SP1

The HR-FAB mass spectrum of SP1 showed the molecular ion peak at m/z 456, 439(M^+-OH) and at 455(M^+-H), which fitted into the molecular formula $C_{30}H_{48}O_3$. The peak at m/z 248 was the characteristic for pentacyclic triterpene with a double bond in between C12-C13. The IR spectrum showed absorptions at 3459.01 and 1694.95 cm^{-1} indicating the presence of hydroxyl and carbonyl functions of carboxylic acid. The ^1H NMR spectrum of the compound showed the presence of an olefinic proton resonating at δ 5.264 (1H, m, J= 3.2 Hz) corresponding to H-12. The ^1H NMR spectrum also showed signal for seven methyl groups at δ 0.750, 0.772, 0.902, 0.912, 0.925, 0.984 and at 1.130 and the sharp singlet nature of signals indicated the tertiary nature of these methyl groups. The ^{13}C NMR spectrum of the compound showed signals, the chemical shifts of these signals were identical to that of oleanolic acid. On the basis of these observations, the compound SP1 was elucidated to be 3 β-hydroxyolea-12-en-28-oic acid, commonly known as 'oleanolic acid' with IR, MS, ^1H NMR and ^{13}C NMR spectral data by

comparing with those reported in literature (Seebacher *et al.*, 2003; Ramos *et al.*, 2003 and Mahato and Kundu, 1994). Sanchez *et al.* (2002) reported that oleanolic acid gave a R_f value of 0.39 in benzene:methanol:ethylacetate (119:14:7) solvent system. Wang and Jiang (1992) reported oleanolic acid (3 β-hydroxyolea-12-en-28-oic acid) to be a triterpenoid compound being widely present in natural medicinal plants in the form of free acid. Liu (1995) reported oleanolic acid to be a triterpenoid compound existing widely in food, medicinal herbs and other plants and effective in protecting against chemically induced liver injury in laboratory animals. He further reported oleanolic acid to have antitumor promotion effect. Kashiwada *et al.* (1998) reported oleanolic acid to be an anti-HIV principle isolated from several plants. Sing and Saxena (1976) isolated oleonolic acid from fruits of *Spondias pinnata*.

5.3.2. Isolation and characterization of active compounds from *Streblus asper*

The methanol extract of the stem bark of the plant partitioned with petroleum ether exhibited antimicrobial activity. Column chromatography of the extract afforded fraction 1 and 2. Thin layer chromatography of the fraction 1 (1.0 g) afforded pure compounds 1a, 1b and 1c; the fraction 2 (1.4 g) afforded pure compounds 2a and 2b. The compound 2b (SA2) exhibited antimicrobial activity. Subsequently, the compound was subjected to structure elucidation.

Structure elucidation

The HR-FABMS of the compound established molecular ion peak at m/z 425 (M^+-H) and at 409 (M^+-OH) corresponding to molecular formula $C_{30}H_{50}O$. The diagnostic fragment ion peaks for pentacyclic triterpene with an isopropenyl group were also obtained at m/z 218 $(M\text{-}C_{15}H_{28})^+$ and at 207 $(M\text{-}C_{16}H_{27})^+$. The IR spectrum of the

compound showed absorption bands for hydroxyl group (3319.19 cm^{-1}) and terminal methylene group (3084.11, 1643.85 and 891.97 cm^{-1}). The ^{1}HNMR spectrum of the compound showed the presence of seven tertiary methyl groups at δ 0.965, 0.758, 0.827, 1.027, 0.941, 0.786 and 1.55 (3H each). All of these appeared as sharp singlets and hence the tertiary nature of methyl groups could be ascertained. A pair of multiplets at δ 4.551 and 4.669 (1H each) was indicative of a terminal isopropenyl group which in turn indicated that the compound belongs to lupane group of terpenoids. The double doublet at δ 3.19 (1H, dd, J= 5.6 and 10.8 Hz) was due to the proton attached to the carbon bearing hydroxyl group which is in β orientation at C-3 position. In the ^{13}CNMR spectrum of the compound having two distinguished peaks at δ 150.79 and 109.23 were attributable to an isopropenyl group. This was in consistent with earlier report by You *et al.* (2003). On the basis of these observations, the compound SA2 was elucidated to be Lup-20(29)-en-3 β-ol, commonly known as 'lupeol' with IR, MS, ^{1}HNMR and ^{13}CNMR spectral data in comparison to those reported in literature (Fotie, 2006 and Aratanechemuge, 2004). Aynilian *et al.* (1972) reported that lupeol had a R_f value of 0.90 in ethylacetate: methanol (4:1) when subjected to TLC on silica gel. Barua *et al.* (1968) also isolated lupeol using petroleum ether extract of the stem bark of *Streblus asper*.

5.4. Determination of antimicrobial activity

5.4.1. Antimicrobial activity of *Spondias pinnata* fruit extract

The compound oleanolic acid (Table 11) was found to contain antimicrobial activity against all the test organisms except *Klebsiella pneumoniae*. The highest activity of 19.0±0.2 mm was recorded against *Staphylococcus aureus* followed by *Bacillus subtilis, Escherichia coli* and *Candida albicans*. This finding was in conformity with the

finding of Woldemichael *et al.* (2003). They reported that oleanolic acid isolated from *Caesalpinia paraguariensis* showed antibacterial activity against *S. aureus* and *B. subtilis*. Murillo-Alvarez *et al.* (2001) reported marked antibacterial activity of the ethanol-extract of *Spondias* sp. against *Bacillus subtilis* and *Streptococcus faecalis*.

.5.4.2. Antimicrobial activity of *Streblus asper* stem bark extract

The compound lupeol (Table 14) showed antimicrobial activity against all the test micro-organisms except *Klebsiella pneumoniae*. The highest activity of 17.0±0.5 mm was recorded against *Bacillus subtilis* followed by *Staphyllococcus aureus, Candida albicans* and *Escherichia coli*. Singh and Singh (2003) reported both antibacterial and antifungal activity of lupeol isolated from *Trichodesma amplexicaule* Roth. Triratana and Thaweboon (1987) observed inhibition of growth of *Streptococcus mutans* while treated with ethanol-extract of sticks and leaves of *Streblus asper*. Taweechaisupapong *et al.* (2005) showed *in vitro* inhibitory effect of *Streblus asper* leaf-extract on the adhesion of *Candida albicans* to human buccal epithelial cells.

5.5. Genome size

5.5.1. Genome size determination by flow cytometry

Streblus asper possessed a higher DNA C-value of 3.93 pg, where as it was 2.36 pg in *Spondias pinnata*. The negligible variation observed between repeated estimations in each species confirms the precise and reproducible estimation of nuclear DNA content. Dolezel and Bartos (2005) reported that the use of Otto I and II buffers could produce histograms of DNA content with unsurpassed resolution in many plant species. This might be due to the use of citric acid in one of the steps, which not only improved chromatin accessibility but also homozenized chromatin structure, thus greatly

eliminating differences in staining intensity amongst populations of nuclei having the same DNA content of different chromatin stages. As the leaf tissues tend to contain cells at different phases of the cycle, the selection of well defined mature leaves thereby eliminated the chance of having major proportion of nuclei in the G_2 stage of the cell cycle. The use of internal standard gave poor peak qualities and to eliminate this problem the external standard was used. Amsellem *et al.* (2001) observed that the use of internal standard might lead to interferences between the staining solution and the genome of the plant species. The plant spcies *Pisum sativum* was used as the standard because its genome size is almost of moderate size in comparison to all known plant genome sizes. Moreover, as reported by Baranyi and Greilhuber (1995) the genome size of pea is stable.

5.5.2. Genome size determination by microscopy

Genomic DNA of *Spondias pinnata* and *Streblus asper* was isolated using a CTAB based isolation protocol. The yield of genomic DNA per gram of fresh leaf tissue of *Spondias pinnata* was 43.1 µg in and that of *Streblus asper* 48.5 µg. The purity as judged by A260 : A280 ratio was 1.77 in *Spondias pinnata* and 1.89 *Streblus asper.* Medicinal plants are the source of bio-active substances and they contain large quantities of secondary metabolites. According to Pirttila *et al.* (2001) the presence of exceptionally high amounts of secondary metabolites interfere with the isolation procedure of DNA. The major problems encountered includes low DNA yield, poor restriction endonuclease digestion and improper polymerase chain reaction (PCR) amplification. Khanuja *et al.* (1999) obtained 15 to 50 µg DNA yield per gram fresh tissue in different medicinal plants. The improved protocol followed was expected to yield more of quality DNA from the concerned medicinal plant species having larger molecular size. In the present

method, CTAB was used as the molecular detergent in the extraction buffer to separate polysaccharides from DNA. The further addition of PVP and β-mercaptoethanol was helpful in removing polyphenols. According to Michiels *et al.* (2003) PVP forms complex hydrogen-bonds with phenolic compounds and coprecipitates cell debris upon lysis. The isolated genomic DNA of the two plant species was incubated with 10 units each of *Eco*RI and *Hin*d III. A double digestion was also performed with both the endonucleases. Digestion of genomic DNAs revealed the high quality of the extracted DNA. The electrophoresis of genomic DNA of *Streblus asper* exhibited the presence of more *Hin*d III restriction sites as compared to *Eco*RI while the genomic DNA of *Spondias pinnata* exhibited the presence of almost equal *Hin*d III and *Eco*RI restriction sites.

5.5.3. Comparison of flow cytometry and microscopy methods of genome size determination

Flow cytometry, Feulgen micro spetcrophotometry and DNA image densitometry are the methods employed in the determination of genome size in plants. But, the methods are found to have some limitations. At present, flow cytometry is becoming popular. The method needs the establishment of the sophisticated and costly equipment, the flow cytometer. The cost is indeed prohibitive for most of the organizations and handling of the equipment needs expert manpower. On the other hand, discrepancies are observed in flow cytometric data of a single sample analysed in different laboratories. Hence, an effort has been made to develop a novel but simple and less expensive method for the determination of genome size of plants without compromising the quality of the work. The leaves for genomic DNA isolation were selected such that most of the cell

nuclei remain at G_0/G_1 stage of interphase. Similar observations were reported by Amsellem *et al.* (2001). In the flow cytometric analysis pea was used as the external standard as the nuclear genome of pea is stable (2C- 9.09 pg). Moreover, high quality nuclei suspension can be easily prepared from pea leaves appearing to be free of compounds interfering with propidium iodide staining (Baranyi and Greilhuber, 1995).

Though, the secondary metabolites commonly present in medicinal plants tend to hamper DNA isolation, the protocol followed could yield quality DNA (Table 16: 43.1-48.5 $\mu g\ g^{-1}$ leaf tissue) as was evident from the restriction digestion of the isolated DNA (Plate 5 and 6) as well as UV-Vis spectrophotometric absorbance ratio at 260: 280 nm being 1.77-1.89 (Table 16). Two critical points were taken into account in this method: firstly, the method for the isolation of genomic DNA was such that it could isolate almost all the DNA from the nuclei; secondly, the accurate determination of the intercellular space in the concerned plant species ($5.1x10^9$ in *Streblus asper* μm^3 and $6.1x10^9$ μm^3 in *Spondias pinnata*). The genome size or C value of the two plants determined by both methods was almost found to be similar having a minor variation of 0.04 pg in *Spondias pinnata* and 0.12 pg in *Streblus asper*. The developing countries in one hand have a vast resource of plant diversity while on the other they face the problem of acquisition and maintenance of flow cytometers (Greilhuber and Temsch, 2001). The flow cytometry method possesses the problems as described above. That is why the simple and less expensive microscopy method is expected to help in characterizing the vast unexplored plant resources of the developing countries. The technique of estimating nuclear DNA content of a nucleus by microscopy is expected to be innovative.

Conclusions

1. *Spondias pinnata* is a moderately tall plant (27.4±2.302 m and girth of 2.42±0.449 m). The longitudinally cracked bark is thick and greyish brown in colour. The leaf size varies from 16.20±2.08 x 6.98±0.92 to 18.70±2.35 x 7.92±0.53 cm with the average leaf area of 95.63±4.73 - 108.19±3.38 cm^2. Fruits are ovoid 4-5 cm long and remain in terminal clusters of 10-15. Flowering occurs from February till June. The inflorescence is a terminal panicle with the length variation of 30-40 cm.

2. The moisture content in fruits is 76.62± 0.785%, crude protein 3.336±0.195%, reducing sugar 69.56±1.060 mg g^{-1}, crude fibre 23.07±0.780 mg g^{-1}, phosphorous 0.483±0.032 mg g^{-1}, iron 0.043±0.005 mg g^{-1}, calcium 5.967±0.472 mg g^{-1} and potassium 83.60±1.520 mg g^{-1}.

3. The yield of the fraction 1c (SP1) separated from the chloroform-extract of fruits was 283 mg or 0.0566% and the R_f value 0.45 in chloroform:methanol (60:40) and 0.39 in benzene:methanol:ethylacetate (119:14:7). The fraction was elucidated to be '3 β-hydroxyolea-12-en-28-oic acid' commonly known as 'oleanolic acid' by IR, MS, ^1H NMR and ^{13}C NMR spectroscopy.

4. Oleanolic acid showed antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Candida*. The highest antimicrobial activity was recorded against *Staphylococcus aureus* (19.0±0.2 mm) followed by *Bacillus subtilis* and *Escherichia coli*.

5. *Streblus asper* Lour is a small evergreen, dioecious and thick foliage bearing tree having height of 7.96±1.533 m and girth of 0.88±0.216 m. The plant possesses a warty, wrinkled and greyish white bark and alternate, entire, obovate-acute leaves.

6. Leaves of *Streblus asper* is rich in protein and fat with 16.73±0.079 and 1.029±0.029%, respectively. The ash content is also high with 8.113±0.256 mg g^{-1}. The content of starch and reducing sugar is 12.05±0.710 and 1.15±0.060 mg g^{-1}, respectively. There is a high content of crude fibre with the value of 17.08±0.120 mg g^{-1}. These indicate a high nutritive quality of leaves of *Streblus asper* as fodder.

7. The yield of the fraction 2b (SA2) separated from the petroleum ether-extract of the stem bark was 263 mg or 0.0526% and the R_f 0.35 in petroleum ether:ethyl acetate (60:40) and 0.90 in ethylacetate:methanol (4:1). The fraction was elucidated to be 'Lup-20(29)-en-3 β-ol' commonly known as 'lupeol' with the analysis of spectral data of IR, MS, ^{1}HNMR and ^{13}CNMR.

8. Lupeol showed antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Candida albicans.* The highest activity was recorded against *B. subtilis* (17.0±0.5 mm) followed by *S. aureus* and *C. albicans.*

9. The genomic DNA of *Spondias pinnata* and *Streblus asper* was isolated with the help of a CTAB based improved protocol. The yield of genomic DNA per gram of fresh leaf tissue of the two medicinal plant species was 43.1 µg and 48.5 µg; the purity as judged by A260 : A280 ratio was 1.77 and 1.89, respectively.

10. The genome size of *Streblus asper* and *Spondias pinnata* were determined to be 3.93 pg and 2.36 pg, respectively by flow cytometry. The genome size or C value of *the plants* was also determined by the microscopy method described by Konwar *et al.* (2007). The genome size of the plants determined by both methods possessed minor variation of 0.04 pg in *Spondias pinnata* and 0.12 pg in *Streblus asper.*

Further works

Research on molecular genetics, cytology and biochemistry will help to understand the biosynthesis of the active compounds by the plants. Research on propagation techniques of the plants particularly by tissue culture will help in the cultivation, improvement and conservation of the plants. The techniques developed for the isolation of active compounds needs confirmation and improvement for their subsequent industrial application. The compound 'lupeol' isolated from *Streblus asper* is expected to be an effective preventive drug against the scale decay disease of fishes. However, this needs further investigation. Pharmacological research on 'oleanolic acid' and 'lupeol' is necessary for confirming their usefulness as medicines for human ailments.

The presence of antioxidant property in fruits of *S. pinnata* could be assessed. The possible hepatoprotective role could also be ascertained through *in vitro* studies with cultured animal cell lines.

Apoptosis is an orchestrated series of events through which the cell precipitates its death. The stages of apoptosis include cell shrinkage, chromatin condensation, nuclear segmentation and inter-nucleosomal fragmentation of DNA, resulting in the generation of apoptotic bodies. The potentiality of lupeol extracted from the bark of *S. asper* in inducing apoptosis leading to suppression of proliferation of cultured human cancerous cells could further be investigated in future. In the light of such experiments, the possibility of developing a new herbal medicine from the bark of *S. asper* could be explored.

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