Phytochemical Screening & *in vitro* Antioxidant and Thrombolytic Activities of *Aphanamixis polystachya* (Wall.) Parker Leaf Extracts

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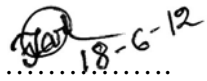
A thesis report, submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the degree of Masters of Clinical Pharmacy and Molecular Pharmacology

RESEARCH REPORT ON

"Phytochemical Screening and in vitro Antioxidant and Thrombolytic Activities of Aphanamixis polystachya (Wall.) Parker Leaf Extracts"

DECLARATION BY THE CANDIDATE

It is my pleasure to submit the Research Report. The report is prepared on "Phytochemical Screening and *in vitro* Antioxidant and Thrombolytic Activities of *Aphanamixis polystachya* (Wall.) Parker Leaf Extracts" which was carried out by me under the guidance of Mr. Apurba Sarker Apu, Senior lecturer, Department of Pharmacy, East West University, Dhaka. I have given my best effort in preparing this report and to make it a worthy one. I have enjoyed working on the report and gained some experience.



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CERTIFICATE BY THE INVIGILATOR

This is to certify that the dissertation, entitled "Phytochemical Screening and *in vitro* Antioxidant and Thrombolytic Activities of *Aphanamixis polystachya* (Wall.) Parker Leaf Extracts" is a bonafide research work done by Ferdous Ara, in partial fulfillment of the requirement for the Degree of Masters of Clinical Pharmacy and Molecular Pharmacology.

Apurba 19.06.12

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Mr. Apurba Sarker Apu

Senior Lecturer

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ENDORSEMENT BY THE CHAIRPERSON

This is to certify that the dissertation, entitled "Phytochemical Screening and *in vitro* Antioxidant and Thrombolytic Activities of *Aphanamixis polystachya* (Wall.) Parker Leaf Extracts" is a bonafide research work done by Ferdous Ara, under the guidance of Mr. Apurba Sarker Apu, Senior Lecturer, Department of Pharmacy, East West University, Dhaka.

Sufia Islam
20.06.2012
.....
Dr. Sufia Islam

Chairperson and Associate Professor

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Acknowledgement

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I express my sincere gratitude to my caring parents for their support, inspiration and guiding me all through my life, including that for my research project. I am very grateful to my sisters who encouraged me enormously.

Dedication

This Research paper is dedicated to
My beloved parents and sisters

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ABSTRACT

Purpose: In present study the leaves extracts of *Aphanamixis polystachya* (Meliaceae) have been screened for their photochemical constituents, antioxidant, total phenolic content and *in vitro* thrombolytic activities.

Methods: The *n*-hexane, ethyl acetate and methanol extracts were screened for the presence of phytochemicals, using *in vitro* assay methods and their inhibition of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical was used to evaluate their free radical scavenging activity at 517 nm. Nitric oxide scavenging capacity was measured by sodium nitroprusside and griss reagent. The total phenolic content (TPC) measured by the Folin-Ciocalteu method. An *in vitro* thrombolytic model was used to check out the clot lysis effects of three extracts using streptokinase as a positive control.

Results: Phytochemical screening of the plant showed the presence of alkaloids, flavonoids, terpenoids, saponins, tannins, cardiac glycosides and anthraquinones. All the extracts inhibited DPPH, indicating their antioxidant activity. In DPPH and nitric oxide scavenging assay both *n*-hexane extract showed highest IC_{50} values 74.84 µg/ml, 76.15 µg/ml and the lowest IC_{50} value was 12.54 µg/ml of methanol extract and 39.90 µg/ml of ethylacetate extract. It appears that, it has greater free radical scavenging capacity. Ethyl acetate extract had the highest total phenolic content of 107.77 mg GAE/100 g extract. All the extracts showed significant % of clot lysis effect ($p<0.001$) with reference to negative control.

Conclusion: The outcomes of the study are an indication of present of phytochemicals and may be responsible for some of the therapeutic uses of these plants.

Keywords: *Aphanamixis polystachya*, Phytochemical screening, DPPH, Nitric oxide, Phenolic content, *In vitro* Thrombolytic.

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INTRODUCTION

Throughout the ages, humans have relied on nature for their basic needs for the production of foodstuffs, shelter, clothing, means of transportation, fertilizers, flavors and not least, medicines. Since origin of human's life, plants continue to play a curative and therapeutic role in preserving human health against disease and decay (Nair *et al*., 2005). Medicinal plants have been used in all cultures as a source of medicine, since times immemorial. Herbal Medicine is still the mainstay of health care in several developing countries. The widespread use of herbal remedies and health care preparations, as those described in ancient texts like the Vedas and the Bible, and obtained from commonly used traditional herbs and medicinal plants, has been traced to the occurrence of natural products with medicinal properties (Nair *et al*., 2005).

The use of herbal medicine has become increasingly popular worldwide and medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects. Approximately, half of the world's 25 best-selling pharmaceutical agents are derived from natural products. Thus, emphasis is now given on the standardization of herbal medication by screening of biological activities of medicinal plants and isolating active principles from them (Arvigo & Balick, 1993).

The World Health Organization (WHO) has estimated that more than 80% of the world's population in developing countries depends primarily on herbal medicine for basic healthcare needs. This scenario is similar to the one occurring in Bangladesh (Guerrero *et al*., 2004). Bangladesh is an Asian country where only 20% of the total populations are provided with modern healthcare services while the rest are dependent on traditional plant-based systems.

Moreover, it is estimated that only 500 medicinal plant species had been recorded in Bangladesh out of approximately 1,900 species regarded as having medicinal value. There are several studies on the botanical aspects of the plants of Bangladesh. However, although plants are used by a great segment of the population, scarce investigation has been done on their biological activities. In more recent years, with considerable research, it has been found that many plants do indeed have medicinal values (Guerrero *et al*., 2004).

In a study it has been shown that at least 119 chemical substances, derived from 90 plant species, can be considered as important drugs that are in use in one or more countries. Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine (Arvigo & Balick, 1993). Various parts of the plant are used by traditional medicine practitioners in Bangladesh in the management and treatment of several disorders which include rheumatism, hypertension, cancer, and inflammatory diseases (Guerrero *et al*., 2004).

Traditional medicine providing leads to bioactive natural products abound. Suffice it to point to some recent confirmations of the wealth of this resource. Artemisine (qinghaosu) is the antimalerial sesquiterpene from a Chinese medicinal herb *Artemisia annua* (wormwood) used in herbal remedies since ancient times (Klaymann & Clark, 1985). Forskolin is the antihypertensive agent from *Coleus forskohlii* Briq. (Labiatae), a plant whose use was described in ancient Hindu Ayurvadic texts (Bhat *et al*., 1977).

Paclitaxel is the most recent example of an important natural product that has made an enormous

impact on medicine. It is interact with tubulin during the mitotic phase of the cell cycle, and thus prevents the disassembly of the microtubules and their by interrupts the cell division (Wani *et al*., 1991). The original target diseases for the compound were ovarian and breast cancers, but now it is used to treat a number of other human tissue proliferating diseases as well (Strobel *et al*., 2004).

New drugs of plant origin and new methods of producing them will continue to be an important parts of the service and thus Plants are considered as are of the most important and interesting subjects that should be explored for the discovery and development of newer and safer drug candidates (Parker, 1997).

Plant Profile

Aphanamixis polystachya is a traditional medicinal plant of the Meliaceae (Neem family) family.

Common name: Amoora, Pithraj Tree

Botanical name: *Aphanamixis polystachya*

Synonyms: *Amoora aphanamixis*, *Aglaia polystachya*, *Amoora rohituka*, *Andersonia rohituka*, *Aphanamixis cumingiana*, *Aphanamixis rohituka*, *Ricinocarpodendron polystachyum* (Parker, 1997). Royna, Tiktaraj, Baddiraj, Pitraj, Pitta-raj, Lashune (Bengali); Rohituka tree (English); Harin-hara, Harinkhana, Mullu munthala, Rohado, Mangai, Khanda, Rohituka (Hindi); Heirangkhoi (Manipuri); Raktharohida (Marathi); Malampuluvan, Sem, Semmaram (Tamil); Chemmaram, Sem (Malayalam); Chevamanu, Rohitaka (Telugu); Mukhyamuttage, Mullumuttaga, Mulluhitthalu, Roheethaka (Kannada); Sahala (Kuki); Dieng rata (Khasi); Agan (Rongmei); Hakhori bakhori (Assamese); Rohitaka, Anavallabha, Ksharayogya, Lakshmi, Lakshmivana, Lohita (Sanskrit); Elahirilla, Amoora, Pitraj (Sri Lanka); Thit-nee (Myanmar);

Chemmaram , Karagil (Malaysia); Bandniphal (Nepal).

Trade names: Amoora, Pitraj (Parker, 1997).

Scientific classification

Kingdom: Plantae

Phyllum: Magnoliophyta

Class: Magnoliatae

Sub Class: Rosidae

Order: Sapindales

Family: Meliaceae

Genus: *Aphanamixis*

Species: *Aphanamixis polystachya*

Distributional Range

The plant of *A. polystachya* is a timber tree mainly growing in the tropical areas of Asia. *A. polystachya* grows in undisturbed mixed dipterocarp and coastal forests usually on hillsides and ridges with sandy to clay soils. Also found on limestone. In secondary forests it usually present as a pre-disturbance remnant.

A. polystachya is distributed mainly in the rain forests of a geographical area spanning from India to South China to the Solomon Islands (Wiart, 2006). In Vietnam, this tree occurs in evergreen tropical rainforest or monsoon forest (Sanyal & Datta, 1981). It can be mainly found in Bangladesh, India, Myanmar, Malaysia, Nepal and Sri Lanka (Wiart, 2006).

Native distribution:

This tree is available in the tropical region of China, also available in Bhutan, Indochina, Thailand, Indonesia, Singapore; Taiwan, Papua New Guinea and Philippines. In Bangladesh this tree is also found in Narshingdi, Tangail, Maymensingh, Sylhet, Chittagong, Chittagong Hill Tracts forests (Banderban, Khagrachari and Rangamati Hill districts) and Cox's Bazaar districts. *A. polystachya* also cultivated in the Neotropics and under glass in Europe (Parker, 1997).

Table 1: Species, local name and local distribution of *A. polystachya* available in Bangladesh

Species	**Local Name**	**Local Distribution**
Amoora chittagonga, Aglaia chittagonga, Aphanamixis chittagonga	Thitpasing	Modhuchara, Chittagong
Amoora cucullata	Amoor, Latmi	Sundarban, Hiron point, Mongla, Khulna B.L. College, Cox's Bazar (Ukhia)
Amoora rohituka Aphanamixis polystachya, Aglaia polystachya	Pitraj, Royna, Titraj, Pitti, Bajor, Baiddiraj, Tiktaraj	Chittagong, CHT, Dhamrai, Northern parts of the country
Amoora wallichii	Lali	Sylhet

Botanical Descriptions of *A. polystachya*

Habit: This tree occurs in evergreen tropical rainforest or monsoon forest. Trees up to 20-30 meter tall (Figure 1).

Figure 2: Bark

Figure 1: Habit

Foliage: Evergreen

Plant shape: Spreading

Trunk & bark: Bark grayish brown to dark brown, rough, exfoliating in circular flakes grey, fissured; blaze cream (Figure 2).

Branches and branchlets: Branchlets terete, glabrous (Figure 3).

Figure3: Branch

Figure 4: Shoot apex

Figure 5: Rachis and leaf insertion

Leaves: Leaves are green color large, odd- or even- pinnate, 30-60 cm long, with 9-21 leaflets. Leaflets are oblong-elliptic, elliptic, or ovate. Alternate, spiral, clustered at twig ends, 50 cm

long; rachis pulvinate (Figure 5), 30 cm or more long, often lepidote scaly; leaflets opposite to subopposite, 4-8 pairs with terminal one (Figure 7), petiolule 0.4 to 1 cm long (Figure 6); lamina oblong-lanceolate, apex acuminate (Figure 4), base asymmetric, margin entire, coriaceous, glabrous; midrib slightly raised above, stout beneath; secondary nerves slender; tertiary nerves broadly reticulate.

Figure 6: Petiolule insertion

Figure 7: Terminal leaflets

Figure 8: Leaflet lower side

Figure 9: Leaflet upper side

Figure 10: Venation

Inflorescence / Flower: Inflorescence panicles; flowers polygamous. Flower clusters occur in leaf axils, less than a foot long. Flowers are 6-7 mm in diameter, with 3 bracteoles. Flowers have 5 nearly curcular sepals, 1-1.5 mm across. Petals are 3-7 mm in diameter, concave. Inner

perianth pale yellow or cream-coloured, some or partly joined (Figure 11); stamens 3-8, present, joined (to form a staminal tube), at base joined to the perianth; ovary superior, carpels joined.

Flowering: May-September.

Figure 11: Flowers

Fruit and seed: The fruits are 4cm in diameter (Figure 12), and exude a white latex after incision. Capsule, subglobose, to 3 cm across, coriaceous, pale reddish, 3-celled, orange red (Figure 13) when mature, seeds 1, greyish brown.

Figure 12: Dehisced fruit

Figure 13: Fruit insertion

Type of stem: Hard wooded

Wood: Reddish brown.

Roots: Shallow roots, Tap roots

Plant utilities: Flower and Garden Plant, Commercial, Medicinal Plant, Timber crop

Season: Perennial

Ecology: Understorey to subcanopy trees in evergreen forests, up to 1300 m (Gamble, 1997).

Major Threat: The wood is used for construction of ships, vehicles, posts and agricultural tools.

Uses of *A. polystachya*

1. Medicinal uses

The bark of this plant is used as an astringent. Ayurveda recommends the decoction of root bark in abdominal complaints like enlargement of glands, liver and spleen disorders and corpulence. Seeds: Refrigerant, laxative, anthelmintic; diseases of the blood, lessen muscular pain. Oil of the seeds is used to treatment of reumatism. Oil has pestisidal character. Folk people of Bangladesh massage their body parts with this oil before laid down into poluted water to prevent insect bite.Extract of bark, leaves and roots have antitumar activity. Ayurveda recommends Rohituka in ulcer, dyspepsia, intestinal worms, skin diseases, leprosy, diabetes, eye diseases, jaundice, haemorrhoids, burning sensation, arthritis and leucorrhoea. Special action: Digestive, carminative, depurative, diuretic. In Ayurveda, Rohitaka infusion mixed with honey is given internally to tackle skin diseases of a fast spreading nature (Sanyal & Datta, 1981). *Amoora rohituka* is use in many Drops, syrups, tablets. Indication: Hepatostimulant, Hepatoprotective and Hepatoregenerative. The herb *Amoora rohituka* (Rohitak) stimulates the liver to produce more bile which in turn relieves the congestion in the liver. *A. rohituka*. containing herbal medicine offers comprehensive coverage for virtually every manifestation of liver dysfunction. It is not only helps impaired functions within the liver, but also related conditions ranging from

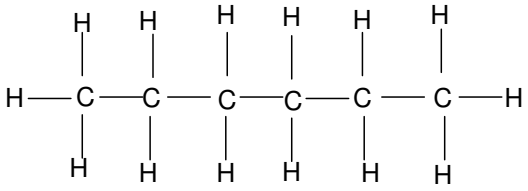
poor appetite to stunted growth and chronic constipation. In the presence of chronic liver disorders such as cirrhosis, *A. rohituka*. containing herbal medicine arrest further progression and salvage existing liver function. Clinically tried and trusted plusprin liver capsule containing 20mg of *Amoora rohituka* (Rohitak) is effective in hepatomegaly and protects against hepatotoxic agents. Rakt rohida (*Amoora rohituka*) liquor is used as a natural Herb for womb-cleansing (Sanyal & Datta, 1981).

2. Common uses

Boat building (general), Boat building: framing, Boxes and crates, Cabinetmaking, Canoes, Furniture, Heavy construction, Joinery, Light construction, Plywood, Poles, Roofing, Shingles, Textile equipment, Turnery.

Solvent System

1. *n*-hexane



Chemical structure of *n*-hexane

Applications

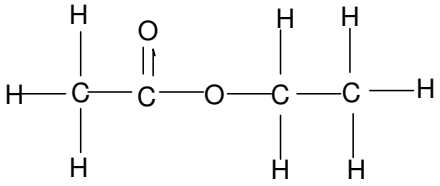
- *n*-hexane is widely used as cheap, relatively safe, largely unreactive, and easily evaporated non-polar solvent.
- It is used in the extraction of mainly lipophilic compounds from many plant extracts.

- It is also used as an alcohol denaturant and as a paint diluent.
- *n*-hexane is also used to extract oil from grains as well as protein from soy and hexane can persist in the final food product created (McCaine, 1990).

Table 2: Physical and chemical properties of *n*-hexane

Appearance	Colorless liquid
Molecular formula	C_6H_{14}
Molecular weight	86.10 g mol^{-1}
Density	0.660 g/cm^3
Boiling point	68.95°C
Melting point	-95.3°C
Vapor pressure	25°C
Solubility in water	13 mg/L at 20°C

2. Ethyl acetate



Chemical structure of Ethyl acetate

Applications

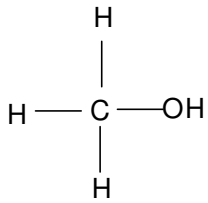
- Ethyl acetate is used as the solvent for various purposes in different sectors, e.g. as electroplating-vapor degreasing solvent, solvent for dilution and extraction in laboratories, solvents for Flexography and Gravure Printing, varnish solvent etc.

- Ethyl acetate is reasonably polar and it is chosen to extract a reasonably polar compound from a mixture, is like a good rule of thumb for extractions.

Table 3: Physical and chemical properties of ethyl acetate

Appearance	Colorless liquid
Molecular formula	$CH_3COOC_2H_5$
Molecular weight	88
Boiling point	77ºC (171ºF)
Melting point	-83ºC (-117ºF)
Vapor pressure (mm Hg)	76 at 20ºC (68ºF)
Solubility	1 ml/10ml water at 25ºC
Odor	Fruity odor
pH	Acidic and about 5-7

3. Methanol



Chemical structure of Methanol

Applications

- Methanol, a common laboratory solvent, is especially useful for HPLC, UV/VIS spectroscopy.
- The largest use of methanol by far is in making other chemicals. About 40% of methanol is converted to formaldehyde, and from there into products as diverse as plastics, plywood,

paints, explosives, and permanent press textiles.

- Other chemical derivatives of methanol include dimethyl ether, which has replaced chlorofluorocarbons as an aerosol spray and propellant, acetic acid. Dimethyl ether (DME) also can be blended with liquefied petroleum gas (LPG) for home heating and cooking, and can be used as a diesel replacement for transportation fuel (Blum, 2010).

Table 4: Physical and chemical properties of methanol

Physical state	Colorless liquid
Molecular formula	CH_3OH
Molecular weight	32.0419 g /mol
Boiling point	65°C, 338 K, 149°F
Melting point	−98--97°C, 175-176 K, -144--143°F
Vapor pressure	13.02 kPa at 20°C
Solubility	Miscible in water
Odor	Pungent
Reactivity	Flammable; may explode when exposed to flame
Lethal dosage LD_{50}	5628 mg/kg (oral dose for rats) (Verschueren, 1983)

Phytochemical Screening

The medicinal value of plants lies in some chemical substances that produce a definite physiologic action on the human body have a beneficial effect on health or play an active role in amelioration of diseases. In fact, some people claim that many of the diseases afflicting human beings are the result of lack of phytonutrients in their diet (Hill, 1952). Phytonutrients have

various health benefits, for example, they may have antimicrobial, anti-inflammatory, cancer preventive, antidiabetic and antihypertensive effects to mention but a few. The phytochemical constituent of a plant will often determine the physiological action on the human body (Pamplona-Roger, 1998). The most important of these bioactive constituents of plants are alkaloids, flavonoids, tannins, phenolic compounds etc. (Hill, 1952).

Knowledge of the chemical constituents of plants is desirable, not only for the discovery of therapeutic agents, but also because such information may be of value in disclosing new sources of such economic materials as tannins, oils, gums, precursors for the synthesis of complex chemical substances. In addition, the knowledge of the chemical constituents of plants would further be valuable in discovering the actual value of folkloric remedies (Mojab *et al*., 2003). Chemically constituents may be therapeutically active or inactive. The ones which are active are called active constituents and the inactive ones are called inert chemical constituents (Iyengar, 1995).

Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity20, and their absence in this plant tend to lower the risk of poisoning by the plant. Flavonoids have been shown to exhibit their actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2. Flavonoids serve as health promoting compound. Steroids which are very important compounds especially due to their relationship with compounds such as sex hormone 19. These phenolic compounds contribute to their anti-oxidative properties and thus the usefulness of these in herbal medicament. Phenols have been found to be useful in the

preparation of some antimicrobial compounds such as dettol and cresol (Shrivastava & Leelavathi, 2010). Saponins, which are present in plants, have been suggested as possible anti-carcinogens. They possess surface-active characteristics that are due to the amphiphilic nature of their chemical structure. The mechanisms of anticarcinogenic properties of saponins include direct cytotoxicity, immune-modulatory effects, bile acid binding and normalization of carcinogen-induced cell proliferation. Tannins are known to be useful in the treatment of inflamed or ulcerated tissues and they have remarkable activity in cancer prevention and anticancer (Shrivastava & Leelavathi, 2010).

Some methods for identification the chemical constituents are given in the following table: (Vinod *et a*l., 2010; Reuben *et a*l., 2008; Shrivastava & Leelavathi, 2010)

Table 5: Methods for identification the chemical constituents

Name of chemical constituents	Name of the methods
Alkaloids	Hager's Test
	Mayer's Test
	Dragendorff's test
	Wagner's test
Flavonoids	Shinoda Test
	Lead Acetate Test
	Sodium Hydroxide Test
	$FeCl_3$ test
	Pew's test
Steroids	Salkowski Test
	Leibermann's Reaction
	Libarman-Burchard's test

	Noller's test
Terpenoids	Salkowski Test
	Leibermann's Reaction
	Noller's test
Carbohydrates	Fehling's Test
	Benedict's test
	Molisch's test
	Burford's test
Saponin	Foam Test
	Frothing test
	NaOH solution
Tannins	$FeCl_3$ Solution Test:
	Lead Acetate Test
	Dil. HNO_3 Test
Cardiac glycosides	Borntrager's Test
	Legal's Test
	Keller-Killani Test
Anthraquinones	Chloroform layer test

Table 6: Some popular methods used for the detection of various phytochemicals

Chemical Constituents	**Test**
Alkaloid	Hager's test
Flavonoid	Ammonia test (modified)
Steroid	Salkowski test
Terpenoid	Salkowski test (modified)
Carbohydrates	Fehling's (Reducing sugar) test (modified)
Saponins	Frothing test
Tannins	$FeCl_3$ test

Cardiacglycoside	Killer-Killani's test
Anthraquinones	Chloroform layer test

Antioxidants

Anti-oxidants are substances that delay or inhibit oxidative damage to a target molecule, also capable to mop up free radicals and prevent them from causing cell damage. Antioxidants cause protective effect by neutralizing free radicals by donating one of their own electrons, ending the carbon-stealing reaction. Which are toxic byproducts of natural cell metabolism. The human body naturally produces antioxidants but the process is not 100 percent effective in case of overwhelming production of free radicals and that effectiveness also declines with age. Increasing the antioxidant intake can prevent diseases and lower the health problems. Phytoconstituents are important source of antioxidant and capable to terminate the free radical chain reactions. Antioxidants prevent cell and tissue damage as they act as scavenger (Sen *et al*., 2010) and thus help body fight against the pathophysiology of aging and a multitude of diseases, such as cancer, Alzheimer's disease and Parkinson's disease.

Free radical formation by oxygen

Oxygen has double-edged properties, being essential for life; it can also aggravate the damage within the cell by oxidative events. Free radicals and its adverse effects were discovered in the last decade. These are dangerous substances produced in the body along with toxins and wastes which are formed during the normal metabolic process of the body (Sen *et al*., 2010). Oxidative stress and impaired antioxidant system have been implicated in the pathophysiology of diverse disease states (Agbafor & Nwachukwu, 2011).

The body obtained energy by the oxidation of carbohydrates, fats and proteins through both aerobic and anaerobic process leads the generation of free radicals. Over production of the free radicals can responsible for tissue injury. Cell membranes are made of unsaturated lipids and these unsaturated lipid molecules of cell membranes are particularly susceptible to free radicals (Sen *et al*., 2010). Oxidative damage can direct to a breakdown or even hardening of lipids, which composition of all cell walls. Breakdown or hardening is due to lipid peroxidation leads to death of cell or it becomes unfeasible for the cell to properly get its nutrients or get signals to achieve another. In addition, other biological molecules including RNA, DNA and protein enzymes are also susceptible to oxidative damage (Sen *et al*., 2010).

Reactive oxygen-free radicals (ROS) have been implicated in many diseases and in aging process. These free radicals, which cause tissue damage via oxidative stress, are generated by aerobic respiration, inflammation, and lipid peroxidation. Antioxidant systems minimize or prevent deleterious effects of the ROS. High concentration of hydrogen peroxide is deleterious to cells, and its accumulation causes oxidation of cellular targets such as DNA, proteins, and lipids, leading to mutagenesis and cell death (Agbafor & Nwachukwu, 2011).

Environmental agents also initiate free radical generation leads different complication in body. The toxicity of lead, pesticides, cadmium, ionizing radiation, alcohol, cigarette smoke, UV light and pollution may all be due to their free radical initiating capability. A free radical may defined as a molecule or molecular fragments containing one or more unpaired electrons in its outermost atomic or molecular orbital and are capable of independent existence (Sen *et al*., 2010).

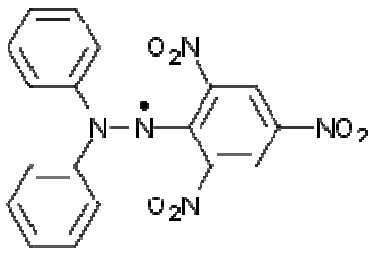
Problem of free radical

Free radical stress leads to a wide number of health problems which include tissue injury and progression of disease conditions such as arthritis, hemorrhagic shock, atherosclerosis, diabetes, hepatic injury, aging, ischemia, reperfusion injury of many tissues, gastritis, tumor promotion, neurodegenerative diseases and carcinogenesis. Safer antioxidants suitable for long term use are needed to prevent or stop the progression of free radical mediated disorders (Krishnaraju *et al*., 2009).

Free radical damage and oxidative stress are the major reasons for liver tissue damage. Lipid peroxidation is regarded as one of the basic mechanisms of tissue damage caused by free radicals. The antioxidants are the first-line defense against such damage and thus provide protection against the deteriorating outcome (Agbafor & Nwachukwu, 2011).

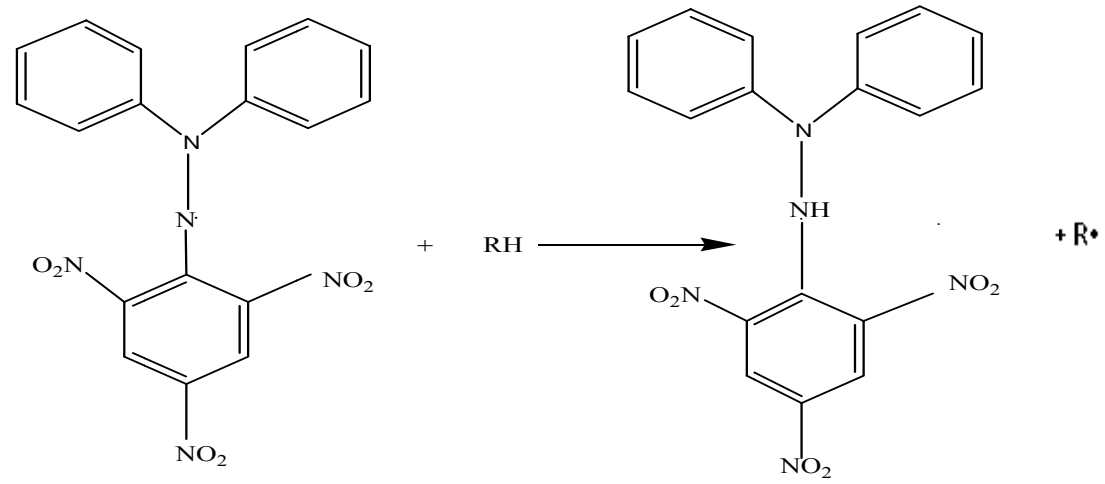
DPPH free radical scavenging assay

Free radical scavenging activity is evaluated with the spectrometric method where the DPPH reagent (2, 2-diphenyl-1-picrylhydrazyl), is mixed with serial dilutions of the extracts, is utilized to determine the antioxidant potential (Guerrero *et al*., 2004). DPPH free radical scavenging was first reported by Alexander Prokhorov in 1963.



DPPH (2,2-diphenyl-1-picrylhydrazyl)

The DPPH assay was used to measure the level of antioxidants in a substance. DPPH is a well-known radical and a trap ("scavenger") for other radicals. Therefore, rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction (Agbafor & Nwachukwu, 2011).



*DPPH (Oxidized form)

DPPH (Reduced form)

The structure of DPPH and its reduction by an antioxidant are shown above. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is deep violet in color. The odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H and the absorption reduces from 9660 to 1640 (Prakash *et al*.). The color turns from deep violet to pale yellow when neutralized. This property allows visual monitoring of the reaction. The absorbance is taken by UV-VIS spectrophotometer and methanol is taken as the solvent. Ascorbic acid is used as the standard (Agbafor & Nwachukwu, 2011).

Nitric oxide scavenging

The procedure is based on the method, where the compound sodium nitropruside is known to decompose in aqueous solution at physiological pH (7.2) and spontaneously generates nitric oxide (NO•). Under aerobic condition, NO• interacts with oxygen to produce stable products nitrate and nitrite ions, which can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with Naphthyl ethylenediamine dihydrochloride can be immediately read at 546 nm (Balakrishnan *et al*., 2009).

Estimation of total phenolic content

The antioxidative effect is mainly due to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes. The antioxidant activity of phenolic compounds is mainly due to their redox properties (Javanmardi *et al*., 2003).

Polyphenols recently have received increasing attention because of some interesting new findings regarding their biological activities. From pharmacological and therapeutic points of view, the antioxidant properties of polyphenols, such as free radical scavenging and inhibition of lipid per oxidation, are the most crucial. The antioxidant activity of apples is highly correlated to the total phenolic content (TPC) measured by the Folin-Ciocalteu method (Tsao *et al*., 2005).

Total phenolic content is determined by the Folin–Ciocalteu method, which was adapted from Swain and Hillis (1959) (Thaipong *et al*., 2006). The extracts oxidized with Folin-Ciocalteu reagent, and the reaction neutralized with sodium carbonate. The absorbance of the resulting blue color is measured at 765 nm (Hodzic *et al*., 2009). The Folin-Ciocalteu method is an electron

transfer (ET) based assay and measures reducing capacity, which has normally been used to expressed as phenolic contents of biological materials (Huang *et al*.,2005).

The Folin–Ciocalteu reagent (FCR) is a mixture of phosphomolybdate and phosphotungstate used for the assay of phenolic and polyphenolic antioxidants. It works by measuring the amount of the substance being tested needed to inhibit the oxidation of the reagent. However, this reagent does not only measure total phenols and will react with any reducing substance. The reagent therefore measures the total reducing capacity of a sample, not just the level of phenolic compounds [48]. Folin-Ciocalteau reagent is use for analysis of total phenol (Wangcharoen & Morasuk, 2007).

Thrombolytic Activity

Medicinal plants contain different therapeutic agents which may have thrombolytic activity. Atherothrombotic diseases such as myocardial or cerebral infarction occur as serious impacts of the thrombus formed in blood vessels. Acute coronary syndrome (ACS) patients are at increased risk of cardiovascular events, despite optimal antiplatelet medication. Thrombotic events depend on the propensity for thrombus formation and the efficacy of endogenous thrombolytic activity in preventing lasting arterial occlusion (Saraf et al., 2009).

Various thrombolytic agents are used to dissolve the clots that have already formed in the blood vessels. One of the major causes of blood circulation problem is the formation of blood clots. Thrombi or emboli can lodge in a blood vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. This can result in damage, destruction or even death of the tissues (necrosis) in that area. Thrombolytic therapy reduces mortality.

A blood clot (thrombus) is developed in the circulatory system due to failure of hemostasis causes vascular blockage which formed from fibrinogen by thrombin and is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator (tPA). Fribrinolytic drugs has been used to dissolve thrombi in acutely occluded coronary arteries there by to restore blood supply to ischaemic myocardium, to limit necrosis and to improve prognosis (Laurence & Bennett, 1992).

Commonly used thrombolytic agents are alteplase, anistreplase, streptokinase, urokinase and tissue plasminogen activator (tPA) to dissolve clots (Anwar *et al*., 2011). Streptokinase is an antigenic thrombolytic agent used for the treatment of acute myocardial infarction. It reduces mortality as effectively as the nonantigenic altreplase in most infarct patients while having the advantages of being much less expensive. Tissue-type Plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators (Khan *et al*., 2011). Streptokinase forms a complex with plasminogen (Figure 14) which then converts plasminogen to plasmin. Plasmin breaks down clots as well as fibrinogen and other plasma proteins (Banerjee *et al*., 2004).

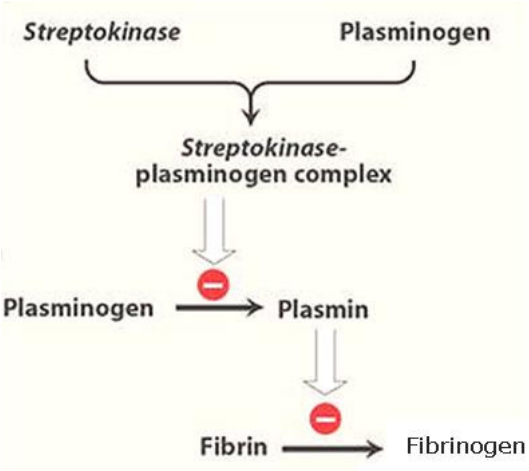


Figure 14: Mechanism of anticoagulation of streptokinase

All available thrombolytic agents still have certain significant shortcomings, including the need for large doses to be maximally effective, limited fibrin specificity and a significant associated bleeding tendency which cause serious and sometimes fatal consequences. All thrombolytic agents work by activating the enzyme plasminogen that clears the cross-linked fibrin mesh (Khan *et al*., 2011).

Thrombolytic drugs are widely used for the management of cerebral venous sinus thrombosis patients. During the last three decades of the 20th century, research activity in antithrombotic field was devoted to compounds showing antiaggregatory potency. Several drugs were explored, but except aspirin, among the huge number of synthetic molecules tested, only very few of them found a clinical use (Dupin *et al*., 2002).

Aspirin (acetyl salicylic acid) a potent inhibitor of platelet aggregation, is therapeutically used extensively both for the prevention and for the treatment of AIHD (acute ischemic heart disease). The inhibition of platelet aggregation by aspirin has been reported to be related to the inhibition of prostaglandin synthesis due to the inhibition of platelet cyclooxygenase (COX) by the compound. Recently it was reported that aspirin through its ability to stimulate NO synthesis in platelets can also inhibit platelet aggregation independent of the inhibition of COX. It is currently believed that the antithrombotic effect of aspirin is limited to the inhibition of platelet aggregation and this compound has no effect on the formed thrombus (Karmohapatra *et al*., 2007).

Ticlopidine [5-(2-chlorophenyl)-methyl]-4,5,6,7-tetrahydro-thieno[3,2-c] pyridine hydrochloride

was first used in 1978 and finds a broad scope of applications. The mechanism of its antiaggregant activity depends on its antagonism to ADP membrane platelet receptors. In 1996 it was first observed that ingestion of ticlopidine, at a dose of 500 mg by patients with peripheral arterial disease, was accompanied by an immediate fibrinolytic action, shown by shortening of euglobulin clot lysis time.

Another synthetic molecule, clopidogrel thieno [3,2-c] pyridine-5(4H)-acetic acid, a(2 chlorophenyl)-6,7-dihydro-,methyl ester,(*S*) is available since 1998 as an antiplatelet drug used in prevention or treatment of myocardial infarction and other diseases associated with atherosclerosis. Clopidogrel and ticlopidine are usually called antiplatelet thienopyridines. Their beneficial actions are linked with their ability to antagonise platelet ADP receptors. The difference between ticlopidine and clopidogrel chemical structure consists in the replacement of a CH_2 group by a $CH\text{-}COOCH_3$ group of atoms (Dupin *et al*., 2002).

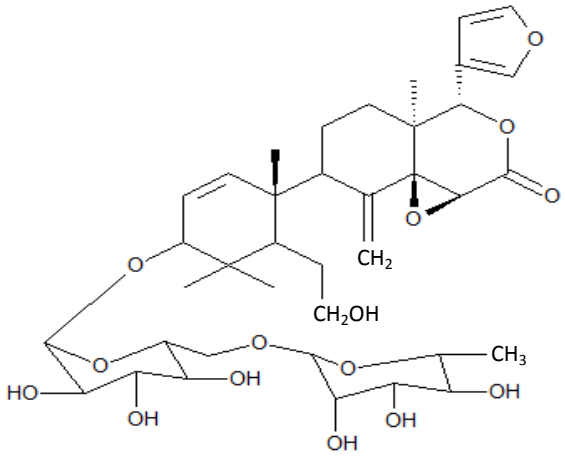
Purpose of the Present Study

The tests were done to find the presence of the active chemical constituents such as alkaloids, terpenoids, steroids, flavonoids, reducing sugar, tannin**,** saponins, cardic glycoside and anthraquinones. The antioxidant property of fresh leaves of *A. polystachya* use in the management and treatment of various diseases. The present study investigates the antioxidant and the total phenolic content property of the plant of *A. polystachya*. Herbs and their components can be use for thrombolysis and possessing antithrombotic activity has been reported before (Prasad *et al*., 2007). In this study, an attempt has been made to investigate whether *A. polystachya* leaves extracts possess thrombolytic activity or not.

LITERATURE REVIEW

Phytochemical Studies

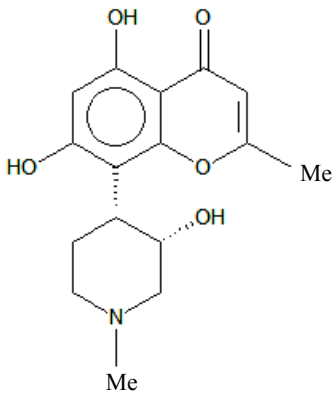
- The study of the plant *Amoorarohituka* has disclosed the presence of a class of limonoid, andirobin. The isolation and characterization of a new limonoid, Amoorinin on the basis of spectral and chemical methods (Agnihotri *et al*., 1987).



Amoorinin.

- Aphanamixin (XVI) and aphanamixinin (XXIX) structures have been derived from the studies of spectroscopic and chemical properties subsequently confirmed by their correlation with compounds of known structure and established stereochemistry (Chatterjee *et al*., 1970).

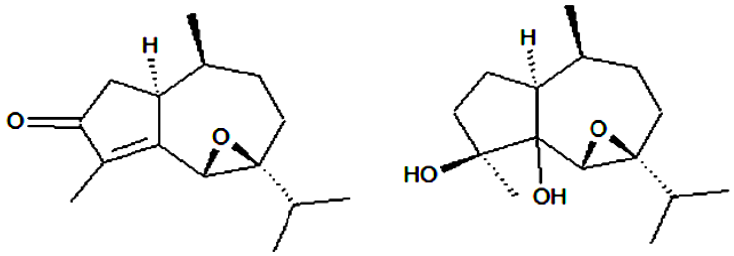
- Rohitukine, a chromane alkaloid, is a precursor of flavopiridol, a promising anti-cancer compound. Currently in Phase III clinical trials, flavopiridol is a potent inhibitor of several cyclin-dependent kinases (CDKs). Rohitukine was first reported from *Amoora rohituka* (0.083% dry weight) followed by that in *Dysoxylum binectariferum* (0.9% dry weight), both belonging to the family Meliaceae (Mohanakumara *et al*., 2010).



Rohitukine

- The structure of a new limonoid, amoorinin, from the stem bark of *Amoora rohituka* is presented on the basis of spectral and chemical evidences (Agnihotri *et al*., 1987).

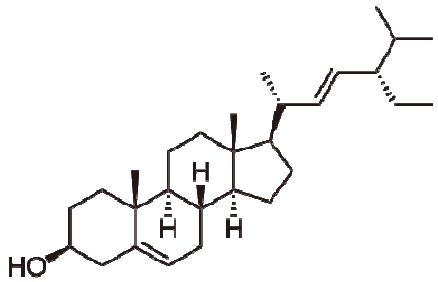
- The petroleum ether extract of the stem bark of *Amoora rohituka* afforded two novel guaiane-derived sesquiterpenoids, 6β, 7β-epoxyguai-4-en-3-one ***(1)*** and 6β, 7β-epoxy-4β, 5-dihydroxy guaiane ***(2)***. The structures ***1*** and ***2*** were determined by extensive NMR and MS analysis and by comparison of their spectral data with related compounds. The relative stereochemistry of the asymmetric centers in ***1*** and ***2***, except at C-5 of ***2***, were determined by selective 1D-NOESY experiments (Chowdhury *et al*., 2003).



1 *2*

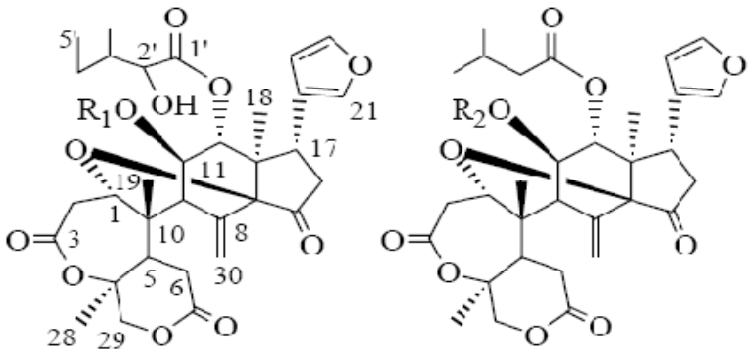
6β,7β-Epoxyguai-4-en-3-one (***1***) and 6β,7β-Epoxy-4β,5-dihydroxyguaiane (***2***)

- From an MeOH extract of the dried bark of *Aphanamixis polystachya* a new lignan, polystachyol, two lignan glycosides, lyoniside and nudiposide, and a sterol, ergosta-4,6,8(14),22-tetraen-3-one, with stigmasterol, and oleic and linoleic acids, have been isolated. The structures of the isolated compounds were elucidated by analysis of 1D and 2D NMR and mass spectroscopic data. The compounds did not have growth inhibitory activity against HeLa cells (Sadhu *et al*., 1980).



Stigmasterol

- From the seed of *Aphanamixis polystachya* (Meliaceae), a new limonoid named rohituka-15, with a known limonoid dregeana-1, was isolated. The ^{13}C NMR data assignment of dregeana-1 and the structural elucidation of the new compounds were based on spectral analysis including ^{1}H-^{1}H COSY, HMQC and HMBC experiments (Zhang *et al*., 2002).

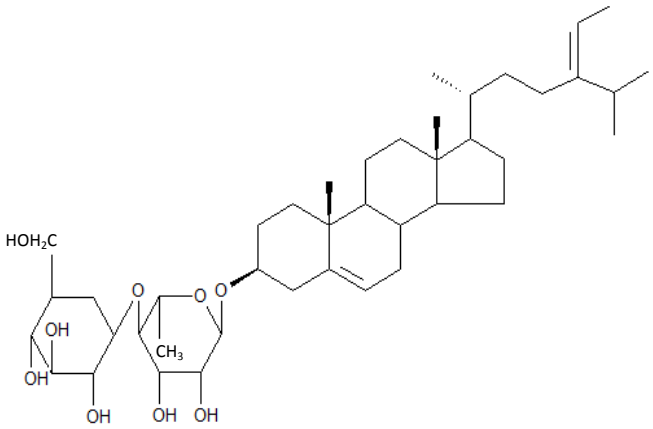


Compounds:- R_1 = H, Rohituka-15; R_1 = HCO, Dregeana-1; R_2 = H, Rohituka-12; R_2 = HCO, Polystachin

- Two novel limonoids, Aphanamolides A and B, along with a structurally related known limonoid, were isolated from the EtOH extract of the seeds of *Aphanamixis polystachya*. Aphanamolide A featured an unprecedented carbon skeleton via the formation of a C-3-C-6 bond. Aphanamolide A was isolated as white amorphous powders. The molecular formula was determined to be $C_{35}H_{44}O_{14}$. Compounds showed cytotoxic activity against two tumor cell lines (Yang *et al*., 2011).

- A number of glycosides were isolate from the seeds and bark of *Aphanamixis polystachya.* The seed oil was found to be comprised of the fatty acids: stearic, palmitic, oleic,α-linoleic, isomeric linoleic and α-linolenic acids. From the ethyl acetate extract of the seeds, new glycosides viz., 3′, 4′, 5′-trihydroxyflavonone-7-*O*-β-Dxylopyranosyl-β-D-arabinopyranoside25; dihydrorobinetin-7-*O*-β-D glucopyranosyl-*O*-α-L-rhamnopyranoside were isolated (Mabberley & Sing, 1931).

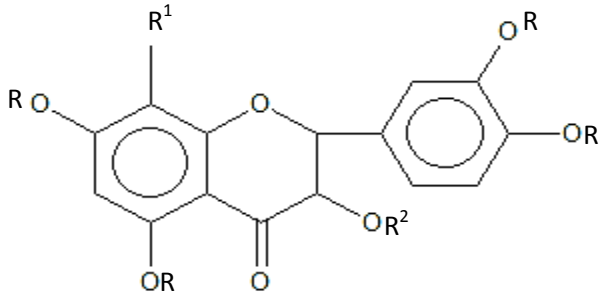
- Isolation of a new Saponin was done from the seeds of *Aphanamixis polystachya*. Phytochemical examination of the seeds of *Aphanamixis polystachya* resulted in the isolation and identification of a new saponin, stigmata-5,24(28)-dien-3-*O*-[β-d-glucopyranosyl-α-L-rhamnopyranoside] (Bhatt *et al*., 1981).



Stigmata-5,24(28)-dien-3-*O*-[β-D-glucopyranosyl-α-L-rhamnopyranoside]

- A new saponin poriferasterol-3-rhamnoside, isolate from the seeds of *Aphanamixis polystachya* (Bhatt *et al*., 1980).

- Isolation and characterization of a new flavone glycoside from the root of *Aphanamixis polystachya* whose structure is 8-C-methyl-quercetin-3-O-β-D-xylopyranoside have been reported (Jain & Srivastava, 1985).



8-C-methyl-quercetin-3-*O*-β-D-xylopyranoside; R = H, $R^1 = CH_3$, R^2=Xylose

- The structure of aphananin isolated from the fruits of *Aphanamixis polystachya* was established as 21,23*S*-epoxytirucall-7-ene-3β,21β,24,25-tetrol 3β-monoacetate from spectral

analysis and chemical transformations (Kundu *et al*., 1985).

- Two new highly oxidized A, B-*seco* limonoids, aphapolynins A and B, were isolated from the fruits of *Aphanamixis polystachya*. Their structures were elucidated by spectroscopic analysis; in particular, the absolute configuration of aphapolynin A was determined by a single-crystal X-ray study using a mirror Cu Kα radiation. Aphapolynin A exhibit moderate cytotoxicities when tested against a panel of tumor cell lines (Zhang *et al*., 2011).

Table 7: Summary of the Phytochemical Studies on *A. polystachya*

Plant part	Findings	Reference
Stem bark	Aphanamixin and Aphanamixinin	Chatterjee *et al*., 1970
Dried bark	Polystachyol, Lignan glycosides, Lyoniside, Nudiposide, a sterol	Sadhu *et al*., 1980
Seed	Flavanone glycoside	Bhatt *et al*., 1981
Seed	Saponin	Bhatt *et al*., 1981
Root	Flavone Glycoside	Jain & Srivastava, 1985
Fruits	Aphananin	Kundu *et al*., 1985
Stem bark	Amoorinin	Agnihotri *et al*., 1987
Seed	Rohituka-15	Zhang *et al*., 2002
Stem bark	Guaiane sesquiterpenes	Chowdhury *et al*., 2003
Whole plant	Rohitukine	Mohanakumara *et al*., 2010
Seed	Aphanamolides A and B	Yang *et al*., 2011
Fruits	Aphapolynins A and B	Zhang *et al*., 2011

Pharmacological Studies

- Free radical stress leads to tissue injury and progression of disease conditions such as arthritis, hemorrhagic shock, atherosclerosis, diabetes, hepatic injury, aging and ischemia, reperfusion injury of many tissues, gastritis, tumor promotion, neurodegenerative diseases and carcinogenesis. Safer antioxidants suitable for long term use are needed to prevent or stop the progression of free radical mediated disorders. Due to its natural origin and potent free-radical scavenging ability *Aphanamixis polystachya* could be used as a potential preventive intervention for free radical-mediated diseases (Krishnaraju *et al*., 2009).

- Preliminary phytochemical investigation revealed the presence of carbohydrates, saponins and triterpenoids in aqueous extract of *Aphanamixis polystachya*. Treatment with aqueous extract of *Aphanamixis polystachya* significantly showed the anti-ulcer activity as compared to control and other extracts. The histopathological study of the stomach also supported the above results. The results were comparable to that of standard drug (Omeprazole). From the literature survey and the work carried out, it may be confirmed that bark of *Aphanamixis polystachya* does possesses anti-ulcer property. Phytochemical investigation suggests the presence of saponins which may be responsible for the anti-ulcer activity (Shinkar, 2007).

- The petroleum ether, dichloromethane and methanol extracts of *Aphanamixis polystachya* demonstrated a good laxative potential at 250 and 400 mg/kg respectively and the data obtained after 1 h of drug administration were statistically significant. The petroleum ether and methanol extracts also showed significant gastrointestinal hypermotility following barium sulphate milk in mice. The data showed dose dependency and were well correlated with the findings of laxative screening. The crude extracts of *Aphanamixis polystachya* have

laxative principles comparable to those of a stimulant laxative, sennosideB (Chowdhury & Rashid, 2003).

- *Aphanamixis polystachya* protect against radiation-induced lethality, lipid peroxidation and DNA damage. The fractional guided evaluation may help to develop new radioprotectors of desired activities. The ethyl acetate fraction of *Aphanamixis polystachya* at a dose of 7.5 mg/kg b. wt. before exposure to 1–5 Gy of whole body gamma-radiation significantly reduced the frequencies of aberrant cells and chromosomal aberrations like acentric fragments, chromatid and chromosome breaks, centric rings, dicentrics, exchanges and total aberrations at all post-irradiation scoring times. It also showed a concentration dependent scavenging of hydroxyl, superoxide, 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radicals and the 2, 2-azino-bis-3-ethyl benzothiazoline-6-sulphonic acid (ABTS) cation radicals in vitro (Jagetia, 2007).

- The effect of radiation on tumor tissue can be optimized by adding radiosensitizing agents, in order to achieve a greater degree of tumor damage than expected from the use of either treatment alone. The ethanolic extract of *Aphanamixis polystachya* (APE) was tested in Swiss albino mice transplanted with Ehrlich ascites carcinoma (EAC) and exposed to various doses of gamma-radiation. The best effect of APE and radiation was observed for 6 Gy gamma-radiation. The APE treatment before irradiation elevated lipid peroxidation followed by a reduction in the glutathione contents. Treatment of tumor bearing mice with APE before irradiation further reduced the activities of various antioxidant enzymes like glutathione peroxidase, glutathione-s-transferase, superoxide dismutase and catalase at different post last

drug administration (PLDA) times (Jagetia & Venkatesha, 2005).

- Chemotherapeutic agents for cancer are highly toxic to healthy tissues and hence alternative medicine avenues are widely researched. Majority of the recent studies on alternative medicine suggested that *Amoora rohituka* possesses considerable antitumor and antibacterial properties. Rohituka fractionated with petroleum ether, dichloromethane, and ethanol, were explored for its anticancer potential against two breast cancer (MCF-7 and HTB-126) and three pancreatic cancers (Panc-1, Mia-Paca2, and Capan1). The human foreskin fibroblast, Hs68, was also included. Cytotoxicity of each extract was analyzed using the MTT assay and label-free photonic crystal biosensor assay (Chan *et al*., 2011).

- Normal tissue radiosensitivity is the major limiting factor in radiotherapy of cancer. The use of phytochemicals may reduce the adverse effects of radiation in normal tissue. The effect of ethyl acetate fraction of *Aphanamixis polystachya* (EAP) was investigated on the radiation-induced chromosome damage in the bone marrow cells of Swiss albino mice exposed to various doses of gamma-radiation. Irradiation of mice to different doses of gamma radiation caused a dose dependent elevation in the frequency of aberrant cells and chromosome aberrations like chromatid breaks, chromosome breaks, dicentrics, acentric fragments and total aberrations at all the post-irradiation times studied (Jagetia & Venkatesha, 2006).

- An invention describes 5-lipoxygenase inhibitory extracts or bio-enriched extracts or fractions of *Aphanamixis polystachya*, methods of making 5-lipoxygenase inhibitory extract, and methods of treating and preventing disease conditions mediated by 5-lipoxygenase. The

invention further discloses pharmaceutical or dietary compositions containing therapeutically effective amounts of the extracts of *Aphanamixis polystachya* in combination with other known anti-inflammatory agents useful for oral, parenteral and topical administration (Gokaraju *et al*., 2010).

- A crude ethanolic extract of the leaf of *Aphanamixis polystachya* shows a beneficial effect on toxic liver injury. Its antihepatotoxic activity was evaluated on carbon tetrachloride (CCl_4)-induced liver injury in a rat model. The assessment of hepatoprotective activity was evaluated by measuring the activities of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (ALP), acid phosphatase (ACP) and lactate dehydrogenase (LDH), serum total bilirubin and albumin and histology of the liver. The crude leaf extract significantly inhibits the enhanced ASAT, ALAT, ALP, ACP and LDH activities released from the CCl_4-intoxicated animals (Gole & Dasgupta, 2002).

- The investigation of the possible CNS depressant and analgesic action of the methanol extract of *Aphanamixis polystachya* leaf was done. Its CNS depressant activity was evaluated by using thiopental sodium-induced sleeping time, hole cross and open field tests. The analgesic activity was also investigated for its central and peripheral pharmacological actions using hot plate and tail immersion test and acetic acid-induced writhing test in mice respectively. The extract significantly maximized the duration of sleeping time when administered with thiopental sodium. The extract increased in pain threshold both in hotplate and tail immersion methods in a dose dependent manner. These results suggest that the extract possesses strong CNS depressant and analgesic activity in mice (Hossain *et al*., 2009).

- Amooranin, 25-hydroxy-3-oxoolean-12-en-28-oic acid, is a triterpene acid isolated from the stem bark of a tropical tree (*Amoora rohituka*). The cytotoxic effects of amooranin and its derivatives were studied. *A. rohituka* stem bark is used for the treatment of human malignancies. Amooranin and its methyl ester showed greater cytotoxicity against MCF-7 and HeLa cells derived from tumour tissues (Rabi *et al*., 2002). The mechanism of cell death was investigated associated with AMR cytotoxicity in human mammary carcinoma MCF-7, multidrug resistant breast carcinoma MCF-7/TH and breast epithelial MCF-10A cell lines. The induction of apoptosis in AMR treated cells was accompanied by the elevation of total caspase and caspase-8 activities (Rabi *et al*., 2003).

- The extracts of *Amoora rohituka* (stem bark), along with siderin, a major coumarin from T. ciliata, exhibited significant in vitro antibacterial activity. The extracts also demonstrated mild antifungal effect (Chowdhury *et al*., 2003).

- *Aphanamixis polystachya* bark was a strong astringent, used for the treatment of liver and spleen diseases, rheumatism and tumors. Antioxidant activity of the crude extracts of bark of *Aphanamixis polystachya* was assessed using NBT, DPPH, ABTS and FRAPS assays. The methanol, aqueous methanol and water extracts exhibited potent antioxidant activity compared to known antioxidants (Krishnaraju *et al*., 2009).

- The dried stem bark of *Aphanamixis polystachya* was extracted with alcoholic, hydroalcoholic and aqueous solvent. All three extracts were further fractionized in to the petroleum ether, ethyl acetate and n-butanol fractions and studied for *in vitro* antimicrobial

activity by Agar cup method using different bacterial strains in nutrient agar media. The extracts dose of 500 mcg/cup were used against the kanamycin, which was used as standard antimicrobial agent at the dose of 30 mcg/cup. The zone of inhibitions indicates that the extracts of dried stem bark of *Aphanamixis polystachya* showed significant antimicrobial activity as comparison to kanamycin (Yadav *et al*., 2010).

- The oil and 20% crude alkaloid solution from *Aphanamixis polystachya* seeds, both at 20 and 40 µl/disc, were tested for antimicrobial activities against human bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Shigella dysenteriae*, *Salmonella typhi*, *Bacillus cereus* and *B. subtilis*) and plant pathogenic fungi (*Alternaria alternata, Cochliobolus lunatus, Colletotrichum corchori, Fusarium equiseti, Macrophomina phaseolina, Drechslera oryzae* and *Botryodiplodia theobromae*). Among the bacteria, *B. cereus, E. coli* and *Staphylococcus aureus* showed mild sensitivity (13-14 mm) to the seed oil while the rest did not exhibit any degree of sensitivity. *S. aureus* and *E. coli* were mildly sensitive (15-16 mm) to the alkaloid solution. The oil and alkaloid solution showed antifungal activities in different degrees against all fungi tested. The highest growth inhibition exhibited by the seed oil (40.28 and 50.24% at 20 and 40 µl, respectively) was recorded for *D. oryzae*. The alkaloid showed the highest inhibition (71.47 and 78.87% at 20 and µl, respectively) on *M. phaseolina* (Bhuyan *et al*., 2000).

- *Aphanamixis polystachya* crude seed extracts were evaluated for their repellency, feeding deterrency, contact toxicity and oviposition deterrency to rice weevils. The extracts had strong repellent and feeding deterrent effects on rice weevils. The extracts were moderately

toxic to rice weevils. An ethanol extract was the most toxic and showed the lowest LD_{50} and LT_{50} values. The ground leaves, bark and seeds at a ratio of 2.5% mixture provided good protection for rice grains by reducing the F1 progeny emergence and the grain infestation rates (Howse, 1994).

- Seed extracts of *Aphanamixis polystachya* Wall and Parker (pithraj) were evaluated for their repellent, anti-feedant and contact toxicity to adults of *Tribolium castaneum*. The crude seed extracts were strong repellents and moderate feeding deterrents to *T. castaneum*. All extracts were toxic to beetles . The ethanol extract was the most toxic and showed the lowest LD_{50} value. Ground leaves, barks and seeds were also tested for oviposition deterrency to *T. castaneum*. The ground leaves, bark and seeds at a ratio of 2.5% mixture provided some protection for wheat flour by reducing F1 progeny (Talukder & Howse, 1995).

- Sub-fractions of an acetone extract of *Aphanamixis polystachya* seeds were evaluated for their feeding deterrent effects on adult *T. castaneum*. The results showed that a sub-fraction was highly deterrent to *T. castaneum* feeding. This fraction was isolated and analyzed by gas chromatography-mass spectrometry. Four compounds were identified: glycerol, 2-methoxy-2-hydroxy propanoic acid, 3-methyl-2-hydroxy pentanoic acid and 2,3,4-trihydroxy butanal. 2-methoxy-2-hydroxy propanoic acid might be the active compound against *T. castaneum* (Talukder & Howse, 2000).

Table 8: Summary of the pharmacological studies on *A. polystachya*

Plant part	Findings	Reference
Seed	Toxic & repellent properties	Howse, 1994
Seed	Repellent, antifeedant and contact toxicity	Talukder & Howse, 1995
Seed	Antimicrobial activity	Bhuyan *et al*., 2000
Seed	Feeding deterrent	Talukder & Howse, 2000
Seed	Deterrent and insecticidal effects	Talukder & Howse 2011
Leaf	Hepatoprotective activity	Gole & Dasgupta, 2002
Stem bark	Cytotoxic effect	Rabi *et al*., 2002
Stem bark	Laxative properties	Chowdhury & Rashid, 2003
Stem bark	Antimicrobial activity	Chowdhury *et al*., 2003
Stem bark	Anticancer activity on human breast carcinoma	Rabi *et al*., 2003
Whole plant	Anticancer activity on ehrlich ascites carcinoma	Jagetia & Venkatesha, 2005
Whole plant	Inhibiting molecular interactions between nuclear factors and target DNA sequences mimicking NF-kappa B binding sites	Lampronti *et al*., 2005
Stem bark	Reduction of radiation-induced chromosome injury	Jagetia & Venkatesha, 2006
Whole plant	Antiulcer activity	Shinkar, 2007
Leaf	CNS depressant and analgesic activity	Hossain *et al*., 2009
Bark	*In vitro* and *in vivo* antioxidant activity	Krishnaraju *et al*., 2009
Stem bark	Antimicrobial activity	Yadav *et al*., 2010
Whole plant	5-lipoxygenase inhibitory product	Gokaraju *et al*., 2010
Whole plant	Found effective as radical scavengers and inhibitors of lipid peroxidation	Talukder & Howse, 2011
Plant	Anticancer potential	Chan *et al*., 2011

MATERIALS AND METHODS

Collection and Identification of Plant Material

Healthy, disease free leaves of *Aphanamixis polystachya* were collected during summer in the month of July 2011 from Sher-e-Bangla Agricultural University, Dhaka, Bangladesh. For taxonomical identification, the specimen of the plant has been submitted in the National Herbarium, Mirpur, Dhaka, Bangladesh. Where identification of the plant was done by a spec ialist. Then sample specimen was deposited in the archive of the National Herbarium under the accession number DACB-35449 (Figure 15).

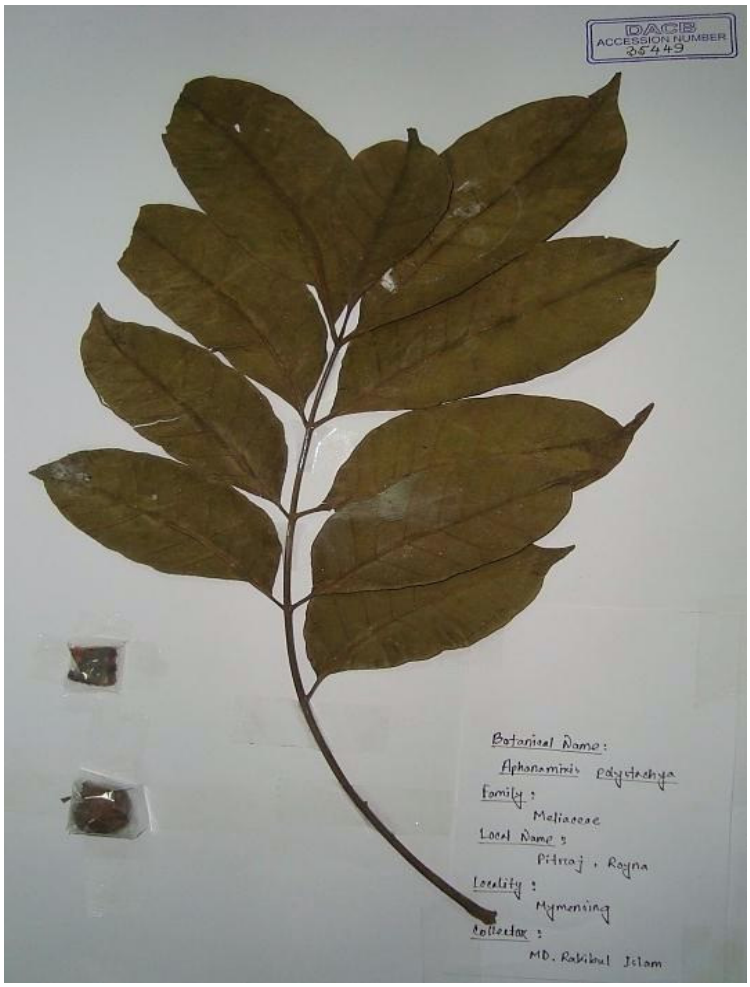


Figure 15: Identification plate of *A. polystachya*.

Materials

Table 9: Name of chemicals, equipments and glass apparatus for extraction

Chemicals	**Equipments**	**Glass apparatus**
n-hexane (Merck, Germany)	Balance (Shimadzu, Japan)	Beaker
Ethyl acetate (Merck, Germany)	Blender (Miyako, Japan)	Measuring cylinder
Methanol (Merck, Germany)	Soxhlet apparatus	Funnel
Dichloromethane (Sigma-Aldrich , Germany)	Rotary evaporator (IKA, Germany)	Conical flask
Acetone (Merck, Germany)	Hot air oven (NUVE)	

Method

Extraction of plant material

Thoroughly clean leaves were shade dried and then powdered (284.3 gm) with the help of blender. Then the powders were preserved for solvent extraction process. The powdered leaf was subjected to successive solvent extraction using solvents from nonpolar to polar like *n*-hexane, ethyl acetate and methanol. (141.5 gm x 2) of powdered leaf material were then packed in the soxhlet apparatus (Figure 16) and extracted with *n*-hexane, ethyl acetate and methanol successively.

Table 10: Time and temperature of extraction

Solvent	**Temperature**	**Time**
n-hexane	40˚C	1 h 57 min
Ethyl acetate	50˚C	3 h 31 min
Methanol	50˚C	2 h 48 min

Figure 16: Soxhlet apparatus

Figure 17: Extracts (before evaporation)

The extracts were transferred in conical flask and opening was wraped by aluminum foil and gelo tep. Then extracts were kept for evaporation to remove the excessive solvents (Figure 17).

Condensation of the leaf extracts

The extracts were transferred to the round bottle flask of rotary evaporator (Figure 18). Then excess amount of solvents in the extracts were removed by rotary evaporator, with reduced pressure which was done by using a vacuum pump. Following criterias (Table 11) were set for evaporation process.

Figure 18: Rotary evaporator

Table 11: Temperature, rpm and time of evaporation

Solvent	Temperature	rpm	Time
n-hexane	50˚C	120	25 min
Ethyl acetate	50˚C	120	1 h 7 min
Methanol	50˚C	120	1 h 7 min

After evaporation extracts were transferred in a beaker (Figure 19). Rest of the extract of leaf was removed from the round bottle flask by using dichloromethane. Then extracts were kept in hot air oven to get more dried extract. All beakers were covered with aluminum foil. The extracts were then collected and stored in a cool (4˚C) dry place for further assay.

Figure 19: Extracts (after evaporation)

Table 12: Characteristic of *A. polystachya*

Characteristics	***n*-hexane extract (APHE)**	**Ethyl acetate extract (APEA)**	**Methanol extract (APME)**
Extractive value	13.7 gm	21.9 gm	24.1 gm
Physical appearance	Semisolid mass	Semisolid mass	Semisolid mass
Color	Dark green	Dark green	Dark green
Odor	Aromatic	Aromatic	Aromatic
Taste	Bitter	Bitter	Bitter

Screening of Phytochemical Constituents

Phytochemical analysis of the extracts were conducted using the following procedures. The tests were carried out to find the presence of the active chemical constituents such as alkaloids, glycosides, terpenoids and steroids, flavonoids, reducing sugars and tannins.

Test for alkaloids: Hager's test

Materials

Table 13: Name of chemicals, equipments and glass apparatus for alkaloid test

Chemicals	**Equipments**	**Glass apparatus**
Methanol (Merck, Germany)	Balance (Shimadzu, Japan)	Volumetric flask
Acetone (Merck, Germany)	Vortex mixture (Bibby sterilin ltd., UK)	Test tubes
H_2SO_4 (BDH ltd., England)	Distillation apparatus (Hamilton	Beaker
Picric acid (Loba chemie, India)	laboratory, USA)	Funnel
		Pipette

Method

Preparation of 2% H_2SO_4 solution

2 ml of 1 N H_2SO_4 was taken in a volumetric flask. Volume was then adjusted to 100 ml using distilled water.

Preparation of Hager´s reagent

0.25 gm of picric acid was weighed in a volumetric flask. 25 ml of distilled water was added to prepare solution.

Procedure (Nobakht *et al*., 2010)

0.02 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed in 3 different test tubes. 10 ml of methanol was added in each test tube and test tubes were placed

in vortex mixture to dissolve the extracts. The extract solutions were then filtered. 2 ml of filtrate were taken and mixed with 4 drops of 2% H_2SO_4. To 1 ml of this mixture 6 drops of Hager´s reagent was added. Yellow (turbid) color indicates presence of alkaloids (Figure 20).

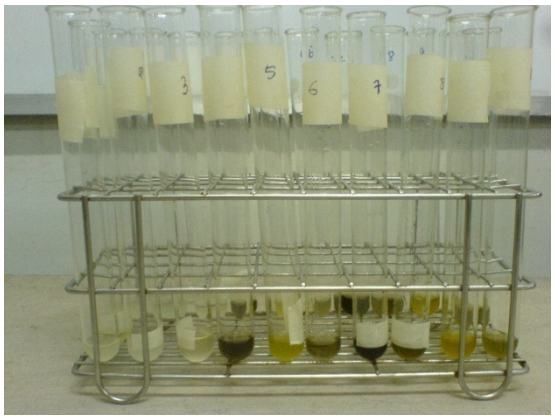


Figure 20: Identification of alkaloids by Hager's reagent

Test for flavonoids: Ammonia test (modified)

Materials

Table 14: Name of chemicals, equipments and glass apparatus for flavonoid test

Chemicals	**Equipments**	**Glass apparatus**
Ethyl acetate (Merck, Germany)	Balance (Shimadzu, Japan)	Volumetric flask
Ammonia (Loba chemie, India)	Water bath (Gemmy industrial corp.,	Test tubes
Acetone (Merck, Germany)	Taiwan)	Funnel
		Pipette

Method

Preparation of 5% ammonia solution

5 ml of ammonia was transferred in a volumetric flask. Volume was then adjusted to 100 ml

using distilled water.

Procedure (Edeoga *et al*., 2005)

0.25 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed in 3 different test tubes. 5 ml of ethyl acetate was added in each test tube. Then test tubes were heated at 40˚C for 3 min in water bath. The mixtures were filtered and 2 ml of filtrate were taken. 0.5 ml of 5% ammonia solution was added. Yellow color shows the presence of flavonoid (Figure 21).

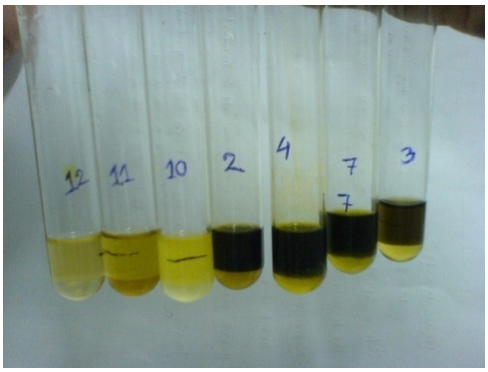


Figure 21: Identification of flavonoids by ammonia test

Test for steroids: Salkowski test

Materials

Table 15: Name of chemicals, equipments and glass apparatus for steroid test

Chemicals	**Equipments**	**Glass apparatus**
Methanol (Merck, Germany)	Balance (Shimadzu, Japan)	Test tubes
Chloroform (Merck, Germany)	UV plate (Whatman biometra, Germany)	Pipette
H_2SO_4 (BDH, England)		Funnel
Acetone (Merck, Germany)		

Method

Procedure (Nobakht *et al*., 2010)

0.02 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed in 3 different test tubes. The extracts were mixed with 2 ml of methanol and filtered. 1 ml chloroform and 1 ml concentrated H_2SO_4 were added into the filtrate. Yellow green fluorescent indicates the presence of steroids (Figure 22).

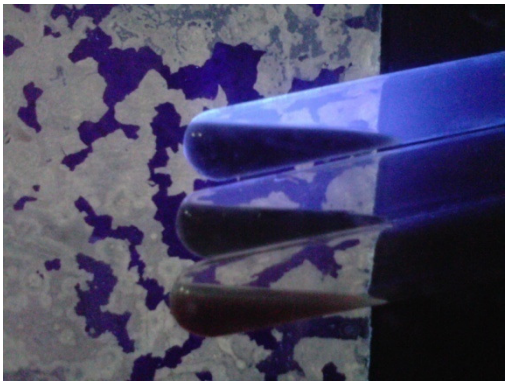


Figure 22: Identification of steroids by salkowski test

Test for terpenoids: Salkowski test (modified)

Materials

Table 16: Name of chemicals, equipments and glass apparatus for terpenoid test

Chemicals	**Equipments**	**Glass apparatus**
Acetic anhydride (BDH, England)	Balance (Shimadzu, Japan)	Test tubes
Chloroform (Merck, Germany)		Pipette
H_2SO_4 (BDH, England)		
Acetone (Merck, Germany)		

Method

Procedure (Chhetri *et al*., 2008)

0.004 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed in 3 different test tubes. The extracts were treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. 3 ml concentrated H_2SO_4 were added slowly. Red violet color represents the presence of terpenoid (Figure 23).

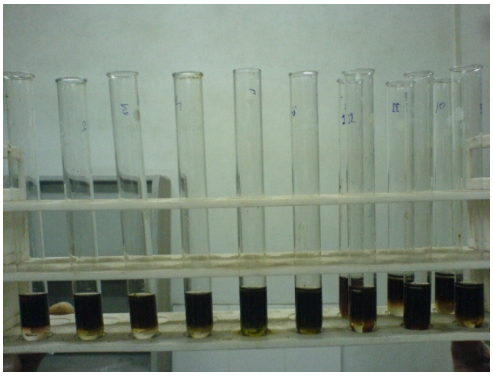


Figure 23: Identification of terpenoids by salkowski test

Test for carbohydrates: Fehling's (Reducing sugar) test (modified)

Materials

Table 17: Name of chemicals, equipments and glass apparatus for carbohydrate test

Chemicals	**Equipments**	**Glass apparatus**
Methanol (Merck, Germany)	Balance (Shimadzu, Japan)	Test tubes
Fehling's solution		Pipette
Acetone (Merck, Germany)		Conical flask
H_2SO_4 (BDH, England)		

Method

Preparation of Fehling I solution

3.436 gm copper II sulphate crystals (Merck, Germany) in water containing a few drops of dilute H_2SO_4 (5-10%) was dissolved in 20 ml distilled water. Final volume of solution was made upto 50 ml.

Preparation of Fehling II solution

6 gm of pure NaOH (Merck, Germany) and 17.1 gm of Na-K tartrate (BDH chemicals ltd., England) was dissolved in 20 ml of distilled water. Final volume of solution was made up to 50 ml.

Preparation of Fehling solution

5 ml of Fehling I solution and 5 ml of Fehling II solution was taken in a conical flask and mixed properly.

Procedure (Chhetri *et al*., 2008)

0.02 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed in 3 different test tubes. The extracts were dissolved in 0.5 ml of methanol. Then 1 ml of water was added in it. 5-8 drops of Fehling solution were added. The samples were heated. Carbohydrate present or absent depends on brick red precipitate.

Test for saponins: Frothing test

Materials

Table 18: Name of chemicals, equipments and glass apparatus for saponin test

Chemicals	**Equipments**	**Glass apparatus**
Olive oil (Acesur, Spain)	Balance (Shimadzu, Japan)	Test tubes
Acetone (Merck, Germany)	Vortex mixture (Bibby sterilin, UK)	Pipette

Method

Procedure (Ayoola *et al*., 2008)

0.5 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed in 3 different test tubes. 5 ml of distilled water was added. The solutions were shaken vigorously and observed for a stable persistent froth. The frothing were mixed with 3 drops of olive oil and shaken vigorously. An emulsion formation indicates the presence of saponin (Figure 24).

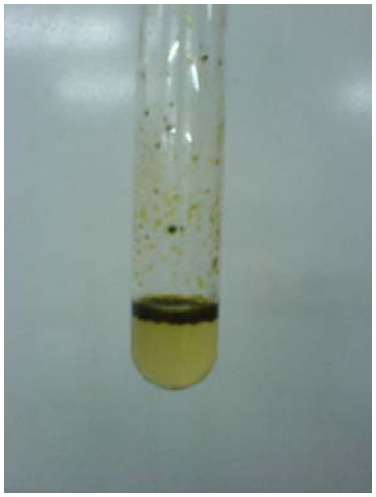


Figure 24: Identification of saponins by frothing test

Test for tannins: $FeCl_3$ test

Materials

Table 19: Name of chemicals, equipments and glass apparatus for tannin test

Chemicals	**Equipments**	**Glass apparatus**
Ferric chloride (Loba chemie, India)	Balance (Shimadzu, Japan)	Test tubes
	Vortex mixture (Bibby sterilin, UK)	Pipette
Acetone (Merck, Germany)	Water bath (Gemmy industrial corp., Taiwan)	Funnel

Method

Preparation of 0.1% ferric chloride solution

0.1 gm of ferric chloride was accurately weighed in a 100 ml volumetric flask and it was dissolved in 50 ml of distilled water. Final volume was made upto 100 ml.

Procedure (Edeoga *et al*., 2005)

0.125 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed in 3 different test tubes. 5 ml of distilled water was added and dissolved by vortex mixture. Then samples were boiled for 3 min. in water bath. The samples were filtered. 3 drops of 0.1% ferric chloride solution were added into the filtrate. Brownish green or blue-black colouration shows the presence of tannin (Figure 25).

Figure 25: Identification of tannins by $FeCl_3$ test

Test for cardiac glycosides: Killer-Killani's test

Materials

Table 20: Name of chemicals, equipments and glass apparatus for cardiac glycoside test

Chemicals	Equipments	Glass apparatus
Methanol (Merck, Germany)	Balance (Shimadzu, Japan)	Test tubes
Glacial acetic acid (Merck, Germany)	Vortex mixture (Bibby sterilin, UK)	Pipette
Ferric chloride (Loba chemie, India)		
H_2SO_4 (BDH, England)		
Acetone (Merck, Germany)		

Method

Procedure (Edeoga *et al*., 2005)

0.02 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed in 3 different test tubes. 5 ml of methanol was added in each test tube, and dissolved by vortex mixture. Then the extract solutions were treated with 2 ml of glacial acetic acid containing 3 drops of ferric chloride solution. They were underlayed with 1 ml of concentrated H_2SO_4. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides (Figure 26).

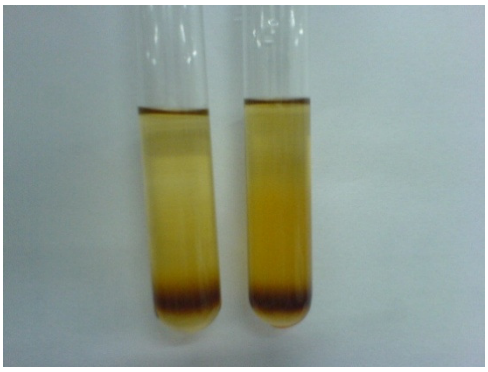


Figure 26: Identification of cardiac glycosides by Killer-Killani's test

Test for anthraquinones: Chloroform layer test

Materials

Table 21: Name of chemicals, equipments and glass apparatus for anthraquinone test

Chemicals	**Equipments**	**Glass apparatus**
H_2SO_4 (BDH, England)	Balance (Shimadzu, Japan)	Test tubes
Chloroform (Merck, Germany)	Water bath (Gemmy industrial corp.,	Pipette
Ammonia (Loba chemie, India)	Taiwan)	Funnel
Acetone (Merck, Germany)		

Method

Procedure (Ayoola *et al*., 2008)

0.5 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed in 3 different test tubes. 10 ml of H_2SO_4 were added in each test tube. The samples were kept in water bath for 3 min. to boil. Then they were filtered while hot. Filtrates were shaken with 5 ml of chloroform. Chloroform layer were pipette into another test tube. 1 ml dilute ammonia was added. Resulting solutions were observed for color changes.

Antioxidant Test with DPPH

Materials

Table 22: Name of chemicals, equipments and glass apparatus for DPPH assay

Chemicals	**Equipments**	**Glass apparatus**
DPPH (Sigma-Aldrich,	Balance (Shimadzu, Japan)	Volumetric flask
USA)	Micropipette (Eppendorf, Germany)	Test tubes
Methanol (Merck, Germany)	UV-VIS spectrophotometer (Shimadzu,	Beaker
Acetone (Merck, Germany)	Japan)	Funnel
	Vortex mixture (Bibby sterilin, UK)	Pipette

Method (Feresin *et al*., 2002)

Preparation of DPPH reagent

0.008 gm of DPPH was accurately weighed and dissolved in 20 ml of methanol. The reagent was then kept in dark place for 30 min. The conc. of this solution was 400 µg/ ml.

Preparation of extract solution

0.005 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed in 3 different volumetric flasks and dissolved in methanol by vortex mixture. Then the volume was made upto 50 ml with methanol. The extract solutions were filtered by using filter paper and the filtrate was collected. The conc. of each solution was 100 µg / ml.

Procedure

1, 2, 3, 4, 5 ml filtrate of plant extract solutions were transferred in 5 test tubes. The volume was then adjusted to 5 ml by adding methanol and the absorbances were taken at 517 nm. Again, in another 5 test tubes 1, 2, 3, 4, 5 ml filtrates were transferred. Conc. of the test solutions in different test tubes were 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml respectively. In each test tube 100 µl of DPPH reagent was added (Figure 27) and mixed properly. Then the solution was kept in dark place for 20 min. After 20 min. the absorbance were taken at 517 nm using UV-VIS spectrophotometer (Figure 28). The radical scavenging activity was expressed as the percentage inhibition calculated using the following formula:

$$\%\text{inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Then % inhibitions were plotted against respective concentrations used and from the graph IC_{50} values were calculated. Ascorbic acid, a potential antioxidant was used as positive control.

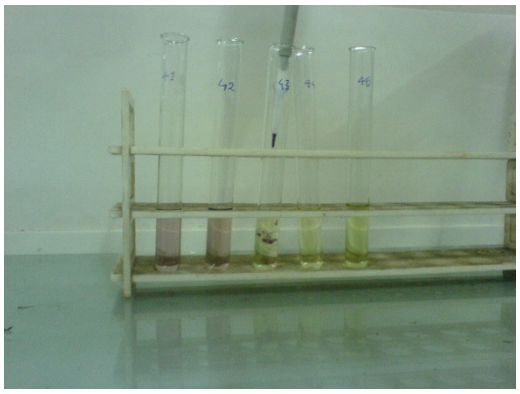


Figure 27: DPPH reagent added in extracts

Figure 28: UV-VIS spectrophotometer

Nitric Oxide Scavenging Capacity Assay

Materials

Table 23: Name of chemicals, equipments and glass apparatus for nitric oxide scavenging assay

Chemicals	**Equipments**	**Glass apparatus**
Sodium nitroprusside (Merck, Germany)	Balance (Shimadzu, Japan)	Volumetric flask
Sulfanilamide (Loba Chemie, India)	UV-VIS spectrophotometer	Test tubes
o-phosphoric acid (Merck, Germany)	(Shimadzu, Japan)	Beaker
N-(1-Naphthyl ethylenediamine	Vortex mixture (Bibby	Funnel
dihydrochloride) (Merck, Germany)	sterilin, UK)	Pipette
Ethanol (BDH, England)		
Acetone (Merck, Germany)		

Method (Balakrishnan *et al*., 2009; Hoque *et al*., 2011)

Preparation of 5 mM solution of sodium nitroprusside

0.075 gm of Sodium nitroprusside was accurately weighed in 50 ml volumetric flask which was dissolved in distilled water. Then the volume was made upto 50 ml with distilled water. The absorbance of this solution was taken at 546 nm as a control solution absorbance against distilled

water as a blank.

Preparation of Griss reagent

0.5 gm Sulfanilamide, 0.05 gm N-(1-Naphthyl ethylenediamine dihydrochloride), were accurately weighed and taken in a volumetric flask. 1 ml o-phosphoric acid was added into the volumetric flask and mixed properly. Then volume was adjusted by distilled water upto 50 ml.

Preparation of stock of extract solution

0.006 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed in 3 different volumetric flasks and it was dissolved in 30 ml of ethanol by vortex mixture. Conc. of each extract solution was 200 µg / ml.

Procedure

The procedure was carried out in 3 steps. In each steps 5 test tubes were used.

Step 1: From the stock solutions 10, 5, 2.5, 1.5, 0.5 ml solutions were transferred to the 5 test tubes marked as 1, 2, 3, 4, and 5. Then volume was adjusted to 10 ml by distilled water. Conc. of the test solutions in different test tubes were 200 µg/ml, 100 µg/ml, 50 µg/ml, 30 µg/ml and 10 µg/ml respectively. Then absorbances were taken at 546 nm.

Step 2: 4 ml of solution was transferred from each previous test tube to another 5 test tubes. 1 ml of sodium nitroprusside was added into each test tube and mixed properly (Figure 29). Then the test tubes were kept in dark for 2 h.

Step 3: After 2 h 2 ml solutions were transferred from each previous test tube to another 5 test tubes accordingly. 1.2 ml of Griss reagent was added in this test tube and mixed properly. Then absorbances were taken at 546 nm using UV-VIS spectrophotometer. The nitric oxide scavenged (%) activity was calculated from the following equation:

$$\text{Nitric oxide scavenged (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

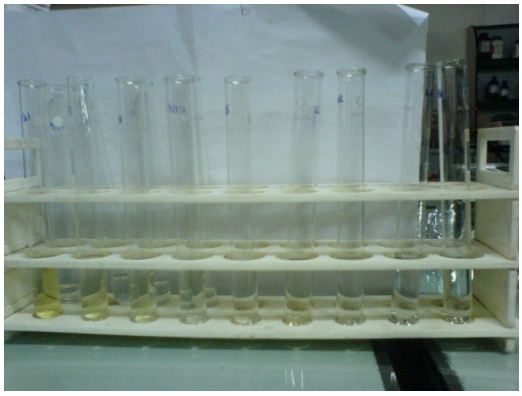


Figure 29: Extract Solutions

Estimation of Total Phenolic Content

Materials

Table 24: Name of chemicals, equipments and glass apparatus for the estimation of total phenolic content

Chemicals	**Equipments**	**Glass apparatus**
Methanol (Merck, Germany)	Balance (Shimadzu, Japan)	Volumetric flasks
Folin-Ciocalteu reagent (Merck, Germany)	Micropipette (Eppendorf, Germany)	Test tubes
Na_2CO_3 (Merck, Germany)	Centrifuge machine (Hettich, Germany)	Screw cap test tubes
Acetone (Merck, Germany)	Vortex mixture (Bibby sterilin, UK)	Pipette
	UV-VIS spectrophotometer (Shimadzu, Japan)	Beaker

Table 25: Composition of Folin-Ciocalteu Reagent

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

Method (Ainsworth & Gillespie, 2007)

Preparation of solutions of plant extracts

0.02 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed in 3 different centrifuge test tubes. 2 ml of methanol was added in each test tube. The extracts were dissolved using vortex mixture. It was kept at room temperature for 48 h in dark.

Preparation of 10% Folin-Ciocalteu phenol reagent

5 ml of F-C reagent was transferred in a volumetric flask. Volume was then adjusted to 50 ml using distilled water.

Preparation of 700 mM Na_2CO_3 solution

7.416 gm of Na_2CO_3 was accurately weighed in a volumetric flask. 100 ml of distilled water was added and dissolved.

Procedure

After 48 h test tubes were centrifuge in 5000 rpm for 5 min. at room temperature. After centrifuge the sample supernatant were transferred in other test tubes. 300 µl sample supernatant

was withdrawn from each plant extract solution to a separate test tube. 300 µl of methanol (blank) was taken as negative control to a separate test tube. 600 µl of 10% F-C reagent solution were added in each test tubes and vortex thoroughly. 2.4 ml of 700 mM Na_2CO_3 solution was added in each test tube (Figure 30) and it was kept at room temperature for 2 h. The absorbance of total volume (3.3 ml) was taken in UV-VIS spectrophotometer at 765 nm. Steps, from transferring 300 µl sample supernatant to taking the absorbance this were done for three times, accordance to constitutive interval. The total phenolic contents were determined from a standard curve prepared with gallic acid and the results were expressed as Mean±SD.

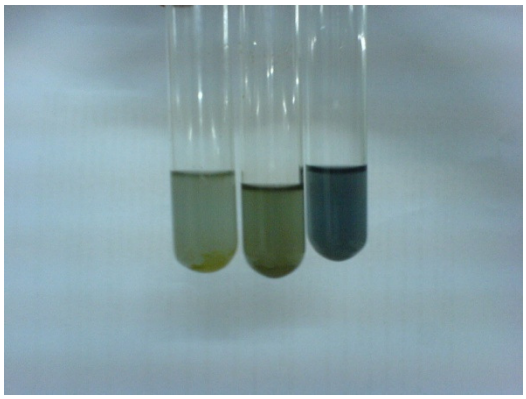


Figure 30: Reagent added in extracts solution

In Vitro Thrombolytic Activity Test

Materials

Table 26: Name of chemicals, equipments and glass apparatus for thrombolytic activity test

Chemicals	Equipments	Glass apparatus
Saline (0.95% NaCl) (Opsonin infusion, Bangladesh)	Balance (Shimadzu , Japan)	Test tubes
Streptokinase (CSL Behring	Vortex mixture (Bibby sterilin, UK)	Beaker
	Incubator (EHRET Laboratory and	

GmbH , Germany)	Pharmatechnical, Germany)
Acetone (Merck , Germany)	Micropipette (Eppendorf , Germany)

Method (Khan *et al*., 2011)

Preparation of stock

5 ml saline (Figure 31) was added to streptokinase vial (15, 00,000 I.U) and mixed properly.

Preparation of extracts solutions

0.1 gm of *n*-hexane, ethyl acetate and methanol extracts of *A. polystachya* leaves were weighed and suspended in 10 ml of saline (Figure 32) and it was shaked vigorously on vortex mixture. It was kept for overnight. After that the solutions were filtered and the filtrate was collected.

Figure 31: Saline taken

Figure 32: Saline added in extracts

Preparation of blood sample

Venous blood was collected from healthy human volunteers (n = 10) without having of oral contraceptives or anticoagulant therapy where male = 5 and female = 5. 500 µl of blood was transferred to previously weighed in 50 Eppendorf tubes. The tubes then kept for incubation to the incubator for 2 h at 37˚C. After clot formation (Figure 33), serum was removed completely

without disturbing the clot (Figure 34). Again weight was taken of each tube with clot. Clot weight was determined for each tube separately.

Clot weight = wt. of clot containing tube – wt. of tube alone

Figure 33: Clot formation

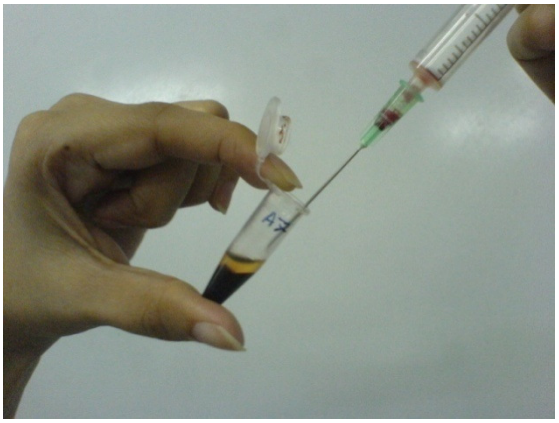


Figure 34: Serum withdrowing

Procedure

100 µl of extract solution was added to each tube containing clot (Figure 35). For positive control 100 µl of streptokinase and for negative control 100 µl of normal saline was added. Again all tubes were incubated for 90 min at 37˚C and it was observed for clot lysis. After incubation release fluid was removed carefully (Figure 36). Again each tubes weighed was taken to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

$$\%\ \text{of clot lysis} = \frac{\text{clot wt.} - \text{wt. of released clot}}{\text{clot wt.}} \times 100$$

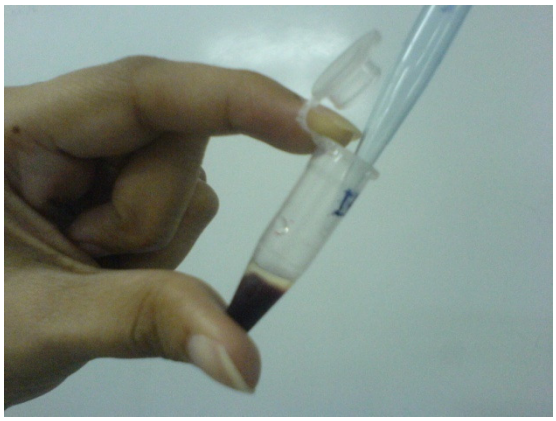


Figure 35: Extract added in clot

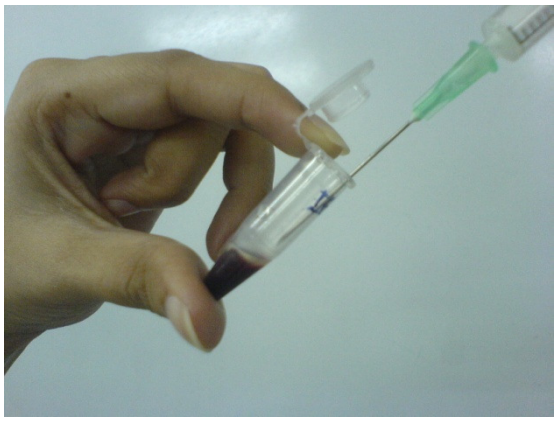


Figure 36: After clot lysis serum withdrowing

Statistical analysis: Statistical comparisons were performed with Student's '*t*' tests using Microsoft Excel 2007. A *p* value of 0.001 or less was considered to be significant. Mean values ± S.D. were calculated for the parameters where applicable.

RESULTS

Phytochemical Screening

Table 27: Data of phytochemical screening of *A. polystachya*

S.No	Chemical Constituents	Test	APHE	APEA	APME
1	Alkaloids	Hager's test	+	+	++
2	Flavonoids	Ammonia test (modified)	++	+	++
3	Steroids	Salkowski test	–	–	–
4	Terpenoids	Salkowski test (modified)	+++	+++	+++
5	Carbohydrates	Fehling's (Reducing sugar) test (modified)	_	_	_
6	Saponins	Frothing test	–	+	–
7	Tannins	$FeCl_3$ test	–	–	+
8	Cardiacglycosides	Killer-Killani's test	++	+	+
9	Anthraquinones	Chloroform layer test	+++	+	+

Key:

– = Negative (absent); + = Positive (slightly present); ++ = Positive (moderately present); +++ = Positive (strongly present)

Phytochemical screening of the plant *A. polystachya* leaf extracts (APHE, APEA and APME) revealed the presence of various bioactive components. Alkaloids, flavonoids, terpenoids, saponins, tannins, cardiac glycosides and anthraquinones were the most prominent (Table 27).

DPPH Free Radical Scavenging Assay

Table 28: IC_{50} value of ascorbic acid (AA)

Test tube no.	Conc.(µg/ml)	Absorbance of AA	Absorbance of blank	% inhibition	Value of IC_{50}
0	0	0.197	0.197	0	
1	20	0.132	0.197	32.99	
2	40	0.119	0.197	39.59	76.11
3	60	0.107	0.197	45.69	
4	80	0.099	0.197	49.75	
5	100	0.086	0.197	56.35	

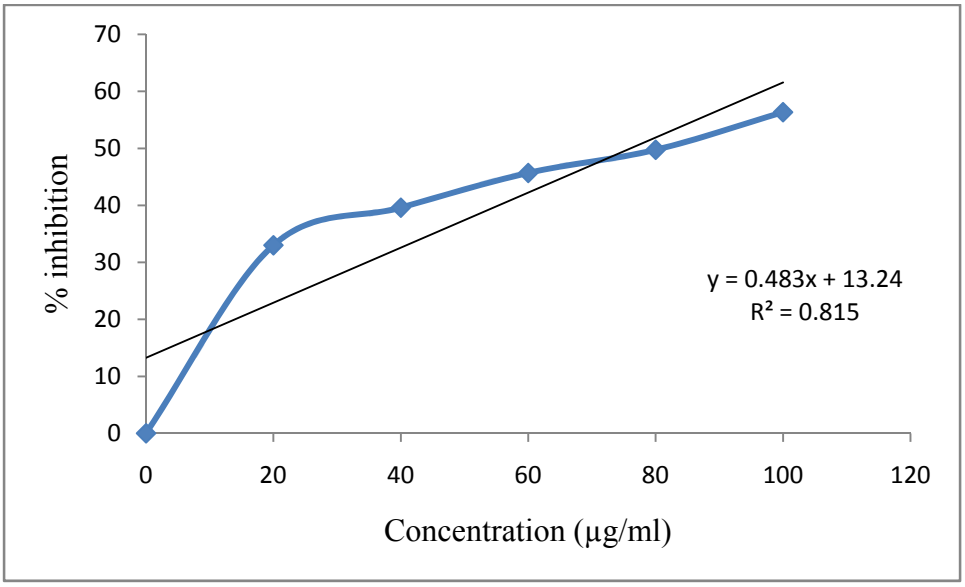


Figure 37: % inhibition curve of ascorbic acid (AA)

Table 29: IC_{50} value of the leaf of APHE

Test tube no.	Conc.(µg/ml)	Absorbance of extract	Absorbance of blank	% inhibition	Value of IC_{50}
0	0	0.197	0.197	0	
1	20	0.118	0.197	40.10	
2	40	0.113	0.197	42.64	74.84
3	60	0.110	0.197	44.16	
4	80	0.093	0.197	52.79	
5	100	0.090	0.197	54.31	

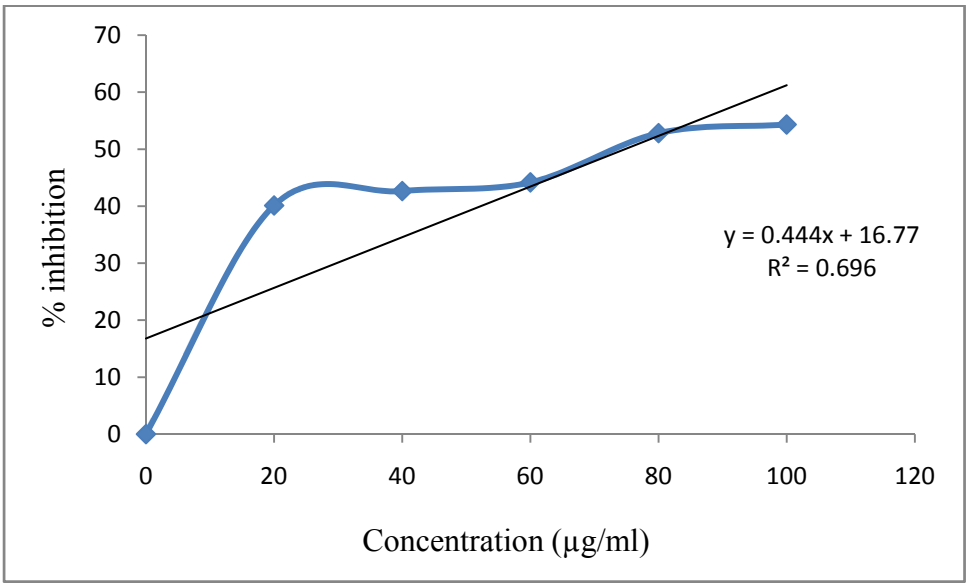


Figure 38: Scavenging effects of the leaf of APHE

Table 30: IC_{50} value of the leaf of APEA

Test tube no.	Conc.(µg/ml)	Absorbance of extract	Absorbance of blank	% inhibition	Value of IC_{50}
0	0	0.197	0.197	0	
1	20	0.092	0.197	53.30	
2	40	0.099	0.197	49.75	50.07
3	60	0.079	0.197	59.90	
4	80	0.052	0.197	73.60	
5	100	0.072	0.197	63.45	

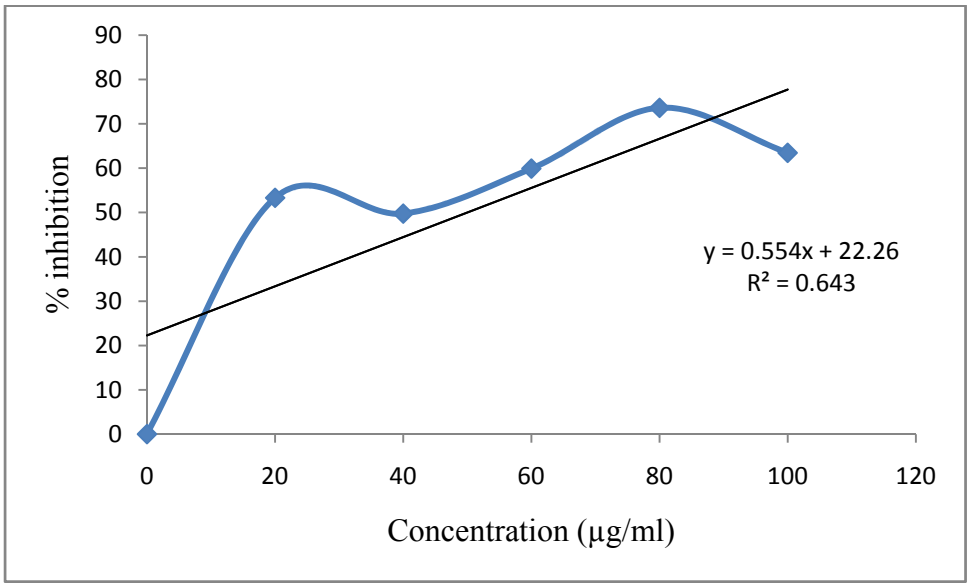


Figure 39: Scavenging effects of the leaf of APEA

Table 31: IC_{50} value of the leaf of APME

Test tube no.	Conc.(µg/ml)	Absorbance of extract	Absorbance of blank	% inhibition	Value of IC_{50}
0	0	0.197	0.197	0	
1	20	0.013	0.197	93.40	
2	40	0.009	0.197	95.43	12.54
3	60	0.007	0.197	96.45	
4	80	0.007	0.197	96.45	
5	100	0.003	0.197	98.48	

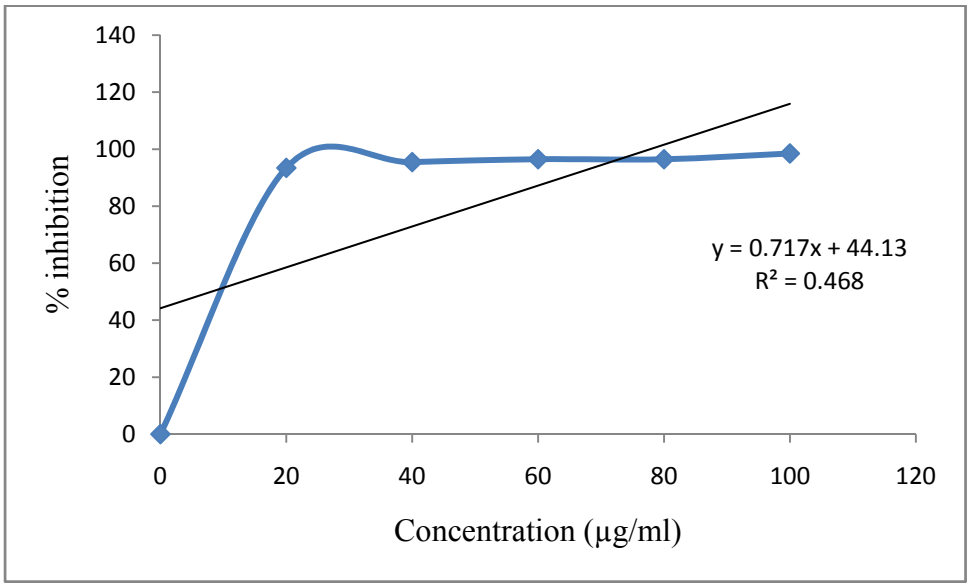


Figure 40: Scavenging effects of the leaf of APME

Table 32: % inhibition of APHE, APEA, APME and AA

Conc.(µg/ml)	% inhibition (APHE)	% inhibition (APEA)	% inhibition (APME)	% inhibition (AA)
0	0.00	0.00	0.00	0.00
20	40.10	53.30	93.40	32.99
40	42.64	49.75	95.43	39.59
60	44.16	59.90	96.45	45.69
80	52.79	73.60	96.45	49.75
100	54.31	63.45	98.48	56.35

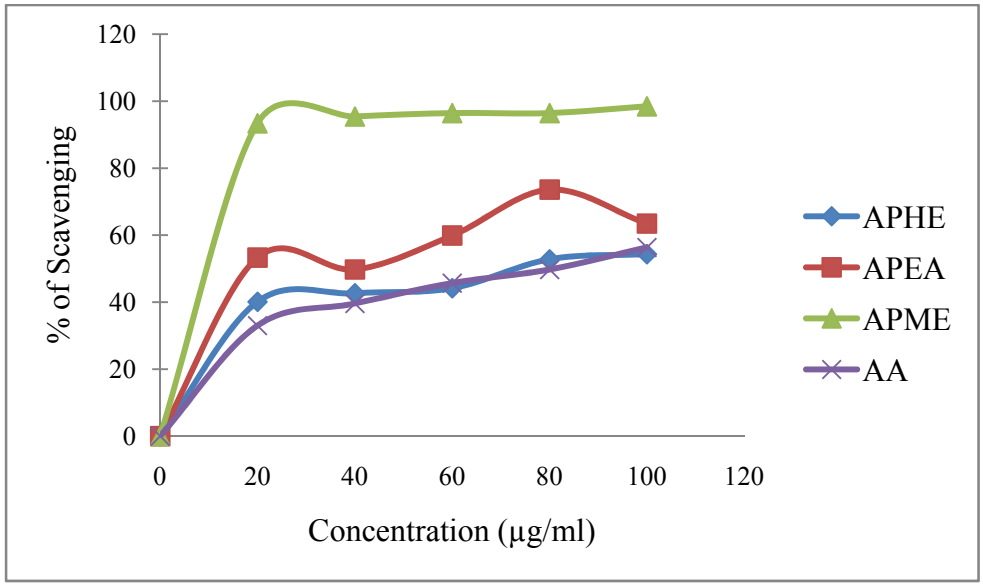


Figure 41: DPPH radical scavenging activity of AA and the APHE, APEA & APME extracts of *A. polystachya* leaf

It was interesting to notice that the extract of *A. polystachia* exhibited free radical scavenging activity in the antioxidant assay. All the extracts inhibited DPPH, indicating their antioxidant activity. The highest IC_{50} values among the extract of *A. polystachia* was 74.84 µg/ml (Table 29) of leaf of *n*-hexane fraction while ascorbic acid had IC_{50} value 76.11 µg/ml (Table 28) indicating the presence of potential candidates of antioxidant compounds. It appears that the APME has greater DPPH free radical scavenging capacity as it has the lowest value of IC_{50} 12.54 µg/ml (Table 31). The IC_{50} value of APEA was 50.07 µg/ml (Table 30).

Nitric Oxide Free Radical Scavenging of Leaf of *A. polystachya*

Table 33: IC_{50} value of ascorbic acid (AA)

Test tube no.	**Conc.(µg/ml)**	**Absorbance of AA**	**Absorbance of blank**	**% inhibition**	**Value of IC_{50}**
1	0	0.096	0.096	0.00	
2	5	0.049	0.096	48.96	
3	25	0.034	0.096	64.58	34.06
4	50	0.026	0.096	72.92	
5	100	0.021	0.096	78.13	
6	200	0.015	0.096	84.38	

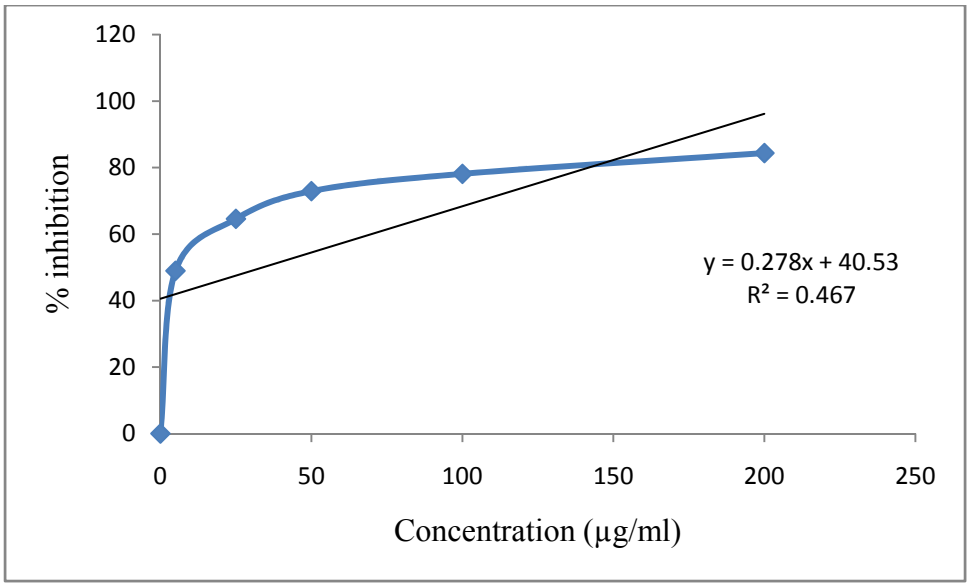


Figure 42: % inhibition curve of Ascorbic acid (AA)

Table 34: IC_{50} value of the leaf of APHE

Test tube no.	**Conc.(µg/ml)**	**Absorbance of extract**	**Absorbance of blank**	**% inhibition**	**Value of IC_{50}**
0	0	0.096	0.096	0.00	
1	5	0.095	0.096	1.04	
2	25	0.089	0.096	7.29	76.15
3	50	0.026	0.096	72.92	
4	100	0.018	0.096	81.25	
5	200	0.002	0.096	97.92	

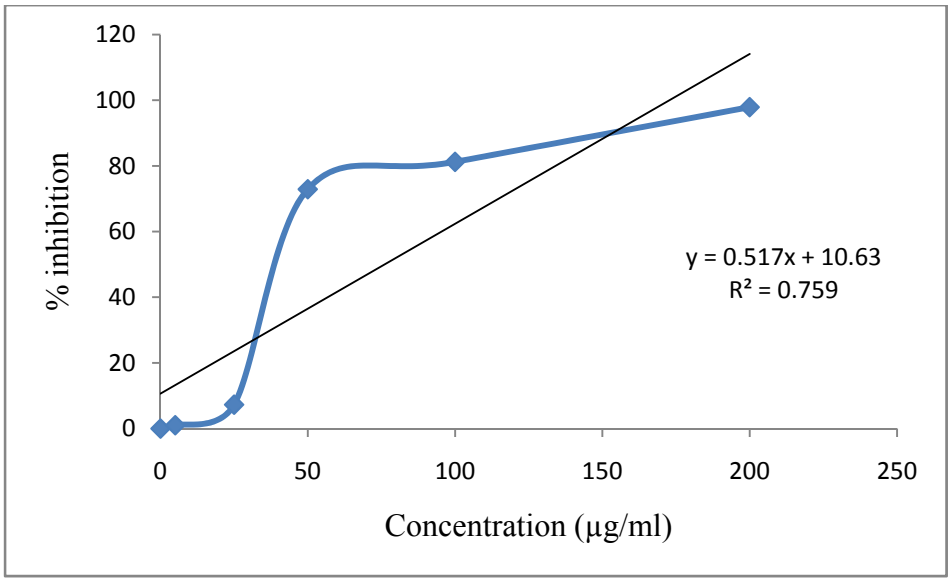


Figure 43: Scavenging effects of the leaf of APHE

Table 35: IC_{50} value of the leaf of APEA

Test tube no.	**Conc.(µg/ml)**	**Absorbance of extract**	**Absorbance of blank**	**% inhibition**	**Value of IC_{50}**
0	0	0.096	0.096	0.00	
1	5	0.070	0.096	27.08	
2	25	0.046	0.096	52.08	39.90
3	50	0.009	0.096	90.63	
4	100	0.008	0.096	91.67	
5	200	0.006	0.096	93.75	

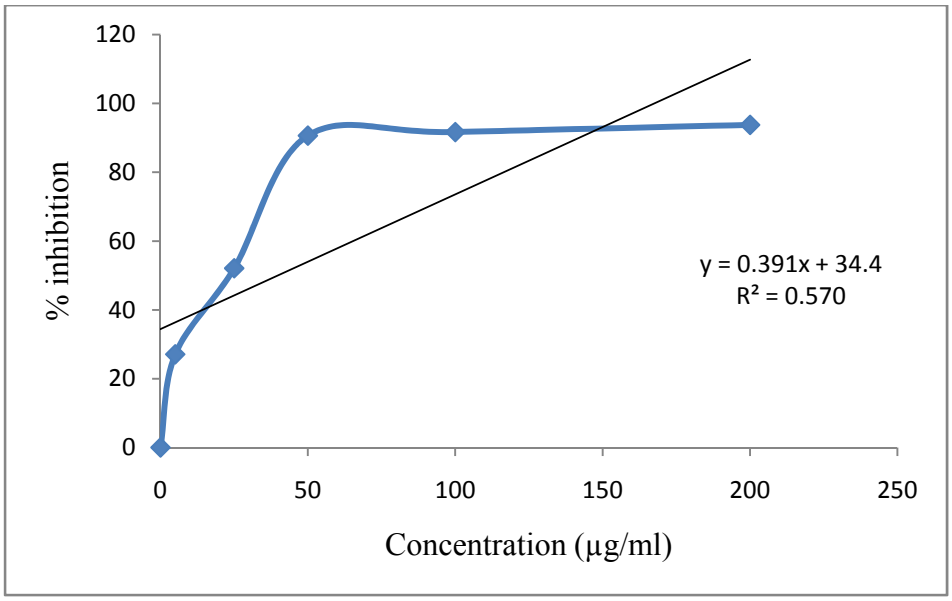


Figure 44: Scavenging effects of the leaf of APEA

Table 36: IC_{50} value of the leaf of APME

Test tube no.	**Conc.(µg/ml)**	**Absorbance of extract**	**Absorbance of blank**	**% inhibition**	**Value of IC_{50}**
0	0	0.096	0.096	0.00	
1	5	0.062	0.096	35.42	
2	25	0.037	0.096	61.46	57.58
3	50	0.031	0.096	67.71	
4	100	0.030	0.096	68.75	
5	200	0.023	0.096	76.04	

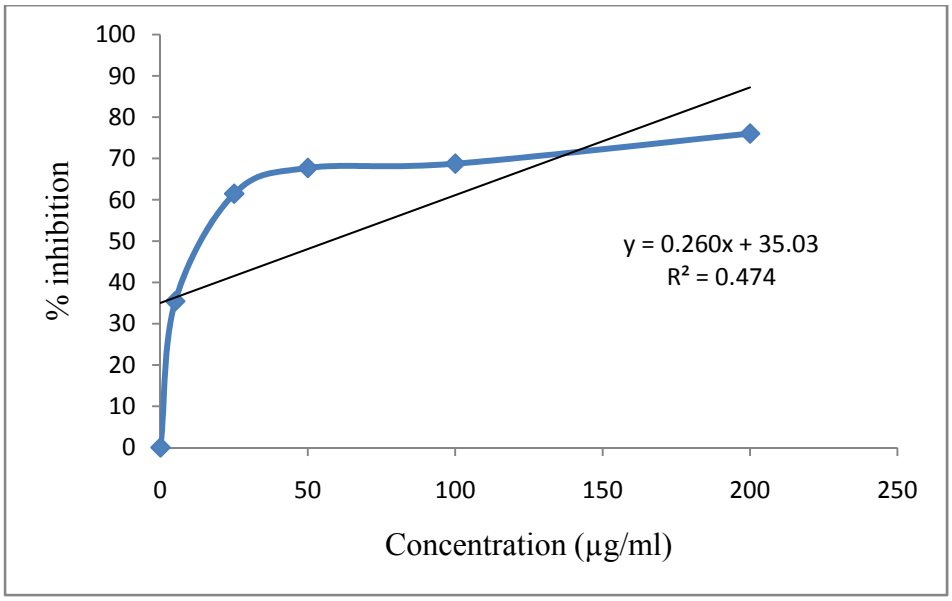


Figure 45: Scavenging effects of the leaf of APME

Table 37: % inhibition of APHE, APEA, APME and AA

Conc.(µg/ml)	**% inhibition (APHE)**	**% inhibition (APEA)**	**% inhibition (APME)**	**% inhibition (AA)**
0	0.00	0.00	0.00	0.00
5	1.04	27.08	35.42	48.96
25	7.29	52.08	61.46	64.58
50	72.92	90.63	67.71	72.92
100	81.25	91.67	68.75	78.13
200	97.92	93.75	76.04	84.38

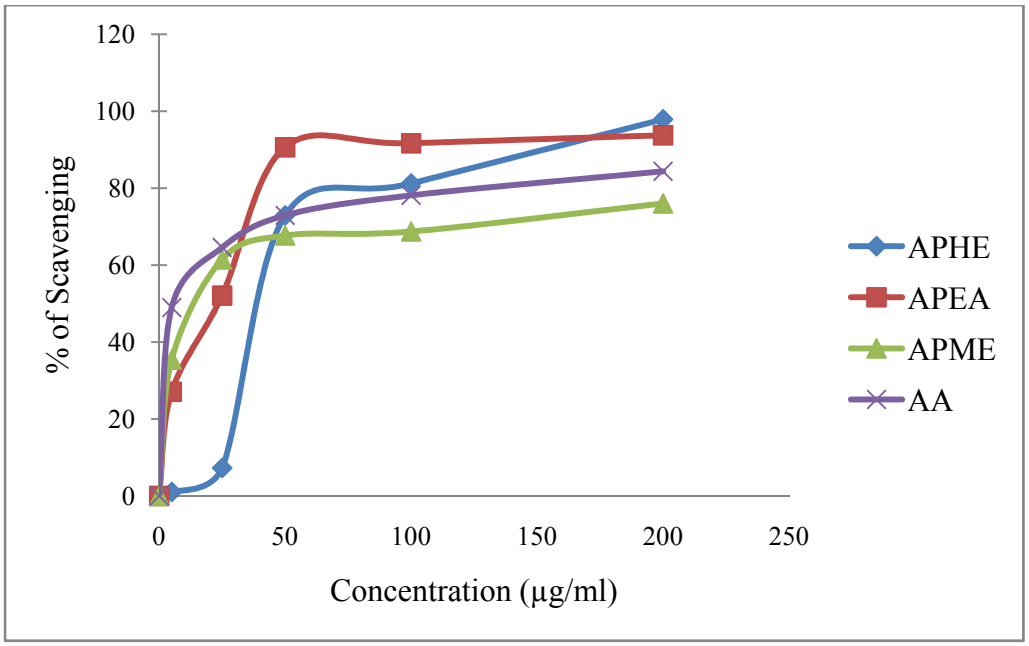


Figure 46: NO scavenging activity of AA and the APHE, APEA & APME extracts of *A. polystachya* leaf

The highest IC_{50} values among the extracts of *A. polystachia* was 76.15 µg/ml (Table 34) of leaf of *n*-hexane fraction while ascorbic acid had IC_{50} value 34.06 µg/ml (Table 33) indicating the presence of potential candidates of antioxidant compounds. It appears that the APEA has greater free radical scavenging capacity as it has the lowest value of IC_{50} 39.90 µg/ml (Table 35). The IC_{50} value of APME was 57.58 µg/ml (Table 36).

Total Phenolic Content

Table 38: Absorbance of gallic acid

SL. No.	Conc. of the Standard (µg/ml)	Absorbance
0	0	0
1	10	0.234
2	20	0.487
3	30	0.718
4	40	1.140
5	50	1.521
6	60	2.294
7	70	2.285
8	80	2.711
9	90	3.188
10	100	3.665

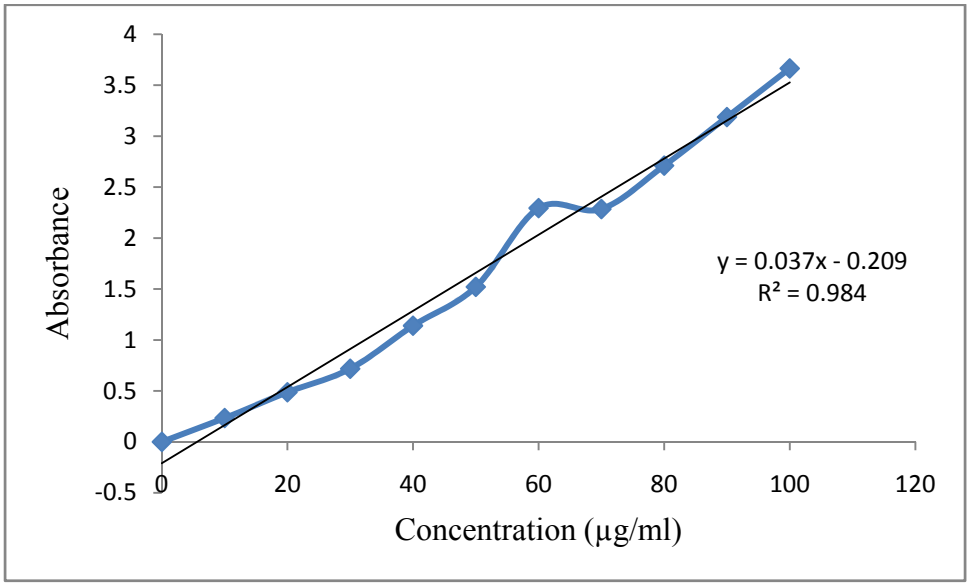


Figure 47: Standard curve of gallic acid

Table 39: Content of total phenol of the extracts of *A. polystachya* leaves

Extracts	Total phenolic content (n=3) (in mg/g,Gallic acid equivalents)			Average	SD
	mg GAE/gm (1)	mg GAE/gm (2)	mg GAE/gm (3)		
APHE	95.11	85.55	95.62	92.09	5.67
APEA	112.12	106.62	104.58	107.77	3.90
APME	65.83	55.34	56.27	59.15	5.81

The total phenolic content of the *A. polystachya* leaves extracts were expressed as gallic acid equivalent and are presented in (Table 39). The data clearly showed that APEA had the highest total phenolic content followed by APHE and APME which mean value of 107.77 mg GAE/gm extract, 92.09 mg GAE/gm extract and 59.15 mg GAE/gm extract, respectively.

***In vitro* Thrombolytic Activity**

Table 40: % Thrombolysis of APHE, APEA, APME, streptokinase and normal saline

Sample no.	% Thrombolysis APHE	% Thrombolysis APEA	% Thrombolysis APME	% Thrombolysis Streptokinase	% Thrombolysis Normal saline
1	15.327	5.294	10.611	43.988	5.892
2	23.574	9.192	8.277	36.431	4.430
3	14.874	8.162	14.600	37.152	3.398
4	15.434	5.029	9.998	45.533	4.211
5	17.944	8.385	16.027	40.146	5.181
6	23.619	7.113	10.572	48.768	5.142
7	20.983	9.860	8.968	46.007	4.508
8	16.432	12.383	7.140	38.811	6.147
9	12.489	5.996	10.111	38.413	3.647
10	18.617	9.698	8.936	32.299	4.559

Table 41: Clot lysis of blood sample data of extracts of *A. polystachya* fruit and streptokinase.

Extracts/ Positive control	Mean±SD (% Clot lysis)
APHE	17.93 ± 3.77*
APEA	8.11 ± 2.30*
APME	10.52 ± 2.76*
Streptokinase	40.75 ± 5.14*

Values are expressed in Mean±SD (n=10), $p<0.001$

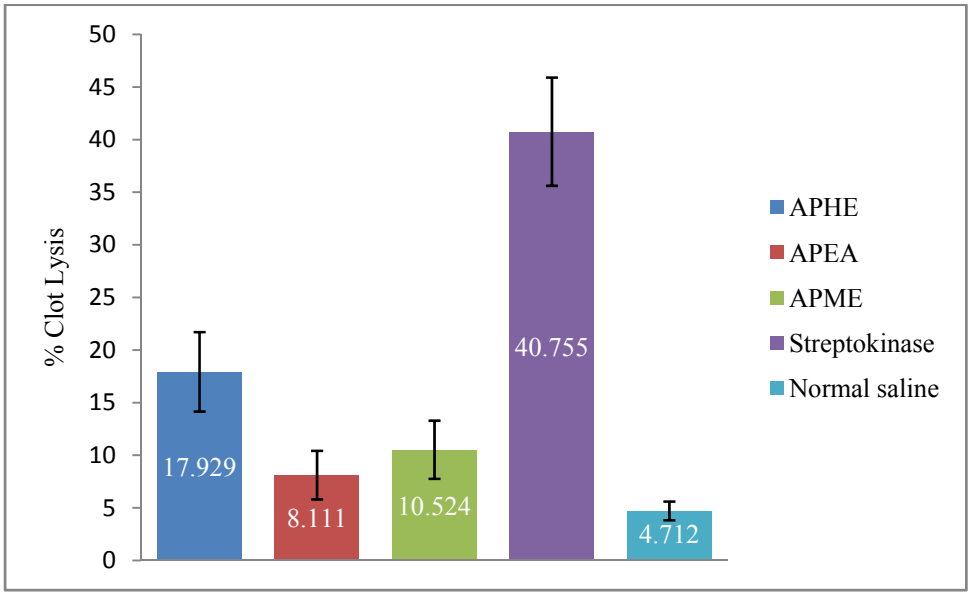


Figure 48: Clot lysis of blood samples of normal subjects by APHE, APEA, and APME extracts of *A. polystachya* leaves, streptokinase and normal saline.

When streptokinase (100 µl) was added to the clots maximum clot lysis was visually seen (Table 41, Figure 48). With normal saline 4.71% weight difference was seen. The *in vitro* thrombolytic

activity study revealed that *n*-hexane (APHE), ethyl acetate (APEA) and methanol (APME) extracts of *A*. *polystachya* leaves showed 17.93%, 8.11% and 10.52% clot lysis respectively and compared with the negative control (normal saline) the mean clot lysis % difference was significant ($p < 0.001$).

DISCUSSION

Phytochemical Screening

Previous researchers reported the presence of alkaloids, tannins, steroids, saponins, glycosides, flavonoids, anthraquinones and terpenoids in *A. polystachya* (Krishnaraju *et al*., 2009; Chowdhury & Rashid, 2003; Jagetia & Venkatesha, 2006; Sarla *et al*., 2011). The current study also correlates with previous studies. Presence of varieties of chemical compounds in the plant is directly or indirectly responsible for the significant amount of biological activities of *A. polystachya* leaf extracts. All the phytochemicals are reported to possess good antioxidant activities and has been reported to show numerous biological effects including anti-inflammatory and antitumor activities (Chowdhury & Rashid, 2003; Jagetia & Venkatesha, 2006; Sarla *et al*., 2011).

Antioxidant Activity

On the basis of the scavenging ability of the free radicals for DPPH, the highest antioxidant activity was found in APME (Table 31) and for NO, the highest antioxidant activity was found in APEA (Table 35). May be, due to the presence of polyphenolic compounds such as flavonoids and tannins (Table 27) the antioxidant activity of the extracts were observed. The outcome is agreed with the findings of the other researchers (Sarla *et al*., 2011; Krishnaraju *et al*., 2005) in which the antioxidant activity of methanol and ethyl acetate extracts of *A. polystachya* was reported and therefore could be used as a potential preventive measure against free radical-mediated diseases. The highest total phenolic content was found in ethyl acetate extract followed by *n*-hexane and methanol extract (Table 39).

Thrombolytic Activity

Leaf extracts of the plant showed significant ($p<0.001$) thrombolytic activity compared to the negative control. The phytochemical analysis showed that the extracts were a rich source of alkaloids, flavonoids, tannins and terpenoids which could be responsible for the clot lysis activity (Chowdhury *et al*., 2011; Dwivedi, 2007).

CONCLUSION

The presence of the identified phytochemicals makes the leaves pharmacologically active. Their antioxidant activity may be responsible for their usefulness in the management and treatment of various diseases. The medicinal values of the plant leaves may be related to their constituent phytochemicals. Due to its natural origin and potent free-radical scavenging ability *A. polystachya* could be used as a potential preventive intervention for free radical-mediated diseases. The presented data for total phenolic content are a basis of assessment of the preventive role of *A. polystachya* against free radicals effect. From *in vitro* clot lysis study, we demonstrated that *A. polystachya* have clot lysis activity. So that, we may assume that these extracts can be considered as a potential source of natural thrombolytic agent.

REFERENCES:

Agbafor KN, Nwachukwu N. Phytochemical Analysis and Antioxidant Property of Leaf Extracts of Vitex doniana and Mucuna pruriens. Biochemistry Research International. Volume 2011 (2011), Article ID 459839, 4 pages doi:10.1155/2011/459839.

Agnihotri VK, Srivastava SD, Srivastava SK. A New Limonoid, Amoorinin from the Stem Bark of *Amoora rohituka*. *Current Science*, 1987; 56(15):770.

Agnihotri VK, Srivastava SD, Srivastava SK. A New Limonoid, Amoorinin, from the Stem Bark of *Amoora rohituka*. *Planta Med,* 1987;53(3): 298-9.

Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nat Protoc* 2007;2(4)875-7.

Anwar MS, Khan IN, Barua S, Kamal ATMM, Hosen SMZ, Kawsar MH. Assessment of Thrombolytic & Cytotoxic Activity of Herbal Preparations Originated from Botanical Source of Bangladesh. *Journal of Applied Pharmaceutical Science* 2011;01(07):77-80.

Arvigo R, Balick M, *Rainforest Remedies*, Lotus Press, Twin Lakes 1993.

Ayoola GA, Coker HAB, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, Atangbayila TO. Phytochemical Screening and Antioxidant Activities of Some Selected Medicinal Plants Used for Malaria Therapy in Southwestern Nigeria. *Tropical Journal of Pharmaceutical Research*, September 2008;7(3):1019-1024.

Banerjee A, Chisti Y, Banerjee UC. Streptokinase—a clinically useful thrombolytic agent. Biotechnology Advances 22 (2004):287-307.

Balakrishnan N, Panda AB, Raj NR, Shrivastava A, Prathani R. The evaluation of nitric oxide scavenging activity of *Acalypha indica* Linn. root. *Asian J Research Chem* 2009;2(2):148-150.

Bhat SV, Bajwa BS, Dornauer H, Souza NJ, Fehlhaber HW, *Tetrahedron Lett.* 1977;1669-72.

Bhatta SK, Saxena VK, Nigam SS. Anew flavanone glycoside from the seeds of Amoora rohituka Roxb. Abstract presented at the Convention of Chemist, IIT Bombay, 1980.

Bhatt SK, Saxena VK, Nigam SS. A New Saponin from Seeds of *Amoorarohituka. Phytochemistry,* 1981;20(7):1749-1750.

Bhuyan MAK, Begum J, Chowdhury JU, Ahmed K, Anwar M N. Antimicrobial activity of oil and crude alkaloids from seeds of *Aphanamixis polystachya* (Wall.) R.N. Parker.; *Bangladesh Journal of Botany;* 2000;29(1):1-5; ISSN-0253-5416; Record Number-20013084157.

Blum D 2010. "The little-told story of how the U.S. government poisoned alcohol during Prohibition. – By Deborah Blum – Slate Magazine".

Chatterjee A, Kundu AB, Chakrabortty T, Chandrasekharan S. Extractives of *Aphanamixis polystachya* wall (Parker): The Structures and Stereochemistry of Aphanamixin and Aphanamixinin. *Tetrahedron*, 1970;26(8):1859-1867.

Chan LL, George S, Ahmad I, Saujanya LG, Abbasi A, Cunningham BT, Watkin KL. Cytotoxicity Effects of *Amoora rohituka* and *chittagonga* on Breast and Pancreatic Cancer Cells. *Evidence-Based Complementary and Alternative Medicine*, 2011:1-8.

Chhetri HP, Yogol NS, Sherchan J, Anupa KC, Mansoor S, Thapa P. P*hytochemical and Antimicrobial evaluations of some medicinal plants of Nepal, Kathmandu University Journal of Science, Engineering and Technology* 2008;i(v):49-54.

Chowdhury R, Hasan CM, Rashid MA. Guaiane Sesquiterpenes from *Amoora rohituka*. *Phytochemistry*, 2003;62(8):1213-1216.

Chowdhury R, Rashid RB. Effect of the crude extracts of *Amoora rohituka* stem bark on gastrointestinal transit in mice. *Indian J of Pharmacol* 2003;35:304-307.

Chowdhury R, Hasan CM, Rashid MA. Antimicrobial Activity of *Toona ciliata* and *Amoora rohituka*. *Fitoterapia,* 2003;74(1-2):155-8.

Chowdhury NS, Alam MB, Haque ASMT, Zahan R, Mazumder MEH, Haque ME. *In vitro* free radical scavenging and thrombolytic activities of Bangladeshi aquatic plant *Aponogeton undulatus Roxb*. *Global J Pharmacol* 2011;5(1):27-32.

Dupin JP, Gryglewski RJ, Gravier D, Hou G, Casadebaig F, Swies J, Chlopicki S. Synthesis and thrombolytic activity of new thienopyrimidinone derivatives. Journal of physiology and pharmacology 2002;53(4):625-634.

Dwivedi S. *Terminalia arjuna* Wight & Arn.-A useful drug for cardiovascular disorders. *J Ethnopharmacol* 2007;114(2):114-129.

Edeoga HO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology* 2005;4(7):685-688.

Feresin GE, Tapia A, Gutierrez RA, Delporte C, Backhouse EN, Schmeda-Hirschmann G. Free radical scavengers, anti-inflammatory and analgesic activity of *Acaena magellanica*. *J Pharm Pharmacol* 2002;54(6):835-44.

Gamble F. Madras 1: 181. 1997 (re. ed); Sasidharan, Indian Forester 57: 486. 1931; *Biodiversity documentation for Kerala- Flowering Plants*, part 6: 88. 2004; Saldanha, Fl. Karnataka 2: 231. 1996.

Gokaraju GR, Gokaraju RR. Golakoti T, Chirravuri, VR, Raju VK, Bhupathiraju AK. Use of *Aphanamixis polystacha* extracts or fractions against 5-lipoxygenase mediated diseases; 2010; Patent application number: 20100178288.

Gole MK, Dasgupta S. Role of plant metabolites in toxic liver injury. *Asia Pacific Journal of Clinical Nutrition*, 2002;11(1):48-50.

Guerrero RO, Khan MTH, Casanas B, Morales M. Specific bioassays with selected plants of Bangladesh. Rev Cubana Plant Med 2004;9(2) *On-line version* ISSN 1028-4796.

Hill AF. Economic Botany. A textbook of useful plants and plant products. 2nd edn. McGarw-Hill Book Company Inc, 1952, New York.

Hodzic Z, Pasalic H, Memisevic A, Srabovic M, Saletovic M, Poljakovic M. The Influence of Total Phenols Content on Antioxidant Capacity in the Whole Grain Extracts. *European Journal of Scientific Research.* ISSN 1450-216X 2009;28(3):471-477.

Hossain M, Biva IJ, Jahangir R, Vhuiyan IM. Central nervous system depressant and analgesic activity of *Aphanamixis polystachya* (Wall.) parker leaf extract in mice. *African Journal of Pharmacy and Pharmacology* 2009,3(5),282-286.

Hoque N, Imam MZ, Akter S, Mazumder MEH, Hasan SMR, Ahmed J, et al. Antioxidant and antihyperglycemic activities of methanolic extract of *Glinus oppositifolius* leaves. *J App Pharm Sci* 2011;1(7):50-3.

Howse PE. Laboratory evaluation of toxic and repellent properties of the pithraj tree, *Aphanamixis polystachya* (Wall & Parker), against *Sitophilusoryzae* (L.). International Journal of Pest Management, 1994;40(3):274-279.

HUANG D, BOXIN OU , RONALD L. PRIOR. The Chemistry behind Antioxidant Capacity Assays. J. Agric. Food Chem. 2005, 53, 184118561841.

Iyengar MA. Study of Crude Drugs. 8th ed., Manipal Power Press, Manipal, India. 1995;2.

Jagetia GC, Venkatesha VA. Enhancement of radiation effect by *Aphanamixis polystachya* in mice transplanted with Ehrlich ascites carcinoma. *Biological and Pharmaceutical Bulletin*, 2005;28(1):69-77.

Jagetia GC, Venkatesha VA. Treatment of mice with stem bark extract of *Aphanamixis polystachya* reduces radiation-induced chromosome damage. *International Journal of Radiation Biology* 2006;82(3):197-209.

Jagetia GC. Radioprotective Potential of Plants and Herbs against the Effects of Ionizing Radiation. *J. Clin. Biochem. Nutr,* 2007;40:74-81.

Jain A, Srivastava SK. 8-C-methyl-quercetin-3-O-β-D-xylopyranoside, A New Flavone Glycoside from the Roots of *Amoorarohituka*. *Journal of Natural Products*, 1985;48(2):299-301.

Javanmardi J, Stushnoff C, Locke E, Vivanco JM, Antioxidant activity and total phenolic content of Iranian Ocimum accessions. Food Chemistry 83 (2003) 547–550.

Karmohapatra SK, Kahn NN, Sinha AK. The thrombolytic effect of aspirin in animal model. *J Thromb* Thrombolysis 2007;24:123–129.

Khan IN, Sarker MI, Almamun A, Mazumder K, Bhuiya MAM, Mannan A. Cytotoxic and Thrombolytic Activity of Ethanolic Extract of Zanthoxylum Budrunga (Fam: Rutaceae) Leaves. European Journal of Scientific Research 2011;66(2):303-310.

Khan IN, Habib MR, Rahman MM, Mannan A, Sarker MMI, Hawlader S. Thrombolytic potential of *Ocimum sanctum* L., *Curcuma longa* L., *Azadirachta indica* L. and *Anacardium occidentale* L. *J Basic Clin Pharm*, 2011;2(3):125-7.

Klaymann DL, Clark AM, *Science* 1985, *228*, 1049. *Pharm. Res.* 1996;13:1133-1144.

Krishnaraju AV, Rao TVN, Sundararaju D, Vanisreeb M, Tsay HS, Subbaraju GV. Assessment of bioactivity of Indian medicinal plants using brine shrimp (*Artemia salina*) lethality assay. *Int J Appl Sci Eng* 2005;3(2):125-134.

Krishnaraju AV, Rao CV, Rao TVN, Reddy KN, Trimurtulu G. *In vitro* and *In vivo* antioxidant activity of *Aphanamixis polystachya* bark. *American Journal of Infectious Diseases*. 2009;5(2):60-67 DOI**:** 10.3844/ajidsp.

Kundu AB, Ray S, Chatterjee A. Aphananin, a triterpene from *Aphanamixis polystachya*; *Phytochemistry* 1985;24(9):2123-2125.

Lampronti I, Khan MT, Bianchi N, Ather A, Borgatti M, VFabbri E, Rizziello LG. Bangladeshi medicinal plant extracts inhibiting molecular interactions between nuclear factors and target DNA sequences mimicking NF-kappaB binding sites 2005;1(4):327-33.

Laurence DR, Bennett PN. Clinical Pharmacology: Seventh Edition 1992: Page 483.

Mabberley DJ, Sing AM. *Indian Forester,*1931;62:486.

McCaine WD. *The Properties of Petroleum Fluids* (2nded.), 1990, Oklahoma: PennWell Publishing Company.

Mohanakumara P, Sreejayan N, Priti V. *Dysoxylumbinectariferum* Hook.F (Meliaceae), a Rich Source of Rohitukine. *Fitoterapia*, 2010;81(2):145-148.

Mojab F, Kamalinejad M, Ghaderi N, Vahidipour H. *Phytochemical Screening of Some Iranian Plants*. Iranian Journal of Pharmaceutical Research. 2003;77-82.

Nair RT, Kalariya J, Chanda S. *Antibacterial activity of some selected Indian medicinal flora.* Tuky J. Biol. 2005;29:41-47.

Nobakht GM, Kadir MA, Stanslas J. Analysis of preliminary phytochemical screening of *Typhonium flagelliforme*. *African Journal of Biotechnology* 2010;9(11):1655-1657.

Pamplona-Roger D. Encyclopedia of Medicinal Plants, Saeliz, 1998, Spain.

Parker R, *Aphanamixis polystachy,* 1997. *Indian Forester* 57:486. 1931, Nomen number: 312562.

Prakash A, Rigelhof F, Miller E. Antioxidant Activity, *A publication of Medallion Labs.*

Prasad S, Kashyap RS, Deopujari JY, Purohit HJ, Taori GM, Daginawala HF. Effect of *Fagonia Arabica* (Dhamasa) on in vitro thrombolysis. *BMC Complementary and Alternative Medicine* 2007;7:36.

Rabi T, Karunagaran D, Nair MK, Bhattathiri VN. Cytotoxic Activity of Amooranin and its Derivatives. *Phytotherapy Research* 2002;16(1):84-86.

Rabi T, Ramachandran C, Fonseca HB, Nair RP, Alamo A, Melnick SJ, Escalon E. Novel Drug Amooranin Induces Apoptosis Through CaspaseActivity in Human Breast Carcinoma Cell Lines. *Breast Cancer Research and Treatment* 2003;80(3):321-330.

Reuben KD, Abdulrahman FI, Akan JC, Usman H, Sodipo OA, Egwu GO. Phytochemical Screening and *In Vitro* Antimicrobial Investigation of the Methanolic Extract of *Croton Zambesicus* Muell ARG. Stem Bark. European Journal of Scientific Research. ISSN 1450-216X. 2008;23(1):134-140.

Sadhu SK, Phattanawasin P, Choudhuri MSK, Ohtsuki T, Ishibashi M. A New Lignan from *Aphanamixis polystachya. Journal of Natural Medicines,* 1980;60(3):258-260.

Sanyal M, Datta PC. Pharmacognosy of *Aphanamixis polystachya* stem bark. *Quart. J Crude Drug Res* 1981;19(2-3):113-126.

Saraf S, Sharma S, Bensalha I, Wellsted D, Gorog D. Impaired endogenous thrombolysis in acute coronary syndrome patients predicts recurrent events: results using a novel thrombosis test. Heart 2009;95(Suppl I):A1–A87.

Sarla S, Prakash MA, Apeksha R, Subhash C. Free radical scavenging (DPPH) and ferric reducing ability (FRAP) of *Aphanamixis polystachya* (Wall) Parker. *Int J Drug Dev Res* 2011;3(4):271-274.

Sen S, Chakraborty R, Sridhar C, Reddy YSR, De B. FREE RADICALS, ANTIOXIDANTS, DISEASES AND PHYTOMEDICINES:CURRENT STATUS AND FUTURE PROSPECT. *International Journal of Pharmaceutical Sciences Review and Research*, 2010;3(1) ISSN 0976 – 044X. Article 021.

Shinkar AS. Phytochemical investigation and antiulcer activity of Aphanamixis polystachya (wall.) Parker, *(Dissertation);* Submitted to: Rajiv Gandhi University of Health sciences, Karnataka, Bangalore, September, 2007.

Shrivastava S, Leelavathi S. PRELIMINARY PHYTOCHEMICAL EVALUATION OF LEAF EXTRACTS OF *CATUNAREGUM SPINOSA* THUNB. *International Journal of Pharmaceutical Sciences Review and Research*. ISSN 0976 – 044X. 2010;3(2) Article 020.

Strobel G, Daisy B, Castillo U, Harper J, *J. Nat. Prod.* 2004;67:257-268.

Talukder FA, Howse PE. Evaluation of *Aphanamixis polystachya* as a source of repellents, antifeedants, toxicants and protectants in storage against *Tribolium castaneum* (Herbst); *Journal of Stored Products Research* 1995;31(1):Pages 55-61.

Talukder FA, Howse PE. Isolation of secondary plant compounds from *Aphanamixis polystachya* as feeding deterrents against adult *Tribolium castaneum* (Coleoptera: Tenebrionidae); *Journal of Stored Products Research* 2000;107(5):498-504; ISSN-0340-8159; Record Number-20003026520.

Talukder FA, Howse PE. Deterrent and insecticidal effects of extracts of pithraj,*Aphanamixis polystachya* (Meliaceae), against *Tribolium castaneum* in storage, *Journal of Chemical Ecology*, 2011;19(11):2463-2471, DOI: 10.1007/BF00980683

Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Byrne DH. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. Journal of Food Composition and Analysis 2006;19:669–675.

Tsao R, Yang R, Xie S, Sockovie E, Khanizadeh S. Which polyphenolic compounds contribute to the total antioxidant activities of apple? J Agric Food Chem. 2005;53(12):4989-95 15941346 Cit:26.

Verschueren KE. Handbook of Environmental Data on Organic Chemicals, 2nd ed. Van Nostrand Reinhold Company, New York, 1983, pp. 818-820.

Vinod SK, Raghuveer I, Alok S, Himanshu G. Phytochemical investigation and chromatographic evaluation of the ethanolic extract of whole plant extract of *dendrophthoe falcata* (l.f.) Ettingsh. International journal of pharmaceutical sciences and research. Ijpsr 2010;1(1)

Wangcharoen W, Morasuk W. Antioxidant capacity and phenolic content of some Thai culinary plants. *Maejo International Journal of Science and Technology.* ISSN 1905-7873. 2007;01(02):100-106.

Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT, *J. Am. Chem. Soc.*1971;93:2325-2327.

Wiart C. *Medicinal Plants of Asia and the Pacific*. New York, 2006 NY: CRC Press.

Yadav R, Chauhan NS, Chouhan AS, Soni VK, Omray L. Antimicrobial screening of various extracts of *Aphanamixis polystachya* stems bark. *International Journal of Advances in Pharmaceutical Sciences*, 2010;1:147-150.

Yang SP, Chen HD, Liao SG, Xie BJ, Miao ZH, Yue JM. Aphanamolide A, a New Limonoid from *Aphanamixis polystachya; American Chemical Society, Organic Letters,* 2011;13(1):150-153.

Zhang H, Chen F, Wang X, Wu D, Chen Q. A New Limonoid from the seed of *Aphanamixis polystachya*. *Chinese Chemical Letters*, 2002;13(4):341-342.

Zhang Y, Wang JS, Wang XB, Wei DD, Luo JG, Luo J, Yang MH, Kong LY. Aphapolynins A and B, two new limonoids from the fruits of *Aphanamixis polystachya;* Tetrahedron Letters 2011;52(20):2590-2593.