Phytochemical analysis, bioactivity screening and characterization of biosynthesized silver nanoparticles on selected species of *Aglaia* Louriero (Meliaceae)

Thesis
submitted to the University of Calicut
for the award of the degree of

DOCTOR OF PHILOSOPHY IN BOTANY

By
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CERTIFICATE

This is to certify that the thesis entitled **"Phytochemical analysis, bioactivity screening and characterization of biosynthesized silver nanoparticles on selected species of *Aglaia* Louriero (Meliaceae)"** submitted to the University of Calicut, for the award of the degree of DOCTOR OF PHILOSOPHY IN BOTANY is a record of original research work done by **Archana E. R.** during the period of study (2015-2020) at the Cell and Molecular Biology Division, Department of Botany, University of Calicut under my supervision and guidance and that it has not formed the basis for award of any degree or diploma. Also certified that the contents in the thesis is subjected to plagiarism check using the software URKUND, that no text or data is reproduced from other works.

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Supervising Teacher

DECLARATION

I, Archana E. R., hereby declare that the thesis entitled **"Phytochemical analysis, bioactivity screening and characterization of biosynthesized silver nanoparticles on selected species of *Aglaia* Louriero (Meliaceae)"** submitted to the University of Calicut, for the award of the degree of DOCTOR OF PHILOSOPHY IN BOTANY is a record of original research work done by me under the supervision and guidance of Dr John E. Thoppil, Professor, Department of Botany, University of Calicut and that it has not formed the basis for the award of any degree/diploma to any candidate of any University.

Date: **Archana E. R.**

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Archana E.R.

CONTENTS

ABBREVIATIONS

µg/mL	:	Microgram/milliliter
µM	:	Micromolar
AFM	:	Atomic Force Microscopy
AgNPs	:	Silver nanoparticles
CA	:	Chromosomal aberrations
cDNA	:	Complementary DNA
DMEM	:	Dulbecco's Modified Eagles's Medium
DMRT	:	Duncan's Multiple Range Test
DMSO	:	Dimethyl sulphoxide
dNTP	:	Deoxyribonucleotide triphosphate
DPPH	:	1, 1-diphenyl-2-picrylhydrazyl
DW	:	Dry weight
EDTA	:	Ethylene diamine tetra acetic acid
FC	:	Folin-Ciocalteu
$FeCl_3$	:	Ferric chloride
FITC	:	Fluorescein isothiocyanate
FTIR	:	Fourier-transform infrared spectroscopy
GAE	:	Gallic acid equivalent
GC/MS	:	Gas Chromatography/Mass Spectrometry
h	:	Hour
H_2O_2	:	Hydrogen peroxide
H_2SO_4	:	Sulphuric acid
HCl	:	Hydrochloric acid
HeLa	:	Henrietta Lacks
HepG2	:	Human hepatocarcinoma cells
HPTLC	:	High Performance Thin Layer Chromatography

IC_{50}	:	Inhibition concentration 50%
IUCN	:	International Union for Conservation of Nature
LD_{50}	:	Lethal dose
LE	:	Linalool equivalent
M	:	Molar
mg	:	milligram
MI	:	Mitotic index
min	:	Minute
mM	:	Millimolar
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
N	:	Normal
Na_2CO_3	:	Sodium carbonate
NaOH	:	Sodium hydroxide
NBT	:	Nitro blue tetrazolium
NCCS	:	National Center for Cell Sciences
NF-κB	:	Nuclear factor kappa light chain enhancer of activated B cells
NIST	:	National Institute of Standards and Technology
nm	:	Nanometer
NO	:	Nitric oxide
NOS	:	Nitric oxide synthase
OD	:	Optical density
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
QE	:	Quercetin equivalent
RAW 264.7	:	Mouse leukemic monocyte macrophage cell line
RNS	:	Reactive nitrogen species
ROS	:	Reactive oxygen species

RT	:	Retention time
SDS	:	Sodium dodecyl sulphate
SE	:	Standard error
SEM	:	Scanning electron microscope
SPR	:	Surface plasmon resonance
TAE	:	Tris acetate EDTA
TBA	:	Thio barbituric acid
TBE	:	Tris borate EDTA
TCA	:	Trichloro acetic acid
TE	:	Tris EDTA
TEM	:	Transmission electron microscope
Tm	:	Annealing temperature
TNF	:	Tumor necrosis factor
UV	:	Ultraviolet
v/v	:	volume per volume
w/v	:	weight per volume
WG	:	The Western Ghats
WHO	:	World Health Organization

INTRODUCTION

Man is bound to nature and natural products from time immemorial and is benefitted with the plant products in all walks of life. The plant kingdom is the treasure house of active drugs and potent therapeutics. Plants are being used in the pharmacotherapy to get rid of the ailments which date back to human origin. The ancient use of specific plants for particular ailments was confirmed by repeated trials and experience. Progressively, man learned to find utility in specific plant parts rather than from the whole plant. Apart from the leaves, other plant parts like seeds, fruits, roots, and bark were also consumed as drugs.

The oldest written evidence of usage of plants in medicine and drug preparation available dates back to 5000 y. a. Twelve recipes of drugs involving the use of 250 plants were engraved on a Sumerian clay slab. In Chinese medicine, the use of dried plant parts in drug preparation is depicted in the book 'Pen T Sao' (2500 BC). Evidence shows that the world's first drug stores were established by the Arabs which included the consumption of herbs and organic products for pharmacotherapy. Archeological evidence points out that herbalism dates back as far as 6000 y. a. in Iraq and 8000 y. a. in China (Pan et al., 2014a). These indicate the involvement of plant-based therapy all over the world since prehistoric ages. Estimates show that three fourth of the world's total population depends predominantly on plants and plant products for basic health care needs. More than 30% of the entire plant species were used for medicine and drug preparation (Petrovska, 2012).

Indian traditional system of medicine dates back to the age of the Vedas. Even today, the majority of the Indian population looks forward to the traditional and folk system of medicine for health care and longevity. The intense belief that 'everything could be a drug' is immersed in the Indian

culture and the traditional physicians utilized extensive modes of medication including herbs/herbal products, marine resources, animal urine, *etc*. (Vaidya & Devasagayam, 2007). Indian herbal medicine is properly processed to balance the three humoral doctrines - Vata, Pitta, and Kapha. Ancestral Indian medicine could be codified into a system called AYUSH, which comprises Ayurveda, Yoga, Unani, and Siddha amongst which Ayurveda, Siddha, and Unani extensively employ the use of plants and plant-derived products. These happen to be the major health care systems relied upon by approximately 70 percent of the population (Mukharjee et al., 2016).

Herbal medicines are gaining immense interest nowadays due to several reasons. The incapability to supply adequate drugs to the increasing population, the enormous cost and side effects of the modern medicine, increasing resistance of contemporary drugs against the growing novel infections and cultural acceptability of the herbs are some of them. However, scientific validation and authentication of the herbal remedies is an indispensable and primary requirement for the industry use and market supply. Toxicological standardization, physical methods, and chemical procedures are employed to standardize herbal drugs. The importance of herbal medicine throughout the world has been recognized by WHO and several resolutions are adopted to preserve and propagate traditional herbalism. As a valuable and readily available resource for primary health care, herbal medicines are promoted and standardized worldwide. An extensive and comprehensive program for identification, cultivation, evaluation, utilization, and conservation of herbal medicines has been developed so that the public will be benefitted by even-handed knowledge and information (http[1]).

Plants are the workhouse and repository of surplus chemical constituents which might be categorized into two - primary and secondary

metabolites. Primary metabolites include essential nutrient components like carbohydrate, protein, lipid, *etc.* while secondary metabolites are the ones produced in the tissues as a result of secondary metabolism. The latter ones provide defense, allelopathic feature or other additional properties to the plant, rather than vital functions. Alkaloids, glycosides, polyphenols, terpenoids, and flavonoids are the prominent classes of secondary metabolites constituting the phytochemicals.

Characterization of the phytoconstituents becomes an important lead in the exploration and validation of a specific property of a particular plant. A broad analysis of the chemical constituents includes preliminary screening or qualitative phytochemical analysis which enables the detection of various classes of phytochemicals or bioactive principles. The quantitative analysis determines the variability of pharmacologically important secondary metabolites in the plant parts. Chemical and spectroscopic methods are employed for the estimation.

Various classes of compounds render different properties in a living system. Flavonoids are water-soluble radical scavengers capable of preventing oxidative cell damages (Okwu, 2004). Tannins are proved to possess stringent properties and fasten the healing of wounds and inflamed mucous membranes. Phenols possess the ability to block specific enzymes that cause inflammation whereas; saponins are active as expectorants useful in upper respiratory tract inflammations. Saponins also regulate cell proliferation and inhibit the growth of cancer cells (Jimoh & Oladji, 2005).

Contemporary phytochemical studies involve detailed analysis of the individual constituents, structure elucidation and identification of the potential bioactivities. Fractional or serial extraction of the components using multiple solvent systems, isolation of individual compounds, and interpretation of the structures are some of the popular methods employed for the same. Modern

techniques like chromatography, spectroscopy, spectrophotometry, and crystallography are the prominent procedures used to characterize the phytochemical compounds.

Modern analytical techniques like HPLC, GC, TLC, *etc.* are performed with high accuracy for metabolite profiling. Currently, these techniques are coupled with mass spectrometry for the precise separation, detailed characterization and the approximate quantification of wide range of compounds in the plant matrices (Jorge et al., 2016). Gas chromatography coupled with mass spectrometry provides profiling of metabolites under large scale with a good balance of sensitivity and reliability, more robust than liquid chromatography - mass spectrometry. Even the trace amounts of volatile and semi-volatile organic contents could be determined and quantified using GC-MS analysis. The technique can be employed for diverse applications like diagnostics, gene annotation and systems biology (Lisec et al., 2006).

Since the past centuries, the trial-and-error approach was employed in drug discovery. Progressively, the search for plant/nature-derived biologically active compounds with curative property became systematic and a step by step process with mere possibilities for “chance” discoveries as if in the case of penicillin. Efficient and cost-effective bioactivity screening in a systematic way reveals the potential of the plant- derived products thereby paving the way for novel drug discovery. The primary step in bioactivity screening is the identification of interesting active component(s) from natural sources which might be a single compound or a group of compounds. The identified components are isolated and purified from the crude extract, followed by a series of tests designed to analyze the effect of the particular component on specific biological functions. The proven candidates showing effective and marked activity in the bioassays performed are subjected to further *in vivo*

testing in animal models. Finally, the component with proven bioactivity and compatibility with the living system is selected for the clinical trials on human subjects, thus completing the prolonged complex process (Katiyar et al., 2012).

Oxidative free radicals are formed naturally in a system as a result of normal metabolic processes or external stimuli. These are highly reactive molecules which are able to initiate dominoes like chain reaction leading to suppressed cellular functions and progressively, cell death. Degenerative diseases are often resulted due to the accumulation of the free radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS). Oxidative species attack several vital molecules like polyunsaturated fatty acids to produce a series of signaling products, thereby hindering the original pathways (Njie-Mbye et al., 2013). It can damage biological molecules like lipid, protein and DNA thereby cause membrane disruption and loss of cellular structural integrity (Palipoch, 2013). Some of the ageing symptoms are resulted from the oxidation of the normal tissues leading to cell death. Development of ageing symptoms like atherosclerosis could be attributed to radical induced oxidation of cholesterol to 7-ketocholesterol (Lyons & Brown, 1999).

The oxidative chain reactions initiated by the free radicals are often terminated by certain components that are collectively known as antioxidants. Oxidative suppressors like glutathione, catalases and superoxide dismutase are formed in the living tissues to balance the oxidative stress. Externally, the antioxidants are administered through diet which is essential for growth and development. The protective effect of the antioxidants is essential for the well-being of man as it rejuvenates the tissues and repairs the damages resulted from oxidative stress. Various drugs used for the treatment of degenerative diseases are rich antioxidants that are either chemically

synthesized or derived from natural products. The requirement for more effective and non-toxic natural antioxidants is increasing nowadays and thus extensive studies are going on around the globe for the same. Biochemical antioxidant assays are carried out as a routine mode of study. Advanced confirmation studies are implemented *in vitro* in isolated mammalian cell systems. Generally, there are two types of antioxidant assays - one is based on the rate of oxidation of lipid content and the other one is based on the rate of radical or electron scavenging ability. The former one includes assays like thiobarbituric acid assay (TBA), malonaldehyde/high-performance liquid chromatography (MA/HPLC) assay, malonaldehyde/gas chromatography (MA/GC) assay, beta-carotene bleaching assay, and conjugated diene assay. On the other hand, the latter one includes 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2, 2'-azinobis (3-ethylbenzothiazoline - 6 - sulfonic acid) (ABTS) assay, ferric reducing/ antioxidant power (FRAP) assay, ferrous oxidation-xylenol orange (FOX) assay, ferric thiocyanate (FTC) assay, and aldehyde/carboxylic acid (ACA) assay (Moon & Shibamoto, 2009).

Plant-derived compounds have been documented as excellent free radical scavengers capable of rectifying the damages caused by the oxidative species in the living system. Recently, naturally occurring antioxidants are gaining interest in pharmaceutical, nutraceutical, food and cosmetic industries due to their multiple actions and negligible side effects thereby providing huge scope in replacing synthetic ones. Moreover, natural antioxidants can terminate the chain reactions initiated by ROS and RNS entities considerably, hence providing a protective effect against tissue degradation (Saeed et al., 2012).

Oxidative stress often leads to the destruction of the structural and functional integrity of the vital organs. The case of hepatotoxicity induced by ROS is increasing nowadays due to the changes in lifestyle, food habits and

medications in addition to genetic factors and pre-existing pathological conditions. The physiological mechanism of hepatic damage is merely understood but is associated with the metabolic conversion of xenobiotics to ROS, which induces oxidative stress (Rašković et al., 2014). Overdose of the drugs like paracetamol, antitubercular drugs, exposure to toxic compounds like carbon tetrachloride (CCl_4), thioacetamide, dimethylnitrosamine (DMN), *D*-galactosamine/lipopolysaccharide and obviously, alcohol consumption leads to hepatotoxic damages (Madrigal-Santillán et al., 2014). Though modern hepatoprotective drugs are available, mere reliability and increased side effects have drawn the attention of researchers to develop natural/herbal preparations for hepatoprotection. Indian traditional health systems include a vast knowledge about antihepatotoxic herbs and the formulations are widely used to cure hepatic disorders. The protective effect rendered by the herbal extracts is primarily due to the presence of active polysaccharides (Wang et al., 2012).

Cytotoxic studies have been an initial step in determining the deleterious or protective effect of a test substance - a plant extract or a biologically active candidate of plant origin. Various test systems are being employed by the researchers to determine the multitude and magnitude of the cytotoxic effect of the herbal extracts/compounds. *Allium cepa* is an excellent plant model used as a preliminary test system in toxicity studies as well as environmental biomonitoring. Due to the effortless handling, ease in availability and rapid propagation, *Allium cepa* root meristem has been used as an immediate test model for genotoxic and cytotoxic evaluation studies since early 1900s. It shows good correlation with the mammalian test systems thus could be used as a primary step in toxicity studies by evaluating the chromosomal aberrations. Reports show that *Allium cepa* assay shows results similar to that of the mammalian test systems (Teixeira et al., 2003).

The cytotoxic effect of the plant extract could be tackled in a beneficial way by implementing them in anti-tumor and anticancer studies to combat dreadful diseases. Thorough investigations on the cytotoxic effect of the plant extract could be carried out using various test systems including mammalian cells. The toxic effect of the same is often used to check the uncontrolled cell proliferation. The antiproliferative products might become the promising candidates in cancer research.

According to WHO, cancer is the second leading cause of death around the globe and counts for every 1 out of 6 deaths. Intake of tobacco and alcohol, reduced consumption of fruits and vegetables, lack of physical exercise, high body mass index and exposure to carcinogenic substances are the leading behavioral and dietary risks resulting in the development of cancer. Cancer develops from a lesion in the body tissue which further develops into malignant lumps followed by metastatic distribution through body fluids. Metastasis and delocalization of the malignant tissue forms the major cause of death by cancer. Accumulation of abnormal cells with uninhibited division grows beyond their usual boundaries thereby hindering the structure and function of the adjacent tissues. The most common types of cancers leading to high mortality rate include lung, colorectal, breast, prostrate, skin and stomach cancers (http[2]).

Cancer has always been a dreadful disease which still lacks a proper drug-based therapy. As ample researches and investigations are going on in this field, the interests in herbal formulations with antiproliferative property are increasing. Herbal formulations make use of the synergistic action of the complex phytoconstituents rather than a single compound, thereby rendering a multifaceted action with minimum side effects. Well established chemotherapeutic compounds like taxol, vincristine, vinblastine, *etc*. are of plant origin, thereby providing a base for herb-based cancer research. These

drugs are capable of inducing death in rapidly dividing tissues using three major mechanisms - Type I - Apoptosis, Type II - Autophagy and Type III - Necrosis; along with other modes of cell death like pyroptosis and mitotic catastrophe (Galluzzi et al. 2007).

Apoptosis is a normal mode of cell death occurring in a programmed manner during the developmental process of multicellular organisms. The cells undergoing apoptotic destruction are characterized by membrane blebs, cell shrinkage and formation of apoptotic bodies. Apoptosis happens *via* either intrinsic or extrinsic pathways. Caspases, the cysteine protease enzymes, are the major factors involved in the apoptotic pathways. The dead cells are removed from the system through phagocytosis without causing the spillage of the cellular contents into the surroundings. Apoptosis is a normal, inevitable and controlled process for removing the damaged, aged and unwanted cells from the tissues. The controllability of the process is welcomed by the workers dealing with cancer research leading to drug discovery/designing to induce apoptosis in the unnecessarily dividing cells.

Necrosis is another major mode of cellular destruction that usually results from an external injury, infections, and trauma. It often refers to the post mortem changes that occur after cell death. Necrosis always results in detrimental effects and leads to further complexities like inflammation, gangrene and sometimes become fatal. This often results from acute hypoxia or anoxia condition developed in the surrounding tissues (Kim et al., 2003). Membrane disruption in the dead cells leads to the discharge of internal contents of the damaged cells, thus provoking inflammatory responses and damaging adjacent cells, all of which are in contrary to apoptosis.

Detection of the mode of destruction can be identified using various techniques. Though cell death occurs in multiple ways, apoptotic death of the unwanted cells is of prime interest in the drug development in cancer

research. Apoptosis can be detected using simple light microscopy to the highly sophisticated modern techniques. A peripheral observation of apoptotic cells is determined by light microscopy but is not reliable as it lacks reliability and accuracy. SEM or TEM analysis provides better details about the sub-cellular changes like chromatin condensation, membrane blebbing, karyorrhexis and formation of apoptotic bodies. Biochemical markers like gel electrophoresis gives additional evidences of sequential degradation of DNA caused due to both, single and double stranded breaks. Pulsed-field and single cell gel electrophoretic techniques clearly distinguish apoptotic cells from live cells by displaying their peculiar comet-like morphology. Sophisticated techniques like flow cytometry gives a vivid picture of the apoptotic cells with marked alterations in the cell morphology. Apoptotic cells are identified by their ability to uptake DNA specific flourochromes like propidium iodide, trypan blue, DAPI, acridine orange, and Hoechst stains. Quantification of the apoptotic cells using flow cytometry is gaining interest nowadays due to its reliability, efficacy and precision. *In-situ* end labeling is another important technique where single apoptotic cells could be identified and imaged. The extent of cell responses and genetic involvement in the apoptotic responses could be clearly unveiled using immunohistochemical analyses. Studies on expression level of apoptotic genes like caspases and p53 are the prevailing common techniques involved. Annexin V is a calcium dependent phospholipid binding protein that specifically binds phosphatidyl serine which is a notable feature of apoptotic cells. Histochemical quantification of Annexin V uptake also determines the quantitative aspects of apoptosis (Archana et al., 2013).

Nanotechnology is an interdisciplinary and convergent area of science that opens up a new realm of diagnostics and treatment. Nanoparticles have very specific biological and physicochemical characteristics because of their unique size (1-100 nm). Their properties like melting point, charge capacity,

tensile strength, *etc*., are desirable to researchers rather than their parent elements. Nanoparticles are having applications in diverse fields such as biomedical, health care, drug delivery, environment, electronic, magnetic, space science, sensors and energy storage. Synthesis of nanoparticles has two approaches; top-bottom, where the larger particles are degraded into particles of nano-size and bottom-top, where the nanoparticles are built by combining the smaller units like atoms and molecules (Nath & Banerjee, 2013). The mode of synthesis might be a physicochemical method or biogenic method.

Biogenic or biological reduction of metallic ions to nanoparticles is of great importance nowadays as it is a rapid, readily conducted, easy to scale up, environmentally benign and cost-effective practice. Physicochemical methods involve the use of toxic chemicals as reducing and stabilizing agents, which is expensive as well as an ecologically lethal process. On the other hand, plant extracts can reduce the metal ions as well as stabilizes the resultant nanoparticles (Kumar & Yadav, 2009). Nanoparticles of noble metals like silver, gold, platinum, other metals like copper, compounds like zinc oxide or elements like carbon, *etc*. are the prevailing ones in the day-to-day research and industries. Among these, silver nanoparticles have pulled considerable researchers' attention because of their attractive properties such as chemical stability, high catalytic activity, high thermal and electrical conductivity and surface-enhanced Raman scattering (Jeyraj et al., 2013).

Microorganisms were also employed for biogenic synthesis of nanoparticles, but the cost of microorganism culture and production is higher than involving plant extracts for the same. A plant extract mediated bio-reduction involves the mixing of aqueous extract with an aqueous solvent of relevant metal salt. The complex process of bio-reduction occurs at room temperature and normal pressure within few minutes. Different plant extracts contain different concentrations and combinations of various reducing agents

and thus the source of plant extract influences the characteristics of nanoparticles (Mukunthan & Balaji, 2012). Other factors also affect the phytosynthesis of metal nanoparticles. pH determines the shape and size of the particles, with low pH (2-4) producing larger particles whereas; high pH facilitates the nucleation and subsequent formation of large number of NPs with smaller diameter. The production of nanoparticles also shows a positive correlation with the temperature as well as time of incubation.

Characterization of the nanoparticles involves various techniques to unravel shape, size, surface area and diversity. Common techniques used nowadays are UV-visible spectrophotometry, dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and energy dispersive spectroscopy (EDS) (Mittal et al., 2013). Identification of the particle element is possible by UV-vis spectrophotometry. Surface charge and size distribution of the particles suspended in a liquid is enabled by DLS (Jiang et al., 2009). SEM and TEM provide the details of the morphological properties of the particles and their size in nano-scale. Surface chemistry is studied by the FTIR studies which provide an account on the organic functional groups like carbonyl and hydroxyl along with the other surface residues attached on the particles (Chithrani et al., 2006). Phase identification and characterization of the crystal structure is detailed using XRD analysis (Sun et al., 2012).

Nanoparticles are being employed in various fields because of the unique structure and properties. Silver nanoparticles are gaining importance in medical and commercial consumer products as antibacterial agents (Rai et al., 2009). Gold NPs also exhibit bactericidal properties against human and animal pathogens along with its application in DNA labeling, biosensor and drug delivery. NPs of platinum show anticancer effect while that of selenium

exhibits antimicrobial activity. Biocatalysis is enabled by palladium NPs. Copper NPs are also having remarkable antimicrobial effect. Iron NPs show considerable anticancer activity and also finds application in molecular imaging. NPs of zinc oxide are widely used as coating agent and in cosmetics (Mittal et al., 2013).

Meliaceae, the mahogany family, is a family comprising of woody plants or trees prevailing in the tropics and subtropics, with 51 genera and about 550 species. The family is extremely useful to man for the high-quality timbers and some species could be easily grown in plantations. Loureiro described the first species of *Aglaia*, the largest genus in the family, in his Flora Cochinchinensis (1790). *Aglaia* is the only natural repository which holds the unique group of potential phytochemical components called the rocaglamides. These are flavaglines characterized with tetrahydrocyclopenta-skeleton which has attracted the researchers with its incredible bioactive potentials. Despite the remarkable utilities, many species remains underexplored and is used only in the timber industry. Bioactive compounds like pannellin, aglaroxin, ponapensin *etc.* have been isolated from the different species of *Aglaia* and were found to be effective as anticancer, antimicrobial and anthelminthic agents (Kim et al., 2006; Proksch et al., 2001; Salim et al., 2007).

The Western Ghats is a rich treasure of several invaluable medicinal and commercially important plants. More than 12 species of *Aglaia* was reported so far from the WG, some of which are *A. edulis, A. lawii, A. elaegnoideae, A. barberi, A. malabarica, A. roxburghiana* and *A. simplicifolia*. Phytochemical studies and bioactive potential screening of only few species are reported so far. *A. barberi, A. simplicifolia,* and *A. malabarica* are some of the extremely underexplored ones which are selected for the present study.

Considering the aforesaid aspects, the present study was designed to evaluate the phytochemical constituents followed by the screening of various bioactivities of the three selected species of *Aglaia* which include *A. barberi*, *A. simplicifolia* and *A. malabarica*. The whole study was divided into three phases and the primary objectives are as follows:

- Qualitative or preliminary phytochemical screening of the selected plant extracts
- Quantitative estimation of major secondary metabolites
- GC-MS analysis of the plant extracts to unravel the volatile components
- Evaluation of the antioxidant capacity of the three plants
- To study the protective efficacy of the plant extracts using HepG2 hepatocytes
- To evaluate the cytotoxic potential of the plant extracts using *Allium cepa* root meristem assay, *in-situ* visualization studies and the effect on human erythrocytes
- Antiproliferative study on HeLa cell line using all the three plant extracts
- Extensive studies on apoptotic effect of the most potent plant of *Aglaia* using flow cytometry and its quantification
- Expression studies of the apoptotic effect at the gene level by quantifying the caspases involved in apoptosis
- Determination of the bio-reduction ability of all the three plant extracts on silver nitrate into elemental silver nanoparticles
- Characterization of AgNPs using UV-Vis spectrophotometry, X-ray diffraction and SEM analyses

REVIEW OF LITERATURE

A. BACKGROUND

Humans rely on plants for their invaluable utilities since the prehistoric age. Plants are harnessed for curative purposes due to their inherent potential to combat human ailments. Indigenous systems of medicines like Ayurveda, Unani, tribal medicines, *etc*. depend largely on plants against human diseases. As time passed, modern medicines took the place of traditional systems, while it was further found to have a deleterious effect on human health. These force man to return to plant-derived medicines, wherever possible. The healing ability of plants can be attributed to the synergistic action of the phytoconstituents present in them. Extensive studies are going on globally to determine the pharmacognostic properties of plants thereby, paving way for drug development.

Family Meliaceae, also known as 'the mahogany family', belongs to the order Sapindales which mostly comprises of shrubs and trees. The family includes 52 genera and more than 600 species distributed in the tropical and sub-tropical regions (http[3]). The plants are used for various purposes like timber, pharmaceuticals, insecticides, *etc*. Most of the genera like *Swietenia*, *Toona*, *Cedrela*, *Carapa*, *Khaya*, *etc*., are widely used in the timber industry. Some genera, like *Azadirachta*, *Melia*, *Aphanomixis*, *etc*., have been reported for its medicinal and insecticidal activities. *Aglaia* is one of the largest genera that comprises of about 120 species with arborescent habit enjoying tropical and subtropical distribution. In India, it is found in the tropical evergreen forest belts. Some of the species observed in the Western Ghats include *A. bourdillonii*, *A. elaeagnoidea*, *A. barberi*, *A. tomentosa*, *A. perviridis*, *A. simplicifolia*, *A. lawii*, *A. malabarica*, *A. cucullata*, *A. edulis*, *A.*

roxburghiana and *A. minutiflora*. Some species of *Aglaia* are well known and utilized for their biological properties like antipyretic, analgesic, anticancer, hepatoprotective, antidiabetic, anti-inflammatory, immunomodulatory and insecticidal activities (Bangajavalli & Ramasubramanian, 2015).

Aglaia is acquiring attention by researchers for the past few years due to the presence of a unique group of flavonoids called, flavaglines or rocaglamides. *Aglaia* is morphologically discriminated from other genera in Meliaceae by the presence of characteristic indumentum of peltate scales or stellate hairs on its vegetative parts. Simple hairs are never found in *Aglaia* (Pannell, 1992). *Aglaia* is often confused with other genera like *Lansium*, *Reinwardtiodendron* and *Amoora* by morphological features but it can be distinguished using DNA data as well as chemical profiles (Muellner et al., 2015), rocaglamide being the phytochemical identifying factor. The genus is well known for timber and the identification is sometimes aided by wood anatomical features (Khaopakro et al., 2015). Most of the species are reported for its medicinal and pharmaceutical effects. Fruits of certain species are edible and used by indigenous people. This led to further exploration of the bioactive potential of the genus.

B. PHYTOCHEMICAL ASPECTS

Phytochemical studies are of prime importance to elucidate the mechanisms by which the drugs act as a bioactive factor. Researchers use several methods for phytoconstituent analysis; from conventional qualitative preliminary phytochemical analysis methods to advanced techniques like GC/MS, HR-LC/MS, HPTLC, *etc*. Quantitative estimation of major classes of bioactive secondary metabolites has been reported from plants, which moreover advanced to compound isolation and identification.

Though studies on the phytochemical aspects and bioactive potential of the genus is undergoing extensively around the globe, most of the species in the Western Ghats remains underexplored. It also includes the ones listed in the IUCN red data book. Immense studies on the phytochemistry of *Aglaia* have been reported to date. New compounds have been reported and isolated from the plant parts, which have also been verified for various bioactivities. Table 1 depicts the recent reports on the phytochemical studies on *Aglaia*.

Aglaia is believed to be the only natural source of the potent bioactive compound, rocaglamide. These are cyclopentabenzofurans/pyrans which are also known as flavaglines. Most of the species of *Aglaia* have been subjected to the isolation of this promising compound and the structural elucidation reveals the variations in cyclopentane part at C-1, C-2, C-8 and phenyl rings. The variations might be resulted due to the presence of hydroxy- or acetoxy-groups as common while, aldehyde, oxime or oxo being rare as the substituent on C-1. The residue found on the C-2 is generally a methyl ester, amide or carboxyl group. In some species of *Aglaia*, rocaglamides are also observed with fused C-1 and C-2 to form a pyrimidinone unit (Pan et al., 2014b).

Preliminary phytochemical screening of various fruit pulp extracts of *A. elaeagnoidea* was done by Rajagopal et al. (2018). Ethanolic and aqueous extracts were reported to contain alkaloids, phenolic compounds, tannins, flavonoids and proteins. Phytosterols were detected in petroleum ether extract while fixed oils and fats were present in benzene extract.

Ravindran and Thoppil (2018) studied the phytochemical constituents of aqueous and methanolic leaf extracts of *A. malabarica* and reported the presence of bioactive secondary metabolites including phenols, flavonoids, terpenoids, tannins, alkaloids and steroids. Methanolic leaf extract exhibited

significant antibacterial activity against both Gram-positive and Gram-negative strains.

Qualitative analysis of various leaf extracts of *A. roxburghiana* revealed the presence of bioactive phytoconstituents like steroids, triterpenes and coumarin in the four extracts like hexane, benzene, chloroform and ethyl alcohol. Phenols, tannins, quinones, sugars and acids were also detected while saponins and furan compounds were absent. These preliminary phytochemical analyses were supported along with fluorescent data as reported by Sasikala et al. (1999).

The ethanolic fraction of fruits of *A. elaeagnoidea* exhibited the presence of carbohydrates and terpenoid compounds. The fruits showed a null effect when tested for other major secondary metabolites like coumarins, phenols, resins, quinones, *etc*. As the fruits were edible, nutritive standards were also determined that revealed the high nutritional indices of the same (Bidari et al., 2017).

Biu et al. (2009) performed the preliminary phytochemical characterization of mature leaves of *Azadirachta indica*. The aqueous extract was used for the same and the study revealed the presence of saponins, terpenes, alkaloids, flavonoids, tannins and glycosides. Anthraquinones and ketones were not detected.

Qualitative phytochemical analysis followed by evaluation of antifungal and cytotoxic studies of various extracts of *Trichilia heudelotii* was reported by Opawale et al. (2015). Aqueous, acetone and ethanolic extracts of leaves and stem bark were used for the study. Qualitative screening of phytochemicals revealed the presence of tannins, alkaloids, glycosides, flavonoids and anthraquinones. Steroids and chalcones were not detected. In the antifungal screening, acetone leaf extract exhibited the highest activity

against *Candida albicans* ATCC1023 which was followed by ethanol and acetone extracts of bark against *Candida albicans* and *Trichophyton rubrum*, respectively. Cytotoxicity assay was conducted using brine shrimp assay which produced significant results. The results thereby authenticate the traditional application of the plant against human fungal skin infection, dermatitis and infections caused by other organisms.

A new rocaglamide derivative named 5′, 6 - dimethoxyisolariciresinol-(3″, 4″-dimethoxy)-3*α*-*O*-*β*-d-glucopyranoside was isolated from *A. exima.* The methanolic extract of the bark of the tree was used for the same. Structure elucidation was done using the data obtained from UV, IR, NMR, ESI-TOFMS as well as comparison with the related compounds that were reported earlier (Sianturi et al., 2016a). Another study on the leaves of *A. exima* resulted in the isolation of six triterpenoids and two steroid compounds. Structure elucidation was done using UV, X-ray spectrometry and NMR data. *In vitro* testing for cytotoxicity of all the compounds showed effective action against eight cancer cell lines *viz.*, A549 (lung), DU-145 (prostate), SK-MEL-5 (skin), BxPC-3 (pancreas), Hep G2 (liver), HT-29 (colon), MCF-7 and MDA-MB-231 (breast). The new cycloartane triterpenoid isolated was 24(E)-cycloart-24-ene-26-ol-3-one, which showed strong activity against HT-29 cells (Awang et al., 2012).

A. lawii is commonly found in the tropical evergreen belts of Western Ghats. Phytochemical aspects have been studied by Lavate et al. (2013). Various solvents (ethyl acetate, acetone, ethyl alcohol and methanol) were used to extract phytoconstituents of the different aerial plant parts followed by the estimation of total phenolic and flavonoid contents. All the extracts were found to contain phenols and flavonoids but the amount differed. The leaves possess abundant phenolic compounds while comparatively less phenolic content were obtained from the bark. Fruits were observed to have maximum

flavonoids. The study suggests that *A. lawii* is a rich source of potential bioactive constituents.

Chloroform soluble extract from the fruits of *A. sylvestris* was found to be cytotoxic against several human cancer cell lines and notably, it was found to be active in P-388 *in vivo* test system. The active compound isolated from the fraction was later named as silvestrol which was found effective against Lu1 (lung), LNCaP (prostate) and MCF-7 (breast) cells. The best part of the study was that it exhibited dose-dependent cytotoxicity with no significant weight loss. Test conducted in the *in vivo* hollow fiber bioassay also displayed the effectiveness of the compound similarly, without weight loss. Studies to elucidate the mechanism of action of silvestrol are ongoing followed by subsequent re-isolation and hopefully, pre-clinical trials might be employed (Hwang et al., 2004).

Othman et al. (2016) isolated five terpenoid compounds belonging to secodammarane class (stellatonins A-E) as well as silvestrol from the methanolic stem extract of *A. stellatopilosa* through bioassay-guided fractionation. Spectroscopic and chemical methods were employed to elucidate the structure of the isolated compounds. Cytotoxic studies on human cancer cells as well as antimicrobial assays were evaluated that provided promising results.

Chromatographic techniques are employed to determine the phytoconstituents that were resolved according to their retention time. Gas chromatography coupled with mass spectrometry (GC-MS) gives a detailed data of volatile compounds along with their quantity. Phytochemical characterization of most of the important genera of Meliaceae, including *Aglaia* has been discussed here.

A. odorata is an economically important plant extensively used in flavouring industry in China. Several reports on its bioactivities and phytochemical aspects are available. Supercritical CO_2 fluid was used to extract the volatile flower compounds by Zhang et al. (2007). The constituents were resolved using GC-MS analysis where 54 peaks were resolved and 48 compounds were identified. 18 terpenes, 12 esters and other volatile compounds were identified which included copaene, caryophyllene, ethyl palmitate, methyl jasmonate, ethyl linolenate, α-humulene, β-elemene *etc*. In another study conducted by Hongxiang (2010), volatile constituents of *A. odorata* essential oil were subjected to GC-MS analysis where, α-humulene, ethyl linolenate, germacrene D *etc*. were identified as the major compounds. The exciting finding in this study was that the essential oil could be used as tobacco flavourant in cigarettes to reduce the offensive odour and to make tobacco smoke smooth and fine.

Gas chromatography-mass spectrometry analysis of the stem-derived essential oil obtained from *A. odorata* revealed 39 compounds. Germacrene-D, β-caryophyllene, α-humulene, *etc*. were identified as the major compounds. Ar-turmerone was isolated from the stem essential oil while eichlerialactone from the ethanolic stem extract. The former exhibited significant antifungal effect whereas, the latter one was found to possess great bactericidal activity when tested for the antimicrobial potential (Joycharat et al., 2014).

The dried fruit pulp of *A. dookkoo* was extracted by steam distillation to get essential oil. This was further subjected to GC-MS analysis which revealed the presence of oleic acid, copaene, germacrene-D, δ-cadinene, τ-muurolol, palmitic acid, *etc*. as prominent compounds. Soxhlet extraction of methanol soluble compounds was done to identify the organic acids which

revealed the presence of malic, citric, glycolic and maleic acids (Chairgulprasert et al., 2006).

A. odoratissima was studied by Maznah et al. (2015) to identify the existing volatile compounds using GC-MS analysis. Serial extraction of the dried leaves was done using four solvents *viz.*, hexane, ethyl acetate, chloroform and methanol. Several compounds that have been previously reported as potential bioactive agents were identified from all the extracts. Hexane extract revealed the presence of the sesquiterpenoid, β-selane along with a significant quantity of α- copaene and lupenone. Chloroform and ethyl acetate extract fractions showed the highest peaks showing rich triterpenoid content while methanolic fraction revealed the presence of organic acid. The insecticidal property of the plant could be attributed to the terpenes present in it.

The GC-MS analysis of leaf extract of an important medicinal plant *Melia dubia*, revealed the presence of unsaturated fatty acids, terpenoids, phenolic derivatives and lipophilic organic compounds. Compounds like linolenic acid, palmitic acid, caryophyllene, humulene, aromadendrene, *etc.* were identified which are biologically active and possess antimicrobial, antioxidant, anti-inflammatory as well as anti-tumor activity (Murugesan et al., 2013).

Indian neem, *Azadirachta indica*, has proven medicinal history from the age of Vedas and is used for treating various treatments in the crude form even now. Further, strong evidence on its excellent insecticidal property along with its medicinal value was reported. By the time, phytoconstituent recognition and isolation of the bioactive compounds became inevitable for elucidating the effect of plant parts/extracts against pathogens and other ailments. Gas chromatography-mass spectrometry analyses of leaves revealed the presence of bioactive compounds like 1, 2-ethendiol monoacetate, aeremanthin, α-bergamotene, isolongifolene methyl ester, *etc*. The detailed

phytochemical data was reported by Dineshkumar & Rajkumar (2015) and the results suggest that the leaves of the tree can be used as the herbal alternative against various diseases.

Trichilia connaroides is another medicinally important plant belonging to the family Meliaceae. Indigenous people in the Western Ghats widely use the plant parts as an effective medicine against several intestinal and skin ailments. Methanolic leaf extract was subjected to GC-MS analysis which unveiled the presence of prominent bioactive compounds like palmitic acid, oleic acid, isochiapin B, servergenin acetate, lycopersen, *etc.* which have been reported for several bioactivities (Senthilkumar et al., 2012).

Table 1: Phytochemicals reported or isolated from various species of *Aglaia*

Species	Plant part	Chemical compound	Reference
A. andamanica	leaves	*Epi*-melianodiol, aglaiodiol, pyramidaglain A and B	Puripattanavong et al., 2000
A. rubiginosa	leaves	Cycloartane triterpenes, dammaranes, stigmastandiol, β- sitisterol and β-D-glucoside	Weber et al., 2000
	twigs & leaves	1-O-acetylrocaglaol, methyl rocaglate, rocagloic acid,1-O-acetylmethylrocaglate	Rivero-Cruz et al., 2004
A. spectabilis	bark	Methylrocaglate and C-3′ hydroxylmethylrocaglate	Schneider et al., 2000
A. forbesii	leaves	Desacetylpyramidaglains A, C, D , 23, 24, 25-trihydroxycycloartan-3-one; 2β, 3β-dihydroxy-5α-pregn-17(*Z*) - en- 16-one; 2β, 3β-dihydroxy-5α-pregn-17(*E*) - en - 16-one	Joycharat et al., 2008
	seed	Isoeichlerialactone, methyl isoeichlerialactone, isocabralealactone,	Joycharat et al., 2010

		isoeichlerianic acid, aglinin A, spathulenol, β-sitosterol and stigmasterol	
A. smithii	bark	Aglinin E (20*S*, 24*S* - epoxy - 25 - hydroxyl -1-en - dammarene)	Harneti et al., 2012
A. odorata	root & bark	Rocaglaol, aglafolin, rocaglamide, desmethyl rocaglamide	Engelmeier et al., 2000
	leaves	8- (7′,8′,9′-Propanetriol-4′-methoxy-3′-O-phenylpropanoid)-7-hydroxy-6-methoxycoumarin	Zhang et al., 2012
	leaves	Dolabellane diterpenoids (1*R*,3*E*,7*E*,10*S*,11*S*,12*R*)-dolabella-3,7-dien-10,18-diol; (1*R*,3*S*,7*E*,11*S*,12*R*) - dolabella-4,7-dien-3,18-diol; (1*R*,7*E*,11*S*,12*R*)-18-hydroxydolabella-4(16),7-dien-3-one ;	Cai et al., 2010
	bark	(1*R*,3*S*,4*S*,7*E*,11*S*,12*R*)-3,4-epoxydolabella -7-en-18-ol; (1*R*,3*R*,7*E*,11*S*,12*R*)-dolabella-4(16),7,18-trien-3-ol Odoratanone A	Liu et al., 2013
A. eximia	bark	3, 4-Epoxy - (22R, 25)-tetrahydrofuran-stigmast - 5 - en	Harneti et al., 2014
	leaves	Cycloart - 24 - ene - 3β, 26 - diol 2 , schizandronic acid ; 24(E) - 3β - hydroxycycloart - 24 - ene -26 - al 4; vaticinone 5 [11], cabraleahydroxylactone 6 [112,13] and β-sitosterol 7	Awang et al., 2012
A. edulis	root bark	Pannellin, Aglaroxin A, Thapoxepine A and Thapsakinacetate A	Engelmeier et al., 2000
A. elaeagnoidaea	stem & bark	Aglalactone	Engelmeier et al., 2000

	fruit pulp	Lupeol	Rajagopal et al., 2018
A. silvestris	fruit & twig	Silvestrol and episilvestrol	Hwang et al., 2004
A. dasyclada	leaves	Dasyclamide	
		Aglain, aglaforbesin, forbaglin,	Chaidir et al., 2001
A. cordata	stem & bark	Aglacins	Wang et al., 2002
A. giganteana	leaves	Gigantamide A and grandiamide D	Duong et al., 2007
		Dasyclamide	
A. cucullata	fruits	1-O-formylrocagloic acid and 3′-hydroxyrocagloic acid	Pan et al., 2014a
A. ponapensis	leaves & twigs	Ponapensin and 5,6-desmethylenedioxy-5-methoxy-aglalactone	Salim et al., 2007
A. erythrosperma	fruits & leaves	Cabraleadiol, cabraleahydroxylactone, ethyl eichlerianoate, eichlerialactone, aglinin A, cabralealactone; 5,6-desmethylenedioxy-5-methoxy-aglalactone, the flavagline 4′-demethoxy-3′,4′-methylenedioxy-methyl rocaglate, scoparone and scopoletin.	Phongmaykin et al., 2011
A. minahassae	stem bark	4(15)-Eudesmen-1b,6a-diol and spathulenol	Kurniasih et al., 2018
A. elliptifolia	leaves	argenteanones C−E, argenteanols B−E	Wang et al., 2001

C. BIOACTIVE POTENTIALS

(i) Antioxidant studies

Metabolic reactions in a biological system result in the formation of free radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS). These are scavenged by the system itself to some extent but if produced in excess amount, accumulation in the tissues occurs that might lead to deleterious effects including membrane damages and genetic material degradation (Fig. 1). Antioxidant agents are the ones who could scavenge free radicals in a system thereby facilitating the cytoprotective effect. Natural antioxidants, particularly plant-derived ones are gaining interest these days due to their negligible side effects and many could be included in the diet. Recent reports on the antioxidant ability of *Aglaia* spp. and other important genera of Meliaceae are enlisted below:

Yu et al. (2016) measured the antioxidant activity of *A. oligophylla* using various extracts of trunk and stem. Cupric reducing antioxidant capacity (CUPRAC) and ferric reducing antioxidant power (FRAP) assays were the techniques employed to evaluate the same. CUPRAC assay results were expressed as Trolox equivalent (TE/g) and the maximum reducing potential was exhibited by ethyl acetate extract of the trunk (1543 mg TE/g) followed by methanolic extract of the stem (1059 mg TE/g). Methanolic extracts showed maximum efficacy in FRAP assay with the reducing power of 1269 mg TE/g (trunk) and 1084 mg TE/g (stem). Isolation of the bioactive components using column chromatography was also performed which resulted in the extraction and identification of stigmasterol, β-sitosterol and oligophyllic acid. The identification of the isolated compounds was done with the aid of previous reports.

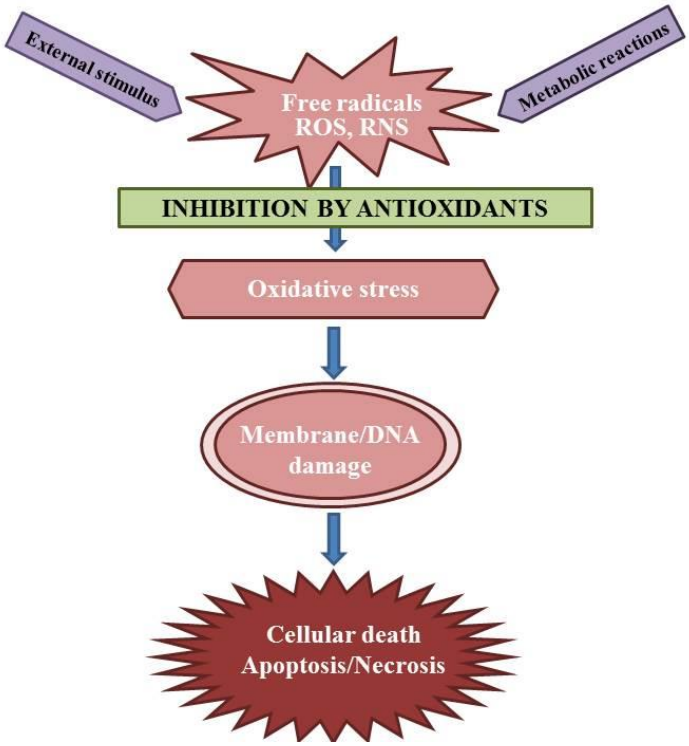

Fig. 1: Protective effect of antioxidants by scavenging free radicals

Measurement of total antioxidant capacities of plants used in Thai herbal medicines was conducted by Palasuwan & Soogarun (2014) including *A. pyramidata*, where a moderate effect was reported.

Evaluation of antioxidant activity along with the phytochemical characterization of a few important medicinal herbs was done by Kaja et al. (2014). Radical scavenging ability was assessed using DPPH, hydroxyl, superoxide radicals scavenging, lipid peroxidation and FRAP assays. A moderate effect was exhibited by *A. elaegnoideae*. Preliminary phytochemical analysis of leaf and bark extracts in various solvents revealed the presence of alkaloids, tannins, polyphenolics, and flavonoids whereas, anthraquinones were not detected. The total phenolic content in the leaf was estimated to be about 1.5 mg GAE/g.

An important tropical medicinal plant belonging to Meliaceae, *Trichilia monadelpha*, was studied for its antioxidant ability which was supported by phytochemical data. The plant extracts in various solvents like petroleum ether, ethyl acetate and ethanol were subjected to antioxidant assays *viz*., reducing power assay and DPPH radical scavenging ability, which revealed a significant activity and the maximum result was shown by ethanol extract. Total phenolic contents were estimated as 7.51±0.87 mg tannic acid equivalent/g in petroleum ether extract, 34.14±0.78 mg tannic acid equivalent/g in ethyl acetate extract and 119.30±3.20 mg tannic acid equivalent/g in the ethyl alcohol extract. Qualitative phytochemical analysis revealed the presence of coumarins, reducing sugars, alkaloids, terpenoids, phytosterols, flavonoids, cardiac glycosides and tannins in various extracts. The study suggests that the plant is a rich source of natural antioxidants and might be used to treat ailments resulting from oxidative stress (Ben et al., 2013).

Stem extracts of *Khaya senegalensis* in various solvents were proved to be potent natural antioxidants. Aqueous and crude ethanolic extract of the stem barks were used for the antioxidant activity analysis employing DPPH radical scavenging assay, where rutin and quercetin were used as standards. Defatted and lyophilized aqueous fractions showed significant activity almost close to that of rutin but less than that of quercetin (Lompo et al., 2007).

Another important tropical plant *Toona sinensis* was studied by Jiang et al. (2009) to determine the radical scavenging ability. Methanolic crude extract along with the sub-fractions in four solvents like hexane, ethyl acetate, n-butanol and water of the old leaves were obtained by sequential partitioning. The antioxidant potential was evaluated using DPPH free radical scavenging assay, FRAP assay, β-carotene bleaching method and stabilizing soybean oil. The extracts exhibited high antioxidant ability by significant

scavenging of the free radicals and preventing oxidation of soybean oil. Ethyl acetate fraction showed maximum activity and the estimation of bioactive secondary metabolites revealed the total flavonoid content as 108.57 mg rutin equivalents/g and total phenol content as 262.09 mg gallic acid equivalents/g. The study suggests that old leaves extract of *T. sinensis* as an alternative natural antioxidant that could be used as a dietary ingredient and as a natural stabilizer for soybean oil.

Antioxidant ability along with the antimalarial effect of *Entandrophragma cylindricum* bark extracts was evaluated using three extracts *viz*., methanolic, ethyl acetate and aqueous extracts. Plant extracts showed significant activity against the malarial parasite *Plasmodium falciparum* with the maximum activity by ethyl acetate extract. Along with this, the antioxidant activity of the extracts was determined *in vitro*, by measuring ferric reducing-antioxidant power, DPPH radical scavenging, ferrous chelating activities and nitric oxide radical scavenging. The maximum radical scavenging activity was shown by ethyl acetate extract itself and thus this could be tapped in the future for further up-regulation of the activity (Nadia et al., 2017).

(ii) Hepatoprotective studies

Protective efficacy of the plant extracts is often exploited in the drug preparation to retain the degrading cells in a biological system. Studies on hepatoprotection, neuroprotection, nephroprotection, *etc*. are some of the studies carried out to analyze the protective efficacy of the drugs. The same is reported in many plants belonging to the family Meliaceae and in several cases, the active compounds are isolated and characterized.

Cao et al. (2019) have isolated two polysaccharide fractions from *Toona sinensis* leaves which were named TSP-1 and TSP-2. Techniques

like GC-MS, NMR, FTIR, HPLC and AFM revealed the chemical composition of the isolate. The study also displayed high hepatoprotective efficacy of the isolated phytoconstituents against CCl_4 induced hepatic toxicity using decreasing lipid peroxidation, increasing antioxidant activity and enhancing anti-inflammatory response, *in vivo*. Elucidation of the possible mechanistic pathway has to be unveiled.

Majumdar et al. (2019) reported that taking 25 to 30 drops of juice of *Azadirachta indica* leaves along with honey in an empty stomach in the early morning for three weeks provided significant results in hepatoprotection. This was attributed to the presence of alkaloids in the plant extract.

The methanolic bark extract of *Swietenia mahagony* was defatted and screened for the hepatoprotective ability against paracetamol-induced liver damage in Wistar rats. Various biochemical and histopathological analyses were done after drug treatment. Total serum protein, total bilirubin content, serum glutamine pyruvate transaminase, serum alkaline phosphatase, reduced glutathione content, *etc.*, are some of the parameters studied. Along with this, histopathological observation of liver cells was also carried out. Results from all these assays showed remarkable hepatoprotective ability of the plant extract against paracetamol-induced hepatic damage. The effect was observed to be dose-dependent and is attributed to the antioxidant defense mechanism and the modulation of lipid peroxidation (Haldar et al., 2011).

The hepatoprotective ability of aqueous and ethanolic leaf extracts of *Azadirachta indica* was reported by Kalaivani et al. (2009). Carbon tetrachloride was injected to induce liver damage in mice. Significant protection by the leaf extracts was evident from the enzyme activities of serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase and alkaline phosphatase. The result confirms the

ethnomedicinal or traditional use of the plant as a potent hepatoprotective agent.

Protective efficacy of *Melia azedarach* leaf extracts against simvastatin induced hepatotoxicity was studied by analyzing biochemical parameters. An increase in serum glutamate pyruvate transaminase, serum glutamate oxalate transaminase, alanine phosphatase and serum bilirubin was induced along with a prominent drop in total protein content in simvastatin treated roots and a progressive normalization was brought by the *M. azederach* leaf extract that reflects the hepatoprotective ability of the extract. Phytochemical analysis revealed the presence of active secondary metabolites like alkaloids, tannins, phenols, flavonoids, glycosides and steroids (Rao et al., 2012).

Aphanamixis polystachya, a traditional medicinal plant belonging to the family Meliaceae, is indigenously used in India. The healing effect of the crude leaf methanolic extract against toxic liver injury was studied. Hepatotoxicity was induced in a rat model using carbon tetrachloride toxicity and the protective effect of the drug was assessed by measuring the enzymatic activities of aspartate aminotransferase, alanine aminotransferase, acid phosphatase, alkaline phosphatase and lactate dehydrogenase. The increased biochemical parameters were lowered significantly in the drug-treated animals thereby revealing the protective effect of the plant extract. Histopathological studies also revealed the protective efficacy against CCl_4 intoxication. The overall results revealed the healing effect of hepatocytes against acute CCl_4 toxicity (Gole & Dasgupta., 2002).

From long back onwards, decoctions and tonic of *Trichilia* spp. are used in the treatment of liver disorders. The plant plays an important role in traditional medicine. In this light, hepatoprotective efficacy screening of an important plant, *Trichilia connaroides* was evaluated. Aqueous extracts of the roots were tested in animals induced with toxicity by carbon tetrachloride and

a comparative study was done with a known protective agent. Intoxication resulted in the elevation of serum enzymes like aminotransferase, alanine aminotransferase, alkaline phosphatase and total protein content. The elevated enzymatic levels were normalized to the optimum level in the animals administered with the plant extract. The biochemical inferences were supported by histopathological studies of rat hepatocytes. The results obtained were comparable to those obtained from the standard hepatoprotective agent and that too in a dose-dependent manner. To substantiate the results, the antioxidant activity of the plant extract was assessed using both *in vitro* and *in vivo* models which included reducing power assay, radical scavenging activity and chelating activity on ferrous ions. The potential of the plant can be attributed to the presence of antioxidant phenolic compounds that was confirmed by the phytochemical analyses (Agarwal et al., 2010).

Asha (2001) studied the hepatoprotective activity of two plants, *Momordica subangulata* of Cucurbitaceae and *Naregamia alata* belonging to the family Meliaceae. Toxicity was induced by paracetamol overdose in rats and the drug was administered intraduodenally. Bile flow was observed along with serum enzyme activities to determine the hepatotoxicity or hepatoprotective effect. The results revealed the hepatoprotective ability of *Momordica subangulata* leaf extracts, both dry and fresh, and the null effect of *Naregamia alata*.

(iii) Cytotoxicity and anticancer studies

The ability of a plant extract to induce cytological aberrations might result in membrane disruption, cytoplasmic deterioration or chromosomal aberrations. Any of these could lead to cell/tissue death. This indicates the cytotoxic effect of the plant and this property could be utilized in the field of drug development against deadly diseases including cancer. Various reports on the investigation of the cytotoxic effect of plants on different test systems are available and the recent ones are listed below.

Allium cepa is a classical test system, often used for the preliminary screening of genotoxic/cytotoxic potential of a drug. Monitoring of the cytological aberrations and morphological alterations could be easily employed for *in-vitro* and *in-situ* studies. Table 2 represents some recent studies on cytotoxic studies using *A. cepa* root meristem reported recently.

Traditionally used five medicinal plants *viz., Azadirachta indica, Morinda lucida, Cymbopogon citratus, Mangifera indica* and *Carica papaya*, were evaluated for their cytotoxic and genotoxic effects using *Allium cepa* assay. Onion bulbs were treated with various concentrations of the aqueous plant extracts which was followed by macroscopic and microscopic evaluation. Dose-dependent cytotoxic effect was observed for the inhibition of root growth in all the extracts, thereby indicating mitodepressive effect as well as disturbances in spindle formation. The genotoxic and cytotoxic effects of these medicinal herbs are evident from the results (Akinboro & Bakare, 2007).

A. malabarica was evaluated for its ability to induce cytotoxicity and apoptosis in *A. cepa* root meristem along with human erythrocytes and cancer cells. Various concentrations of leaf methanolic extract were used for the treatment of *A. cepa* bulbs. The macroscopic observation was done for *in situ* evaluation of cell death and a dose-dependent effect was observed. Mitotic squash preparations were done to analyze the apoptotic features induced by the extract which revealed the formation of chromosomal fragments, micronuclei, chromatin disintegration, nuclear lesions, *etc*. Mitotic index and percentage of chromosomal aberrations were found to be dose-dependent. Erythrocytes treated with the plant extract were observed with morphological alterations like membrane blebs, disrupted membrane and formation of apoptotic bodies. Cytotoxic effect of the plant extract was also evident in HeLa cells and was further confirmed that the effect is brought by inducing apoptosis (Ravindran & Thoppil, 2019).

Table 2: Reports of cytotoxicity analysis of plant extracts using *Allium cepa* assay

Plant	Study	Reference
Amaranthus spinosus	Genotoxicity & antigenotoxicity	Prajitha & Thoppil, 2016a
Inula viscosa	Cytotoxicity & genotoxicity	Çelik & Aslantürk, 2010
Pogostemon auricularius	Cytotoxicity	Anjana & Thoppil, 2013
Amomum pterocarpum	Cytotoxicity & Apoptosis	Sinitha & Thoppil, 2016
Punica granatum	Antiproliferative	Kuhn et al., 2015a
Hyptis suaveolens & *Leucas indica*	Genotoxicity	Sumitha & Thoppil, 2016
Peltodon longipes	Genotoxicity	Kuhn et al., 2015b
Cynanchum sarcomedium	Pro-apoptotic activity	Bhagyanathan & Thoppil, 2016
Clerodendrum viscosum	Cytotoxicity & Micronuclei induction	Kundu & Ray, 2017
Heracleum sosnowskyi	Genotoxicity	Pesnya et al., 2017
Rubus fruticosus, *Vaccinium myrtillus, Potentilla erecta,* *Geum urbanum* and *Phaseolus vulgaris*	Cytotoxicity	Madić et al., 2018
Plinia peruviana	Cytoprotective efficacy	Franscescon et al., 2018

An odorine compound and a bisamide were isolated from the ethyl acetate fraction extracted from *Aglaia odorata* by bioassay-guided fractionation method. Odorine was studied for its effect on *Echinochloa crus-galli* and compared with that of ethyl acetate fraction. Though both the test phytochemicals were able to inhibit seed germination and seedling growth of *E. crus-galli*, ethyl acetate fraction was found to be more potent. Further studies were carried out with the ethyl acetate fraction. The use of ethyl acetate fraction in wettable powder formulation caused the seeds to show lower imbibition and α - amylase activity. The same fraction was determined for its effect on *Allium cepa* root meristem. Mitodepressive activity of the

ethyl acetate fraction was revealed by the dose-dependent decrease in the mitotic index of *Allium cepa* root meristem. The extract could also induce mitotic abnormalities which might be due to the disturbances on chromatin organization and mitotic spindles in the treated roots. The results unveiled the cytotoxic and genotoxic effect of the ethyl acetate fraction of the leaves of *Aglaia odorata* (Teerarak et al., 2012).

Allium cepa assay was conducted to evaluate the cytotoxic and genotoxic effects of aqueous leaf extracts of *Azadirachta indica*. Several chromosomal aberrations were observed in cells of various mitotic stages. Sticky metaphase, C-banding, binucleate cells, lagging chromosomes, anaphase bridges and tripolar anaphase are a few aberrations noted. These anomalies might be the signs of the genotoxic and cytotoxic effects of the plant extract (Akaneme & Amaefule, 2012).

Azadirachtin containing kernel extracts of *Melia azedarach* was subjected to cytogenetic analysis. The kernel extracts are used as the active insect anti-feedant and growth regulators. The cytogenetic studies were done using *the Allium cepa* root meristem assay. The recommended doses for agricultural purposes were used as the test concentrations for the assay. A significant reduction in the mitotic index was observed and that too in a dose-dependent manner. The extract also induced chromosomal aberrations as well as mitotic disturbances including breaks, stickiness, micronuclei, pole deviation, *etc*. The observations revealed the toxic effect of the kernel extracts on the plants as well as the non-target organisms (Özmen & Sümer, 2004).

Liu & Xu (2016) isolated twelve active compounds from the roots of *A. odorata viz*., rocaglaol, rocaglamide, eichlerialactone, sapelins A, isofouquierone, eichlerianic acid, shoreic acid, agladupol E, epimliantriol, cleomiscosins B, pregnene and two methyl pyrrole compounds. Rocaglaol and rocaglamide exhibited maximum cytotoxic activity against human cancer

cells like HL-60, MCF-7, A-549, SW480 and SMMC-7721. All other compounds showed moderate to no cytotoxic effect. This study suggested that the anticancer effect of the plant *A. odorata* can be attributed to the presence of flavaglines and terpenoids.

Cytotoxic effect of *Aglaia roxburghiana* and *Chukrasia tubularis*, plants belonging to Meliaceae, was conducted by Islam et al. (2009). The antitumour effect of various concentrations of methanolic crude extracts was done using brine shrimp lethality assay where vincristine sulfate was used as the standard for reference. Significant cytotoxic activity was observed by both the plants with an LC_{50} of 1.58 μg/mL for *C. tubularis* and 2.21 μg/mL for *A. roxburghiana*.

To study the cytotoxic effect of *A. erythrosperma* against human cancer cells, NCI-H187, KB, and BC cell lines were treated with the leaf ethanolic extract and the IC_{50} values of 2.4, 7.6 and 4.9 mg/mL, respectively were obtained. The extract was also active against *Mycobacterium tuberculosis* with a minimum inhibitory concentration (MIC) value of 3.1 mg/mL as well as against *Plasmodium falciparum* (malarial parasite) showing an IC_{50} value of 2.5 mg/mL. Apart from the leaf extract, ethanolic extract of the seeds also showed cytotoxicity against the cell lines mentioned above as well as the malarial parasite (Phongmaykin et al., 2011).

Aphanamixis polystachya stem extract was screened for anticancer as well as antioxidant activities. Methanolic as well as n-hexane extracts were subjected to radical scavenging assays like nitric oxide scavenging activity, FRAP, and hydroxyl radical scavenging ability. This unveiled the potential scavenging ability of the plant extracts thereby proving the plant as a promising antioxidant. *Allium cepa* assay of the extracts revealed the mitodepressive ability of the plant with petroleum ether, chloroform, and Methanol. SRB assay was done to determine the anticancer activity against

HeLa cells and MCF-7. The extracts were found to be much effective against HeLa cells while moderate activity against MCF-7 (Rani et al., 2014).

Antiplasmodial effect and cytotoxic efficacy of the sesquiterpenoid, kurubasch aldehyde isolated from *Trichilia emetica*, belonging to Meliaceae (order Sapindales), was reported by Traore et al. (2007). The sesquiterpenoid was isolated as free alcohol consisting of a hydroxylated humulene skeleton. Some of the related compounds have been previously reported from plants belonging to Apiales. This is the first report of humulenes in the genus *Trichilia*. The compound showed modest growth inhibition against *Plasmodium falciparum* with an IC_{50} of 76 mM. Cytotoxic effect was studied on MCF-7 cells as well as on S180 cancer cells, which revealed a potent inhibitory effect of the isolate.

Meliaceae plants are rich sources of limonoids and a number of them have been isolated from several plants. Five limonoids isolated from the seeds of *Chisocheton siamensis* were reported to have antimalarial, antimicrobial and cytotoxic activities. All of the five limonoids showed toxicity against *Plasmodium falciparum*, thereby revealing the antimalarial ability. Azadiradione is the only limonoid that exhibited a strong mycobacterial effect. Four limonoids exhibited significant cytotoxic activity against NCI-H187, KB and MCF-7 cell lines (Maneerat et al., 2008).

Another study on the cytotoxic effect of limonoids along with the anti-inflammatory screening was reported by Dzoyem et al. (2015). Three limonoids: trichilia lactone D5, rohituka3, and dregeanin DM4, was isolated from *Trichilia welwitschii* and tested for nitric oxide inhibition and acetylcholine esterase inhibitory activity. Limonoids showed considerable inhibition of both inflammatory responses when compared to that of the standard galantamine. The compound showed very low toxicity against Vero cells but showed significant inhibition or antiproliferative activity against

RAW264.7 cancer cells. The study proved that the isolated limonoid possesses anti-inflammatory potential along with cytotoxicity against cancer cells but no toxicity against normal cells. Three new triterpenoids, aglaiaglabretols A-C, along with nine known compounds like stigmasterol, rocaglaol, epicotillol, scopoletin, *etc*., were isolated from *A. crassinervia.* Chloroform soluble partition of methanolic bark extract was subjected to activity-guided fractionation to isolate the compounds. Spectroscopic evaluation and chemical methods were used to elucidate the structure of the new compounds and were confirmed by single-crystal X-ray studies. The isolates were subjected to cytotoxic studies and the known cyclopenta benzofuran and rocaglaol, showed significant activity comparable to that of positive controls used in the study, paclitaxel, and camptothecin. Further testing of the activity of aglaiaglabretol B was done using the *in vivo* hollow fiber model (Su et al., 2006).

(v) Apoptotic studies

Researches are focusing on plant derived products to develop therapeutic agents against rapidly proliferating cells. Mostly studies are focused on bringing cell death *via* apoptosis so that the cellular contents could be eliminated from the system without spillage. Two major pathways involved in apoptosis are intrinsic and extrinsic (Fig. 2). Each pathway is coordinated by several apoptotic factors including caspases and cytochromes. Several reports are available on the studies targeting on the effect of plant extracts and plant derived proteins on these apoptotic factors. Recent studies on the plants belonging to Meliaceae are as follows:

Favaglines or flavonols are cinnamate derived cyclopentabenzofurans which naturally occurs only in the genus *Aglaia*. This has been reported to be a potent antileukemic agent. The most potent one reported so far is aglaiastatin. The antiproliferative effect of the compound against colorectal

tumor cells was evident from the apoptotic death of SW480 and HT29/HI1 carcinoma cells. An interesting outcome was that apoptosis was induced at nanomolar concentrations of the compound. Meanwhile, the premalignant adenoma cells VACO235 and LT97 as well as normal intestinal cells were 1000 times less sensitive to aglaiastatin. A decreased level of cyclin A, an increase in cyclin B along with the formation of characteristic apoptotic morphology of apoptotic nuclei, down-regulation of Bcl proteins and decreased MMP was observed in the treated cells, thereby indicating apoptotic death (Hausott et al., 2004).

The tumor that arises from the epithelium of nasopharynx, called nasopharyngeal carcinoma (NPC) is usually treated using chemotherapeutic drugs like cisplatin and 5- fluorouracil. To discover a natural alternative therapeutic solution, extensive studies on the same are going on. Silvestrol and epi-silvestrol are the compounds isolated from *A. stellatopilosa* and these compounds were tested for their ability to inhibit cell proliferation in human NPC. The monitoring of the cells was done using real-time, impedance-based cell analyzer and colorimeter. Flow cytometry analysis was done to detect apoptosis and no effect was found in the cells treated with silvestrol and epi-silvestrol alone, while an arrest at the G2/M phase was observed. The effect of the combined therapy of isolates and the standard drugs was examined and a tremendous effect was observed. A significant rise in the number of apoptotic cells, as well as arrest in cell cycle stages, was obtained. Several fold enhanced antitumor effect of the compounds is evident in a combined form (Daker et al., 2016).

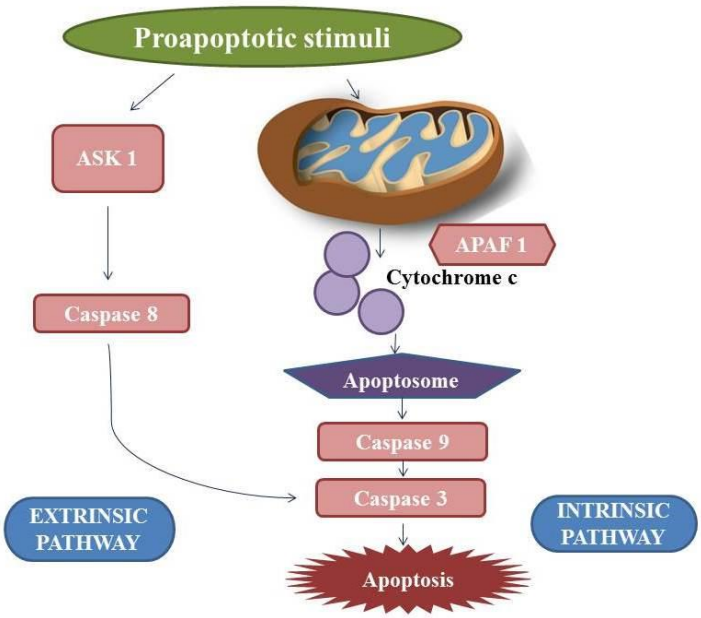

Fig. 2: Apoptotic pathways

Betulonic acid isolated from the bark of *Walsura pinnata*, belonging to the family Meliaceae, was investigated for the pro-apoptotic effects on Leukemia stem cells (LSC) in *in vivo* and *in vitro* systems. Betulonic acid is a terpenoid with a pentacyclic lupine group and was reported for exhibiting cytotoxic effects against various cancer cell lines. The ability to induce apoptosis by intrinsic mechanism in solid tumors was also evident from several studies. Magnetic sorting was the method employed in the isolation of high purity LSC from the Kusumi-1 cell line. The cells were treated with various concentrations of betulonic acid for various time intervals. Treated cells were characterized by flow cytometer and viability was tested using the MTT assay and was found to be directly dependent on dose and time. MethoCult® H4435 enriched media was used for growing the cells and colony formation was assessed. A significant inhibition against the formation of colonies by LSC was observed. Detection of apoptosis was carried out by Annexin-V and propidium iodide double staining. JC-1 staining was done to determine mitochondrial transmembrane potential and the same was found to

drop significantly. RT-PCR was carried out to determine the expression level of apoptotic genes *viz*., BAX, Bcl-2 and surviving. Apoptosis was triggered by betulonic acid through the up-regulation of BAX gene and suppression of Bcl-2 and survivin genes. Caspase 9 and 3/7 activities were also monitored and were observed that the former gets activated leading to the further activation of downstream latter caspases. LSC was xenoplanted to zebrafish and the antileukemia activity was analyzed by TUNEL assay. The formation of leukemia colonies and proliferation in the *in vivo* model was found to get suppressed by triggered apoptotic effect by butelonic acid treatment. The study suggests the development of chemotherapeutic drugs from butelonic acid against leukemia (Leong et al., 2017).

Compounds of *Toona sinensis* were extracted in different solvents and were tested for anti-cancer activity on ovarian cells. SKOV3 cells were separately treated with the extracts and viability was determined using MTT assay and morphological analyses were done using flow cytometry and immunoblotting. The aqueous extract was found to be the most active extract and the cell cycle arrest was found at the G2/M phase leading to apoptosis. The cells were inoculated as a xenograft in nude mice and were observed to suppress the proliferation of ovarian cancer cells without much toxicity against neuro-, hepatic or bone marrow cells (Chang et al., 2006).

Chen et al. (2014) reported the antiproliferative effect of *Toona sinensis* on two clear cell types of renal cell carcinoma. The toxicity effect was found to be dose and time-dependent. Apoptotic induction in the cells was facilitated with the accumulation of reactive oxygen species and a reduction in the mitochondrial membrane potential. The treated cells also showed increased expression of genes coding for the apoptotic proteins like caspase-3,-7, -9 and PARP. Release of cytochromes from mitochondria and downregulation of Bcl-2 and heat shock proteins was also observed. The

study suggests that the extract might be inducing apoptosis by enabling the mitochondria-mediated apoptotic pathway.

The antioxidant ability of *Toona sinensis* has been reported by several researchers. In this background, the apoptotic efficacy of the same was evaluated by Yang et al. (2006). Apoptosis-inducing ability of various concentrations of aqueous extract of *T. sinensis* as well as gallic acid isolated from the extract was tested against promyelocytic leukemia LH-60 cells. The effect was found to be dose and time-dependent and apoptosis was marked by the loss of cell viability as well as internucleosomal DNA degradation. Moreover, the release of cytochrome c, caspase 3 activation and proteolytic cleavage of PARP was also facilitated. On increasing the concentration of the extract, reduction in the level of Bcl-2 and an increase in Bax protein were also observed. A notable part of the research was that the extract and gallic acid induced the generation of ROS in the cells in a dose-dependent manner. All these factors lead to apoptosis thereby leading to antiproliferation of HL-60 cells. The extract could be a valuable anticancer agent and may be used in food and drug products.

Another compound that was reported to exhibit an apoptotic effect on HL-60 cells is 12-*O*-Acetylazedarachin B which is isolated from the fruits of *Melia azedarach*. It also shows a potent antiproliferative effect against stomach cancer cells, AZ521. A phospholipid called phosphatidylserine is exposed on the apoptotic cells that provide a signal for phagocytosis. The cells treated with the isolate were detected by the observation of phospholipid exposure on their membrane along with the fragmentation of genetic material. Both these are the signs of proapoptotic cells and these were analyzed using flow cytometer. Apoptotic genes like procaspase-3, -8 and -9 were found to drop considerably while caspase-3, -8 and -9 were elevated which was revealed by Western blot. Accordingly, a prominent increase was noted in the

Bax/Bcl-2 ratio. Apoptotic death thereby is brought in Hl-60 by both mitochondrial and death receptor-mediated pathways. Flow cytometric analyses showed that cell death is predominantly due to apoptosis, though necrosis was also induced in a considerably low quantity. The study suggests that the compound might be a promising lead to the development of an effective antiproliferative drug against HL-60 cells (Kikuchi et al., 2013).

Toosendanin is a triterpenoid compound isolated from *Melia toosendan* which has been reported for pharmacological and agricultural applications. Anticancer studies revealed the ability of the compound to induce apoptosis in human hepatocellular carcinoma cells. Induction of apoptosis was confirmed to be *via* a mitochondria-dependent apoptotic pathway and this was inferred from the results obtained from morphological observations, annexin V staining, detection of caspase activity and the gene level expression studies of BAX, Bcl and Fas proteins. The same was also demonstrated *in vivo* using the rat model (He et al., 2010).

Limonoids like azadirachtin and nimbolide isolated from *Azadirachta indica* was reported to have a potential antiproliferative effect against HBP carcinogenesis. Cell death was confirmed to be due to apoptosis enabled *via* both intrinsic as well as extrinsic apoptotic pathways. Expression level of genes coding for PCNA, p21waf1, cyclin D1, glutathione S-transferase pi GST-P, NF-κB, p53, Fas, Bcl-2, Bax, Bid, Apaf-1, cytochrome C, survivin, caspases-3, −6, −8 and −9, and PARP was studied along with immunoblotting and cell cycle analyses (Kumar et al., 2010a).

A rare phytosterol 7α-hydroxy-β-sitosterol isolated from *Chisocheton tomentosus* was found to be cytotoxic against three different human cancer cell lines with minimum toxicity to normal cells. Apoptosis was induced by down-regulating of ERK1/2 signaling pathway. It also elevated BAX protein levels and suppressing Bcl-2 protein thereby triggering the intrinsic apoptotic

pathway. Reduced levels of pro-caspases and rise in the level of caspases indicated that the mitochondrial pathway enabled the apoptotic effect of the isolate on the cancer cells. The study suggests the compound as a potential antiproliferative agent against breast adenocarcinoma cells (Tasyriq et al., 2012).

Gunadharini et al. (2011) reported that the antiproliferative or apoptotic effect of ethanolic leaf extract of neem on PC-3 and LNCaP cells is caused by inhibiting PI3K/Akt pathway. mRNA expression studies, Western blot, and gene expression studies were done to confirm the same. Immunoblotting of cyclin D1 and p21 unveiled the inhibition pathways of cell proliferation.

(iv) Other bioactivities

Extensive studies on several species of *Aglaia* are going on and several bioactivities have been reported so far. Recent reports on the important biological potential of *Aglaia* spp. are listed below.

The anti-inflammatory potential of the compounds isolated from leaves of *A. odorata* was studied by Yodsaoue et. al. (2012). Dolabellane diterpenoids, dammarane diterpenoids, and protostane triterpenoids along with 20 known compounds were isolated from the leaves and the structural elucidation was done using extensive spectroscopic analysis and analyzing previous NMR reports. All the compounds were evaluated for anti-inflammatory activity by measuring the activity against lipopolysaccharide-induced nitric oxide (NO) production in RAW264.7 cells. 11 compounds were found to show potent NO inhibitory activity with IC50 values even better than the positive control (indomethacin). This study confirms the anti-inflammatory effect of the widely used ornamental, medicinal tree *A. odorata.*

Isolates from *Aglaia* sp. were screened for antiviral activity by evaluating the inhibition of early steps of the lentiviral replication cycle. Potent inhibition of HIV-1 infection was exhibited by a 3, 4 - secodammarane triterpenoid, ignT1 with an IC50 of 0.48 μg/mL. Inhibition of cell proliferation and cytotoxic potential was evaluated for which the activity was observed for the concentration above 10.69 μg/mL. Vector-based antiviral studies of dammarenolic acid showed potent inhibition of *in vitro* replication of retroviruses including Simian immune deficiency virus and Murine leukemia virus while, the methyl ester analog of dammarenolic acid - methyldammarenolate- did not affect HIV-1. Cell cycle arrest at S and G2/M phase in ignT1 treated HeLa cells was identified by cell cycle analysis and these results suggest dammarenolic acid as a promising compound for anti-retroviral drug development (Esimone et al., 2010).

A new compound was isolated from the bark of *A. abbreviata* and was named as 2,3-seco-12-oleanene-2,3-dioic acid. Along with this, 10 known compounds were also isolated. The new compound was tested for cytotoxicity and anti-inflammatory potential. Cytotoxicity was tested against human leukemia cells (K562), hepatocellular carcinoma cells (SMMC-7721), breast cancer cells (MCF-7) and oral epithelium cancer (KB) along with multi-drug resistant cells of MCF-7/ADM and KB/VCR and a significant activity was observed. Promising results were also obtained in the anti-inflammatory assay done using the isolates in lipopolysaccharide-stimulated RAW264.7 cells. The new compound showed the highest anti-inflammatory activity with a maximum inhibition of nitrite production in the RAM macrophages (Zhang et al., 2016).

D. BIOGENIC SYNTHESIS OF NANOMATERIALS

Nanoparticles are the ones that have a size of about 1 to 100 nm (1 nm = 10^{-9} m). The particles exist in a colloidal state when in a liquid phase rather

than a solution. They show enormous variation in their basic properties when compared with that of the parent element/compound. Nanoparticles of elemental silver (Ag), gold (Au), copper (Cu), carbon (C), *etc*., are gaining great interest among researchers due to their potential activities in biological as well as industrial fields. In the medicinal ground, nanomaterials are attaining curiosity for drug delivery, activity enhancement of medicines as well as their ease to handle. Eco-friendly, energy-efficient, non-toxic and cost-effective novel methods for the production of nanomaterials are the next stage research interest and various reports are available for the same. Recent reports on green synthesis of nanomaterials using plants and their characterization are enlisted (Table 3).

A. elaeagnoidea is an underexplored plant distributed in the Western Ghats. The flower extracts of the same were used for the biogenic synthesis of Ag and Au nanoparticles. These were characterized by UV-visible spec., XRD analysis, EDAX, and TEM imaging. Resultant nanoparticles were robust and free from agglomeration, crystalline and spherical. Rapid production of nanoparticles and quick degradation of methylene blue and congo red were a notable part of this research. Conversion of 4-nitrophenol to 4-aminophenol was obtained within a few minutes in the homogenous method while the same was obtained within a few seconds in the heterogeneous method. These promising results suggest the application of the biosynthesized nanoparticles as a catalyst in wastewater treatment plants (Manjari et al., 2017a).

Biologically synthesized silver nanoparticles using the aqueous leaf extract of *A. elaeagnoidea* was tested for its larvicidal activity against mosquito larvae. The synthesized particles were subjected to characterization using spectrophotometric techniques along with TEM imaging. The nanoparticles were found to be toxic against larvae of mosquito vectors

like *Anopheles stephansi, Aedes aegypti* and *Culex uinquefasciatus*. The ease in production, greater stability over long time storage, cost-effectiveness along with negligible toxicity against non-target vectors encourages the application of these nanoparticles as a larvicide. Moreover, the particles showed effective larvicidal activity even at very low doses with a significant decline in the malarial and arboviral mosquito larvae and that too without affecting the natural predatory organisms like fishes, water bugs and backswimmers (Benelli et al., 2018).

Biosynthesized copper oxide nanoparticles were generated and characterized by Manjari et al. (2017b). The authors reported this as highly active, recoverable and recyclable nanocatalyst that could be used in the wastewater treatment. The CuO nanoparticles are nontoxic catalysts that could act as stabilizing and reducing agents. The characterization of the nanoparticles performed using FESEM, TEM, XRD, EDX, and TGA-DSC analyses revealed 20-45 nm-sized stable, agglomeration free particles. The aqueous suspension of nanoparticles showed a high catalytic property for the reduction of congo red, methylene blue, and 4-nitrophenol. Easy recovery and reuse for 6 cycles were also confirmed thereby enabling cost-effective, eco-friendly reduction of dyes and organic pollutants in waste-water treatment.

A novel study on the preparation of nanoparticles in the form of quantum dots using plant extract was reported by Mariselvam et al. (2014a). Aqueous extracts of the roots of *Rubia cardifolia* was used to synthesize copper nanoparticles in the form of quantum dots. These particles were further characterized by SEM, TEM, AFM, UV-Vis spectrophotometer and fluorescence microscopy. Analyses revealed the spherical shape and rough surface of the particles and their size was determined as 22.68 nm. The particles also revealed a green fluorescent nature. When subjected to

antibacterial assay, the quantum dots showed significant activity against strains like *E. coli*, *Klebsiella*, *Streptococcus*, *Pseudomonas*, *etc*.

Table 3: Reports of phytogenic synthesis of nanoparticles

Plant	Nanoparticle component	Activities & Application	References
Cola nitida	Silver	Antibacterial and antioxidant activities Antimicrobial additive in paints	Lateef et al., 2016
Cochlospermum religiosum	Gold	Heterogeneous catalyst Waste water treatment	Maity et al., 2012
Cocos nucifera	Silver	Antibacterial activity against human pathogens	Mariselvam et al., 2014b
Camellia sinensis	Silver	Compatible in human cell systems	Moulton et al., 2010
Mimusops elengi	Silver	Antibacterial against multidrug resistant human pathogens	Prakash et al., 2013a
Ziziphus oenoplia	Silver	Antibacterial activity	Soman & Ray, 2016
Magnolia kobus	Gold	Medicine, food and cosmetics	Song et al., 2009
Diopyros kaki	Gold	Products that comes in direct contact with human body	Song et al., 2009
Morinda citrifolia	Gold	Anticancer and anti-diabetic activities	Suman et al., 2014
Saraca indica	Silver	Catalytic activity	Vidhu & Philip, 2014
Acacia farnesiana	Silver	Antibacterial and antioxidant activities	Yallappa et al., 2013a
Terminalia arjuna	Copper	Antimicrobial and antioxidant activities	Yallappa et al., 2013b

The literatures suggest that Meliaceae is the family comprising of plants with high potential for various bioactivities and these could be attributed to the presence of certain unique class of compounds found as their phytochemical constituents. It strengthens the necessity for conducting more studies on further exploration of the phytochemical components, isolation of the active components, screening of bioactivities and elucidating the pathway of beneficial effects of the underexplored plants in the family.

MATERIALS AND METHODS

Aglaia is an important tree genus from the family Meliaceae which enjoys worldwide distribution. Almost 12 species are found in the Western Ghats and the present study targets on the 3 taxa namely, *A. barberi*, *A. simplicifolia* and *A. malabarica*. The present study comprises of 3 major phases:

Phase I: Phytochemical analysis

Phase II: Bioactivity screening

Phase III: Biogenic/green synthesis of silver nanoparticles.

Phase I deals with the qualitative analysis, quantitative estimation and GC/MS analysis of the plant extracts. The second phase includes the antioxidant potential screening, hepatoprotective ability analysis, cytotoxic potential and detailed anticancer studies. The final phase is about the ability of the plant extract to reduce the molecular silver into elemental nanoparticles. The studies were carried out at the Department of Botany, University of Calicut, Kerala, India; Biogenix Research Center, Thiruvananthapuram; National Institute of Technology, Calicut, Kerala and Rajiv Gandhi Center for Biotechnology, Thiruvananthapuram, Kerala, India.

A. PLANT MATERIALS

a. Collection and authentication:

The selected species of *Aglaia* used for the study were *A. barberi*, *A. simplicifolia* and *A. malabarica*. These trees are endemic to the Western Ghats and were collected from various regions of Kerala which include Wayanad, Thrissur and Idukki districts. The collected plants were authenticated by Dr N. Sasidharan, Emeritus Scientist, Kerala Forest Research

Institute, Thrissur, Kerala and Dr A. K. Pradeep, Angiosperm Taxonomy Division, Department of Botany, University of Calicut, Kerala. Voucher specimens were deposited at the Herbarium (CALI), Department of Botany, University of Calicut, Kerala, India.

b. Taxonomic description:

1. *Aglaia barberi* Gamble

Habit: Tree, slightly buttressed

Habitat: Evergreen forests and semi - evergreen forests

Flowering & Fruiting: December - June

Distribution: Western Ghats (Endemic)

Description: Trees with reddish brown bark. Leaves are imparipinnate, exstipulate, and alternate; leaflets are 7, sub-opposite, lanceolate, acutely oblique, acuminate at apex with approximately 12 lateral nerves, almost at right angles to the mid-nerve, lepidote, obscure reticulation. Inflorescence is axillary, panicle with cymose branching, lepidote with minute bracts. Flowers are arranged close together. Sepals are shortly ciliate. Petals are oblong-ovate, glabrous, staminal tube globose, undulate at apex and not contracted at base. Stamens are long as a tube that included anthers. Ovary is villous, short styled with capitate stigma. Berries are globose, depressed at apex with arillated seeds.

2. *Aglaia simplicifolia* (Bedd.) Harms

Habit: Tree

Habitat: Evergreen forests

Flowering & Fruiting: November - June

Distribution: Indo- Malayan region, Western Ghats, Philippines and Thailand

Description: Small tree, twigs greyish-brown, with stellate hairs. Leaves are simple, alternate, 15 to 30 cm long and 4 to 8 cm wide acuminate or caudate at the apex, cuneate and slightly asymmetrical at the base; reticulation sub-prominent on the dorsal surface; petiole up to 4 cm. Inflorescence is up to 15 cm long. Flowers are sub-globose, pedicel up to 2mm, calyx deeply divided into 5 sub-rotund lobes densely covered with stellate scales. Petals are yellow and obovate with quinquintial aestivation. Staminal tube is as long as the petals, obovoid with a small aperture, anthers half the length of the tube and broadly ovoid. Ovary is sub-globose, densely covered with stellate scales; stigma ovoid with two apical lobes; ovary and stigma together. Fruits are up to 4 cm long, obovoid or sub-globose, brown, indehiscent with a thick woody pericarp up to 5 mm thick and densely covered with stellate hairs on the outside; pericarp is longitudinally ridged; internally it is unilocular containing one seed along with gelatinous and transparent aril.

3. *Aglaia malabarica* Sasidh.

Habit: Tree

Habitat: Evergreen forests

Flowering & Fruiting: December - July

Distribution: Western Ghats (Endemic)

Description: Trees grow up to 25 m, bark smooth and brown; young shoots are golden brown covered with dense peltate scales. Leaves are imparipinnate, alternate; rachis is long, stout and covered with peltate scales, swollen at the base and grooved above; leaflets are alternate, 7-11, petiole covered with scales, lamina oblong with oblique base and acuminate apex, entire margin

having upper surface pitted with peltate scales and lower surface with numerous peltate scales on the mid rib as well as lateral ribs. Flowers are polygamodioecious and yellow in colour. Male inflorescence densely covered with peltate scales; petals 5, stamens 5, staminal tube 2 mm long, anthers are included and 5 in number. Female inflorescence covered with reddish brown peltate scales; calyx is 5 lobed which is thick and fleshy at the base and densely scaly, corolla with 5 petals, included staminodes 5 in number, ovary superior and depressed globose with reddish brown scales, 3 celled ovules in each cell; stigma is having an apical depression and is sub-globose. Fruit is a capsule with an apical depression; pericarp is having longitudinal ridges and dense peltate scales; seeds completely surrounded by a pink translucent aril.

B. METHODS

Preparation of methanolic extract

The aerial parts of the plants were collected and washed thoroughly to remove the dirt and other contaminants. Leaves were excised from the washed twigs, shade dried, powdered using an electric blender and stored in a sealed air tight container. Methanolic extract was prepared from each plant. 10 g of the powdered material was extracted in 100 ml absolute methanol using Soxhlet apparatus for 6 h. Extract thus obtained was cooled, filtered and evaporated to dryness under vacuum. This dried extract was stored at 4 ^{o}C in air tight glass vials for further experimental analyses.

PHASE I – PHYTOCHEMICAL ANALYSES

In order to determine the phytoconstituents in a broad approach, qualitative phytochemical analyses were done. Some of the important secondary metabolites like terpenoids, flavonoids and phenols were quantitatively estimated. GC/MS analysis was done to determine the volatile components present in the extracts.

(i) Qualitative phytochemical screening

Screening of the various secondary metabolites was done to obtain an overall idea of chemical composition of the plants. Preliminary screening paves the way for further studies including quantitative estimation, separation of bioactive chemical constituents and compound isolation. The analyses were carried out using modified protocols (Edeoga et al, 2005; Harborne, 1998; Trease and Evans, 1989).

1. Test for saponins

Foam test:

1 mL of 1 g/10mL aqueous extract of the leaves was taken in a test tube, diluted 20 times and vigorously shaken for a few minutes. This was kept for a while after the formation of foam on the top layer. The retention of the foam for 15 min and more indicates the presence of saponins.

2. Test for quinones

Borntrager's test:

In a test tube, 1 mL of the extract was taken along with 1 mL of 10% ferric chloride and 1 mL of conc. HCl. The mixture was heated, then gradually cooled and filtered. To the filtrate, equal amount of diethyl ether

was added and shaken. The ethyl extract obtained was exposed to ammonia and the development of pinkish or red colour in the aqueous layer reveals the presence of quinones.

3. Test for phenols

Spot test:

A filter paper was spotted with plant extract followed by the addition of few drops of phosphomolybdic acid, on the same spot. This was exposed to ammonia vapour and the presence of phenol was indicated by the formation of blue colouration.

Ferric chloride test:

1 mL of the plant extract was treated with 5% aqueous ferric chloride solution. Presence of phenol was indicated by the formation of deep blue or black colour or precipitate.

4. Test for flavonoids

Alkaline reagent test:

2 mL of plant extract was taken to which 1 mL of 20% sodium hydroxide (aqueous) solution was added. Intense yellow colour formation which becomes colourless on the addition of dilute hydrochloric acid indicates the presence of flavonoids.

Lead acetate test:

The plant extract was treated with a few drops of 10% lead acetate (aqueous). The formation of yellow/white precipitate or colour indicates the presence of flavonoids.

5. Test for terpenoids

Salkowski test:

5 mL of the plant extract was taken in a test tube. To this, 2 mL of chloroform was added and shaken well. 3 mL of concentrated sulphuric acid was added to this mixture very carefully along the sides of the test tube, so as to form a separate layer. The presence of terpenoids was indicated by the formation of reddish brown colour at the interface of the solutions.

Acetate test:

Powdered plant material was shaken well along with chloroform, filtered and used as the sample extract. 2 mL of this extract was taken in a test tube, to which 1 mL of acetic anhydride was added, followed by 1 mL of concentrated sulphuric acid. The presence of terpenoids was confirmed by the formation of reddish blue or violet colour formation.

6. Test for alkaloids

Hager's test:

Few drops of Hager's reagent were added to 1 mL methanolic extract taken in a test tube. The formation of yellow precipitate reveals the presence of alkaloids.

Wagner's test:

2 mL of the methanolic plant extract was taken in a test tube to which Wagner's reagent was mixed well. Formation of reddish brown precipitate or colour indicates the presence of alkaloid.

Mayer's test:

A portion of the methanolic extract was treated with 2 mL of 1% HCl followed by gentle heating. This mixture was then treated with Mayer's reagent. The presence of alkaloids was identified by turbidity formation.

7. Test for steroids

Double layer test:

To 1 mL of methanolic extract, 10 mL chloroform was added. This was followed by the addition of equal volume of concentrated sulphuric acid along the sides of the test tube. Two separate layers are obtained, in which upper layer turns red and lower one turns yellow with green fluorescence, if steroids are present.

Liebermann-Burchard test:

To 1 mL of methanolic extract, few drops of chloroform, acetic anhydride and concentrated sulphuric acid were added and observed. The formation of dark pink or red colour indicates the presence of steroids.

8. Test for tannins

Braymer's test:

A portion of the sample was treated with 10% alcoholic ferric chloride solution. The development of blue or greenish colouration indicates the presence of tannins in the samples.

9. Test for carotenoids

Interfacial ring test:

1 g of the dried sample was extracted with 10 mL chloroform by vigorous shaking in a test tube. This was filtered and 85% sulphuric acid was

added to the filtrate along the sides of the test tube. Development of blue colour in the interface of the two solutions indicates the presence of carotenoids.

10. Test for cardiac glycosides

Keller Kiliani's test:

To 5 mL of methanolic plant extract, 2 mL of glacial acetic acid was added followed by a drop of ferric chloride solution. To this mixture concentrated sulphuric acid was added through the sides of the test tube. Formation of a brown ring at the interface of the solutions indicates the presence of cardenolides.

(ii) Quantitative phytochemical estimation

Quantitative estimation of some of the important secondary metabolites like phenols, flavonoids and terpenoids were done using standard protocols as mentioned below:

1. Estimation of total phenolic content:

Modified protocol of Oueslati et al. (2012) was used to determine the total phenolic content in each plant extract. The prime reagent used was Folin - Ciocalteu reagent (FC). Gallic acid was used as the standard and the samples taken were 1 mg/10 mL of methanolic leaf extracts. Analyses of the samples were done in triplicates. The sample was shaken well along with 0.5 mL of distilled water and 0.125 mL of 1N FC reagent. This mixture was incubated for 5 minutes. 1.25 mL of 7% Na_2CO_3 was added and then the final volume was made up to 3 mL using distilled water. The mixture was made uniform by proper shaking and then incubated in the dark for 90 min. Absorbance of the standard as well as the samples, against the blank, were measured at 760 nm. Calibration curve of the standard was plotted and the total phenolic content in

the samples were expressed as milligrams of gallic acid equivalent per gram of dry weight of the sample (mg GAE/g DW) using the derived regression equation.

2. Estimation of total terpenoid content:

For the estimation of the total terpenoid content of the plant extracts, method according to Ghorai et al. (2012) was used. Linalool was used as the standard for reference. 1 mL of the sample (1 mg/ 10 mL methanolic extract) was treated with 1 mL of chloroform and 1 mL of concentrated sulphuric acid. The absorbance was measured against the blank at 538 nm. Assays of the samples were performed in triplicates. Calibration curve was made using the standard. The total terpenoid content of the samples were measured using the regression equation thus obtained and expressed as milligrams of linalool equivalent per gram of dry weight of the sample (mg LE/g DW).

3. Estimation of total flavonoid content:

Method described by Chang et al. (2002) was used to determine the total flavonoid content in the plants. Quercetin was used as the standard. The samples used were 1 mg/10 mL methanolic plant extracts. An aliquot of standard/sample was treated with 75 µL of 7% $NaNO_2$ and kept for 6 min. To this, 0.15 mL of 10% $AlCl_3$ was added followed by incubation for 5 min. After incubation, 1 M NaOH was added and the final volume was made up to 3 mL. Absorbance was read at 510 nm against the blank. Calibration curve of the standard was plotted from which regression equation was obtained. Total flavonoid content in the samples were calculated using the regression equation and were expressed as milligrams of quercetin equivalent per gram of dry weight of the sample (mg QE/g DW). Assays were performed in triplicates of the sample.

(iii) Gas chromatography/mass spectrometry (GC/MS) analysis

Gas chromatography is an extensively used technique to study the phytochemical constitution of the plant extracts in detail. Detection of the specific compounds in the extracts can also be determined using the technique. Even the trace amounts of the volatile bioactive compounds can be detected using the technique when coupled with mass spectrometry (GC/MS).

All the three taxa used in the present study were subjected to GC/MS analysis. Identification and quantification of the volatile components in the methanolic plant extracts were carried out by the same. Varian model CP-3800 GC interfaced with Varian Saturn 2200 Ion Trap Spectrometer (ITSM) was used for GC analysis. This operates at 70eV and 250 ^{o}C which are equipped with a CP-1177 Split/Splitless capillary injector as well as Combi PAL auto sampler. Capillary column was equipped with a cross linked factor, VF 5ms having 30 m × 0.25 mm ID and 0.25 µm film thickness. Helium gas at a flow rate of 1 mL/min was used as the carrier or vehicle while the injection volume of the sample was 1 µL at a split ratio of 1:20. The total run time was programmed for 60 min where the initial temperature was set as 60 ^{o}C for 1 min followed by a gradual rise at a rate of 3 ^{o}C/min up to 280 ^{o}C. Constituents got resolved according to their retention time. Comparison of their linear retention indices was done and identification of the individual components was carried out by matching the fragmentation patterns with NIST-MS (National Institute of Standards and Technology and Mass Spectra) libraries. Peak area percentage of each isolated constituents were calculated in order to quantify the same.

PHASE II – BIOACTIVITY SCREENING

A. ANTIOXIDANT ABILITY

Antioxidant potential of the plant extracts was screened in order to determine its ability to scavenge free radicals in a system. Methanolic plant extracts of all the three species of *Aglaia* were used for the experiments. Four different assays were performed using various concentrations of each plant extract *viz*., 125, 250, 500, 1000 and 2000 µg/mL. The scavenging potential of the extracts were analyzed in triplicates in all the four assays. IC_{50} values were determined using ED50 PLUS V1.0 software.

(i) DPPH radical scavenging assay

Assessment of the radical scavenging activity by DPPH method was done using the method described by Chang et al (2001). DPPH is a stable free radical which is pink in color in the solution, and rapidly turns yellow if it encounters a scavenging agent. This ability was detected by the decrease in the absorbance spectrum which was read at 517 nm. 10 mg/mL stock solutions of the samples were prepared. Experiments were carried out in amber coloured vials. Various volumes of the sample (12.5, 25, 50, 100 and 200 µL) were treated with 1.5 µL of 0.1mM DPPH. The final volume was made up to 3 mL using DMSO. The solution without the test sample was taken as the control. The mixture was incubated in the dark for 20 min. Absorbance was read at 517 nm after incubation against the reference solution. Ascorbic acid was used as the standard. Assays were performed in triplicates. Percentage inhibition was determined using the following equation:

$$\% \text{ Inhibition } = \frac{\text{Ac} - \text{At}}{\text{Ac}} \times 100$$

Where, Ac is the absorbance of control and At is the absorbance of the test sample.

(ii) ABTS radical scavenging assay

Free radicals generated by the oxidation of ABTS by potassium persulphate, a blue chromogen, get reduced by the hydrogen releasing antioxidants. The scavenging ability of all the three plant extracts was analyzed by this assay according to the method described by Re et al. (1999). Reagent was prepared by adding 0.3 mL of 17 mM potassium persulphate to 50 mL of 20 mM aqueous ABTS solution. The mixture was kept overnight under room temperature. Various concentrations (125, 250, 500, 1000 and 2000 µg) of the plant extract was prepared from a stock of 10 mg/mL. To 0.45 mL of the test sample, 0.35 mL reagent was added. Final volume was made up to 3 mL. The same mixture was taken as the control without the test sample, but an equivalent amount of distilled water. The mixture was incubated for 20 min under room temperature. Ascorbic acid was used as the standard. Absorbance was measured at 734 nm. Triplicates of the samples were analyzed. Percentage inhibition was obtained by comparing the absorbance values of the samples with that of the control as mentioned below:

$$\% \text{ Inhibition } = \frac{\text{Ac} - \text{At}}{\text{Ac}} \times 100$$

Where, Ac is the absorbance of control and At is the absorbance of the test sample.

(iii) Hydroxyl radical scavenging assay

Hydroxyl ions are the free radicals used in the assay which was generated by Fenton reaction from Fe^{3+}/Ascorbate/EDTA/H_2O_2 system. Assays were carried out by using the method according to Kunchandy and Rao (1990). Gallic acid was used as the standard. Antioxidants as well as

deoxy ribose binds with the free radical. Hydroxyl radicals attack deoxy ribose, thereby producing TBA (thiobarbituric acid) reacting substances. Stock solution of the sample made was 10 mg/mL, out of which various concentrations were prepared. The reaction mixture was prepared using deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1.0 mM), ascorbic acid (100 μM) in KH_2PO_4 - KOH buffer (20 mM, pH 7.4) and various concentrations of the sample. The final volume was made up to 1 mL and incubated for 1 h at 37 ^oC. To this, 1 mL of 2.8% TCA and 1 mL of 1% aqueous TBA were added and incubated for another 15 min at 90 ^oC. After cooling, the absorbance values were measured against appropriate blank at 532 nm. Samples were analyzed in triplicates of each concentration. Percentage inhibition was obtained by comparing the absorbance values of the samples with that of the control as mentioned below:

$$\% \text{ Inhibition } = \frac{\text{Ac} - \text{At}}{\text{Ac}} \times 100$$

Where, Ac is the absorbance of control and At is the absorbance of the test sample.

(iv) Superoxide radical scavenging assay

Reduction of nitroblue tetrazolium (NBT) by the superoxide generated in riboflavin - NADH system was assayed to determine the radical scavenging ability of the plant extract. The method described by Valentao et al. (2002) was used for the assay. Ascorbic acid was taken as the standard. Varying concentrations of the samples were prepared. Reagent was prepared by mixing 0.05 mL of 0.12 mM riboflavin, 0.2 mL of 0.1 M EDTA and 0.1 mL of 1.5 mM nitroblue tetrazolium, and diluted up to 2.64 mL using 0.067 M phosphate buffer. To this, 0.36 mL of test sample was added. A control was kept without adding the sample but an equivalent amount of distilled water. The reaction mixture was incubated in fluorescent light for 5 min and

absorbance was measured at 560 nm. Again the reaction mixture was illuminated for 30 min and absorbance was measured. The difference in the absorbance values was taken as the OD. Samples were analyzed in triplicates. Percentage inhibition was obtained by comparing the absorbance values of the samples with that of the control as mentioned below:

$$\% \text{ Inhibition } = \frac{\text{Ac} - \text{At}}{\text{Ac}} \times 100$$

Where, Ac is the absorbance of control and At is the absorbance of the test sample.

B. HEPATOPROTECTIVE ABILITY

The protective ability of all the three species of *Aglaia* was studied using Hep G2 hepatocytes. Various concentrations of each plant extract were used for the experiment. Toxicity was induced in the hepatocytes using 40% alcohol and these cells were subjected to treatment with the various concentrations of plant extracts. The protective ability of the extracts was analyzed by direct microscopic observation as well as by MTT method.

a. Cell culture

Human hepatocellular carcinoma (Hep G2) cells were purchased from National Center for Cell Sciences (NCCS), Pune, India. The cells were cultured in culture flasks in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, sodium bicarbonate and antibiotic solution which contained penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (2.5 µg/mL). Culture medium before adding the supplements, vials, tubes, micropipette tips and cotton were autoclaved, prior to use. Cultured cells were placed in a humidified 5% CO_2 incubator at 37 ^{o}C for 48 h. Monolayer of 48 h confluence was trypsinized and suspended in 10% growth medium to get a homogenous suspension. 100

µL of this suspension was seeded in 96 well plate and incubated under the same conditions as mentioned above.

b. Preparation of the sample

1 mg of the dried methanolic plant extract was weighed and dissolved in 1 mL of 5% DMEM and mixed well using a cyclomixer. The extract solution was filtered using a syringe filter of pore size 0.22 µm in order to sterilize it. The filtered extracts were five times diluted by two fold dilution using 5% DMEM so as to get 100 µg, 50 µg, 25 µg, 12.5 µg and 6.25 µg per 500 µL of 5% DMEM.

c. Induction of cytotoxicity and treatment

To induce cytotoxicity in the cell lines 40% ethyl alcohol was used. After the cells attaining sufficient growth in the 96 well plate, 40% ethyl alcohol was added and incubated for 1 h under 5% humidity in a CO_2 incubator at 37 ^oC.

To the cytotoxicity induced cells, prepared plant extracts of different concentrations were added in triplicates of each in respective wells. This was again incubated under the same conditions. Control was kept without adding the extract but with an equivalent amount of microsieved 5% DMEM.

d. Hepatoprotective activity evaluation

Direct microscopic observation:

Entire plate was observed at an interval of each 24 hours up to 72 hours in an inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera). Aberrations in the morphology of the cells such as cell shrinkage, cytoplasmic or nuclear granulation and cytoplasmic vacuolization were considered as the signs of cytotoxicity.

MTT method:

Live cells convert 3- (4, 5- dimethyl-2-thiazolyl) -2, 5-diphenyl- 2H-tetrazolium bromide (MTT) into a coloured product, formazan. Quantification of this formazan was done to determine the percentage of viable cells.

15 mg of MTT stain was reconstituted in 3 mL PBS and mixed well using a cyclomixture. This was filtered using syringe filter of pore size 0.22 µm prior to use.

After 24 h of incubation period, the supernatant in wells which contained the samples were removed and 30 µL of MTT was added. The plate was gently shaken for a few minutes for proper mixing and incubated for 4 h at 37ºC in CO2 incubator. After the incubation period, the supernatant was removed and 100 µL of DMSO was added in order to dissolve the formazan crystals formed from MTT. The mixture in each well was gently pipetted up and down for the complete dissolution. The absorbance values were measured at 540 nm by using ELISA microplate reader (Talarico et al., 2004). Samples were analyzed in triplicates. Percentage of viable cells was calculated using the equation as follows:

$$\% \text{ viability } = \frac{\text{Mean OD of samples}}{\text{Mean OD of control}} \times 100$$

C. CYTOTOXIC ACTIVITY

Preliminary screening of the cytotoxic potential of all the 3 plant extracts were done using *Allium cepa* assay. This was followed by the *in-situ* visualization of the apoptotic activity induced by the extracts using Evans blue staining method. Cytotoxicity induced in human erythrocytes was also evaluated for all the 3 plant extracts.

(i) *Allium cepa* assay

1 mg/mL stock solution of the methanolic plant extracts were prepared using distilled water. From the stock, various test concentrations were made *viz*., 5, 10, 15, 20 and 30 µg/mL, using distilled water.

Fresh and healthy *Allium cepa* bulbs were collected and washed thoroughly. Outer dried scales were removed carefully. Dried off roots and debris were excised without destroying the root primordia. Washed bulbs were placed for rooting in autoclaved sand for 2 days. The rooted bulbs possessing healthy roots of approximately 2 to 4 cm were used for the experiment. The extracts of various concentrations was filled in glass vials and the bulbs were kept on the rim of the vials so that the roots were immersed completely, but leaving the bulb portion outside the extracts. The bulbs kept in distilled water was taken as the negative control while, those kept in 0.1% malathion served as the positive control. The experimental set up was kept undisturbed for different time periods *viz*., 1, 12 and 24 h. After the specific time periods, the bulbs were collected at the time of peak mitotic activity (almost between 9.10 am and 9.40 am, during normal sunshine condition). Roots from the treated bulbs were excised and fixed in modified Carnoy's fluid for 1 h. Mitotic squash preparation was done according to the modified protocol of Sharma and Sharma (1990). Hydrolysis of the fixed roots were done using 1 N HCl for 3 min. and washed properly in distilled water. These roots were stained using acetocarmine for 3 h followed by destaining in 45% acetic acid. Root tips were mounted and slides were prepared for examination. Cells were observed at 40x light microscope (Leica DM2000) and microphotographs were taken using the top mount camera (Leica DMC2900). The parameters like number of dividing cells, non-dividing cells and aberrant cells were noted. From these, percentage of aberrant cells and mitotic index were calculated as follows:

$$\text{Aberration \%} = \frac{\text{Number of aberrant cells}}{\text{Total number of cells}} \times 100$$

$$\text{Mitotic index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100$$

(ii) *In-situ* visualization of cell death

Evans blue staining technique served the purpose of visualizing cell death in the treated root meristem. The method proposed by Baker and Mock (1994) was followed. Various concentrations of the plant extracts (5, 15 and 30 µg/mL) were used for the study. *Allium cepa* bulbs were kept for rooting in distilled water until it acquires a length of about 5 to 8 cm. Washed bulbs were treated with the various concentrations of the plant extracts for 24 h. Malathion and distilled water were kept as positive and negative control, respectively. After incubation, the bulbs were washed thoroughly and placed in Evans blue solution (0.25% w/v) for 15 min, washed with distilled water and photographed. Equal numbers of roots with almost same length, were excised from the treated and control bulbs. These were kept in 3 mL of *N*, *N* - dimethyl formamide in a test tube for 1 h at room temperature in order to release the stain. Absorbance values of the supernatant after removing the roots were measured at 600 nm.

(iii) Cytotoxicity induction in human erythrocytes

Human blood was collected from Health center, University of Calicut in a vial coated with the anti-coagulant, ethylene diamine tetraacetic acid (EDTA). Modified method of Bhagyanathan and Thoppil (2016) was followed for the assay. Collected blood was centrifuged at 10,000 rpm for 10 min. Blood serum obtained as the supernatant was discarded using a pipette and the residual cells are retained. The cells were repeatedly washed with

normal saline (0.9% NaCl, pH 7.4) for at least 3 times. Cells were stored as suspension in normal saline at 4 ^{o}C for further use.

0.1% (w/v) of all the 3 plant extracts was prepared using distilled water. 500 µL of the blood cell suspension was treated with equal volume of this sample stock for 1 h. Cells incubated without the sample but an equivalent amount of saline solution served as the control for reference. After incubation, the mixture was subjected to a short spin of 1 min and smears of the suspended cells were prepared on glass slides. Slides were air dried, fixed and stained using May-Grünwald-Giemsa method (Junqueira & Carneiro 2004; Maiworm et al., 2008). Microscopic observation was done using 40x objective lens of light microscope (Leica DM2000) and photographs were taken using Leica DMC 2900 camera as top mount.

D. ANTIPROLIFERATIVE ACTIVITY

Antiproliferative activity of all the 3 selected *Aglaia* spp. were carried out using cervical cancer cell line (HeLa). Screening of the cytotoxic activity on the cell lines was done using MTT assay and the most active extract was assayed for confirming apoptotic/necrotic induction ability followed by the quantification of apoptotic/necrotic cells by means of flow cytometry. This was in turn analyzed at gene level by studying the expression of apoptotic/necrotic genes.

(i) MTT method

a. Cell culture

Human cervical cancer cells (HeLa) as well as the normal cells (L929 fibroblast cells) were procured from National Center for Cell Sciences (NCCS), Pune, India. The cells were cultured in 25 cm^2 culture flasks in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal

bovine serum (FBS), L-glutamine, sodium bicarbonate and antibiotic solution which contained penicillin (100 U/mL), streptomycin (100 µg/mL) and amphoteracin B (2.5 µg/mL). The culture medium before adding the supplements, vials, tubes, micropipette tips and cotton were autoclaved before use. Cultured cells were placed in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany) at 37 ^oC for 48 h. The monolayer of 70% confluence was trypsinized and suspended in 10% growth medium to get a homogenous suspension. 100 µL of this suspension was seeded in each well of 96 well plate and incubated under 37^oC in a CO_2 incubator.

b. Preparation of plant extracts

1 mg/mL stock solution of the methanolic plant extract was prepared using PBS and dissolved well using a vortex mixer. From this stock, various concentrations *viz.*, 6.25, 12.5, 25, 50 and 100 µg/mL, were prepared using cell culture medium DMEM and mixed thoroughly by means of a cyclomixer. In order to ensure sterility, the samples were syringe filtered through a pore size of 0.22 µm.

c. Antiproliferative assay

MTT stain was prepared by reconstituting 15 mg of it in 3 mL PBS, dissolved completely and filtered through 0.22 µm syringe filter.

Freshly prepared sample solutions were added into each well in triplicates as the treatment and incubated for 24 h. After incubation, the plates were observed under the phase contrast inverted microscope (Olympus CKX41) for examining the cytological aberrations induced by the plant extract. The microphotographs were captured using the top mount camera (Optika Pro5 CCD camera).

Quantification of cytotoxicity was done by MTT method (Talarico et al., 2004). The supernatant in wells which contained the samples were removed and 30 µl of the reconstituted MTT stain was added. The plate was gently shaken for a few minutes for proper mixing and incubated for another 4 h at 37ºC in 5% humidity CO_2 incubator. After incubation, the supernatant was removed and 100 µL of DMSO (formazan dissolving solvent) was added to mobilize the formazan crystals formed from MTT. The mixture in each well is gently pipetted up and down for the complete dissolution. The absorbance values were measured at 540 nm by using a microplate reader. Samples were analyzed in triplicates. Percentage of viable cells was calculated using the equation as follows:

$$\% \text{ cell viability } = \frac{\text{Mean OD of samples}}{\text{Mean OD of control}} \times 100$$

(ii) Detection of apoptosis/necrosis

The cytotoxic potential of the plant might induce cell death due to either apoptosis or necrosis. In order to verify the same, double staining of the treated cells with acridine orange (AO) and ethidium bromide (EB) was done. The most active one among the plant extracts was used for the treatment of the HeLa cells and assayed to detect the mode of toxicity induction. The plant extract with lowest LD_{50} value was considered as the one with maximum apoptotic/necrotic potential and the same concentration was used for the treatment. HeLa cells maintained in DMEM was treated with the LD_{50} concentration of the most potent extract and incubated for 24 h. After incubation, the cells were washed repeatedly twice using PBS after removing the extract portion in the supernatant. Stain was prepared by mixing acridine orange (100 µg/mL) and ethidium bromide (100 µg/mL). After washing the treated cells, the fluorescent dye combination was added to the wells and kept for 10 min under room temperature. The cells were again washed twice with

PBS and observed under inverted fluorescence microscope (Olympus CKX41) and images were captured using the top mount camera (Optika Pro5 CCD camera).

(iii) Quantitative apoptotic evaluation

HeLa (cervical carcinoma) cells were cultured as per the procedures described above and treated with the LC_{50} concentration of the most potent plant extract. Untreated cells were taken as the control group for reference. Incubation was done for 24 h. The cells were trypsinized after incubation and washed with PBS. 100 µL of the cell suspension was transferred to separate tube. To this, 100 µL of FITC-Annexin V reagent (FITC Annexin V/Dead cell apoptosis kit) was added. The tubes were mixed thoroughly by pipetting up and down for 3 to 5 seconds followed by incubation for 20 minutes at room temperature in the dark. The cells were analyzed in a flow cytometer (MUSE^{TM}) by means of an argon-ion laser at an excitation at 488 nm. The resultant plots were analyzed using Muse flow cytometry software. Cells were gated against untreated control cells and analyzed for apoptosis using Muse FCS 3.0 software.

(iv) Cell cycle analysis

Flow cytometric analysis was carried out to study the mechanism of apoptotic effect of the plant extract on cancer cells. This was done to identify the stage at which cell cycle arrest was brought by the extract. HeLa cells treated with LC_{50} concentration of the most potent extract (*A. malabarica* methanolic extract) were transferred to 50 mL conical flask and centrifuged at 3000 rpm for 5 min. The supernatant was discarded to obtain a white film at the bottom. PBS was added and the flask was gently vortexed to mobilize the cells and centrifuged then followed by removal of the supernatant. The cells were resuspended with approximately 50 µL of PBS per 1×10^6 cells. The

suspended cells were transferred to tubes containing 1 mL of chilled ethanol (70% v/v), vortexed at medium speed and stored at -20ºC. The samples were incubated overnight and further centrifuged for 5min at 3000 rpm. After the removal of the supernatant, 250 µL of PBS was added and washed thrice. Finally, the obtained pellet was treated with 250 µL of cell cycle reagent (contains propidium iodide stain that differentiates viable and non-viable cells) and incubated in the dark for 30 min. After incubation, the mixture was subjected to cell sorting using MUSE^{TM} Cell Analyser (Merck-millipore, Germany) with an excitation of 480 nm Argon-ion laser beam. Using the untreated cells as reference, gating was performed and the plots obtained were analyzed using Muse FCS 3.0 software.

(v) Gene expression analysis

The effect of *Aglaia* extract on the regulation of apoptotic genes was analyzed by studying its expression level in the treated cancer cells. Expression levels of two apoptotic genes - caspase 3 and caspase 9, along with a house keeping gene - B-actin, were studied.

a. Total RNA isolation

Isolation of the total RNA content in the treated cells was done using Trizol method. Total RNA isolation kit was used according to the protocol suggested by the manufacturer (Invitrogen). Trizol induces membrane disruption that enables the release of RNA out of the cell.

On reaching 70% confluence, HeLa cells cultured in a 6 well microtitre plate was treated with LD_{50} concentration of the most potent *Aglaia* sp. and incubated for 24 h in a 5% humid CO_2 incubator under 37 ºC. Control group was also kept under the same condition without the extract but with an equivalent quantity of DMEM. After incubation, medium in the wells were pipetted out aseptically followed by an addition of 200 µL Trizol reagent. The

set up was kept for 5 min and the contents were transferred to sterilized microfuge tubes. To this, 200 µL of chloroform was added and shaken vigorously for 12 to 15 seconds and kept for 2 - 3 min at room temperature. After incubation, it was centrifuged at 12,000 rpm for 20 min at 4 ^{o}C. The supernatant including the chloroform layer was discarded which was followed by the addition of 500 µL of absolute isopropanol to the aqueous layer. This was incubated for 15 min at room temperature and centrifuged at 12,000 rpm for 20 min at 4 ^{o}C. Supernatant was discarded and the pellet was washed with 75% ethanol followed by centrifugation and decantation. The pellet thus obtained was dried and suspended in Tris - EDTA (TE) buffer for further use.

b. cDNA synthesis

Reverse transcriptase polymerase chain reaction (RT-PCR) was done to generate several copies of cDNA sequences. cDNA synthesis kit (Thermo scientific verso) was used for amplification. The PCR reaction mixture contained 4 µL of buffer solution, 2 µL of dNTPs, 1 µL of anchored oligonucleotide or primer, 1 µL of verso enzyme mix and 5 µL of RNA template (approximately 1 ng of the isolated total RNA) taken in an RNAse free PCR vial. Total PCR mixture volume was made up to 20 µL using sterile milli Q water and mixed thoroughly by gently pipetting up and down. PCR thermal cycler (Eppendorf) was programmed to undertake cDNA synthesis and the cycling conditions are mentioned below.

Reaction conditions:

Step	Temperature ^{o}C	Time (min)	No. of cycles
cDNA synthesis	42	30	1
Inactivation	95	2	1

d. Amplification of cDNA

The synthesized cDNA was amplified into considerable amount by using amplification kit (Thermo scientific). To a fresh PCR tube new reaction mixture was added which contained 25 µL of PCR master mix, 2 µL of forward primer (0.1-1.0 µM), 2 µL of reverse primer (0.1-1.0 µM) and 5 µL of template cDNA (10 pg-1 µg). The reaction mixture was made up to a final volume of 50 µL using nuclease free sterile milli Q water. Primary denaturation was enabled at 95 ^{o}C for 3 min. Further denaturation was done at 95 ^{o}C for 30 sec. The annealing temperature (Tm) was given for 30 sec and the extension temperature of 72 ^{o}C for 1 min. The process was repeated for 35 cycles and the final extension temperature of 72 ^{o}C was provided for 5 min. After the completion of the reaction, the PCR product was separated using agarose gel electrophoresis.

Reaction conditions set in the thermocycler for amplification using PCR are:

Step	**Temperature in ^{o}C**	**Time**	**Number of cycles**
Initial denaturation	95	3 min	1
Denaturation	95	30 sec	
Annealing	50-65	30 sec	35
Extension	72	1 min/kb	
Final extension	72	5 min	1

Primer sequences used for amplification and their annealing temperatures (Tm) are listed below:

Primer	Sequence	Tm (^{0}C)
B-Actin (forward)	5'- TCACCCACACTGTGCCCATCTACGA - 3'	66.3
B-Actin (reverse)	5'- CAGCGGAACCGCTCATTGCCAATGG - 3'	67.9
Caspase 3 (forward)	5'- TTCAGGGGGATCGTTGTAGAAGTC - 3'	63.0
Caspase 3(reverse)	5'- CAAGCTTGTCGGCATACTGTTTCAG - 3'	63.0
Caspase 9 (forward)	5'- ATGGACGAAGCGGATCGGCGGCTCC - 3'	71.3
Caspase 9 (reverse)	5'- GCACCACTGGGGGTAAGGTTTTCTAG - 3'	66.4

e. Agarose gel electrophoresis

The amplified cDNA obtained as a result of PCR was subjected to agarose gel electrophoresis. 1.5% agarose gel was prepared using TBE (Tris borate EDTA) buffer. Agarose powder was weighed and dissolved in 1x TBE and heated until it becomes a clear solution. The melted agarose was cooled up to 45 ^{o}C and to this 6 µL of 10 mg/mL ethidium bromide dye was added. This was poured into a casting tray with comb for creating wells. The gel was allowed to set for at least 1 h. Electrophoresis tank was filled with 1x TBE electrophoresis buffer and the gel cast tray along with the gel was placed on the platform. The wells were loaded with the samples and run at 50 V for 30 min. The gel was removed from the tray and viewed under UV rays through a gel documentation system (BIORAD). Images of the same were captured. Raw intensity of the expressed genes was calculated using Image J analysis software and relative expression were plotted.

PHASE III - GREEN SYNTHESIS OF SILVER NANOPARTICLES

For the further exploration of the reducing ability, all the three selected plants were analyzed for their potential to reduce the compound silver into elemental silver nanoparticles. The characterization of the synthesized nanomaterials was carried out using X-ray diffraction and scanning electron microscopic imaging.

a. Preparation of plant extracts

10 mg of the dried methanolic plant extract was dissolved in 10 mL of deionized water. This was well blended into a uniform solution using a cyclomixer. To remove the large particles, it was sieved through Whatman No.1 filter paper.

b. Synthesis of silver nanoparticles

2 mM of silver nitrate solution was prepared using deionized water and stored in an amber coloured bottle. 10 mL of the prepared plant extract was mixed with 90 mL of silver nitrate solution and boiled at 80 ^{o}C for 10 min. The pH was adjusted to 9. Development of a brown colour in the solution marks the production of nanoparticles (Gnanadesigan et al., 2011). The solution was centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. The particles were reconstituted in sterile water and centrifuged again using the same conditions as above. This step was repeated thrice for a thorough washing. The final pellet obtained was air dried in a laminar air flow chamber and stored in a dark coloured vial under room temperature for further analyses.

c. Characterization of nanoparticles

(i) UV-visible spectroscopy:

1 µg of the dried particles is resuspended in 3 mL of deionized water, transferred into the vial and analyzed using UV-visible spectroscope [JASCO V 550]. The excel data thus obtained was evaluated using ORIGIN 8.0 software and plots are prepared to find the broadest and prominent peaks specific for elemental silver particles.

(ii) X-ray diffraction analysis

Determination of the purity level of nanomaterial could be done most efficiently using XRD spectrum analysis. The nanomaterial pellet obtained was reconstituted in few drops of sterile distilled water and coated on a glass slide. This was subjected to XRD analysis using X-ray diffractometer [Rigaku MiniFlex 600]. The values thus obtained were analyzed using the ORIGIN 8.0 software and graphs were prepared accordingly. Crystal size was calculated using Debye-Scherrer equation:

$$D = \frac{k\lambda}{\beta \cos \theta}$$

where, D is the crystal size, k is the geometric factor (0.9), λ is the wavelength of the X-ray beam (1.54), β is the angular FWHM at the diffracted X-ray at an angle θ.

(iii) SEM imaging

The pellet of nanoparticles obtained was resuspended in a drop of deionized water and subjected to scanning electron microscopic imaging. The pellets were mounted on the stub with the help of a carbon tape and kept in

the desiccator for 1 h to dehydrate completely. The dried sample on the stub was sputter coated with gold particles for 5 seconds and examined using scanning electron microscope (Hitachi SU 6600, Japan) and images of the nanomaterial were recorded in various resolutions.

RESULTS

The three selected species of *Aglaia* Lour. were subjected to phytochemical characterization followed by the screening of its bioactive potentials. Antioxidant ability, hepatoprotective ability and cytotoxicity of all the selected species were analyzed. Antiproliferative ability of the three species was screened and a detailed study of the most active plant extract was performed. Test for the ability of the plant extracts to reduce silver nitrate to elemental silver nanoparticles was also carried out. This was followed by characterization of the biosynthesized nanoparticles.

PHASE I – PHYTOCHEMICAL ANALYSES

Phytochemical characterization of the selected taxa of *Aglaia* was carried out using various methods like preliminary qualitative analysis, quantitative estimation of some major secondary metabolites and GC/MS analysis of the methanolic plant extracts.

(i) Qualitative phytochemical screening

Preliminary screening for the detection of major secondary metabolites of all the three selected taxa was done (Table 4). Saponins were absent in all the three plants. All the other phytoconstituents screened like, quinones, phenols, flavonoids, terpenoids, alkaloids, steroids, tannins, carotenoids and cardiac glycosides were present in all the three plant extracts.

(ii) Quantitative phytochemical estimation

The amount of major potential secondary metabolites like phenols, terpenoids and flavonoids was estimated and the results are listed in Table 5.

The total amount of phenolic content was estimated using Folin - Ciocalteu reagent. The amount of phenolic compounds estimated in the plant

extracts were expressed as milligrams of gallic acid equivalent per grams of dry weight of the sample (mg GAE/g DW). The quantity was calculated using the regression equation obtained from the graph of the standard ($y = 0.006x$, $R^2 = 0.9939$; Fig. 3). Total phenolic content was estimated to be highest in *A. simplicifolia* (126.09 ± 8.08 mg GAE/g DW) followed by *A. malabarica* (118.59 ± 13.03 mg GAE/g DW) and *A. barberi* (35.89 ± 4.54 mg GAE/g DW) (Table 5).

Total terpenoids in the samples were extracted into the chloroform fraction of the reaction mixture and was estimated spectrophotometrically. Linear graph was plotted using linalool as the standard and regression equation was derived ($y = 0.0042x$, $R^2 = 0.8601$; Fig. 4). This equation was used to estimate the total terpenoids in the plant extracts and was expressed as milligrams of linalool equivalent per gram of dry weight of the sample (mg LE/g DW). Highest amount of terpenoids was observed in *A. malabarica* (162.32 ± 22.8 mg LE/g DW). This was followed by *A. barberi* (65.08 ± 3.05 mg LE/g DW) and *A. simplicifolia* (61.74 ± 2.45 mg LE/g DW) (Table 5).

Estimation of total flavonoid content was done using the aluminium chloride method where, quercetin was used as the standard. Regression equation was obtained from the calibration curve plotted using the values obtained from the standard ($y = 0.0097x$, $R^2 = 0.9713$; Fig 5). Total flavonoid content in the plant extracts were expressed as milligrams of quercetin equivalent per gram of dry weight of the sample (mg QE/g DW). *A. malabarica* was observed to have comparatively highest amount of flavonoids *i.e.*, 95.67 ± 5.09 mg QE/g DW. This was followed by *A. simplicifolia* which was estimated to possess total flavonoids of about 29.56 ± 0.64 mg QE/g DW and *A. barberi* with 21.73 ± 0.74 mg QE/g DW (Table 5).

All the three plant extracts studied were found to have considerable amounts of potential secondary metabolites.

(iii) Gas chromatography/ mass spectrometry (GC/MS) analysis

In order to determine the volatile components of the plant extracts, all the three selected taxa of *Aglaia* were subjected to GC/MS analysis. Compounds got resolved according to their retention time and even those present in trace amounts were detected. A sum total of 112 compounds were detected from all the three extracts. Chromatograms of the samples as well as mass spectrum of each compound were plotted. Compounds were grouped into various phytoconstituent classes like alkaloids, phenols, terpenoids, sterols, esters, fatty acids, alcohols *etc*. Percentage peak area of each compound facilitated comparative quantification.

GC/MS analysis of the methanolic plant extract of *A. barberi* revealed 37 compounds along with their mass spectra (Fig. 6 [i-vii]). The majority of the compounds belonged to the classes like sesquiterpenes, triterpenes, organic compounds, alkaloids, steroids, esters *etc*. An organic compound N-methyl pyrrole (13. 91%) was found in highest amount. This was followed by a steroid compound gitoxigenin (9.37%) and 2- acetyl-5-(5-methyl furfuryl) furan (8.83%). Sesquiterpenes were represented by epizonarene (7.71%), cubebene (1.59%) and guainene (1.21%) in considerable amounts as well as, α-bulnesene (0.92%), α-silenene (0.77%), copaene (0.67%) *etc.* in trace amounts. The triterpene compounds identified in the extract were digitoxigenin (5.77%), erythrodiol (0.91%) and oleanolic acid (0.68%). Compounds with potential bioactivities like 4-hydroxytryptophan (amino acid - 7.09%), vincadifformine (alkaloid - 3.28%), squalene (organic compound - 1.05%) and 3-myristoylolean-12-en-28-ol (ester - 5.80%) were detected in considerable amounts. Along with it, trace amounts of other compounds like 2- methoxy-4-vinylphenol (phenolic compound - 1.29%), isorhoifolin (flavonoid - 0.34%), 8-Chloro neoisolongifolene (organic compound - 2%)

were also identified (Table 6). Chromatogram showing the compound specific peaks was also obtained (Fig. 7).

GC/MS analysis of *A. simplicifolia* leaf methanolic extract unveiled 37 compounds which belonged to various classes of phytoconstituents. Mass spectra are shown in the figure 8 [i-vii]. 3 - (dimethylamino) phenyl methanol (14.13%) and ethyl 3-methyl-2-butenoate (13.92%) were found in highest amounts. A potential alkaloid O- methyl psychotrine (7.17%) as well as organic compounds like D-alphatocopherol (10.17%) along with 4-morpholine butyric acid (7.82%) were identified as other prominent constituents. Bioactive steroids like gitoxigenin (1.79%) and γ- sitosterol (4.37%) were also observed in notable amounts. Phenolic compounds were represented by mequinol (2.03%) and cis-isoeugenol (1.43%). Numerous terpenoid compounds like abieta-6, 13-diene (2.77%), aciphyllene (2.02%), lupeol acetate (1.06%), urs-12-en-28-al, (3-acetoxy) -3β (1.03%) *etc.* were detected. 2- naphthyl-β-D-galactopyranoside (0.71%) was the glycoside identified from the extract. Trace amounts of numerous organic compounds and alcohols were also resolved along with the above compounds (Table 7). Peaks obtained for each compound was observed using the chromatogram (Fig. 9).

In the GC/MS analysis of methanolic leaf extract of *A. malabarica*, *N* - methyl pyrrole (15.91%) and the alkaloid pectenin (13.81%) were observed in highest amounts. Other alkaloids identified like anosmine (5.34%) and dehydroheliamine (5.46%) were also found to have considerable peak area percentage. The potential organic compounds like 5-penta flouroethyl histamine (4.88%) and squalene (3.1%) showed a notable percentage. A diverse array of terpenes was found in considerable amounts which were represented by α - cubebene (3.45%), ledene alcohol (1.62%), alloaromadendrene (1.23%), ekeberin C1 (1.18%) *etc*. Significant amounts of

bioactive ester, ochratoxin B-methyl ester (5.69%) and fatty acid, octadecyl palmitate (2.39%) were also identified from the extract. Moreover, trace amounts of organic compounds, phenolic compounds and alcohols were resolved according to their retention times (Table 8). Mass spectra of all the compounds are represented in Figure 10 [i-vi] and the chromatogram obtained is shown in Figure 11.

GC/MS analysis of the three selected plant methanolic extracts comprises a wide array of various phytoconstituents, belonging to various classes like terpenes, phenolic compounds, alkaloids, glycosides *etc.* Chromatograms gave an overall view on compound peaks of each extract. All the three extracts showed a diverse collection of potential secondary metabolites. Though few compounds observed were common in three extracts, their percentage peak area differed.

PHASE II – BIOACTIVITY SCREENING

A. ANTIOXIDANT ABILITY

Ability to scavenge the reactive free radicals in a system of all the three selected taxa of *Aglaia* was analyzed. Four assays were done to determine the same which included DPPH radical scavenging assay, ABTS radical scavenging assay, Hydroxyl radical scavenging assay and super oxide radical scavenging assay. Standard antioxidants like ascorbic or gallic acid were used for the comparative estimation of antioxidant ability of the sample plant extracts.

(i) DPPH radical scavenging assay

Five concentrations (12.5, 25, 50, 100 and 200 µg/mL) of all the three methanolic plant extracts were taken for DPPH radical scavenging assay. The calibration curve was prepared by using ascorbic acid as the standard (Fig.

12). The means of the triplicate values (percentage inhibition) obtained from the samples were plotted against concentration and were found to be in a dose dependent manner (Fig. 13).

Percentage inhibition of *A. barberi* was found to increase from 19.47 ± 1.2 to 51.39 ± 3.4 with the increasing concentration from 12.5 to 200 µg/mL (Fig. 13). In a similar manner, a dose dependent activity was observed in other two plant extracts also. *A. simplicifolia* showed percentage inhibition of 14.15 ± 2.8 to 64.09 ± 2.9 while that of *A. malabarica* was observed as ranging from 20.16 ± 2.93 to 58.94 ± 1.41, as the concentration was increased from 12.5 to 200 µg/mL (Fig. 13). At lower concentrations, highest activity was shown by *A. malabarica*, while at increased concentrations most prominent activity was shown by *A. simplicifolia*. IC_{50} values were calculated using ED50 PLUS V1.0 software. Most effective IC_{50} value was observed for *A. simplicifolia* (131.87 µg/mL). It was followed by *A. malabarica* (153.22 µg/mL) and *A. barberi* (165.13 µg/mL) (Table 9).

(ii) ABTS radical scavenging assay

ABTS radical scavenging assay of all the three samples were carried out using five different concentrations (12.5, 25, 50, 100, 200 µg/mL). Triplicates of each concentration were done and the results were expressed as mean value ± standard error. Ascorbic acid was used as the standard for the preparation of calibration curve (Fig. 14). Lowest percentage inhibition was observed for *A. barberi* extract (13.37 ± 3.85 to 77.26 ± 4.71). This was followed by *A. simplicifolia* (19.23 ± 6.08 to 82.24 ± 1.79) while the highest activity was shown by *A. malabarica* (43.25 ± 7.93 to 83.14 ± 2.89) (Fig. 15). IC_{50} values were obtained using ED50 PLUS V1.0 software. Highest activity of *A. malabarica* was exhibited by the smallest IC_{50} value of 33.68 µg/mL. *A. simplicifolia* showed a medium activity with an IC_{50} value 50.98 µg/mL and

the highest IC_{50} value was obtained for *A. barberi* (64.40 µg/mL) (Table 9). All these values mark considerable antioxidant activity of the extracts.

(iii) Hydroxyl radical scavenging assay

The ability of all the selected plant extracts to scavenge free hydroxyl radicals were analyzed using five different concentrations (12.5, 25, 50, 100 and 200 µg/mL). The standard used was gallic acid and the calibration curve was plotted using the mean values obtained (Fig. 16). Percentage inhibition was calculated and IC_{50} values were determined using ED50 PLUS V1.0 software. Maximum percentage inhibition was exhibited by *A. simplicifolia* which ranges between 33.88 ± 1.75 to 79.44 ± 1.36. Though minimal inhibition was observed at lower concentrations, *A. malabarica* showed a drastic hike in scavenging ability at higher concentrations (9.64 ± 1.88 to 77.24 ±1.74). Comparatively minimum activity was observed in *A. barberi* extract where, the percentage inhibition obtained ranges from 8.86 ± 2.44 to 60.39 ± 1.43 (Fig. 17). IC_{50} values of *A. barberi*, *A. simplicifolia* and *A. malabarica* were obtained as 152.23 µg/mL, 154.34 µg/mL and 83.76 µg/mL, respectively (Table 9).

(iv) Superoxide radical scavenging assay

All the three selected taxa of *Aglaia* were analyzed for their superoxide radical scavenging ability. Five concentrations of each sample (12.5 to 200 µg/mL) were assayed. Ascorbic acid being the standard, calibration curve was plotted (Fig. 18). Percentage inhibition of *A. barberi* was observed between 12.39 ± 1.5 and 85.89 ± 2.2. *A. simplicifolia* exhibited the maximum percentage ranging from 9.23 ± 2.77 to 62.27 ± 10.12. Percentage inhibition obtained for *A. malabarica* was between 5.2 ± 1.26 and 57.27 ± 2.37 (Fig. 19). Maximum scavenging ability was shown by *A. simplicifolia* with an IC_{50} value of 71.99 µg/mL. It was followed by *A. barberi* with IC_{50} 83.26 µg/mL

and a considerable activity was shown by *A. malabarica* also (IC_{50} = 152.71 µg/mL). The results are recorded in Table 9.

All the three selected taxa of *Aglaia* showed considerably high radical scavenging ability and thus, further studies on the cytoprotective as well as cytotoxic abilities might be done in order to explore their mode of therapeutic action in a living system.

B. HEPATOPROTECTIVE ACTIVITY

In order to determine the protective ability of the selected taxa of *Aglaia* on normal living cells, human hepatocytes were used. Toxicity was induced in HepG2 cells using ethyl alcohol and then subjected to treatment with the plant extracts. Toxicity induced cells were observed with aberrations like nuclear disintegration, echinoid spikes, dead cells *etc.* Triplicates of five different concentrations (6.25, 12.5, 25, 50 and 100 µg/mL) of each plant extract were used for the treatment, after inducing alcohol toxicity. Mean values of percentage viability was derived from the absorbance values obtained.

Percentage viability of control cells: 99.1%

Percentage viability of alcohol treated cells: 42.83%

Direct microscopic observation revealed the protective effect of extracts (Plates 2-4). Aberrations and cell death were observed less in the treated cells and that too in a dose dependent manner for all the three extracts. Comparatively minimum protective ability was observed in *A. malabarica* extract (Plate 4). It showed protective ability at lower concentrations while deteriorating effect at higher. Percentage viability of the same ranged from 88.28 ± 2.2 to 24.29 ± 4.1 as the concentration increases from 6.25 to 100 µg/mL (Fig. 20). *A. barberi* and *A. simplicifolia* showed almost similar and

considerably higher protective effect on the toxicity induced cells. *A. barberi* showed the percentage viability which ranged within 94.96 ± 4.5 and 67.35 ± 3.3 on increasing the concentration from 6.25 to100 µg/mL while, that of *A. simplicifolia* was observed as 96.65 ± 7.3 to 67.55 ± 6.3.

C. CYTOTOXIC ACTIVITY

Though the lower concentrations of the plant extracts were having protective ability, a cytotoxic effect was observed in the cells treated with the higher concentrations. In order to reveal the cytotoxic effect of the extracts on rapidly dividing cells, cytotoxic assays were carried out. Three methods were used for the same.

(i) *Allium cepa* assay

Ability to induce cytotoxicity of all the three selected plant taxa was carried out using *Allium cepa* assay. *Allium* bulbs were treated with different concentrations (5, 10, 15, 20 and 30 µg/mL) of all the plant extracts for various time periods (1, 12 and 24 h). For reference, malathion treated ones were taken as the positive control and the bulbs kept in distilled water was considered as negative control. The effect of the extracts on the *Allium* root meristem was studied by direct microscopic observation as well as by determining various parameters like mitotic index and percentage aberration. The effects were found to be dose dependent as well as time dependent.

The aberrations at all the stages of cell cycle (interphase, prophase, metaphase, anaphase, telophase and cytokinesis) were identified (Plates 6-17) which were different from that of the normal cells (Plate 5). Direct microscopic observation revealed both clastogenic (caused due to direct effect on chromosome) and non-clastogenic aberrations (resulted due to effect on spindle fibers) during all the stages of cell cycle. Some of the important clastogenic aberrations include nuclear disintegration, nuclear peak, nuclear

appendage formation, nuclear budding, chromosome erosion, pulverized chromosome, chromosome fragments, sticky chromosomes *etc.* whereas the non-clastogenic ones include displaced chromosomes, chromosomal vagrants, laggards, shift in MTOC, tropokinesis, multiple ploidy (hyperploidy and polyploidy), *etc*. Along with these, giant cells and macronuclei formation, strap cells, binucleate cells and cytomixis were also observed. Some aberrations were observed to be recurrent for a particular extract; *eg.* Multiple ploidy or polyploidy was very frequent in the cells treated with *A. barberi* extracts. Interestingly, large number of cells with cytostatic metaphase chromosomes was also observed in the root tips treated with *A. barberi* extract. Similarly, *A. simplicifolia* was able to induce vacuolation at a high frequency whereas; *A. malabarica* induced prominent nuclear and cytoplasmic lesions in almost all the treated cells. Most of the cells were detected with multiple or large lesions.

The parameters like mitotic index (MI) and percentage chromosomal aberration (CA) was used to determine the cytotoxic potential of the plant extracts on *A. cepa* root meristem. Mitotic index is the measure of dividing cells in a particular field. Six such fields were selected for counting the cells for each treatment and the mean values were determined. MI was found to be inversely proportional to concentration as well as time period for all the three plant extracts.

After the treatment of *A. cepa* bulbs for 1 h, MI of the positive control was obtained as 9.76 ± 1.3 and that of negative control was noted to be 83.76 ± 2.0 (Fig. 21A). MI of *A. barberi* was obtained in a range of 84.4 ± 3.5 to 60.85 ± 3.0. However, MI of *A. simplicifolia* was obtained between 87.68 ± 1.6 to 66.54 ± 1.2 and *A. malabarica* in between 88.79 ± 3.3 to 37.73 ± 2.5 with the range of concentration from 5 to 30 µg/mL (Fig. 21A). For the 12 h treatment, positive control showed MI of 8.41 ± 2.2 and that of negative

control was obtained as 89.11 ± 1.9. A comparatively lower MI was observed in the 12 h extract treated roots than that of 1 h treatment. After 12 h treatment, *A. barberi* exhibited MI ranging from 77.39 ± 2.1 to 21.85 ± 1.4, *A. simplicifolia* showed MI between 84.78 ± 1.7 and 49.75 ± 3.4 and *A. malabarica* had MI in a range of 82.29 ± 3.3 to 24.17 ± 2.5 as the concentration was increased from 5 to 30 µg/mL (Fig. 21B). For the treatment for 24 h, positive control displayed MI of 7.12 ± 2.2 and negative control showed MI of 88.46 ± 2.3. All the 24 h plant extracts treated cells showed considerable decrease in MI than short time treatments. MI was observed to be dropped in *A. barberi* treated root meristem from 72.24 ± 3.6 to 21.71 ± 2.2, in *A. simplicifolia* extract treated ones from 77.48 ± 1.7 to 36.49 ± 2.3 and that of *A. malabarica* decreased from 60.76 ± 6.2 to 15.53 ± 2.1 (Fig. 21C). MI was found to be decreasing with increasing concentration thereby revealing the mitodepressive activity of all the plant extracts studied.

A wide range of chromosomal aberrations were obtained as the result of treatment of *Allium* root meristem with plant extracts of selected taxa of *Aglaia*. Quantitatively, the aberrations were expressed as percentage chromosomal aberration (CA). After 1 h treatment, malathion treated *Allium* bulbs (positive control) showed CA as 92.92 ± 1.1 and the bulbs kept in distilled water (negative control) had CA to be 1.06 ± 0.5. Dose dependent activity was exhibited in all the extract treated bulbs. Maximum aberration percentages were noted in the bulbs treated with *A. malabarica i. e.*, 33.40 ± 2.6 to 83.54 ± 1.7 as the concentration increased from 5 to 30 µg/mL. It was followed by *A. simplicifolia* (23.18 ± 0.8 to 77.65 ± 1.9) and *A. barberi* (22.24 ± 3.7 to 63.12 ± 1.1) as represented in Fig. 22A. *Allium* bulbs subjected to a half day treatment (12 h) showed comparatively higher aberration frequency than 1 h treated ones. The positive control had CA as 94.55 ± 1.8 and the negative control with CA as 2.41 ± 1.6. Here also, the bulbs treated with *A. malabarica* exhibited maximum aberration frequency (46.62 ± 2.0 to 86.43 ±

2.6). This was followed by *A. simplicifolia* (33.45 ± 4.1 to 84.87 ± 2.0) and *A. barberi* (23.25 ± 2.3 to 74.22 ± 1.2) (Fig. 22B). The same pattern of aberration induction was observed in the root meristem treated for the time period 24 h, but at a higher frequency than 1 h and 12 h treated ones. Highest aberration percentage was displayed by *A. malabarica* (54.09 ± 6.9 to 89.32 ± 1.5), a comparatively moderate effect by *A. simplicifolia* (44.12 ± 2.5 to 81.35 ± 1.0) and lowest by *A. barberi* (37.38 ± 2.3 to 78.69 ± 3.6) as explained in Fig. 22C. Though there were differences in the effect caused by different extracts, all of them showed dose as well as time dependent activity.

(ii) *In-situ* visualization of cell death

Cell death which occurred in the treated root meristem was visualized by Evans blue staining. The stain penetrates into the non-viable cells which might be resulted from the cytotoxic effect of the plant extracts. The amount of stain that entered into the root meristem was spectrophotometrically quantified in order to determine the extent of cell death. Various concentrations (5, 10 and 30 µg/mL) of each extract were used to treat the *Allium* bulbs. The stained roots were incubated in *N*, *N* - dimethyl formamide so as to release the penetrated stain and spectrophotometric data of this enables a comparative quantification of cell death (Fig. 23). Positive control (malathion treated bulbs) showed the maximum uptake of stain with absorbance value 0.52 ± 0.02 while, negative control (distilled water treated bulbs) remained least stained thereby producing absorbance value of 0.02 ± 0.01. Maximum stain uptake was seen in the roots treated with *A. malabarica* (0.13 ± 0.012 to 0.31 ± 0.009) extract which was followed by *A. simplicifolia* (0.05 ± 0.009 to 0.21 ± 0.016) and lowest uptake was seen in *A. barberi* treated roots (0.03 ± 0.012 to 0.18 ± 0.015) as the concentration was increased from 5 µg/mL to 30 µg/mL. The uptake of the stain by the treated roots was found to be dose dependent in all the three samples (Plate 18).

(iii) Cytotoxicity induction in human erythrocytes

Cytotoxic potential of all the three selected taxa of *Aglaia* were performed using human RBC or erythrocytes as test model, to study their effect on a membrane system. Serum was removed and washed cells were treated with a particular concentration of test sample to induce membrane toxicity. The microscopic observation revealed the presence of membrane blebs, distorted membranes and apoptotic bodies in the treated cells (Plate 19). The treated cells lost their spherical shape and transformed into echinoid spike like structures. The control cells, which were incubated under the same conditions without adding plant extract, remained intact and circular. Comparatively maximum membrane damages were observed in the cells treated with *A. malabarica* extract than that with *A. simplicifolia* and *A. barberi* (Plate 19).

D. ANTIPROLIFERATIVE ACTIVITY

The methanolic extracts of all the three selected taxa of *Aglaia* were subjected to antiproliferative assay on HeLa cell lines. MTT assay was used for the screening. The most active one was subjected to apoptotic/necrotic activity detection. Quantification of the same and analysis of the cell cycle stages were done using flow cytometry and this was followed by the gene expression studies.

(i) MTT method

Five different concentrations of each extract *viz*., 6.25, 12.5, 25, 50 and 100 µg/mL, were used for the treatment of HeLa cells and incubated for 24 h. The direct microscopic observation revealed the toxic effect of all the plant extracts on the cancer cells. Aberrations like formation of membrane blebs, apoptotic bodies, nuclear condensation, membrane distortion, formation of echinoid spikes, budding, fragmentation and cell shrinkage were clearly

visible (Plates 20-22). All these are remarkable characteristics showing cell death.

Percentage viability of each test cell group was determined using the absorbance values obtained. A dose dependent effect on cell death was observed. Maximum antiproliferative activity was exhibited by *A. malabarica* with the lowest percentage viability at a range of 82.77 ± 3.43 to 42.84 ± 1.93 as the concentration was increased from 6.25 to 100 µg/mL (Fig. 24). The aberrations noted during microscopic examination were also very prominent in the cells treated with the same. LD_{50} value of *A. malabarica* was calculated as 42.14 ± 3.6 µg/mL using ED50 PLUS V1.0 software (Table 10). A moderate activity was shown by *A. simplicifolia* with a higher percentage viability of 84.87 ± 2.07 during the lowest concentration of 6.25 µg/mL. It was dropped up to 56.63 ± 0.62 with the increase in concentration to 100 µg/mL (Fig. 24). LD_{50} value of *A. simplicifolia* was calculated and obtained as 57.48 ± 4.1 µg/mL. Minimum yet a considerable antiproliferative activity was observed in *A. barberi* treated HeLa cells. Percentage viability dropped from 95.48 ± 0.76 to 51.17 ± 3.48 as the concentration was increased from 6.25 to 100 µg/mL (Fig. 24). LD_{50} was calculated as 101.54 ± 5.6 µg/mL (Table 10).

Interestingly, no significant alterations were observed in the normal L929 fibroblast cells after the treatment with various concentrations of *A. malabarica*. Only a negligible cytotoxic effect was observed even in the cells treated with the highest concentration of the extract. LD_{50} value of 212.482 µg/mL was obtained which indicated the absence of significant cytotoxic effect. The percentage of viable cells after the treatment with the extract ranged between 96.42 ± 0.24 to 75.10 ± 0.24 with an increasing concentration gradient (Table 11). This indicates the specific toxicity of the plant extract towards the malignant cells rather than the normal cells (Plate 23).

(ii) Detection of apoptosis/necrosis

Cell death inducing ability of all the three plant extracts was revealed in the previous assay. In order to determine the mode of cell death, apoptosis or necrosis, double staining technique was employed. Acridine orange/ethidium bromide combination was used to stain the HeLa cells treated with LD_{50} concentration of the most active plant extract *i.e*., 42.14 µg/mL of *A. malabarica*. Viable cells were stained green while non-viable ones were visible as orange coloured bodies. Microscopic evaluation of the fluorescent stained cells revealed the morphological alterations in the treated cells. The loss of membrane integrity lead to cell shrinkage, apoptotic bodies were formed, membrane blebs appeared and nuclear fragmentation was observed in the treated cells. All these are marked as the sign of apoptosis thereby, unveiling the apoptotic ability of *A. malabarica.* Along with these aberrations, almost half of the treated cells were stained orange that proved the occurrence of cell death. At the same time, the control or untreated cells appeared intact without any membrane distortions and stained green with uniform intensity which showed their viability (Plate 24). From the morphological features observed in the treated HeLa cells, it is proved that the cell death caused by *A. malabarica* extract might be due to apoptosis.

(iii) Quantitative apoptotic evaluation

In order to determine the extent of apoptosis induced by *A. malabarica* extract on HeLa cells, quantitative estimation of apoptosis using flow cytometer was employed. LD_{50} concentration of *A. malabarica* extract was used to treat HeLa cells and incubated for 24 h. These cells were stained with recombinant Annexin V FITC - propidium iodide dye combination. Live cells do not take up dye thus, little or no fluorescence was observed. Annexin V specifically binds to apoptotic cells due to their characteristic membrane property and emits green fluorescence while other dead cells/debris shows red

fluorescence due to the binding of propidium iodide. In the control system, the percentage of viable cells was observed as 89.15 % and apoptotic cells comprised of 9.95% of the total population (approximately 5.09×10^5 cells per mL). Percentage of dead cell matter or debris formed 0.67% (Fig. 25 [iii]). In the test sample, the cells treated with 42 µg/mL of *A. malabarica* extract, the percentage of live cell was dropped to 74.46% and percentage of apoptotic cell population increased to 10.72%. Dead cell matter was found to be approximately 5.8×10^5 cells per mL which occupies 14.82% of the total population along with 10.72% late apoptotic cells (approximately 4.19×10^5 cells per mL) and this clearly indicates the toxic effect of the plant extract (Fig. 25 [iii]). The population profile indicated the increase in the dead cell content by the increased amount of Annexin V uptake (Fig. 25 [ii]) while, scattering was found minimum in control cells (Fig. 25 [ii]). Quadrant I and Quadrant IV showed increased scattering of cells in the treated sample than in the control (Fig. 25 [i]) which proved the hike in cellular death or apoptosis which might be the result of cytotoxic potential of the extract.

(iv) Cell cycle analysis

The apoptotic effect of *A. malabarica* leaf extract on cell cycle progression was analyzed using flow cytometric cell sorting system. Plots detailing the variation in the DNA content and the cell count/population profile of the treated and untreated control cells were obtained (Fig. 26). The histogram denoting DNA content as well as the population profile indicated the amount of cells at each stage of the cell cycle. The percentage of cells found at each stage, as the cell cycle progresses, is represented in the figure 26. As the DNA content was analyzed, a prominent increase in the same was observed at G0/G1 phase in the treated cells when compared to the untreated control. The increase in the sub-G0 DNA content indicates the apoptotic cell population. A corresponding decrease in the DNA content at G2/M phase in

the treated cells was also noted. DNA content index during G0/G1 phase was obtained as 75.1% in the treated cells while that in the control was 57.9%. Meanwhile, DNA content index at G2/M phase was observed as 5.6% in the treated cells and 11.1% in the control. Population profile showed a scatter in the initial stage and the subsequent reduction of cells in the further stages in the treated vials (Fig. 26 [ii]) thereby indicating the arrest of the cell cycle during G0/G1 phase. HeLa cells treated with *A. malabarica* extract showed a prominent death rate and the histograms revealed the apoptotic death during G1 phase of the cell cycle. A comparison of the DNA content during various stages of cell cycle is depicted in the figure 27.

(v) Gene expression analysis

Regulation of the pro-apoptotic/apoptotic genes in the HeLa cells by *A. malabarica* extract was studied by relative quantification of their expression in treated cells *vs*. untreated control cells. Two genes involved in apoptosis like caspase 3 and caspase 9 were selected along with B-actin, a house keeping gene. It is clearly evident from agarose gel images that the apoptotic genes are significantly up regulated in the extract treated cells than the control. Both the genes, caspase 3 and caspase 9, showed intense fluorescence in the agarose gel indicating their presence in high quantity in the treated cells, while in the control cells expression of caspase genes are not much prominent. At the same time, B-actin remains equal in both the treated and control cells. (Fig. 28).

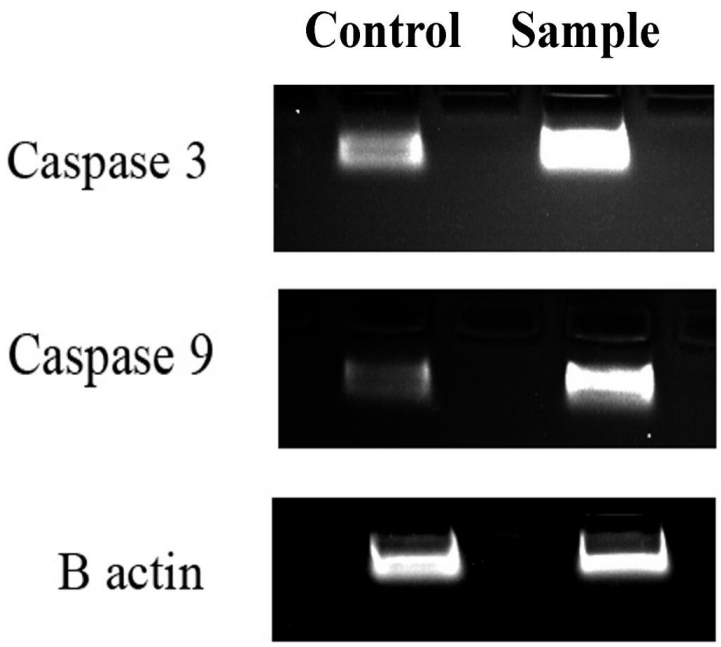

Figure 28: The effect of methanolic extract of *A. malabarica* on apoptotic gene regulation in HeLa cells.

The relative expression of the genes was quantified using the agarose gel images by means of Image J analysis software. The relative expression of caspase 3 for the treated cells was obtained as 2.184 while that of control was 0.975 (Fig. 29). Similarly, caspase 9 exhibited a relative expression of 1.342 in the treated cells and 0.214 in the control cells (Fig. 30).

PHASE III - GREEN SYNTHESIS OF SILVER NANOPARTICLES

Biologically mediated silver nanomaterial synthesis is a non-toxic, environmentally benign and cost-effective method of obtaining nanoparticles of desirable properties. The resultant particles are characterized by better shapes, sizes and stability even without providing an external capping agent. Here, an attempt was done to synthesize silver nanoparticles from silver nitrate solution by using the methanolic plant extract of selected species of *Aglaia* as the natural reducing agent. This was followed by the characterization of the resultant silver nanoparticles *via* UV-visible

spectrometry, X-ray diffraction analysis and scanning electron microscopic imaging.

(i) Biosynthesis of nanoparticles:

1 mg/mL methanolic extract was incubated along with 2 mM $AgNO_3$ solution in a specific proportion (1:9) and incubated under optimum reaction conditions of pH - 9 and temperature of 80 ^oC for 10 min. The reaction mixture was observed which revealed the change in colour from pale green to brown after incubation, indicating the reduction of metallic silver in the solution. *A. barberi* showed a pale brown colour formation which was more intense than that formed by *A. simplicifolia* while *A. malabarica* exhibited the most prominent brown colour formation, after the incubation period. At the same time, the control solution remained colourless throughout the experiment (Plate 25).

(ii) UV-visible spectrum analysis:

The synthesized nanoparticles were dispersed in deionized water and subjected to UV-visible spectroscopy. *A. barberi* yielded the broadest and prominent peak with 476 nm as the peak point along with a narrow one at 246 nm (Fig. 31 A). *A. simplicifolia* produced the noticeable peak at 426 nm and a short one at 248 nm (Fig. 31 B). The broadest and considerable peak obtained was at 446 nm and a narrow one at 248 nm for *A. malabarica* (Fig. 31 C). Though multiple peaks were obtained, the broad and strong surface plasmon resonance (SPR) peaks were obtained between 420-480 nm in all the three extracts which indicate the presence of elemental silver nanoparticles.

(iii) X-ray diffraction analysis

X-rays diffracted from the crystals reveals the angle of diffraction which is used to calculate the crystallite size. FWHM is obtained from the

plot (Fig. 32) and the crystal size is calculated using Debye-Scherrer equation. The approximate crystal size obtained from A. barberi extract is 40.96 nm, whereas that in A. simplicifolia is 23.39 nm and A. malabarica is 5.99 nm (Table 12).

Table 12. Approximate crystal size of nano-crystal obtained from XRD data

Plant	2θ	FWHM	Approximate crystal size (nm)
A. barberi	81.86	2.68	40.96
A. simplicifolia	82.08	4.70	23.39
A. malabarica	23.35	14.13	5.99

(iv) SEM imaging

Scanning electron microscopy enabled imaging of the nanoparticles was conducted to further study their morphology, size and shape. The images were taken in several magnifications and were measured using the scale bar. The silver nanoparticles obtained using the extract of *A. barberi* were observed to have a somewhat cubical shape with a particle size ranging around 33.1 - 71 nm (Plate 26). Largest nanoparticles were produced in the biogenic reduction mediated by *A. simplicifolia* extract. SEM images revealed the perfect cube morphology of the particles with an approximate size between 76.3 - 101 nm (Plate 27). Most reduced ones or the smallest particles were produced from *A. malabarica* induced reduction of metallic silver with their size ranging from 18.1 - 30.7 nm and showed cuboid like morphology (Plate 28). The silver nanoparticles production involving the use of plant extracts of selected species of *Aglaia* thereby provides an efficient method for green synthesis of nanoparticles.

DISCUSSION

Plants and other natural products have won great interest in drug discovery and improvement from ancient time onwards which is still increasing eventually. The incomparable benefits of the plant derived products such as unique diversity in chemical composition and structures, effective biological properties, negligible side effects, cost-effectiveness and immense clinical experiences are explored with great concern. Modern treatment and pharmacotherapy employs specific compounds against a particular ailment. On the other hand, traditional medicine takes the benefit of the synergistic action of the multiple and unique chemical composition in the crude plant extracts which might deliver better results in several therapeutic cases including that of diabetes, cardiac diseases, hypertension, cancer, *etc*. Efficient treatment of these complex diseases have made possible by employing synergism with the concept of "multi-drug and multi-targets" rather than "one disease, one target, one drug" approach. In many cases plant derived medicinal preparations have provided cue for the specific compound for the drug development against several severe diseases. The inimitable chemical constitution of the herbal drugs is an important resource for developing new lead compounds and scaffolds for the development of effective drugs against critical human ailments.

The concerted action of the complex chemical constituents in the herbal products is hard to elucidate while exploration and related researches become necessary for the better treatment. Interpretation of the pathways and possible mechanisms involved in the therapeutic action of the multi-constituent herbal drugs helps in the combination therapies. Modern drug development could be enhanced with the precious experience from the traditional knowledge and natural products. This enables the blending of

traditional medicinal knowledge with the modern therapeutic approach in a scientific way which can make breakthroughs in human health care.

PHASE I - PHYTOCHEMICAL ANALYSIS

In the path of knowledge generation, human interest to explore the reasons behind healing properties of plants leads to the need of phytochemistry. Several methodologies are employed for the identification of the chemical constituents, their quantification and elucidation of the unique structure and pathways by which they enact as drugs. A proper knowledge or understanding on phytochemicals is essential for drug discovery or innovation of novel therapeutic agents against severe diseases.

The primary step in the phytochemistry is the collection of plants in the adequate quantity and its processing in appropriate way. Various phytoconstituents dissolves in different solvents according to their polarity. Hexane, ethyl acetate, chloroform, methanol, water, *etc*., can be used as the solvents for extracting phytochemicals. However, methanol is a medium polar solvent in which most of the important bioactive phytoconstituents dissolves in considerable quantity (Ngo et al., 2017). Considering the efficacy of methanol as an excellent solvent for bioactive principles, all the three plants under study were extracted using the same.

A wide array of methods for plant extraction is being used conventionally, such as cold extraction, overnight dissolution, decoction, steam distillation, *etc*. Soxhlet extraction is one among the most efficient and reliable conventional modes of plant extract preparation which efficiently recycles a small amount of solvent to dissolve large amounts of constituents with an unmonitored operation (De Castro & Priego-Capote, 2010). The same was used to extract phytoconstituents from the dried leaves of the selected taxa of *Aglaia*. The methanolic leaf extracts were subjected to three level

phytochemical analyses - Qualitative analysis as a preliminary study, followed by the quantification of some important secondary metabolites and GC/MS analysis for the identification of the volatile constituents.

(i) Qualitative phytochemical analysis

A broad look in to the phytochemical classes present in the plant extracts was done using preliminary qualitative screening. Tests for the detection of bioactive principles or the anti-nutritional factors were done using the methanolic and aqueous extracts of the selected taxa of *Aglaia*. All the three plants showed the presence of secondary metabolites like quinones, phenols, flavonoids, terpenoids, alkaloids, steroids, tannins, carotenoids and cardiac glycosides but all of them were found to be devoid of saponins (Table 4). The results obtained for *A. malabarica* have been published (Ravindran & Thoppil, 2018). Previous reports on the phytochemical constituents of *A. roxburghiana* (Sasikala et al., 1999) and *A. lawii* (Lavate et al., 2014) also unveil similar results.

Quinones are the class of organic compounds which are found naturally in plants. Most of the common plants belonging to the family Meliaceae have been reported to possess the same composition with regard to their important phytocomponents. The hypoglycemic activity of *Khaya senegalensis* was attributed to the presence of quinones and the studies in the Wistar rats thereby suggest the plant as an anti-diabetic agent (Franck et al., 2016). An anti-diabetic plant *Entandrophragma angolense* was reported to possess considerable amount of quinones (Degaulle et al., 2018). Quinones are well known for their anticancer and antitumor effects. Quinone derivatives and quinone containing drugs are widely used for this purpose nowadays. Quinones bind to eukaryotic 80S ribosomes thereby inhibiting the binding of aminoacyl-tRNA as well as translocation of peptidyl-tRNA which are

essential steps in protein synthesis. This inhibition finally results in the antitumor effect (Prakash et al., 2013b).

The aromatic organic component, phenols and its derivatives are found extensively in the plants that renders multiple functions and bioactivities. The phenolic compounds, especially polyphenols, act as strong antioxidants thereby scavenging the reactive free radicals and stabilizing the biological system (Hsieh et al., 2006). Phenolics are excellent hydrogen donors and quenches reactive oxygen and nitrogen species thereby terminating the radical chain reaction. Polyphenols are also involved in chelating metal ions involved in the oxidative reactions. Due to their hydrophobic ring, they bind to the membrane proteins which inhibit the action of reactive ions, leading to membrane stabilization (Pereira et al., 2009). All the three selected species of *Aglaia* exhibited the presence of phenolics in their extracts.

Flavonoids are derived from the phenolic compounds which are well known for their antibacterial and anticancer effects. *Aglaia* is a well-established source of naturally occurring highly potent flavonoids. The cytotoxic activity of *A. eximia* against P-388 leukemia cells is attributed to the potent flavonoids which were isolated from its bark (Sianturi et al., 2015). The hydroxyl moiety at specific positions in the aromatic ring of the flavonoid enhances its bactericidal ability. Metabolic inhibition of various bacterial functions such as nucleic acid synthesis, energy metabolism, attachment and biofilm formation, alteration of porins on cell membrane and membrane permeability are brought by flavonoids (Xie et al., 2015). One of the reasons for the potential bactericidal ability of *A. malabarica* might be attributed to the presence of considerable quantity of flavonoids (Ravindran & Thoppil, 2018).

Terpenoids are the class of organic compounds that are composed of isoprene units. Several types of terpenes and terpenoid derivatives are found

naturally that contribute to the anticancer (Phongmaykin et al., 2008), antioxidant (Nakatani et. al., 2000) and anti-feedant (Kiranmai et al., 2011) activities of the plants belonging to Meliaceae. Anticancer activity of terpenoids is well noted and this property is facilitated by inhibiting angiogenesis in the newly formed tissues (He et al., 2009). The present study revealed the presence of terpenoids in all the three plants (Table 4).

The organic compounds with a basic nitrogen, alkaloid, is found naturally in various plants and accounts for a wide range of potential bioactivities. As the alkaloids are poorly soluble in water, organic solvent extraction (chloroform) was carried out and tested for the presence of the same. All the three species of *Aglaia* under study revealed the presence of alkaloids (Table 4). Alkaloids and alkaloid containing taxa plays an important role on drug development and discovery of new therapeutic agents ever from the ancient days. From the self-experimentation in the past to the clinical trials of the present time, alkaloids have been a matter of interest (Amirkia & Heinrich, 2014). Most of the Meliaceae members have been proved to possess alkaloids, so as *Aglaia*.

Steroids are a diverse group of organic compounds that play pivotal roles in the membrane stabilization and cell signaling. Plant steroids are derived from the terpenoid compound squalene. Hypocholesterolemic action of phytosterols is appreciable (Gunaherath & Gunatilaka, 2006). Many *Aglaia* spp. are reported to possess cytotoxic and bioactive steroids. Similarly, the three selected taxa of *Aglaia* also exhibited the presence of steroids. Steroids from the leaves of *A. eximia* show cytotoxic effect against several human cancer cell lines (Awang et al., 2012). But low or moderate cytotoxic activity is observed from the isolated steroids from *A. abbreviata* (Zhang et al., 2016). Crude extracts may give a better result as the synergism would act.

The presence of a 3β-hydroxyl group enhances the cytotoxic potential while, sugar and fatty acid lowers the same (Farabi et al., 2017).

The water soluble polyphenolic derivatives, tannins, are considered to lower the nutritional value of the plants. Various kinds of tannins are present in various plants and possess different activities, either beneficial or adverse. Some might be carcinogenic while others act as anti-carcinogenic agents. Antimicrobial properties of tannins in the ripened fruits provides them defense. This property is enabled by the hydrolysis of the ester linkage in gallic acid and polyols after ripening of these fruits (Chung et al., 1998). The three taxa revealed the presence of tannins (Table 4) and further studies are required to isolate and explore their bioactivities and applications.

The tertraterpenoid vital pigment in plants, the carotenoid, was screened and found to be present in all the three taxa studied. Carotenoids are reported to be beneficial antioxidants thus scavenging ROS and protecting from degenerative diseases (Cramer et al., 2001). Their role in apoptosis, gene regulation and angiogenesis has led to new advancement in the regulation of immune function and cancer (Chew & Park, 2004). The presence of carotenoids was detected in all the selected plants under study.

Cardiac glycosides are potent bioactive organic compounds that are clinically important against heart diseases. They act in the Na-K ATPase pump in the cardiac muscles, thereby regulating the heart function (Patel, 2016). Many plants possess the same as a phytoconstituent. The three species of *Aglaia* studied also revealed the presence of cardiac glycosides. Previously, the leaves of *A. lawii* have been reported to be the source of potent cardiac glycosides (Lavate et al., 2014).

The three selected species of *Aglaia* like *A. barberi*, *A. simplicifolia* and *A. malabarica*, were found to hold potential secondary metabolites or

bioactive principles in their extracts. Further study was conducted to determine the approximate quantity of some of the most important phytoconstituents with comparison to the standard pure compounds belonging to the same class.

(ii) Quantitative phytochemical estimation

In order to determine the amount of major phytoconstituents, quantitative estimation of phenols, terpenoids and flavonoids of the selected taxa of *Aglaia* was performed (Table 5). Total phenolic content present in the methanolic leaf extracts was quantified using gallic acid as the standard and expressed in terms of milligrams of gallic acid equivalent per gram of dry weight of the sample. The highest amount of phenolics was observed in *A. simplicifolia* (126.09 ± 8.08 mg GAE/g DW) and the lowest in *A. barberi* (35.89 ± 4.54 mg GAE/g DW) (Table 5). Phenolics are the prime factors responsible for the antioxidant activity of the plants. They donate hydrogen atoms from the O-H moiety or C-H center, which readily react with the reactive oxidative ions and thus terminate the progressing chain reaction. Quenching of the free electrons in the system is an alternative way to stabilize the biological system (Barzegar, 2012). Maximum antioxidant ability of *A. simplicifolia* extract might be attributed to the rich phenolic content in the same.

Total terpenoid content in the plant extracts was determined using linalool as the standard and was expressed as milligrams of linalool equivalent per gram of dry weight of the sample. Estimated amount of terpenoids in the selected taxa revealed the highest quantity in *A. malabarica* (162.32 ± 22.8 mg LE/g DW). The same plant extract exhibited most potent anticancer activity. Terpenoids act as anti-cancer agents either by inhibiting inflammatory responses or by inducing apoptosis in human cancer cells (Li et al., 2016). They are also able to overcome multi-drug resistance of cancer

cells by inhibiting overexpression of glycoprotein on the surface of cancer cells, thus suggesting a co-administration along with chemotherapy (Saeidnia, 2015).

Aluminium chloride method was done to quantify the total flavonoid content in the leaf extracts of the three taxa of *Aglaia*, using quercetin as the standard. The maximum quantity of flavonoids was determined to be in *A. malabarica* (95.67 ± 5.09 mg QE/g DW). Flavonoids are excellent reducing agents and are nowadays employed for the green synthesis of colloidal nanoparticles of several metallic elements. They serve as both reducing agents as well as capping agents thereby stabilizing the produced NPs (Hussain et al., 2019). The uniform sized bioactive nanoparticles show exceptional antimicrobial effect and this property is attributed to the flavonoids that form the capping agents (Sahu et al., 2016). Antiviral, anti-thrombogenic effect and anti-atherosclerotic effects of flavonoids are also reported (Nijveldt et al., 2001).

The selected taxa of *Aglaia* showed considerable amount of potential secondary metabolites in their extracts that render potential bioactivities to them.

(iii) Gas chromatography/mass spectrometry (GC/MS) analysis

The technique employs the qualitative and quantitative analysis of the volatile constituents at a broad level. Specificity and sensitivity of the method made it reliable and common among researchers. The compounds are identified by comparing the individual mass spectrum with the spectral database or library. GC/MS analysis of the methanolic plant extracts of the selected taxa of *Aglaia* was carried out and the compounds got resolved according to their retention time. A total of 112 compounds belonging to several classes were identified. The partitioning of specific molecules in the

gaseous and liquid phases occurs at specific temperature on the basis of which they move through the column. Thus, the temperature is applied on an increasing gradient enabling the separation of compounds accordingly. The polarity and volatility of the metabolites or compounds determine the type of column to be used (Villas-Bôas et al., 2005).

GC/MS profile of *A. barberi* unveiled the presence of several classes of compounds where, terpenes formed the major constituent, which agrees with the quantitative data (Table 6). Along with the terpenes, steroids, alkaloids, flavonoids and other organic compounds were also detected. The sesquiterpenes found were epizonarene, α-cubebene, guaiene, α-selinene, β-selinene, α-bulnesene and copaene which form a total relative percentage of 13.22. Potential terpenoid constituents like digitoxigenin, erythrodiol, oleanolic acid and squalene are the triterpenes identified which constituted about a relative percentage of 8.41. Gitoxigenin, fluocinolone acetonide and dehydro diosgenin are the steroids identified that amounts to a relative percentage of 12.04. The bioactive alkaloid, vincadifformine (3.28%) was found as a prominent component along with an amino acid, 4-hydroxytryptophan (4.09%). The compound with highest peak area percentage obtained was N-methyl pyrrole (13.91%).

Gas chromatographic profile of *A. simplicifolia* revealed 37 compounds belonging to several classes. Diterpenes, sesquiterpenes and triterpenes were identified with a cumulative relative percentage of 14.76. Isocaryophyllene, γ-himachalene, aciphyllene, cedrelanol and 9-methoxy calamenene are the major sesquiterpenes identified that composed of 5.36%. Urs-12-en-28-al (3-acetoxy)-3β, octahydropicenone and lupeol acetate are the triterpenes (6.02%) whereas, abieta-6, 13-diene and agathic acid are the diterpenes (3.38%) resolved from the extract. The ester compound, ethylmethyl butanoate, was found in a considerable amount with a relative

percentage of 13.92. Sterols were found to form around 8.56% of the total content and some of them are γ-sitosterol, gitoxigenin, dimethyl cholestadienol and 3β-pregnanetriol. Other major components were D - alphatocopherol, 4-morpholine butyric acid and 5-hydroxypipecolic acid. The potential alkaloid O-methylpsychotrine was found in a considerable amount that occupied a relative percentage of 7.71.

GC/MS profile of *A. malabarica* revealed 34 compounds belonging to several classes. Diversity in alkaloids was observed in the extract in which pectenin was the one found with the highest relative peak area percentage of 13.81. Along with this, anosmine, dehydroheliamine, and N-trimethyl phenyldiamine were found as alkaloids contributing to the total alkaloid content of 27.13%. Some of the sesquiterpenes identified are α-cubebene, alloaromadendrene, γ-gurjunene, elixene and ledene alcohol. Phytol and thunbergol are the diterpenes whereas; Ekeberin C1, squalene and urs-12-en-28-al are the triterpenes. 13.4% of the total constituents identified were contributed by the terpenes.

Most of the compounds detected in the plant extracts were formerly reported to possess significant bioactivity and therapeutic potentials. The phytoconstituents were also reported from other plants belonging to the same family (Meliaceae) as well as those belonging to other families. Pyrroles are present in many natural products, drugs and chemical catalysts and the derivatives have been reported to possess hepatoprotective ability, cholesterol level lowering property in hypercholesterolemia and hyperlipidemia cases (Chin et al., 2003). Pyrroles along with furan compounds facilitate excellent free radical scavenging activity (Shiratsuchi et al., 2012). The non-conducting polymer of pyrrole is used in batteries and solar cells for insulation (Michlik & Kempe, 2013).

Terpenoids are the most abundant secondary metabolite found among the constituents of natural products. The diverse effects and complex structure makes them interesting to the researchers and provides wide use in pharmaceuticals, food and cosmetic industries. In plants, terpenoids play a key role in growth and development, as well as response to external stimuli and physiological processes. Triterpenoids are of much interest in the anticancer research due to their potent cytotoxicity against the malignant cells while, least or null effect on the normal tissues (Žiberna et al., 2017). *A. barberi* was found to possess a wide array of terpenoids and detailed studies of some of them along with some other major compounds are as follows:

Epizonarene is the cadinane sesquiterpene which is thermodynamically the most stable compound. It imparts bactericidal activity of the plants belonging to Meliaceae. Surplus reports on the occurrence of epizonarene in Meliacean members are available. Leaf essential oil of *Eugenia cotinifolia* (Sarvesan et al., 2015), essential oil from the fruits of *Harungana madagascariensis* (Gbolade et al., 2009), *Carapa guianensis* (Meccia et al., 2013) and leaf essential oil from *Cedrela fissilis* (Setzer, 2008) possess epizonarene. The sesquiterpenoid obtained with highest peak area percentage of 7.71% in *A. simplicifolia* was epizonarene (Table 6).

Gitoxigenin is the steroid formed as a result of hydrolysis of cardiac glycoside gitoxin, which belongs to a group of cardio-active steroids with an unsaturated F-lactone. The compounds are collectively known as cardenolides which act as Na+/K+-ATPase inhibitor which makes them clinically useful in the treatment of heart failure. The potent anticancer activity of the compound is believed to be brought about by the activation of PMC (promyelocytic leukemia protein) nuclear body which is a tumor suppressor protein. It also induces apoptosis in the renal adenocarcinoma cancer cells (Lopez-Lazaro et al., 2003).

Vincadifformine is a natural product which is quite common in Apocynaceae plants as a functional plant metabolite. The monoterpenoid alkaloid methyl ester has a complex structure with a cage-like fused pentacyclic skeleton. The compound shows a pronounced anti-plasmodial effect against chloroquine resistant strain and also possesses antimalarial activity (Mohammed, 2009). The cytotoxic efficacy of the compound against HeLa cells (Mustofa et al., 2006) and vincristine-resistant human cancer cells make it therapeutically important (Lim et al., 2007). The compound as well as the analogues exhibit significant cytotoxic effect against 60 types of human tumor cell lines obtained from nine cancer types. Thus, great efforts have been devoted for its chemical synthesis considering the invaluable therapeutic value (Pan et al., 2019).

The phytosteroid, dehydrodiosgenin is the precursor for several hormones including progesterone. The compound decreases the level of murine induced obesity which is enabled by the reduction of resistin and adiponectin levels (Perez-Garcia et al., 2017). Dehydrodiosgenin was found to be present in *Gnaphalium uliginosum* (Leonidovna et al., 2019), *Anastatica hierochuntica* (Saranya et al., 2019), *Pennisetum purpureum* (Strezov et al., 2008) and *Tephrosia vogeii* (Antonio et al., 2019).

The sesquiterpenoid compound, guaiene, is formed by the C2-C6 cyclization. Naturally found guaienes are of three types - α, β and δ amongst which α-guaiene is the most common one. Commercially, it is used in fragrance and flavoring industries since long time. Aerial extracts of *Inula helenium* (Jiang et al., 2011) and essential oil from the seeds of *Aframomum melegueta* (Menut et al., 1991) are some of the richest sources of natural guaiene. Use of agar wood as the source of fragrance and medicines is due to the presence of guaiene like products as the flavoring agents in them (Kumeta & Ito, 2011).

Cubebene is a sesquiterpene with two forms of natural existence-α and β. Essential oil of *Azadirachta indica* (Aromdee et al., 2003), volatile oil from the stem bark of *Guarea macrophylla* (Lago et al., 2007) along with *A. malabarica* (Table 8), the plant under study, possess α-cubebene. Leaves of *Magnolia grandiflora* (Lee & Chappel, 2008) and volatile oil from the aerial parts of *Stachys byzantina* (Khanavi et al., 2003) had their major component as β-cubebene. α-iso-cubebene, the analogue of cubebene, was isolated from *Schisandra chinensis* and was reported to exhibit anti-neuroinflammation and neuro-protective property against amyloid induced inflammation in microglia (Park et al., 2013). The tangy smell of *Toona sinensis* may be due to the terpenes including cubebene (Hsu et al., 2012). Another sesquiterpene, selinene, also occurs in α, β and δ confirmations. Sometimes α and β-forms exist together in a plant, as in *Cyperus rotundus*. It provides cytoprotection against gastric ulcers. Selinene also provides the unique odor to the plant (El-Gohary, 2004). β-silenene was detected in the oil of *Artemisia annua* (Stephanache et al., 2017). δ-silenene is found in the essential oil of *Aster albescens* (Kumar et al., 2019).

Bulnesene with α-conformation have potent biological role as a nutrient component, membrane stabilizer and as a source of energy storage. Industrial applications include their use as surfactant and emulsifier. Platelet activating factor (PAF) level increases as a result of inflammatory action. The α-conformation of bulnesene, isolated from *Pogostemon cablin* provided anti-PAF activity and decreased the intensity of inflammation (Tsai et al., 2007). Allelopathic effect of the constituents against oats, rye, grass and onions are also reported due to the presence of this specific compound (Komai et al., 1981).

Digitoxigenin, the cardenolide is an aglyacone, formed by the replacement of glycosyl group of the glycoside (digitoxin) by hydrogen. The

compound is being reported in many plants which are of great therapeutic use. The cardio-active property of *Digitalis* might be attributed to the presence of the same along with other glycosides. Chloroform soluble fraction of methanolic extract of bark of *Thespesia populnea* (Gopalakrishnan et al., 2019) and extracts of *Corchorus olitorius* (Yamaki et al., 1999) contained digitoxigenin as revealed by GC/MS analysis.

The pentacyclic triterpene, erythrodiol, is a very important phytocomponent which have been explored much extensively due to the valuable therapeutic actions. In the present study, *A. barberi* was found to contain the same in the methanolic leaf extract (Table 6). It was also reported to be isolated from the bark extract of *Toona sinensis* (Meng et al., 2016). Erythrodiol is able to prevent lipid peroxidation and suppress superoxide anion generation, thereby rendering antioxidant effect. It exhibit both pro-inflammatory and anti-inflammatory activities based on the chemical structure and dosage thus, useful in modulating the immune responses (Marquez-Martin et al., 2006; Rodriguez-Rodriguez et al., 2004). Further studies on the antiproliferative and apoptotic activity against human adenocarcinoma cells were also reported. This property was made possible either by induction of cell cycle progression or by the induction of apoptosis, or both. Increase in caspase-3 activity and loss of cell adhesion in a dose dependent manner indicated the pro-apoptotic activity induced by erythrodiol (Juan et al., 2008). Antioxidant and antithrombotic effects of erythrodiol are also reported. Atherosclerosis is formed by the oxidation of LDL and thrombin, which incorporates as atherosclerotic plaques, leading to thrombosis which might be often fatal. Hydroxyl moieties at the C-3 and C-17 position of erythrodiol effectively prevent LDL assisted thrombin generation, finally leading to antithrombotic effect (Allouche et al., 2010). The primary hydroxyl group at C-28 influences the anti-inflammatory effect which is effective against chronic inflammation of skin (Meng et al., 2016).

Oleanolic acid is a pentacyclic compound with high biological and therapeutic significance including antioxidant, anti-inflammatory, hepatoprotective, cardioprotective, antipruritic and anti-allergic properties (Parikh et al., 2014). The antihypertensive activity was enabled by decreasing the mean arterial pressure and elevation of the urinary Na+ outputs. Moreover, antioxidant activity in cardiac, hepatic and renal tissues was made possible by increasing the activity of the enzymes like superoxide dismutase and glutathione peroxidase (Madlala et al., 2015). Oleanolic acid is gaining wide acceptance due to its ability to inhibit the multi-drug resistance associated with chemotherapy in cancer treatment (Braga et al., 2007). The compound induces apoptosis which is mediated by caspases as well as elicits DNA fragmentation in human cancer cells (Yan et al., 2010). The anti-inflammatory effect of oleanolic acid is characterized by inhibiting the release of high mobility group box-1 (HMGB-1) protein, which if reaches extracellular fluid, act as potent inflammatory agent finally, resulting in the suppression of sepsis and other inflammatory responses (Kawahara et al., 2009). Camer et al. (2014) explained the anti-inflammatory effect in a different approach. It is capable of inhibiting NF-κB and cytokines associated with inflammation. Oleanolic acid promotes the uptake of glucose from the blood stream to the peripheral tissue through the up-regulation of GLUT-4 or by inhibiting endogenous glucose production by means of gluconeogenesis in liver (Ovesna et al., 2006). GC/MS studies of the methanolic leaf extracts of *A. barberi* (Table 6) reveals the presence of considerable amount of oleanolic acid in it. Oleanolic acid was detected and isolated from the stem and fruits of *Cedrela fissilis* (Leite et al., 2005) belonging to the same family, Meliaceae.

Squalene, an important triterpenoid organic compound, forms the precursor of all the plant and animal sterols, including cholesterol and human steroid hormones. Cyclization of squalene leads to the development of other triterpenoids thereby, forming the precursor for the synthesis of steroids

(Phillips et al., 2006). Interesting feature of the compound is that it behaves as cytoprotective agent for normal cells while cytotoxic to the neoplastic and tumoral tissues, hence could be used as combination drug along with chemotherapeutic agent like ACNA for a significant positive effect. Squalene also optimizes immune system by stimulating the macrophages. (Ronco & De Stefani, 2013). Smith (2000) found that squalene is effective in inhibiting chemically induced colon, lung and skin malignancies. The compound was able to inhibit Ras-farnesylation, modulation of carcinogen activation and antioxidant activities. The intense ability to acquire or release electrons without affecting the molecular structure makes squalene a potent antioxidant. It protects acids like linoleic, linolenic, docasahexanoic and eicosapentanoic acid from temperature induced self-oxidation (Ranco & De Stefani, 2013). Squalene isolated from dichloromethane leaf extract of *Dysoxylum gaudichaudianum* was observed to exhibit cytotoxicity against human cancer cells like MCF-7 and HT-29 (Ragasa et al., 2014). Presence of squalene was also reported in the extracts of aerial parts of *Aglaia argentea* (Hidayat et al., 2018). In the present study, *A. barberi* (Table 6) and *A. malabarica* (Table 8) revealed the presence of considerable amounts of squalene with 1.07 and 3.10 peak area percentages, respectively.

Isorhoifolin is a flavonoid component with 5-o-methylation. The chemical structure is composed of flavone moiety linked through glycosidic bond to two sugar units (Diculescu et al., 2012). Commercially available Daflon @ 500mg used for chronic venous disease (CVD) and hemorrhoidal disorders is the purified form of micronized purified flavonoid fraction (MPFF) with isorhoifolin as the primary component (Paysant et al., 2008). Efficient anti-diabetic property of the compound is also well reported. *Pilea microphylla*, rich in isorhoifolin and other flavonoids is used as anti-diabetic agent in Chinese traditional medicine. The flavonoid rich fraction of the plant attenuated metabolic abnormalities and improves pancreatic functions (Bansal

et al., 2011). The anti-diabetic effect of the compound suggests everyday intake of the same as dietary component (Jaitak, 2019). Boghrati et al. (2016) reported the antioxidant and anti-tyrosinase activity of isorhoifolin isolated from *Teucrium polium*. The present study revealed the presence of isorhoifolin in the methanolic leaf extract of *A. barberi* (Table 6) by means of GC/MS analysis.

Isocaryophyllene, the aromatic sesquiterpene, was reported in several members of the family Meliaceae. It was reported to be present in the wood essential oil of *Swietenia macrophylla* (Saurez et al., 2019), stem essential oil of *Guarea guidonia* (Nunèz et al., 2005), seed volatile oil of *Azadirachta indica* (Ramalakshmi & Sankar, 2018), leaf essential oil of *Toona ciliata* (Duan et al., 2015), whole plant hexane extract of *Munronia pinnata* (Napagoda et al., 2014), flower extracts of *Melia azedarach* (Kharkwala et al., 2015), *etc.* Cytotoxic potential of the compound isolated from *Khaya senegalensis* and *K. grandiflora* flower extracts showed cytotoxicity against HepG2, MCF-7 and HCT-116 cancer cell lines (El-Souda et al., 2016). Anticancer activity of the compound was proved against human cancer cells like MCF-7 and DLD-1 while, non-toxic effect was observed against L929 at minor levels (Legault & Pichette, 2007). It provides somewhat protective effect at the lower concentrations and allows membrane permeability as well as lipid peroxidation at higher concentrations (Legault et al., 2013). Thus, cytotoxic effect was enabled by increasing the membrane permeability and cell shrinkage. Isocaryophyllene blocks the electron transport chain by directly inhibiting the complex-I function (Monzote et al., 2009).

Himachalene is a sesquiterpene that exist in three isomeric forms - α, β and γ. Himachalene was found to be the prominent component in the root essential oil of *Naregamia alata* and was proved to exhibit excellent anti-bacterial activity (Xavier et al., 2011). Himachalene forms aromatic rings and

phenolic hydroxyl groups through cyclization of C10 and C15 moieties. They are able to bind with the active sites of the target bacterial enzymes. Alongside, other terpenes, alcohols, aldehydes and esters can contribute to the overall antimicrobial effect of the essential oils (Oyedeji et al., 2005). The anti-inflammatory and anti-tumor effects of the compound are also well reported (Menichini et al., 2009). Other plants like *Swietenia macrophylla*, *Guarea macrophylla* (Oliveira et al., 2019), *Cedrela odorata* and *Toona ciliata* (Soares et al., 2003) contain himachalene in their extracts. Another terpene, Himachala-2, 4-diene, was identified through GC/MS analysis of *A. simplicifolia.* Interestingly, this compound has also been previously reported from the liverwort *Bazzania japonica* (Lu et al., 2003). It was found to be the prominent component of the leaf essential oil of *Toona sinensis* and have weak antioxidant effect (Congjin et al., 2013). The same compound isolated from *Abies alba* was reported for its wound healing property (Yarmolinski et al., 2017).

Aciphyllene is a guaniene sesquiterpene first isolated from the root essential oil of *Lindera glauca* and later confirmed in the essential oils of *Shorea robusta*, *Toona sinensis* (Srikrishna & Pardeshi, 2010), *Croton oblongifolius* (Athikomkulchai et al., 2015) and *Pogostemon cablin* (Swamy & Sinniah, 2015). It is also one among the major constituents of the oil from *Stachys tibetica* and the anti-inflammatory effect of the oil is attributed to the presence of the aciphyllene (Kumar et al., 2012). Aciphyllene was found to be responsible for attracting specific pollinating wasps by the *Ficus* spp. (Grison-Pigé et al., 2002). GC/MS results revealed the presence of aciphyllene in the methanolic leaf extracts of *Aglaia simplicifolia* (Table 7).

O-methyl psychotrine is an isoquinoline alkaloid found to be present in *A. simplicifolia* leaf extract. It was reported to be a selective inhibitor of human immunodeficiency virus-1 reverse transcriptase. It can inhibit DNA

polymerase activity of HIV but shows negligible effect on human and bacterial genetic material and polymerases. At the same time, the analogues were found to be active against human polymerases. Thus, the unique structural properties enable the unique interaction with the specific viral polymerases (Tan et al., 1991).

Mequinol, methoxy phenol was found to be present in the methanolic leaf extract of *A. simplicifolia*. It is one among the prominent ingredients of the topical drugs used for skin depigmentation. It is often used in combination with tretinion for the treatment of solar lentigens and skin lightening by ethnic populations (Draelos, 2006). The tyrosine inhibiting ability of mequinol is appreciable and is available in the market as cosmaceuticals, in combination with other constituents (Fleischer et al., 2000). *Cis*-isoeugenol is another phenolic compound or phenyl propenoid which forms an important constituent of many natural volatile oils. The woody odor of the toasted oak wood is due to the abundance of mequinol (Diaz-Maroto et al., 2008). Iso-eugenol and the isomeric phenols are often used as chemical probes to elucidate various enzymatic pathways including laccase catalysis, which is the initial step in lignification and delignification (Qi et al., 2015). The presence of the compound was detected in the wood of *Cryptomeria japonica,* treated with super critical water (Takada et al., 2004). It is also a component of the essential oil of *Pimpinella anisum* (Saibi et al., 2012).

Lupeol acetate is a triterpenoid compound well reported for its potential bioactivities and in the present study, it was detected from the extract of *A. simplicifolia*. Anti-inflammatory efficacy of the compound is enabled by inhibiting MPO release from the stimulated human neutrophils and decreasing the number of cells expressing iNOS activity, COX2 expression and NO production (Lucetti et al., 2010; Chen et al., 2012). Anti-venom activity of the compound was studied against neurotoxic as well as

hemotoxic venoms. Lupeol acetate significantly neutralizes lethality, haemorrhage, defibrinogenation and edema as well as cardio-toxicity, neurotoxicity and respiratory changes. It also counteracts venom induced lipid peroxidation and superoxide dismutase activity (Chatterjee et al., 2006). The compound showed an efficient anti-arthritic effect by increasing osteoblastic activity in the arthritic rodents. This activity was enhanced by reducing serum creatinine, preventing the reduction and maintaining the normal level of alkaline phosphatase. All these lead to the reduction in ankle swelling (Kweifio-Okai & Caroll, 1993). The compound was reported in the roots of the hepatoprotective plant, *Hemidesmus indicus* (Girish & Pradhan, 2012), *Aerva lanata* (Taranisen, 2014) and the anti-malarial plant *Gongronema latifolium* (Adebajo et al., 2013).

Agathic acid, the diterpenoid compound is the starting material of many bioactive principles including the limonoid azadiradione (Fernandez-Mateos, 1990). Agathic acid and its derivatives are capable of inhibiting superoxide anion (Chinou, 2005). It also shows excellent anti-leishmanial activity with the toxicity in a margin of 12-fold against the parasite rather than to the host. This activity was brought by rapidly increasing the plasma membrane permeability and mitochondrial depolarization in the cells of the parasitic protozoan (Santos et al., 2013a). Agathic acid and its derivatives have also been reported to be present in the copal gum of *Bursera bipinnata* (Choudhary & Pawar, 2014), bark of *Juniperus osteosperma* (Gardner et al., 2010) *etc.*

Sitosterol is the naturally occurring plant steroid which is widely distributed. *Azadirachta indica* leaf extracts (Akpuaka et al., 2013), *Bauhinia platypetala* (Dos Santos et al., 2012), crude oils of *Terminalia catappa* (Adu et al., 2013) *etc*., were reported to contain sitosterol of different natural conformations. The antioxidant, antibacterial and prophylactic activities of the

Azadirachta extracts are attributed to the action of γ- sitosterol. Leaves of *Moringa oleifera* possess allelopathic action against wild mustard seeds that could be due to sitosterol (Tahir et al., 2018). Human ailment like hyperlipidemia was observed to get suppressed significantly and this was confirmed by docking studies. Sitosterol was suggested as a significant molecule which docks well with least binging energy and inhibition constant values. Thus, it could be effectively developed into a potent hypolipidemic agent (Balamurugan et al., 2015). A considerable relative percentage composition of γ-sitosterol was detected in the methanolic leaf extract of *A. simplicifolia*.

D-Alpha tocopherol is a type of biologically active, naturally occurring vitamin E. The compound is well known for the anti-atherogenic activity (Verlangieri & Bush, 1992). It is able to decrease the release of reactive oxygen species, lipid peroxidation, IL-1β secretion and endothelial-monocyte cell adhesion. All these suggest the compound to be an effective agent against atherosclerosis and can be used as an antioxidant molecule. The inhibition of the aforesaid processes is made possible by the inhibition of protein kinase C (Devaraj et al., 1996). Ability to prevent the activation of protein kinase C as well as suppress the diacyl glycerol levels is often associated with renal dysfunctions in diabetic cases. Thus, the compound could also be employed in the prevention of diabetic glomerular dysfunction (Koya et al., 1997). Comparably huge amount of D-alpha tocopherol was detected in the methanolic leaf extract of *A. simplicifolia* (Table 7) with a relative peak area percentage of 10.17 in the GC/MS spectrum analysis.

Carnegine is a tyrosine derived organic compound which is a tetrahydroquinoline alkaloid. It has been isolated from several plants like, *Pachycereus pectin-arborigium*, other Cactales, Chenopodiales, *etc.* Existence of carnegine along with anhalidine is of high chemotaxonomic value.

Hallucinogenic drug like properties of the compound makes it interesting. Toxic effect to the nervous system was revealed by the strychnine-like tono-clonic convulsions. As a result of toxic intake of the compound, synsual bradycardia might happen. Meanwhile, the lower concentrations of carnegine would bring a counteraction against the chronotropic effect of the adrenaline and noradrenaline even without affecting their inotropic properties. Carnegine is also effective in stimulating the rate and amplitude of respiration, eliciting slight spasmolytic and vasodilator activities in total leading to a hypotensive effect whereas, no effect was observed on phosphodiesterase activity in brain (Bracca & Kaufman, 2004). Another alkaloid found in a trace amount was dehydroheliamine which is a dihydroquinoline compound. The bioactivities reported are when the compound is in combination with carnegine (Ordaz et al., 1983).

Anosmine is an imidazole alkaloid derived from two lysine units- one providing C-5 N unit and the other one providing C-6 N, leading to the formation of pipecolic acid and cadaverine. These compounds cyclize and give rise to the active alkaloid, anosmine (Hemscheidt & Spenser, 1991). Anosmine is the only water-soluble imidazole-type compound found in nature. Plant extract fraction that contains anosmine shows effective antioxidant property, α-glucosidase inhibition and anti-inflammatory effect (Kongkatitham et al., 2018). Leaf methanolic extracts of *A. malabarica* contained ample quantity of alkaloids like carnegine (13.81%), dehydroheliamine (5.46%) and anosmine (5.34%) (Table 8), thereby making it an interesting candidate for toxicity studies. Thus, the present study also aims to determine the toxicity actions and consequences of the plant extract on different test systems.

Alloaromadendrene is a sesquiterpenoid compound with aromadendrene carbon skeleton and is a precursor molecule for several

bioactive natural compounds. Natural volatile oils of several plants have been reported to contain this terpenoid compound and most of these plants have been studied for various bioactivities. The anti-termitic and insect repulsive property of *Dipterocarpus* wood is explained by the presence of alloaromadendrene along with humulene and caryophyllene (Messer et al., 1990). Due to the efficient antioxidant property, it is effective in delaying the aging of cells by reducing oxidative stress (Yu et al., 2014). The compound was found to possess significant cytotoxicity and inhibitory effect against the malignant mammary epithelial cell growth which was evidenced by reduced number of viable cells (Sawant et al., 2007). The cytotoxic effect of essential oil from *Piper gaudichaudianum* against V-79 cells was evident to occur without the induction of mutagenicity. This could be related to ROS and DNA single strand breaks developed in the presence of oxidative lesions (Sperotto et al., 2013). The cytotoxic effect of the compound was made possible by inducing apoptosis in the human cancer cells like C6, A549 and MCF-7 (Duymus et al., 2014). Considerable quantity of alloaromadendrene was found in *A. malabarica* (Table 8) and thus cytotoxic studies using the same becomes relevant. Ledene alcohol is an oxygenated seqsuiterpene which brings strong and effective antioxidant activity when in combination with other sesquiterpenes (Li et al., 2013). Strong allelopathic activity of the compound was also reported (El-Gawad et al., 2019).

Phytol, the acyclic diterpene alcohol is the precursor of the synthesis of vitamin E and K1 which is a branched chain unsaturated alcohol. Naturally, it is well distributed in all the plants as a structural unit of chlorophyll. Anti-nociceptive effect and *in vitro* antioxidant activity of phytol is reported in several models. Reduction in nociception is brought by significantly reducing abdominal contortions and may be associated with an anti-inflammatory effect (Santos et al., 2013b). Phytol metabolites derived from the phytyl chain of chlorophyll exhibit anticancer and antimicrobial activities (Kumar et al.,

2010b). Phytol has the ability to activate the nuclear hormone receptors which affects gene expression and cell differentiation. Neuronal functions are altered which influence neurotransmitter systems, thereby affecting seizure process. This anti-convulsant effect can be thereby made use for the development of the drug against epilepsy (Costa et al., 2012). Other meliacean plants like *Cedrela sinensis*, *Toona ciliata*, *Trichilia connaroides*, *etc*. were reported to possess considerable quantity of phytol (Luo et al., 2000; Negi et al., 2011; Senthilkumar et al., 2012). Strong antibacterial activity against *Pseudomonas aeruginosa* induced by phytol was observed by Lee et al. (2016). Phytol induces oxidation of GSH and elevates oxidative stress which is resulted from the accumulation of reactive oxygen species in the bacterial cells. It also inhibits bacterial cell division and in total, the whole population gets destroyed by DNA damage and membrane depolarization (Lee et al., 2016). Phytol as well as the nanoemulsion developed from the same exhibited cytotoxic and genotoxic effects on *Allium cepa* (Islam et al., 2017). Intense cytotoxic effect of phytol, observed against MCF-7 and HeLa cells, was believed to occur by two ways - by affecting the cell growth and viability or by inducing apoptosis (Thakor et al., 2016). Antitumor effect on hepatic carcinoma cell lines like Huh 7 and Hep G2 were also reported. Phytol is able to cleave PARP, activate caspases-9/3 and bax along with the attenuation of bcl-2 & c-My c, suppress cell adhesion and produce fibroblast-like mesenchymal cells. All these effects suggest that, antitumor effect of phytol is enabled *via* induction of apoptosis as well as inhibition of epithelial mesenchymal transition signaling (Kim et al., 2015). Antiproliferative effect is made possible without disturbing the membrane integrity but, by depolarizing mitochondrial membrane, elevating cell population at sub-G0 phase, down-regulating bcl-2 as well as activation of caspases. Inhibition of CAM vascular tissue development was inhibited by phytol and this suggests anti-angiogenic property of the compound (Sakthivel et al., 2018). All these

characteristics are highly desirable for the active principles to be developed as anticancer drugs.

The bioactivities exhibited by the plant extracts might be attributed to the different active principles present in them in different quantities. In the above mentioned context, the three selected species of *Aglaia* contained biologically potent phytochemicals as their components. The rest of the study deals with the detailed investigation on the bioactivities displayed by these plants.

PHASE II - BIOACTIVITY SCREENING

A. ANTIOXIDANT ABILITY

Redox reactions form the core of the metabolic machinery of a living system which involves the relocation of electrons and hydrogen atoms among the reactant molecules. Evolution has selected oxygen as the carrier or reactant molecule in the living system due to the unique distribution of electrons in its outer shell. This makes it unable to accept a spin-matched pair of electrons until one of its electrons undergoes spin reversal. But, under normal conditions, the time of contact is too less for the chance of a spin reversal which makes a high kinetic barrier. In other words, the activation energy required for the electron transfer or the reaction to take place is very high, making it a stable component. Thus, the barrier creates a condition of high thermodynamic oxidizing potential by suppressing the chance of an explosive reaction. This makes oxygen ideal in a living system and so it acts as a terminal electron acceptor in many biological reactions. Enzymes in the living system can overcome this barrier by lowering the activation energy. The breaking up of electron pairs in an oxygen atom to accept the electrons results in the formation of free radicals. Chemically, a free radical is a molecule with a single unpaired electron which is highly reactive (McCord,

2000). Oxidative free radicals are the products of electron reduction which includes superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), peroxy radical ($ROO^{\bullet}$) and alkoxy radical ($RO^{\bullet}$), together known as reactive oxygen species (ROS). Though some of the ROS are beneficial, overproduction of the same makes the living system unable to build an antioxidant defense system and results in oxidative stress. These reactive entities can initiate and propagate free radical chain reactions which ultimately results in extreme cellular damages (Riley, 1994). Oxidative stress is believed to make a significant contribution to inflammatory diseases like arthritis, vasculitis, glomerular nephritis, heart diseases, emphysema, gastric ulcers, Parkinson's syndrome, multiple sclerosis, *etc*. (McCord, 2000).

Another set of destructive molecules include peroxynitrite, nitric oxide, nitrosyl radical, *etc*., which are collectively known as reactive nitrogen species (RNS). The reactive atmosphere leading to the physiological stress called nitrosative stress is created by the accumulation of RNS. Metabolic alterations like lipid nitration, protein tyrosine nitration, protein-s-nitrosylation, *etc.* are the consequences of nitrosative stress. Cumulative effects of the redox stress result in damages like DNA strand breaks, oxidation of vital organic molecules, membrane disintegration and ultimately, necrosis. Cellular enzymes like catalases, superoxide dismutase, glutathione peroxidase, *etc*. can break down or scavenge these free radicals (Riley, 1994). The loss of balance in the free radical synthesis and scavenging by the in-built antioxidant defense system occurs due to the over-accumulation of reactive species which finally results in the stressed condition. This necessitates the external supply of additional antioxidants to restore metastasis.

Antioxidants are the molecules that are able to scavenge these accumulated reactive species thereby, reducing the chance of necrotic tissue damage and associated inflammation. Plants are excellent repositories of

compounds that can annihilate the reactive free radicals in a biological system. They provide cellular protection against DNA damage, membrane destruction, and other damages. Surplus reports are available that explain the antioxidant ability of the plants. Many synthetic antioxidant products have their initial origin from nature. The antioxidant and protective ability of the three selected species of *Aglaia* was determined using four different assays.

(i) DPPH radical scavenging assay

Being a simple, inexpensive and quick method, DPPH radical scavenging assay was used to determine the antioxidant ability of the plant extracts of all the three selected taxa of *Aglaia*. Unlike other free radicals, DPPH does not get dimerized thus remains as a stable free radical. The technique was developed by Blois (1958) where, the reduction of the stable free radical DPPH is measured. The electrons are delocalized around the molecule in the alcoholic solution and this imparts pink or violet color to the solution. The odd or unpaired electron in the nitrogen atom of the DPPH radical is reduced by the active antioxidant compounds in the plant extracts by the corresponding release of the hydrogen atom to the hydrazine moiety (Contreras-Guzmán & Strong, 1982). On the addition of the plant extract to the alcoholic solution of DPPH, active antioxidant components in the extracts are released to the system where the free electrons in the reagent (DPPH) are paired off. This subsequently leads to the discoloration of the reaction mixture and the same is measured spectrophotometrically after a definite period. The reaction is so sensitive that it occurs even in the presence of a weak antioxidant (Kedare & Singh, 2011). Highest scavenging activity was obtained for *A. simplicifolia* with an IC_{50} value of 131.87 µg/mL which was followed by *A. malabarica* (IC_{50}=153.22 µg/mL). Only mild scavenging ability was showcased by *A. barberi* leaf extract. Reports of antioxidant ability of other taxa of *Aglaia* are also available. Considerable antioxidant

activity was observed by the bark constituents of *A. eximia* (Sianturi et al., 2016b) while, a strong scavenging property was observed by the fruits of *A. elaeagnoidea* (Bidari et al., 2017). These differences in the scavenging ability in the same genus might be due to the diversity in the phytoconstituents or the variation in the concentrations of the active principles in different taxa.

(ii) ABTS radical scavenging assay

The assay is used in food and chemical industries to determine the scavenging ability of a particular antioxidant. ABTS is a stable compound that becomes a radical monocation when oxidized in the presence of potassium persulfate. A monocation is a cation formed by the removal of a single electron from a neutral entity. The ABTS radical monocation thus formed is reduced in the presence of the hydrogen factor donated by the active antioxidants. The extent of reduction depends on the concentration of the antioxidants as well as duration of the reaction. This is also a colorimetric assay, where the reduction reaction is quantified using spectrophotometric measurements. The assay is useful to determine the reducing potential of both the aqueous and the lipophilic systems and thus, used for a broad spectrum analysis (Re et al., 1999).

All the three selected taxa of *Aglaia* were analyzed using ABTS assay for its reducing potential, which revealed a gradual increase in the activity of *A. malabarica* with increasing concentration. At low concentration, the highest activity was observed for *A. malabarica* but as the concentration was increased, the equivalent reducing ability was observed for *A. malabarica* and *A. simplicifolia*. Comparatively a lower reduction potential was observed for *A. barberi*. The antioxidant potential of the plant could be attributed to the presence of phenolic components in the plant extract. These compounds can quench the free electrons in the solution. The methanolic leaf extract of *A. barberi* was explored to possess comparatively less amount of phenolic

compounds than the other two taxa. Thus, the antioxidant activity results were obtained in accordance with their phytochemical composition.

ABTS radical scavenging assay is not completely reliable always. The reaction between the antioxidants and the ABTS radical may not be completely under the conditions provided in the reacting atmosphere. The total antioxidant capacity of the samples may vary when analyzed *via* other assays. For the effective determination of antioxidant ability, ABTS radical scavenging assays are suggested to carry out coupled along with Trolox equivalent antioxidant capacity (TEAC) assay (Arts et al., 2004). In total, the results from this particular assay have only limited reliability and require further confirmative analysis to establish the antioxidant ability of the plant extracts.

(iii) Hydroxyl radical scavenging assay

Hydroxyl radical is the most reactive oxygen centered free radical species that causes severe and deleterious effects on the adjacent biomolecules. The radical is the reduction product of molecular oxygen with three free electrons, making it the most powerful and highly reactive entity. The hydroxyl radical ($HO^{\bullet}$) can react with any biological micro or macromolecule thereby inactivates or disrupts the membrane and cellular proteins, lipids, DNA and RNA (Cederbaum, 2017).

Hydroxyl radical scavenging assay is a reliable method to determine the antioxidant efficacy of a sample. In the present study, the Fenton reaction was employed for the generation of reactive hydroxyl radicals. Fe^{3+}-ascorbate-EDTA-H_2O_2 reaction mixture at a particular pH enabled the production of hydroxyl radicals which when treated with the extracts undergo reduction, and the extent of reduction was measured spectrophotometrically at the wavelength of 532 nm. Among the selected plants, *A. malabarica* and *A.*

simplicifolia showed considerable hydroxyl radical scavenging potential when compared to that of *A. barberi* (Table 9). The phenolics and the terpenoids in the extracts might be the reason for the reducing ability. Quantitative estimation of the phenolic compounds in the plant extracts was also observed to serve accordingly. *A. malabarica* and *A. simplicifolia* showed a considerable amount of the active phenols in the quantitative studies.

(iv) Superoxide radical scavenging assay

A superoxide radical is a potent free radical which is a product of one-electron reduction of molecular dioxygen. It is an anion with a single unpaired electron and a net charge of -1, widely occurring in nature. In a living system, superoxide free radicals exert severe metabolic damages like deactivation of vital mammalian enzymes (*eg.* myofibrillar ATPase, glutamine synthase, creatine phosphokinase, *etc.*). It is also involved in the destructive events like the initiation of DNA damage and enhancing the rate of lipid peroxidation.

In the present study, superoxide radicals were generated in a riboflavin-NADH system where NADH acts as the oxidizing agent. On the addition of the plant extract, the reducing agents in it react with the superoxide radicals and releases H+. NBT in the reaction mixture gets reduced by this release of H+ producing a blue-colored formazan product. This color formation is recognized as the antioxidant ability of the plant extract and is measured spectrophotometrically, at 560 nm. The maximum reducing ability was observed when treated with *A. simplicifolia* extract agreeing with the results of most of the previous antioxidant assays. *A. malabarica* also showed a considerable antioxidant effect while only a mild effect was displayed by *A. barberi*.

The antioxidant ability of the plant extracts is often attributed to the presence of potential secondary metabolites, primarily the phenolic

components. Phenolic compounds are excellent hydrogen donors and thus can react with reactive oxygen and nitrogen species thereby, terminating the chain reaction. The hydroxyl moiety interacts with the pi-electrons of the benzene ring which imparts unique properties to it (Pereira et al., 2009). Terpenoids also acts as reducing agents to a certain extent. All the three plants screened exhibited moderate antioxidant ability where *A. simplicifolia* gave comparatively better results. Among the three plants screened, *A. simplicifolia* was found to contain the highest quantity of phenolic compounds which could be the possible reason for the antioxidant or protective efficacy of the same. *A. barberi* was assessed with comparatively lower amounts of phenols as well as other active ingredients like flavonoids and terpenoids.

Many reports of the earlier times have correlated antioxidant and anticancer studies, suggesting that free radical accumulation leads to cancerous effects. But recent reports provides explanation in another view. Accumulation of free radicals in a system brings a destructive effect that leads to degenerative diseases like Alzheimer's disease, Parkinson's syndrome, hepatic degenerative diseases, *etc*. These might often lead to necrotic death and finally, inflammation. Antioxidants play a significant role in the removal of the destructive free radicals, thus rejuvenating the system with healthier tissues and initiating the production of new cells. The strong anti-oxidant effect of the seed extract of *Azadirachta indica* was proved to be the beneficial factor for the neuro-protective, anti-ischemic and anti-apoptotic effect of the same (Vaibhav et al., 2013). This suggests that the anti-oxidants have protective effect and checks the accumulation of the free radicals in the system. Meanwhile, cancer is characterized by the production of abnormal cells and so, the antioxidant effect could merely be correlated with the anticancer studies. To study both the consequences, the protective ability of the *Aglaia* extracts was studied by analyzing the hepatoprotective activity which was followed by the determination of anticancer effect of the same.

B. HEPATOPROTECTIVE ABILITY

The liver is one of the important vital organs which perform fundamental physiological activities like metabolism of carbohydrates and fats, secretion of bile, and storage of vitamins. The capacity of the liver to detoxify endogenous metabolic waste products, as well as exogenous toxic compounds, makes them often exposed to the substances responsible for degradation which progressively lead to liver diseases. Liver diseases are very common nowadays due to the increasing intoxication *via* food and drink intake as well as exposure to synthetic drugs and medicines in an increasing quantity. Free radical accumulation is the major reason for the hepatic damages and cell death as they might involve in covalent bonding and lipid peroxidation.

Undoubtedly, excessive consumption of alcohol is one of the prime reasons that cause dreadful hepatic disorders. Alcohol metabolism occurs in the liver in the presence of alcohol dehydrogenase resulting in the formation of toxic acetaldehyde. This is a reactive intermediate compound which has two-way action. It can form DNA adduct thereby causing DNA damage as well as generates free radicals, thereby inducing extreme stress and reactive environment, finally resulting in tissue damage (Madrigal-Santillán et al., 2014). Most of the damages caused in the cells are due to the accumulation of free radicals and the subsequent induction of oxidative stress by the toxic agent. The reactions against this oxidative stress often results in inflammatory responses and finally, unorganized cell death.

The present study was designed for the *in vitro* determination of the hepatoprotective ability of the selected taxa of *Aglaia* against alcohol-induced hepatotoxicity. Human hepatic carcinoma cells, HepG2, were intoxicated with ethyl alcohol and the effect of the plant extract for the protective activity was observed. The intoxicated cells showed extreme degradation and variation in

their morphology and number. These changes might be due to the alteration in their metabolic pathways caused due to the attack of alcohol. Viability of the cells was determined by MTT assay where it indicates the mitochondrial function/dysfunction by the formation of the stable insoluble compound formazan.

Cell death might have occurred due to the accumulation of free radicals resulted from alcohol metabolism. The percentage of viable cells was dropped to more than half the number of cells in the untreated control plate. After the treatment with plant extracts, cell viability was found to be increased and the percentage viability was found to be dose-dependent. *A. simplicifolia* exhibited maximum protective effect at both the lower as well as higher concentrations (Plate 3). Almost equivalent protection was shown by *A. barberi* extract (Plate 2). *A. malabarica* did not show a protective effect instead; it induced cell death in the altered or damaged cells (Plate 4).

The hepatoprotective ability of the extracts of *Trichilia emetica* against CCl_4 induced hepatotoxicity was attributed to the presence of polyphenols and other antioxidant phenolic compounds including tannins and flavonoids (Germano et al., 2005). Protective ability of *Azadirachta indica* was also attributed to its efficacy to scavenge the oxidative free radicals accumulated in the hepatocytes. Together with this, the extract was able to inhibit the blockage of Na+-K+ ATPase system caused by the toxicant thus, restoring the membrane properties (Chattopadhyay, 2003). A previous report on the hepatoprotective ability of *A. elaeagnoidea* was attributed to the presence of phytochemicals like sitosterol, stigmasterol and unidentified polyphenols (Kumari & Padmaja, 2012). The hepatoprotective ability of most of the reported plants like *Camellia sinensis* (Chaudhari et al., 2005), *Clitoria ternatea* (Zingare et al., 2013), *Ficus carica* (Ali et al., 2012), *Moringa oleifera* (Sreelatha & Padma, 2009), *etc*., were proved to be due to the anti-

inflammatory and antioxidant activities. *A. simplicifolia* was found to possess considerable antioxidant activity and the presence of immense quantity of active phenolic compounds, which might be the reason for its protective efficacy. *A. barberi* didn't show considerable antioxidant ability but, displayed hepatoprotective activity which was almost equivalent to that of *A. simplicifolia* (Fig. 20). This property might be due to the presence of unidentified polyphenols in the same. Phytochemical studies of the same revealed the presence of phenolic compounds in a significant amount along with terpenoids. For confirmation, further studies on various cell lines and animal models are required. *A. malabarica* showed destructive effect rather than a protective efficacy. The damaged cells were brought to death instantly on the application of *A. malabarica* extract even at the lowest concentration. The mode and extent of cell death have to be determined in detail and thus, the further portion of the study was dealt with the toxicity studies on various models/cell systems.

C. CYTOTOXICITY STUDIES

Any factor when comes in contact with a cell that could elicit a destructive effect on the membrane, cellular contents, or genetic material, leading to cell death is considered to be cytotoxic. Cells might undergo death by two means - necrosis and apoptosis. Necrosis leads to membrane disruption and often leads to inflammation. Apoptosis is the programmed cell death which is a normal process in the body to remove the unwanted cells. Cytotoxic agents are deleterious to the cells and might cause degenerative diseases but, this destructive efficacy of the cytotoxic compounds is beneficial in the treatment against proliferative diseases like cancer. Cytotoxic effect specific against rapidly dividing cells is often utilized for therapeutic purposes. These effects not only inhibit the division but also suppress the viability ultimately leading to cell death.

Cytotoxicity is assayed using various models from the basic model test organisms to specific cells cultured *in vitro*. Several methods and staining procedures are employed depending upon the toxicity nature, extent and the test system used. *Allium cepa* is a commonly used basic test system for toxicity studies. It is a simple, cost-effective, and sensitive assay in which the root meristem is treated with the test sample. *Tradescantia paludosa* and *Vicia faba* are other such established test systems used widely for the cytotoxic and genotoxic studies. The ease to handle and convenience for macroscopic as well as microscopic evaluation of the roots makes them a matter of interest in the toxicity assays especially, *Allium cepa*. The relevance of using *A. cepa* becomes prominent as the *in vivo* growth of roots happens in direct contact with the plant extracts comprising of genotoxic/cytotoxic phytochemicals able to elicit the degradation and damages to cellular components and genetic material. The most attractive benefit of the test material is that the results obtained from the *A. cepa* assay could be correlated with other test systems including bacterial system as well as those belonging to higher ranks of mammals including humans (Fiskesjö, 1988, Özkara et al., 2015). This is because the plant chromosomes are structurally and morphologically similar to those of animals and respond in a similar way to the treatment with mutagens (Nefic et al., 2013).

(i) *Allium cepa* assay

All the three selected taxa of *Aglaia* were subjected to cytotoxic evaluation using *Allium cepa* assay. Various concentrations of the plant extracts were used to treat the *A. cepa* root meristem to evaluate its toxic effect. The treatments were carried out at various times of exposure. All the three plant extracts showed notable cytological abnormalities including both clastogenic and non-clastogenic aberrations. Disturbances in membranes as well as the genetic material of *A. cepa* root meristem revealed the cytotoxic

property of the extracts. To determine the factors affecting the toxic effect of the extracts, five different concentrations *viz*., 5, 10, 15, 20, and 30 µg/mL were used for the treatment and the same was analyzed for different durations of 1 h, 12 h and 24 h. For each treatment, six sets of microscopic observations were done, analyzed quantitatively and the significance of the obtained results were determined statistically. Negative control is the one provided with the normal growth conditions and negligible probability of stress. The *A. cepa* bulbs grown in the distilled water served as the negative control. Positive control was also employed where the bulbs were grown in 0.1% aqueous malathion. Malathion is an organophosphate insecticide having direct inhibitory effect on mitosis and is able to induce immense chromosomal aberrations in the treated roots (Santiago & Cannen, 1999). Thus, the positive control, malathion used in the experiment exhibits extreme toxicity features.

The level of cytotoxicity was determined using two parameters - mitotic index (MI) and percentage of chromosomal aberrations (CA). MI is the approximate count of dividing cells in a meristem and this determines the viability and dividing ability of the cells after treating with the plant extract. It denotes the frequency of mitosis or cell division. Inhibition of mitotic activity indicates inhibition of protein synthesis, DNA synthesis or blocking of G1 or G2 phases (Majewska et al., 2003). Mitodepressive activity also results from the disturbances caused in ROS homeostasis (Livanos et al., 2012). CA reveals the capacity of the plant extract to induce toxicity which might be cytotoxic or genotoxic. CA results from the changes in chromosomal structure often followed either from a break or exchange of chromosomal material, or the degradation and loss of genetic material (Kumari et al., 2009).

A drastic drop in the mitotic index in *A. cepa* meristem was observed after treating with the plant extracts. All the three plants influenced the division of the *A. cepa* root cells. This was confirmed by keeping an

experimental control of an untreated *Allium* bulb in distilled water where MI was noted to be high and unaffected. For the bulbs treated with *A. barberi* extract for 1 h, the MI was found to drop from 72.24±3.5 to 21.72±3.0 as the concentration was increased from 5 to 30 µg/mL (Fig. 20). The shortest duration of exposure of the roots to the extract provided was 1 h and the reduction in the mitotic activity was observed even during the same. A dose-dependent mitotic activity was observed where MI was observed to decrease with the increase in concentration. Under the same conditions, MI of *A. simplicifolia* treated roots dropped from 87.68±1.6 to 66.54±1.2 and that of *A. malabarica* treated ones ranged from 60.76±6.2 to 15.53±2.1. MI values were greatly affected in the roots treated with *A. malabarica* extract indicating the most potent cytotoxic activity of the same when compared to the other two plants under study. The decrease in the mitotic index or mitotic activity indicates the mitodepressive effect of the extracts and its ability to block the cells from entering into the divisional phase. This in turn indicates the antiproliferative effect of the test material. The plant extract might prevent the cells from entering the prophase stage during the interphase itself by inhibiting DNA and protein synthesis (El-Ghamery et al., 2000). Reduction in the MI may also occur due to reducing the mitotic progression by increasing the duration of G2 phase or S phase or delaying the onset of prophase (Kumari et al., 2009). If MI falls below 22% than that of the control (the negative control), the treatment agent is considered to have a lethal impact while, if the same drops below 50% a sub-lethal effect is reflected which is considered as the cytotoxic limit value (Prajitha & Thoppil, 2016b). None of the extracts showed a lethal effect for the tested doses while all the three plants showed a sub-lethal effect for its highest tested concentration (30 µg/mL), irrespective of the time of exposure. These actions might be triggered by the potential phytoconstituents in the plant extracts especially the secondary metabolites.

Chromosomal aberrations (CA) are the impairments in the chromatin or chromosomes in the treated cells as a result of the action of the extract on either the spindle or the chromosome itself. On this basis, CA is classified as clastogenic and non-clastogenic. A wide array of CA belonging to both types was noted while carrying out the microscopic observations. The percentage of chromosomal aberrations was also observed to be dose-dependent. The approximate count of the aberrant cells was observed to increase proportionately with the concentration. Other than the chromosomal abnormalities, aberrations caused due to loss of membrane integrity, uneven cytoplasmic/nuclear division, cell enlargement/shrinkage, *etc*., were also noted. All these aberrations were quantitatively affected by the concentration of the extract, while the aberration types observed were the same. Certain aberrations observed were often found to be specific for each plant extract used for the treatment. All the above-mentioned events indicate that the plant extracts have penetrated through the membrane and acted on the genetic material as well as other cytological components resulting in the aberrations. The percentage of CA did not show a consistent increase with the time of exposure thus suggesting a time-independent induction of aberration by the plant extracts.

The highest percentage of chromosomal aberrations was noted in the *Allium* bulbs treated with *A. malabarica* extracts (55.33±6.9 to 92.62±1.5 for 24 h treatment). The chromosomal aberrations were found to be common in cells in the progressive divisional phases of metaphase, anaphase, and telophase. The aberrations included clastogenic as well as non-clastogenic ones. The aberrations might be either caused by the direct influence of the extract on the chromosomes or due to spindle disturbances. Membrane distortions, nuclear disturbances, chromosomal alterations, and spindle dislocation might be the major causes for the aberrations observed. A great diversity was observed in the cytological aberrations formed in the root

meristem after the treatment with the plant extracts at all the exposure durations and concentrations employed in the present study. The most relevant ones are detailed below.

Nuclear aberrations result from the disintegration of genetic material or chromosomal proteins like histones. The phytoconstituents in the extract might penetrate through the membrane and influence the structural and functional integrity of the chromatin and other nuclear components and these aberrations indicate the presence of genotoxic constituents. Nuclear lesions are the primary indications of the genetic material loss or degradation and the same was observed in high frequency in almost all the roots treated with the selected plant extracts. Nuclear lesions and dissolution are considered as the cytological evidence of inhibition of DNA synthesis. This kind of inhibition might occur either by the direct action of the phytoconstituents on the incorporation of DNA synthesis precursors onto the DNA strand or by influencing the biosynthesis of the precursors. The inhibition of the action of precursors results in the total failure of mitosis (Akaneme & Amaefule, 2012).

Nuclear erosion results from the irreversible toxicity effect of the plant extracts. Distortions and degradation of nuclear material were evident from the aberrations like nuclear budding, extrusion, erosion, micronuclei formation and pulverization. Premature condensation of chromosomes is the important reason for pulverization of the chromatin or nucleus. Nuclear buds/blebs often happen simultaneously with cytoplasmic membrane blebbing forming an important trigger for apoptosis. All these aberrations indicate the fragmentation of nuclei which forms the thread to cell death (Utani et al., 2011). Micronuclei formation is a remarkable aberration which was found very frequent in the root cells treated with *A. malabarica* extract. Many explanations are given by the researchers for the formation of these small sized nuclei. These might be the extra-chromosomal bodies emerged out from

a lagging chromosome or a chromosome fragment which might not get incorporated into the daughter nuclei thus, appearing in the cytoplasm (Badr & Ibrahim, 1987). Micronuclei are formed due to the extreme mutagenic property of the treated extract. Nuclear budding, nuclear appendages and nuclear peaks are the protrusions of genetic material from the nucleus and these often buds off to form independent structures. This might be a measure taken by the cell to expel the excessive nuclear material formed due to polyploidy or laggards and these aberrations are attributed to the cytotoxic action of the phytochemicals found in the plant extract.

Stickiness was a very common aberration observed during metaphase and anaphase stages of the mitotic cells. The chromosomes get connected to each other randomly rather than existing independently. Several explanations have been reported for the same. Stickiness might arise from the enlargement of inter-chromosomal chromatin fibers thereby leading to the formation of sub-chromatid linkages between the chromosomes (Chauhan et al., 1986; Liman et al., 2010). Stickiness might also develop due to the loss of telomeric regions of the chromosomes resulting in their joining with other broken fragments (Nefic et al., 2013). Extreme toxicity of the plant extract have caused this irreversible aberration by depolymerization of DNA, DNA condensation and physical adhesion of chromosomal proteins (Patil & Bhatt, 1992; El-Ghamery et al., 2000; Kumari et al., 2009) which might ultimately lead to cell death.

Spindle distortions take place due to the disturbances in the activity of its polymerization and depolymerization during specific time. The movement of chromosomes is thereby affected adversely leading to aberrations and finally to cell death. The non-clastogenic aberrations or those arising from the spindle disturbances comprises of laggards, vagrants, polyploid cells, chromosome rosette, scattered meta- and anaphase, diagonal and dislocated

chromosomes. Abnormal movement of the chromosomes rather than the specific path is the result of the severe disturbances in the spindle fiber mechanism or microtubule organization. Laggards or the lagging chromosomes are often seen in the anaphase stage of the mitotic cells where a few chromosomes fail to spread apart to the distal ends of the mother cell. Vagrants and laggards are observed due to the toxic effect induced on the spindle apparatus by the treated plant extract. The same could also result from the failure of the chromosomes to remain attached to the spindle while moving to either poles (Khanna & Sharma, 2013). All the three plant extracts tested showed the induction of laggards and vagrants especially during the higher concentrations used indicating their role in spindle poisoning.

Ball metaphase is a condition where either the sister chromatids separate into a hollow ball of chromosomes resulting from the early cleavage divisions, or due to the destruction of the spindle that results in the clustering of chromosomes into a tight spherical ball-like structure (Morgan, 2006). C-metaphase is also a condition resulting from the abnormal spindle organization and inactivation of spindle apparatus which might result in the delay of division of centromeres. Prolonged inhibition of spindle activity leads to polyploidy. Sometimes, the microtubule activity of the spindle is lost due to the effect of the extract which might also lead to polyploid condition (Minija et al., 1999). Fatal aberrations like stellate metaphase, multipolar anaphase, and diagonal anaphase are the fore-step of complete disturbance of spindle, effecting in a condition where spindle is absent or remain together in the middle of the cell rather than moving to the poles. This might also result from the unequal spindle movement (Mercykutty & Stephen, 1980).

Chromosomal bridges during anaphase stage of cell division arise due to the stickiness of the chromosomes resulting in the delay of their separation, thus inhibiting the free movement. The bridges result either from the non-

disjunction of these sticky chromosomes or the breakage and reunion during the separation of sticky chromosomes at anaphase (Feretti et al., 2007). Sudhakar et al. (2001) explains the formation of anaphase bridges as a result of the breakage of both the chromatids of a chromosome and rejoining of them with the non-sister chromatids. This aberration often results from the sticky nature of the chromosomes which subsequently leads to the failure of independent anaphase separation. The same is considered as a lethal aberration as the cells are subjected to apoptotic death soon. *A. malabarica* extract was the most potent one observed to induce single (Plate 13) and multiple (Plate 14) chromosome bridges as well as the broken bridges in a considerable number of cells.

Binucleate cells (Plate 6) results from malfunction of spindle movement/division during early anaphase or the absence of cytokinesis after the completion of telophase. Binucleate and multinucleate cells also arise due to the suppression of phragmoblast formation in the early telophase as a result of the action of the extract treated. Thus the cells after nuclear division are devoid of cell plate or cell wall (Satô, 1974; Shehab et al., 1978). Polyploids and aneuploids might also result from the abnormal segregation of chromosomes. All these cells are marked as the aberrant ones which are subsequently subjected to apoptotic death. All the three selected plants were able to suppress cytokinesis thereby leading to the bi/multi-nucleation of the cells among which *A. malabarica* showed the maximum toxicity. *A. simplicifolia* was capable of inducing polyploid cells very frequently and even up to octaploid cells were obtained after the treatment of roots with the same (Plate 12).

Cytostasis is the condition where the cells are arrested suddenly during a particular stage without causing immediate cell death (Rixe & Fojo, 2007). Cytostatic effect during metaphase is beneficial for karyotyping purposes.

Sudden freezing of the microtubules and cellular proteins involved in cell division might be the possible trigger to induce cytostasis in the treated root cells. The phytochemicals present in the plant extract could have induced this static pressure on the chromosomes and spindle, thereby restricting their free movement in the dividing cell. *A. barberi* was found to induce perfect cytostatic arrest of the treated cells very frequently (Plate 11) and this property could be exploited in a beneficial manner for cytological studies. The other two plants under study were not observed to induce the same.

Cytological aberrations caused due to other reasons instead of the spindle disturbances were also observed. Cytomixis is such an aberration which involves the migration of nuclear material from one plant cell to another by means of intercellular connections called cytomictic channels (Plate 17). These channels differ much from the intercellular plasmodesmatal connections morphologically in size and structure (Mursalimov et al., 2013). The phenomenon could bring changes in the ploidy level as the migration of the nuclear material happens (Sidorchuk et al., 2007). Some workers suggests that other aberrant conditions like micronuclei formation, nuclear bridges, binucleate cells, enucleate cells, and polyploidy are the commonly obtained products of cytomixis (Negron-Ortiz, 2007). Cytomixis was previously considered as a normal process happening in the microsporangial cells but later on evidences for the phenomenon occurring in the somatic cells were displayed by many researchers. Exposure to certain chemicals, herbicides and invasion of pathogens are some of the reasons for cytomixis to occur in somatic cells (Ravindran & Thoppil, 2020).

Giant cells are the excessively large cells which arise due to polyploidization resulting from endomitosis or endoreplication (Bonciu et al., 2018). This internal replication might be induced by means of physical trigger or chemical factors able to influence cell cycle at S-phase. Complete stoppage

of division or cell cycle arrest during the S-phase is the resultant consequence (Mercykutty & Stephen, 1980). Another explanation reported for the formation of giant cells reveal the failure to complete the cytoplasmic division even though the cell enters to the divisional phase. The cell enlarges in size, carries out DNA replication and nuclear division while fails to undergo cytoplasmic division subsequently leading to cell death (Prajitha & Thoppil, 2016b). Giant cells were observed to be induced by all the three selected plant taxa while the frequent induction of the same was observed in the *Allium* roots treated with *A. malabarica* extract when compared to the other two plants under study.

A wide array of multiple chromosomal aberrations was observed during the present study. Most of the nuclear aberrations were accompanied with nuclear lesions, *eg*., stickiness along with a nuclear lesion at prophase (Plate 7), cell with pulverized nucleus as well as lesion (Plate 7), nuclear peak along with nuclear lesion (Plate 8), *etc*. The difference in the ploidy level of the root cells after the treatment and multiple aberrations in these aneuploid cells were also observed. Partial C-metaphase in a hypoploid cell (Plate 10), pole to pole metaphase in a hypoploid cell (Plate 10), disturbed metaphase in a hyperploid cell (Plate 10) and somatic pairing in polyploid cell (Plate 12) are some of them. Multiple anaphase bridges with vagrants and laggards (Plate 14), formation of macronucleus as well as micronucleus along with a nuclear peak in a giant cell (Plate 17), strap cell possessing a strap nucleus with a lesion, *etc.* are some of the other multiple aberrations observed. Most of the multiple aberrations were noted in the roots after treatment with *A. malabarica* extract. A large number of cells were observed with multiple aberrations rather than a single aberration. This suggests the synergistic action of large number of phytochemicals present in the extract capable of inducing disturbance in the normal cell cycle. Cytological aberrations, single or multiple, interferes with the normal cell cycle process of the cells. The

aberrant cells are not sustained in the system as it might induce complications in the ploidy level of the offspring as well as the normal developmental processes. Thus the system tries to eliminate the aberrant cells immediately by employing apoptotic cell death mechanism. Most of the cells showed the signs of apoptotic activity after the treatment thereby suggesting the cytotoxic potential of all the three plant extracts. Many cells were observed with multiple aberrations and this reveals the combined or concerted action of the various phytochemicals present in the selected plant extracts on the cell metabolism and division. As the treated cells showed vivid apoptotic signs, the desirable feature of anticancer drugs, the plant extracts could be employed as a therapeutic agent in anticancer studies and further in drug development.

(ii) *In-situ* apoptotic detection

Apoptotic cell death is a normal phenomenon in the living system for eliminating the aberrant, aged or deformed cells without causing disturbances to the adjacent cells. Apoptotic death of the uncontrollably dividing cells is the desirable mechanism in the treatment of cancer. As a preliminary examination of the apoptotic activity of the selected plant extracts, they were subjected to *in situ* analysis using Evans blue staining of the *A. cepa* bulbs rooted in the various concentrations of the plant extracts.

Evans blue is an exclusion azo-dye which could be effectively employed to visualize cell death in a tissue and is much reliable than vital dyes like TTC. Using vital dyes to examine the cell death cannot be relied up on always as they measures the total metabolic process of the cells, but some enzymes remains alive for some time even after the occurrence of cell death. Another possible matter of deviation is that some enzymes in the living cells might have attenuated thereby not responding to the vital dyes. Thus exclusion dyes are often preferred rather than the vital dyes for the exact measure of viable cells (Baker & Mock, 1994). Exclusion dyes depend on the

membrane integrity to measure the cell viability. Evans blue can penetrate through the plasma membrane of dead cells and thus the stained cells mark the membrane degradation which leads to cell death (Peterson et al., 2008).

A. cepa bulbs were rooted in the various concentrations of the selected plant extracts and then subjected to Evans blue staining. The roots took up the stain in a dose-dependent manner that marks the concentration dependent cell death of the root cells. This unveils the cytotoxic property of the sample extracts used. The property of the plant extract to induce membrane damages and subsequent cell death is measured using the quantification of the dye penetrated into the *A. cepa* roots. The stained roots after washing in distilled water shows varied colour retention according to the cell death occurred. The roots treated with higher concentrations were observed to stain more intensely thereby indicating more number of dead cells and heavy toxicity induced by the extract (Plate 18). The quantification of the penetrated dye was done by the exclusion of the same from the dead cells into the organic solvent *N*, *N*-dimethyl formamide which was measured spectrophotometrically. Evans blue gets readily dissolved in the solvent as soon as the stained roots are incubated in it. The higher the cell death, the more will be the stain entered into the roots and subsequently more will be the amount of stain excluded into the solvent. Thus, the spectrophotometric data provides the exact quantity of the cell death which reveals the extent of cytotoxic potential of the plant extract. The overall data obtained reveal the dose-dependent cytotoxic activity of the extracts as the absorbance values were maximum for the highest concentration and minimum for the lowest concentrations, for all the three plant extracts used in the study. Specifically, *A. malabarica* showed maximum absorbance values at all the concentrations than the other two plants thus, suggesting the highest cytotoxic activity of the same (Fig. 23). This assay could be used as a procedure to confirm the apoptotic death occurring in the roots of *Allium cepa* as a result of treatment with the plant extracts.

(iii) Cytotoxicity induction in human erythrocytes

Efficacy of the plant extract as a potent cytotoxic agent on the membranes was analyzed using erythrocytes as the test model. The study of interaction of the plant extracts on the erythrocytes sounds less specialized but is of high significance as the membranes are the key components in the production of chemical and electronic gradients across the membrane which supports the active and passive flow of ions and other molecules (Bhagyanathan & Thoppil, 2016). Though erythrocytes lack nuclei and mitochondria, it is an excellent model for studying the features affecting the membrane integrity like cell shrinkage, membrane blebbing and deformities caused due to the defects in the cytoskeleton. Erythrocytes are capable of ingesting endogenous substances which include the reducing substances that cause oxidative damages to the cytoskeleton, ultimately leading to the shape change and cell death. Alteration in the bi-lipid layer of the cells and oxidation of the labile factors of the cytoskeletal proteins often leads to increased rigidity of the membrane (Richards et al., 2000).

In the present study, erythrocytes were subjected to treatment with all the three selected plant extracts of *Aglaia* leaves and the morphological alterations were observed after the incubation. Giemsa stain is a differential stain which is a combination of acidic and basic dyes. This stains the cytoplasm, membrane and nuclear material differentially thus enables the observation of normal and distorted cells. Giemsa stain was used to stain the erythrocytes after treating with the plant extracts and the cells were observed with the signs of apoptotic properties like membrane blebs, distorted membranes and formation of apoptotic bodies (Plate 19). Apoptotic signs were most evident in the cells treated with *A. malabarica* and in considerable way in those treated with *A. simplicifolia* and *A. barberi.* Marked difference in the morphology of the erythrocytes was resulted after the treatment with

the plant extracts. The discoid shape of the erythrocytes was altered to echinoid like structures due to the loss of membrane integrity. The changes in the shape of the cells might be due to the differential expansion of the bi-lipid layer as well as due to the depolymerization of the microtubules and fibrils in the cytoskeleton. Perturbations in the membranes and the bilipid structure alterations result from the oxidative modification of the lipid structure and composition in the membranes (Zavodnik et al., 2001). Meanwhile, the control cells were intact and observed with negligible aberrations. Certain natural polyphenolic substance like tannins along with flavonoids and terpenoids often absorb proteins and metal ions from the cytoskeletal elements and the membranes, which results in the loss of shape and function of the erythrocytes and ultimately leads to the appearance of apoptotic signs (Taraphdar, 2001).

The cytotoxic studies revealed the potential of the plant extracts to induce apoptotic death in the rapidly growing cells *via* two broad methods - degradation of the genetic material as well as by breaking down the membrane integrity. This efficacy of the plant extracts to lead the treated cells to undergo apoptotic death is highly desirable in the drug development against proliferative diseases and thus the selected taxa of *Aglaia* provides a significant lead to the antiproliferative therapeutics. As to confirm the same *in vitro*, the further study was dealt with the determination of the activity of the plant extracts on the human carcinoma cell line (HeLa cells).

D. ANTIPROLIFERATIVE ACTIVITY

Cancer has been a dreadful disease since decades and vast researches are still active for finding out the root causes, elucidation of the pathways involved, and development of remedies which could be employed for its relief and to check its further progress. Though the domination of the synthetic drugs exists, the necessity for the novel herbal therapeutic agents is growing

due to the adverse side effects of the former one. The uncontrolled proliferation of the cells is often checked and the cells are killed by the herbal agents that are actually proven to be cytotoxic. The present study revealed the cytotoxic potential of the selected taxa of *Aglaia* against the rapidly dividing cells in various models and thus the potential of the same as an antiproliferative agent was tested *in vitro* in HeLa cells. The most potent one among the studied plant extracts was subjected to further confirmation analyses to establish its antiproliferative property.

(i) MTT method

The human cervical cancer cell line, HeLa was used for the analysis of the antiproliferative effect of the plant extracts. Cervical cancer is the second major case of cancer in females globally and researches on antiproliferative therapies are ongoing at a great pace. HeLa cells are being used extensively in cancer research since decades and still receives wide acceptance due to its peculiar immortal features. All the three plants were subjected to *in vitro* cytotoxic assay using the cultured HeLa cells.

The cytotoxicity analysis using MTT assay is prevalent nowadays for the *in vitro* cell viability studies due to its reliability, repeatability and sensitivity. In the presence of live cells, the tetrazolium salt MTT is converted into a coloured stable compound called formazan which is quantified for the assessment of cell viability. The reaction happens due to the activity of mitochondrial enzymes which is possible only in the presence of viable cells. The formazan crystals thus formed are solubilized in an appropriate solvent and quantified by means of spectrophotometry which gives an approximate count of viable cells in percentage. The method provides an easy and quick quantification rather than the direct counting method.

Here, five different concentrations *viz*., 6.25, 12.5, 25, 50 and 100 µg/mL, of each plant extract were screened for its antiproliferative property yielding half-maximal (LD_{50}) values of 101.538 ± 5.6, 57.483 ± 4.1 and 42.137 ± 3.6 µg/mL for *A. barberi*, *A. simplicifolia* and *A. malabarica*, respectively. All the three extracts showed a dose dependent effect on the viability of the treated cells (Plates 20-22). The viability of the treated cells was found to be decreasing with the increase in the concentration of the extract. Very prominent morphological and genetic alterations were also observed in the cancer cells. The major alterations displayed were nuclear fragmentation, condensation of the nuclei, cellular shrinkage, blebs on the membranes, formation of apoptotic bodies, cellular budding and formation of echinoid spike like bodies (Plates 20-22). All these are the characteristic features indicating the occurrence of apoptosis. The hallmark feature of apoptosis, chromatin/nuclear fragmentation, could be the result of the inter-nucleosomal cleavage of DNA induced by the active compounds in the plant extract (Liao et al., 2015). The development of membrane blebs and cellular budding marks the initiation of disintegration of cells into vesicular structures or apoptotic bodies which get further engulfed by the macrophages. The formation of echinoid spikes indicates the loss of basic cellular structure which might be caused by the disturbances in the cytoskeleton. Condensed nuclei results from the loss of chromatin integrity induced by the bioactive elements in the plant extract and this might often result in cell death. These features too indicate the progress of apoptotic stages. *A. malabarica* extract treated cells showed all the above mentioned aberrations in the highest frequency than the other two studied plant extracts. The half maximal values obtained from the colorimetric estimation of cytotoxicity also displayed the best results for *A. malabarica*. Thus, further assays were performed to identify and confirm the antiproliferative activity of the same as well as to elucidate the mechanism of cell death induction.

The cytotoxic effect of the methanolic leaf extract of *A. malabarica* was also analysed on the L929 cells in order to determine its effect on normal cells. The immortalized mouse fibroblast cells, L929 are commonly used as the normal cells in the *in vitro* cytotoxic studies. The resultant effect of the sample extract on these cells can be correlated with that of normally dividing mammalian cells/tissues. There were no significant morphological changes observed in the L929 cells after the treatment with *A. malabarica* extract even at the highest test concentration. Meanwhile, the HeLa cells showed remarkable aberrant morphology along with loss of adhesion to the surface at all the tested concentrations. The viability of the L929 cells after the treatment was also not much affected and was observed with a half maximal concentration of 212.482 µg/mL (Table 11), whereas the same for the HeLa cells was obtained as 42.137 ± 3.6 µg/mL (Table 10). The HeLa cells after treatment with the plant extract showed remarkable morphological changes indicating typical apoptotic signs like loss of surface adhesion, nuclear budding, cell membrane blebbing, formation of apoptotic bodies and echinoid like spikes (Plate 22). No prominent signs of apoptosis were observed in the L929 cells after the treatment (Plate 23) while only a slight decrease was seen in the viability of these cells treated with the highest concentration of 100 µg/mL. This indicates a mild and negligible cytotoxic effect of *A. malabarica* leaf extract on the normal mammalian cells while a significant toxicity against the malignant cells (HeLa) and thus, could be developed as an effective antiproliferative drug. The bioactive components in the plant extract might be able to interfere with the unique biochemical pathways existing in the malignant cells and this could be the lead cause for its selective toxicity against the same.

(ii) Detection of apoptosis/necrosis

A. malabarica plant extract was found to be capable of inducing cytotoxic cell death significantly. The morphological features induced in the treated cells indicated the signs of apoptotic cell death and a further assessment of the same was done using acridine orange/ethidium bromide double staining. Acridine orange (AO) is a DNA intercalating fluorescent dye that can penetrate through both viable and non-viable cells and emits green fluorescence under excitation of 500-505 nm. Ethidium bromide (EB) is also a fluorescent, nucleic acid specific dye which is capable of penetrating through the membranes of the non-viable cells emitting orange-red fluorescence on excitation of 525-530 nm. This double staining technique is a reliable, easy and inexpensive method for cell viability analysis where, viable cells are green and non-viable ones are displayed orange-red. This distinct fluorescence is observed as the viable cells take up only AO and appears green while, the non-viable cells also picks up EB thereby suppressing the green emission of AO and appearing orange-red. Necrotic cells are displayed in intense red fluorescence. The HeLa cells treated with the half maximal concentration of the methanolic extract of *A. malabarica* were observed to get stained both green as well as orange-red in approximately equal numbers. The green viable cells were intact and almost with no morphological aberrations. Most of the non-viable cells (orange-red) were observed with disintegrated nuclear material, loss of membrane integrity and development of membrane blebs along with the formation of apoptotic bodies. All these morphological aberrations indicate the loss of cell viability due to apoptosis. At the same time, the control cells were observed to emit green fluorescence alone which reveals that all are viable cells with no considerable morphological alterations (Plate 24). The signs of necrotic death like formation of vesicles and cell debris were not observed and red coloured cells were almost absent which in

turn confirms that the non-viable cells are resulted from apoptosis and not necrosis.

The action induced by *A. malabarica* on the malignant cells to induce death was confirmed to be apoptosis based on the above mentioned observations and analyses. The ability of an agent to induce apoptosis on rapidly dividing cells is the most desirable feature for antiproliferative drug development. Researchers nowadays focus on the strategies to develop this feature by customising and modulating apoptotic effect of the drugs for an effective control of malignancy. The quantification and extend of apoptotic death and its mechanism is dealt in the forthcoming portion of the present study.

(iii) Quantitative apoptotic evaluation

Apoptosis being the key mechanism involved in the antiproliferative activity of *A. malabarica* extract, was quantified using flow cytometry. Conventional methods like light and fluorescence microscopy can be used for visualization of the cells but is not convenient for quantification of the cells. DNA profiling and similar biochemical techniques provide a rough idea about the quantity of dead cells but distinction between apoptotic and necrotic cells often gets muddled. An efficient estimation of the number of apoptotic cells could be done using flow cytometer as a large number of single cells are counted using fluorescence activated cell sorting (Ormerod et al., 1993).

Annexin V in combination with FITC was used for the flow cytometric estimation of apoptotic cells in the present investigation. The phospholipid called phosphatidyl serine is usually located in the inner surface of the plasma membrane. The externalization of the same on to the outer plasma membrane providing an ‘eat me’ signal is a common feature of the cells undergoing apoptosis. This signal triggers the macrophages to engulf the cells resulting in

phagocytosis thereby eliminating it from the system. In flow cytometry, this feature of exposing phosphatidyl serine is used to detect the cells undergoing apoptosis (Pietkiewicz et al., 2015). The fluorescent dye conjugate binds with the phospholipid thereby indicating the apoptotic stage of the cells and this in turn emphasize the ability of the extract to initiate apoptosis.

The LD_{50} concentration of *A. malabarica* methanolic extract, obtained from the MTT assay, was used to treat HeLa cells resulting in the reduction of live/viable cells to 74.46% which was 89.15 % in the untreated control. There was no significant increase in the number of cells in the Qudrant I that indicates early apoptotic cells. While, the increase of fluorescence in the Quadrant II reveals considerably high number of dead cells (Fig. 25). These could have accumulated either due to necrotic death or as a result of apoptosis. The actual detection of the apoptotic cells should be accompanied with the morphological studies in order to filter out the apoptotic cells from the dead cell debris (Zamai et al., 1993). The dead cells might have also resulted due to the lack of clearance of the apoptotic cells by phagocytosis in the *in vitro* system. The morphological alterations and signs observed in the microscopic observation clearly indicate cell death due to apoptosis and thus it can be concluded that in the present investigation the increased dead cells might be the result of apoptosis induced by the extract of *A. malabarica*.

(iv) Cell cycle analysis

The major treatment against proliferative diseases employs the use of the drugs which checks the cell cycle progression and arrests division. This is often followed by apoptotic action thereby reducing the proliferation rate and suppressing the tissue growth. Various therapeutic agents affect the cell cycle at different checkpoints and in varying extend. Flow cytometry is one of the most reliable and a common method used to determine the cell cycle progression and checkpoints. Cell cycle progression in the cervical cancer

cells treated with the methanolic extract of *A. malabarica* along with the cells before the treatment was analyzed in the present study.

Regulation of cell cycle is the core mechanism that regulates cell proliferation in a tissue system. Cell cycle is a complex phenomenon which is thoroughly mediated by two classes of factors such as cyclin-dependent kinases (CDK) which up-regulate division and cyclin-dependent kinase inhibitors (CDK-I) which acts contrarily against the former one. Plant extracts and other chemotherapeutic agents regulate the action of these kinases thereby arresting cell cycle in malignant cells and terminating proliferation (Li et al., 2009). The various stages of cell cycle are regulated by different factors belonging to the CDK class of proteins. The G0/G1 transition is governed by CDK3, CDK4/6-cyclin D, CDK2-cyclin E; S-phase is regulated by CDK2-cyclin A and G2/M transition is regulated by CDK1-cyclin A, CDK1-cyclin B and CDK7-cyclin H complexes. These along with the CDK-I proteins regulates the various checkpoints in the cell cycle where DNA damages, cell/nuclear size, growth factors and chromosomal aberrations are counted (Arellano & Moreno, 1997). When treated with an antiproliferative agent, the arrest might occur during any of the stages of the cell cycle which unveils the specific cellular factor on which the agent binds or inhibits. In the present investigation, the cell cycle arrest was found in the G0/G1 transition stage which was evident from the DNA content profile (Fig. 26). A striking increase in the DNA content during G0/G1 transition, *i.e.,* in the sub-G1 stage in the treated cells indicating the arrest of the cell during the same (Fig. 27). This proves the action of the plant extract on the cell cycle regulators involved in the G0/G1 transition stage. Most probably, D-type cyclins might have been deactivated by the extract which would lead to the attenuation of the partner kinases - CDK4 or CDK6 inhibiting the cells to progress to the G1 phase. This can be substantiated by the previous report on the cell cycle arrest brought by the acetone extract of *Buxus sempervirens* in a comparable manner

(Cheng et al., 2004). If the cells involved in division are subjected to DNA damage, the cell cycle might get arrested at the initial phase *i.e*., G0/G1 where p53-mediated transcription of the CDK-inhibitor p21 is involved in the inhibition (Lam et al., 2012). The active biomolecules or the phytoconstituents in the plant extract might have inhibited the action of the cell-cycle regulatory proteins. The cells with damaged genetic content or non-functional stage are often removed from the tissues by means of apoptosis (Leong et al., 2016).

(v) Gene expression analysis

Apoptosis or the programmed cell death is one of the best studied modes of cell death which is regulated by a class of cysteine-dependent aspartate-specific proteases known as caspases. These endoproteases have essential role in the regulation of various pathways involved in apoptosis as well as the other cell death mechanisms like pyroptosis and necroptosis. The caspases involved in the apoptotic pathways in humans include initiators like caspase-2, 8, 9 and 10 along with the executioners like caspase-3, 6 and 7. The initial inactive form of initiator caspase called pro-caspase, gets immediately cleaved and activated on receiving an apoptotic stimuli. This further activates the downstream executioner caspases leading to the degradation of nucleic acids and membrane proteins ultimately leading to cell death (Pietkiewicz et al., 2015).

Two major pathways are involved in apoptosis - intrinsic pathway when the cell senses an apoptotic stimuli and extrinsic pathway when it gets signal from other adjacent cells. Intrinsic pathway includes the action of mitochondrial and sub-cellular proteins like bcl-2, bcl-xl and bax/bak. Mitochondrial cytochrome 3 is released to the cell matrix associates with another cytoplasmic factor, Apaf-1, to form a complex called apoptosome. This in turn activates caspase-9 followed by the cleavage and activation of

caspase-3 thus leading to a sequence of cytological events ultimately resulting in cell death. Extrinsic pathway is initiated when the death receptor on the plasma membrane receives the apoptotic signal. Caspase-8 is activated by this which further leads to the activation of caspase-3 and the degradation of cytological contents is initiated. The determination of the gene level expression folds of the caspases reveals the pathways involved and the extent of apoptosis. Caspase-9 and -3 have been identified as the major regulators of apoptosis which are involved in initiation as well as execution phases by cleaving around 400 substrate factors. The biochemical and morphological alterations in the apoptotic cells like cell shrinkage, chromatin condensation, and DNA fragmentation were identified to result from the cleavage enabled by these caspases.

Caspase-9 is an initiator caspase that switches on the cascade of apoptotic reactions on receiving a cytological trigger. It plays a crucial role in the mitochondrial or intrinsic pathway. The activation of caspase-9 is enabled by dimerization rather than cleavage. The expression of caspase-9 could be observed in almost all mammalian tissues and any abnormal suppression of the same could lead to developmental anomaly. The studies reveal the inevitable role of caspase-9 in maintaining cell homeostasis which is enabled by the cleavage of multiple factors having key roles in apoptosis. Caspase-9 also eliminates the cells by inducing apoptotic death during early developmental stages which is a vital step to inhibit proliferative diseases. This is made possible by the continuous elimination of defective and irreparable cells in the cell cycle (Li et al., 2017).

Caspase-3 is an important executioner caspase coded with *CASP3* gene and is involved in both intrinsic and extrinsic pathways of apoptosis. The activation of procaspase-3 is enabled by the interaction with both caspase 9 (intrinsic pathway) as well as with caspase 8 (extrinsic pathway) and this in

turn activates caspase-6 and -7 thereby progressing the apoptotic reaction cascade. Caspase-3 was proved to be the key factor responsible for DNA fragmentation which is the pivotal step in apoptosis. The fragmentation of genetic material is brought by specific cleavage and resultant inactivation of the inhibitor of the caspase activated DNase (iCAD). Thus, the crucial role of caspase-3 in apoptosis was evident from the extensive double stranded DNA fragments, inter-nucleosomal DNA laddering and other morphological changes which are hall marks of apoptosis (Jänicke et al., 1998; Davoli et al., 2002).

The HeLa cells treated with the LD_{50} concentration of *A. malabarica* leaf extract was determined for the caspase activity. The mRNA specific for the caspases-9 and -3 along with that of a house keeping gene (B-actin) were isolated for which cDNA were prepared, amplified and examined after electrophoresis. A prominent increase in the gene level expression of both the caspases were found to be elevated in the cells after the treatment with the extract (Fig. 29 & 30). No expression level changes in the B-actin gene was observed in the treated cells thereby revealing that differential expression of caspases alone has happened. As these caspases are the ones who play pivotal role in apoptotic pathways, the expression of the same was studied in the treated cells. The elevated level of caspase-9 gene suggests that intrinsic pathway is involved in the apoptosis and increase in caspase-3 reveals DNA fragmentation in the treated cells. Gedunin, a prominent terpenoid compound in the Meliaceae plants have been previously reported to induce the activation and elevated levels of caspase 3 in cancer stem cells (Tharmarajah et al., 2017). Phytochemical studies have revealed that *A. malabarica* is also a rich source of potent terpenoids which might be involved in the triggering the activation of these specific caspases. Thus, the results suggest that the antiproliferative effect of the plant extract is due to the inhibition of growth

and induction of apoptosis by means of caspase-dependent mechanism in the proliferating cells.

In the present investigation, the cytotoxic potential of the methanolic leaf extract of *A. malabarica* on HeLa cells was revealed by the MTT assay and the cell death was confirmed to be due to apoptosis by AO/EB fluorescent imaging technique. The cytotoxic effect was observed only against the malignant cells. This was confirmed by studying the effect of the plant extract on the normal L929 cells which revealed the negligible cytotoxic effect of the extract on the same. The cell division was observed to be arrested in the G0/G1 transition phase of the cell cycle thereby indicating the DNA degradation, fragmentation and morphological aberrations. The cells with erroneous genetic material are eliminated from the system by means of programmed cell death and the morphological features observed were remarkable hallmarks of apoptosis. The gene expression studies revealed a prominent shoot up in the levels of caspases like caspase-3 and -9 which confirm the intrinsic mode of apoptosis that happened as a result of the action of the plant extract. All these are the desired features for the drugs employed against the proliferative diseases particularly, cancer.

Flavonoids and sesquiterpenoid compounds are reported to possess antiproliferative effect on cervical cancer cells (Priyadarsini et al., 2010; Khantamat et al., 2004; Hahm et al., 2015). The anticancer activity of *Aglaia longkong* fruit was attributed to the presence of *α*-cubebene along with other organic compounds. Elixene was identified as one of the important anti-tumor factor in the extract of *Euphorbia macrorrhiza* (Lin et al., 2012). Antiproliferative activity of 5-hydroxypipecolic acid in *Morus alba* leaf extract (Chan et al., 2020), and cytotoxic effect of ekeberin and alloaromadendrene in *Melia azedarach* (Wu et al., 2009) were also reported.

The leaf extract of *A. malabarica* was observed to be a rich source of phytoconstituents which included the above mentioned ones in considerable quantities. Either the action of these potent components in single or a synergistic action of altogether might be the reason for the antiproliferative effect of the *A. malabarica* extract on the tested human carcinoma cells.

All the three selected taxa of *Aglaia* were found to be rich sources of diverse array of potent phytochemicals and exhibited a varying range of bioactivities. Phytochemical characterization revealed the active components in the plant extracts and some of the important classes were quantified too. Among the studied ones, *A. simplicifolia* was observed to hold a protective effect on the mammalian cells which was evident from the free radical scavenging assays and hepatoprotective studies. The extract of *A. simplicifolia* possessed a moderate antioxidant effect and a strong protection for the human hepatocytes. *A. barberi* also showed a comparable protective effect but much less than that of *A. simplicifolia*. The protective effect of these plants could be attributed to the unique chemical constitution in the leaf methanolic extract of the same. Cytotoxic, apoptotic and excellent antiproliferative effects were displayed by the leaf extracts of *A. malabarica.* The extract was proved to induce apoptosis in the human cervical cancer cells thereby inhibiting the division and destroying the malignant tissues by means of programmed cell death. Thus the selected plants are proved to possess potential clinical utilities which shall be further implemented by drug development and clinical trials.

PHASE III - GREEN SYNTHESIS OF SILVER NANOPARTICLES

The scientific world is curious about the tiny materials having their size in nanometer scale due to their peculiar properties differing from that of larger particles. The chemical, electronic and physical properties of the nanoparticles differ highly from that of their source element/compound which is possible due to the increased surface area to volume ratio of the smaller particles. These diverse features enable the application of the same for various purposes. Nano-materials have gained wide acceptance nowadays in healthcare, biomedicine, textiles, food, environmental monitoring, electronics and energy resources (Malik et al., 2017). The biomedical applications include the use of nanoparticles for its antimicrobial property, as an agent for drug delivery, for cancer therapy, and controlled drug release. The synthesis of the nanoparticles is possible by means of physico-chemical as well as biogenic methods. The biogenic synthesis of nanoparticles is environmentally benign, easier, cheap and a single step process, and hence it is extensively employed rather than the physico-chemical method (Ahmed et al., 2016). The biological agents like plant extracts, fungi, yeast, and bacteria could be used to reduce the elements/compounds into nanoparticles. Among these, the plant extracts are employed widely as they are reliable and healthier than the other biomaterials (Mousavi et al., 2018).

The properties of the various nanoparticles rely upon their size, shape and their agglomerative ability. Assigning a particular application to a kind of nanoparticles is based on their physical and chemical properties. The particles are capped using appropriate capping agents in order to stabilize the particles and preserve its properties. The advantage of biogenic synthesis of the nanoparticles is that the phytocomponents itself acts as the capping agents and thus the synthesis could be done in a single step. The factors affecting the morphology and agglomeration of the nanoparticles during the process of its

synthesis are pH, temperature and concentrations of the source element and the plant extract (Kaviya et al., 2011).

(i) Nanoparticle biosynthesis

In the present investigation, an attempt was done to synthesize silver nanoparticles from silver nitrate solution using the methanolic leaf extracts of the selected species of *Aglaia*. The components in the plant extract acts as the natural reducing agents which reduces the elemental silver to the particles in nano-scale. The phytoconstituents also functions as the capping agents for the stabilization of the synthesized nanoparticles and thus the whole process takes a single step action. The experimental part included the standardization of the incubation temperature, pH of the reaction mixture and the ratio of $AgNO_3$ to sample plant extract, by means of repeated trial and error attempts. The efficient synthesis of the nanoparticles was observed to happen with a solution mixture of 1 mg/mL methanolic plant extract and 2 mM $AgNO_3$ in a ratio of 1:9, at pH 9 and incubated at a boiling temperature of 80 ^{0}C. The reduction process of the compound silver into elemental nanoparticles was indicated by conversion of the colourless reaction mixture to brown (Plate 25). The colour of the reaction mixture after the incubation depends on the properties of the nanoparticles suspended in the solution. The size and shape of the particles affects the light scatter and absorbance and this imparts yellowish to dark brown colour to the solution which is not exhibited by the source ions/elements of larger size (Jafari et al., 2016). All the three selected taxa of *Aglaia* were observed to be efficient in synthesizing nanoparticles from the silver nitrate solution. The reducing agents present in the plant extracts might be the responsible factors to induce the production of silver nanoparticles. The detailed characteristics of the synthesized nanoparticles were revealed from UV-Vis spectrum, XRD data and SEM images.

(ii) UV-visible spectrum analysis:

The silver nanoparticles synthesized using the methanolic plant extracts were subjected to UV-visible spectral analysis to identify and characterize the presence of the elemental silver particles. The nanoparticles have characteristic optical properties governed by its shape, size, agglomeration and refractive index. These parameters enable the analysis of the nanoparticles by means of UV-visible spectroscopy (Mallick et al., 2007).

All the three selected taxa of *Aglaia* yielded nanoparticles which on UV-vis spectrum analysis revealed a prominent peak at the range of 420-480 nm and a smaller peak in between 240-250 nm. The broadest peaks obtained for the biosynthesized nanoparticles were at the wavelength of 476 nm, 426 nm and 446 nm for *A. barberi*, *A. simplicifolia* and *A. malabarica*, respectively. The prominent band is due to the characteristic surface plasmon resonance (SPR) of the nanoparticles and this particular wavelength indicates the presence of elemental silver particles. The difference in the absorbance wavelength in different extracts indicates the diversity in shape and size of the nanoparticles in the suspension (Roy et al., 2017). The colour transition according to the progress of reaction is often associated with the specific SPR and these might have resulted from the coherent oscillation of free conducting electrons in the suspension (Mulvaney, 1996).

An additional peak with smaller band width was produced in the UV-Vis spectrum between 240-250 nm. Literatures show the reason for the additional peak due to the formation of nanoclusters and resultant decrease in the absorbance (Zhang et al., 2003). The presence of the polyphenolic or antioxidant compounds or the phytoconstituents acting as the capping agents might also result in the formation of additional peaks. The appearance of an

SPR peak at 260 nm indicates the excitation of tryptophan and tyrosine residues in the proteins (Singhal et al., 2011). Mie's theory explains that a single SPR band is resulted from the spherical morphology of the nanoparticles and multiple SPR bands indicate anisotropic particles due to the diversity in shape (Murphy et al., 2005). Probably, the additional peaks obtained in the present investigation at 240-250 nm might be due to the presence of active phytoconstituents which would have capped the nanoparticles in the aqueous suspension.

(ii) X-ray diffraction analysis

The crystalline structure analysis using X-ray diffraction is a non-destructive method to determine the crystal size, texture and strain. The biosynthesized silver nanoparticles in the powder form were subjected to X-ray diffraction and the FWHM values were used to determine the crystal size. The FWHM values are obtained from the peak intensities which could be determined by the atomic positions within the lattice planes of the crystals (http[4]). The approximate crystallite size of the particles was calculated as 40.96 nm, 23.39 nm, and 5.99 nm for *A. barberi*, *A. simplicifolia* and *A. malabarica* respectively (Table 12).

The calculation of the size as well as determination of the crystal shape is possible from the XRD data. Unfortunately, broadening of the peaks might not help in the determination of the shape. The broadening of the peak results from the analysis of extremely small crystals and anisotropic strain (Ungár, 2004). In the present study, somewhat broad peaks were obtained which is not completely reliable for the determination of the crystal shape (Fig. 32). Thus in order to confirm the size of the nanoparticles and to determine the shape, SEM imaging technique was employed.

(iii) SEM imaging

The silver nanoparticles obtained as a result of reduction by all the three extracts of the selected taxa of *Aglaia* were analyzed using SEM imaging. The size of the nanoparticles obtained by the reducing action of *A. barberi* was observed to range between 33.1-71 nm which almost agrees with that obtained from the XRD data. The particles were observed to have somewhat cubical morphology when in crystalline form (Plate 26). In the case of *A. simplicifolia*, perfect cube was the morphology displayed which revealed a size of around 76.3-101 nm (Plate 27). The XRD data of the same provided a size of approximately 23.39 nm (Table 12) which is different from that obtained from SEM images. Most probable reasons for the same might be either the aggregation of the particles in the crystalline form or the anisotropic strain developed due to the presence of the active phytoconstituents as the capping agents. Thus the visually measured data from the SEM images could be taken into account as a more reliable and valid one. The smallest silver nanoparticles were obtained as a result of the reduction by the plant extract of *A. malabarica* with a size of 18.1-30.7 nm (Plate 28). This indicates the high reducing ability of the plant which could be attributed to the presence of potent phenolic compounds and flavonoids (Francis et al., 2017).

All the three plant extracts were proved as rich sources of phenolic compounds that hold carboxyl and hydroxyl ions which could bind and reduce the metallic elements into smaller particles. The phenolic compounds along with other reducing agents in the plant extracts might donate either electrons or hydrogen atoms thereby reducing the complex particles into the particles of nanoscale. The sugars and proteins in the reaction mixture might

act as the capping agents thus preserving the robustness of the nanoparticles formed (Manjari et al., 2017a).

As the nanoparticles have great applications in several fields and the green synthesis of the same is a cheap, eco-friendly approach, this method shall be adopted as the major method for its production. In spite of the tremendous advantages, the nanoparticles have been reported to be hazardous in many aspects during drug delivery and treatments (Kim et al., 2010). Thus extensive researches on the eradication of the adverse effects of nanoparticles and its judicious utilization become a necessity.

SUMMARY AND CONCLUSIONS

Plant-based system of medicine has been the core of human health care from the pre-historical period onwards. In spite of the tremendous developments in the techniques and technologies in the medical system, traditional or herbal medicines still finds wide acceptance among the human communities around the globe. The diverse chemical constitution and incomparable biological activities have made them promising candidates in curing the ailments and the maintenance of human wellbeing. Though extensive researches are going on to explore the bioactivities and chemical constitution of the plants, immense number of flora remains underexplored and unidentified. This necessitates more researches to be conducted for identifying the beneficial properties in plants/plant parts and subsequent drug development.

Meliaceae is the angiosperm family well known for the plants having incredible biological activities and incomparable phytochemical constitution. Numerous species belonging to the family have been reported for its valuable properties. *Aglaia* is the genus that has been a matter of interest for the pharmacology investigators due to the unique chemical constitution and the extensive studies on many species are going on. But still, most of the endemic taxa under the genus *Aglaia* remain underexplored in both phytochemical as well as pharmacological aspects. Thus the present investigation was designed to study the phytochemical constitution and the important bioactivities of some selected species of *Aglaia* that are endemic to Western Ghats. The three taxa of *Aglaia* selected and studied include *A. barberi*, *A. simplicifolia* and *A. malabarica*. The plants were collected from the tropical evergreen forests of the Western Ghats, leaves were dried, powdered and extracted in methanol

using Soxhlet apparatus for the further studies. The major findings of the study are listed below:

Phytochemical characterization

All the three selected taxa of *Aglaia* revealed the presence of biologically potent phytoconstituents. The qualitative analysis displayed the presence of quinones, phenols, flavonoids, terpenoids, alkaloids, steroids, tannins, carotenoids and cardiac glycosides. Quantitative estimation revealed the highest amount of phenolic compounds in *A. simplicifolia* and the highest amount of terpenoids and flavonoids in *A. malabarica*. The volatile compounds were determined by GC-MS analysis of the plant extracts. A total of 112 compounds were resolved according to their retention time and the compounds belonging to several biological classes were identified. Squalene, gitoxigenin, phytol, alloaromadendrene, O-methyl psychotrine, ledene alcohol, α - cubebene, *N* - methyl pyrrole, *etc.* are some of the most prominent volatile compounds identified from the plant extracts. The phytochemical characterization revealed the presence of potent phytochemicals that lead to the screening of the bioactivities of these plants.

Antioxidant activity

The free radical scavenging ability of the selected taxa of *Aglaia* were carried out using four assays, *viz.*, DPPH radical scavenging assay, ABTS radical scavenging assay, hydroxyl radical scavenging assay and superoxide radical scavenging assays. *A. simplicifolia* and *A. malabarica* revealed the maximum antioxidant ability in all these assays. Meanwhile, weak radical scavenging ability was observed for *A. barberi*.

Hepatoprotective activity

Evaluation of the protective effect of the plant extracts on the human hepatocytes against alcohol-induced toxicity was done. Lower doses of *A. barberi* and *A. simplicifolia* showed considerable protection against alcohol-induced toxicity on HepG2 cells. Meanwhile, *A. malabarica* was proved to have a destructive effect even during the lowest concentration. The polyphenols, tannins and flavonoids might have rendered the protective effect of the extracts.

Cytotoxic activity

Allium cepa assay was performed to study the cytotoxic activity of all the three selected plant extracts. The percentage of chromosomal aberrations and mitotic indices were the parameters noted. A dose-dependent and time dependent effect was found in almost all the cases indicating the mitodepressive effect of the plant extracts. Cytological aberrations resulting from chromosomal and spindle disturbances were observed after the treatment. Formation of hypoploid cells, hyperploid cells, mis-orientation of the chromosomes, shift in MTOC, *etc*., indicates the ability of the extracts to disturb the spindle organization. Chromosome fragments, micronuclei formation, chromatin pulverization disintegrated chromatin, formation of nuclear buds, *etc*., indicates the ability of the plant extracts to induce apoptosis. *In-situ* evaluation of cell death in *A. cepa* root meristem was visualized using Evans blue staining, followed by its spectrophotometric quantification. The effect of the extracts on the membranes was studied using human erythrocytes as a test model. The morphological changes like membrane distortions, blebs and formation of apoptotic bodies after the treatment revealed the cytotoxic effect of the extracts. The results suggested the ability of the extracts to induce apoptosis in the dividing cells which gave

a strong lead to the antiproliferative studies. Among the three taxa studied, *A. malabarica* displayed the best results.

Antiproliferative study

The preliminary analysis of the antiproliferative effect of the selected taxa of *Aglaia* was carried out using MTT assay on HeLa cells. The plant extracts were applied in five different concentrations for 24 h and the cytological alterations were imaged and analyzed. Quantitative estimation was also undergone spectrophotometrically. The apoptotic signs like membrane blebs, nuclear disintegration, formation of echinoid spike like structures, fragmented nuclei, *etc*., were observed in the treated cancer cells. The most remarkable activity was exhibited by *A. malabarica* with an LD_{50} of 42.137 ± 3.6 µg/mL. In order to determine the effect of *A. malabarica* extract on normal human cells, the extract was tested for its activity on L929 cells. A mild or negligible effect of the extract was evident with an LD_{50} of 212.482 µg/mL. This reveals the specific toxicity of *A. malabarica* extract against the malignant cells and negligible effect on the normal human cells. From the preliminary studies, *A. malabarica* was found to be the most effective antiproliferative agent among the selected plants and thus the further assays were limited to the same. AO/EB double staining revealed the occurrence of apoptosis as the mode of cell death in HeLa cells as a result of treatment with the half maximal concentration of *A. malabarica* extract.

The quantification of the apoptotic HeLa cells as a result of treatment with *A. malabarica* extract was carried out using flow cytometer. This was followed by the detection of cell cycle stage during which the division was arrested. From the number of cells in the different quadrants, the population profile and the histograms obtained, it was evident that the cell cycle arrest has occurred during the G0/G1 transition phase. The involvement of caspases (-3 and -9) was proved by the gene expression analysis in the treated cells and

hence, the mode of action involved was proved to be apoptosis. These activities of the plant extracts could be attributed to the presence of potential phytoconstituents as the bioactive principles.

Biogenic synthesis of silver nanoparticles

All the three selected plant extracts were employed to reduce the metallic silver in the silver nitrate solution to elemental silver particles in nano-scale. A positive result was obtained from all the extracts but in a varying extent. The UV-Visible spectral analysis resulted in the presence of the broadest peak at the range of 420-480 nm. This range of SPR is the characteristic feature of the elemental silver particles. XRD analysis of the crystal structure was carried out from which the approximate crystal size of the nanoparticles was carried out. SEM analysis unveiled the shape and almost exact particle size of the synthesized nanoparticles. The sizes of the nanoparticles were obtained as 33.1 - 71 nm, 76.3 - 101 nm and 18.1 - 30.7 nm for *A. barberi*, *A. simplicifolia* and *A. malabarica*, respectively. A cubical morphology was the common pattern observed.

Conclusions and deliberations

The present investigation unveiled the phytochemical composition and bioactivities of some endemic and under-utilized taxa of *Aglaia* in the Western Ghats. The phytochemical characterization provided a lead for the characterization of some important bioactivities. *A. simplicifolia* and *A. barberi* exhibited the protective effect on the mammalian cells. This was proved from the anti-oxidant and hepatoprotective assays carried out using the methanolic leaf extracts of the plants. Meanwhile, *A. malabarica* showed a destructive effect on the rapidly dividing cells and that too by means of apoptosis. This particular feature is highly desirable for the development of drugs against proliferative diseases. The present investigation revealed the

antiproliferative effect of *A. malabarica* extracts on the human cancer cells and the analysis of cell cycle progression revealed apoptotic death in the dividing cells during the initial phase itself. Thus the efficacy of the plant extract as an anticancer candidate is established. All the three plant extracts were also proved to be excellent reducers capable of reducing bound silver metal ions into silver nanoparticles in an environmentally safe and benign method. The excellent bioactive potentials of the plant could be attributed to the concerted action of the potent compounds in the plants. Identification, isolation and extensive studies on the bioactivities of these compounds might be required. The effective utilization of these benefits of the plant is possible by the compound isolation, extensive clinical trials and drug development studies on live mammalian models. Advanced studies on the characterization of the biogenic synthesis of nanoparticles and investigation on the bioactive potentials of the synthesized nanoparticles are also recommended.

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Appendix 1

Acetocarmine

Carmine - 2g

45% Acetic acid - 100 mL

The solution is heated to dissolve carmine and is filtered to remove undissolved stain.

Appendix 2

DMEM medium

Sodium bicarbonate - 1.85g

HEPES - 2.95g

DMEM powder - 1 packet

Distilled water - 1L

Vacuum sterilized and stored at 4 ^{o}C.

Appendix 3

Electrophoresis buffer (300mM NaOH/ 1 mM EDTA)

Stock solutions:

10 N NaOH - 200 g/500 mL distilled water

200 mM EDTA - 14. 89g/200 mL distilled water

pH - 10

Store the stock solutions at room temperature.

For 1X Buffer (make fresh buffer before each electrophoresis run) add 30 mL NaOH and EDTA, per 1L and mix well. Ensure pH as > 13 prior to use.

Appendix 4

Ethidium bromide stain

Ethidium bromide - 20

Add 10 mg to 50 mL distilled water and store at room temperature (10X).

For making 1X stock, mix 1 mL with 9 mL of distilled water. Handle Ethidium bromide with caution as it is a known carcinogen.

Appendix 5

Evans Blue (0.25%)

Evans Blue - 0.25 g

Distilled water - 100 mL

Appendix 6

Lysing solution (1000 mL)

2.5 M NaOH - 146.1 g

EDTA - 37.2 g (for 100 mM solution)

Trizma base - 1.2 g (10 mM)

1% SDS - 10 g

Add ingredients to about 700 mL of distilled water and stir the mixture. Add 8g NaOH and allow the mixture to dissolve for about 20 min. Adjust the pH to 10 using concentrated HCl or NaOH and store at room temperature. To this mixture, 10% DMSO and 1% Triton X 100 are added prior to use.

Appendix 7

Mayer's reagent

Mercuric chloride - 1.36 g

Potassium iodide - 5 g

Distilled water - 100 mL

1.36 g of mercuric chloride and 5 g of potassium iodide were dissolved separately in distilled water, both solutions were mixed and made up to 100 mL with distilled water.

Appendix 8

Modified Carnoy's fluid

Acetic acid - 10 mL

Ethanol - 30 mL

Appendix 9

Neutralization buffer

0.4 M Tris - 48.5 g

The above quantity of Tris is added to 800 mL distilled water and pH adjusted to 7.5 with concentrated HCl. The final volume is made to 1000 mL with distilled water and stored at room temperature.

Appendix 10

Phosphate buffer saline (PBS)

NaCl - 8g

KCl - 0.2 g

Na_2HPO_4 - 1.44 g

KH_2PO_4 - 0.24 g

Distilled water - 1 L

pH - 7.4

The above ingredients were mixed in 800 mL of distilled water and pH was adjusted to 7.4 with HCl. The total volume was then made up to 1 L with distilled water. The solution was sterilized by autoclaving (20 min, 121 ^{o}C) and stored at room temperature.

Appendix 11

TAE buffer (50x)

Tris base - 242 g

EDTA - 18.61 g

Glacial acetic acid - 57.1 mL

Add the Tris base and EDTA to approximately 700 mL of distilled water and dissolved. Add the acetic acid and adjust the volume to 1 L. The 1 x TAE solution is 40 mM Tris, 20 mM acetate and 1 mM EDTA and typically has a pH around 8.6.

Appendix 12

Tris-EDTA buffer

Tris-HCl - 10 mM

EDTA - 1 mM

Appendix 13

Tris-HCl buffer

Tris base - 242.2 g

HCl - 80-85 mL

Appendix 14

Wagner's reagent

Iodine - 2 g

Potassium iodide - 6 g

Distilled water - 100 mL

2 g of iodine and potassium iodide was dissolved in 100 mL of distilled water to prepare Wagner's reagent.

Research Publication

1. Archana E. R., & Thoppil, J. E. (2018). Phytochemical profiling and antibacterial efficacy screening of *Aglaia malabarica* Sasidh. *International Journal of Current Pharmaceutical Research*, *10*(1), 20-22.

2. Archana E. R., & Thoppil, J. E. (2019). *Aglaia malabarica* induced apoptosis evaluation in plant meristem, erythrocytes and human cancer cell lines. *Journal of Drug Delivery and Therapeutics*, *9*(2), 118-124.

Paper presentations

1. Archana E. R., & Thoppil, J. E. (2016).Qualitative phytochemical profile of *Aglaia* Lour. Paper presented in the National seminar on Sustainable Agricultural solutions for food security at Govt. college, Madappally, Kerala from 19th to 20th January 2016.

2. Archana E. R., & Thoppil, J. E. (2016). Cytomixis induced by *Aglaia edulis* Roxb. on *Allium cepa* root tips and its phytochemical profiling. Paper presented in the International Seminar on New frontiers in Cytogenetics & XIIIth Conference of The Society of Cytologists & Geneticists (SOCG) at University of Kerala, Thiruvananthapuram, Kerala from 15th to 17th December 2016.

3. Archana E. R., & Thoppil, J. E. (2017). Comparative evaluation of the phytoconstituents of a few rare species of *Aglaia* Louriero. Paper presented in the 4th Indian Biodiversity Congress at Pondicherry University, Puducherry from 10th to 12th March, 2017.

4. Archana E. R., & Thoppil, J. E. (2017). Bactericidal potential analysis and phytoconstituent estimation of *Aglaia malabarica* Sasidh. Poster presented in the National conference on ‘New Frontiers in Life

Sciences and Environment', Goa University, Goa from 15th to 17th March 2017.

5. Archana E. R., & Thoppil, J. E. (2017). *Aglaia malabarica* Sasidh. as a potential apoptosis inducer in eukaryotic nuclear as well as membrane model systems. Paper presented in the third Malaysian Congress on Toxicology, Malaysian Society of Toxicology, National University of Malaysia (UKM), Malaysia from 5th to 7th December 2017.