Evaluation of the antioxidant and *in vitro* cytotoxic activity of *Averrhoa carambola, Aegle marmelos* and *Spondias pinnata* leaf extracts

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Submitted by-

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Dedicated

To

My amazing parents

DECLARATION

The research work embodying the results reported in this thesis entitled "**Evaluation of the antioxidant and *in vitro* cytotoxic activity of** *Averrhoa carambola L*, *Aegle marmelos* **and** *Spondias pinnata* **leaf extracts"** submitted by Anahita Tanzia Zaman in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology, Department of Mathematics and Natural Science, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma. The work was performed at Laboratories of BRAC University, The University of Dhaka and The University of Asia Pacific, Dhaka, Bangladesh.

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ABSTRACT

Three typically grown Bangladeshi fruit trees, *Averrhoa carambola L, Spondias pinnata* and *Aegle marmelos* possess various therapeutically important properties and their different parts have been experimented for antioxidant and cytotoxicity activities in various studies. Antioxidants help prevent free radicals from harming our body with various degenerative diseases by donating electrons. Antioxidants neutralize the free radicals and reduce the damage in cells before oxidants can attack. The objective of this study was to evaluate the three fruit plants leaf extracts and to find their antioxidant content and also *In vitro* cytotoxic activity using HeLa cell line. This study may allow us to get a comprehensive analysis on their individual activities and show susceptible results which will help in further research prospective. As they are typically grown in Bangladeshi environment and are readily available, they can be screened to find out which of them contain the highest amount of antioxidant and cytotoxic properties. Dried powdered leaves of *Averrhoa carambola L, Spondias pinnata* and *A. marmelos* were extracted using a Soxhlet apparatus using, n-hexane, petroleum ether, methanol and chloroform. The extracts were tested for CUPRAC reducing capacity, total antioxidant capacity, total phenolic content and total flavonoid content. Cytotoxic analysis was done using HeLa cell line and cell viability was checked. All three methanolic leaf extracts of the fruits plants showed the highest CUPRAC reducing capacity when compared against the standard (BHT). For both *Averrhoa carambola L* and *A. marmelos*, petroleum ether extract showed the highest antioxidant content; 105.8mg/gm, 89.4mg/gm; n-hexane extract showed the highest phenol content; 212.4mg/gm, 686mg/gm and methanol extract showed the highest flavonoid content; 171.6mg/gm, 146mg/gm. For *Spondias pinnata*, chloroform extract showed the highest total antioxidant content; 122mg/gm, petroleum ether extract showed the highest phenol content; 280.6mg/gm and methanol extract showed the highest flavonoid content; 189.5mg/gm. *In vitro* cytotoxicity test revealed that all extracts of *A. marmelos* showed <1% viability of HeLa cells, followed by *Averrhoa carambola L* extracts, while *Spondias pinnata* extracts showed the least cell death. It can be suggested that both *Averrhoa carambola L* and *A. marmelos* extracts showed promising results than *Spondias pinnata* and they can be further analysed in a more diverse manner.

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ABBREVIATIONS

DMSO	**Dimethyl Sulfoxide**
Spp.	**Species**
RNS	**Reactive Nitrogen Species**
ROS	**Reactive Oxygen Species**
Nc.	**Neucuproine**
BHT	**Butylated Hydroxytoluene**
Mo	**Molybdenum**
GAE	**Gallic Acid Equivalent**
HEPA	**High efficiency particulate air**
HeLa	**Human Epitheloid Cervix Carcinoma**
PBS	**Phosphate buffered saline**
DMEM	**Dulbecco's modified Eagle's Medium**
NH	**N-Hexane**
PEE	**Petroleum ether extract**
ME	**Methanol Extract**
CHE	**Chloroform extract**
AA	**Ascorbic Acid**
GA	**Gallic Acid**
Quer	**Quercertine**
WHO	**World Health Organization**
UV	**Ultraviolet**

Chapter 1

Introduction

Fruits have always been a major part of diet and an important source of health promoting agents. Of these agents, antioxidants are most important because they inhibit the initiation of lipid peroxidation, which is related to aging and the pathogenesis of diseases such as cardiovascular disorders, cancer, inflammation, and brain dysfunction (Ames, 1983; Shon *et al.*, 2004). Various epidemiological studies have suggested that consumption of fruit and vegetables is associated with reduced risk of cardiovascular diseases and cancers (Etherton *et al.*, 2002). Bangladesh is abundant in supply of its typical fruits which possess functional characteristics such as antioxidant content, cytotoxicity content, antitumor content etc. Even though individual works of these plants' extracts of various parts exist, this document is the only one where all three plants' leaf extracts are evaluated to record their antioxidant value and cytotoxic content on cancer cells. People of Bangladesh are mainly poor and are in dire need of good but cheaper medicines for cancer. The fruit plants used in this research are all very cheap and are readily available in rural areas as well as the urban. The aim of this study is to provide a document investigating the leaf extracts of these three fruit trees and provide suitable information regarding the experiments done.

According to the journal published in October 2013 by the department of oncology of Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh, cancer-related death rate in Bangladesh was 7.5% in 2005 and is expected to hit 13% in 2030. Cancer is a deadly disease all over the world. But unlike the first world countries Bangladesh is far behind in treating and controlling it and is in need of alternative drugs which are cheaper and available in the country. There is 13 to 15 lakh cancer patients in Bangladesh as per the journal published by the oncology department of Bangabandhu Sheikh Mujib Medical University, with about 2 lakh are being newly diagnosed with cancer each year. In the near future, the increase in population will result in an increase in the number of cancer patients in Bangladesh.

People all over the country consume these fruits either raw or by making juice by various other means. So the purpose of this study is to find whether these fruit leaves extract contain antitumor effect and how to find a way to use these results produced to make cheaper drugs in the pharmaceutical world.

1.1 *Spondias pinnata* (Hogplum/HogApple; Local name Aamra)

Spondias pinnata is also known as Hogplum or Aamra in Bengali is a deciduous tree which is distributed throughout Bangladesh, India, Srilanka and South-East Asian countries. Its accession number is 40254 as per the Bangladesh National Herbarium. The tree has a strong, stout trunk having a smooth ash-coloured bark; growing up to 27 meters in height. Leaves are observed to be compound and alternate and clustered at twig ends and are about 30-45 cm long; leaflets 3-5 pairs and a terminal one 7.5-18 cm by 3.5-7.8 cm oblong or elliptical oblong, acuminate, quite entire, more or less oblique. Flowers are bisexual and fruits are yellow in colour; fleshy with a pulp which is finely flavoured and edible. The seeds bear ridges and are having a hard and fibrous surface. *S. pinnata* is a species which grows well in light abundant areas. The plant *Spondias pinnata* ; also called as Indian hog-plum (English), amara(hindi), aamra(Bengali), amora (Assamese), amrataka (Sanskrit), ambalam (Tamil), avimamadi (Telegu), ambula(Oriya) is a medicinally, nutritionally and economically important plant. Fruits, leaves, bark of *S. pinnata* are strong anti-scorbutic agents. Roots of the plants are traditionally used for regulating menstruation. All parts of this plant have been used in folkloric medicine as an anti-tubercular agent, while the unripe fruits were used as an aphrodisiac (Bora *et al*., IJPSR, 2014).

1.1.1 Taxonomy of the plant:

Figure 1 - *Spondias pinnata* fruit

Figure 2 - *Spondias pinnata* leaves

Kingdom : Plantae

Subkingdom : Viridaeplantae

Infrakingdom : Streptophyta

Division : Tracheophyta

Subdivision : Spermatophytina

Infradivision : Angiospermae

Class : Magnoliopsida

Superorder : Rosanae

Order : Sapindales

Family : Anacardiaceae

Genus : Spondias

Species : *Spondias pinnata* (Bora et al., IJPSR, 2014).

Table 1 – Taxonomy of *Spondias pinnata*

1.1.2 Importance of *Spondias pinnata*

S. pinnata is found to contain many chemical constituents of important pharmacological activities.

- **Hypoglycemic activity:** The hypoglycemic activity of *S. pinnata* was tested for their hypoglycemic activity using normoglycemic study and oral glucose tolerance test in adult Wistar rats (Bora *et al*., IJPSR, 2014).
- **Anthelmintic activity:** The ethanolic and acetone extracts of the bark of the plant *Spondias pinnata* contain different glycosides which exhibited potent anthelmintic properties (Bora *et al*., IJPSR, 2014).
- **Anti-cancer activity:** In a recent study; the bark of *S. pinnata* is also reported to have an anti-cancer activity. 70% methanolic extract of the bark of *S. pinnata* was tested for its anti-cancer activity and it showed a significant result. (Bora *et al*., IJPSR, 2014).
- **Anti-microbial activity:** The methanolic bark extract of *S. pinnata* exhibited good antibacterial activity while the aqueous extract of the bark showed only mild antibacterial activity against *Escherichia coli, Salmonella typhimurium* and *Vibrio Cholera* (Bora *et al*., IJPSR, 2014).
- **Anti-oxidant activity:** In a study 70% methanolic extract of the stem bark of *S. pinnata* has been found to be a potent source of antioxidants (Bora *et al*., IJPSR, 2014).
- **Cytotoxic activity:** Preliminary cytotoxic activity of the extract of the fruit of *S. pinnata* has been reported. It was determined by using Brine shrimp lethality test (Bora *et al*., IJPSR, 2014). It showed a significant result in cell mortality.
- **Hepatoprotective activity:** The hepatoprotective effect of the ethyl acetate and methanolic extracts of stem wood of *S. pinnata* were carried out against carbon tetrachloride induced rats (Bora *et al*., IJPSR, 2014).

1.1.3 Other commercial uses

- Wood is used extensively in temporary constructions, mouldings, interior finishing, drawers, turnery, articles, carvings and core stock of plywood. The wood is also suitable in the manufacture of matchsticks, matchboxes, boxes and crates; owing to their lightness and softness (Badoni and Bisht, 2009).
- Leaves and fruits of the plants are also used as fodder for pigs in farms. The trees when cultivated, serves as living fences and also provides adequate shade (Badoni and Bisht, 2009).
- Through value addition of this wild edible fruit tree plant the local people make chutney, jam and pickle. By production and marketing of these products, the local people may increase their socio-economic status. (Badoni and Bisht, 2009).

1.2 *Averrhoa carambola* **(Star Fruit/Carambola/Kamranga)**

Averrhoa carambola is a species of woody plant in the family Oxalidaceae; it has a number of common names including **Carambola** and **Starfruit**. This evergreen tree is from Malaysia and surrounding areas, and might originally have been endemic to the island of Java in Indonesia. *A. carambola* is a small tree or shrub that grows 5-12 meters tall, with rose to red-purple flowers. The flowers are small and bell-shaped, with five petals that have whitish edges. The flowers are often produced year round under tropical conditions (Mia *et al.,* 2007).

Averrhoa carambola, with an accession number 40253 as per the Bangladesh National Herbarium is an attractive, small, slow-growing evergreen tree with a short-trunk or a shrub. The branches are drooping and the wood is white and turns reddish. It has a bushy shape with many branches producing a broad, rounded crown. The compound leaves are soft, medium-green; they are spirally arranged around the branches in an alternate fashion (Chakraborty *et al*., 2012). The top sides of the leaves are smooth and the undersides are finely hairy and whitish. The leaflets are reactive to light and tend to fold together at night, they are also sensitive to abrupt shock and when shaken tend to close up also. The lilac or purple-streaked, downy, flowers are produced in the axils of leaves at the end of twigs. The flowers are arranged in small clusters on the ends of the branches or sometimes on the larger stems and trunk, each cluster is attached to the tree with red stalks (Chakraborty *et al*., 2012). The bell shaped, perfect flowers is produced in loose panicles that are much-branched with pedicellate flowers. The fruits are showy with an oblong shape: they are longitudinally 5- to 6 angled and 6.35-15 cm long and up to 9 cm wide. The fruits have a thin, waxy skin that is orange-yellow colored. The juicy fruits are yellow inside when ripe and have a crisp texture and when cut in cross-section are star shaped. The fruits have an oxalic acid odor, which varies between plants from strong to mild; the taste also varies from very sour to mildly sweetish. Each fruit may have up to twelve 6-12.5 mm long seeds, which are flat, thin and brown. Some cultivated forms produce fruits with no seeds (Chakraborty *et al*., 2012).

Averrhoa carambola (Bengali name- Kamranga;Family, Oxalidaceae) is medium sized tree. It is planted in all over Bangladesh. The fruit juice can be used as antioxidant, astringent, to treat diarrhoea, vomiting, dysentery, hepatic colic, bleeding piles and relieving thirst. The leaves are

antipruritic, antipyretic and anthelmintic and are also useful in scabies, fractured bones, and various types of poisoning intermittent fevers and intestinal worms (Mia *et al.*, 2007).

1.2.1 Taxonomy of the Plant

Figure 3 - *Averrhoa carambola*

Classification of *Averrhoa carambola*
Scientific Classification
Kingdom – Plantae
Phylum – Tracheophyta
Class – Magnoliopsida
Order – Oxalidales
Family – Oxalidaceae
Genus – *Averrhoa*
Species - *Averrhoa Carambola*
(Chakraborty Manodeep *et al*., 2012).

Table 2 – Taxonomy of *Averrhoa carambola*

1.2.2 Importance of *Averrhoa carambola*

- **Antioxidant capacity:** Luximon-Ramma *et al.*, 2003 examined the antioxidant capacity, total phenolics, flavonoids and vitamin C contents of *Averrhoa carambola.* The antioxidant capacity of *A. carambola* was found to be among the highest in the study group and the study concluded *A. carambola* to be a substantial source of phenolic antioxidant, thus it may exhibit potent health benefits (Saghir *et al*., 2013).
- **Anti-inflammatory activity:** Cabrini *et al*., 2011 examined the ethanol extract of *A. carambola* leaves, its hexane, ethyl acetate and butanol fractions and two isolated flavonoids, for anti-inflammatory activity. The ethanol extracts reduced edema in a dose-dependent manner (Saghir *et al*., 2013).
- **Acetylcholinesterase inhibitory activity:** Teh *et al*., 2010 investigated the effects of *A. carambola* juice kept at different storage conditions on the activity of acetylcholinesterase, the results showed a substantial decrease in the hepatic acetyl cholinesterase activity in rats treated with star fruit juice. Highest activity was observed for the freshly prepared juice (Saghir *et al*., 2013).
- **Antimicrobial activity:** Mia *et al*., 2007 isolated two compounds from the bark of *A. carambola*. The petroleum ether, carbon tetrachloride and chloroform fractions of the methanol extract of *A. carambola* were subjected to antimicrobial screening. The petroleum ether extract moderately inhibited the growth of *E. coli* and on the other hand; the chloroform soluble fraction strongly inhibited the growth of *E. coli.* (Saghir *et al*., 2013).
- **Anti-ulcer activity:** Goncalves *et al*., 2006 examined the water-alcohol extract of leaves of *A. carambola* for its antiulcerogenic potential. The study concluded that ethanolic extract of *A. carambola* has low antiulcer activity (Saghir *et al*., 2013).
- **Hypoglycaemic activity:** Ferreira *et al*., 2008 examined the effects of the hydro-alcoholic extract of leaves of *A. carambola L*. on fasting blood glucose. The hydro alcoholic extract treated animals showed significantly lower fasting blood glucose level (Saghir *et al*., 2013).

- **Cytotoxicity effect:** Its cytotoxic action on Brine shrimp nauplii was closely monitored using Brine shrimp lethality assay. Here DMSO was used as a solvent. Whether DMSO had any effect on brine shrimp lethality or not, control was used in this study. The control group of brine shrimp nauplii with and without DMSO exhibited no mortality. The number of nauplii died and percent mortality was counted for the subjected extract. The study clearly indicated that, in terms of cytotoxicity, this species was able to show good cytotoxic activity (Joysree *et al*., 2013).

- **Commercial and Other Uses** Tart-sweet fruit eaten out of hand and in salads. Used as garnishes, in puddings, tarts, stews, curries, as relish, sherbet, pickles and desserts. Green fruit is used to serve with boiled fish. Flowers can be used in salad. Also, beautiful to look at in full fruit (Chakraborty *et al*., 2012).

1.3 Introduction to *Aegle marmelos*

Aegle marmelos, commonly known as Bael in Bengali, belonging to the family Rutaceae, with an accession number 40252 as per the Bangladesh National Herbarium has been widely used in indigenous systems of Indian medicine due to its various medicinal properties. *A. marmelos* is native to Northern India, but widely found throughout the Indian Peninsula and in Ceylon, Burma, Bangladesh, Thailand and Indo-China. It is a medium to large sized deciduous, armed tree with the axillary and 2.5 cm long alternate trifoliate leaves, short flower and globular fruits (Rahman and Parvin, Asian Pac J Trop Dis, 2014).

Aegle marmelos is a medium sized decidous tree bearing strong axillary thorns and leaves with 3 or 5 leaflets. Bael leaves are extremely useful for treating diabetes, jaundice, cholera and asthma. Bael leaf poultice is applied to inflammations–with black pepper for edema, constipation, and jaundice. (Chavda *et al.,* 2012).

The deciduous, alternate leaves, borne singly or in 2's or 3's, are composed of 3 to 5 oval, pointed, shallowly toothed leaflets, 4 to 10 cm long, 2 to 5 cm wide, the terminal one with a long petiole. New foliage is glossy and pinkish-maroon. Mature leaves emit a disagreeable odor when bruised. Fragrant flowers, in clusters of 4 to 7 along the young branch-lets, have 4 recurved, fleshy petals, green outside, yellowish inside, and 50 or more greenish-yellow stamens. The fruit, round, pyriform, oval, or oblong, 5 to 20 cm in diameter, may have a thin, hard, woody shell or a more or less soft rind, gray-green until the fruit is fully ripe, when it turns yellowish. It is dotted with aromatic, minute oil glands. Inside, there is a hard central core and 8 to 20 faintly defined triangular segments, with thin, dark-orange walls, filled with aromatic, pale orange, pasty, sweet, resinous, more or less astringent, pulp. Embedded in the pulp are 10 to 15 seeds, flattened-oblong, about 1 cm long, bearing woolly hairs and each enclosed in a sack of adhesive, transparent mucilage that solidifies on drying. *Aegle marmelos* has enormous traditional uses against various diseases and many bioactive compounds have been isolated from this plant also (Maity *et al*., 2009).

Recently, the stem bark of *Aegle marmelos* has been reported to exhibit potent cytotoxic activities in human tumor cell lines.10) It has also been reported to possess cytotoxic effect on human breast cancer cell lines *in vitro* (Chandra *et al.,* 2005).

Aegle marmelos has been used as an herbal medicine for the management of diabetes mellitus in Ayurvedic, Unani and Siddha systems of medicine in India, Bangladesh and Srilanka. The main usage of the parts of this tree is for medicinal purposes. The unripe dried fruit is astringent, digestive, stomachic and used to cure diarrhea and dysentery. Sweet drink prepared from the pulp of fruits produce a soothing effect on the patients who have just recovered from bacillary dysentery (Sharma *et.al*, 2011).

1.3.1 Taxonomy

Figure 4 – Leaves of *A. marmelos*

Figure 5 - *A. marmelos* **Fruit**

Classification of *Aegle marmelos*	
Kingdom:-	Plantae
Division:-	Magnoliophyta
Class:-	Magnoliopsida
Order:-	Sapindales
Family:-	Rutaceae
Genus:-	*Aegle*
Species:-	*A.marmelos*

Table 3 – Taxonomy of *A. marmelos*

1.3.2 Importance of *Aegle marmelos*

Aegle marmelos has enormous traditional uses against various diseases and many bioactive compounds have been isolated from this plant also. The ripe fruit and unripe fruit, as well as the roots, leaves and branches have all been used in traditional medicine. In Ayurveda, the ripe fruit has been used for chronic diarrhea and dysentery, as a tonic for the heart and brain, and as adjuvant treatment of dysentery. A decoction of the root has been used to treat melancholia, intermittent fevers and palpitation; the roots have mainly been used as an ingredient of the Ayurvedic medicine. The leaves have been given as febrifuge and as a poultice for the treatment of eye disorders and ulcer and administration of fresh leaves have been used for weakness of the heart, dropsy and beriberi. (Maity *et al*., 2009).

The different parts of *Aegle marmelos* are used for various therapeutic purposes, such as for treatment of asthma, anaemia, fractures, healing of wounds, swollen joints, high blood pressure, jaundice, diarrhea and typhoid troubles during pregnancy. The main usage of the parts of this tree is for medicinal purposes. The unripe dried fruit is astringent, digestive, stomachic and used to cure diarrhea and dysentery. Sweet drink prepared from the pulp of fruits produce a soothing effect on the patients who have just recovered from bacillary dysentery. The ripe fruit is a good and simple cure for dyspepsia (Sharma *et.al*, 2011). The pulp of unripe fruit is soaked in gingerly oil for a week and this oil is smeared over the body before bathing. This oil is said to be useful in removing the peculiar burning sensation in the soles. The roots and the bark of the tree are used in the treatment of fever by making a decoction of them. The leaves are made into a poultice and used in the treatment of ophthalmic. The leaves of the plant have been claimed to be used for the treatment of inflammation, asthma, hypoglycemia, febrifuge, and hepatitis (Sharma *et.al*, 2011). The mucilage of the seed is a cementing material. The wood takes a fine polish and is used in building houses, constructing carts, agricultural implements. A yellow dye is obtained from the rind of the unripe fruits. The dried fruits, after their pulp separated from the rind are used as pill boxes for keeping valuable medicines, sacred ashes and tobacco. In Homeopathic treatments it is largely used for conjunctivitis and styes, rhinitis, coccygodynia, nocturnal seminal emission with amorous dreams, chronic dysentery, etc. Ayurveda prescribes the fruit of the herb for heart, stomach, intestinal tonic, chronic constipation and dysentery; some forms of indigestion,

typhoid, debility, cholera, hemorrhoids, intermittent fever, hypochondria, melancholia and for palpitation. The unripe fruit is medicinally better than the ripe fruit. Leaf poultice is applied to inflammation; with black pepper for edema, constipation and jaundice (Sharma *et.al*, 2011).

- **Antidiabetic Activity:** Sunderam *et al.*, 2009 worked on alcoholic extract of *Aegle Marmelos, Momordica Charantia and Eugenia Jambolana* separately; against Streptozotocine induced diabetic rats and confirmed their protective activity against laboratory induced cell necrosis. Whereas, Kuttan & Sabu., 2004 studied on leaf extract of *Aegle Marmelos* on Alloxane induced diabetes and reported that used extract was capable to reduce oxidative stress by scavenging lipid peroxidation and enhancing certain Anti-oxidant levels which causes lowering of elevated blood glucose level. Beside of all above cited work, Hema and Lalithakumari, 1999 had presented tremendous results of *Aegle Marmelos* and documented its hypoglycemic effect along with other pharmacological effects at molecular level (Sharma *et.al*, 2011).

- **Hepatoprotective activity:** Singanan *et al.*, 2007 worked on *Aegle Marmelos* leaf extract on alcohol induced liver injury in albino rats and presented data of excellent hepatoprotective effects. Similarly, Ramnik, 2008, also demontrated that aqueous extract of *Aegle Marmelos* fruit pulp and seeds are effective in the treatment and prevention of CCl_4 induced hepatic toxicity (Sharma *et.al*, 2011).

- **Antimicrobial Activity:** Maheshwari *et al*, (2009) studied on ethnolic extract of dried fruit pulp of *Aegle Marmelos* against various intestinal pathogens i.e. *Shigella boydii*,; Sonnei and Flexneri and proposed that certain phytochemicals including phenols, tannins and flavonoids were effective against all. Citarasu *et al.*, 2003, experimented on *Aegle Marmelos* on certain pathogenic bacteria like *Salmonella typhi, Pseudomonas aeruginosa, Aeromonas hydrophyla & Vibrio sp*., and concluded its positive bactericidal effects (Sharma *et.al*, 2011).

- **Antipyretic Activity, Anti-inflammatory & Analgesic:** Arul *et al.*, 2005 presented anti-inflammatory, antipyretic & analgesic properties of serial extract of leaves of *Aegle*

Marmelos, and presented that most of the extract caused a significant inhibition of the carrageenan-induced paw oedema and cotton-pellet granuloma in rats. The extracts also produced marked analgesic activity by reducing the early and late phases of paw licking in mice. A significant reduction in hyperpyrexia in rats was also produced by the most of the extracts. Similarly, Ghangale, 2008 also evaluated aqueous extract of *Aegle mannelos* for anti-inflammatory activity by using rat paw edema model and proposed that *Aegle marmelos* possesses anti-inflammatory activity. Shankharananth., 2007, demonstrated that methanolic extract of leaves of *Aegle marmelos* at a dose levels of 200mg/kg and 300 mg/ kg show significant analgesic activity on acetic acid induced writhing and tail flick test in mice (Sharma *et.al*, 2011).

- **Antifungal Activity:** Patil, 2009 reported the antifungal activity of ethanolic extract of the *Aegle marmelos* leaves including antidiarrheal, and antimicrobial, activities. Rana, 1997, evaluated anti-fungal activity of essential oils isolated from the leaves of *Aegle marmelos* using spore germination assay. The oil exhibited variable efficacy against different fungal isolates and 100% inhibition of spore germination of all the fungi tested was observed at 500ppm.They proposed that essential oil from *Aegle marmelos* leaves may interfere with the Ca^{2+}-dipicolonic acid metabolism pathway and possibly inhibit the spore formation. Pitre S and Srivastava, 1987, demonstrated the antifungal activity of ethanolic root extract against *Aspergillus fumiganus and Trichphyton mentagrophytes* (Sharma *et.al*, 2011).

- **Cytotoxic and Anticancer Activity:** Costa *et al.*, 2005, evaluated the anticancer potential of folk medicine used in Bangladeshi and used extracts of *Aegle marmelos* for cytotoxic action using brine shrimp lethality assay using tumor cell lines. The extract of *Aegle marmelos* was found to exhibited toxicity significantly. Similarly, Gagetia *et al*., 2005 reported the anticancer effect of hydro alcoholic extract of *Aegle marmelos* leaves in the animal model of Ehrlich ascites carcinoma and proposed that induction of apoptosis may be due the presence of skimmianine in extract (Sharma *et.al*, 2011).

- **Radioprotective Activity:** Radioprotective effect of *Aegle marmelos* extact was studied by Jagetia GC and Venkatesh P, 2005 by exposing to different doses of gamma-radiation in mice and found that oral administration of extract resulted in an increase in radiation tolerance by 1.6 Gray. Again, Jagetia GC *et al*., 2006, studied effects of plant extract on the peripheral blood and small intestine of Swiss albino mice. They exposed the animals to gamma radiation and data were collected against radiation-induced changes in the peripheral blood, spleen colony forming units, and intestinal mucosa, reported that *Aegle marmelos* extract significantly reduces the deleterious effect of radiation in intestine and bone marrow of mouse (Sharma *et.al*, 2011).

- **Antispermatogenic Activity:** Pramanik *et al*., 1999 reported antispermatogenic acitivity of ethanolic extract of *Aegle marmelos* leaves in rats. Bhattacharya, 2002 presented data of anti-motility of rat sperms through *in vitro* study. Similarly, Sharma *et al*., 2009, studied the effect of ethanol extracts of leaves of *A. marmelos* for their *in vitro* effect on sperm motility and suggested that the extracts had a considerable effect on the motility of sperm. It was also proposed that an increase in concentration of the extracts decreased the motility of sperms (Sharma *et.al*, 2011).

- **Antiulcer Activity:** Goel, 1997 reported that oral administration of pyranocoumarin isolated from the seeds of *Aegle marmelos*, showed significant protection against pylorus-ligated and aspirin-induced gastric ulcers in rats and cold restraint stress-induced gastric ulcers in rats and guinea pigs. Dhuley, 2007, reported that pretreatment of rats with unripe *Aegle marmelos* fruit extract produce a significant inhibition of absolute ethanol induced gastric mucosal damage (Sharma *et.al*, 2011).

- **Anti-thyroid Activity:** Panda and Kar, 2006, isolated Scopoletin (7-hydroxy-6-methoxy coumarin) from *Aegle marmelos* leaves and evaluated for its potential to regulate hyperthyroidism. It was observed that scopoletin (at 1.00 mg / kg, p.o. for 7 days) to levo-thyroxine treated animals, decreased serum thyroid hormones level. It was also proved that the scopoletin have superior therapeutic activity than the standard antithyroid drug, propylthiouracil (Sharma *et.al*, 2011).

- **Other reported medicinal values:** The antidiarrheal effect of aqueous extract of *Aegle marmelos* fruit has been reported by effecting outer membrane protein C of *Enteropathogenic Escherichia Coli***.** Besides these activities, insecticidal activity, anti-lipid peroxidative activity. Antioxidant property has also been reported (Sharma *et.al*, 2011).

- **Commercial uses**

- **Food:** *A. marmelos* fruits may be cut in half, or the soft type's broken open, and the pulp, dressed with palm sugar, eaten for breakfast, as is a common practice in Indonesia. The pulp is often processed as nectar. Beating the seeded pulp together with milk and sugar makes a popular drink called sherbet in India. A beverage is also made by combining *Aegle marmelos* fruit pulp with that of tamarind. Mature but still unripe fruits are made into jam, with the addition of citric acid. Confection, *Aegle marmelos* fruit toffee, is prepared by combining the pulp with sugar, glucose, skim milk powder and hydrogenated fat. Indian food technologists view the prospects for expanded *Aegle marmelos* fruit processing as highly promising. The young leaves and shoots are eaten as a vegetable in Thailand and used to season food in Indonesia. They are said to reduce the appetite. An infusion of the flowers is a cooling drink. The food value per 100 g of fresh *Aegle marmelos* fruit as analyzed in India and the Philippines is: water 54.96-61.5 g, protein 1.8- 2.62 g, fat 0.2-0.39 g, carbohydrates 28.11-31.8 g, ash 1.04-1.7 g, carotene55 mg, thiamine 0.13 mg, riboflavin1.19 mg, niacin 1.1 mg, ascorbic acid 8-60 mg and tartaric acid 2.11 mg (Chavda *et al.*, 2012).

- **Fodder:** The leaves and twigs are lopped for fodder (Chavda *et al.*, 2012).

- **Timber:** The wood is strongly aromatic when freshly cut. It is gray-white, hard, but not durable; has been used for carts and construction, though it is inclined to warp and crack during curing. It is best utilized for carving, small-scale turnery, tool and knife handles, pestles and combs, taking a fine polish (Chavda *et al.*, 2012).

- **Gum or Resins:** The gum enveloping the seeds is most abundant in wild fruits and especially when they are unripe. It is commonly used as household glue and is employed as an adhesive by jewelers. Sometimes it is resorted to as a soap-substitute. It is mixed with lime plaster for waterproofing wells and is added to cement when building walls. Artists add it to their watercolors, and it may be applied as a protective coating on paintings (Chavda *et al.,* 2012).

- **Tannin or dyestuff:** There is as much as 9% tannin in the pulp of wild fruits, less in the cultivated types. The rind contains up to 20%. Tannin is also present in the leaves. The rind of the unripe fruit is employed in tanning and also yields a yellow dye for calico and silk fabrics (Chavda *et al.,* 2012).

1.4 Objective of this study

The objective of this study was to evaluate the antioxidant content and *in vitro* cytotoxic activity of the three fruit plants leaf extracts using HeLa cell line. This study may allow us to get a comprehensive analysis on their individual activities and show susceptible results which will help in further research prospective.

Chapter 2

Materials and Methods

2.1 Collection of *Spondias pinnata* leaves

The plant material used in this experiment was leaves. The leaves of *Spondias pinnata* were collected from Ashulia and it was identified and authenticated by taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka.

2.1.1 Preparation of *Spondias pinnata* leaves

The leaves were washed properly and sun dried till crisp for a week or so and then using a grinder the leaves were grinded into fine powder. The coarse powder was then stored in an air-tight container with necessary markings for identification and stored in cool, dark and dry place.

2.1.2 Extraction of the plant material

Here hot solvent extraction process was used for extraction of the plant material. Four solvents were used for the extraction of the plant material. They were as follows

i) N hexane
ii) Petroleum ether
iii) Methanol
iv) Chloroform

The powdered plant material (50 gm) was successively extracted in a Soxhlet extractor with an elevated temperature using 250 ml of n-hexane, followed by petroleum ether, methanol and chloroform, according to increasing solubility. All extracts were filtered individually and poured on petri dishes to evaporate the solvents from the extracted material. After drying, crude extracts were stored in stock vials and kept in refrigerator for further use.

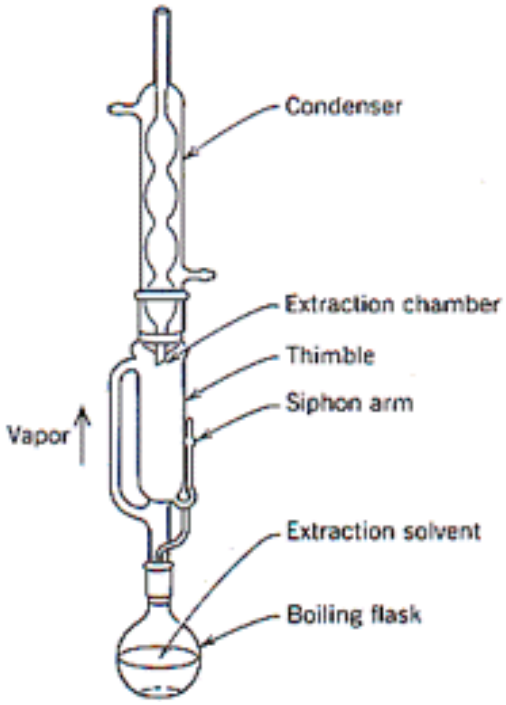


Figure 6 – Soxhlet Apparatus

2.2 Collection of *Averrhoa carambola* leaves

The plant material used in this experiment was leaves. The leaves of *Averrhoa carambola* were collected from Ashulia and it was identified and authenticated by taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka.

2.2.1 Preparation of *Averrhoa carambola* leaves

The leaves were washed properly and sun dried till crisp for a week or so and then using a grinder the leaves were grinded into fine powder. The coarse powder was then stored in an air-tight container with necessary markings for identification and stored in cool, dark and dry place.

2.2.2 Extraction of the plant material

Here hot solvent extraction process was used for extraction of the plant material. Four solvents were used for the extraction of the plant material. They were as follows

i) N hexane
ii) Petroleum ether
iii) Methanol
iv) Chloroform

The powdered plant material (50 gm) was successively extracted in a Soxhlet extractor with an elevated temperature using 250 ml of n-hexane, followed by petroleum ether, methanol and chloroform, according to increasing solubility. All extracts were filtered individually and poured on petri dishes to evaporate the solvents from the extracted material. After drying, crude extracts were stored in stock vials and kept in refrigerator for further use.

2.3 Collection of *Aegle marmelos* ***leaves***

The plant material used in this experiment was leaves. The leaves of *Aegle marmelos* were collected from Ashulia and it was identified and authenticated by taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka.

2.3.1 Preparation of *Aegle marmelos* leaves

The leaves were washed properly and sun dried till crisp for a week or so and then using a grinder the leaves were grinded into fine powder. The coarse powder was then stored in an air-tight container with necessary markings for identification and stored in cool, dark and dry place.

2.3.2 Extraction of the plant material

Here hot solvent extraction process was used for extraction of the plant material. Four solvents were used for the extraction of the plant material. They were as follows

i) N hexane
ii) Petroleum ether
iii) Methanol
iv) Chloroform

The powdered plant material (50 gm) was successively extracted in a Soxhlet extractor with an elevated temperature using 250 ml of n-hexane, followed by petroleum ether, methanol and chloroform, according to increasing solubility. All extracts were filtered individually and poured on petri dishes to evaporate the solvents from the extracted material. After drying, crude extracts were stored in stock vials and kept in refrigerator for further use.

2.4 Antioxidant Capacity

An antioxidant can be defined as: "any substance that when present in low concentrations compared to that of an oxidizable substrate significantly delays or inhibits the oxidation of that substrate". The physiological role of antioxidants, as this definition suggests, is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals (Young and Woodside, 2001). Free radical is constantly generated in all living cells and is a part of normal cellular function. However, excess free radical originating from endogenous or exogenous sources are responsible for aging and causing various human diseases. Free radicals cause oxidative damage to different molecules, such as lipids, proteins and nucleic acids and thus are involved in the initiation phase of some degenerative diseases. Research has shown that free radical mediated oxidative stress is among the major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others. Antioxidants prevent free radicals from doing harm to our DNA, proteins, and cells by donating electrons to stabilize and neutralize the harmful effects of the free radicals. This action helps in

protecting the body from degenerative diseases. With that, the role of antioxidants has drawn much attention as a candidate to combat certain diseases and prevent the aging process. An antioxidant can be defined as: any substance that when present in low concentrations compared to that of an oxidizable substrate significantly delays or inhibits the oxidation of molecules, by inhibiting the initiation or propagation of oxidizing chain reactions (Khatoon *et al*., 2013).

A free radical is defined as any atom or molecule possessing unpaired electrons. Antioxidants are agents which scavenge the free radicals and prevent the damage caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). Antioxidants can greatly reduce the damage due to oxidants by neutralizing the free radicals before they can attack the cells prevent damage to lipids, proteins, enzymes, carbohydrates DNA. A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases (Kalita *et al*., 2013).

The antioxidant activities were determined by using the four following methods:

1. Cupric Reducing Antioxidant Capacity (CUPRAC).

2. Determination of Total Antioxidant Content.

3. Determination of Total Phenolic Content.

4. Determination of Total Flavonoid Content.

2.4.1 Cupric Reducing Antioxidant Capacity (CUPRAC)

Principle

This assay is based on the changes in absorption characteristics of the neocuproine (Nc) copper (II) complex when it is reduced by an antioxidant. The reduction potential of the sample or standard effectively converts Cu^{+2} to Cu^{+1}. This reduced complex absorption is measured at 450nm. This is a colorimetric assay with the reagents changing colour from blue-green to yellow in the presence of an antioxidant (Apak *et al*., 2004).

2.4.1.1 Materials needed for Cupric reducing antioxidant capacity

Reagents

- Cupric chloride *(*$CuCl_2.2H_2O$*)*
- Ammonium acetate buffer, pH 7.0
- Neocuproine
- Butylated hydroxytoluene (BHT) (standard)
- Distilled water
- Ethanol (solvent)

Apparatus

- Screw cap test tubes
- Test tubes
- Pipettes (1ml, 5ml, 10ml)
- Micropipette (10-100µl)
- Spatula
- Beaker
- Volumetric flasks (250 ml, 100ml)
- Test tube racks
- Pipette filler
- Electronic Balance

2.4.1.2 Preparation of reagents

- **Preparation of 0.01 M solution of cupric chloride**
  0.4262 gm of $CuCl_2.2H_2O$, after measuring in an electric balance was taken in a 250ml of volumetric flask and distilled water was added to adjust the volume.
- **Preparation of 1 M ammonium acetate buffer, pH 7.0**
  19.27 gm of ammonium acetate was taken and placed in a 250 ml volumetric flask and the volume was adjusted with distilled water.

- **Preparation of 0.0075 M solution of Neocuproine**

  0.156 gm of neocaprom was taken and placed in a 100ml volumetric flask and the volume was adjusted with 96% ethanol.

- **Preparation of Standard and Extract solution**

  The standard and extract solutions were prepared separately by weighing them and adding ethanol to a volume to yield a concentration of 5mg/ml. The experimental concentrations were prepared by serial dilution of this stock solution.

2.4.1.3 Experimental Procedure

80µl of each stock solution (containing 400µg of standard or extracts) was mixed with 1.92ml of distilled water to achieve a concentration of 200µg/ml. Serial dilutions were done to get concentrations of 100µg/ml, 50µg/ml, 25µg/ml and 12.5µg/ml. A final volume of 500µl per test tube was adjusted to which, 1 ml of copper *(II)* chloride solution (0.01 M prepared from $CuCl_2.2H_20$), 1 ml of ammonium acetate buffer at pH 7.0 and 1 ml of neocuproine solution (0.0075 M) and 600µl of distilled water were mixed. The test tubes were incubated for 1 hour at room temperature. Then the absorbance of the solution was measured at 450 nm using a spectrophotometer against a blank. BHT was used as the standard.

2.4.2 Total antioxidant content

Principle

The phospho-molybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, α-tocopherol, and carotenoids. The phospho-molybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and subsequent formation of green phosphate/ Mo (V) complex. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI) and the formation of a green phosphate/Mo (V) complex with maximal absorption at 695 nm (Prieto *et al.,* 1999).

$$Mo\ (VI) + e \longrightarrow Mo\ (V)$$

2.4.2.1 Materials needed for total antioxidant content

Reagents

- 96 % Sulphuric Acid (H_2SO_4)
- Sodium Phosphate Na_3P0_4
- Ammonium Molybdate
- Ascorbic acid (standard)
- Ethanol (solvent)
- Distilled Water

Apparatus

- Screw cap test tubes
- Test tubes
- Pipettes (1ml, 5ml, 10ml)
- Micropipette (10-100µl)
- Spatula
- Beaker
- Volumetric flasks (250 ml, 100ml)
- Test tube racks
- Pipette filler
- Electronic Balance

2.4.2.2 Preparation of reagents

- **Preparation of 96% Sulphuric Acid (H_2SO_4)**
  0.333ml of H_2SO_4 of 96% concentration was taken in a 100ml volumetric flask and distilled water was added to adjust the volume.
- **Preparation of 0.02295 M Sodium Phosphate (Na_3P0_4)**
  0.459 gm of Na_3P0_4 was taken in a 100ml volumetric flask and distilled water was added to adjust the volume.

- **Preparation of 0.004 M Ammonium Molybdate**

  0.4943 gm of ammonium molybdate was taken in a 100ml volumetric flask and distilled water was added to adjust the volume.

- **Preparation of standard and stock solution**

  The standard and extract solutions were prepared separately by weighing them and adding ethanol to a volume to yield a concentration of 5mg/ml. The experimental concentrations were prepared by serial dilution of this stock solution.

2.4.2.3 Experimental procedure

80µl of each stock solution (containing 400µg of standard or extracts) was mixed with 1.92ml of distilled water to achieve a concentration of 200µg/ml. Serial dilutions were done to get concentrations of 100µg/ml, 50µg/ml, 25µg/ml and 12.5µg/ml. A final volume of 300µl per test tube was adjusted. Then 1 ml of concentrated Sulphuric Acid (H_2SO_4), 1ml of 0.02295M Sodium Phosphate (Na_3P0_4) and 1ml of 0.004M Ammonium Molybdate were added. The test tubes were incubated at 95°C for 90 minutes. After cooling to room temperature, the absorbance of each sample was measured at 695 nm against a blank. Ascorbic acid was used as the standard.

2.4.2.4 Calculation

The antioxidant activity is expressed as the number of equivalents of ascorbic acid and was calculated by' the following equation:

$A = (c \times V)/m$

Where,

A = total content of Antioxidant compounds in mg/gm plant extract, in Ascorbic acid Equivalent.

c = the concentration of Ascorbic acid established from the calibration curve in mg/ml

v = the volume of extract in ml

m = the weight of crude plant extract in gm

2.4.3 Total phenolic content

Principle

Total phenolic content of all the extracts was evaluated with Folin-Ciocalteu method. Samples containing polyphenols are reduced by the Folin-Ciocalteu reagent by producing blue colored complex. The phenolic concentration of extracts was evaluated from a Gallic acid calibration curve. After incubation at 20°C for 60 min, the quantitative phenolic estimation was performed at 765 nm against reagent blank by UV Spectrophotometer (1650 Shimadzu, Japan). Total phenolic content was expressed as milligrams of Gallic acid equivalent (GAE) per gm of extract (Khatoon *et al*., 2013).

2.4.3.1 Materials needed for total phenolic content

Reagents

- Folin -ciocalteu reagent
- 7.5% Sodium carbonate solution
- Methanol (solvent)
- Gallic acid (standard)
- Distilled water

Apparatus

- Screw cap test tubes
- Test tubes
- Pipettes (1ml, 5ml, 10ml)
- Micropipette (10-100µl)
- Spatula
- Beaker
- Volumetric flasks (250 ml, 100ml)
- Test tube racks
- Pipette filler
- Electronic Balance

2.4.3.2 Method

- **Preparation of 7.5% Sodium Carbonate solution**

  7.5 gm of Na_2C0_3 was taken into a 100 ml of a volumetric flask and the volume was adjusted by distilled water.

- **Preparation of 1:10 v/v Ratio Folin-ciocalteu slolution**

  20 ml Folin & ciocalteu phenol reagent was dissolved in 200 ml of distilled water to get a ratio of 1:10

- **Preparation of standard and stock solution**

  The standard and extract solutions were prepared separately by weighing them and adding methanol to a volume to yield a concentration of 5mg/ml. The experimental concentrations were prepared by serial dilution of this stock solution.

2.4.3.3 Experimental procedure

80µl of each stock solution (containing 400µg of standard or extracts) was mixed with 1.92ml of distilled water to achieve a concentration of 200µg/ml. Serial dilutions were done to get concentrations of 100µg/ml, 50µg/ml, 25µg/ml and 12.5µg/ml. A final volume of 1ml per test tube was adjusted. Then 5 mL Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 mL (7.5% sodium carbonate) of sodium carbonate solution were added tp each test tube. They were allowed to stand for 60 min at 20°C for color development. Absorbance of samples and standard were measured at 765 nm using spectrophotometer against blank (methanol). Gallic acid was used as the standard.

2.4.3.4 Calculation

The total content of phenolic compounds plant extracts in gallic acid equivalents (GAE) calculated using the following equation:

$$C = (c \times V)/m$$

Where;

C = total content of phenolic compounds in mg/gm plant extract, in GAE

c = the concentration of gallic acid established from the calibration curve (mg/ml)

V =the volume of extract in ml

m = the weight of crude plant extract in gm

2.4.4 Total flavonoid content

Principle

Determining the total flavonoids by using aluminum chloride method is based upon the formation of stable complex between aluminum chloride and keto and hydroxyl groups of flavones and flavonoids. For building the calibration curve, quercetin was used as standard. Various concentrations of standard quercetin solution were used to make a standard calibration curve (Pallab *et al*., 2013).

2.4.4.1 Materials needed for Total Flavonoid content

Reagents

- 10% Aluminium Chloride solution
- 1M Potassium Acetate solution
- Methanol (solvent)
- Quercertine (standard)
- Distilled water

Apparatus

- Screw cap test tubes
- Test tubes
- Pipettes (1ml, 5ml, 10ml)
- Micropipette (10-100µl)
- Spatula
- Beaker
- Volumetric flasks (250 ml, 100ml)
- Test tube racks
- Pipette filler
- Electronic Balance

2.4.4.2 Preparation of reagents

- **Preparation of 10% Aluminum Chloride ($AlCl_{3)}$ solution**

  10 gm of $AlCl_3$ was taken into a 100 ml of a volumetric flask and the volume was made up to 100ml using distilled water.

- **Preparation of 1M Potassium Acetate solution**

  9.815 gm of potassium acetate was taken into a 100 ml of ii volumetric flask and the volume adjusted up to 100ml by distilled water.

- **Preparation of standard and stock solution**

  The standard and extract solutions were prepared separately by weighing them and adding methanol to a volume to yield a concentration of 5mg/ml. The experimental concentrations were prepared by serial dilution of this stock solution.

2.4.4.3 Experimental procedure

80µl of each stock solution (containing 400µg of standard or extracts) was mixed with 1.92ml of distilled water to achieve a concentration of 200µg/ml. Serial dilutions were done to get concentrations of 100µg/ml, 50µg/ml, 25µg/ml and 12.5µg/ml. A final volume of 1ml per tube was adjusted to which, 3 ml of methanol, 200µl of aluminum chloride, 200µl of 1M potassium acetate and 5.6 ml of distilled water. The test tubes remained at room temperature for 30 min; the absorbance of the mixture was measured at 415 nm with spectrophotometer against blank. Quercertin was used as the standard.

2.4.4.4 Calculation

The total content of flavonoid compounds in plant methanol extracts in quercetin equivalents was calculated by the following formula equation:

$$C = \{c \times V)/m$$

Where;

C = total content of flavonoid compounds in mg/gm plant extract, in quercetin equivalent

c = the concentration of quercetin established from the calibration curve (mg/ml)

V =the volume of extract in ml

m = the weight of crude plant extract in gm

2.5 In vitro Cytotoxicity using Animal cell culture

Principle of *in vitro* cytotoxic activity

In vitro Cytotoxicity involves a cell line which is maintained in specific medium containing antibiotics and nutrients. These allow the cells to remain healthy, anchored to the surface and remain uncontaminated. Cytotoxicity assays vary depending upon the choice of experiment. To a specific amount of cells per well of a cell culture or micro titer plate, the extracts are added after which cell viability is checked under a microscope. Cultured cells that are undergoing apoptosis *in vitro* eventually undergo secondary necrosis. After extended incubation, apoptotic cells ultimately shut down metabolism, lose membrane integrity and release their cytoplasmic contents into the culture medium. The amount of cell viability determines whether the extracts had any effect on the cell line (Nema and Khare., 2012).

2.5.1 Applications of Animal Cell Culture

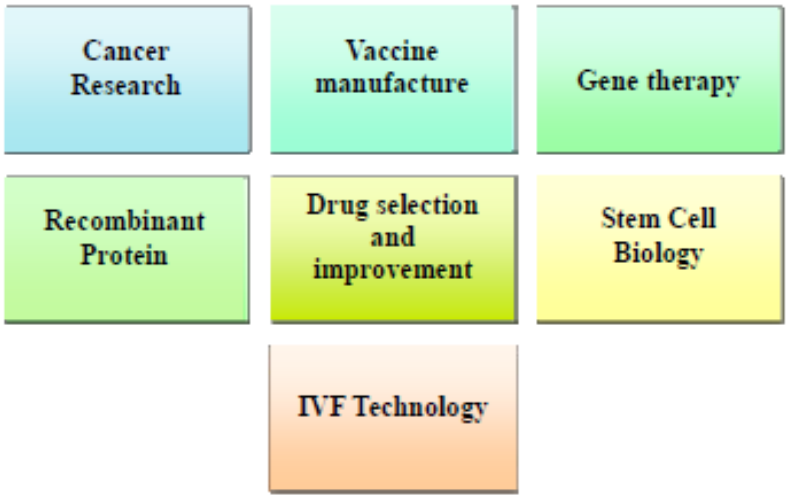


Figure 7 Applications of Animal Cell culture technique

2.5.2 Experimental Procedure

2.5.2.1 Preparation of samples

The extracted samples were taken, measured and dissolved in DMSO in such a manner that, in 24 well micro titer plate, the concentration of the DMSO would be 1.5% in each well.

2.5.2.2 Used Consumables:

24-well plate, 15-ml tubes, Tips, Gloves, Culture flask, Cell culture media, Antibiotics (Penicillin and Streptomycin), Gentamycin, Serological pipette, Trypsin etc.

2.5.2.3 Thawing of cells

The cryovial containing the frozen cells from liquid nitrogen storage were immediately placed into a 37°C water bath and quickly thawed for (< 1 minute) by gently swirling the vial in the 37°C water bath until there is just a small bit of ice left in the vial. Then the vial was transferred into a laminar flow hood. After that the thawed cells were transferred drop wise into the centrifuge tube containing the desired amount of pre-warmed complete growth medium appropriate for the cell line. It was centrifuged for 1 min at 1000rpm and after which the clarity of supernatant was checked for a complete pellet. Then gently, the cells were re suspended in complete growth medium, and transferred into the culture vessel.

2.5.2.4 Cell passage

Cell passage was done to get a fresh cell suspension. The used culture media in the vessel was discarded and washed with PBS. Then PBS was discarded and 800µl of trypsin was added to detach the cells from the surface of the vessel. After a few minutes of incubation the cells were observed under the microscope for detachment and after 90% of cells were seen to be detached, 5ml of fresh media (DMEM) was added to the vessel and was thoroughly mixed with a pipette. Then, 1ml of this solution was taken in another vessel and 4ml of DMEM was added and was kept in the incubator for future use.

2.5.2.5 Cell counting

Cell counting was done using a hemocytometer. To prepare the hemocytometer, the mirror-like polished surface was carefully cleaned with lens paper and ethanol. The coverslip was also cleaned. The coverslip was placed over the counting surface prior to adding the cell suspension. The fresh cell suspension after cell passage was introduced into the hemocytometer using a Pasteur pipet. Enough suspension was introduced so that the mirrored surface was just flooded. The counting chamber was then placed on the microscope stage and the counting grid was

brought into focus at low power. One entire grid on standard hemocytometers with Neubauer rulings can be seen at 40X magnification. Each large square of the hemocytometer has a volume of is $0.1mm^3$. Counting of the cells was done from the 4 large squares. Those cells touching either upper and left borders or lower and right borders were counted.

Calculation

Cell count in large square A= 13 cells
Cell count in large square B= 12 cells
Cell count in large square C= 14 cells
Cell count in large square D= 11 cells

The average no. of cells was be found out by dividing the total no. of cells counted by the no. of squares.

$$\text{Therefore, Average no. of cells} = \frac{13+12+14+11}{4} \times 10^4 \text{cells/ml}$$

$$= 12.5 \times 10^4 \text{ cells/ml}$$

In 1000µl, there are 12.5×10^4 cells

So, in 400µl, there are 5×10^4 cells/400µl/well.

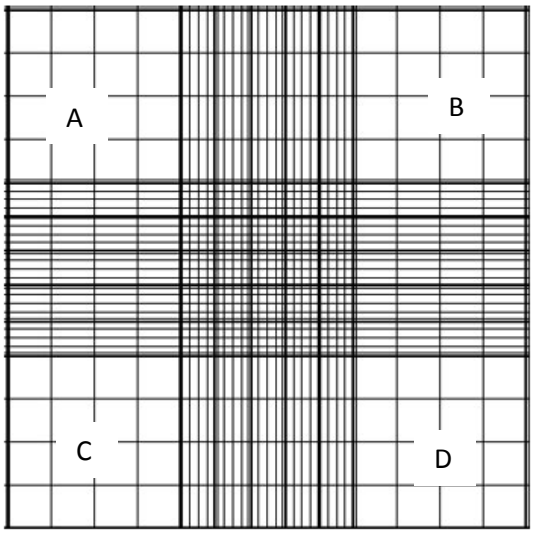


Figure 8 Showing a Hemocytometer

2.5.2.6 Determination of Cytotoxic activity

In theory 2% of DMSO and 20% of water is cytotoxic to animal cells. So keeping that in mind the experimental procedure was developed to determine the cytotoxic activity of the plant extracts. Cytotoxic effect was examined in The Centre for Advanced Research in Sciences using their services. In this study, the *in vitro* cytotoxic effects of the leaf extracts were evaluated using HeLa cell line, where HeLa, a human cervical carcinoma cell line, was maintained in DMEM (Dulbecco's Modified Eagles' medium) containing 1% penicillin- streptomycin (1:1), 0.2% gentamycin and 10% fetal bovine Serum (FBS). After counting the cells in hemocytometer, a 5×10^4 HeLa cells were seeded onto a 24-well cell culture plate at a concentration of 5×10^4/400µl/well and incubated at 37°C and 5% CO_2. Next day, 100 µl of sample (filtered through 0.45µM previously) was added to each well. Duplicate wells were used for each sample. A control was made using DMSO and water. The concentration of DMSO in each well was 1.5%, the concentration of water was 18.5% and the concentration of plant extracts were 45%. After 48 hours of incubation, the cells were taken under the microscope to check their percentage viability for each given extract and the results were recorded.

Chapter 3

Results

3.1 Curpic Reducing Antioxidant Capacities

3.1.1 Curpic Reducing Antioxidant Capacity of *Averrhoa carambola*

Reduction of Cu^{2+} ion to Cu^{1+} was found to rise with increasing concentrations of the different extracts. The standard BHT showed highest reducing capacity. Among the extracts the methanol extract of *Averrhoa carambola* showed maximum reducing capacity that is comparable to BHT (Figure 9).

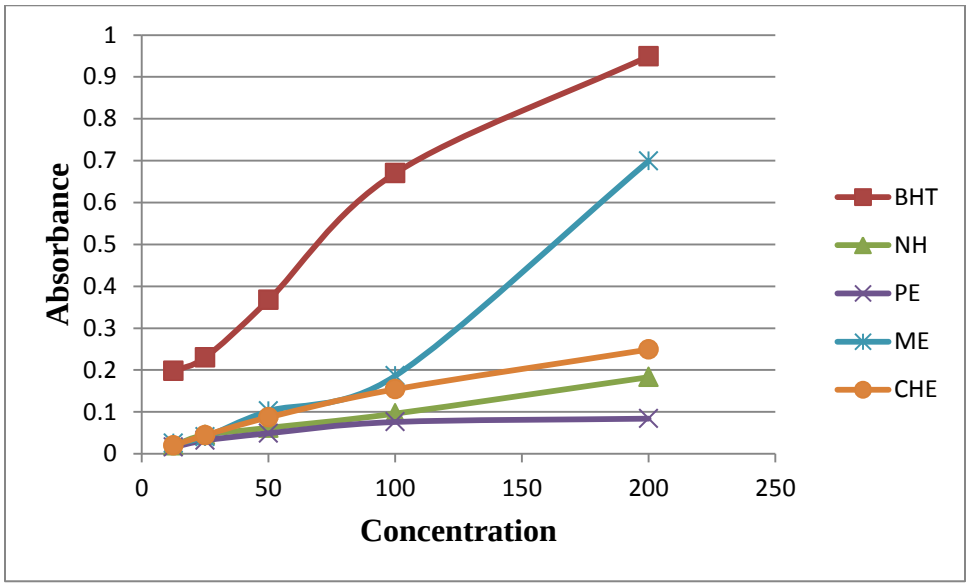


Figure 9 Comparative reducing powers of *Averrhoa carambola* extracts and Butylated hydroxyl toluene (BHT)

3.1.2 Curpic Antioxidant Capacity of extracts of *A. marmelos*

Reduction of Cu2+ ion to Cu+ was found to rise with increasing concentrations of the different extracts. The standard BHT showed highest reducing capacity. Among the extracts the methanol extract of *A. marmelos* showed maximum reducing capacity that is comparable to BHT (Figure 10).

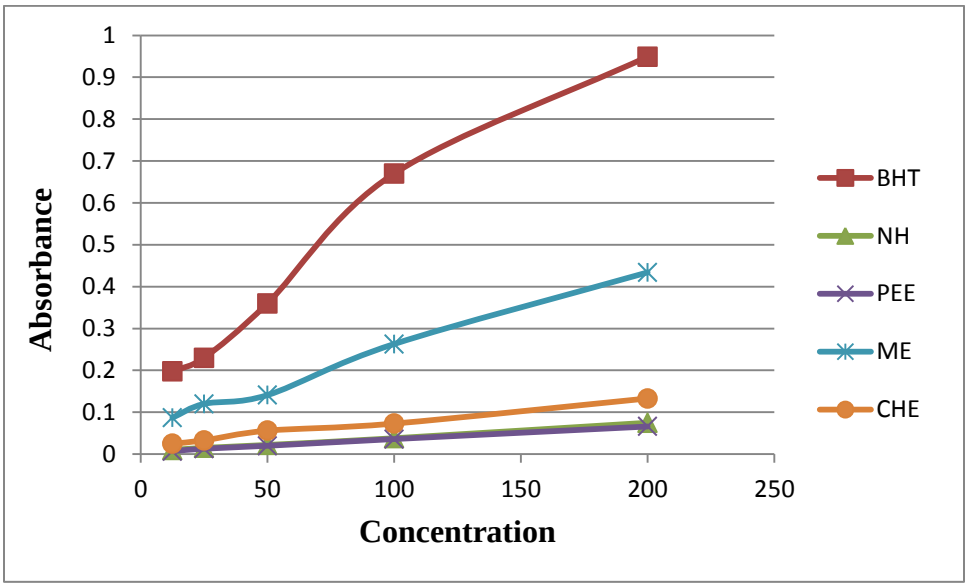


Figure 10 Comparative reducing powers of *A. marmelos* extracts and Butylated hydroxyl toluene (BHT)

3.1.3 Cupric Reducing Antioxidant Capacity of extracts of *Spondias pinnata*

Reduction of Cu2+ ion to Cu+ was found to rise with increasing concentrations of the different extracts. The standard ascorbic acid and BHT showed highest reducing capacity. Among the extracts the methanol extract of *Spondias pinnata* showed maximum reducing capacity that is comparable to BHT (Figure 11).

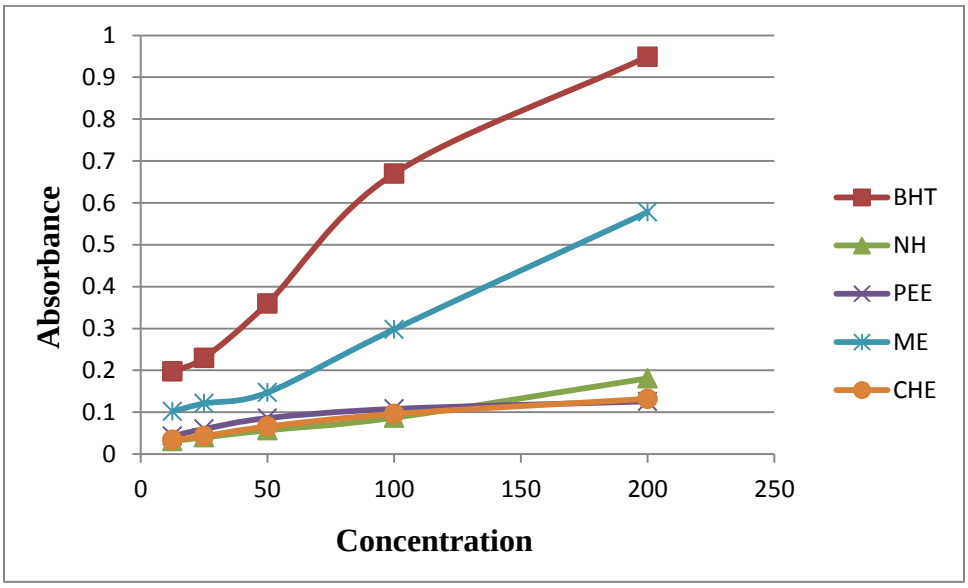


Figure 11 Comparative reducing powers of *Spondias pinnata* extracts and Butylated hydroxyl toluene (BHT)

This method is based on the principle of increase in the absorbance of the reaction mixtures. Increase in the absorbance indicates increase in the antioxidant activity. Increase in absorbance of the reaction mixture indicates the reducing power of the samples (Jayaprakasha *et al.*, 2001). Reducing power is associated with antioxidant activity and may serve as a significant reflection· of the antioxidant activity (Oktay *et al*, 2003). Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995).

3.2 Total antioxidant Contents

3.2.1 Total antioxidant content of *Averrhoa carambola* extracts

Total antioxidant capacity of the different extracts of *Averrhoa carambola* was evaluated by the phospho molybdenum method and was expressed as ascorbic acid equivalents (AAE) per gram of plant extract. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid (y = 0.0025x + 0.0492; R2=0.9561) **(Figure 12).** Petroleum ether extract of *Averrhoa carambola* was found to possess the highest total antioxidant capacity **(Table 1)**. Total antioxidant capacity of the extracts was found to decrease in the following order: Petroleum ether extract > Methanol extract > N hexane extract > Chloroform extract (Figure 13).

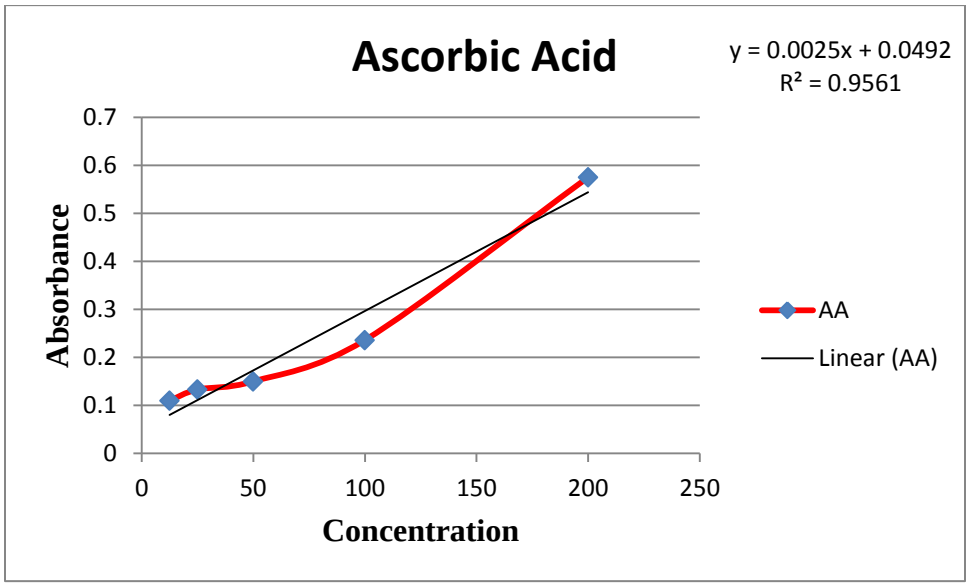


Figure 12 Calibration curve of ascorbic acid for *Averrhoa carambola*

Table 4 Total antioxidant content of the different extracts of *Averrhoa carambola* extracts

Extract	Total Antioxidant Capacity (mg/gm, Ascorbic Acid Equivalent)
N Hexane	**54 ± 14.9**
Petroleum Ether	**105.8 ± 20.1**
Methanol	**62.6 ± 16.5**
Chloroform	**44.5 ± 14.2**

Values are the mean of duplicate experiments and represented as mean ±

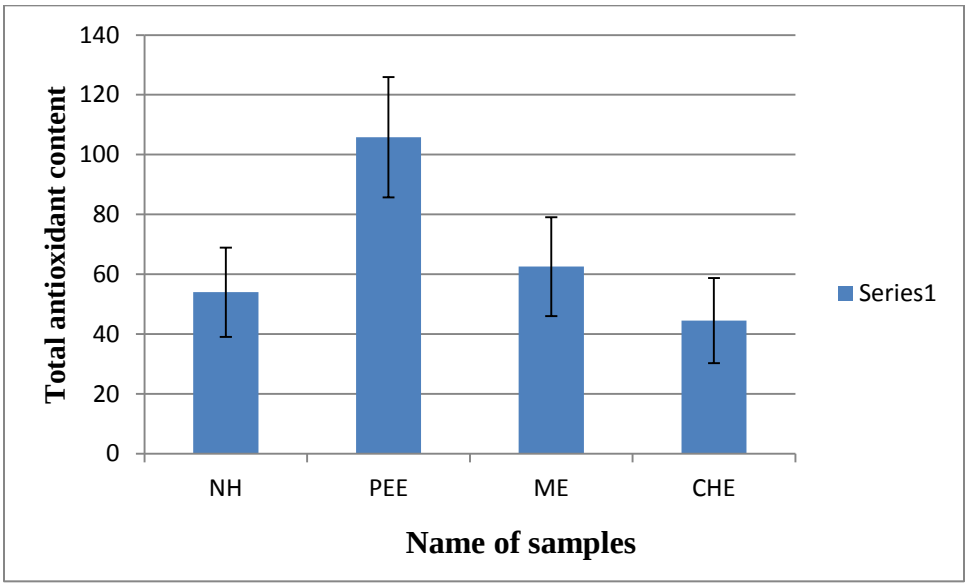


Figure 13 Total antioxidant content of the different extracts of *Averrhoa carambola* extracts

3.2.1 Total antioxidant content of *A. marmelos* extracts

Total antioxidant capacity of the different extracts of *A. marmelos* was evaluated by the phospho molybdenum method and was expressed as ascorbic acid equivalents (AAE) per gram of plant extract. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid (y = 0.0025x + 0.0492; R2=0.9561) **(Figure 14).** Petroleum ether extract of *A. marmelos* was found to possess the highest total antioxidant capacity **(Table 2)**. Total antioxidant capacity of the extracts was found to decrease in the following order: Petroleum ether extract > N hexane extract > Chloroform extract > Methanol extract (Figure 15).

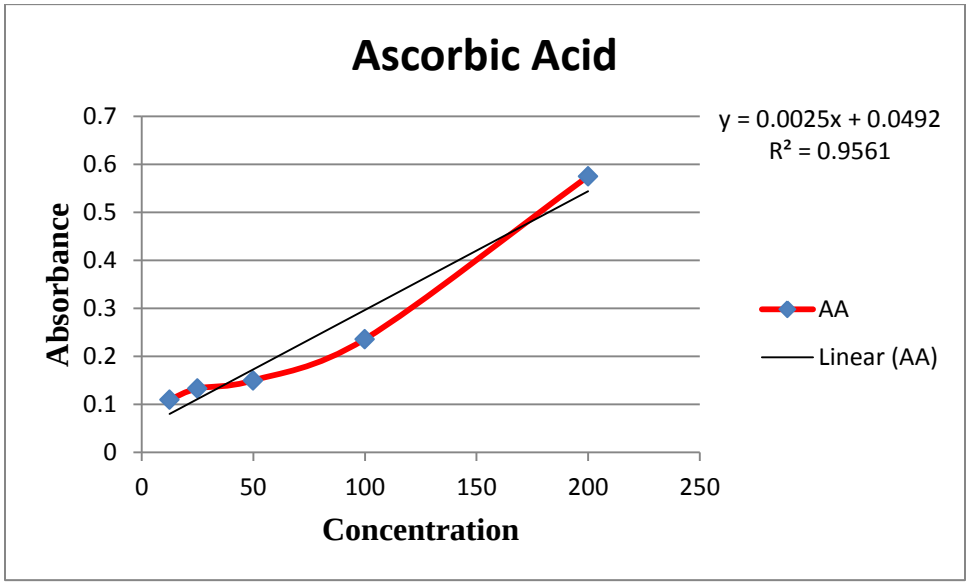


Figure 14 Calibration curve of ascorbic acid for *A. marmelos*

Table 5 Total antioxidant content of the different extracts of *A. marmelos* extracts

Extract	Total Antioxidant Capacity (mg/gm, Ascorbic Acid Equivalent)
N Hexane	**88.6 ± 20.5**
Petroleum Ether	**89.4 ± 34.7**
Methanol	**58.2± 14.3**
Chloroform	**65.4 ± 12.8**

Values are the mean of duplicate experiments and represented as mean ± SD

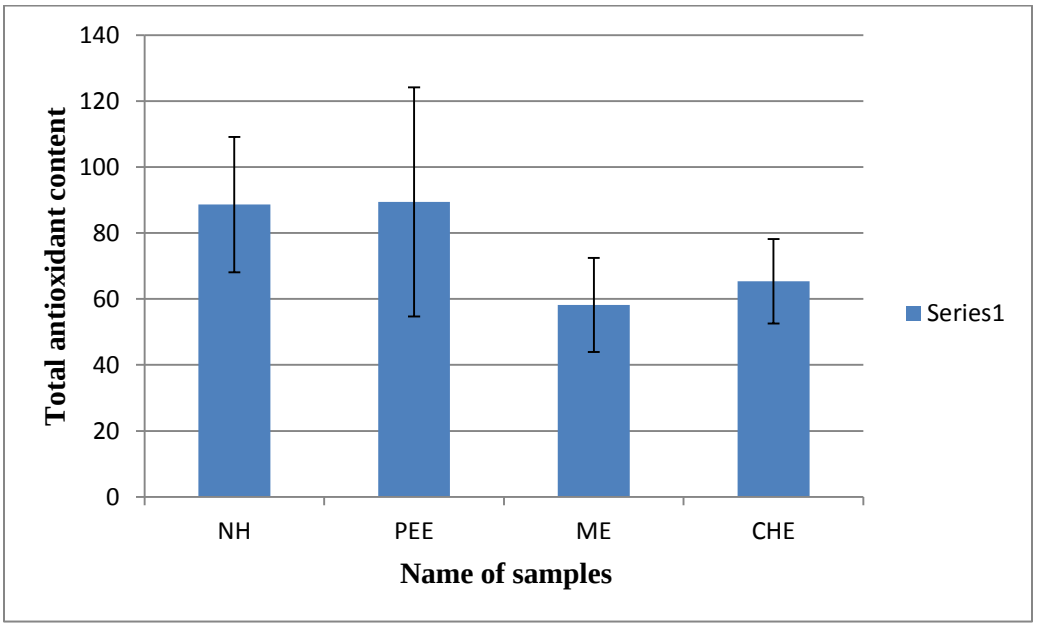


Figure 15 Total antioxidant content of the different extracts of *A. marmelos* extracts

3.2.1 Total antioxidant content of *Spondias pinnata* extracts

Total antioxidant capacity of the different extracts of *Spondias pinnata* was evaluated by the phospho molybdenum method and was expressed as ascorbic acid equivalents (AAE) per gram of plant extract. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid (y = 0.0025x + 0.0492; R2=0.9561) **(Figure 16).** Choloform extract of *Spondias pinnata* was found to possess the highest total antioxidant capacity **(Table 3)**. Total antioxidant capacity of the extracts was found to decrease in the following order: Chloroform extract > Methanol extract >Petroleum ether extract > N hexane extract > (Figure 17).

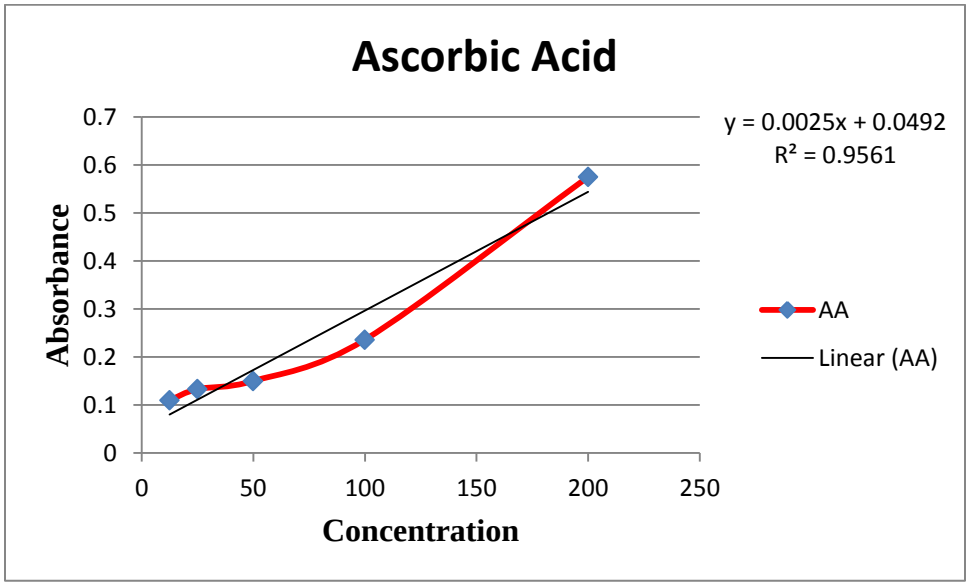


Figure 16 Calibration curve of ascorbic acid for *Spondias pinnata* extracts

Table 6 Total antioxidant content of the different extracts of *Spondias pinnata* extracts

Extract	Total Antioxidant Capacity (mg/gm, Ascorbic Acid Equivalent)
N Hexane	**60.6 ± 13.5**
Petroleum Ether	**63 ± 17.7**
Methanol	**92 ± 19.7**
Chloroform	**122 ± 21.9**

Values are the mean of duplicate experiments and represented as mean ± SD.

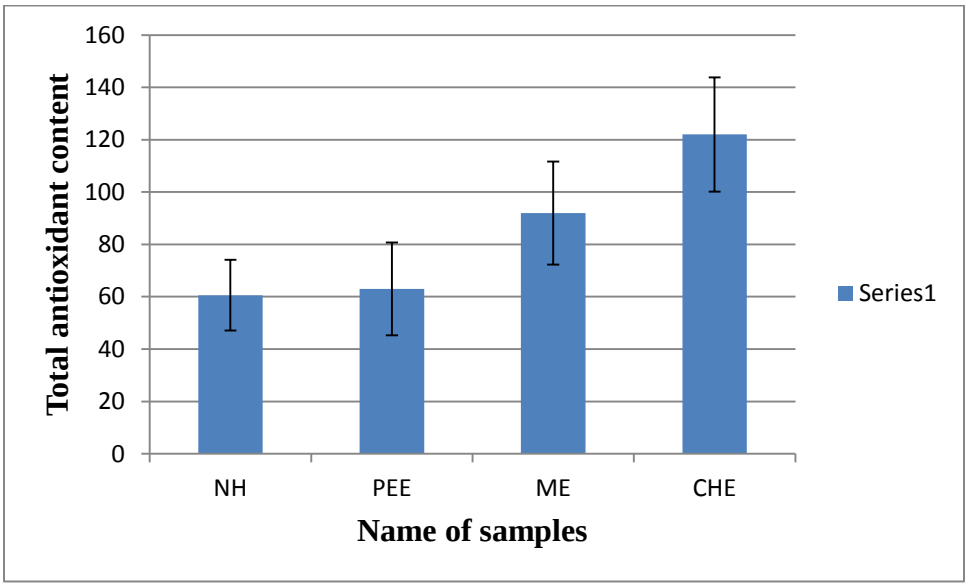


Figure 17 Total antioxidant content of the different extracts of *Spondias pinnata* extract

3.3 Determination of Total Phenolic content

3.3.1 Total Phenolic content of *Averrhoa carambola* extracts

Total phenolic content of the different extracts of *Averrhoa carambola* were determined by using the Folin Ciocalteu reagent and were expressed as Gallic acid equivalents (GAE) per gram of plant extract. The total phenolic contents of the test fractions were calculated using the standard curve of Gallic acid (y = 0.0034x + 0.3777; R2 = 0.8555) (Figure 18). N Hexane extract of *Averrhoa carambola L.* was found to contain the highest amount of phenols (Table 4). Phenol contents of the extracts were found to decrease in the following order: N hexane extract> Petroleum ether> Methanol extract> Chloroform extract (Figure 19).

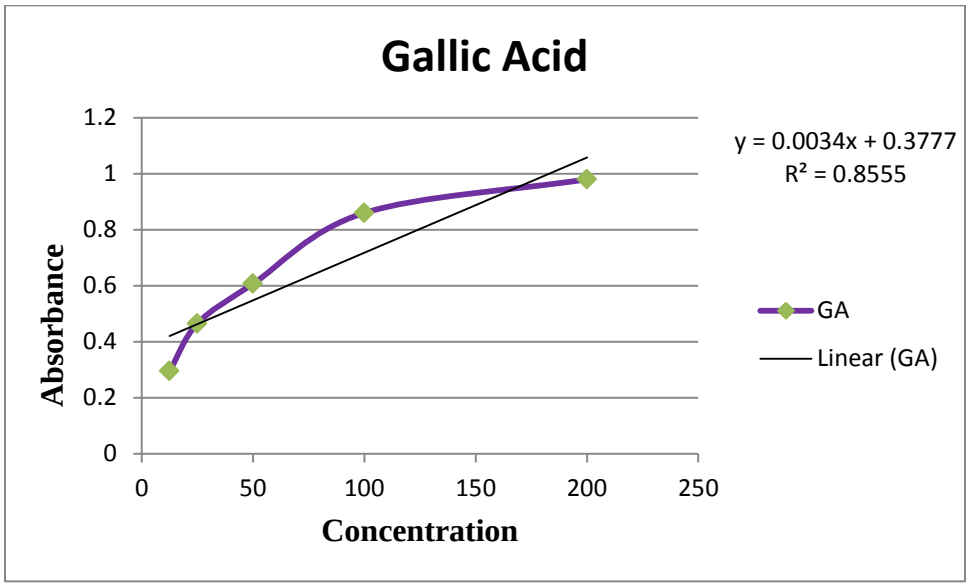


Figure 18 Calibration curve of Gallic Acid for *Averrhoa carambola*

Table 7 Total phenol contents of the different extracts of *Averrhoa carambola*

Extract	Total Phenol contents mg/gm, GallicAcid
N Hexane	**212.4 ± 14.3**
Petroleum Ether	**208 ± 13.3**
Methanol	**79.6 ± 15.1**
Chloroform	**12.4 ± 1.8**

Values are the mean of duplicate experiments and represented as mean ± SD.

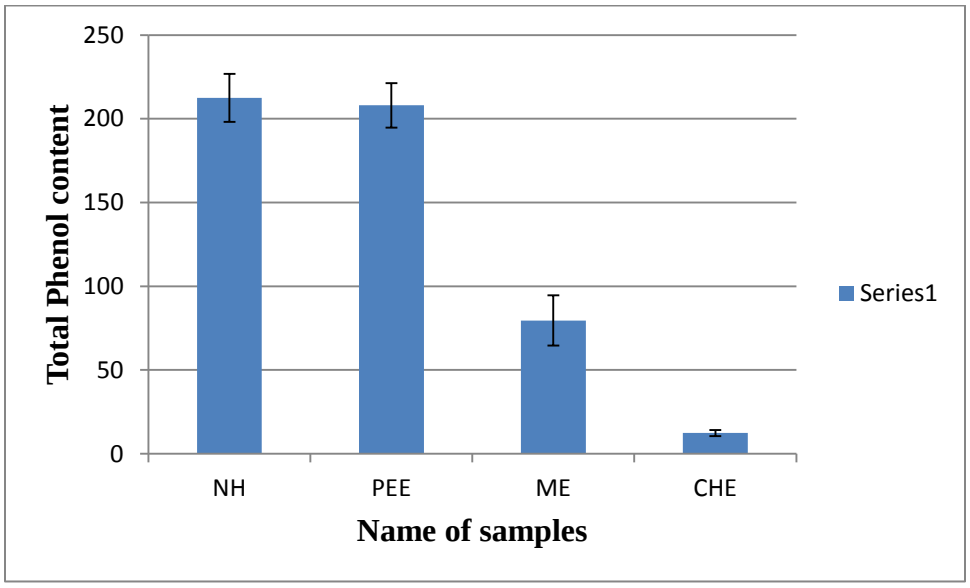


Figure 19 Total phenol contents of the different extracts of *Averrhoa carambola*

3.3.2 Total Phenolic content of *A. marmelos* extracts

Total phenolic content of the different extracts of *A. marmelos* were determined by using the Folin Ciocalteu reagent and were expressed as Gallic acid equivalents (GAE) per gram of plant extract. The total phenolic contents of the test fractions were calculated using the standard curve of Gallic acid (y = 0.0033x + 0.396; R2 = 0.8215) (Figure 20). N Hexane extract of *A. marmelos* was found to contain the highest amount of phenols (Table 5). Phenol contents of the extracts were found to decrease in the following order: N hexane extract> Chloroform extract > Petroleum Ether extract >Methanol extract (Figure 21).

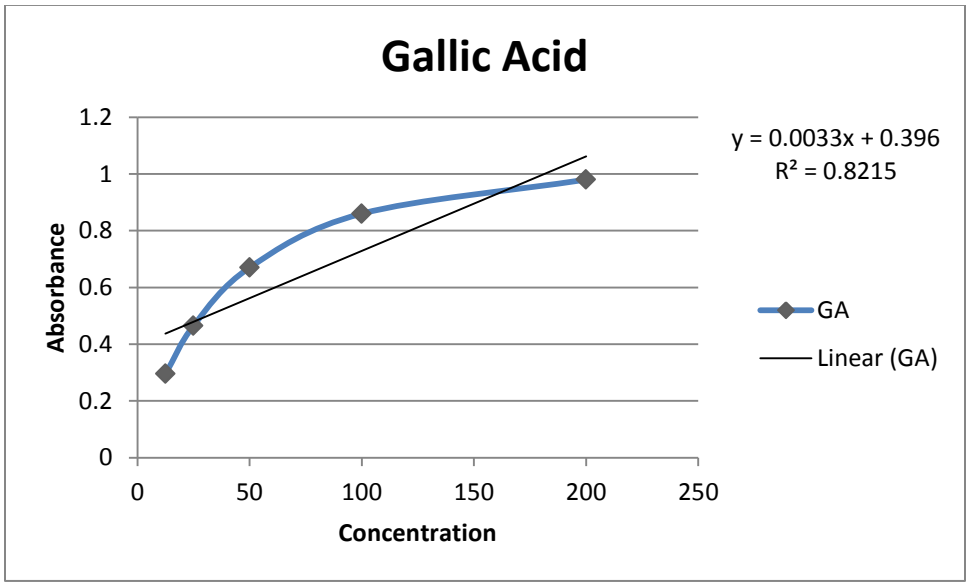


Figure 20 Calibration curve of Gallic Acid for *A. marmelos*

Table 8 Total phenol contents of the different extracts of *A. marmelos*

Extract	Total Phenol contents mg/gm, GallicAcid
N Hexane	**686 ± 32.3**
Petroleum Ether	**192 ± 17.1**
Methanol	**67 ± 15.4**
Chloroform	**195 ± 17.8**

Values are the mean of duplicate experiments and represented as mean ± SD.

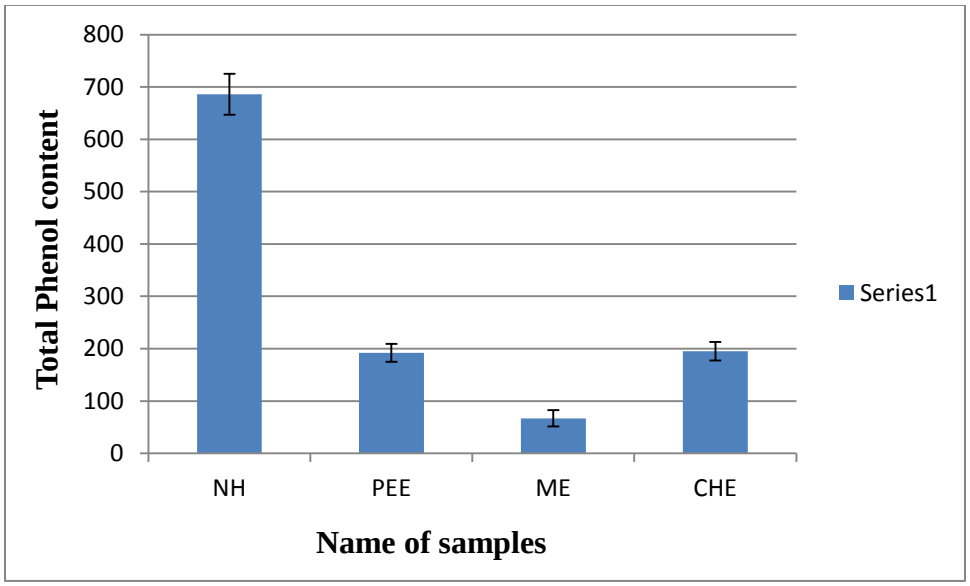


Figure 21 Total phenol contents of the different extracts of *A. marmelos*

3.3.3 Total Phenolic content of *Spondias pinnata* extracts

Total phenolic content of the different extracts of *Spondias pinnata* were determined by using the Folin Ciocalteu reagent and were expressed as Gallic acid equivalents (GAE) per gram of plant extract. The total phenolic contents of the test fractions were calculated using the standard curve of Gallic acid (y = 0.0034x + 0.3777; R2 = 0.8555) (Figure 22). Petroleum ether extract of *Spondias pinnata* was found to contain the highest amount of phenols (Table 6). Phenol contents of the extracts were found to decrease in the following order: Petroleum Ether extract> N hexane > Chloroform extract > Methanol extract (Figure 23).

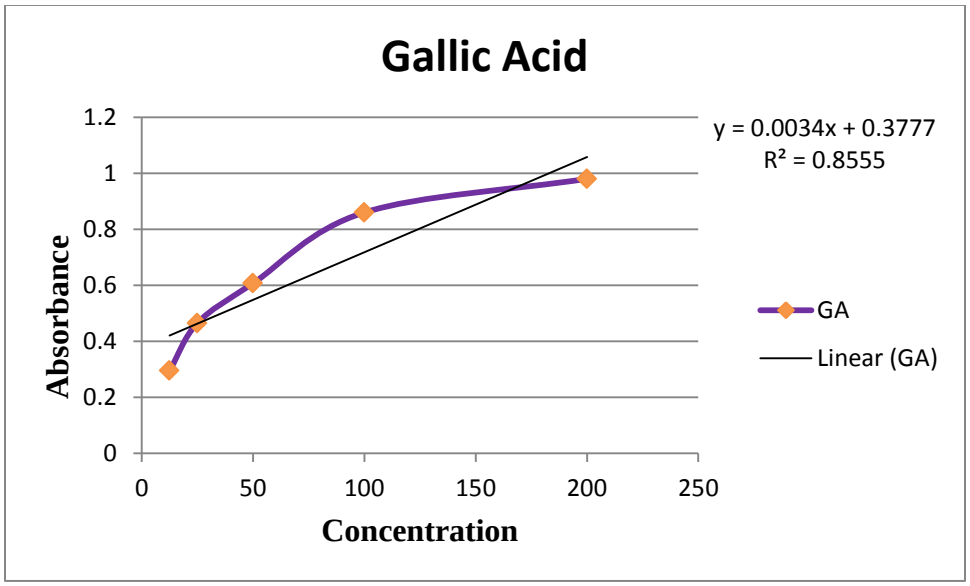


Figure 22 Calibration curve of Gallic Acid for *Spondias pinnata*

Table 9 Total phenol contents of the different extracts of *Spondias pinnata*

Extract	Total Phenol contents mg/gm, GallicAcid
N Hexane	**251.6 ± 20.6**
Petroleum Ether	**280.6 ± 16.4**
Methanol	**76 ± 15.8**
Chloroform	**238.8 ± 15.8**

Values are the mean of duplicate experiments and represented as mean ± SD.

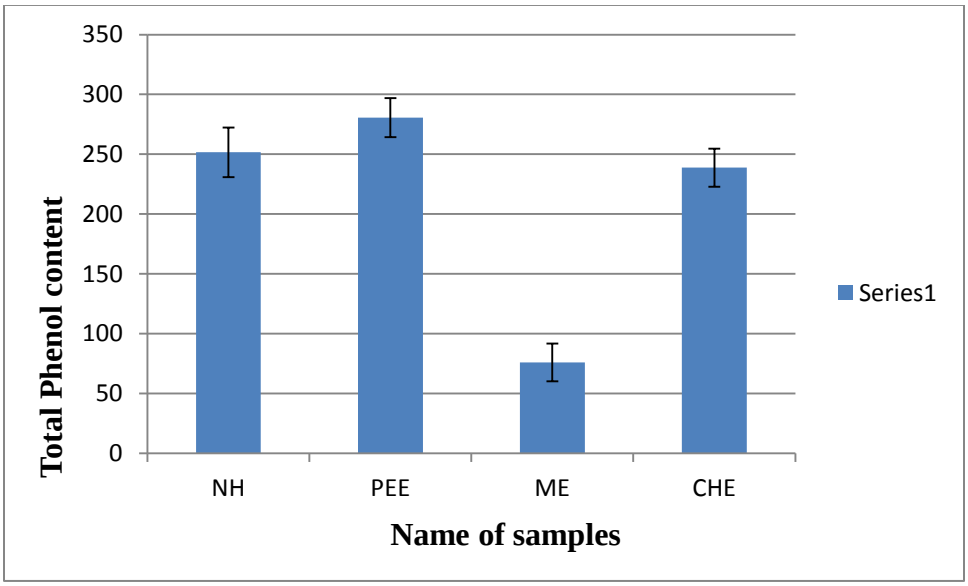


Figure 23 Total phenol contents of the different extracts of *Spondias pinnata*

3.4 Determination of Total Flavonoid content

3.4.1 Total Phenolic content of *Averrhoa carambola* extracts

Aluminium chloride colorimetric method was used to determine the total flavonoid contents of the different extracts of *Averrhoa carambola*. Total flavonoid contents were calculated using the standard curve of quercetin (y = 0.0028x + 0.0959; R2 = 0.9853) **(Figure24)** and was expressed as quercetin equivalents (QE) per gram of the plant extract. Petroleum ether of *Averrhoa carambola* was found to contain the highest amount of flavonoid **(Table 7).** Flavonoid contents of the extracts were found to decrease in the following order: Methanol extract > Chloroform extract > Petroleum ether extract > N Hexane extract **(Figure 25).**

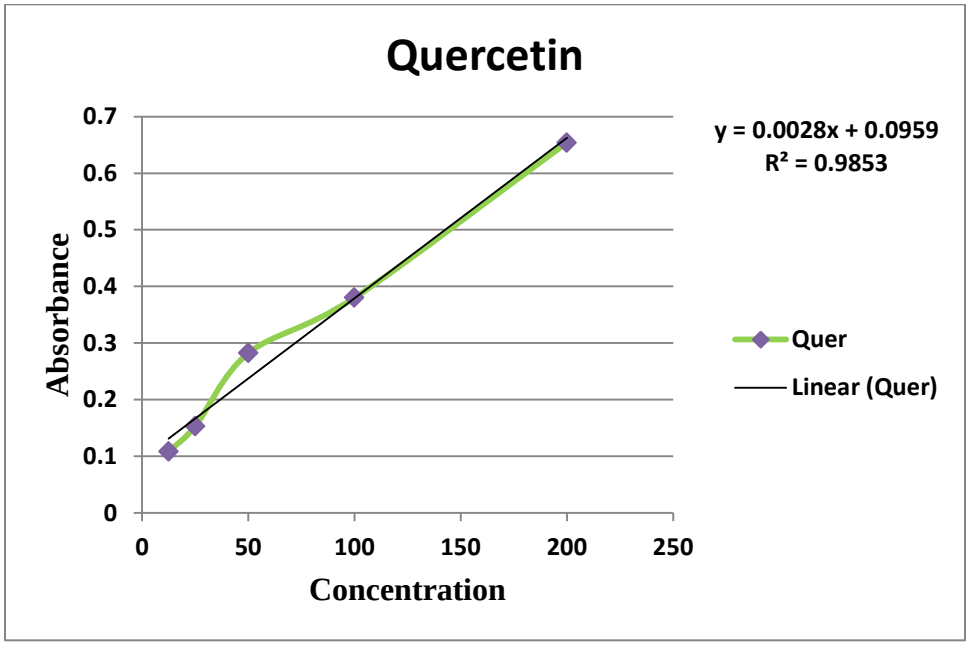


Figure 24 Calibration curve of Quercetin for *Averrhoa carambola*

Table 10 Total flavonoid contents of the different extracts of *Averrhoa carambola*

Extract	Total Flavonoid contents,mg/gm, Quercetin
N Hexane	**55.6 ± 14.9**
Petroleum Ether	**113 ± 16.0**
Methanol	**171.6 ± 18.7**
Chloroform	**132 ± 18.9**

Values are the mean of duplicate experiments and represented as mean ± SD.

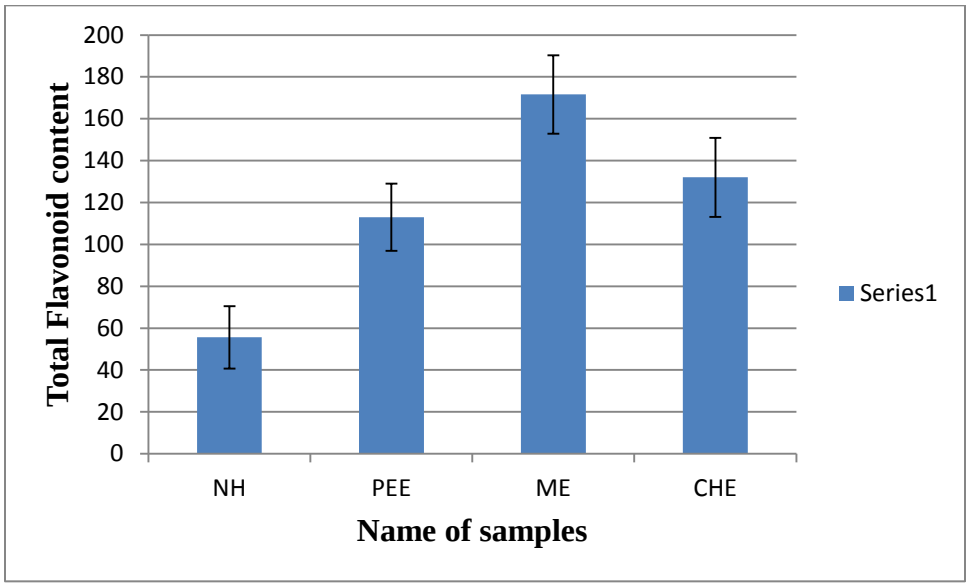


Figure 25 Total flavonoid contents of the different extracts of *Averrhoa carambola*

3.4.2 Total Flavonoid content of *A. marmelos* extracts

Aluminium chloride colorimetric method was used to determine the total flavonoid contents of the different extracts of *A. marmelos*. Total flavonoid contents were calculated using the standard curve of quercetin (y = 0.0028x + 0.0956; R2 = 0.9858) **(Figure26)** and was expressed as quercetin equivalents (QE) per gram of the plant extract. Petroleum ether of *A. marmelos* was found to contain the highest amount of flavonoid **(Table 8).** Flavonoid contents of the extracts were found to decrease in the following order: Methanol extract > Chloroform extract > N Hexane extract > Petroleum ether extract **(Figure 27).**

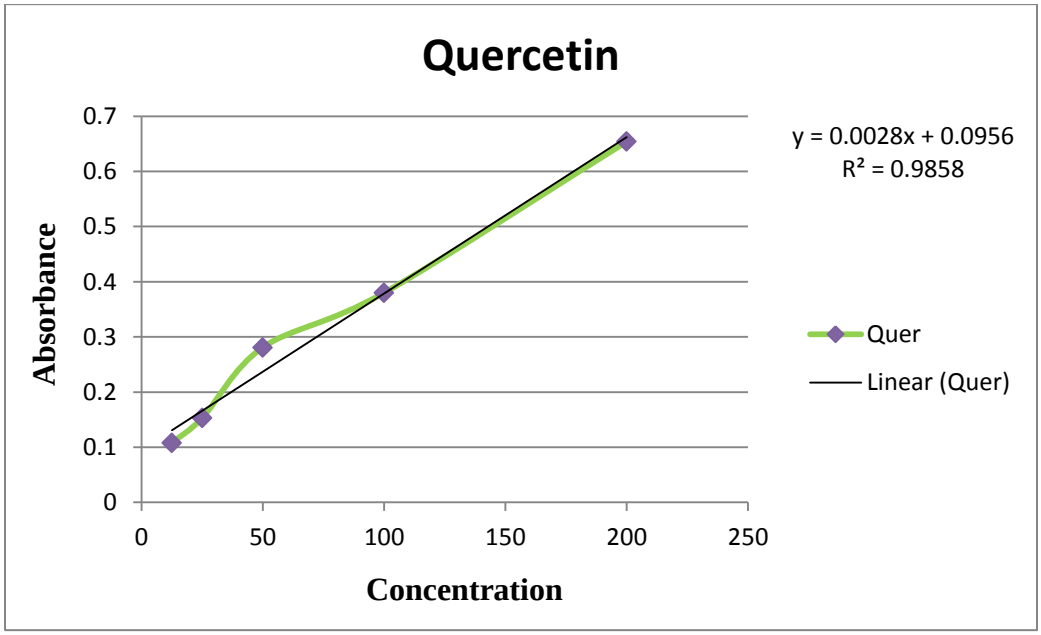


Figure 26 Calibration curve of Quercetin for *A. marmelos*

Table 11 Total flavonoid contents of the different extracts of *A. marmelos*

Extract	Total Flavonoid contents,mg/gm, Quercetin
N Hexane	**68.5 ± 16.8**
Petroleum Ether	**51.9 ± 18.8**
Methanol	**145 ± 19.2**
Chloroform	**74.6 ± 20.9**

Values are the mean of duplicate experiments and represented as mean ± SD.

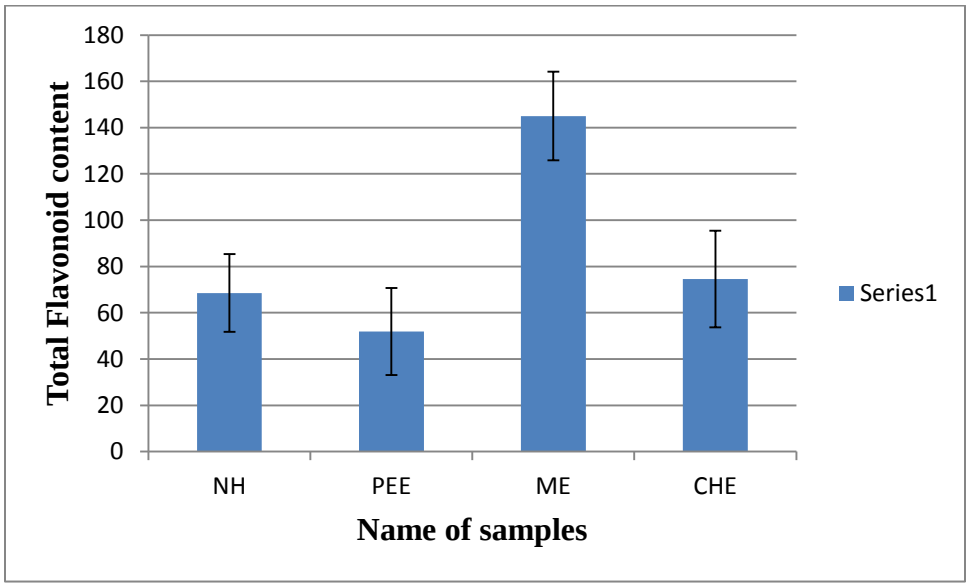


Figure 27 Total flavonoid contents of the different extracts of *A. marmelos*

3.4.3 Total Flavonoid content of *Spondias pinnata* extracts

Aluminium chloride colorimetric method was used to determine the total flavonoid contents of the different extracts of *Spondias pinnata*. Total flavonoid contents were calculated using the standard curve of quercetin (y = 0.0028x + 0.0961; R2 = 0.9857) **(Figure28)** and was expressed as quercetin equivalents (QE) per gram of the plant extract. Petroleum ether of *Spondias pinnata* was found to contain the highest amount of flavonoid **(Table 9).** Flavonoid contents of the extracts were found to decrease in the following order: Methanol extract > Chloroform extract > N Hexane extract > Petroleum ether extract **(Figure 29).**

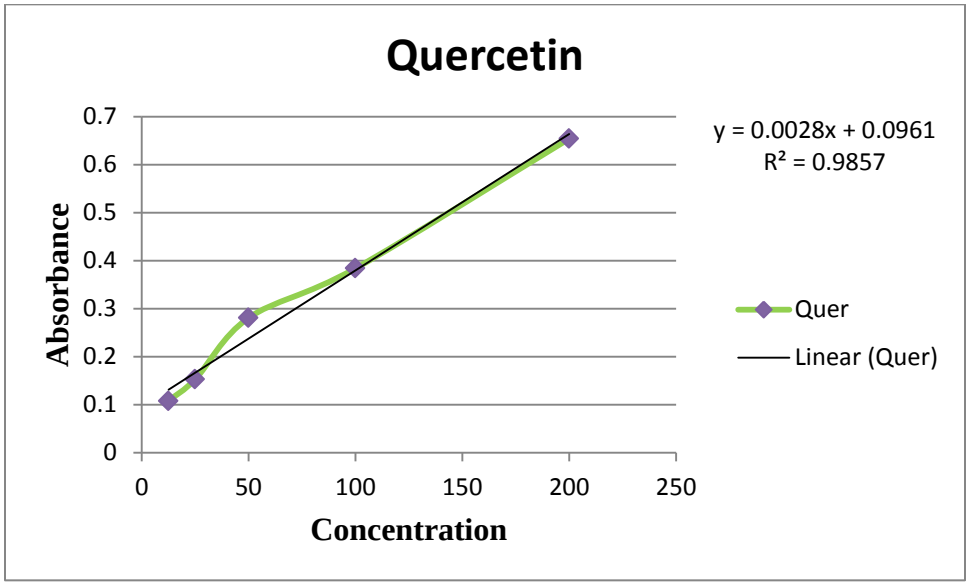


Figure 28 Calibration curve of Quercetin for *Spondias pinnata*

Table 12 Total flavonoid contents of the different extracts of *Spondias pinnata*

Extract	Total Flavonoid contents,mg/gm, Quercetin
N Hexane	**121 ± 26.6**
Petroleum Ether	**113 ± 34.2**
Methanol	**189.5 ± 15.2**
Chloroform	**130 ± 18.8**

Values are the mean of duplicate experiments and represented as mean ± SD.

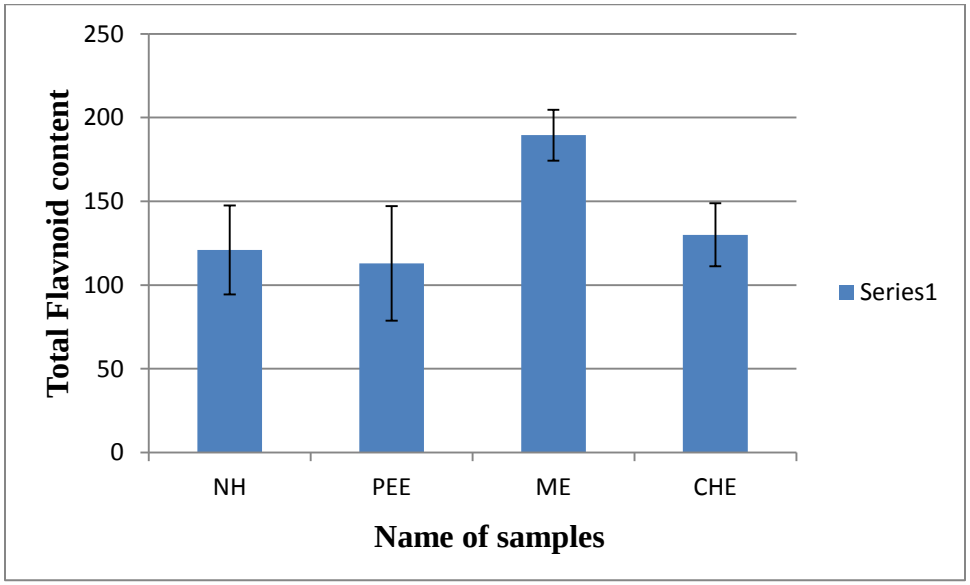


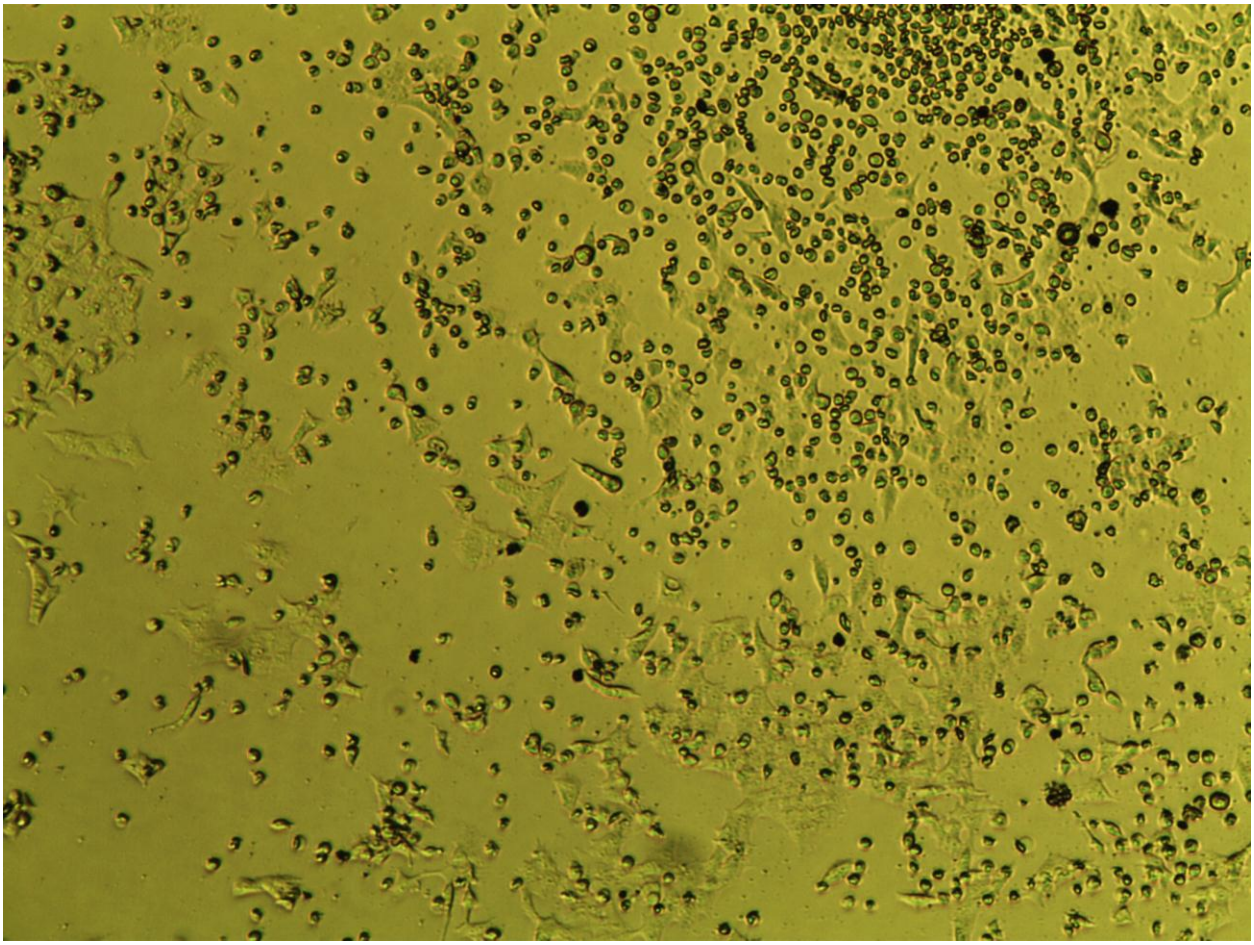
Figure 29 Total flavonoid contents of the different extracts of *Spondias pinnata*

3.5 Determination of Cytotoxicity of the extracts

Cytotoxicity was examined under an inverted light microscope after 48 hours of incubation. The results after 48 hours of incubation the cytotoxicity results are as follows:-

3.5.1 For *Averrhoa carambola L* leaf extracts

3.5.1.1 N hexane extract of *Averrhoa carambola L* showed 10% viability of HeLa cells (Figure 30) where 100 µl of sample (filtered through 0.45µM previously) and 400 µl of media were added to each well in a 24 well plate.



.**Figure 30 showing cell viability of N hexane extract of** *Averrhoa carambola L* **after 48 hours**

3.5.1.2 Petroleum ether extract of *Averrhoa carambola L* showed 1% viability of HeLa cells (Figure 30) where 100 µl of sample (filtered through 0.45µM previously) and 400 µl of media were added to each well in a 24 well plate.

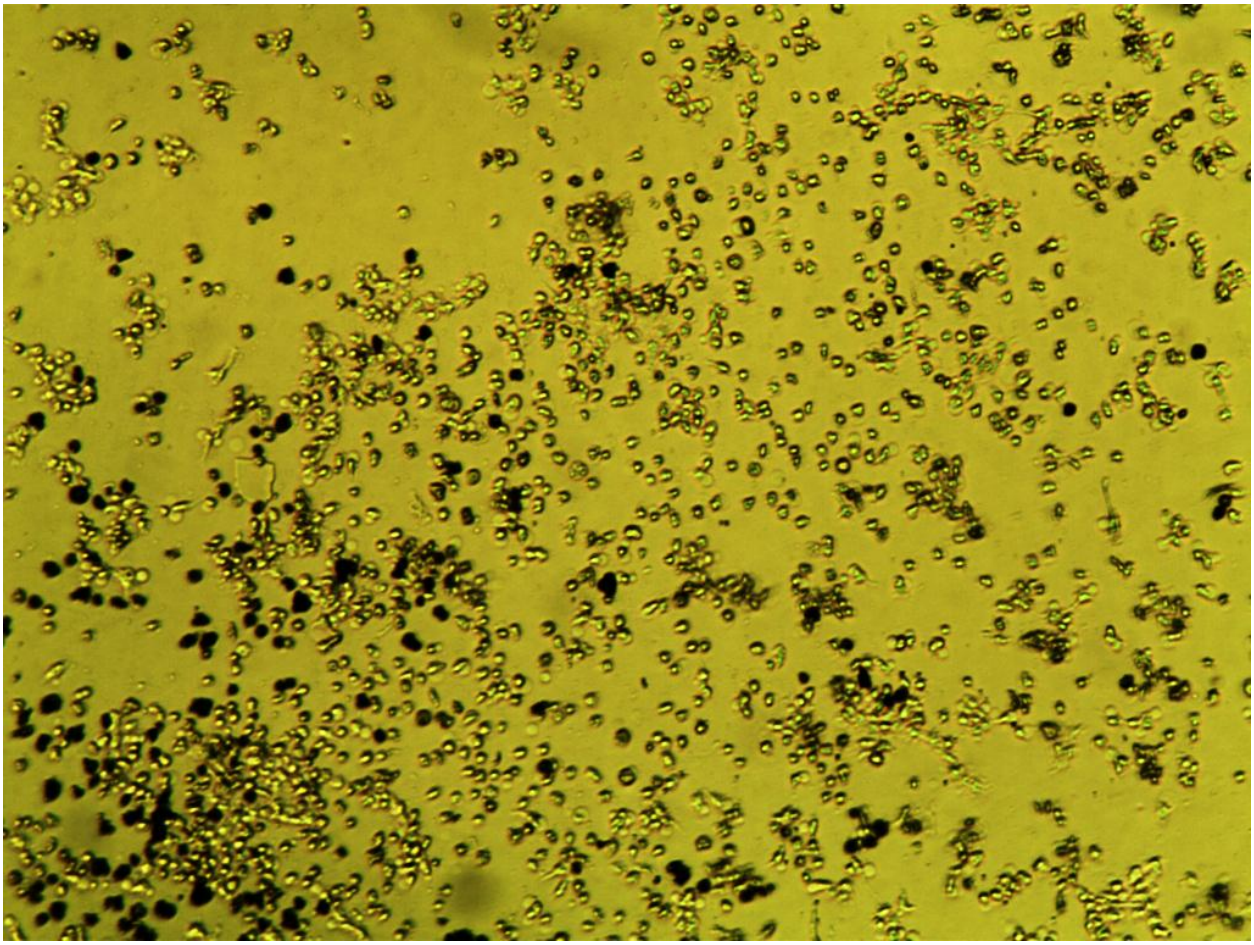


Figure 31 showing cell viability of Petroleum ether extract of *Averrhoa carambola L* **after 48 hours**

3.5.1.3 Methanol extract of *Averrhoa carambola L* showed 2% viability of HeLa cells (Figure 30) where 100 µl of sample (filtered through 0.45µM previously) and 400 µl of media were added to each well in a 24 well plate.

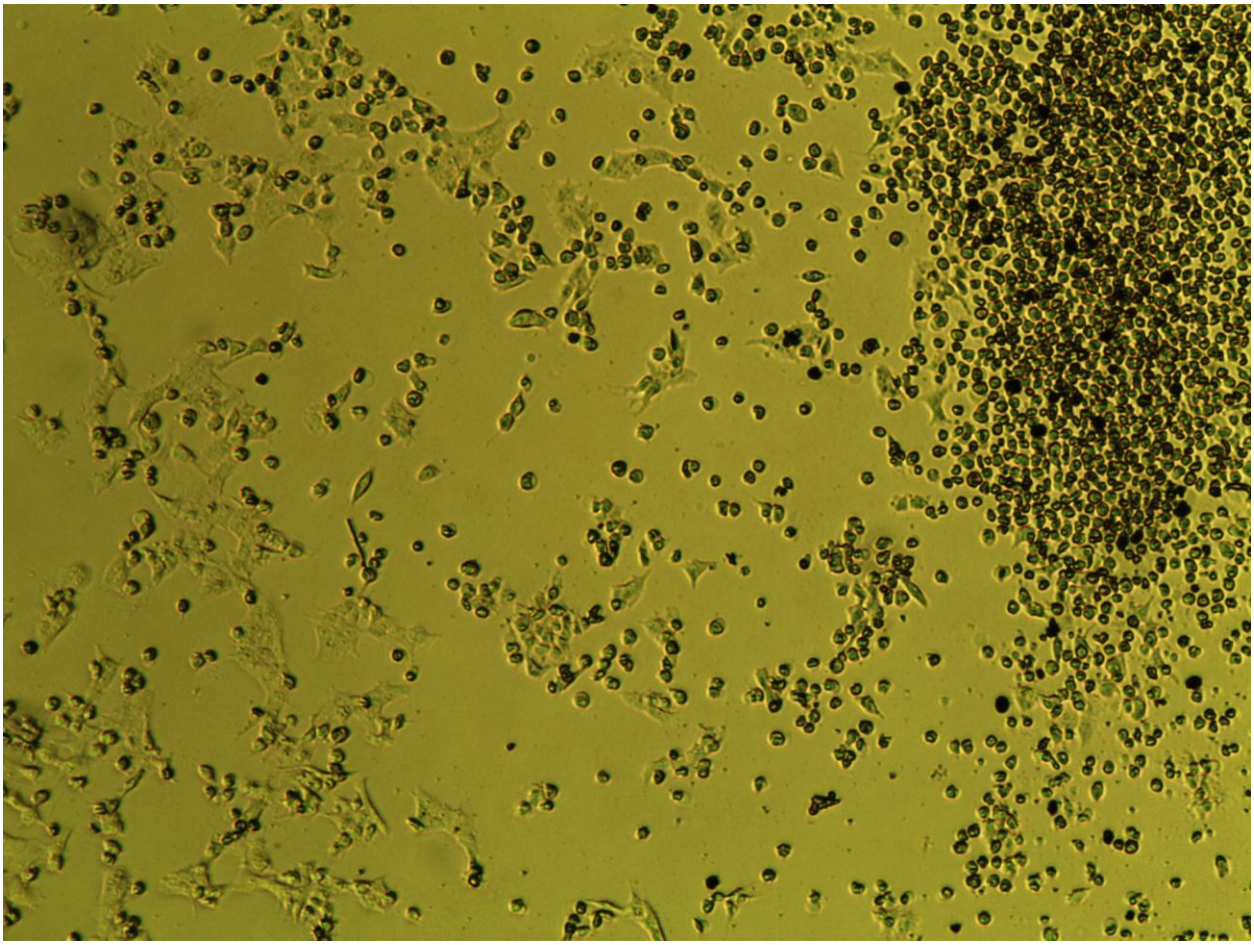


Figure 32 showing cell viability of Methanol extract of *Averrhoa carambola L* **after 48 hours**

3.5.1.4 Chloroform extract of *Averrhoa carambola L* showed 1% viability of HeLa cells (Figure 30) where 100 µl of sample (filtered through 0.45µM previously) and 400 µl of media were added to each well in a 24 well plate.

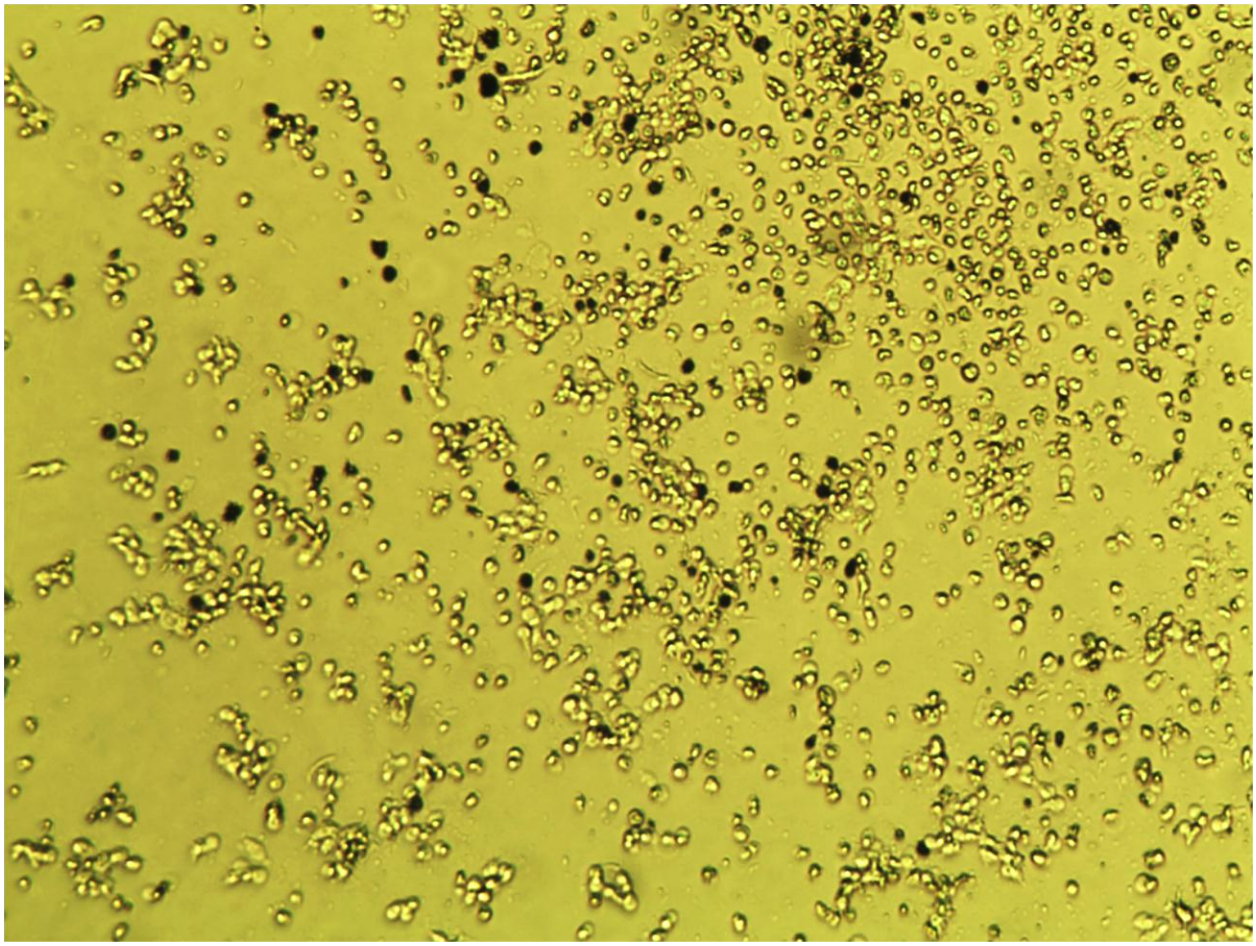


Figure 33 showing cell viability of Chloroform extract of *Averrhoa carambola L* after 48 hours

3.5.2 For *A. marmelos* leaf extracts

3.5.2.1 N hexane extract of *A. marmelos* showed <1% viability of HeLa cells (Figure 30) where 100 µl of sample (filtered through 0.45µM previously) and 400 µl of media were added to each well in a 24 well plate.

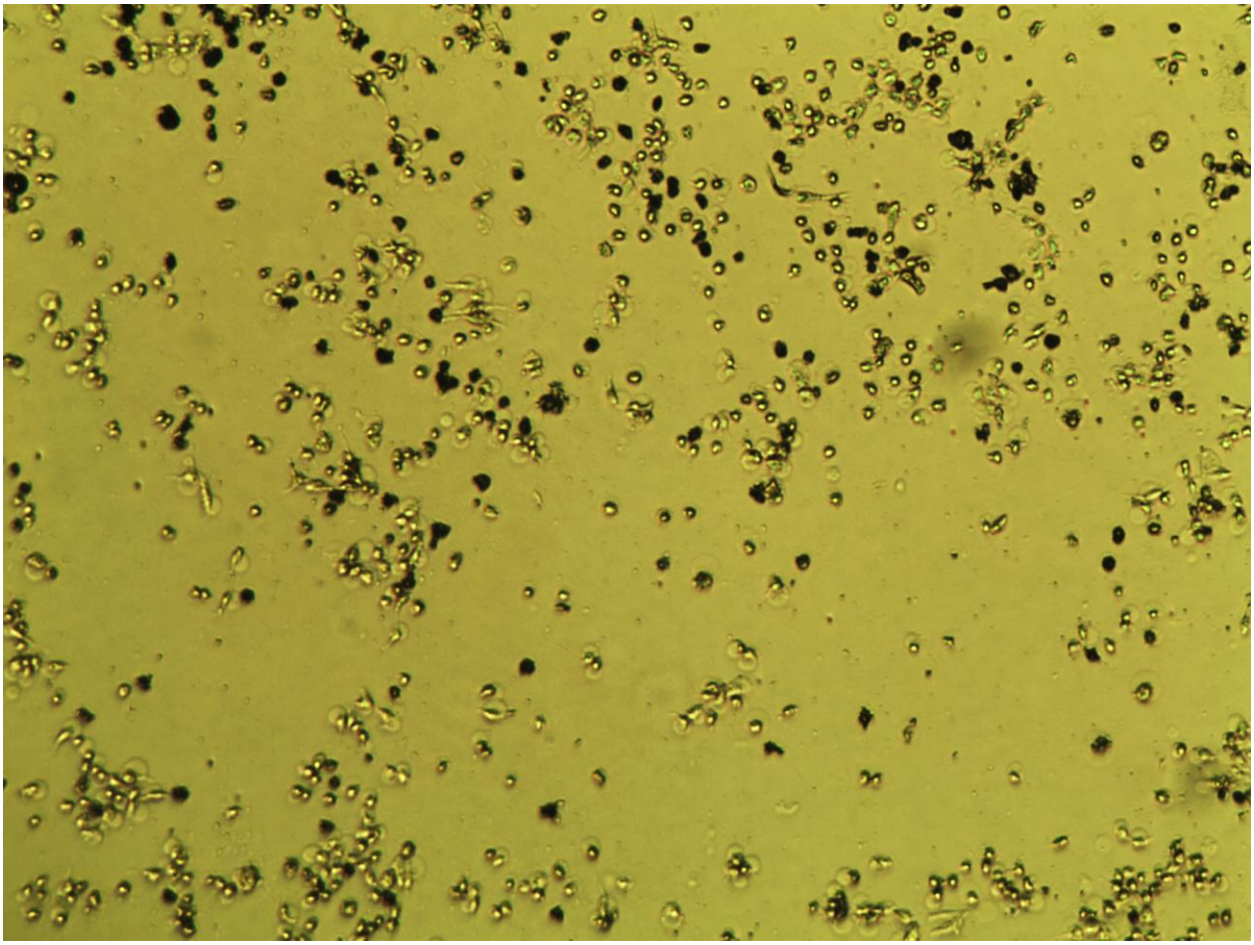


Figure 34 showing cell viability of N hexane extract of *A. marmelos* after 48 hours

3.5.2.2 Petroleum ether extract of *A. marmelos* showed <1% viability of HeLa cells (Figure 30) where 100 µl of sample (filtered through 0.45µM previously) and 400 µl of media were added to each well in a 24 well plate.

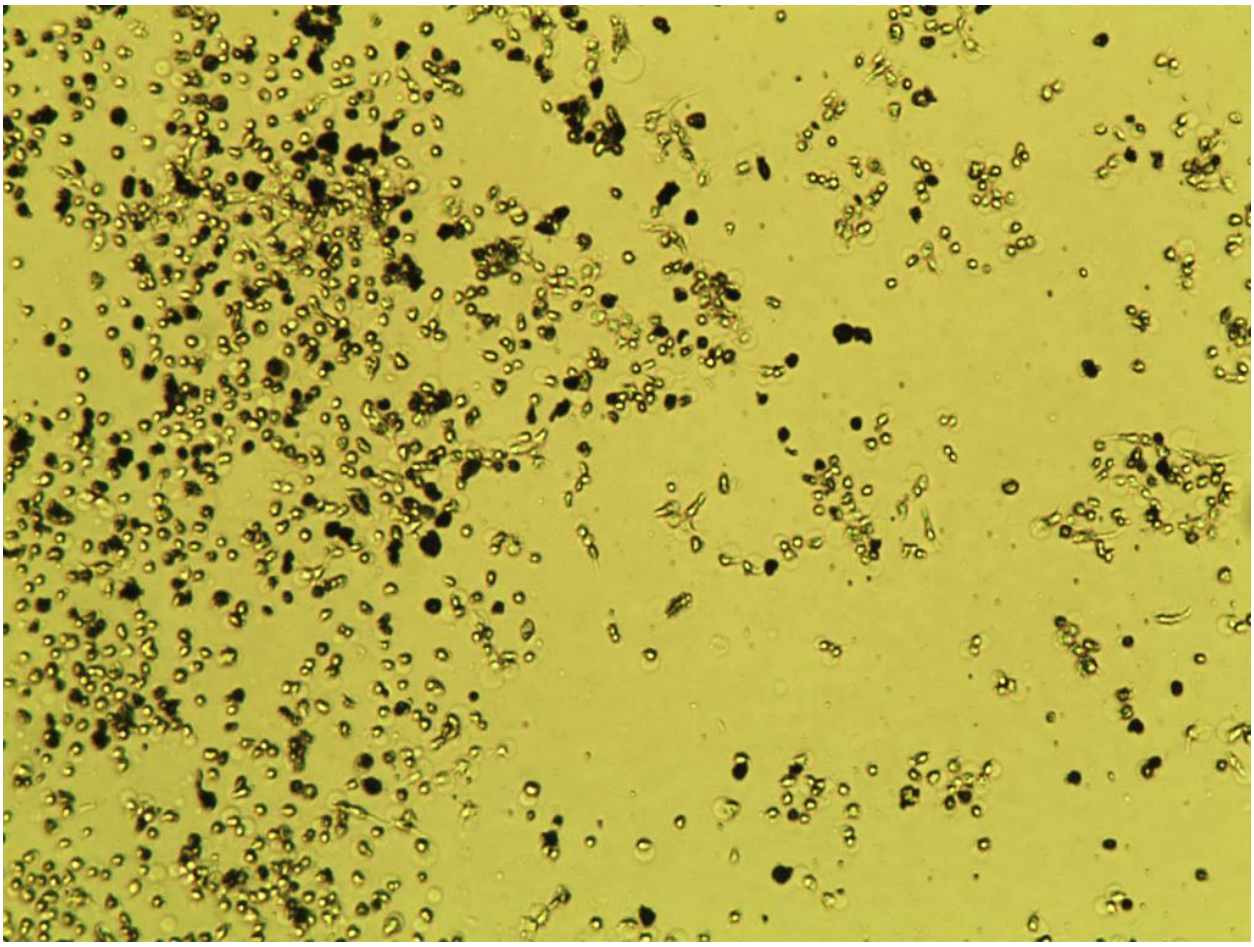


Figure 35 showing cell viability of Petroleum ether extract of *A. marmelos* after 48 hours

3.5.2.3 Methanol extract of *A. marmelos* showed <1% viability of HeLa cells (Figure 30) where 100 µl of sample (filtered through 0.45µM previously) and 400 µl of media were added to each well in a 24 well plate.

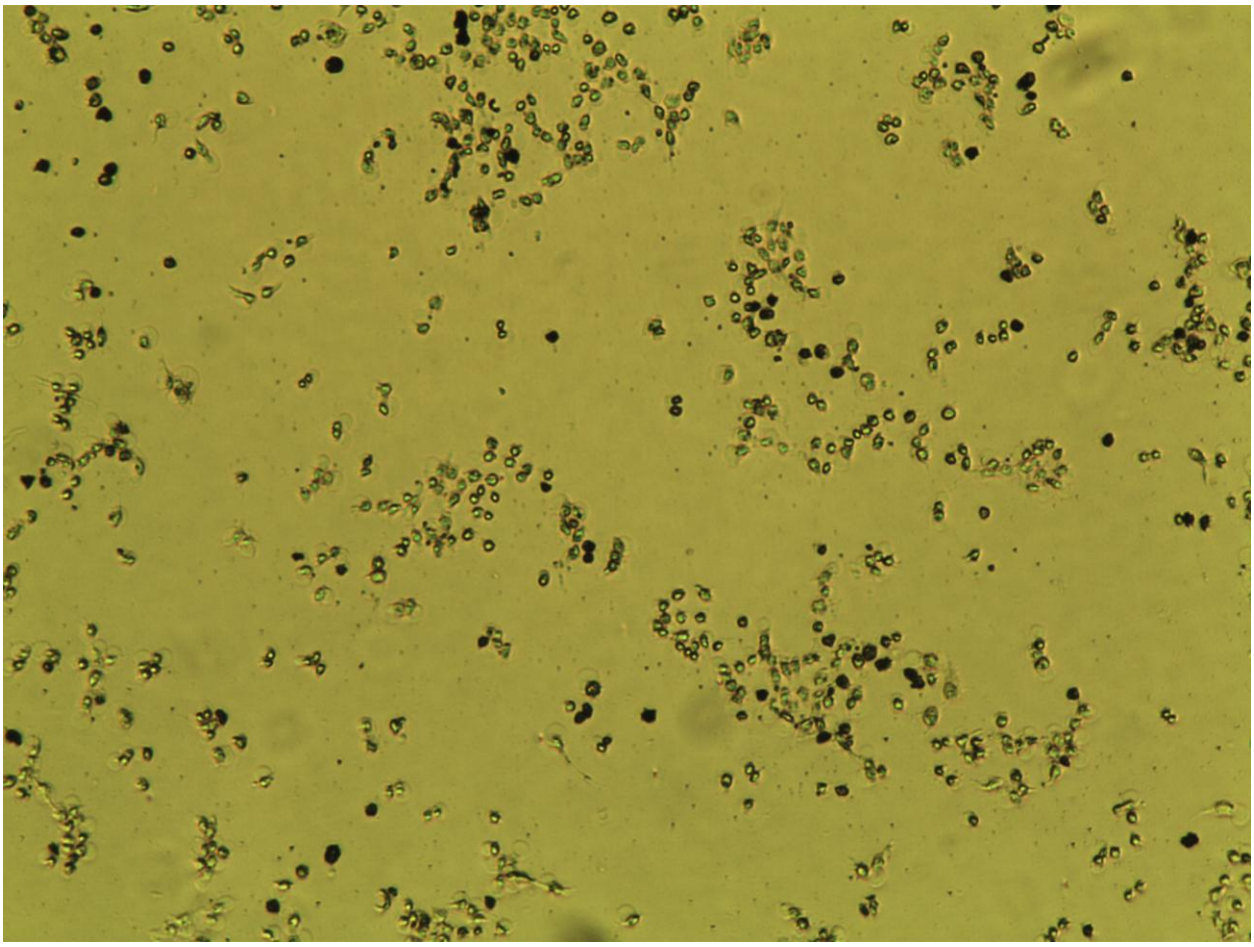


Figure 36 showing cell viability of Methanol extract of *A. marmelos* after 48 hours

3.5.2.4 Chloroform extract of *A. marmelos* showed <1% viability of HeLa cells (Figure 30) where 100 µl of sample (filtered through 0.45µM previously) and 400 µl of media were added to each well in a 24 well plate.

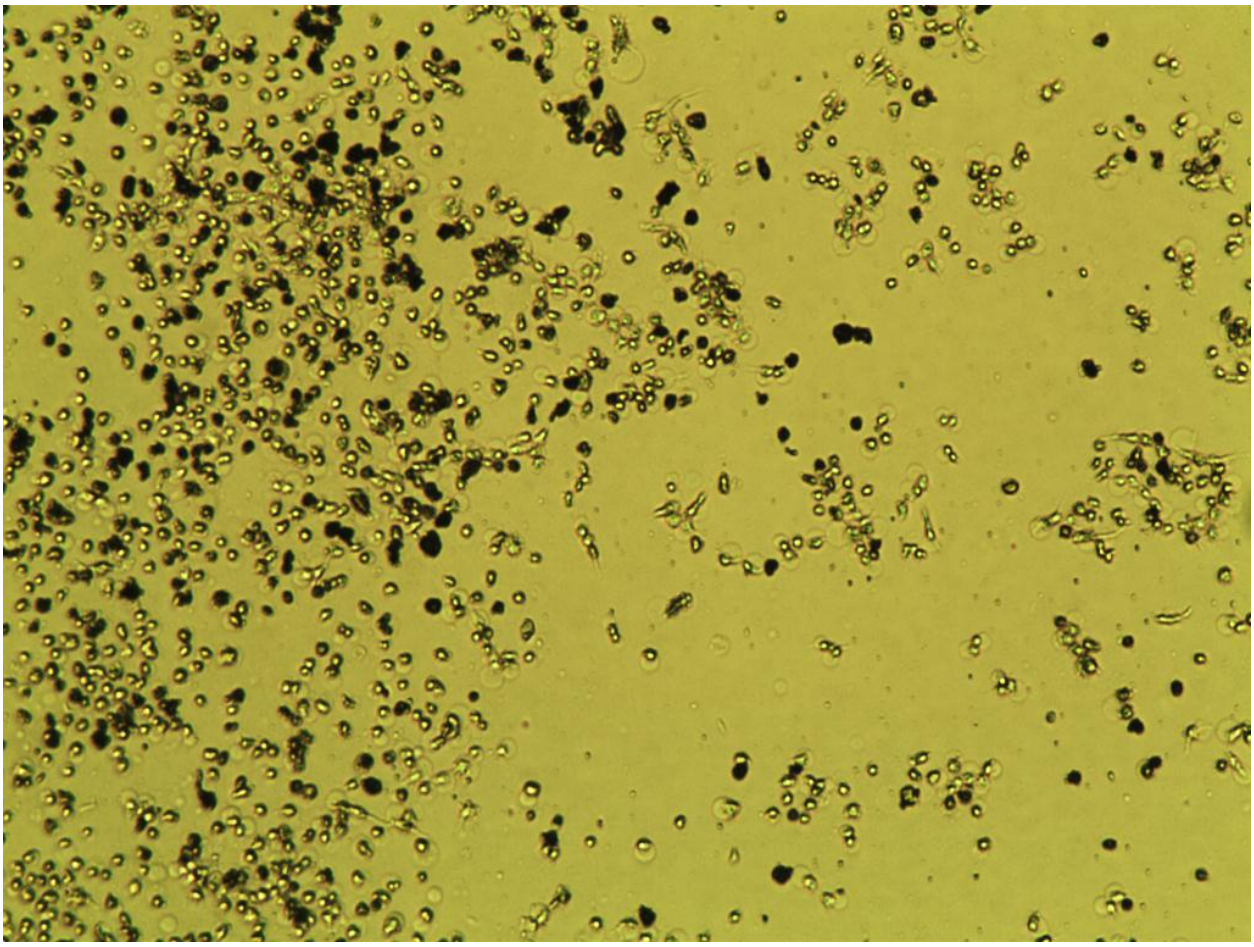


Figure 37 showing cell viability of Chloroform extract of *A. marmelos* after 48 hours

3.5.3 For *Spondias pinnata* leaf extracts

3.5.3.1 N hexane extract of *Spondias pinnata* showed 40% viability of HeLa cells (Figure 30) where 100 µl of sample (filtered through 0.45µM previously) and 400 µl of media were added to each well in a 24 well plate.

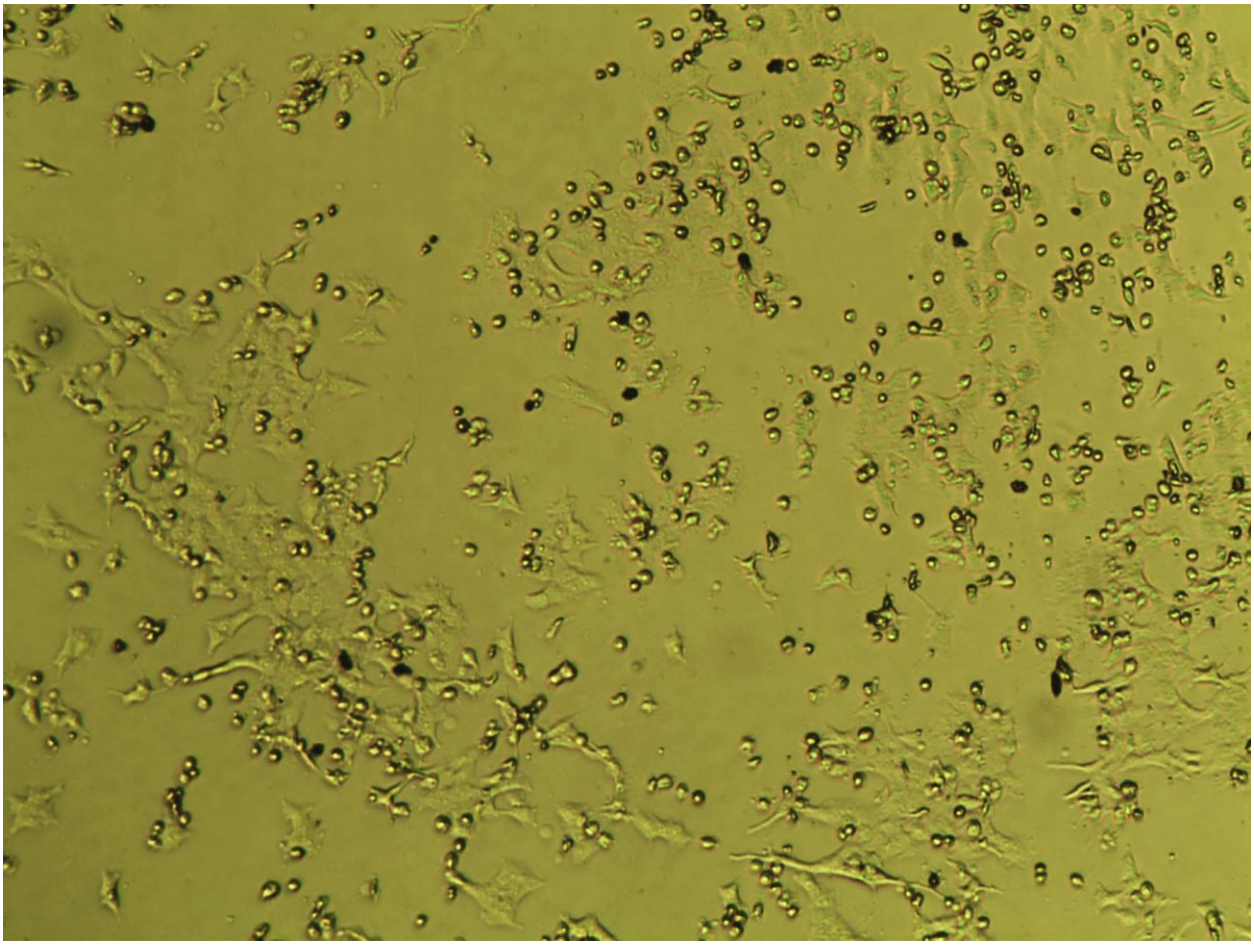


Figure 38 showing cell viability of N hexane extract of *Spondias pinnata* after 48 hours

3.5.3.2 Petroleum ether extract of *Spondias pinnata* showed <10% viability of HeLa cells (Figure 30) where 100 µl of sample (filtered through 0.45µM previously) and 400 µl of media were added to each well in a 24 well plate.

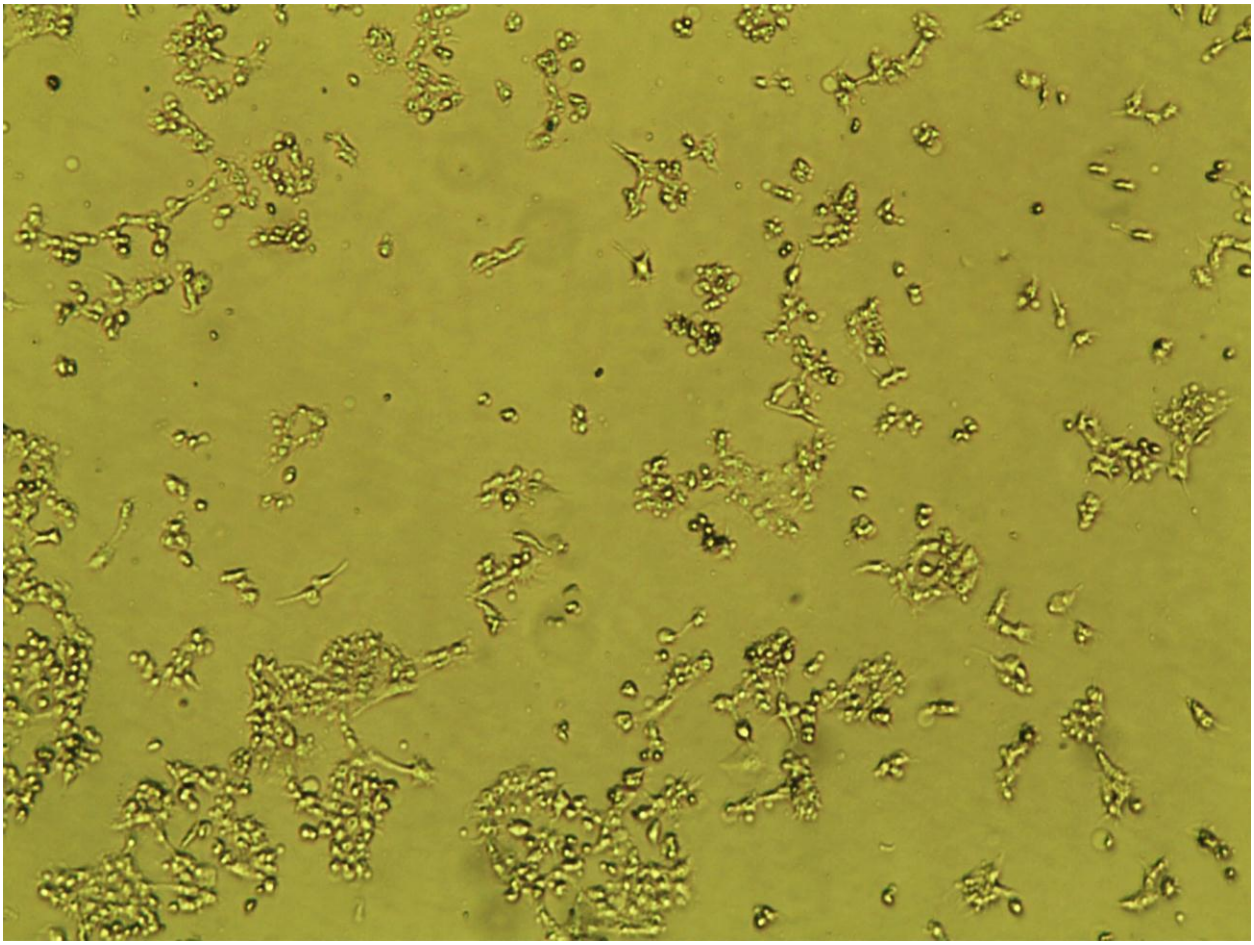


Figure 39 showing cell viability of Petroleum ether extract of *Spondias pinnata* after 48 hours

3.5.3.3 Methanol extract of *Spondias pinnata* showed 60% viability of HeLa cells (Figure 30) where 100 µl of sample (filtered through 0.45µM previously) and 400 µl of media were added to each well in a 24 well plate.

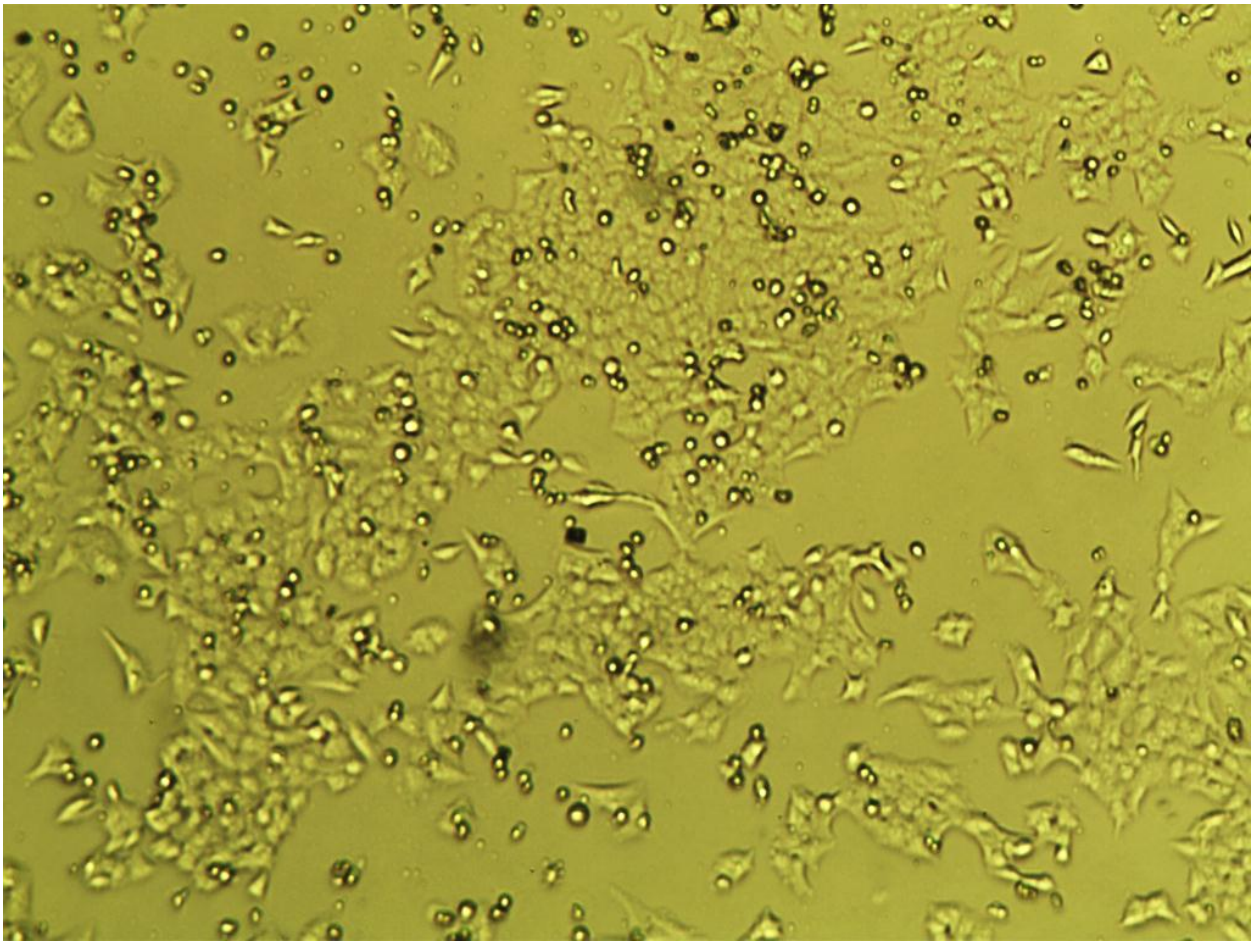


Figure 40 showing cell viability of Methanol extract of *Spondias pinnata* after 48 hours

3.5.3.4 Chloroform extract of *Spondias pinnata* showed 80% viability of HeLa cells (Figure 30) where 100 µl of sample (filtered through 0.45µM previously) and 400 µl of media were added to each well in a 24 well plate.

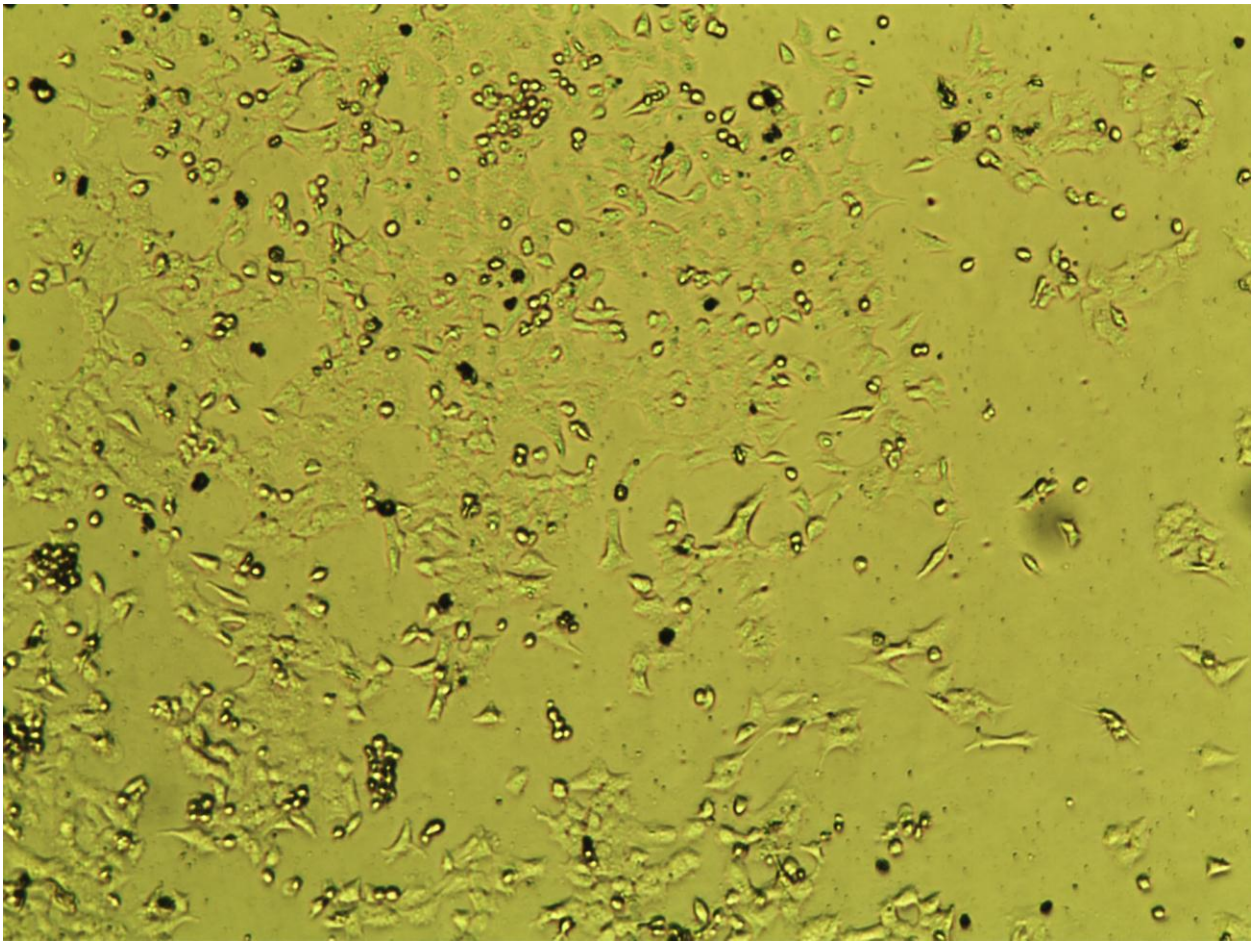


Figure 41 showing cell viability of Chloroform extract of *Spondias pinnata* after 48 hours

3.5.4 Control

Control containing 400 µl of media plus 100 µl of DMSO and water had nearly 80% survival which was considered as 100%.

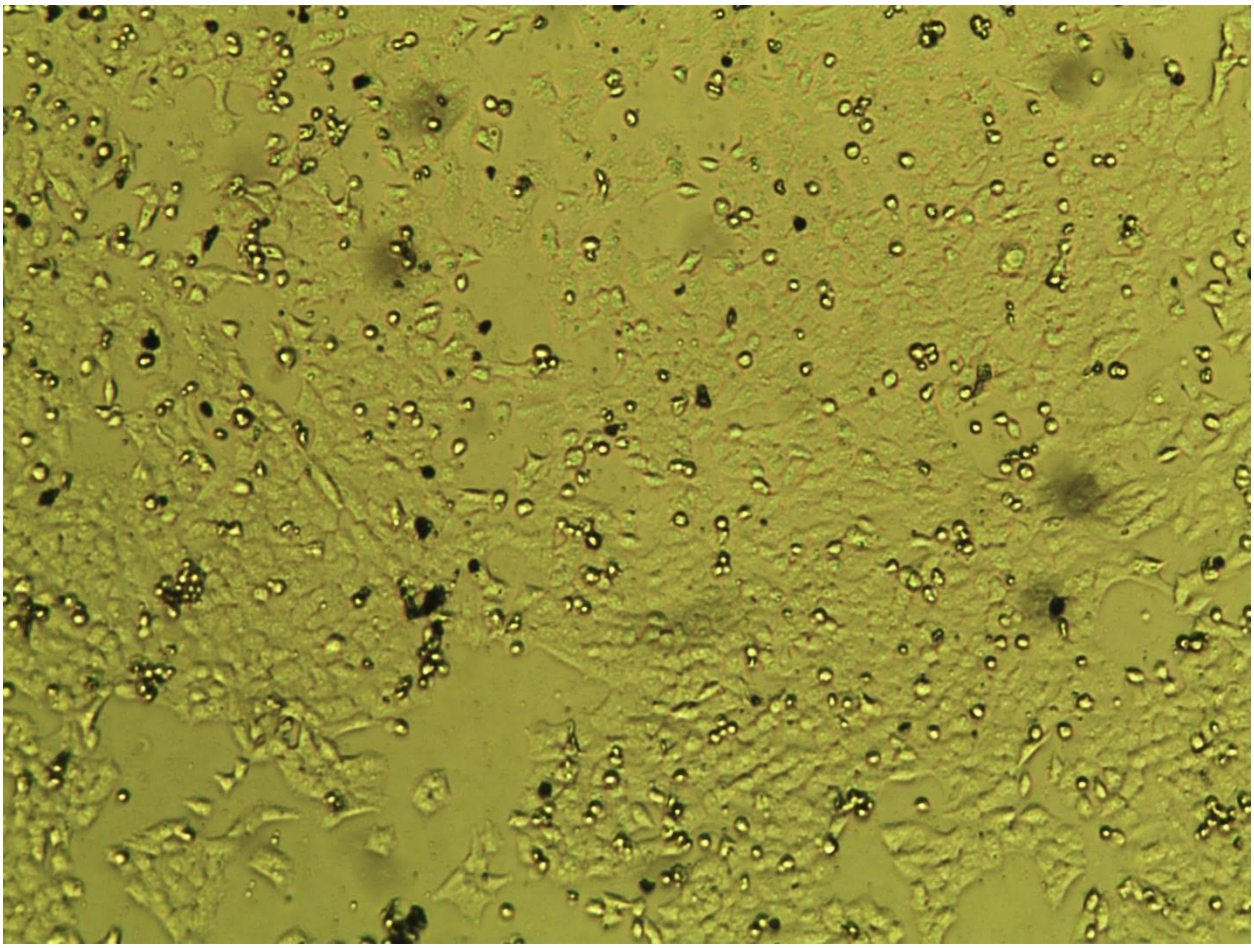


Figure 42 – Control showing cell viability in DMSO and water after 48 hours

Chapter 4

Discussion

Spondias pinnata is a well-known plant indigenous to South East Asian countries. The plant has been used intensively in many traditional herbal medicines across the globe. This plant has been known to possess antimicrobial, anti-diabetic, ulcer-protective, anti-cancer, anti-diarrhea, anthelmintic, and cytotoxic activity. It has also been reported that different parts of the plant is used as anti-thirst, anti-emetic and as an anti-tubercular agent (Bora *et al*., IJPSR, 2014). According to Hafiz., 2010, ethyl acetate extracts of barks of *Spondias pinnata* showed significant antioxidant capacity in the total phenol content experiment. In another study, individually, n-hexane, ethyl acetate and dichloromethane extracts of fruits of *Spondias pinnata* were screened for total phenol content, total flavonoid content and total antioxidant capacity. The results showed *Spondias pinnata* fruit extract containing ethyl acetate to have the highest phenol content followed by dichloromethane and n hexane. In total flavonoid and total antioxidant content experiment it was found that ethyl acetate extract showed the highest value followed by n-hexane and dichloromethane (Manik *et al*., 2013). Another study conducted ethanol and chloroform extracts of bark of *Spondias pinnata* to perform the total phenol and flavonoid content experiment where in both cases the ethanol extract showed the highest value (Das *et* al., 2011). In this study, *Spondias pinnata* leaf extracts, extracted individually containing n-hexane, petroleum ether, methanol and chloroform according to increasing solubility was used to screen for total antioxidant, total phenol and total flavonoid content. The results obtained showed *Spondias pinnata* chloroform extract to have the highest total antioxidant content followed by methanol extract, petroleum ether and n-hexane to have the least, while for both total phenol and flavonoid content the results showed n hexane to have the highest value followed by petroleum ether extract, chloroform and methanol extract to have the least value. It can be said that, different extracts of different parts of *Spondias pinnata* tree have significant antioxidant values from different fractions and varies accordingly.

Cytotoxic study has been done for *Spondias pinnata* in previous studies where brine shrimp lethality assay has been used to find the % mortality rate against a standard. According to Hafiz., 2010, the % mortality rate for ethyl acetate extract of bark of *Spondias pinnata* was more than the standard. In another study by Bora *et al*., it was found that ethanolic extracts of fruit of

Spondias pinnata against Vincristine Sulphate as a standard showed a significant increase in % mortality of nauplii than the standard after 24 hours of exposure. Another study (Das *et al*., 2011) showed chloroform extract of *Spondias pinnata* barks to have a high mortality rate of the nauplii against DMSO as the control. In this current study, in vtiro animal cell culture was performed in cancer cell lines using HeLa cells where DMSO was used as the control and individual extracts of *Spondias pinnata* leaves containing n-hexane, petroleum ether, methanol and chloroform was screened for cell viability under a microscope after 48 hours of incubaton. The results obtained showed petroleum ether extracts to have the highest mortality rate, >10% survival, followed by n hexane, 40% survival, methanol, 60% survival and the chloroform extract showed the least amount of mortality rate >80% survival. It can be said that, *Spondias pinnata* leaf extracts have the potential to kill cancer cells *in vitro* which can be efficiently done using animal cell culture. This is an easy, feasible and time saving process compared to brime shrimp lethality assay, because getting a supply of shrimp nauplii and maintaining them may be a bit of a hassle.

Bael, *Aegle marmelos,* is another medicinal plant; which has enormous traditional values against various diseases and many bioactive compounds have been isolated from this plant. The main usage of the parts of this tree is for medicinal purposes. The leaf part of the plants have been claimed to be used for the treatment of inflammation, asthma, hypoglycemia, hepatitis and analgesic (Sharma *et al*., 2011). Ethyl acetate leaf extracts of *Aegle marmelos* showed antioxidant properties such as flavonoids, phenols, etc as reported by Maity *et al*., 2009. Another study conducted by Rajan *et al*., 2010 showed alcoholic extracts of fruit pulps of *Aegle marmelos* to exhibit the highest total phenol and flavonoid content. Methanolic extracts of leaves, barks and stems of *Aegle marmelos* were screened for total phenol and flavonoid content in a study by Siddique *et al*., 2009, where the leaf extracts showed the highest for both the tests. Similarly another study was conducted by Sathya *et al*., 2013 where leaves of *Aegle marmelos* were extracted individually using ethanol, ethyl acetate and distilled water, where ethanol showed the highest phenol content. In this study, four solvents were used individually according to their increase in solubility to extract the leaves of *Aegle marmelos* and used to test its antioxidant activity. Total antioxidant, total phenol content and total flavonoid content test was done where the petroleum ether extract showed the highest total antioxidant value, n-hexane extract showed the highest phenol content and chloroform extract showed the highest flavonoid content.

Various studies involving various tests for antioxidant activity have been done with leaf extracts of *Aegle marmelos* using different solvents, which show the leaves, contain very high antioxidant properties and has significant values in varying ranges.

Previous studies shown by Leticia V and Costa L. (2005), the anticancer potential of folk medicine used in Bangladeshi and used extracts of *Aegle marmelos* for cytotoxic action using brine shrimp lethality assay and sea urchin eggs assay, using tumor cell lines, it exhibited toxicity in all used assays. Also, cytotoxicity in, *in vitro* cell culture using normal cell line, Vero and HEP2 showed nontoxic potential of ethyl acetate leaf extracts of *Aegle marmelos*, showing nontoxic to normal cells, the compound exhibited potent inhibitory activity against HEP2 and Vero cell line (Rejiniemon *et al*., 2014). Another study by Urmi *et al*., screened ethanol, ethyl acetate and n hexane leaf extracts of *Aegle marmelos* for cytotoxicity using brine shrimp lethality assay where the methanol extract showed the highest mortality rate followed by ethyl acetate and ethanol, against the standard Vincristine. In this study, cytotoxicity was done *in vitro* using animal cell culture involving cancer cell lines, HeLa and DMSO as the control. Four extracts of *Aegle marmelos* leaves containing n hexane, petroleum ether, methanol and chloroform were screen for cytotoxicity by seeing the cell viability under a microscope after 48 hours of incubation. The results showed, all four extracts seemed to work efficiently against the cancer cells and the mortality rate was >1% for each. This shows that *Aegle marmelos* leaf extracts have the potential to kill cancer cells using different solvent extractions, also, a stated previously it is also nontoxic to normal cells, so a very feasible and logical comparison can be found between cancer and normal cells even though further investigation is required.

Averrhoa carambola (Oxalidaceae) is grown in the warmer parts of India, Bangladesh and other areas of the world with the same climate (Ghani, 2003). In addition, the extract obtained through drying the leaves of *A.carambola* has been used in the treatment of diabetes (Provasi et al, 2001). Phytochemistry studies have shown that the fruit of *A. carambola* is rich in antioxidants, especially polyphenolic compounds, which act against reactive oxygen species (Tiwari *et al,* 1979). In a previous study, green and ripe fruit peel and pulp methanolic extract of *Averrhoa carambola* were tested for total phenol content where the ripe fruit and peel extract showed highest phenol content compared with green fruit and peel extract (Lim and Lee., 2013). Another

study, two types of *Averrhoa carambola* fruits were taken (tart and honey type) and extracted with methanol to test their total phenol and flavonoid content. The result showed the tart fruit extract to have both the highest phenol and flavonoid content than the honey type, this is probably due to the colour, shape, maturity and environment of the fruits (Asna and Noriham., 2014). In a different study conducted by Patil *et al*., 2011, fresh fruits of *Averrhoa carambola* were powdered and extracted using distilled water and were tested for total phenol and antioxidant content. The result obtained showed *Averrhoa carambola* aqueous fruit extracts to possess a high amount of both phenol and total antioxidant content, but when compared to *Averrhoa bilimbi* aqueous fruit extract, it was slightly less (Patil *et al*., 2011). Das *et al.,* 2013 also conducted a study on bark extracts of *Averrhoa carambola* using chloroform and ethanol to test the phenol and flavonoid content, where, a moderate amount of both phenol and flavonoid content was found in the extracts but which showed the highest was not mentioned. In this study, four solvents were used individually according to their increase in solubility to extract the leaves of *Averrhoa carambola* and used to test its antioxidant activity. Total antioxidant, total phenol content and total flavonoid content test was done where; petroleum ether extract showed the highest total antioxidant content, methanol extract showed the highest phenol content and chloroform extract showed the highest flavonoid content. Compared to the studies mentioned above, it can be said that, with the fruits containing significant antioxidant properties, similarly, the leaves also contain potential antioxidant properties which can be further screened with the fruit extracts to find out a probable source of medicine.

Cytotoxicity of *Averrhoa carambola* has been done in various studies (Das *et al.,* 2013) using the brine shrimp lethality assay using ethanol and chloroform bark extracts showed a high mortality rate compared to the standard (DMSO), where ethanol showed a higher amount than chloroform. Crude methanolic fruit extracts were taken and further fractionated, portioning with n-hexane, water and dicloromethane (Das and Ahmed., 2012). They were screened for cytotoxicity using brine shrimp lethality assay, where after 24 hours of exposure to sample and positive control (Vincristine sulphate), dichloromethane fruit extracts showed significant mortality to the nauplii than the water and n hexane extracts (Das and Ahmed., 2012). In this present study, cytotoxicity screening was done using animal cell culture *in vitro* involving cancer cell lines HeLa, where the four individual solvent leaf extracts of *Averrhoa carambola* were incubated for 48 hours with the

cells and later was checked for cell viability against a positive control (DMSO). The result obtained showed significant mortality in cancer cell, where, the petroleum ether and chloroform extract presented 1% survival rate followed by 2% by methanol extract and 10% by n hexane extract. When compared to the standard, they showed very high percentage of cell mortality, where it can be said that it wasn't the DMSO but the potent cytotoxic properties of the leaf extracts which caused the cells to die.

Even though various results and screening studies have been shown previously in different journals using different parts of these plants, no record of antioxidant with the used solvents in this particular study and *in vitro* cell cytotoxicity using cancer cell lines was done for leaf extractions of the three tropical fruit trees. Although more toxicological and pharmacological studies are required to positively declare the potential development and execute the results perfectly, it can be stated that, the leaves of these three evaluated fruit trees do contain high antioxidant and cytotoxic properties which can be considered for future references in producing susceptible cancer medicines in a cheap but effective way.

Chapter 5

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APPENDICES

A. Reagents

N-Hexane	Merck-Reagent and Standard Substance.
Petroleum Ether	Merck-Reagent and Standard Substance.
Methanol	Merck-Reagent and Standard Substance, Germany.
Chloroform	Merck-Reagent and Standard Substance.
DMSO	Sigma, Aldrich Chemistry
Cupric Chloride	Merck
Ammonium Acetate	Merck
Neocuproine	Loba chemie
Ascorbic Acid	Loba chemie
BHT	Merck
Ethanol	Merck
Sodium Phosphate	Merck

Ammonium Molybdate	BDH Chemicals LTD.
Concentrated Sulphuric Acid	Sigma, Aldrich
Folin-ciocalteu reagent	Loba chemie
Sodium Carbonate	Merck
Gallic Acid	Merck
Aluminum Chloride	Unichem chemicals
Potassium Acetate	Envirolab
Quercertin	Sigma, Aldrich
DMEM(Dulbecco's Modified Eagles' medium)	Gibco
FBS (Fetal Bovine Serum)	Gibco
Antibiotics (Penicillin and Streptomycin)	Gibco
PBS (Phosphate buffer Saline)	Gibco
Trypsin	Gibco

B. Preparation of Reagents

i) 0.01Molar Cupric Chloride solution composition

Reagent	Amount
Cupric Chloride	0.4262gm
Distilled water	up to 250ml

ii) 1Molar Ammonium Acetate solution composition

Reagent	Amount
Potassium acetate	19.27gm
Distilled Water	up to 250ml

iii) 0.0075M Neocuproine solution composition

Reagent	Amount
Neocuproine	0.156gm
Ethanol	up to 100ml

iv) 0.333% Concentrated Sulphuric Acid solution

Reagent	Amount
Concentrated Sulphuric Acid	0.333ml
Distilled Water	up to 100ml

v) 0.004 Molar Ammonium Molybdate Solution

Reagent	Amount
Ammonium Molybdate	0.4943gm
Distilled Water	up to 100ml

vi) 0.2295Molar Sodium Phosphate Solution

Reagent	Amount
Sodium Phosphate	0.459gm
Distilled Water	up to 100ml

vii) 7.5% Sodium Carbonate Solution

Reagent	Amount
Sodium Carbonate	7.5gm
Distilled Water	up to 100ml

viii) 1:10v/v Ratio Folin Ciocalteu Solution

Reagent	Amount
Folin Ciocalteu	20ml
Distilled Water	up to 200ml

ix) 10% Aluminum chloride solution

Reagent	Amount
Aluminum chloride	10gm
Distilled Water	up to 100ml

x) 1 Molar Potassium Acetate Solution

Reagent	Amount
Potassium Acetate	9.815gm
Distilled Water	up to 100ml