# Bioactivity studies and phytochemical characterization of some selected dye-yielding plants of Kerala

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**DOCTOR OF PHILOSOPHY IN BOTANY**

by

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# CERTIFICATE

This is to certify that the thesis entitled **"Bioactivity studies and phytochemical characterization of some selected dye-yielding plants of Kerala"** 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 **Aswathi P.,** during the period of study (2019-2023) 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 the award of any degree or diploma. I also certify that the contents in the thesis are subjected to plagiarism check using the software OURIGINAL and that no text or data is reproduced from other works.

**Dr JOHN E. THOPPIL**
Supervising Teacher

# DECLARATION

I, Aswathi P, hereby declare that the thesis entitled **"Bioactivity studies and phytochemical characterization of some selected dye-yielding plants of Kerala"** 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, Senior 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.

Calicut University
Date:

**Aswathi P.**

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***Aswathi P.***

*Dedicated to my family and friends*

# CONTENTS

# ABBREVIATIONS

| | | |
|---|---|---|
| ABTS | : | 2, 2'- Azino-bis [3-ethylbenzothiozoline-6-sulfonic acid] |
| Ag | : | Silver |
| AgNP | : | Silver nanoparticle |
| $AlCl_3$ | : | Aluminium Chloride |
| amu | : | Atomic Mass Unit |
| ANOVA | : | Analysis of Variance |
| AO | : | Acridine Orange |
| Bcl-2 | : | B Cell Lymphoma 2 |
| $CaCl_2$ | : | Calcium Chloride |
| cDNA | : | Complementary DNA |
| cm | : | Centimetre |
| $CO_2$ | : | Carbon Dioxide |
| COX | : | Cyclooxygenase |
| CUPRAC | : | Cupric ion Reducing Antioxidant Capacity |
| DLS | : | Dynamic Light Scattering |
| DMEM | : | Dulbecco's Modified Eagle's medium |
| DMRT | : | Duncan's Multiple Range Test |
| DMSO | : | Dimethyl Sulphoxide |
| dNTP | : | Deoxy Nucleotide Triphosphate |
| DPPH | : | 2, 2-Diphenyl-1- Picrylhydrazyl |
| EDAX | : | Energy Dispersive X-ray Spectroscopy |
| EDTA | : | Ethylenediamine Tetra Acetic Acid |
| ELISA | : | Enzyme Linked Immunosorbent Assay |
| EMT | : | Epithelial Mesenchymal Transition |

| | | |
|---|---|---|
| ER | : | Estrogen Receptor |
| EtBr | : | Ethidium Bromide |
| eV | : | Electron Volt |
| FCC | : | Face Centred Cubic |
| $FeCl_3$ | : | Ferric Chloride |
| Fig. | : | Figure |
| FRAP | : | Ferric Reducing Ability of Power |
| FTIR | : | Fourier Transform Infrared Spectroscopy |
| FWHM | : | Full Width at Half Maximum Intensity |
| g | : | gram |
| GC-MS | : | Gas Chromatography - Mass Spectrometry |
| h | : | Hour |
| $H_2O_2$ | : | Hydrogen Peroxide |
| $H_2SO_4$ | : | Sulphuric Acid |
| HCl | : | Hydrochloric Acid |
| HPLC | : | High Performance Liquid Chromatography |
| HR LC-MS | : | High Resolution Liquid Chromatography - Mass Spectrometry |
| $IC_{50}$ | : | Inhibition Concentration 50% |
| KCl | : | Potassium Chloride |
| KOH | : | Potassium Hydroxide |
| L | : | Litre |
| L929 | : | Normal Fibroblast Cell Line |
| $LC_{50}$ | : | Least Concentration 50% |
| LDH | : | Lactate Dehydrogenase |
| LDLs | : | Low Density Lipoproteins |

| | | |
|---|---|---|
| m/z | : | Mass-to-Charge |
| M | : | Molar |
| MAPK | : | Mitogen-Activated Protein Kinase |
| MCF-7 | : | Michigan Cancer Foundation-7 (Human breast cancer cell line) |
| MDA-MB 231 | : | M. D. Anderson-Metastatic Breast 231 (Human breast cancer cell line) |
| mg | : | Milligram |
| min | : | Minute |
| mL | : | Millilitre |
| mm | : | Millimetre |
| mM | : | Millimolar |
| MTT | : | 3-(4,5-Dimethylthiazol-2-yl),2-5-Diphenyltetrazolium Bromide |
| N | : | Normal |
| NaCl | : | Sodium Chloride |
| $Na_2CO_3$ | : | Sodium Carbonate |
| $NaNO_2$ | : | Sodium Nitrate |
| NaOH | : | Sodium Hydroxide |
| NBT | : | Nitroblue Tetrazolium |
| NCCS | : | National Centre for Cell Sciences |
| NIST | : | National Institute of Standards and Technology |
| nm | : | Nanometre |
| NO | : | Nitric Oxide |
| ORAC | : | Oxygen Radical Absorbance Capacity |
| PBS | : | Phosphate Buffered Saline |

| | | |
|---|---|---|
| PR | : | Progesterone Receptor |
| RNS | : | Reactive Nitrogen Species |
| ROS | : | Reactive Oxygen Species |
| rpm | : | Rotations Per Minute |
| RT | : | Retention Time |
| RT-qPCR | : | Reverse Transcription-Quantitative Real-Time Polymerase Chain Reaction |
| s | : | Seconds |
| SE | : | Standard Error |
| SEM | : | Scanning Electron Microscopy |
| SPR | : | Surface Plasmon Resonance |
| SPSS | : | Statistical Package for Social Sciences |
| TBA | : | Thio Barbituric Acid |
| TBARS | : | Thiobarbituric Acid Reactive Substances |
| TCA | : | Trichloro Acetic Acid |
| TE | : | Tris EDTA |
| TEAC | : | Trolox Equivalent Antioxidant Capacity |
| TEM | : | Transmission Electron Microscopy |
| TGA | : | Thermogravimetric Analysis |
| TGF-β | : | Transforming Growth Factor-β |
| TNF | : | Tumour Necrosis Factor |
| TRAIL | : | TNF Related Apoptosis Inducing Ligand |
| TRAP | : | Total Radical Trapping Antioxidant Parameter |
| UV | : | Ultraviolet |
| v/v | : | Volume Per Volume |
| V | : | Volt |

| | | |
|---|---|---|
| w/v | : | Weight Per Volume |
| WHO | : | World Health Organization |
| XRD | : | X-ray Diffraction |
| µg/mL | : | Microgram Per Millilitre |
| µg | : | Microgram |
| µl | : | Microlitre |
| µm | : | Micrometre |
| µM | : | Micromolar |
| $^{o}$C | : | Degree Celsius |

# INTRODUCTION

Dyes are coloured compounds that can be usually applied in various substrates like cosmetics, textiles, food, hair, paper etc. Natural dyes or colourants are commonly derived from plants, invertebrates, minerals, etc. Among them, plants are the major sources of natural dyes and these plants are generally called dye-yielding plants. The dye-yielding parts for each plant are different in various species. The majority of the dyes are extracted from the roots, fruits, leaves, flowers, bark, etc. Besides, most of the dye-yielding plants have great medicinal value and can be used for various other purposes as well. Natural dyes extracted from plants have a great influence on human life because of their non-toxic characteristics. But nowadays the use of cheaper synthetic dyes is increasing day by day. The frequent use of these synthetic dyes can cause big health issues. Therefore, awareness of such dye-yielding plants and their products will act as a stepping stone in the field of commercial as well as therapeutical industries.

Plants are not only used for the basic needs of human life like food, fibre, fuel, and shelter but also used as natural colourants. These natural colours are formed due to the absorption of light in the visible region of 400–800 nm by various organic and inorganic molecules and their mixture in plants (Chengaiah et al., 2010). Indian Vedas are the oldest written religious scriptures which originated from priameval India and comprise four divisions namely, Rig Veda, Sama Veda, Yajur Veda, and Atharva Veda. In Atharva Veda, we can find major descriptions of natural dyes. Natural dyeing materials are also used in the wall paintings of Ajanta, Ellora and Sithanavasal and they still establish the effectiveness of dyeing craft that had been inherited from ancient times in India (Ravi & Choudhary 2012). In recent years, the importance of natural dyes has to be taken seriously because

the frequent use of synthetic dyes is polluting the environment. Moreover, the carcinogenicity of certain diazo dyes makes synthetic dyes highly unpopular as well as poorly accepted. Due to this reason, a global ban has been imposed by EU, USA, and India on the use of several toxic synthetic dyes. Additionally, natural dyes are biodegradable as well as highly compatible with the environment and their uses can reduce the defects caused by synthetic dyes. These reasons point towards the need to investigate natural and eco-friendly dyes with a view to increase their commercialization. According to Chandramouli (1995), various plant parts can produce more than 2,000 colour pigments, out of these only about 150 have been commercially exploited. Some plants may synthesize more than one colour depending on the plant part (Sinha et al., 2012). Barks and roots are the common sources of red colourants (Vankar, 2000). In the natural dye class, yellow is one of the most common and abundant ones (Gulrajani et al., 1992; Roshan, 1996). Plants that produce green dyes are rare. Also, there is virtually no limit to the natural sources for producing brown colour. Red and yellow dyes can also have the capacity to produce orange (Tayade & Adivarekar, 2013). Black shades are generally formed from tannin-rich plants and are appreciably applicable for cellulosic and protein fibres (Hao et al., 2006). Soxhlet extraction is one of the common methods for the extraction of dyes from plant parts. For this method, organic solvents are needed which include acetone, petroleum ether, chloroform, ethanol, methanol, or a mixture of solvents such as a mixture of ethanol and methanol, a mixture of water with alcohol, and so on. The water/alcohol extraction method is used to extract both water-soluble as well as water-insoluble substances from the plant resources. The extraction yield is very higher than the aqueous method because a larger number of chemicals and colouring materials are extracted (Aggarwal, 2021).

The present study is mainly focused on the bioactive studies and phytochemical characterization of selected dye-yielding plants, which include *Terminalia paniculata*, *Mallotus philippensis* and *Albizia odoratissima*. The methanolic extract of the dye-yielding plant parts for each one is selected for the study. *T. paniculata* produces a yellow colour extract and *M. philippensis* produce an orange-coloured extract from its fruits. But in the case of *A. odoratissima*, the bark is used as the major source of the dye. It produces a brick red coloured extract. All these extracts could be obtained with the help of the Soxhlet extraction technique. In plants, a wide spectrum of essential and non-essential phytochemicals is available. These phytochemicals include primary as well as secondary metabolites. Primary metabolites are very common in all plants and help to maintain and build plant cells. Whereas, secondary metabolites are usually responsible for the normal growth and development of plants. They are involved in the offense and defense mechanisms and other miscellaneous activities of the plants. These secondary metabolites can be used for the production of medicines, flavourings and recreational drugs. Also, their medicinal values are very well-known all over the world (Prabhu et al., 2011). Phenols, flavonoids, alkaloids, saponins, phlobatannins, terpenoids, coumarins etc. are the major secondary metabolites found in plants. Synergistic actions of these phytochemicals have the capacity to boost the bioactivities and pharmacological properties of a plant. In this study, the presence of such phytochemicals was evaluated with the help of qualitative and quantitative analysis. Moreover, phytochemical screening is a very important method to identify the new sources of phytoconstituents that are significant in therapeutical as well as industrial purposes. Also, plants are very rich in secondary metabolites and are widely used in traditional medicines and for curing various ailments like anti-inflammatory, antispasmodic, analgesic and diuretic activities (Savithramma et al., 2013). The presence of saponins, tannins, alkaloids and steroids in a plant represents

the pharmacological importance of that plant (Hostettmann, 1995). According to Ndukwe et al. (2005), the presence of saponins and aglycones in a plant extract reveals its ability to cure antiulcerogenic, anti-inflammatory, fibrinolytic, antipyretic, analgesic and antioedematous issues. Different solvent systems such as water, ethanol, methanol, petroleum ether etc. are also responsible for the extraction of secondary metabolites like tannins, terpenoids, alkaloids, flavonoids, phenols and quinones (Tiwari et al., 2011). The amount of these secondary metabolites is varying plant to plant due to the variations in climates and ecological factors (Baquar, 1989). Additionally, phytoconstituents also play an important role in protecting plants from microorganisms, insects and other phytophagous organisms (Farnsworth & Morris, 1976). GC-MS, LC-MS, HR LC-MS etc. are routine analytical techniques used for the identification, monitoring and screening purposes of phytochemicals (Zwiener & Frimmel, 2004). GC-MS analysis is mainly responsible for high-resolution compound separation, especially for the volatile compounds. Hence in the field of plant metabolomics, the exploration using this technique is highly appreciable. LC-MS techniques are commonly used for the identification of non-volatile compounds and are not well categorized by other methods of metabolomics. It is a promising tool for the identification of structural information of new or uncommon compounds (Kasture et al., 2012).

Antioxidants are substances that prevent the oxidation of other compounds or neutralize toxic free radicals. Biological systems hold destructive free radicals due to several metabolic processes. Antioxidant activity defines the ability of a drug to scavenge these free radicals. Oxidation is a noteworthy process in living organisms for the crucial performing of catabolism. However, oxygen-centered free radicals and some reactive oxygen species (ROS) were produced inside the body that may cause severe diseases like atherosclerosis, diabetes, cancer, and cirrhosis (Halliwell &

Gutteridge, 1995). Environmental pollutants involve automobile exhaust, radiation, air pollution, cigarette smoke, and pesticides. They can play a vital role to generate free radicals (Li & Trush, 1994). Generally, there is a balance between the number of free radicals produced in the body and the antioxidant defense mechanisms. By scavenging, antioxidants will protect the body from the deleterious effects of these toxic free radicals (Nose, 2000). Antioxidants from plants are a large group of bioactive constituents that comprises flavonoids, phenolic compounds, sulphur-containing compounds, alkaloids, tannins, terpenoids and vitamins (Yashin et al., 2017). These compounds show different antioxidant properties. Flavonoids have the capacity to scavenge free radicals and can form complexes with catalytic metal ions for inactivating them. Also, the efficacy of plant-derived secondary metabolites as a good source of antioxidants against various diseases is induced by ROS. Phenolic compounds, such as flavonoids and phenolic acids are responsible for the antioxidant property (Kumar et al., 2018). Moreover, antioxidants have the ability to protect lipids and oils in food from oxidative degradation. When antioxidants are added to food, it controls the rancidity development, reduces the formation of toxic oxidation products, and sustains the nutritional quality, and increase the shelf life of products. The increased level of free radicals in cells and tissues can be induced by several negative factors like gamma, UV, X-rays, psycho-emotional stress, toxic food, adverse environmental conditions, severe physical exertion, smoking, alcoholism, and drug addiction. Body macromolecules that include lipids, proteins, deoxyribonucleic acid (DNA), and carbohydrates are susceptible to damage by the high concentration of free radicals (Olugbami et al., 2015). Furthermore, diseases like cancer, heart-related problems, and the acceleration of aging are common issues caused by chronic oxidative stress (Bi et al., 2017; Serafini & Peluso, 2016). Also, oxidative stress possessed an active part in the physiology of very common diseases such as diabetes, high blood

pressure, pre-eclampsia, atherosclerosis, acute renal failure, Alzheimer's, and Parkinson's Diseases (Munteanu & Apetrei, 2021). The uncontrolled increase in intracellular ROS can induce cancer cell cycle arrest, senescence, and apoptosis. Hence, it can be checked effectively with chemotherapy (Liou & Storz, 2010).

Antioxidant capacity is mainly determined by different principles which include peroxyl radical scavenging (Oxygen radical absorbance capacity, ORAC), total radical-trapping antioxidant parameter (TRAP), metal-reducing power (Ferric reducing antioxidant power, FRAP), cupric reducing antioxidant capacity (CUPRAC), hydroxyl radical scavenging (deoxyribose assay), organic radical scavenging [(2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) ($ABTS^{\bullet+}$)], 2,2-Diphenyl-1- picrylhydrazyl (DPPH), quantification of the products formed during the lipid peroxidation (Thiobarbituric acid reactive substances, TBARS), low-density lipoproteins (LDLs) oxidation, etc. Among these, the most commonly used procedures for evaluating the antioxidant capacity are FRAP, ABTS, TEAC (Trolox equivalent antioxidant capacity), DPPH, and ORAC (Pérez-Jiménez et al., 2008). The DPPH method is a rapid, simple, accurate, and very cost-effective technique for evaluating the ability of different compounds to act as free radical scavengers (Prakash et al., 2001). Hence the effect of antioxidant activity in selected dye-yielding plants will make a milestone in the therapeutic field for curing various ailments. Also, it reveals a perfect scavenging ability to scavenge free radicals produced by various toxic synthetic products including colorants.

Increased concentrations of toxic free radicals as well as oxidative stress will destruct the structural and functional properties of vital organs. This is mainly due to modern food habits and lifestyle. The liver is one of the prime victims of this. The addition of synthetic colourants and toxic

preservatives will adversely affect the functioning of the liver and may ultimately lead to cancer. About 20,000 deaths occur every year due to liver diseases. Hepatocellular carcinoma is one of the ten most common tumors with 2,50,000 new cases reported every year (Kshirsagar et al., 2011). Many plants and polyherbal formulations are used to cure liver diseases. But in many cases, treatments are not satisfactory. As a result, experimental evaluation of the hepatoprotective activity of various plants is increasing day by day. Common antioxidants will protect the liver from damage caused by oxidative mechanisms of toxic chemicals (Kumar et al., 2011a). Due to their high antioxidant property, dye-yielding plants are sufficiently active against certain hepatotoxins (Adewusi & Afolayan, 2010). Plants and their bioactive compounds play a vital role in the treatment of hepatic disorders. Bioactive compounds including polyphenol-containing plants exhibited antioxidant and hepatoprotective properties (van Wyk & Wink, 2015). The high amount of alkaloids, flavonoids, and saponins in an extract possesses high hepatoprotective activity (Meshack & Gupta, 2022). Moreover, Patel et al. (2017) reported that plants containing active constituents like flavonoids, glycosides, monoterpenes, coumarins, lignans, essential oils, carotenoids, organic acids, alkaloids, and xanthenes are also responsible for the hepatoprotective activity. Reactive oxygen/nitrogen species (ROS/RNS) are the free radicals that exert oxidative stress and cause several hepatic abnormalities such as degeneration, necrosis, apoptosis, swelling, etc. (Kumar et al., 2019). Liver transplantation is now common and improves the survival rate of patients, but due to the non-availability of compatible donors, this treatment is limited. Hence, there is an imperative need for possible remedies to improve liver regeneration as well as to overcome liver failure. This problem can counteract by the use of natural products and plant extracts having antioxidant and hepatoprotective properties (van Wyk & Wink, 2015). Antimicrobials, anticonvulsants, corticosteroids, NSAIDs, and analgesics are

some synthetic compounds available as hepatoprotective agents. But these compounds are not completely safe and expose several side effects and disadvantages. Therefore, investigations of natural therapeutic benefits are helpful for liver disease patients. Besides, a number of herbal preparations are available in the market. Triphala is one of the commonly used polyherbal formulations in the Indian system of medicine mainly in Ayurveda with known hepatoprotective activities (Gupta et al., 2015). Several investigations are conducted on various kinds of plant species to discover their possible hepatoprotective activity to cure liver diseases.

Induction of chromosomal aberration is one of the easy and cost-effective techniques, where among plant tissues primarily the root tips are used (Auti et al., 2010). *Allium cepa* is very easy to use and its macroscopic and microscopic parameters can be easily managed. Besides, this system will preferably correlate the data obtained from the eukaryotic and prokaryotic systems (Özkara et al., 2015). Mitotic index (MI) is the best parameter to evaluate the cytotoxicity of various agents (Gonzalez et al., 2012). With the help of the *Allium cepa* root tip assay, the determination of cytotoxic and/or genotoxic effects of various substances can be easily understood (Timothy et al., 2014). This assay has many advantages including the investigation of the effect of physical and chemical mutagenesis, cytotoxicity of polluting agents, plant extracts, and cytogenetic effects of similar active materials in mitotic cell division (Türkoğlu, 2007; Rank, 2003). Cytotoxic agents have a destructive action in certain cells ([1]http). For estimating the effect of mutagens, the degree of cytological aberrations either in mitosis or meiosis is a dependable criterion (Seetharami Reddi & Reddi, 1985). Also, cytotoxicity is used to describe the immune activity as well as the toxicity of certain drugs which limit the development of cancer cells. It is also responsible for the pharmaceutical effects, especially in the area of cancer research. A reliable indicator of mutagenic activity is the chromosomal aberrations and there are

evidences for the correlation between chromosomal damage and the cytotoxic effects of different plants (Mohandas and Grant, 1972). When cells were treated with cytotoxic compounds, it will result in various cell fates and the aberrations are mainly due to the effect of plant extract as well as other chemicals. As a result, scientists use these extracts as herbal cytotoxicants ([2]http).

Among one-third of all malignancies, breast cancer is the most commonly occurring cancer in females. About 25-30% of females with breast cancer will die from this invasive disease. Also, mortality rates are very high in women below the age of 35 and also in old ones especially those greater than the age of 75. It occurs as a very aggressive disease in young ones and may not be treated assertively. For the old, it may increase breast cancer fatality. Moreover, about 95% of breast cancers are carcinomas and they emerged from the breast epithelial elements (Richie & Swanson, 2003). There are numerous causes associated with breast cancer including oxidative stress and is operating in most of the intercellular pathways concerned with cell proliferation (Sanches et al., 2017). The level of oxidative stress in breast cancer is higher in breast cancer patients when compared to healthy people because of genetic abnormalities (Cuchra et al., 2016; Cramer et al., 2017). Commonly used breast cancer cell lines were established in the late 1970s. The available number of breast cancer cell lines is very less and a few of them were studied effectively. Widely used breast cancer cell lines for various studies are T-47D, BT-474, SK-BR-3, MDA-MB 231, Hs578T etc., which are used as tumour models (Lacroix & Leclercq, 2004). Most of the available breast cancer cell lines are delivered from metastatic tumours, especially from pleural effusions. These effusions will provide usually large numbers of dissociated, viable tumour cells with less or no contamination by fibroblasts and other tumour stroma cells (Cailleau et al., 1978). MDA-MB 231 breast cancer cell line is generally used to model late-stage breast cancer. They are

ER (estrogen receptor), PR (progesterone receptor), and E-cadherin negative and with a mutated p53. Also, these cells lack the growth factor receptor HER2 and are the best model for triple-negative breast cancer (Welsh, 2013).

The cell cycle involves DNA replication and cell division. The analysis of the cell cycle and the measurement of cellular DNA content can be unveiled by flow cytometry. It is a very fast method in which the nuclear DNA content of a cell can be easily measured quantitatively. In order to determine the cellular DNA content, flow cytometry also identifies the cell distribution during the various phases of the cell cycle. G1, S (DNA synthesis phase), G2, and M phase (Mitosis) are the four different phases that could be recognized in a proliferating cell population (Nunez, 2001). Cell cycle control mechanisms are of two types in which the first one involves kinase activation to relay a cell from one stage to the next. Whereas the second one is a type of checkpoint control by which the flaws in critical events activate a delay in cell cycle progression. Although toxicants may cause cell damage or stress, cellular proteins were involved in cell cycle control and apoptosis, which are the final arbiters of cell fate. After stress, the biochemical pathways that restrain cell cycle transition and/or induce cell death are known as cell cycle checkpoints. The fidelity of DNA replication, repair, and division can be maintained by these checkpoints (Pietenpol & Stewart, 2002). The genes involved in apoptosis can be found with the help of cell cycle analysis and further studies in gene expression can be performed.

Apoptosis is a defense mechanism and is described as a programmed type of cell death. Various types of cellular death mechanisms are there which include pyroptosis, necrosis, or autophagy, and some others are not yet been discovered (Thompson, 1995). Mitochondria play an active role by interacting with various apoptotic as well as anti-apoptotic proteins and they release signal molecules (Rosenblatt et al., 2001). Moreover, mitochondria provide

distinct pro-apoptotic signals and which create a downstream cascade of apoptosis activation. There is a balance between the pro-apoptotic and anti-apoptotic molecules, which maintain the stability of cellular homeostasis and defines the cell fate, whether it is apoptosis or proliferation. Besides, mitochondria release apoptosis-inducing molecules like cytochrome C, SMAC, apoptosis-inducing factor, or endonuclease G due to the permeabilization of the mitochondrial membrane. Pro-apoptotic B cell lymphoma (Bcl)-2 family proteins triggered the permeabilization and the anti-apoptotic members of Bcl-2 family maintained the integrity of the mitochondrial membrane (Ferri & Kroemer, 2001). p53, p16, p21, p27, E2F genes, FHIT, PTEN etc. were the apoptotic genes, whereas Bcl-XL, Bcl-w, Bcl-2a. Mc11 etc. are the anti-apoptotic genes. The pro-apoptotic genes include Bax, Bak 1, Bim, Bid, etc. (Horvitz, 1994).

Apoptosis can be stimulated by two different pathways which include the intrinsic pathway and the extrinsic pathway. The intrinsic pathway mainly occurred through the release of cytochrome C from the mitochondria. Also, it activates the distinct caspases as downstream signals. Extrinsic pathway occurred when the Fas death receptor is stimulated by a signal entering from the outside of the cell. Both these pathways will coincide at the final step of the caspase activation after the stimulation of intermediate molecules by signaling cascade and causes the cleavage of different proteins (Ghobrial et al., 2005). The Bcl-2 family of proteins and p53 tumour suppressor protein control all the intrinsic apoptosis events and are involved in the activation of Bcl-2 family proteins. Bcl-2 protein family members can act either as pro-apoptotic (Bax, Bak, Bid, Bim, Puma, Noxa, Bad, and Blk) ones or anti-apoptotic (Bcl-2, Bcl-XL, Bcl-X, and BAG) involved in the process of cytochrome C release (Elmore, 2007; Cory & Adams 2002). Apoptosis generated by the extrinsic pathway occurs by the signaling through membrane-bound death receptors that belong to the tumour necrosis factor

(TNF) gene superfamily. The primary signal is provided by the interaction between the ligands and the cell membrane death receptors like Fas ligand/ FasR, TNF/TNF R1, Apo2L/DR4, or TNF-related apoptosis-inducing ligand (TRAIL) R1, which can happen on attachment of ligands to death domains of these receptors (Locksley et al., 2001). Bid is the pro-apoptotic member of Bcl-2 family, which exhibit a common molecule between intrinsic and extrinsic pathways of apoptosis (Sax et al., 2002).

Epithelial-mesenchymal transition (EMT) defines the rapid and often reversible change of cell phenotype. Throughout EMT, epithelial cells loosen cell-cell adhesion, which modulates their polarity, rearranges their cytoskeleton organization and express vimentin filaments and downregulate cytokeratin. Here, cells become motile, isolated, and become resistant to apoptosis (Savagner, 2010). EMT concepts have been extended to the physiological process of partial EMT that occurs during wound healing and mammary tubulogenesis based on the similarities between transducing pathways (Arnoux et al., 2004). Also, EMT like process is induced during tumour progression and metastasis emergence (Tarin et al., 2005). Many of the genes and pathways are involved to induce EMT in tumour cells. These pathways are also responsible for various processes such as cell proliferation, apoptosis and differentiation during early developmental stages, tissue morphogenesis, and wound healing (Savagner, 2010). Moreover, the EMT phenomenon has been a useful explanation of distant metastases for epithelial cancers including breast cancer (Wu et al., 2016). Three different analyses *viz*., cell migration assay, cell aggregation, and clonogenic assay are favourable methods to describe the EMT in cancer cell lines. Cell migration assay is a simple and cost-effective method to know the directional cell migration *in vitro*. It is mainly helpful to know the effects of cell-matrix and cell-cell interactions during cell migration (Rodriguez et al., 2005). Cell aggregation assay is mainly helpful to test the functionality of the complex in

epithelioid tumour cells. The functional integrity of the complex is a basic criterion for the cell-cell adhesion between epithelial cells and for measuring the aggregation of cells *in vitro*. Also, it is a fruitful method to study the differences between invasive and non-invasive cell types (Boterberg et al., 2001). The clonogenic assay is a favourable tool to check whether a given cancer therapy can reduce the clonogenic survival of cancer cells. It is an *in vitro* cell survival assay used to know the ability of a single cell to grow into a colony. Also, it is used to determine cell reproductive death after treatment with ionizing radiation and for getting information regarding the effectiveness of other cytotoxic agents (Yang, 2012).

Nanotechnology is an emerging technology with diverse applications. In biological sciences, the use of metallic nanoparticles is very renowned for its applications in drug delivery, bio-imaging, bio-sensing, catalysis, etc. Recently, a new biological approach has been attempted for the synthesis of nanoparticles with negative toxicity. In this technique, biological materials are used as the reducing bioactive agents including plants and microorganisms. Nanoparticle synthesis by green technology is an inexpensive, acceptable, efficient, and eco-friendly safe method when compared to other physical and chemical methods (Prabhu & Poulose, 2012). Nanomaterials are promising candidates for substituting conventional chemotherapy (Jeevanandam et al., 2018). The development of nanomaterials also allows new scientific methods for diagnosis, treatment, monitoring, cancer prevention, and treatment of inflammatory and neurological diseases (El-Sayed & Kamel, 2020). They have an appreciable penetrating capacity in epithelial and inflammatory cells, which leads to significant effectiveness and better persistence in the treatment (Agarwal et al., 2019). Silver nanoparticles are one of the highly utilized nanomaterials among nanoparticles due to its high antimicrobial activity, high electrical conductivity as well as optical properties. Besides, silver nanoparticles have a wide range of applications in pharmaceutical research.

Also, there are several reports that they are helpful in the purification of drinking water or effluent water by the removal of water pathogens (Lin et al., 2013; Krishnaraj et al., 2010). Furthermore, the applications and activity of silver nanoparticles are not only depending on their metallic constituents but also their size and shape. Small-sized nanoparticles with spherical shape are more active than the other morphologies with larger sizes (Tripathi et al., 2019). In pharmacological studies, nanoparticles are used as a carrier for site-specific targeting and drug delivery which enhance bioavailability (Bonifacio et al., 2014). Dye-yielding plants act as strong candidates for the synthesis of silver nanoparticles. So, the in-depth study of silver nanoparticles in such plants is very necessary due to their impact on biological systems (Aswathi & Thoppil, 2022).

*Terminalia paniculata* Roth. is a tropical tree belonging to Combretaceae family with a large natural distribution in Western Ghats, India. It is a deciduous tree, widely spread over tropical semi-evergreen and moist deciduous forests. Flowers have ten stamens, which remain inside the bud, and anthesis takes place at different times of the day (Srinivasan et al., 2016; Thangaraja & Ganesan, 2008).

**Systematic position (APG IV, 2016)**

| | | |
|---|---|---|
| Kingdom | : | Plantae |
| Clade | : | Tracheophytes |
| Clade | : | Angiosperms |
| Clade | : | Eudicots |
| Clade | : | Rosids |
| Order | : | Myrtales |

Family : Combretaceae

Genus : *Terminalia*

Species : *paniculata*

Conventionally, flower juice and bark of *T. paniculata* have been used as a treatment for cholera, diabetes, inflamed parotid glands, and in menstrual disorders. It has been broadly utilized as wood and non-wood products for construction and is often used as a substitute for teak. Moreover, *T. paniculata* is used in indigenous drug preparations. Tannins, gums, oils, fodder, and various organic compounds are extracted from its leaves, trunk, bark, and fruits.

*Mallotus philippensis* L. is commonly known as Kamala and belongs to the family Euphorbiaceae. It is a medium-sized much branched, tolerant and soil-improving small tree and is widely distributed throughout tropical India along the Himalayas from Kashmir eastwards up to 5000 ft, mainly found in Uttar-Pradesh, Bengal, Assam, Burma, Singapore, and from Sind Southwards to Mumbai and Ceylon. Also, it is reported in China, the Malaya islands, Australia, Pakistan, and Andaman Islands (Sharma & Varma, 2011).

**Systematic position (APG IV, 2016)**

Kingdom : Plantae

Clade : Tracheophytes

Clade : Angiosperms

Clade : Eudicots

Clade : Rosids

Order : Malpighiales

Family : Euphorbiaceae

Genus : *Mallotus*

Species : *philippensis*

Biologically active phytocompounds are very rich in this plant and is also considered as a common dye-yielding plant. In the Indian system of medicine, it is one of the inevitable common plants. Its various parts are used to cure different diseases that include, skin problem, bronchitis, antifungal disease, tapeworm infection, eye disease, cancer, diabetes, diarrhoea, jaundice, malaria, urinogenital infection, etc. *Mallotus* is a highly cross-pollinated plant and differences among the same species are limited. It consists of both male and female plants. Anciently, Kamala, the plant dye produced from *Mallotus* was used in India for dyeing silk and wool to a bright orange colour. Now-a-days it is still used for the same purpose to a limited extent. For soaps, oils, ice cream, and drinks, Kamala is used as a colouring agent. Especially, rottlerin and its derivatives are employed for colouring foodstuffs, lemonades, lime juice, and other beverages. Also, the woman in India used Kamala as a sindhur or Kumkum. Generally, fruits are used for preparing dyes (Sharma & Varma, 2011).

*Albizia odoratissima* (L. f.) Benth., commonly known as 'Black Siris' and 'Ceylon rosewood,' is a fast-growing deciduous tree. It belongs to the family Fabaceae and is a large-sized tree distributed throughout India, Sri Lanka, and Nepal. It has been widely used in Indian folk medicine to cure various ailments that include inflammatory pathologies, like arthritis, dysentery, sepsis, burns, asthma, allergic rhinitis, and helminth infections (Banothu et al., 2017).

**Systematic position (APG IV, 2016)**

| | | |
|---|---|---|
| Kingdom | : | Plantae |
| Clade | : | Tracheophytes |
| Clade | : | Angiosperms |
| Clade | : | Eudicots |
| Clade | : | Rosids |
| Order | : | Fabales |
| Family | : | Fabaceae |
| Genus | : | *Albizia* |
| Species | : | *odoratissima* |

It is a large erect tree spread over the sub-Himalayan tracts, slopes, and valleys up to 1,500 m, common in peninsular India, Assam, West Bengal, and also throughout the Western Ghats of South India. Almost all the plant parts are used for anxiety and depression issues. The flower head can treat digestive disorders, insomnia, and diuretic problems. It is used as a sedative and anthelmintic. Often, the stem has the power as an analgesic and stimulant, used to cure swelling, injuries, abscesses, and also used as a diuretic, anthelmintic, and to relieve diabetic problems. In the conventional Indian Medicine, its bark is used for the treatment of leprosy, ulcers, and cough. Also, the bark is used as an astringent, depurative, acrid, and cooling expectorant. It is helpful to treat skin diseases, rheumatism, erysipelas, cough, bronchitis, diabetes, and burning sensation (Kumar et al., 2011b).

By considering these aspects, the present study was mainly focused on the exploration of selected dye-yielding plants *viz*., *T. paniculata*, *M.*

*philippensis*, and *A. odoratissima,* especially in their phytochemical as well as bioactivity characterizations.

The important objectives of the study are as follows:

- Phytochemical characterization of selected dye-yielding plants by qualitative, quantitative, GC-MS, and HR LC-MS analyses
- To screen the free radical scavenging activity of the selected dye-yielding plants
- To assess the hepatoprotective activity of the plant that exhibits high antioxidant potential
- To evaluate the cytotoxic activity of the plants by *Allium cepa* root tip assay
- Determination of the antiproliferative activity of the selected dye-yielding plants on MDA-MB 231 (Human breast cancer) cell lines
- Elucidation of antiproliferative mechanism by gene expression studies
- Epithelial-mesenchymal transition (EMT) screening on MDA-MB 231 cell lines
- Biosynthesis of silver nanoparticles and their physicochemical characterization

# REVIEW OF LITERATURE

## Phytochemistry

Phytochemical screening is a developed technique that is used to determine the presence of chemical constituents in plants. It is mainly responsible for the medicinal activity of plant species. In food plants, these phytochemicals have a reliable function in human biology and have health benefits. Moreover, all the plant parts can produce immensely active compounds and have very much importance both therapeutically and industrially.

The phytochemical analysis is very important in identifying prime compounds like alkaloids, phenolic compounds, saponins, steroids, flavonoids, tannins, terpenoids, etc. (Akindele & Adeyemi, 2007). Anciently the crude drugs were identified by the comparison only based on the standard descriptions available, but now various techniques have been followed for the standardization of crude drugs by the improvement in the field of pharmacognosy (Savithramma et al., 2010). Dubey et al. (2004), carried out the complete phytochemical investigations of medicinal plants and secondary metabolites that are responsible for the medicinal activity of these plants. From ancient times, plant products have been a part of phytomedicines. These can be synthesized from any part of the plant like bark, leaves, flowers, seeds, etc i.e., any part of the plant may contain active components. Detailed knowledge is essential regarding the chemical constituents of plants because such information will be providing a positive awareness about the synthesis of complex chemical substances (Cragg & Newman, 2001).

The genus *Terminalia* includes about 200 tropical trees and shrubs belonging to the family Combretaceae (Mongalo et al., 2016). The

phytochemistry of *Terminalia* and its medicinal uses have been reported worldwide (Cock, 2015). Commonly, most compounds have been isolated from the roots and stem bark of the plants. In the majority of plants, phenolic acids are isolated from the roots. Other important phytoconstituents like tannins, flavonoids, saponins, etc. are detected in high quantities from the methanolic extract of both roots and leaves (Viol et al., 2013). Moreover, a few useful phytocompounds were also isolated from *Terminalia arjuna* such as terpenoids, tannins, and flavonoids. All these compounds are biologically effective in various fields like triterpenoids for cardiovascular activities, whereas tannins and flavonoids for the anticancer and antimicrobial properties (Nema et al., 2012). The ethanolic bark extract of *T. paniculata* exhibited a maximum quantity of phytoconstituents like alkaloids, glycosides, saponins, flavonoids, coumarins, tannins, and carbohydrates (Gangwar & Ghosh, 2016). Moreover, several reports revealed that preliminary phytochemical analysis of the methanolic bark extract of *T. arjuna* possesses phytosterols, lactones, flavonoids, phenolic compounds, tannins, and glycosides in high quantities (Mandal et al., 2013). Some phenolic compounds like flavonoids, phenolic acids, and tocopherols are the major natural antioxidants that originated from plants (Ali et al., 2008).

Phytochemical screening of *T. glaucescens* revealed the presence of alkaloids, tannins, saponins, steroids, flavonoids, anthraquinones, and phlobatannins (Adebayo & Ishola, 2009). Similarly, the reports of *T. avicennioides* revealed that biologically active phytochemicals like phenols, steroids, glycosides, flavonoids, tannins, and ellagic acids were present in its bark, leaves, and roots. But anthraquinones were not detected when all the plant parts of *T. avicennioides* were phytochemically screened and the spotted compounds possess a significant influence on antimicrobial activity (Mann et al., 2008).

*Mallotus philippensis* (Kamala tree) is conventionally used by various tribal groups as a remedial agent for various diseases. So, the scientific evidence for the medicinal potential of the plant should be needed to develop drugs from this plant. To accomplish this, the phytochemical analysis of the plant was carried out by Kumar et al. (2020) and revealed about 50 different phytochemicals. These phytochemicals hold various biological activities like anthelmintic, antibacterial, anti-inflammatory, antioxidant, anti-cancerous, anti-tuberculosis, anti-parasitic, analgesic, anti-urolithiatic and anti-viral activities. Several pentacyclic triterpenoids were isolated from the heartwood and bark of *M. philippensis*. Moreover, 15 different tannin compounds were identified from the leaves. Its fruits revealed countless phytochemicals like phenolic compounds, flavonoids, phloroglucinol derivatives, chalcone derivatives, etc.

The principal constituents of *M. philippensis* are the derivatives of phloroglucinol such as rottlerin, 4'-hydroxyrottlerin, isorottlerin, 4'-hydroxy-isorottlerin, isoallorottlerin, etc. Several red and yellow compounds (Kamalins) are very rich in *M. philippensis* fruit powder (Hong et al., 2010; Kulkarni et al., 2014). Flavonoid compounds were also isolated from the flowers and fruits of the plant such as 5, 7-dihydroxy-8-methyl-6-prenylflavanone; 6, 6-dimethylpyrano (2'', 3'': 7, 6)-5-hydroxy-8- methyl flavanone (Furusawa et al., 2005), 3'-prenylrubranine (Zaidi et al., 2009), and 8-cinnamoyl-2, 2-dimethyl-7-hydroxy-5- methoxychromene (Hong et al., 2010). According to Kumar et al. (2020) phytochemicals of *M. philippensis* were identified and categorized from the fruits (21%), followed by leaves (13%), bark (12%), seeds (11%), wood (6%) and flowers (3%).

*Mallotus philippensis* have substantial therapeutic values which are recommended by the effect of biologically important secondary metabolites like alkaloids and glycosides (Cheng et al., 1998). Chemical compounds

isolated from *M. philippensis* such as diterpenoids, steroids, flavonoids, cardenolides, triterpenoids, coumarins, isocoumarins, and phenolic compounds *viz*., bergenin, mallotophilippinens, rottlerin, and isorottlerin conveyed fascinating biological activities like antimicrobial, antiviral, cytotoxicity, antioxidant, anti-inflammatory, immunoregulatory activity as well as protein inhibition against cancer cells. The seeds of *M. philippensis* contain cardenolides which include corotoxigenin L-rhamnoside, and coroglaucigenin L-rhamnoside. However, petroleum ether extract of heartwood can prominently yield triterpenoids like betulin-3-acetate, lupeol acetate, and lupeol. Hence from the bark a friedelane-type triterpenoid *i.e.,* friedelin was identified. It is very common in plants and moreover, a pentacyclic terpenoid called acetylaleuritolic acid was also isolated from the petroleum and ether extract of the stem bark (Gangwar et al., 2014).

There are several reports which revealed that *Albizia odoratissima* was used in countless medicinal aspects and the importance of their leaves are enlightened in various ancient traditional systems of medicine. The preliminary phytochemical screening of these leaves is carried out in different solvents like petroleum ether, chloroform, ethyl acetate, methanol, ethanol, and water. Carbohydrates, proteins, amino acids, steroids, glycosides, alkaloids, flavonoids, tannins, phenolic compounds, fixed oils, etc. are the important phytochemicals obtained by the phytochemical analysis (Rajan et al., 2011). Similarly, the phytochemical investigation of *A. odoratissima* bark revealed a high quantity of secondary metabolites like flavonoids, tannins, carbohydrates, saponins, and triterpenoids (Chandra & Tarasingh, 2011).

Literature revealed that most of the species of *Albizia* are very rich in triterpenoid saponins which include oleanolic acid, echinocystic acid, and acacic acid lactone or machaerinic acid γ-lactone. Moreover, the presence of sugar residue such as arabinose, xylose, rhamnose, fucose, glucose or 2-

acetamido-2-deoxy glucose could provide an incredible position in traditional medicine systems, especially in folk medicine. Countless pharmacological activities were reported in *Albizia* extracts with saponins. Pure isolated saponins from the extracts also possess significant pharmacological activities (Singab et al., 2015). The majority of triterpenoids are active components in *Albizia* species and generally total saponins were extracted in 70% ethanol. The structural complexity of saponins in *Albizia* species might be due to the existence of pharmacological and biological properties (He et al., 2020).

Exploration in *Albizia* species revealed flavonoid components such as flavones, isoflavones, flavonols, chalcone, flavanes, etc. They have been used in diverse bioactivities like anti-inflammatory (Yadava & Tripathi, 2000), sedative (Kang et al., 2000) and antiproliferative (Fotso et al., 2017) activities. *Albizia* species have been widely used for the treatment of fever, wounds, insomnia, diabetes, rheumatism, snake bite, etc. In traditional and local medicines, it has been used against parasitic infection. The major phytoconstituents detected in *Albizia* species are triterpenoid saponins, flavonoids, lignanoids, alkaloids, phenolic glycosides, etc. and they actively participate in various pharmacological activities. Moreover, the antidiabetic, anti-inflammatory, antifertility, antianxiety, antidepressant and anti-fever properties are dependable on the local applications of *Albizia* species (He et al., 2020). It was reported that in South African medicine, the bark of *Albizia adianthifolia* has been used to cure neurodegenerative disorders like Alzheimer's as well as to improve memory (Maroyi, 2018).

GC-MS analysis of *T. arjuna* extract unveiled 21 phytocompounds. From these, anethole was proved to be the most prominent compound having 61.41% of the total area and its peak area was found to be having an RT value of 9.15. Similarly, lowest peak area was observed at 21.21 RT for heptadecyl ester, 3-chloropropanoate, palmitoleic acid. Other bioactive components

obtained include 1-monolinoleoylglycerol trimethylsilyl ether (8.13%), 8, 14-Seco-3, 19-epoxyandrostane-8, 14- dione, 17-acetoxy-3á-methoxy-4, 4-dimethyl (4.40%), Methyl (9Z)- 9-hexadecenoate (3.92%), heptadecyl ester, 3-chloropropanoate and palmitoleic acid (3.89%). Anethole exhibits tremendous pharmacological activities and is widely used as a flavoring agent in the food industry mostly used in cakes, ice-creams as well as alcoholic beverages (Gupta & Kumar, 2017). A wide range of plant sterols are obtained through GC-MS studies of *T. arjuna* extracts (Subramaniam et al., 2011). Similarly, about 25 different bioactive compounds are reported on *T. arjuna* by Ramesh and Dhanraj, (2015) through GC-MS analysis, which includes phenolic derivatives, alcoholic compounds, carbohydrates, ketones, fatty acids, alkaloids, etc. Additionally, fatty acids and phenylpropanes are commonly used as flavour and fragrance agents as well as in cosmetic industries (Gupta & Kumar, 2017).

The characteristic feature of *T. chebula* fruits is the presence of phenolic acids, tannins, and flavonoids. Moreover, fruits are the major source of vitamin C (ascorbic acid) (Nigam et al., 2020). HPLC was carried out on *T. chebula* fruits, which revealed 14 phenolic components. Furthermore, 48 phenolic compounds are also revealed from the methanolic extract of *T. chebula* (Lee et al., 2017). The phenolic compounds and tannins are very common in *Terminalia* species (Cock, 2015). Also, innumerable phenolic acids such as gallic acid, ellagic acid, hydroxycinnamic acids, and their derivatives are widely distributed in *Terminalia* species. Moreover, flavonoids are rich in fruits of *Terminalia* in the form of rutin, quercetin as well as methylated derivatives of quercetin (Nigam et al., 2020).

*M. philippensis* is commonly called the Kamala tree and it is found to be a rich source of cardenolides, flavonoids, tannins, fatty acids, chalcone, and phloroglucinol derivatives (Rivière et al., 2010). Kamala oil is the

characteristic feature of Kamala seeds, which involves unsaturated fatty α- and β-kamlolenic acid (18-hydroxy-Δ 9 cis,11 trans,13 transoctadecatrienoic acid) with a little amount of oleic, linoleic and eicosenoic acid. Similarly, saturated fatty acids like myristic acid, palmitic acid, and stearic acid are also seen in *M. philippensis* seeds (Smith et al., 2013). Tanaka et al. (2008) reported that heartwood and stem bark of *M. philippensis* yield various pentacyclic triterpenoids like friedelin, 2β-hydroxy-D: A-friedooleanan-3-one; 3-hydroxy-D: A-friedoolean- 3-en-2-one and 3α-hydroxy-D A- friedooleanan-2-one. Additionally, acetylaleuritolic acid, α-amyrine, 3β-acetoxy-22-β-hydroxyolean-18-ene, and kamaladiol are other major triterpenoids found in *M. philippensis*. β-sitosterol, daucosterol, and 11-O-galloyl bergenin are the major steroids reported in wood and bark of *M. philippensis* by Khan et al. (2016). Phytocompounds like phenolics, flavonoids, phloroglucinol derivatives, chalcone derivatives, etc. are commonly found in the fruits of *M. philippensis*. There are only 5 different chalcone derivatives (A, B, C, D, and E) are determined so far (Kulkarni et al., 2014). Likewise, Mallotophilippens (A, B, C, D, E, and F) another class of compounds isolated from the fruits of *M. philippensis* and there is a red and yellow compound present in fruit powder called Kamalins (Hong et al., 2010). Moreover, leaves have bilariciresinol, platanoside, isovitexin, dihydromyricetin, bergenin, 4-O-galloylbergenin, and pachysandiol A (Mai et al., 2010). Whereas flowers and fruits have plenty of flavanones like 5, 7-dihydroxy-8-methyl-6-prenylflavanone; 6, 6-dimethylpyrano (2'', 3'': 7, 6)-5-hydroxy-8- methyl flavanone, 3'-prenylrubranine, and 8-cinnamoyl-2, 2-dimethyl-7-hydroxy-5- methoxychromene (Furusawa et al., 2005). Kumar et al. (2020) inferred that phytocompounds have been isolated from the *M. philippensis* fruits showing a comparatively high percentage, (21%) followed by leaves (13%), bark (12%), seeds (11%), wood (6%), and flowers (3%).

*Albizia* species were characteristic with triterpenoid saponins. Triterpenoid saponins can be truthfully obtained by the HPTLC method. So far, about 149 saponin compounds have been isolated from the *Albizia* species. Aglycones are the major triterpenoid saponin group found in *Albizia*. Saponins exhibited structural diversity and which might be the reason for various biological as well as pharmacological properties (He et al., 2020). Albibrissinosides A and B are the two new phenolic glycosides isolated from the stem bark of *A. julibrissin*. From which, albibrissinoside B is able to act as a good radical scavenger on 1,1-diphenyl-2-picrylhydrazyl radical (Jung et al., 2004). Leaf and bark extracts of several *Albizia* species like *A. procera, A. odoratissima*, *A. lebbeck* and *A. chinensis* revealed 15 different phytocompounds through GC-MS analysis with antioxidant properties. Whereas beta-amyrin, phytol, squalene, vitamin E and 31 other chemical compounds possess antimicrobial, antibacterial, antifungal, and antiviral activities (Ghosh et al., 2021). 2-hydroxy-2-methyl-4-pentanone diacetone alcohol and alpha-bisabolol oxide are two phytocompounds with antioxidant properties that are found in the bark of *A. odoratissima*, *A. lebbeck* and *A. chinensis*. (Srinivasan et al., 2014; Thenmozhi & Rajan 2015). Longiborneol, 1-heptanol, 2-propyl-9-octadecenamide, Z-13-hexyloxacyclotridec-10-en-2-one, β-amyrone, γ-linolenic acid methyl ester, dehydro-abietyl amine, acetophenone, lupenone, methyl commate B, neophytadiene, pentafluoropropionic acid, heptadecyl ester, bis(2-ethylhexyl) phthalate, 1,2-benzene dicarboxylic acid diethyl ester, 1,4-dimethyl-3-2-methyl-1-propenyl-4-vinyl, eucalyptol, oleoyl chloride, nonacosane, etc. are the other important phytocompounds found in *Albizia* species (Ghosh et al., 2021). Major compounds found in *Terminalia*, *Mallotus,* and *Albizia* were summarized in **Table 1** and the biological activities of various compounds reported were described in **Table 2**.

**Table 1: Summary of phytochemical compounds isolated from species of *Terminalia*, *Mallotus* and *Albizia***

| Compounds | Class of compound | Plant | Plant part used | Solvent used for extraction | References |
|---|---|---|---|---|---|
| Benzoic acid<br>Hydrocinnamic acid<br>Ferulinic acid<br>Galacturonic acid<br>Caffeic acid<br>p-coumaric acid<br>Vanillic acid | Phenolics | *T. sericea* | Roots | Methanol | Mongalo et al., 2016 |
| Dihydromyricetin<br>Bergenin<br>Platanoside | Glycosides | *M. philippensis* | Leaves | Methanol | Mai et al., 2010 |
| β-sitosterol<br>Isocoumarins | Steroids<br>Coumarins | *M. philippensis* | Heartwood | Petroleum ether | Gangwar et al., 2014 |
| Albizoside D<br>Albizoside E<br>Julibroside J8 | Saponins | *A. chinensis* | Bark | 1-Butanol | Liu et al., 2017 |
| Anethole | Phenylpropanoid | *T. arjuna* | Leaves | Chloroform: Methanol | Gupta & Kumar, 2017 |
| Phthalic acid | Carboxylic acid | *T. arjuna* | Leaves | Chloroform: Methanol | Gupta & Kumar, 2017 |
| Chebulagic acid | Tannin | *T. chebula* | Fruits | Methanol | Jung et al., 2004 |
| Corilagin | Tannin | *T. chebula* | Fruits | Methanol | Lee et al., 2017 |

| Compounds | Class of compound | Plant | Plant part used | Solvent used for extraction | References |
|---|---|---|---|---|---|
| Quercetin | Flavonoid | *T. chebula* | Fruits | Methanol | Kumar et al., 2012 |
| Luteolin | Flavonoid | *T. chebula* | Fruits | Methanol | Prakash et al., 2012 |
| Isovitexin | Flavone | *M. philippensis* | Leaves | Methanol | Mai et al., 2010 |
| Phytol<br>Squalene<br>Vitamin E<br>Longifolene | Diterpene<br>Triterpene<br>Phenol<br>Sesquiterpene | *A.odoratissima* | Leaves | Acetone | Ghosh et al., 2021 |
| Alpha tocospiro B<br>β-amyrin<br>Stigmasterol | Terpenoids<br>Triterpenoid<br>Phytosterol | *A. procera* | Leaves | Acetone | Ghosh et al., 2021 |
| Tetracontane | Hydrocarbon | *A. lebbeck* | Leaves | Acetone | Ghosh et al., 2021 |

**Table 2: Various phytocompounds and their biological activity reported in species of *Terminalia*, *Mallotus* and *Albizia***

| Plant | Compounds | Biological Activity | References |
|---|---|---|---|
| *T. chebula* | Hydroxycinnamic acid<br>Hydroxybenzoic acid derivatives<br>Flavonol aglycones | Antioxidant activity | Saha et al., 2008 |
| | Chebulic acid<br>Gallic acid<br>Punicalagin | Hepatoprotective activity | Choi et al., 2015 |
| | Chebulagic acid | Neuroprotective effect | Kim et al., 2014 |
| | α-glucosidase | Anti-diabetic activity | Li et al., 2014 |
| | Ellagic acid<br>Tannic acid<br>Chebulinic acid | Antitumor activity | Saleem et al., 2002 |
| *T. sericea* | Anolignan B | Anti-inflammatory activity | Eldeen et al., 2006 |
| *T. arjuna* | Arjunic acid<br>Arjunetin<br>Arjungenin<br>Luteolin | Antimicrobial activity | Kadam & Ghosh, 2008 |
| *M. japonicus* | Bergenin | Hepatoprotective activity | Kim et al., 2000 |
| *M. philippensis* | Mallotophilippen A<br>Mallotophilippen B<br>Rottlerin | Antibacterial activity | Hemachandran et al., 2018 |
| | Bergenin | Antioxidant activity | Khan et al., 2016 |

| Plant | Compounds | Biological Activity | References |
|---|---|---|---|
| | 11-O-galloylbergenin | | |
| | 3α-hydroxy-D: Afriedooleanan-2-one 4'-hydroxyrottlerin | Anti-cancer activity | Kumar et al., 2020 |
| *M. apelta* | benzopyrans | Cytotoxicity | Rivière et al., 2010 |
| *A. subdimidiata* | Albiziatrioside A Albiziatrioside B | Cytotoxicity | Abdel-Kader et al., 2001 |
| *A. coriaria* | Coriarioside A Coriarioside B Gummiferaoside C | Cytotoxicity | Noté et al., 2009 |
| *A. procera* | 1-Heptanol, 2-propyl | Antimicrobial activity | Ghosh et al., 2021 |

**Free radical scavenging potential**

Oxidative stress makes an obstacle in the balance between the free radical oxygen species and antioxidant defense. Free radicals include reactive oxygen species (ROS) and reactive nitrogen species (RNS). The generation of these free radicals occurs in the body through metabolic processes. Usually, they are responsible for the defense mechanism, but the imbalance of these free radicals will adversely affect the body. Plants have the ability to solve these problems through their antioxidant property (**Figure 1**). The excess generation of ROS is resolved by the enzymatic and nonenzymatic defense mechanism of living organisms. Smoking, diet, alcohol, some drugs, etc. are the common external factors, which will decrease the capability of such protecting factors. So, to prevent the adverse effect of oxidative diseases, antioxidants are involved in scavenging the ROS (Willet, 1994). Phenolic components like flavonoids (Pietta, 1998), phenolic acids, and phenolic diterpenes are the major cause of antioxidant effect of plants (Shahidi et al., 1992). Hydroxyl radical, superoxide anion, singlet oxygen, and lipid peroxides are the specific ROS found in plants (Masaki et al., 1995; Yen & Chen, 1995), and the ability to quench these ROS has been evaluated by spectrophotometry (Nishikimi et al., 1972).

According to Kulisic et al. (2004), DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a stable and nitrogen-centered free radical which produces a violet or purple colour in ethanol and forms yellow colour in the presence of antioxidants. Both lipophilic and hydrophilic compounds were tested by this method. Based on these evidences, DPPH assay is one of the most commonly used methods for revealing the antioxidant activities of plant extracts (Nanjo et al., 1996). This assay revealed the capability of the compounds in the extract to donate a hydrogen atom which results in antioxidant activity. When antioxidants react with DPPH, it reduces it to a stable free radical DPPH-H

form. The discoloration specifies the scavenging activity of the antioxidant compound (Venkatalakshmi, et al., 2015). Hydroxyl radical (HO•) is one of the most influential free radicals directly formed by the irreversible damage created by oxidative stress. It is produced by the Fenton reaction; and other ways like the reaction between hypochlorous acid and superoxide anion as well as the decomposition of peroxynitrous acid. Besides, hydroxyl radicals are the major reason for mutagenesis, carcinogenesis, and aging (Halliwell & Gutteridge, 1995).

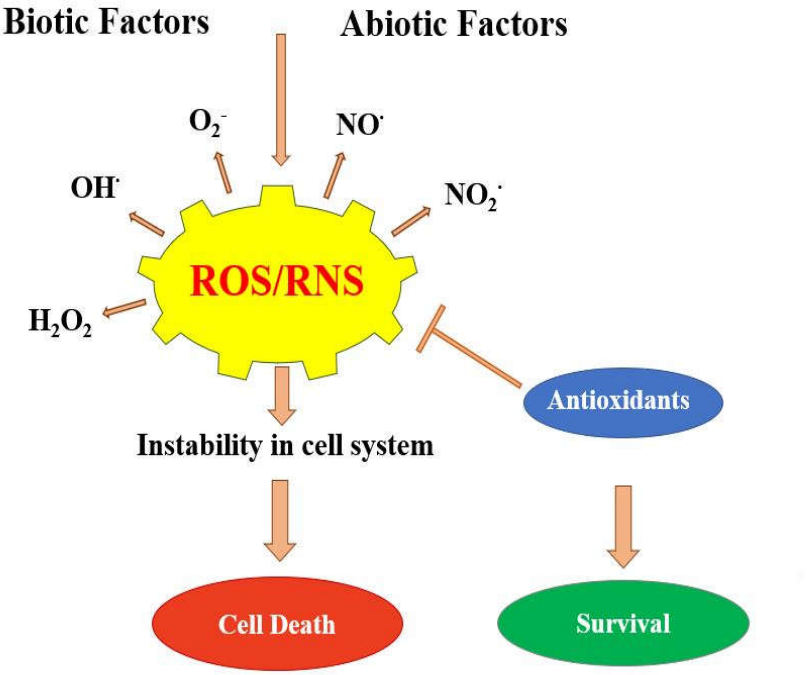



**Figure 1: Schematic diagram of antioxidant activity**

Various metabolic actions in the body will generate multiple destructive free radicals. The methanolic fruit extract of *Terminalia paniculata* showed an appreciable ability to scavenge these free radicals. The four different antioxidant assays, *viz*., DPPH free radical scavenging assay, hydroxyl free radical scavenging assay, nitric oxide free radical scavenging assay, and superoxide free radical scavenging assay revealed significant

results about the scavenging effect of the plant. Dose-dependent scavenging activity was observed in all assays. In DPPH radical scavenging assay, the $IC_{50}$ was obtained as 23.068 µg/mL, which was almost similar to that of the standard ascorbic acid used (20.399 µg/mL). Plants, especially fruits, vegetables, medicinal herbs, etc., have a vivid variety of free radical scavenging molecules such as phenolic acids, flavonoids, quinones, coumarins, and tannins. Furthermore, they may contain some other endogenous metabolites with high antioxidant activity. Similarly, the preliminary phytochemical studies of the fruit extract of *T. paniculata* revealed a significant occurrence of several phytochemical compounds, which are of great therapeutic value (Aswathi & Thoppil, 2020). The bark extract of *T. arjuna* is very rich in antioxidant compounds. Furthermore, antioxidant potential showed variations depending upon the nature of extracting solvents as well as the nature of raw materials to be extracted. For the effective accessibility of antioxidant components, methanol extraction of the plant material should be desirable. These results focus on the efficacy of *T. arjuna* extracts to prevent oxidation. So, the extracts of *T. arjuna* can be used as a possible source of antioxidants in the food and pharmaceutical industries (Chatha et al., 2014). Free radical scavenging activity of five different extracts of the bark of *T. arjuna* was performed by Mohammad et al. (2012). DPPH, hydrogen peroxide radical, nitric oxide radicals (NO), etc. are the adopted methods to identify the antioxidant potential. The antioxidant capacity of the five different extracts was detected by spectrophotometry. In the DPPH assay, the highest $IC_{50}$ value was observed in the methanolic extract followed by ethanol and petroleum ether extract. Also, they reported that methanolic extract showed the highest activity in hydrogen peroxide as well as nitric oxide scavenging assay. So, they inferred that methanolic extract possesses highest antioxidant capacity than ethanolic extract. Based on the DPPH assay, the ethanolic extract of the stem bark of *T. glaucescens* displayed higher

scavenging potential when compared to that of the standard. So, the findings of Olugbami et al. (2015) revealed the promising presence of phytoconstituents in the bark extract of *T. glaucescens.* It also has the ability to quench free radicals and can act as an antioxidant. Antioxidant efficacy of the methanolic fruit extract of *T. chebula* and *T. bellerica* was carried out by Hazra et al. (2010). They reported that various free radical scavenging systems showed antioxidant activity against different ROS at different magnitudes of potency. The isolated compounds from these plants have antioxidant potential and can be taken up in modern drugs. Moreover, studies on the antioxidant activity of these plants are essential because of their usage in a well-known Ayurvedic formulation called Triphala. The methanolic leaf extract of *T. bellerica* and *T. sericea* also possess a prominent antioxidant activity, which was reported by Sobeh et al. (2019). *T. catappa* is another species belonging to Combretaceae, that exhibits a promising antioxidant potential, which was proved by Venkatalakshmi et al. (2015). Various extracts of leaves and bark of *T. catappa* exhibited a significant antioxidant potential (Venkatalakshmi et al., 2001). Furthermore, Venkatalakshmi et al. (2015) suggested that the various parts of *T. catappa* could serve as a novel herbal antioxidant agent. Similarly, Chanda et al. (2013) reported that the methanolic leaf extract of *T. catappa* showed good antioxidant potential. The ethanolic extract derived from the fruits of *T. catappa* and the aqueous fraction of crude extract and its subfractions includes petroleum ether and ethyl acetate, which exhibit significant antioxidant potential (Siddiqi et al., 2011). The dried leaves of *Terminalia* species were extracted with acetone, hexane, dichloromethane, and methanol, which was used to evaluate the antioxidant potential by Masoko and Eloff, 2007. It was observed that all *Terminalia* species extracted with acetone and methanol displayed promising antioxidant potential. Moreover, hexane and dichloromethane extracts didn't exhibit any antioxidant activity. Akter et al. (2019) found out the antioxidant

capacity of the extracts prepared from the fruits, leaves, seedcoats, and bark of *T. ferdinandiana*. They suggested that fruits and leaf extract of *T. ferdinandiana* are rich in antioxidants and have tremendous applications as natural antimicrobials in food preservation.

The genus *Mallotus* belongs to the Euphorbiaceae family and most of the species exhibit antioxidant properties (Hong et al., 2011). The antioxidant activity of the methanolic leaf extract of *M. oppositifolius* was reported by Nwaehujor et al. (2014). They revealed that *M. oppositifolius* showed a dose-dependent antioxidant activity comparing favourably with the standard ascorbic acid through DPPH analysis. These results suggested that *M. oppositifolius* may be used for maintaining health and reducing the adverse effect of degenerative diseases like cancer, diabetes, coronary heart disease, and mountain sickness that are intensified by the generation of ROS in the body. The presence of phytocompounds such as tannins, flavonoids, saponins, and alkaloids might have contributed to the observed antioxidant activity (Adekunle & Ikumapayi, 2006). The antioxidant activity of the crude methanolic leaf extract of *M. oppositifolius* was also reported by Peter Onyeka et al. (2021). They carried out the experiment with a different solvent such as hexane, ethyl-acetate, butanol, and water. The result further displayed that the ethyl-acetate and butanol extracts had the prominent antioxidant capability. ABTS, FRAP and nitric acid radical scavenging assays are the other usual methods used to evaluate the quantitative values of antioxidant activity. The scavenging ability and the reducing effects of the various solvent leaf extracts of *M. rhamnifolius* were analysed through this method by Loganathan and Selvakumar, (2018). For their experiments, a standard antioxidant *viz*., rutin has used for comparing the free radical scavenging activities. They reported that ethanol:water extract of the leaves of *M. rhamnifolius* showed maximum antioxidant capacity and this ability of the extract may be exploited as a therapeutic agent for treating radical-induced

pathological damage. Bergenin and 11-O-galloylbergenin are the two isolated compounds from *M. philippensis*. The *in vitro* antioxidant activity of these compounds was carried out by Khan et al. (2016). DPPH radical scavenging activity and reducing power assay are the adopted protocols used to find out the antioxidant potential of these compounds. The results revealed that 11-O-galloylbergenin exhibits more antioxidant potential than bergenin. The authors suggested that these compounds can be used in drug design and development due to their vital medicinal properties. Moreover, acetone extracts of the fruit and bark of *M. philippensis* have proved a higher antioxidant capacity. Among the extracts, the bark extract exposed a prominent antioxidant potential due to its high content of condensed tannins (Arfan et al., 2007). DPPH, ABTS, hydroxyl, superoxide anion radical and reducing power methods are the adopted protocols for evaluating the antioxidant activity of the polyphenols extracted from *M. oblongifolius* (Duan et al., 2021). The extracted polyphenols had good antioxidant potential and have a strong inhibitory effect on four bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis,* and *Pseudomonas aeruginosa*. Additionally, the alcoholic extract of *M. roxburghianus* leaves extract showed significant antioxidant activity, which was proved by Rana et al. (2005). Ramalakshmi and Muthuchelian (2012) reported that the bark extract of *M. tetracoccus* possess significant antioxidant activity and was found to be very useful in traditional medicines.

The powdered stem bark of *Albizia lebbeck* was extracted with different solvents such as petroleum ether, ethyl acetate, and methanol. Ali et al. (2018) evaluated the antioxidant activity of each extract and they inferred that ethyl acetate extract showed the highest antioxidant activity, when compared to petroleum ether and methanol extracts. All the extracts possess a higher scavenging activity than that of ascorbic acid, which was used as the standard. Many reports revealed that polyphenolic compounds have

characteristic scavenging activity and can be used for treating free radical-related diseases (Siahpoosh & Mehrpeyma 2014). They also reported the antioxidant efficacy of polyphenols extracted from *A. lebbeck*. The methanolic bark extract of the plant was used for the same. Furthermore, *A. lebbeck* has chemical compounds with antioxidant properties and exhibited good protective effect against free radicals. Similarly, the crude extract and its fractions of *A. chinensis* exhibited strong DPPH free radical scavenging activity (Sharmin et al. 2014). Besides, antioxidant activity has been reported in several *Albizia* species, which include *A. anthelmintica*, *A. adiantifolia*, *A. procera*, *A. zygia*, *A. chevalieri*, *A. coriaria*, *A. amara*, *A. gummifera*, *A. richardiana*, *A. harveyi,* etc. In *A. anthelmintica,* the roots and bark of the plant were extracted with various solvents such as hexane, chloroform, and ethanol, which were used to identify the antioxidant potential (Wale et al., 2018). From this study it was clear that in bark the highest antioxidant activity was observed in chloroform and ethanolic extract. While, a little activity was shown in hexane extract. Also, a similar trend was found in the root extract of the plant. Findings from this study revealed that the antioxidant potential of *A. anthelmintica* can be used for the development of novel drugs. A compound known as quercetin-3-O-β-D- glucopyranoside isolated from *A. anthelmintica* exhibited potent free radical scavenging activity towards DPPH radical, which was proved by Mohamed et al. (2013). In *A. adiantifolia*, ethyl acetate extract of the stem bark possesses antioxidant potential. The fractionation of the stem bark extract produces two known compounds called lupeol and aurantiamide acetate with high antioxidant properties. These discoveries provide significant information regarding the potent use of *A. adiantifolia* as well as the compound aurantiamide acetate for curing oxidative damage (Tamokou et al., 2012). The scavenging activity of different solvent extracts of *A. procera* leaves disclosed a significant result. Methanolic extract and its fractions like petroleum ether, carbon tetrachloride, dichloromethane, ethyl acetate, and the

aqueous fraction of the leaves were used for the study of antioxidant potential. From these fractions, ethyl acetate showed maximum antioxidant efficacy compared to that of the standard ascorbic acid. So, these findings have suggested that ethyl acetate extract from *A. procera* leaf can be used as a natural antioxidant that can prevent free radical-associated diseases (Islam et al., 2013). Also, Kokila et al. (2013) contemplated the antioxidant property of the ethanolic leaf extract of *A. procera*. They displayed a strong quenching activity of *A. procera* towards free radicals and may be useful against oxidative stresses. Furthermore, the methanolic stem bark extract of *A. zygia* exhibited a characteristic antioxidant potential, which was proved by Obonga et al. (2017). They suggested that the existence of phytochemical compounds such as flavonoids, reducing sugars, terpenoids, alkaloids, and tannins are responsible for the antioxidant activity. Likewise, previous studies reported that the presence of phenolic compounds from *A. chevalieri* and *A. amara* was responsible for the antioxidant activity (Muchuweti et al., 2006). *A. chevalieri* leaf extract exhibited high free radical scavenging activity as compared to that of standard ascorbic acid. The DPPH assay showed a concentration-dependent antioxidant activity. When the concentration of the extract increases, the scavenging activity also increases (Garba et al., 2019). Also, there are reports available on the antioxidant efficacy of crude extract and fractions from the stem bark of *A. gummifera* (Atsafack et al., 2016). The antioxidant activity of ethanolic, ethyl acetate and aqueous extract of *A. coriaria* leaves was also proved in several reports (Omara et al., 2021). The findings of these experiments proved that there are characteristic differences between the antioxidant activity of ethyl acetate, ethanol, and aqueous extracts of *A. coriaria* leaves. These differences might have happened by the differences in the presence of phytochemical composition of different extracts due to geographical, soil, climate, and genetic variations. Also, they found that ethanolic extracts showed maximum antioxidant efficacy due to their high

content of phenolic compounds present in them. Phenols are the major phytoconstituents found in plants with effective radical scavenging ability due to their hydroxyl groups (Saha et al., 2008). In *A. amara*, there is a significant relationship, prevailing between total phenols and antioxidant activity (Rajkumar et al., 2010).

**Hepatoprotective activity**

The liver is one of the chief organs in the body. It helps with metabolism, secretion, and storage. The liver is such a vulnerable organ to several exogenous substances like drugs, alcohol, environmental toxins, etc., and cause diseases. Liver diseases are the main health problem faced by humans due to their food habits.

Hepatotoxicity is the injury or damage occurred in the liver by drugs, herbal agents, industrial chemical agents, or nutritional supplements (**Figure 2**). About 900 drugs were found to be causing injury to the liver and it is the most common reason for a drug to be withdrawn from the market (Pandit et al., 2012). Acetaminophen, paracetamol, tyrosine kinase inhibitors, antitubercular drugs, simvastatin, carbon tetrachloride, etc. are some of the hepatotoxicity-inducing agents (Meshack & Gupta, 2022). These hepatotoxic agents will increase the serum levels of alanine transaminase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, direct bilirubin, serum triglycerides, cholesterol, and urea. Whereas, it decreases the levels of albumin, GSH glutathione reductase, and total protein (Marianne et al., 2020). Furthermore, tartrazine and sunset yellow are the most commonly used synthetic food colorants, which are frequently used in bakery items, cereals, beverages, candies, gelatine, and other various commodities. Also, the drinks and juices will be more attractive with the use of tartrazine and metanil yellow. Extensive application of these food colorants can cause hepatotoxicity (El-Malky et al., 2014). Exposure to the food colours is harmful to humans

and causes hepatic damage due to metabolic activation of the compounds to strong free radical products. It may lead to lipid peroxidation, reducing the activities of antioxidant enzymes like superoxide dismutase, catalase, non-enzymatic reduced glutathione, and elevation of hepatic enzyme, alkaline phosphatase (Escobar et al., 1996). So, reports clearly display that use of artificial food colorants with aromatic azo compounds can impair normal hepatic functions and cause more risk to the health (Saxena & Sharma, 2015).

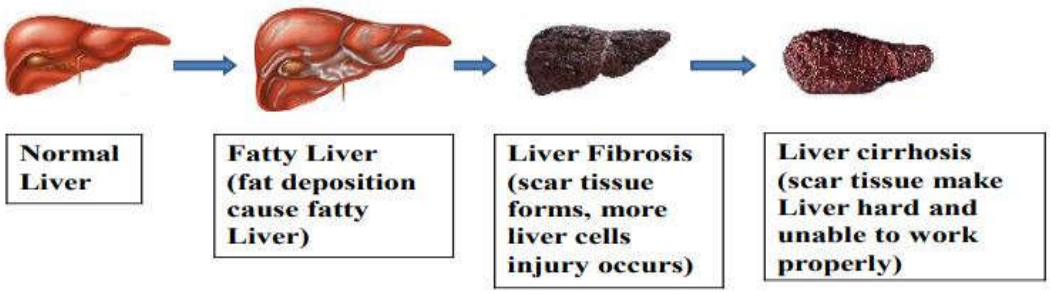



**Figure 2:** Progressive changes in the liver during hepatotoxicity

(Source: Gupta et al., 2015)

Oxidative stress and free radicals increase hepatic damage and it can be prevented by the antioxidant mechanism. Plants having antioxidant property are widely used to cure liver diseases since ancient times. So, the preventive action, as well as the therapeutic activity of plants with hepatoprotective efficacy is a good topic for researchers. Plants are a rich source of biologically active phytoconstituents like alkaloids, flavonoids, phenols, terpenoids, coumarins, carotenoids, glycosides, etc. which proves the hepatoprotective activity. Reports demonstrated that if an extract contains higher alkaloids, flavonoids and saponins, then it possesses higher hepatoprotective activity. Flavonoids are polyphenolic compounds that have been responsible to scavenge free radicals as well as protecting the hepatocytes (Nerdy & Ritarwan, 2019). Polyphenols are the proven phytocompounds in plants with high antioxidant potential. The antioxidant ability is mostly due to their redox

potential, which permits the neutralization of free radicals, singlet oxygen, or decomposing peroxides (Saague et al., 2019). Due to its high antioxidant capacity, most of the *Terminalia* spp. displayed a promising hepatoprotective efficacy. β–sitosterol, stigmasterol, 1H-inden-1-one,2,3-dihydro-3,3,5,6, tetramethyl, n-hexadecanoic acid, flavonoids, and tannins are the hepatoprotective agents found in *Terminalia coriacea* (Patel et al., 2017). There are reports on the hepatoprotective activity of several *Terminalia* spp., which involves aqueous bark extract of *T. arjuna* that exhibited significant hepatoprotective activity. In addition, the ethanolic fruit extract of *T. bellerica* and *T. chebula* as well as the aqueous leaf extract of *T. catappa* also showed a characteristic hepatoprotective activity (Adewusi & Afolayan, 2010). Also, there is a supporting report revealing the hepatoprotective activity of *T. arjuna*. They investigated the efficacy of an aqueous extract of *T. arjuna* using a standard pro-oxidant (tertiary butyl hydroperoxide) in HepG2 cells (Shivananjappa et al., 2013). About 85 phytocompounds were identified in the leaf extract of both *T. bellerica* and *T. sericea*. Ellagitannins, chebulagic acid, galloylpunicalagin and digalloyl-hexahydroxydiphenoyl-hexoside were the major secondary metabolites found in *T. bellerica.* Whereas, flavonoid glycosides like quercetin rutinoside, and quercetin galloyl-glucoside were found to be present in *T. sericea*. The studied extracts having these phytoconstituents revealed a promising hepatoprotective activity. Also, they concluded that hepatoprotective efficacy might be attributed to the high content of polyphenols (Sobeh et al., 2019).

The use of herbal drugs is increasing day by day. A wide spectrum of herbal medicines is available in the market. Triphala is one of the best examples of a good ayurvedic preparation with known hepatoprotective action. It consists of a combination of *T. chebula* and *T. bellerica* along with *Phyllanthus emblica*. Additionally, Triphala is a rich source of antioxidant agents. Reports clearly described that the aqueous extract of Triphala has the

capability to inhibit paracetamol-induced hepatotoxicity. Moreover, *T. chebula* showed a protective effect against hepatotoxic agents like paracetamol, carbon tetra chloride, gentamicin, etc. and *T. bellerica* proved its protective action against ethanol (Gupta et al., 2015).

**Cytotoxic study**

The *Allium cepa* study is a simple method used to analyze the cytotoxicity in plants (Fiskesjö, 1985). *Allium cepa* root tip assay was proposed by Levan (1938) and it was later considered as a standard method to study genotoxicity (Fiskesjö, 1985). Studies on cytotoxicity have been a stepping stone to the development of anticancer drugs (Mukhopadhyay et al., 2004). Sharma and Sharma (1960, 1962) studied the importance of spontaneous and chemically induced chromosome breaks and emphasized the importance of nucleic acids in controlling chromosome breaks induced by different compounds. Hadder and Wilson (1957) carried out a cytological assay to find the action of C-mitotic and prophase poisons. The potential of the plant extract to produce cytological aberrations might result in membrane disruption and chromosomal aberrations. Most of these abnormalities cause cell or tissue death. This means that the cytotoxic capability of the plant can be used for drug development to cure various diseases including cancer.

Micronuclei are an important chromosomal aberration, which is generated not only from chromosomal materials but also from extrachromosomal elements called double minutes (DMs). They are mainly detected in many human cancer cells. Elimination of DMs from cancer cells results in the loss of malignant phenotypes. It is mainly due to the amplified genes on DMs. The malignant phenotype of cancer cells was determined by these DMs (Von Hoff et al., 1992; Shimizu et al., 1994; Eckhardt et al., 1994). Micronuclei are formed as a consequence of chromosome breakage due to clastogenic agents or from whole chromosomes due to aneugenic

agents that were not incorporated into the main nucleus during the cell division cycle (Fenech, 2007; Leme et al., 2008). Moreover, the nuclear budding process is a mechanism by which cells remove amplified DNA and is therefore a marker of gene amplification. The strong correlation between micronucleus formation, nuclear budding and nucleoplasmic bridges ($r = 0.75 – 0.77$, $P < 0.001$) is supportive of the hypothesis that folic acid deficiency causes genomic instability and gene amplification by the initiation of breakage-fusion-bridge (BFB) cycles (Fenech & Crott, 2002). Nuclear lesions, the widely observed aberrations in *A. cepa* root tips may be due to the disintegration of a portion of nuclear material by the effect of the plant extracts (Mercykutty & Stephen, 1980). Pasqualini et al. (2003) discovered that nuclear lesions are associated with programmed cell death in plants. Chromosome fragmentation (Chauhan & Chuahan, 1999), nuclear disintegration (Liu et al., 2000), chromosome stickiness (Panda & Sahu, 1985) and nuclear distortion (Choi et al., 2001) are the major clastogenic aberrations observed. Whereas misorientation of chromosomes (Selim et al., 1981), the diagonal orientation of chromosomes (Deena & Thoppil, 2000), cytostasis (Ahumada et al., 1995), stathmo-anaphase (Shehab, 1979), etc. are the major non-clastogenic abnormalities observed through the cytotoxic studies. Binucleate and trinucleate cells are another important anomaly and these conditions are the particularities of cancer cells (Oksala & Therman, 1974; Graham et al., 1978). There are a few recent reports regarding the studies of the cytotoxic effect of the selected genera which are described below.

The cytotoxic effect of the bark extract of *Terminalia arjuna* was studied by Debnath et al. (2016). At very low concentrations (0.5-10 mg/ml), a characteristic cell cycle inhibition was obtained. Moreover, all the experimental concentrations possessed cytotoxic effects on cell division. Sticky metaphase, clumped metaphases, chromosome bridges at anaphase and

telophase, laggard chromosome, vagrant chromosome, etc. are the major chromosomal aberrations occurred. Also, it was observed that the lower mitotic rate was due to the inhibition of DNA synthesis or may be due to the blockage in the G2 phase of the cell cycle and thus inhibiting the cell to enter the divisional phases when *T. arjuna* bark extract was used in the treatment. The observed chromosomal aberrations that are in the various stages of mitosis might have occurred due to the blockage of DNA synthesis or spindle formation. So, the cytotoxicity exhibited by the aqueous extract of the bark of *T. arjuna* revealed its beneficial effect in herbal medicine. It was a dose-dependent result and they suggested that unsuitable doses of these herbal medicines may lead to health complications. The studies on the toxic effect of *T. actinophylla,* are still incipient. But, de Sousa et al. (2020) proposed the cytotoxic effect of the ethanolic extract of the *T. actinophylla* bark using the *Allium cepa* assay. From their experiments, there was an increase in chromosomal variations (1.25 and 2.5 mg/ml) compared to distilled water (negative control). Also, they found that the existence of phytochemicals such as tannins, saponins, and reducing sugars in the extract interferes with the cell cycle and cause DNA damage in *A. cepa* in lower concentrations. The major chromosomal aberrations such as metaphase with chromosome adherences and C-metaphases, polyploid cells, etc. were observed but binucleated cells were not yet significant. Alterations in the chromosome segregation like chromosome loss, bridges, and multipolarity during anaphase and/or telophase were analysed. According to de Sousa et al. (2020), chromosome bridges and loss were the most significant aberrations observed. Moreover, micronuclei, nuclear buds, and chromosome fragments were also prominent. Reports are very less on the cytotoxic studies of the extracts of *Mallotus* and *Albizia* in *A. cepa* root meristem.

**Anticancer activity**

Cancer is a disease associated with uncontrolled cell division. In the majority of cases, it is caused by the loss of cell cycle control. According to WHO, cancer is a leading cause of death worldwide. Breast, lung, colon, rectum, and prostate are the most common cancers. Breast cancer is diagnosed among women and the number of breast cancer patients increases day by day. It is the foremost cause of death among women worldwide. The progression and establishment of breast cancer happen by many roots, while oxidative stress could make intercellular pathways suffer from cellular proliferation (Sanches et al., 2017). When compared to normal people, patients who suffer from breast cancer have higher oxidative stress due to genetic abnormalities (Cuchra et al., 2016; Cramer et al., 2017). Higher oxidative stress help to the malignant cells to grow and it rises the ROS-mediated signalling pathways which promote cell growth, cell differentiation, glucose synthesis, protein synthesis, and also cell survival (Fink & Chipuk, 2013). Apoptosis is a process in which morphological changes occur in both nuclei and specific growth-related genes that may lead to cellular self-killing. Generally, it is regulated by two different pathways called extrinsic and intrinsic pathways (**Figure 3**). Death receptor-induced pathways account for the extrinsic pathway whereas the mitochondria-apoptosome-mediated pathway is the intrinsic pathway (Hu & Kavanagh, 2003).

Plant extracts contain a large number of phytochemicals that may synergistically act on diseases (Sharma, 2016). There are so many anticancer drugs like Sulphoraphane, Paclitaxel, Epipodophyllotoxin, Vincristine, Vinblastin, Vinorelbine, Vindesine, Vinflunine, Pomiferin, Roscovitine, etc., that were isolated from various plants. So, plant extract research paves the way in the medical field for the treatment of cancer (Greenwell & Rahman, 2015; Pereira et al., 2016; Basu & Maier, 2018).

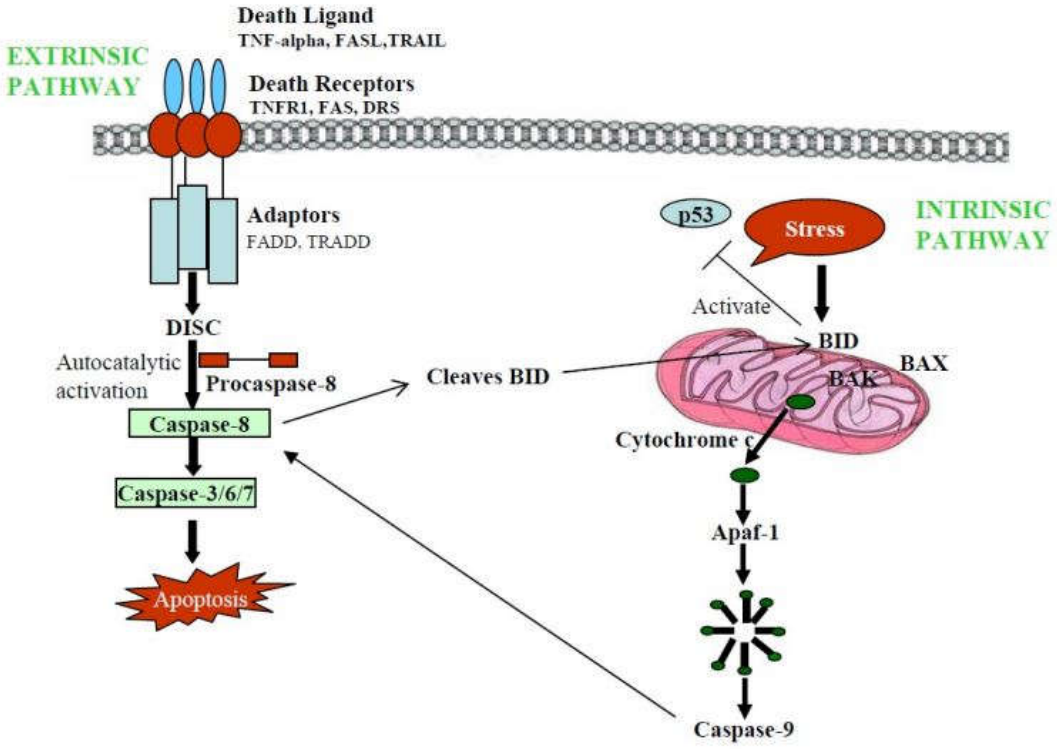



**Figure 3: Extrinsic and intrinsic pathway of apoptosis.** Source: (Rampal et al., 2012)

**Previous reports on breast cancer cell lines**

Plants have been a major source of anticancer compounds. Based on several reports, a large number of phenolics and flavonoids in plants are associated with higher cytotoxicity (Shankara et al., 2016). *Terminalia* spp. has been widely used for healing in various medical systems including the treatment of cancer. Commonly, *T. chebula*, *T. bellerica,* and *T. ferdinandiana* were reported to have significant anticancer activity against several cell lines. Studies displayed that these genera exhibited high antioxidant efficacy and tannin contents, which may be correlated with the anticancer property. Besides, there are notable compounds like combretastatins and other stilbenes with anticancer properties, which is also a good feature of *Terminalia* spp. for promoting antiproliferative activity (Cock & Cheesman, 2021). According to Nurhanan et al. (2008), the methanolic

stem bark and leaf extract of *T. catappa* showed a promising antiproliferative activity on two different breast cancer cell lines, i.e. MCF-7 and T47D. Chebulinic acid is a hydrolysable polyphenol found in most of the *Terminalia* spp. like *T. chebula*, *T. arborea* etc. (Yi et al., 2009). Reports revealed that chebulinic acid possesses the potent anticancer activity and exhibited significant cytotoxicity to MDA-MB 231 breast cancer cells through MTT assay. *In vitro* anticancer activity was also proved in *T. bellerica* against human breast (MCF-7) carcinoma. Methanolic extract from air-dried fruits is used for the study. The outcomes of the study demonstrated that MCF-7 cells formed a time and concentration-dependent inhibition of cell proliferation. The anticancer activity of *T. bellerica* against MCF-7 cells may be due to the free radical scavenging activity of the plant. Moreover, the phytochemicals including phenolics, flavonoids, etc. were found to be responsible for the apoptotic activity. HPLC analysis revealed several compounds having an anticancer activity that includes reserpine, tannic acid, quercetin, catechin, and gallic acid/ascorbic acid (Ghate et al., 2014). Also, octyl gallate and gallic acid are two biologically active compounds isolated from the methanolic fruit extract of *T. bellerica*. These two compounds have the ability to inhibit the growth of breast cancer cells such as MCF-7 and MDA-MB 231 and they induce apoptosis. There is no adverse effect on normal breast cells. The key responsible factor for cell cycle progression and apoptosis is cell cycle regulation. The progression of cell cycle through G1 phase to S phase encompasses the activation of cyclin D (D1, D2 and D 3) with their catalytic associates CDK 4 and CDK 6 (Hall & Peters, 1996). The expression of cell cycle regulators like cyclin D1, D3, CDK-4, CDK-6, p18 INK4, p21Waf-1, and p27 KIP are altered by the inhibition of cell cycle progression with these compounds. The findings demonstrated that octyl gallate is more active than gallic acid. So, it is determined that apoptosis occurred in breast cancer cells treated with compounds by the downregulation of positive cell cycle

regulators and upregulation of negative cell cycle regulators. Hence, the results revealed that both these compounds can act as good anticancer agents and could be established for breast cancer drugs after detailed explorations (Sales et al., 2018). Apoptosis encompasses loss of plasma membrane integrity, fragmentation of DNA, and formation of apoptotic bodies (Cotter, 2009). According to Sales et al. (2018), the cytotoxic effect of octyl gallate and gallic acid on MCF-7 and MDA-MB 231 breast cancer cell lines is established with the reduction of cell viability and high LDH (Lactate dehydrogenase) leakage by the compounds, which represent the loss of membrane integrity. So, the progressive action of these two compounds could be used as a natural therapeutic agent in breast cancer therapy. Antiproliferative activity of the methanolic root extract of *T. sambesiaca* and *T. sericea* was observed by Fyhrquist et al. (2006). They carried out the experiment in MCF-7 breast cancer cells. The dried fruits of *T. chebula* and *T. bellerica* along with *Emblica officinalis* are the major three components of Triphala, an Indian ayurvedic formulation. There are several reports available on the anticancer properties of Triphala on two human breast cancer cell lines called MCF-7 and T47D. Loss of cell viability is observed by MTT assay. Furthermore, the clonogenic survival of MCF-7 cells is inhibited by Triphala, which has been meaningfully recovered by pifithrin-a, the p53 inhibitor. Yet, pifithrin-a will not modify Triphala-induced cytotoxicity in T47D breast cancer cells. During 2h double staining treatment of cells with Triphala, it was revealed that promising apoptosis was found on both cell lines in a dose-dependent manner. The scale of apoptosis was higher in MCF-7 when compared to that of T47D cells. Also, Triphala is accountable for the increase in intracellular ROS in both cell lines. Both MCF-7 and T47D showed a differential sensitivity to Triphala, which may be dependent on their p53 status. Hence, apoptosis has been induced by the antioxidants generated by Triphala and inferred that the p53 status of cancer cells formed a significant

factor that could predict the effect of cancer cells to prooxidant drugs (Sandhya & Mishra, 2006).

*Mallotus philippensis* has renowned therapeutic uses in 18 different states of India. Almost all the plant parts were used either internally or externally for 67 disease conditions. Among these, anticancer activity is one of the most important pharmacological activities ever reported (Buha & Acharya, 2020). According to Gangwar et al. (2014), *M. philippensis* is a major source of natural molecules with medicinal properties and it should be established as a milestone species for modern medicine in the future. Yet, the prevailing knowledge regarding the stem, leaves, and fruits of *M. philippensis* is limited. Hence, research on these plant parts should make a good result in pharmacology. Mallotoxin or rottlerin is a biologically active compound isolated from *M. philippensis*. Due to the high anticancer activity of rottlerin, it can be used in chemotherapy for cancer treatment. Reports revealed that in the future it will become a potential anticancerous agent as well as affect cell machinery involved in apoptosis, survival, and autophagy (Gangwar et al., 2014). For suppressing tumour cell growth in various cancer cells, rottlerin regulates multiple signalling pathways, however, the whole mechanisms are not clear. Depending on the cancer cell type, rottlerin induced apoptosis either by intrinsic pathway or *via* extrinsic pathway of cell death. Nevertheless, the ability of rottlerin to inhibit a class of protein kinases called protein kinase C (PKCδ) is usually responsible for the antitumour activity and keeps it safe from apoptotic cell death (Maioli et al., 2012). MDA-MB 231 is a human breast cancer cell line in which, the p38 mitogen-activated protein kinase (MAPK) signalling pathway is activated by rottlerin and it boosts the expression of IL-1β-induced COX-2. Additionally, rottlerin also enhances the expression of COX-2 when multiple reagents such as tumour necrosis factor-α (TNF-α), phorbol myristate acetate, and lipopolysaccharide were induced (Park & Kwon, 2011).

*Mallotus macrostachyus* is another plant found in Euphorbiaceae and the crude extract of its leaf, stem, and bark unveiled a characteristic antiproliferative activity in two breast cancer cells i. e. MCF-7 and T47D (Nurhanan et al., 2008). Additionally, the leaves of *M. macrostachyus* also showed promising antiproliferative activity in various cancer cell lines (Xu et al., 1991; Kiem et al., 2005). According to Bahaman et al. (2020), *M. paniculatus* is a medicinal plant, widely used in rural areas for the treatment of various diseases. Ethanolic, ethyl acetate, and hexane extract of the plant were used for the study. They proved that *M. paniculatus* exposed a significant antioxidant, antibacterial, antifungal as well as anticancer activity. From their study, both ethanolic and ethyl acetate extracts showed cytotoxic activity against breast cancer cell lines (MCF-7). The cytotoxic efficacy of *M. paniculatus* against cancer cell lines has been demonstrated by the availability of flavonoids and other phytocompounds present in it (Tistaert et al., 2012). Reports confirmed that flavonoids exhibit high antiproliferative activity and apoptosis-promoting effects against cancer cells. Besides, they have the ability to make an obstacle to the cell cycle followed by apoptosis (Singh & Agarwal, 2006). The effect of flavonoids was not only applicable to breast cancer but also prostate, pancreatic, cervical, and ovarian cancers (Brusselmans et al., 2005). Moreover, previous reports established that flavonoids could induce cell cycle arrest in G2 and the mobile phase of the cell cycle, inhibited heat-shock protein, tyrosine kinase, and Ras protein as well as downregulated oestrogen receptor-binding capacity. Most of the reason behind the pathogenesis of cancer includes genetic abnormalities induced by the p53-mutated proteins. The intake of flavonoids involves in the downregulation of these proteins and affects cancer cell growth (Veeramuthu et al., 2017). This infers that *M. paniculatus* is having significant medicinal properties with potent bioactive compounds. Interestingly, *M. apelta* is also a potent medicinal plant belonging to Euphorbiaceae having high cytotoxic

activity against cancer cell lines. Similarly, several species of *Mallotus* like *M. mollissimus, M. japonicus*, *M. peltatus,* etc. exhibit cytotoxic activities against human breast cancer cell lines (Bahaman et al., 2020).

Antiproliferative studies on *Albizia* spp. are very meager. But the existing reports revealed that the genus *Albizia* could create a stepping stone in the field of cancer studies. Previous research reveals that *Albizia coriaria*, can be widely used for the treatment of breast cancers. The bark and leaves of the plant are usually used for the treatment (Ochwang'i et al., 2014). *A. adianthifolia* is another species that has proven antiproliferative activity against both MCF-7 and MDA-MB 231 breast cancer cell lines (Maroyi, 2018). Similarly, the bark and leaves of *A. lebbeck* displayed significant anticancer activity on various cell lines. A tremendous saponin-rich fraction was obtained from the bark of the plant, which was employed for the anticancer activity through MTT assay in the human breast cancer cell line MCF-7 (Balkrishna et al., 2022). According to Ganesan and Subramanian (2015), the bark extract of *A. lebbeck* have diverse bioactive components, which may be attributed to the apoptotic pathways in MCF-7 cells. Also, they inferred that the *in vitro* cytotoxic and apoptotic activity of *A. lebbeck* against human breast cancer cells will provide a new potential chemotherapeutic agent for breast cancer treatment. **Table 3** represents the antiproliferative potential of some members of *Terminalia*, *Mallotus,* and *Albizia*.

**Table 3: Antiproliferative activity shown by some species of *Terminalia*, *Mallotus* and *Albizia* on cell lines**

| No. | Plant | Cell lines | Reference |
|---|---|---|---|
| 1 | *T. bellerica* | A549, OSCC | Ghate et al., 2014; Patra et al., 2020 |
| 2 | *T. combretum* | HeLa, T 24 | Fyhrquist et al., 2006 |
| 3 | *T. chebula* | A549, HOS-1, PC-3 | Shendge et al., 2020; Saleem et al., 2002 |
| 4 | *T. arjuna* | MCF-7 | Kuo et al., 2005 |
| 5 | *T. ferdinandiana* | Caco2, HeLa, JEG-3, JAR, MG63, MC3T3-E1 | Shalom et al., 2018; Shalom & Cock, 2018 |
| 6 | *T. sericea* | Caco2, HeLa | Gu et al., 2018 |
| 7 | *T. avicennioides* | HepG2 | Aliyu-Amoo et al., 2021 |
| 8 | *M. paniculatus* | HT-29, HeLa | Bahaman et al., 2020 |
| 9 | *M. mollissimus* | HeLa, CaOV3 | Ismail et al., 2021 |
| 10 | *M. philippinensis* | HL-60, Thp-1 | Gangwar et al., 2014 |
| 11 | *M. apelta* | TOV-21G, KB, HeLa, FL, Hep-2 | Van Kiem et al., 2020; Lu et al., 2014; Van Kiemi et al., 2005 |
| 12 | *M. japonicus* | KB, L-5 178Y | Anh et al., 2022 |
| 13 | *M. macrostachyus* | KB, LU-1 | Nam et al., 2011 |
| 14 | *M.oppositifolius* | A2780 | Tchangoue et al., 2020; Harinantenaina et al., 2013 |
| 15 | *M. conspurcatus* | HeLa | Zhang et al., 2019 |
| 16 | *A. chinensis* | HeLa | Manosroi et al., 2012 |

| No. | Plant | Cell lines | Reference |
|---|---|---|---|
| 17 | *A. gummifera* | A2780, HCC1395, DU145, Hep2 | Cao et al., 2007; Mbugua et al., 2019 |
| 18 | *A. coriaria* | HELF, HCT116, HT-29 | Ochwang'i et al., 2014 |
| 19 | *A. lebbeck* | U-87 MG, TG1, HepG2, HCT-116, MIA PaCa-2, HeLa, HepG2, AGS, U373MG, A431 | Noté et al., 2015; Singh et al., 2016b; Malaikolundhan et al., 2020; Ganesan & Subramanian, 2015 |
| 20 | *A. adianthifolia* | CCRF-CEM, HCT116, AMJ-13, A549 | Kuete et al., 2016; Sulaiman et al., 2018; Gengan et al., 2013 |
| 21 | *A. zygia* | MCF-7 | Mainasara et al., 2018 |

**Epithelial-mesenchymal transition (EMT) Screening**

In epithelial-mesenchymal transitions (EMTs), the mesenchymal features from epithelial cells may arise due to some biological processes. It defines a rapid and often reversible modulation of phenotype by epithelial cells. Throughout EMT, epithelial cells loosen the cell-cell adhesion structures (Savagner, 2010).

It is classified into three types:

i) EMT that occurs during embryonic development

ii) EMT associated with adult tissue regeneration

iii) EMT that occurs in cancer progression

EMT occurs during embryonic development in gastrulation, renal development, and during the origin and fate of neural crest. It is a highly regulated process but during tumour progression, it is highly deregulated. EMT causes solid tumours and become more malignant as well as speeds up their invasiveness and metastatic activity. EMT modulation could establish an approach to prevent metastasis. The antiproliferative agents have the ability to inhibit EMT initiation because it is regulated by signalling pathways (Ribatti et al., 2020).

**EMT in Cancer**

The correlation between EMT and cancer was reported in the early 80s. Due to EMT, the benign tumour cells gain infiltration and metastasizing effects during the tumour progression. During the activation of EMT, tumour epithelial cells lose their cell polarity and cell-to-cell adhesion and accomplish a migratory and invasive feature, becoming mesenchymal cells (Thiery et al., 2009). It has been confirmed that the transforming growth factor beta (TGF-

β)/Smads pathway is the potential EMT inducer by the upregulation of EMT-related transcription factors (Xu et al., 2009). In tumorigenesis, EMT makes a critical role in various cancers involving prostate, lung, liver, pancreatic, and breast cancers (Hugo et al., 2007; Lee et al., 2006).

**EMT and breast cancer metastasis**

Reports revealed that EMT may contribute to breast tumour metastasis. According to Yu et al. (2013), a single cell responsible for EMT in the cluster of cells, or the pre-existing clusters of circulating tumour cells causes mesenchymal transformation in the bloodstream. The results could demonstrate clearcut evidence of EMT taking place in relation to blood-borne dissemination of human breast cancer. The breast cancer patients exposed to both epithelial and mesenchymal markers reveal that a transition had occurred. In metastatic breast cancer patients, EMT markers like pan-cytokeratin, Twist, and vimentin in circulating tumor cells are also detected (Papadaki et al., 2014; Kallergi et al., 2011). Hence, it can be concluded that EMT is involved in the metastatic process of human breast cancer. However, reports regarding the EMT screening in breast cancer cell lines using the extracts of *Mallotus* are very meagre.

**Nanoparticle biosynthesis**

Nanobiotechnology is a vast developing research area due to its extensive applications in pharmaceutics, the medical field, cosmetics, etc. Metal ions have the capacity to reduce into metal nanoparticles. Silver, gold, platinum, copper, etc. have been widely used for the synthesis of its nanoparticles. Amongst various nanoparticles, biosynthesis of silver nanoparticles (**Figure 4**) is the most sought-after method due to its cost-effectiveness and the ability to inhibit the growth of microorganisms including bacteria, fungi, viruses, etc. It exhibits various biological and

biomedical applications including wound healing, tissue regeneration, anti-inflammatory, antioxidant, antibacterial, antifungal, anticancer, and antidiabetic activities. As a well-known antimicrobial agent, silver nanoparticles have been used for wound dressings, bone fracture, women's hygienic products, disinfectants, artificial ligaments, and antiseptics. Target-specific delivery of imaging and anticancer agents for screening and treatment of human cancer is the efficient nanoplatform developed by silver nanoparticles (Hussain et al., 2020). Besides conductivity, chemical stability and catalytic properties are the unique characteristic features of silver nanoparticles (Zomorodian et al., 2016). Dye-yielding plants are a great source of silver nanoparticles and their exploration is not only in food, cosmetics, and textiles but also in the medical field (Aswathi & Thoppil, 2022).

Phytochemical-mediated biosynthesis or plant product synthesis or microbial synthesis is the desirable method for synthesizing nanoparticles, which has drawn much attention due to its availability, reproducibility, and reliability (Nakkala et al., 2014; Geetha et al., 2013). Typical nano characteristics of silver can be revealed through various spectroscopic and microscopic analyses. UV-vis spectroscopy, scanning electron microscopy (SEM) and energy dispersive X-Ray spectroscopy (EDAX), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), X-Ray diffraction (XRD), thermogravimetric analysis (TGA), zeta potential analysis, etc. are important techniques adopted for the physicochemical characterization of nanoparticles (Selvaraj et al., 2019; Geethalakshmi & Sarada, 2010; Kasthuri et al., 2009; Kirthika et al., 2014).

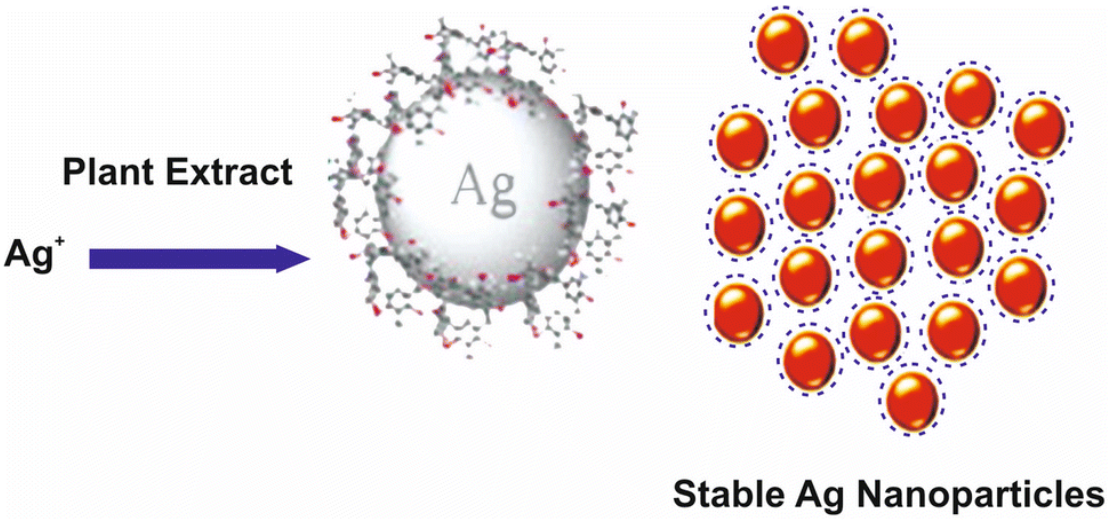



**Figure 4: Schematic representation of the synthesis of silver nanoparticles (Source: Selvaraj et al., 2019)**

UV-Visible spectrum had been analyzed to monitor the reduction of pure Ag+ ions and nanoparticle formation at a wavelength between 400 - 450 nm for silver nanoparticles (Pal et al., 2007). Silver nanoparticles were detected by UV spectra of the Surface Plasmon Resonance (SPR) band observed at 442 nm (Ulug et al., 2015). The SPR of silver nanoparticles was monitored by UV-Visible spectra for optical properties and it depends upon the collective oscillation of free electrons within the silver nanoparticles or other metallic nanoparticles. Moreover, the shift of plasmon resonance depends on the size of the silver nanoparticles (Mogensen & Kneipp 2014). So, UV-Visible spectroscopy is an authenticate technique for the detection of metal nanoparticles, particularly in the aqueous solution (Arunkumar et al., 2013). Morphological characterization of nanoparticles can be comprehended through the scanning electron microscope and EDAX analysis is used to identify the elemental silver of a sample. TEM is a microscopic technique that is generally used to visualize the size, shape, and morphological features of nanoparticles. The possible functional groups which are involved in nanoparticle synthesis can be analysed through a technique called FTIR (Tripathi et al., 2019). XRD is a commonly used technique for analysing the

crystalline structure of nanoparticles by evaluating the diffraction peaks of the synthesized nanoparticles and the crystallite size of the nanoparticle is examined using Debye-Scherrer's formula (Selvaraj et al., 2019). Selvaraj et al. (2019) have also mentioned the 2θ values that assigned the face-centred cubic (FCC) symmetrical structure for silver nanoparticles. The physical and chemical properties of nanomaterials are measured by the TGA method with an increase in temperature (with constant heating rate), or as a function of time (with constant temperature and/or constant loss of mass) (Kasthuri et al., 2009). Besides, the surface charge of nanoparticles in a solution (colloids) can be determined by zeta potential analysis. Each nanoparticle possesses a surface charge and which gets attracted to a thin layer of ions of opposite charge to the nanoparticle surface. Usually, zeta potential between -30 mV and +30 mV depicts an unstable suspension whereas the sample exists in values higher than +30 mV or lower than -30 Mv and is assumed to be a stable suspension (Kirthika et al., 2014).

According to Ahmed and Ikram (2015) the aqueous leaf extract of *Terminalia arjuna* exhibited the reduction of silver ions and produce stable silver nanoparticles. Synthesized silver nanoparticles were confirmed by using UV-Visible spectrophotometer analysis. They performed the reaction with different time intervals like 1 h, 2 h, 3 h, 4 h, 5 h, 18 h, and 24 h indicating the formation of silver nanoparticles due to the excitation of surface plasmon vibrations in silver nanoparticles (Manikandan et al., 2015). A dynamic light scattering (DLS) pattern of the suspension with silver nanoparticles has been visualized from the aqueous leaf extract of *T. arjuna*. Asymmetric distribution of silver nanoparticles was observed and about 3 to 50 nm size of particles were found. Also, the formation of silver nanoparticles was confirmed with the help of TEM and the size, shape, and morphology of the synthesized nanoparticles were elucidated. About 8 - 16 nm size of nanoparticles were identified in the leaf extract and they have a spherical

shape. The reduction of silver ions into silver nanoparticles was confirmed by FTIR and UV-Visible analysis and is exhibited by the support of plant extract as a capping agent. Moreover, the phytoconstituents found in *T. arjuna* leaf extract were responsible to act as the reducing agents. Furthermore, the produced silver nanoparticles revealed promising antimicrobial activities (Ahmed & Ikram, 2015). Likewise, *T. chebula* unveiled a characteristic presence of silver nanoparticles from the aqueous extract of their fruits (Edison & Sethuraman, 2012). They have characterized the synthesized silver nanoparticles with the help of FTIR, XRD, HR-TEM with EDS, and DLS with zeta potential. According to them, the stability of silver nanoparticles with high negative values of zeta potential reveals the capping potential with phytoconstituents in the aqueous fruit extract of *T. chebula*. Also, the crystalline structure with face-centred cubic geometry was observed by XRD and EDS. Silver nanoparticles with a size of 25 nm were observed through HR-TEM and DLS studies.

Green synthesis of silver nanoparticles in the ethanolic fruit extract of *Mallotus philippensis* revealed a significant production. The synthesized silver nanoparticles were characterised by UV, SEM, FTIR and XRD. Exposure to UV reveals that the maximum absorption peak was observed at 356 - 436 nm. The major functional groups responsible for nanoparticle synthesis are alcohol, alkane, hydroxyl, and ether groups as proved by FTIR. The morphological features of nanoparticles were analysed through SEM analysis with an average size of 70 nm. Furthermore, the crystalline structure of silver nanoparticles is unveiled through XRD analysis (Velraj, et al., 2018). The exploration of *Mallotus* species in the field of nanotechnology is meager. So, it will be a good area for future research.

Plant-mediated synthesis of silver nanoparticles using leaf extract of *Albizia procera* was carried out by Rafique et al. (2019). UV–Visible

spectroscopy, XRD, and Atomic Force Microscopy (AFM) were used to analyse the optical, structural and physical properties of the particles respectively. They inferred that the particles exhibit a spherical shape, as well as crystalline nature with an average size of 6.18 nm. According to Gengan et al. (2013), the aqueous leaf extract of *A. adianthifolia* revealed the presence of silver nanoparticles. Characterisation of particles was performed with the help of UV-visible spectroscopy, TEM, XRD, DLS, and FTIR. The SPR at 448 nm is obtained by the UV- vis analysis and the absorbance gradually increased in intensity as a function of reaction time. About 4 - 35 nm-sized particles were synthesized by the extract and XRD revealed the FCC geometry. Hydrodynamic size with 80.27 nm and zeta potential was recorded as −24.7 mV. Synthesised nanoparticles were capped with several protein molecules and other water-soluble phytocompounds like saponins and glycosides, which is proved by the FTIR technique. Also, they suggested that these particles act as stabilizing agents. Similarly, there is another record for the production of silver nanoparticles in *A. julibrissin* flower extract (Awwad et al., 2015). The synthesized silver nanoparticles were characterized with XRD, UV-vis Spectroscopy, SEM, and FTIR. UV-visible spectroscopy revealed the SPR peak at 410-430 nm, confirming the presence of silver nanoparticles. The average particle size is around 5-20 nm. The particle size is depending upon the flower extract, silver ion concentration as well as temperature. FT-IR spectra thus helps to determine the identification of possible functional groups responsible for the reduction of silver ions to silver nanoparticles. Reports on the biosynthesis of silver nanoparticles from various species of the genus *Terminalia, Mallotus,* and *Albizia* are summarised in **Table 4**.

**Table 4: Summary of the biosynthesis of silver nanoparticles from various species of *Terminalia, Mallotus* and *Albizia***

| Sl. No. | Plant | Plant part | Solvent used | Size and shape | Reference |
|---|---|---|---|---|---|
| 1 | *T. cuneata* | Bark | Aqueous | 25 - 50 nm, Spherical | Edison et al., 2016 |
| 2 | *T. bellerica* | Kernel | Aqueous | 19.09 - 47.91 nm, Spherical | Sherin et al., 2020 |
| 3 | *T. chebula* | Leaves | Aqueous | 60 nm, Spherical | Espenti et al., 2016 |
| 4 | *T. arjuna* | Bark | Aqueous | 30 - 50 nm, Spherical | Ahmed et al., 2017 |
| 5 | *T. catappa* | Leaves | Aqueous | 4.56 - 20 nm, Spherical | Devadiga et al., 2017 |
| 6 | *T. chebula* | Seed | Aqueous | 66.7 - 91.2 nm, Spherical | Nandagopal et al., 2014 |
| 7 | *T. brownii* | Bark | Aqueous | 20 - 67 nm, Spherical | Berihu et al., 2021 |
| 8 | *M. philippensis* | Fruit | Ethanol | 30 - 100 nm, Cubic | Velraj et al., 2017 |
| 9 | *A. chevalier* | Bark | Aqueous | 30 nm, Spherical | Khan et al., 2018 |
| 10 | *A. saman* | Leaves | Aqueous | 55 - 83 nm, Spherical, triangular and irregular | Daphedar & Taranath, 2017 |
| 11 | *A. lebbeck* | Bark | Aqueous | 10 - 60 nm, Cubic | Das et al., 2020 |

# MATERIALS AND METHODS

The present study is mainly focused on the bioactivity studies and phytochemical characterization of some selected dye-yielding plants of Kerala. Some common dye-yielding plants like *Terminalia paniculata* Roth., *Mallotus philippensis* (Lam.) Muell. Arg. and *Albizia odoratissima* (L. f.) Benth. were analyzed. The methanolic extract of specific dye-yielding plant parts was used to conduct the study. The materials and methods used for the entire study are presented in this part.

The methodology part involves three different sections.

**Part I-** Phytochemical characterization by GC-MS and HR LC-MS.

**Part II**- Bioactivity studies of the selected dye-yielding plants: Antioxidant potential and the evaluation of free radical scavenging activity; Hepatotoxic screening of selected dye-yielding plant that exhibits high antioxidant potential and corresponding synthetic colourant available in the market; Hepatoprotective screening on the selected dye-yielding plant with lesser hepatotoxicity; Cytotoxicity studies on *Allium cepa*; Antiproliferative assay on breast cancer cell line MDA-MB 231; EMT screening on MDA-MB 231 cell line.

**Part III-** Biosynthesis of silver nanoparticles and their characterization.

## I. PLANT MATERIALS

*Terminalia paniculata* and *Albizia odoratissima* were collected from Malappuram, Kerala and *Mallotus philippensis* from Calicut, Kerala (**Plate 1**). Selected plants were authenticated by Dr A. K. Pradeep, Assistant Professor, Department of Botany, University of Calicut. Voucher specimens

were deposited at the Herbarium of Department of Botany, University of Calicut (CALI).

**1. Botanical Name: *Terminalia paniculata* Roth. (CALI No. 7107)**

**Synonyms:** *Pentaptera alata* Banks ex G. Don, *Pentaptera huliva* Buch.-Ham. ex Wall., *Pentaptera paniculata* Roxb., *Terminalia monaptera* Roth.

**Family:** Combretaceae

**Common Name:** Flowering Murdah, Maruthu, Manjamaruthu, Peimaruthu, Pillamaruthu, Pullamaruthu, Venmaruthu

**Habitat:** Moist and dry deciduous forests, also in the plains

**Distribution:** Peninsular India

**Flowering & Fruiting:** August-February

**Plant Description:** Deciduous trees, rough, young parts are silky pubescent and branchlets are reddish in colour. Leaves are simple, lower leaves subopposite whereas the upper are alternate, 10-24 x 5-10 cm, elliptic, oblong, ovate, elliptic-oblong or ovate-oblong, apex acute or acuminate, base round, obtuse, cordate or oblique with entire margin. At the base of the lamina or at the junction of the petiole and lamina, 2 sessile glands are present. White bisexual flowers are seen in axillary and terminal panicles, tomentose; bracteoles 2-5 mm, linear-lanceolate and recurved. Calyx tube 1-1.2 × 0.8-1 mm, constricted above the ovary, pubescent, lobes 5, cream coloured and triangular. Petals 0 and 10 stamens are arranged in 2 rows, Ovary 1 mm, inferior, 1-celled; ovules 2 or 3, pendulous; style to 4 mm, subulate, stigma terminal. Fruit a drupe 13 x 12 mm, reddish-brown, rusty tomentose, with one large and 2 small wings with one seed (http3).

**2. Botanical Name: *Mallotus philippensis* (Lam.) Muell. Arg. (CALI No. 7105)**

**Synonyms:** *Aconceveibum trinerve* Miq., *Croton coccineus* Vahl, *Croton philippensis* Lam.

**Family:** Euphorbiaceae

**Common Name:** Monkey face tree, Kamala tree, Chenkolli, Kapila, Kuramaddaku, Kurangumanjal, Manjanampottu, Noorimaram, Ponoo, Ponnakam, Shenkolli, Sindooram, Thavatta.

**Habitat:** Semi-evergreen, moist deciduous, evergreen, and dry deciduous forests, also in the plains.

**Distribution:** Indo-Malaysia and Australia

**Flowering & Fruiting:** October - March

**Plant Description:** Dioecious trees that are pale brown in colour; branchlets, young leaves, and inflorescence are yellowish-brown or rusty pubescent. Leaves are simple, alternate, 5-20 × 2-8 cm, ovate or ovate-lanceolate, apex acuminate or acute, base acute or round, margin entire or slightly serrate, glabrous above, greyish pubescent to fulvous tomentose with minute red glands beneath; petiole is 7-60 mm long, stout, swollen at the base. Each side of the leaves has fulvous-pubescence with 2 small sessile glands. Flowers are unisexual, brick red, in rusty puberulous and terminal spicate panicles. Male and female flowers are grown on separate trees. Male flowers are 4.5 mm across; tepals 4, lanceolate and stamens are many. Female flowers are 4 mm across, tepals 3 or 4, thicker than the males, ovate-lanceolate; ovary with red glands, superior, 3-celled, ovules one in each cell; styles 3, up to 3.5 mm long, papillose. Fruit is a capsule, 7-8 mm across, globose, 3-lobed, loculicidally 3 valved, densely red-glandular, pubescent; seeds 1-4, globose, glabrous, black (http4).

**3. Botanical Name: *Albizia odoratissima* (L. f.) Benth. (CALI No. 7104)**

**Synonym:** *Albizia micrantha* B. Boivin, *Feuilleea odoratissima* (L. f.) Kuntze, *Mimosa odoratissima* L. f.

**Family:** Fabaceae

**Common Name:** Black siris, Kala siris, Ceylon rosewood, Karivaka, Karinthakara, Kunnivaka, Mellivaka, Nellivaga, Pulivaka

**Habitat:** Deciduous forests, also in the plains

**Distribution:** Indo-Malaysia

**Flowering & Fruiting:** April-January

**Plant Descriptions:** Deciduous trees with rough and greyish-brown to dark brown surface, irregularly cracked; branchlets blackish to brown, primarily yellowish-brown pubescent, finely glabrous. Leaves are bipinnate, alternate, pinnae 2-8 pairs, opposite, even pinnate, 5-13 cm long, slender, puberulent, glands between the junctions of 1-2 distal pairs of pinnae, leaflets 14-40, opposite, even pinnate, sessile, 1.8-2.5 x 0.5-1.2 cm, oblong, apex obtuse and apiculate, base oblique, with entire margin. Stipule free, lateral, caducous; rachis 20-30 cm long, stout, grooved above, pulvinate, brown pubescent, with a gland at the base. White bisexual flowers are seen with globose heads. Calyx tube cupular, 1-1.5 x 1-1.3 mm, teeth minute, deltoid and pubescent. Petals are connate, generally funnel-shaped, lobes 5, ovate-lanceolate and pubescent. Stamens are many, 1.2-2 cm long, monadelphous condition and tube as long as or shorter than the corolla tube. Ovary stipitate, glabrous or pilose, style filiform, stigma terminal. Fruit a pod 15-20 x 2.5-3.7 cm, flat, strap-shaped, with parallel margin or often some portion constricted, rounded to rostrate at apex, glabrous, often glossy, reddish-brown to dark brown, finely reticulate and veined; seeds are 6-12, oblong, orbicular and compressed (http5).

## II. METHODOLOGY

## PART I- PHYTOCHEMICAL PROFILING

Several standard qualitative tests are adopted to detect the presence or absence of metabolites in the methanolic extract of the dye-yielding plant part. Volatile and non-volatile compounds were identified with the help of GC-MS and HR LC-MS techniques.

The dye-yielding plant part was collected, shade dried and cut into small pieces. 10 g of the plant part was extracted in 100 ml methanol. About 6-8 h of 72 extraction cycles are needed to complete the extraction of phytochemical constituents of the desired plant parts. The obtained methanolic extract was cooled, filtered through a Millipore filter of pore size 0.45 µm and evaporated to remove the whole solvent content present in it. After that, the crude extract was stored in an amber-coloured glass bottle at 4°C for further experiments. The percentage yield of the extract was also calculated.

### a. Qualitative phytochemical screening

Innumerable chemical tests are used for the phytochemical analysis of the methanolic plant extract. These chemical tests could give detailed information regarding the phytoconstituents present in each plant extract as well as the respective class of compounds.

#### i. Test for alkaloids

The dried methanolic extract of selected dye-yielding plants (50 mg) was dissolved in dilute hydrochloric acid and filtered. This filtrate was used for further chemical tests to detect the presence of alkaloids. The tests are given below.

- **Wagner's test** (Shanmugam et al., 2019)

About 3-5 drops of Wagner's reagent (**Appendix 1**) were added to an aliquot of the extract. The formation of a reddish-brown precipitate indicated the presence of alkaloid content.

- **Hager's test**

A fraction of the methanolic extract was treated with a saturated picric acid solution. An orange-yellow coloured precipitate formed confirms the presence of alkaloids.

**ii. Test for flavonoids** (Harborne, 1998)

- **Alkaline reagent test**

Sodium hydroxide solution was treated with a small quantity of extracts. A yellow-coloured solution will be formed. Then a few drops of dilute hydrochloric acid were added to it and the colour disappears, which indicates the presence of flavonoids.

- **Lead acetate test**

A few drops of 10% lead acetate solution were added to the extract. A yellow-coloured precipitate confirms the presence of flavonoid content in the extract.

**ii. Test for phenolics** (Harborne, 1998; Kumar et al., 2007)

- **$FeCl_3$ test**

A few drops of 0.1 % ferric chloride solution were added to the extract. A deep blue colour indicated the presence of phenolics.

- **Phenol test**

The solvent-free extract is spotted on a filter paper and a drop of phosphomolybdic acid reagent is added. Exposure of the spot to ammonia vapours produces blue colouration which indicates the presence of phenols.

**iii. Test for tannins** (Evans & Evans, 2002)

The solvent-free extract (0.5 g) was mixed with 10 ml of distilled water and filtered. The obtained filtrate was used to detect the presence of tannins.

- **Ferric chloride test**

A few drops of 1% ferric chloride solution were added to 2 ml of the filtrate. The presence of a blue-black, green or blue-green precipitate denotes the occurrence of tannins.

**iv. Test for terpenoids**

- **Salkowski test** (Shanmugam et al., 2019)

About 2 ml chloroform was added to 1 ml of the sample, along the sides of the test tube. After that 2 ml of concentrated sulphuric acid was added to it and carefully permitted to stand. The presence of yellow colour with green fluorescence depicts the occurrence of terpenoids.

**v. Test for phytosterols**

- **Liebermann-Burchard reaction** (Siddiqui & Ali, 1997)

The methanolic extract was treated with chloroform and then filtered. About 2ml of acetic anhydride was added to it and 0.5 g of the extract along with 2 ml of sulphuric acid was also added. Colour changes from violet to blue indicates the presence of phytosterols.

**vi. Test for saponins**

- **Foam test** (Adegoke et al., 2010)

About 0.5 g methanolic extract was mixed with 5 ml of distilled water in a test tube. Thus, the obtained suspension was shaken strongly and then observed for a stable persistent froth. A layer of foam was sustained which indicates the presence of saponins.

**vii. Test for cardiac glycosides** (Harborne, 1998)

- **Keller Killiani's test**

A test tube having 5 ml of extract was treated with 2 ml of glacial acetic acid and added a drop of ferric chloride solution. It was cautiously treated with 1 ml of concentrated sulphuric acid. The presence of a brown ring at the interface indicated the deoxy sugar which distinguishes the presence of cardiac glycosides.

**viii. Test for phlobatannins** (Edeoga et al., 2005)

- **HCl test**

About 2 ml of extract was boiled with 1 ml of 1% aqueous hydrochloric acid. A red precipitate indicates the formation of phlobatannins.

**ix. Test for anthraquinones** (Kumar et al., 2007)

- **Borntrager's test**

50 mg of the solvent-free extract was treated with 1 ml ferric chloride solution (10%) and also add 1 ml of conc. HCl. After that, the solution was heated, cooled and filtered. Then add an equal amount of diethyl ether to the filtrate and shake well. The obtained ether extract was then treated with strong

ammonia. A pink or deep red colouration in the aqueous layer forms evidence for the presence of anthraquinones.

**x. Test for proteins and amino acids**

To 2 ml of the extract, 2 drops of ninhydrin solution was added. The formation of a purple colour indicated the presence of proteins and amino acids.

**xi. Test for coumarins**

- **Sodium hydroxide test**

About 1 ml of the extract was treated with 1 ml of 10% sodium hydroxide solution. The presence of a yellow colour indicated the sign of coumarins.

**xii. Test for resins**

About 0.5 g of the extract was mixed with 10 ml of distilled water. A turbidity was formed which indicates the presence of resins.

**b. Quantification of major phytocomponents**

Quantification of major classes of compounds like phenols, flavonoids, alkaloids, and terpenoids was done as per the standard protocols described below.

**i. Total phenolic content**

The total phenolic content of the plant extract was determined by the method of Oueslati et al. (2012). The reagent used for the technique was Folin-Ciocalteu. Gallic acid was used as the standard for the study. A small amount of the sample as well as the standard solution (gallic acid) were mixed with 0.5 mL distilled water and add 0.125 mL of Folin-Ciocalteu reagent

(1N). It was shaken well and allowed for 6 min incubation. Then add 1.25 mL of 7% $Na_2CO_3$ and made up to a final volume of 3 mL with distilled water and mixed well. After that, the reaction mixture was incubated in dark at ambient temperature for 90 min. The absorbance of both sample and standard was measured against blank (distilled water) at 760 nm in a spectrophotometer (Model Cary 5000). The calibration curve of gallic acid was plotted and the phenolic content was expressed in mg of gallic acid equivalents per gram of dry weight (mg GAE/g DW) by using a regression equation. Triplicates of the samples were analysed.

**ii. Total flavonoid content**

The total flavonoid content of the plant extracts was determined by the method of Dewanto et al. (2002) with a slight modification. Aluminium chloride colorimetric method was the preferred way for the total flavonoid content estimation. Quercetin was considered as the standard for the estimation. A little amount of diluted sample as well as a standard solution of quercetin was mixed with 75 µL of $NaNO_2$ solution (7%) for 6 min. Then add 0.15 mL of $AlCl_3$ (10%) to it. After 5 min, add 0.5 mL of 1 M NaOH solution to the reaction mixture and made up to the final volume of 2.5 mL. After mixing well, the absorbance was read at 510 nm against a blank (methanol). The calibration curve of quercetin was plotted and flavonoid content was expressed in mg of quercetin equivalents per gram of dry weight (mg QE/g DW) by using a regression equation. Triplicates of the samples were analysed.

**iii. Total terpenoid content**

The total terpenoid content of the methanolic extract of the dye-yielding part of the selected plants was determined on the basis of the method demonstrated by Ghorai et al. (2012) with slight modifications. Linalool was

considered as the standard for the estimation of total terpenoid content. The Salkowski test was carried out for the qualitative analysis of terpenoids. A reaction mixture was obtained. An aliquot of the reaction mixture was transferred to a cuvette for the spectrophotometric analysis. The absorbance was read at 538 nm against a blank (95% v/v methanol). For the standard curve, 200 µL of linalool solution in methanol was mixed with 1.5 mL chloroform. After that, serial dilutions [dilution level-100 mg/200 µL to 1 mg/200 linalool Conc.] were prepared and a final volume was made up by the addition of 95% (v/v) methanol. The calibration curve of linalool was plotted and the terpenoid content was expressed in mg of linalool equivalents per gram of dry weight (mg linalool/g DW) by using a regression equation. Triplicates of the samples were analysed.

**ii. Total alkaloid content**

The total alkaloid content of plant extract was identified based on an assay demonstrated by Harborne (1973). About 5g of plant sample was taken in a 250 mL beaker and add 200 mL of 10% of alcoholic acetic acid to it. The beaker was covered properly and kept as such for 4h. After the filtration process, the extract was placed in a water bath to concentrate upto one-quarter of the original volume. Then add concentrated ammonium hydroxide dropwise to the extract till the completion of precipitation. The solution was kept for a while to settle and the precipitate was collected. After that dilute ammonium hydroxide was added and filtered. The obtained residue was identified as an alkaloid. It was dried, weighed and the triplicates of the samples were analysed.

**v. Total tannin content**

The total tannin content of the extract was quantified based on the assay proposed by Bainbridge et al. (1996). About 1 mL of the plant extract

was mixed with 5 mL of vanillin reagent. The reaction mixture was kept for incubation at ambient temperature for 30 min. Then the absorbance was measured at 500 nm. Tannic acid was used as the standard and its calibration curve was plotted. The total tannin content was expressed in mg of tannic acid equivalents per gram of dry weight (mg tannic acid/g DW) by using a regression equation. Triplicates of the samples were analysed.

**c. Gas Chromatography-Mass Spectrometry (GC-MS) analysis**

Agilent Model 8890 GC system with a single quadrupole mass spectrometer (5977B MSD) analyzer was used for the identification and separation of volatile components present in the extract. GC oven temperature was near ambient 450°C. For the chromatographic performance, area repeatability was <0.5% and retention time repeatability was <0.008%. Inlet split ratio was 7500:1. Mass accuracy - 1µL injection of 100 pg/µL OFN; standard scanning from 50.35 u will give its monoisotope at m/z 271±0.005. Its ion source temperature was 150-350°C and its quadrupole temperature was 106-200°C. Licensed NIST 2017 Library was used as the spectral library and referral database for compound detection.

**d. High Resolution Liquid Chromatography-Mass Spectrometry (HR LC-MS) analysis**

Q-Exactive Plus Biopharma - High-Resolution Orbitrap was used for the identification and separation of non-volatile components. Direct infusion mass with ESI & APCI (Positive and negative mode ionization) was involved. Its resolving power was up to 280,000. Maximum scan speed was 12 Hz. RF-Lens ion source was used for increased sensitivity. Advanced active beam guide intelligent ion beam management was used for high-flux ion sources. Its mass range (m/z) was 50-8000 amu and its resolution was 1,40,000 @ m/z 200.

## PART II- BIOACTIVITY SCREENING

### A. Free Radical Scavenging Activity

Antioxidants are compounds, which have the property to scavenge free radicals produced by various metabolic activities. For balancing oxidative stress, plants and animals will generate these compounds. Several *in vitro* antioxidant assays are used to govern the effect of plant extract for scavenging free radicals produced by innumerable substrates like DPPH, $Fe^{3+}$ - ascorbate - EDTA - $H_2O_2$ system, sodium nitroprusside, and potassium ferricyanide.

#### i. DPPH free radical scavenging assay

DPPH assay was carried out to determine the radical scavenging activity of selected plant extracts (Chang et al, 2001). After the addition of an antioxidant, there is a considerable decline in the absorption of the DPPH solution was noticed at 517 nm. Ascorbic acid (10 mg/mL DMSO) was used as a reference.

A stable free radical 1, 1-diphenyl-2-picryl hydrazyl with pink colour turns yellow when scavenged. It is the basic nature of DPPH showing free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as,

$$DPPH + [H\text{-}A] \rightarrow DPPH\text{-}H + (A)$$

When antioxidants react with DPPH, they may get reduced to DPPH-H and subsequently results in the decrease of absorbance. After that, the discolouration specifies the effect of the scavenging potential of the extracts or antioxidants through hydrogen donating ability.

The preferred concentrations of the samples, *i.e.,* 12.5 µg/mL – 200 µg/mL taken from the stock concentration (10 mg/mL) and made up to the final volume of 20 µl with DMSO and 1.48 ml of 0.1 mM DPPH solution was added to it. An equivalent amount of distilled water without a test solution was taken as the control whereas ascorbic acid was used as the standard. The reaction mixture was incubated at room temperature for 20 minutes under dark conditions. After that, the absorbance of the reaction mixture was read at 517 nm with 3 ml of DPPH as the blank.

The percentage of inhibition was calculated as per the equation given below:

$$\text{Percentage inhibition} = \frac{\text{AC}-\text{AS}}{\text{AC}} \times 100$$

Where AC is the absorbance of the control and AS is the absorbance of the sample.

Triplicates of each extract were tested and the mean values were calculated.

**ii. Hydroxyl radical scavenging assay**

The condition of the degradation product of 2 deoxyribose by condensation with TBA is the basic scheme of this assay (Elizabeth and Rao, 1990). Fe3+ - ascorbate - EDT - $H_2O_2$ system (The Fenton reaction) generates the hydroxyl radical. Gallic acid (10 mg/mL DMSO) was used as the reference. The samples of different concentrations like 12.5 - 200 µL were taken from a stock concentration (10 mg/mL). After which it was mixed with 500 µL reaction mixture [(2 deoxy 2 ribose (2.8 mM), $FeCl_3$ (100 µM), EDTA (100 µM), $H_2O_2$ (1.0 mM), ascorbic acid (100 µM) in $KH_2PO_4$ - KOH buffer (20 mM pH 7.4)] and made up to a final volume (1 mL). An equivalent amount of distilled water without the test solution was taken as the

control. After that, the reaction mixture was incubated for 1h at 37°C and added 1 mL of 2.8% TCA followed by 1 ml of 1% aqueous TBA. Again, the mixture was incubated at 90°C for 15 min. to develop the colour. After cooling, the absorbance of the mixture was measured at 532 nm in contradiction to a suitable blank solution.

The percentage of inhibition was calculated as follows:

$$\text{Percentage inhibition} = \frac{\text{AC}-\text{AS}}{\text{AC}} \times 100$$

Where, AC is the absorbance of the control and AS is the absorbance of the sample.

**iii. Nitric oxide free radical scavenging assay**

The basic principle behind the nitric oxide scavenging assay is that the sodium nitroprusside in an aqueous solution at physiological pH impulsively generates nitric oxide which interacts with oxygen to produce nitrite ions and can be estimated using Griess reagent (**Appendix 2**). The effective scavengers of nitric oxide compete with oxygen and reduce the nitrite ions.

Different concentrations of the sample such as 125 µg/ml - 2000 µg/ml from a stock concentration of 10 mg/ml was mixed with sodium nitroprusside (5 mM) in phosphate-buffered saline (**Appendix 3**) (pH = 7.4), and incubated at 25°C for 150 min. (Kumaran & Karunakaran, 2006). The reagent without test solution was taken as the control. After 30 min., 1.5 mL of the incubated solution was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% N-1-naphthyl ethylene diamine dihydrochloride). Gallic acid was used as a reference and the absorbance was read at 546 nm.

The percentage of scavenging activity was calculated as follows:

$$\text{Percentage inhibition} = \frac{\text{AC}-\text{AS}}{\text{AC}} \times 100$$

Where AC is the absorbance of the control and AS is the absorbance of the sample.

**iv Superoxide free radical scavenging assay**

Superoxide is biologically significant and it can produce singlet oxygen and hydroxyl radicals. Redox imbalance might be due to the overproduction of superoxide anion and may lead to harmful physiological consequences. Superoxide anion is generated in riboflavin-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of a blue formazan product. Ascorbic acid (10 mg/mL) was used as the reference. In a test tube the selected concentrations of the samples such as 125 – 2000 µg/mL from a stock solution of 10 mg/ml was mixed with 0.05 ml of riboflavin solution (0.12 mM) and 0.2 ml of EDTA solution (0.1 M), and 0.1 ml NBT (Nitro-blue tetrazolium) solution (1.5 mM). The reaction mixture was then diluted up to 2.64 ml with phosphate buffer (0.067 M). Distilled water was taken as the control. Absorbance was read at 560 nm under illumination after 5 minutes of incubation in fluorescent light. Measurement was also taken after illumination for 30 min. at 560 nm on UV visible spectrophotometer. Finally, OD was calculated (Valentão et al., 2002).

The percentage inhibition was calculated as follows:

$$\text{Percentage inhibition} = \frac{\text{AC}-\text{AS}}{\text{AC}} \times 100$$

Where AC is the absorbance of the control and AS is the absorbance of the sample.

## B. Hepatotoxicity Screening

The plant extract which exhibited maximum antioxidant potential was selected to screen for its hepatotoxicity. The synthetic food colorant (Lemon Yellow-19140) was also taken as its corresponding dye for comparison. Hep G2 (Liver Hepatic cells) cell line was purchased from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco's Modified Eagles medium (DMEM) (Sigma Aldrich, USA).

The cell line was cultured in a 25 $cm^2$ tissue culture flask with DMEM (**Appendix 4**) with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany), and an antibiotic solution encompassing penicillin (100 U/mL), Streptomycin (100 µg/mL), and Amphotericin B (2.5 µg/mL). Cultured cell lines were kept at 37ºC in a humidified 5% $CO_2$ incubator (NBS Eppendorf, Germany). Cell viability was assessed by direct observation of cells using an inverted phase contrast microscope. After that MTT assay method was carried out.

**Cells seeding in 96 well plate:**

The confluent monolayer of cells was trypsinized after two days and these cells were transferred to 10% growth medium. Then 100 µl cell suspension ($5x10^3$ cells/well) was seeded in 96 well tissue culture plate and incubated in a humidified 5% $CO_2$ incubator at 37ºC.

**Preparation of samples:**

Methanolic plant extract and its corresponding synthetic food colorant lemon yellow were weighed (1 mg) and both were dissolved in 1 ml DMEM via a cyclomixer. The samples were filtered through a 0.22 µm Millipore syringe filter.

**Cytotoxicity evaluation:**

The growth medium was removed after 24 hours and 100 µL of each concentration of the extract (100, 50, 25, 12.5 and 6.25 µg in 500 µL of DMEM) were added in triplicates to the corresponding wells and incubated in a humified 5% $CO_2$ incubator at 37°C. Untreated control cells were also kept.

**Cytotoxicity assay by direct microscopic observation:**

After 24 hours of treatment, the whole plate was observed in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and was documented as images. If any noticeable changes in the morphology of the cells *viz*., rounding or shrinking of cells, granulation, vacuolization in the cytoplasm of the cells etc. occurred, then they were considered as the repercussions of cytotoxicity.

**Cytotoxicity assay by MTT method:**

About 15 mg of MTT (Sigma, M-5655) was mixed in 3 ml of PBS till it gets completely dissolved and followed by filter sterilization. The sample content in wells was removed after 24 hours of the incubation period, and 30 µl of newly reconstituted MTT solution was added to all test and control wells. The plate was shaken thoroughly and incubated in a humidified 5% $CO_2$ incubator at 37°C for 4 hours. The supernatant was removed after the incubation period, and 100 µl of MTT solubilization solution (Dimethyl sulphoxide: DMSO, Sigma Aldrich, USA) was also added to the wells and mixed moderately by pipetting up and down for solubilizing the formazan crystals. A microplate reader was used to measure the absorbance values at 540 nm (Talarico et al., 2004).

The percentage of growth inhibition was calculated using the formula:

$$\text{Percentage of growth inhibition } = \frac{\text{Mean OD of samples}}{\text{Mean OD of control}} \times 100$$

**C. Hepatoprotective Screening**

After hepatotoxic screening, the plant extract with high antioxidant potential was further subjected to hepatoprotective activity. For this analysis, Hep G2 (Liver Hepatic cells) cell line was purchased from National Centre for Cell Sciences (NCCS), Pune, India and was maintained in Dulbecco's Modified Eagle Medium (DMEM) from NCCS (Sigma Aldrich, USA). The cell line was cultured in a 25 $cm^2$ tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate, and an antibiotic solution encompassing penicillin (100 U/mL), Streptomycin (100 µg/mL), and Amphotericin B (2.5 µg/mL). Cultured cell lines were kept at 37°C in a humidified 5% $CO_2$ incubator (NBS Eppendorf, Germany).

The viability of cells was evaluated by direct observation of cells by inverted phase contrast microscope and followed by MTT assay method as mentioned earlier.

Triplicates were finished to calculate the percentage of viable cells as per the equation described below:

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

**D. Cytotoxicity Studies on *Allium cepa***

For the cytotoxicity assay, the unwanted dry scales of *A. cepa* bulbs were removed without affecting the root primordia. The collected bulbs were kept in distilled water for producing roots. After 1-2 days the roots develop and the bulbs with healthy roots (1-2 cm) were collected at the period of maximum mitotic activity (between 9 am and 10 am on sunny days). The collected roots were washed thoroughly in distilled water. After that, the

bases of bulbs with roots were placed in vials having different concentrations [0.005, 0.01, 0.05, and 0.1% (w/v)] of the plant extract. The root tips were kept immersed in the samples. For this assay, hydrogen peroxide and distilled water were taken as the positive and negative control respectively. The time intervals taken for the study were 12 h, 24 h, and 48 h. Then the treated roots were fixed in modified Carnoy's fluid (**Appendix 5**) for 1 h. After completion of the fixation process, the root tips were transferred to 1N HCl for the separation of cells. After 5-10 minutes, the washed root tips were stained with 2% acetocarmine (**Appendix 6**) for 4 h. Then the root tips were destained with 45% acetic acid, squashed, and mounted on clean slides (Sharama & Sharma, 1990). Mounted slides were observed under a microscope (LEICA DM 2000 LED) for enumerating the aberrated and total cells located on 6 different fields.

**E**. **Antiproliferative activity**

**i. *In vitro* cytotoxicity on normal cell line (L929) by MTT assay**

L929 (Fibroblast) cells were collected from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco's modified Eagles medium, DMEM (Sigma Aldrich, USA). The cell line was cultured in a 25 $cm^2$ tissue culture flask with DMEM added with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution encompassing Pencillin (100 U/mL), Streptomycin (100 µg/mL) and Amphotericin B (2.5 µg/mL). Cultured cell lines were kept at 37°C in a humidified 5% $CO_2$ incubator (NBS Eppendorf, Germany). Cell viability was assessed by direct observation of cells using an inverted phase contrast microscope. After MTT assay method was carried out.

**Cells seeding in 96 well plate:**

The confluent monolayer of cells was trypsinized after two days and these cells were transferred to 10% growth medium. Then 100 µl cell suspension ($5x10^3$ cells/well) was seeded in 96 well tissue culture plate and incubated in a humidified 5% $CO_2$ incubator at 37°C.

**Preparation of the stock:**

The methanolic plant extract was weighed (1 mg) and dissolved in 1 ml DMEM using a cyclomixer. The sample solution was filtered through 0.22 µm Millipore syringe filter.

**Anticancer assessment:**

The growth medium was removed after 24 hours and 100 µL of each concentration of the extract (100, 50, 25, 12.5 and 6.25 µg in 500 µL of DMEM) was added in triplicate to the corresponding wells and incubated in a humified 5% CO2 incubator at 37°C. Untreated control cells were also kept.

**Anticancer assay by direct microscopic observation:**

After 24 hours of treatment, the whole plate was observed in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and was documented as images. If any noticeable changes in the morphology of the cells including rounding or shrinking of cells, granulation, and vacuolization in the cytoplasm of the cells were observed, then they were considered as the repercussions of cytotoxicity.

**Anticancer assay by MTT method:**

About 15 mg of MTT (Sigma, M-5655) was mixed in 3 ml PBS till it gets completely dissolved, followed by filter sterilization. The sample content in wells was removed after 24 hours of the incubation period, and 30 µl of

newly reconstituted MTT solution was added to all test and control wells. The plate was shaken thoroughly and incubated in a humidified 5% $CO_2$ incubator at 37°C for 4 hours. The supernatant was removed after the incubation period, and 100 µl of MTT solubilization solution [Dimethyl sulphoxide (DMSO) Sigma Aldrich, USA] was also added to the wells and mixed moderately by pipetting up and down for solubilizing the formazan crystals. A microplate reader was used to measure the absorbance values at 540 nm (Talarico et al., 2004).

The percentage of growth inhibition was calculated using the formula:

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

**ii. *In vitro* anticancer activity on MDA-MB 231 cell line using MTT assay**

MDA-MB 231 (Human breast cancer) cell line was collected from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's modified Eagles medium (DMEM Sigma Aldrich, USA). The cell line was cultured in a 25 $cm^2$ tissue culture flask with DMEM augmented with 10% FBS, L-glutamine, sodium bicarbonate, and an antibiotic solution containing: Pencillin (100 U/mL), Streptomycin (100 µg/mL) and Amphotericin B (2.5 µg/mL). Cultured cell lines were kept at 37°C in a humidified 5% $CO_2$ incubator (NBS Eppendorf, Germany). Cell viability was assessed by direct observation of cells using an inverted phase contrast microscope. After that MTT assay was carried out as per the method mentioned earlier.

### iii. Double staining using acridine orange (AO) and ethidium bromide (EtBr)

The morphological determination of apoptotic and necrotic cells was performed using DNA-binding dyes such as acridine orange (AO) and ethidium bromide (EtBr) (**Appendix 7**) (Sigma, USA) (Zhang et al, 1998). When AO gets intercalated into the double-stranded nucleic acid (DNA), it emits green fluorescence and was revealed by both viable and non-viable cells. Whereas, EtBr is absorbed only by non-viable cells and emits red fluorescence when intercalated into DNA. After 24 h treatment with $LC_{50}$ concentration of the extract, cells were gently washed with cold PBS and then stained with a mixture of AO (100 µg/mL) and EtBr (100 µg/mL) for 10 min at room temperature. The stained cells were then washed with 1 x PBS and observed by using a fluorescence microscope with a blue filter (Olympus CKX41 with Optika Pro5 camera). The cells can be separately scored into four groups viz. living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation), and necrotic cells (uniformly orange-stained cell nuclei).

### iv. Cell cycle analysis using flow cytometry

The capacity of cells to multiply is very critical for judging the cell's health in toxicity studies. The most reliable method for doing this is by directly measuring DNA synthesis. Standard ethanol fixation and detergent permeabilization is the fundamental principle of the MUSE cell cycle kit and it is necessary to attain access to the DNA during the active cell cycle. The kit includes a premixed reagent with nuclear DNA intercalating stain propidium iodide (PI) which distinguish cells at various stages of the cell cycle. It is mainly based on the differential DNA content in the presence of RNAase to increase the specificity of DNA staining in each phase ($G_0/G_1$, S and $G_2/M$).

MDA-MB 231 (Human breast cancer) cells were cultured based on the standard protocols mentioned earlier. The cells were grown to 60% confluency and it was treated with $LC_{50}$ concentration of the sample. It requires 24 h incubation. Then cell sample was transferred to a 12 × 75 mm polystyrene tube or 50 ml conical flask. The minimum suggested number of cells for fixation in a tube is 1 × 106 cells. After that, samples were centrifuged at 3000 rpm for 5 minutes. Then decant the supernatant without affecting the pellet. Following the centrifugation, the cell pellets may be visible as pellets or as a white film on the bottom of the tube. A suitable volume of PBS was added to each tube (i.e., 1 ml of PBS per 1 × 106 cells) and the contents were mixed by pipetting several times or gently vortexing. After that centrifugation was done at 3000 rpm for 5 minutes. The supernatant was removed without affecting the pellets, leaving about 50 µl of PBS per 1 × 106 cells. The pellets were resuspended in the residual PBS by repeated pipetting many times as well as vortexing. The resuspended cells were supplemented into the tube drop-wise having 1 ml of ice-cold 70% ethanol though vortexing at medium speed. Cap and freeze the tube at -20ºC.

**Staining of cell cycle**

The samples were centrifuged after the overnight incubation at 3000 rpm for 5 minutes at room temperature. The supernatant was discarded and 250 µl PBS was added to the pellet. Again, centrifuged at the same rpm and time. The supernatant was removed and collected the pellet. After that 250 µl of cell cycle reagent was added to it. Due to its light sensitivity, it was incubated in dark for 30 minutes. Finally, it was assessed through a Flow Cytometer. Gating was completed with reference to the untreated control cells and samples were analysed.

**v. Gene expression study using RT-qPCR**

- **Isolation of total RNA (TRIzol method)**

Total RNA was isolated using the total RNA isolation kit based on the manufacturer's instruction (Invitrogen-Product Code 10296010). After the addition of TRIzol solution, disruption of cells and release of the RNA occurs. During chloroform extraction followed by centrifugation, RNA exists in the aqueous phase while proteins are in the interphase and organic phase. RNA gets precipitated as a white pellet on the side and bottom of the tube due to the mixing with isopropanol. MDA-MB 231 was cultured as per the standard protocol mentioned earlier. The $LC_{50}$ concentration of the most effective extract from a stock of 1 mg/mL was added to the cells and kept for 24 h incubation at 37°C in a $CO_2$ incubator. Untreated control cells were also maintained in the same condition. After incubation, DMEM media was aseptically removed and about 1 mL of TRIzol reagent was added to the culture well plate and subjected to 5-minute incubation. The contents were then shifted to a fresh sterile Eppendorf tube. After that, about 200 µL chloroform was added and vigorously shaken for 15 seconds. It was kept for a 2 - 3-minute incubation period at room temperature followed by centrifugation at 14000 rpm for 15 minutes at 4°C. The aqueous layer was separated and 500 µL of 100% isopropanol was added to it. It was then centrifuged at 14000 rpm for 15 minutes at 4°C followed by the 10-minute incubation at room temperature. The supernatant was removed and collected the pellet and was washed with 200 µL of 75% ethanol (Merck). Again, it was centrifuged at 14000 rpm at 4°C for 5 minutes in a cooling centrifuge (Remi CM12). Finally, the RNA pellet was dried and suspended in TE buffer (**Appendix 8**).

- **cDNA synthesis**

Total RNA was extracted by using Trizol (Invitrogen, USA). The concentration and purity of total RNA were determined. With the help of a cDNA preparation kit (G BIOSCIENCES, Product code- 786-5019s,786-5020, master premix for first-strand cDNA synthesis) template complementary DNA was synthesized. After that, about 5 µl of RT Easy mix, 0.5 µl of oligo dT, and 2 µl of RNA template (0.5 µg of total RNA) were added to an RNAse-free tube. By the addition of sterile distilled water, total reaction volume was made up to 10 µL. The solution was then mixed by pipetting gently up and down. A Thermal cycler (Eppendorf Master Cycler) was programmed for the synthesis of cDNA. The following cycling conditions were worked out for 20 minutes at 42°C and 5 minutes at 85°C.

| Steps | Temperature (°C) | Time (min.) | Number of cycles |
|---|---|---|---|
| cDNA synthesis | 42 | 20 | 1 |
| Inactivation | 85 | 5 | 1 |

- **Gene expression analysis by RT- qPCR**

Real-Time qRT-PCR analysis was done by SYBR Green Master Mix (G BIOSCIENCES, Product code-786-5062) using Light cycler 96 (Roche). All reactions were carried out in triplicates and data were analysed by $\Delta\Delta C_t$ method (using Light Cycler 96 SW 1.1 Software).

The steps, time, and temperature involved for the analysis is given in the table.

| **Steps** | **Time required** | **Temperature** |
|---|---|---|
| Initial activation step | 2 minutes | 95°C |
| 3 step cycling: | | |
| Denaturation | 10 seconds | 95°C |
| Annealing | 1 minute | 58°C |
| Extension | 1 min/kb | 72°C |
| Number of cycles | 40 cycles | 68°C |
| End of PCR cycling | Indefinite | 4°C |

**The primer sequences used:**

| | |
|---|---|
| Human GAPDH forward | 5’ - ACTCAGAAGACTGTGGATGG |
| Human GAPDH backward | 3’ - GTCATCATACTTGGCAGGTT |
| p53 forward | 5’ - CCACCATGAGCGCTGCTCA |
| p53 backward | 3’ - GCAGGGGAGGGAGAGATG |
| TGF beta forward | 5’ - TGGAGCAACATGTGGAACTC |
| TGF beta backward | 3’ - TGCCGTACAACTCCAGTGAC |

- **Agarose gel electrophoresis**

It is a method in which DNA fragments can be separated and visualized. Charge and size are the common parameters for the separation of fragments that move through the agarose gel matrix with the effect of an electric field. The electric field is generated by setting up a potential across an electrolyte solution (buffer). Agar gets dissolved when boiled in an aqueous buffer and solidifies to a gel while cooling. Agarose gel (1.5%) was prepared in 1 x TE buffer and melted in a hot water bath at 90°C. It was then cooled down to 45°C. About 6 µL of 10 mg/mL of ethidium bromide was added and poured into the gel casting apparatus with the gel comb. The comb was then

removed from the gel after setting. The gel tank was filled with the electrophoresis buffer and the gel was placed in the platform as it immerses in the buffer. The sample was loaded on to the gel and run at 50 V for 30 minutes. By using a gel documentation system (E gel imager, Invitrogen) the stained gel was visualized.

## F. Epithelial Mesenchymal Transition (EMT) screening

EMT screening was analyzed using three different assays.

- ❖ Cell Migration assay
- ❖ Cell aggregation assay
- ❖ Clonogenic assay

### i. Cell Migration assay

The human breast cancer cell line (MDA-MB 231) was procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco's modified Eagles medium, DMEM (Sigma Aldrich, USA). The cell line was cultured in a 25 $cm^2$ tissue culture flask with DMEM and was supplemented with 10% FBS, L-glutamine, sodium bicarbonate, and an antibiotic solution containing: Pencillin (100 U/mL), Streptomycin (100 µg/mL) and Amphotericin B (2.5 µg/mL). Cultured cell lines were kept at 37°C in a humidified 5% $CO_2$ incubator (NBS Eppendorf, Germany).

**Procedure**

First of all, the growing cells were trypsinized and seeded at a density of 200,000 cells per well into a 12-well plate for 24 h incubation (~90% confluence). A sterile 1 mL pipette tip was used to make scratch wounds through a pre-marked line. The resulting debris was removed from the five linear scratches and the cell monolayer was rinsed three times with PBS

followed by incubation with the extract of the most effective plant for 0 h, 24 h, 48 h, and 72 h. After incubation, wound areas were displayed by taking images just above the interchanges between scratched wound areas and pre-marked lines, and the effect of the sample on wound closure was analysed microscopically (4X magnification, Olympus CKX41). By using MRI-ImageJ analysis software, the effect of the sample on wound closure was measured in terms of area.

**ii. Cell aggregation assay**

For the cell aggregation assay, human breast cancer cell line (MDA-MB 231) was cultured as mentioned earlier. After attaining 80% confluency, cells were exposed to the most effective extract with $LD_{50}$ concentration and incubated. Control cells were also maintained.

**Chemicals used**:

1. Ringer's salt solution (**Appendix 9**): About 8.6 g of NaCl, 330 mg of $CaCl_2$. $2H_2O$ and 300 mg of KCl were dissolved in 900 mL of distilled water. The pH of the solution was adjusted to 7.4 with NaOH and made up the volume to 1L using distilled water. The solution was sterilized by filtration and stored at 4°C. All filtrations are carried out using 0.22 µm filters.

2. Semi-solid agar medium: About 100 mg of Bacto-agar (Difco Laboratories, Detroit, MI, USA) was dissolved in 15 mL of sterile Ringer's salt solution in a sterile 50-mL Erlenmeyer flask. For sterilizing the solution, it was boiled 3 times for 10 s. Cool the solution to about 40–50°C and immediately poured into a 96-well microtiter plate.

3. An appropriate culture medium for the cells was used.

4. Moscona solution (**Appendix 10**): About 8 g of NaCl, 0.3 g of KCl, 0.05 g of $Na_2HPO_4 \cdot H_2O$, 0.025 g of $KH_2PO_4$, 1.0 g of $NaHCO_3$ and 2 g of D (+)-glucose (dextrose) was dissolved in 900 mL of distilled water. Adjust the pH of the solution to 7.0 –7.4 with normal HCl and make upto1 L using distilled water. It was sterilized by filtration and stored at 20°C.

5. Calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS) (**Appendix 11**): About 8 g of NaCl, 0.4 g of KCl, 0.06 g of $KH_2PO_4$, 0.35 g of $NaHCO_3$ and 0.112 g of $Na_2HPO_4 \cdot 12H_2O$ was dissolved in 900 mL of distilled water. Adjusted the pH to 7.4 with 2 M NaOH and added distilled water to make up to 1 L. It was sterilized by filtration and stored at 4°C.

6. Trypsin–EDTA solution (e.g., Gibco BRL, Paisley, Scotland): It involves 0.5 g of trypsin and 0.2 g of ethylenediaminetetraacetic acid tetrasodium salt ($Na_4EDTA$) per liter of CMF-HBSS. It was stored at -20°C.

**Procedure**

About 50 µL of the agar solution (40–50°C) was transferred into each well of a 96-well microtiter plate and kept at 4°C on a horizontal surface for about 1 h to get the agar solidified. Cells were washed twice with 3 mL of Moscona solution. After that cells were detached by standard trypsinization procedures and 100 µL of cell suspension was added to (20,000 cells) in the agar-coated wells. 100 µL of DMEM media was added to both treated and untreated wells and kept at 37°C incubation in a humidified atmosphere. After 24, 48, and 72 hours of incubation, cell aggregates were evaluated. The formation of the large compact aggregates, small loose aggregates and absence of aggregates was assessed using a microscope (4X magnification,

Olympus CKX41). After 72 hours, the cells were stained with crystal violet and the aggregates were first fixed with formaldehyde (4%) for 2 hours and then stained with 0.005% crystal violet for 30 minutes.

### iii. Clonogenic assay

For the clonogenic assay, the human breast cancer cell line (MDA-MB 231) was procured and cultured as mentioned earlier. After attaining 80% confluency, cells were exposed to the most effective extract with $LD_{50}$ concentration and incubated. Control cells were also maintained.

**Procedure**

Clonogenic assay was carried out in a 6-well plate. 1% agarose and 0.8% agarose was prepared and kept in $56^0$C water bath. A bottom layer was made by adding 2 ml of 1% agarose mixed equally with DMEM. It was then kept uninterrupted for a few minutes till it solidifies. About 5 ml of 0.8% agarose was mixed with 6 ml of DMEM. The top layer was made by the addition of 2 ml of the above mixture. It was then kept for 15 minutes. The treated cells were trypsinized and spread over the top layer very carefully. Dishes were incubated for colony formation for 14 days. Colonies were first fixed with formaldehyde (4%) for 2 hours and then stained with 0.005% crystal violet for 30 minutes. Each assay was performed as triplicates and only colonies containing at least 50 cells were counted (Yang, 2012).

## PART III – GREEEN SYNTHESIS OF SILVER NANOPARTICLES

About 10 mg of the methanolic residue of selected dye-yielding plants (*T. paniculata*, *M. philippensis* and *A. odoratissima*) was dissolved in 20 mL of deionized water. After that the extract was filtered by using Whatman No. 1 filter paper.

### i. Synthesis of Silver Nanoparticles

Collected filtrate (10 mL) was treated with 2 mM silver nitrate solution (90 mL) and boiled at 80°C for 10 min. The formation of yellowish-brown colour indicates the presence of silver nanoparticles (Gnanadesigan et al., 2012).

❖ **Collection of silver nanoparticle pellets**

The obtained coloured solutions were centrifuged using an ultra-centrifuge (Thermo Scientific, Sorvall Wx + Ultra series centrifuge) at 12,000 rpm for 10 min. and washed with sterile distilled water. The centrifugation process was continued for 3 times to make sure that all the silver nanoparticles were separated. The obtained nano pellets were kept at room temperature for further characterization studies.

### ii. AgNP Characterization

### a. UV-VIS-NIR spectral analysis

About 1 mL of the synthesized solution was taken to detect how much Ag+ ions were reduced. The absorbance was read at 200 - 700 nm in a UV-Vis-NIR spectrophotometer (model Cary 5000)

**b. FE-SEM analysis**

Air-dried pellets of silver nanoparticles were examined using a field emission scanning electron microscope (FE-SEM: Geminisem 300) with a magnification of 12X – 2,000,000X and acceleration voltage of 0.02 – 30 kV.

**c. EDAX analysis**

The elemental composition of silver was evaluated by energy-dispersive X-ray (EDAX) analysis (Octane Plus model). The active area of the EDAX was 30 $mm^2$.

**d. XRD analysis**

The crystallite structure of silver nanoparticles was evaluated with the help of an X-ray diffractometer (Panalytical, X'Pert3 powder) furnished with Cu Kα radiation source with Ni filter at a setting of 45 kV/30 mA. Complete XRD data were inspected under the experimental conditions in the angular range $10° \leq 2\theta \leq 90°$. The average size of the crystallites was calculated by using the Scherrer formula

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

where D means the crystallite size of the nanoparticle, K is a constant related to the crystallite shape of nanoparticles, normally taken as 0.9. λ means wavelength of X-ray, β represents the full width at half maximum intensity (FWHM) and θ denotes Bragg's angle. Experiments that require replicas were carried out in triplicates.

# RESULTS

## PART I – PHYTOCHEMICAL PROFILING

Qualitative phytochemical tests were carried out to detect the presence of phytochemical constituents in the methanolic extracts of *T. paniculata*, *M. philippensis* and *A. odoratissima*. Additionally, GC-MS and HR LC-MS analyses are the adopted analytical techniques used to identify the potential volatile and non-volatile compounds respectively. Another aspect includes quantitative phytochemical analyses, which revealed how much of the phytochemical constituents are available within the extract.

### a) Qualitative phytochemical screening

Preliminary phytochemical analysis revealed the presence of secondary metabolites in dye-yielding plants *viz*., *T. paniculata*, *M. philippensis* and *A. odoratissima* based on the colour formation as well as precipitation reactions. Methanolic extract of dye-yielding plant part was used for the study. Methanolic fruit extracts were collected from *T. paniculata* and *M. philippensis*, whereas the bark of *A. odoratissima* was used for the extraction procedure. Alkaloids, flavonoids, phenols, tannins, terpenoids, steroids, saponins, glycosides, phlobatannins, anthraquinones, coumarins, resins, proteins, and amino acids are the major classes of compounds obtained through the qualitative estimation tests. All these compounds are identified in the methanolic fruit extract of *T. paniculata*. Among the 13 classes of compounds, saponins and phlobatannins are absent in *M. philippensis*. Similarly, coumarins and resins were not detected in the methanolic bark extract of *A. odoratissima*. The results of the phytochemical analysis of these three plants are described in **Table 5.**

**Table 5: Preliminary phytochemical screening of *T. paniculata*, *M. philippensis* and *A. odoratissima***

| Sl. No. | Class of compounds | Chemical Tests | *T. paniculata* | *M. philippensis* | *A. odoratissima* |
|---|---|---|---|---|---|
| 1 | Alkaloids | Wagner's test | + | + | + |
| | | Hager's test | + | + | + |
| 2 | Flavonoids | Alkaline reagent test | + | + | + |
| | | Lead acetate test | + | + | + |
| 3 | Phenols | Ferric chloride test | + | + | + |
| 4 | Tannins | Braymer's test | + | + | + |
| 5 | Terpenoids | Salkowski's test | + | + | + |
| 6 | Steroids | Liebermann Burchard test | + | + | + |
| 7 | Saponins | Foam test | + | - | + |
| 8 | Glycosides | Keller Killiani test | + | + | + |
| 9 | Phlobatannins | Precipitation test | + | - | + |
| 10 | Anthraquinones | Borntrager's test | + | + | + |
| 11 | Proteins and amino acids | Ninhydrin test | + | + | + |
| 12 | Coumarins | Alcoholic NaOH test | + | + | - |
| 13 | Resin | Turbidity test | + | + | - |

(+: Presence; -: absence)

**b) Quantification of major phytocomponents**

The quantitative phytochemical study revealed the total content of compounds present in the extract. The major classes of compounds obtained through the preliminary analysis were used for the quantitative study. The exact content of phenols, flavonoids, alkaloids, terpenoids, and tannins was evaluated. In total phenolic content, *T. paniculata* showed the highest value (133 ± 1.14 mg GAE/g DW) than the standard used, *viz.* gallic acid (**Figure 5**). This was followed by *A. odoratissima* and *M. philippensis,* which showed characteristic amounts of phenol content (**Figure 6**). But in the case of flavonoid quantification, *A. odoratissima* recorded the high content of flavonoid (98.66 ± 2.98 mg QE/g DW) than *T. paniculata* and *M. philippensis* (**Figure 8**). Quercetin was used as the standard for flavonoid estimation (**Figure 7**). For the quantification of the alkaloid content, caffeine was used as the reference (**Figure 9**), where all three plant species possessed a characteristic amount of alkaloid content. But the highest value (24.99 ± 0.31 mg CE/g DW) was observed in *T. paniculata* fruit extract (**Figure 10**). Moreover, high terpenoid content was found in *T. paniculata* and it was 158.45 ± 2.31 mg against linalool (**Figure 12**). All three species recorded a high amount of terpenoid content than the reference (**Figure 11**). Among all these experiments, the exact content of tannin was observed to be very less when compared to other classes of compounds. The total tannin content was tested against tannic acid (**Figure 13**). The highest value was observed in *A. odoratissima* (6.06 ± 0.26 mg TAE/g DW) followed by *T. paniculata* and *M. philippensis* (**Figure 14**).

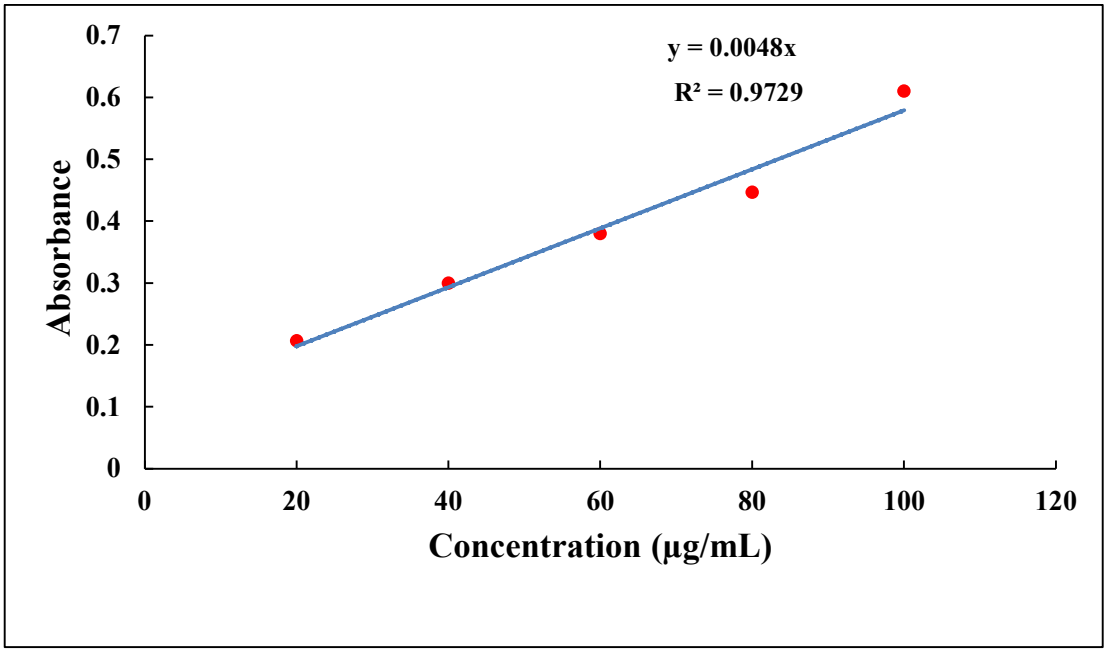



**Figure 5: Standard calibration curve of gallic acid for the determination of total phenolic content**

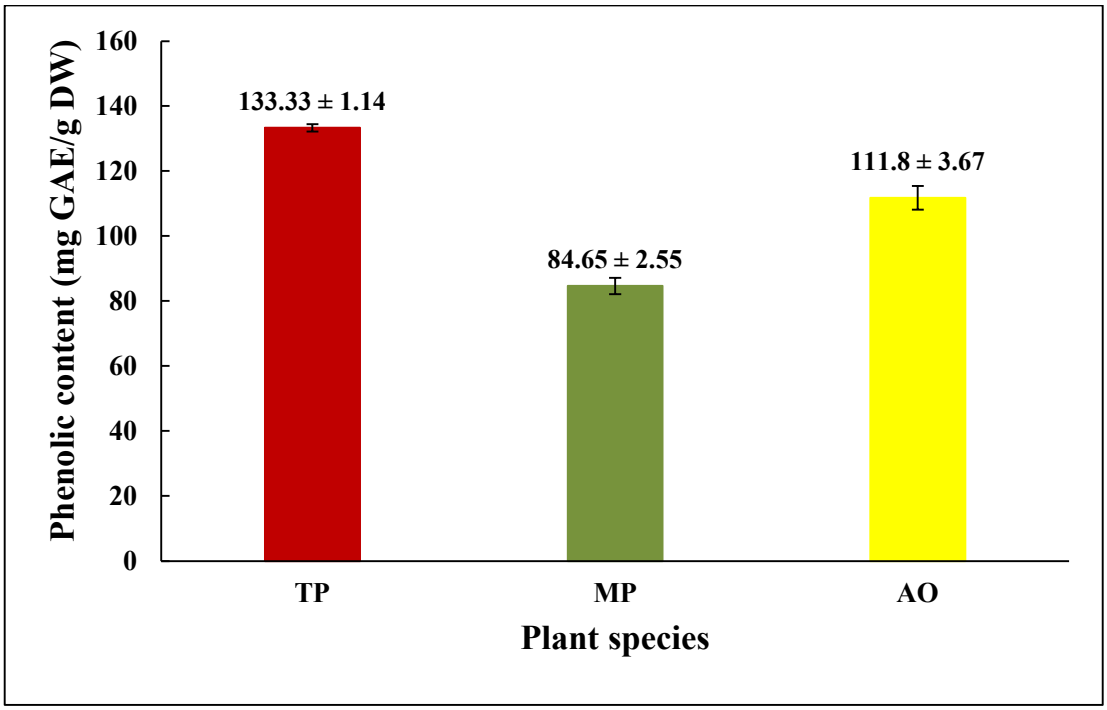



**Figure 6: Quantitative estimation of total phenolic content of selected dye-yielding plants. TP – *Terminalia paniculata*; MP – *Mallotus philippensis*; AO – *Albizia odoratissima***

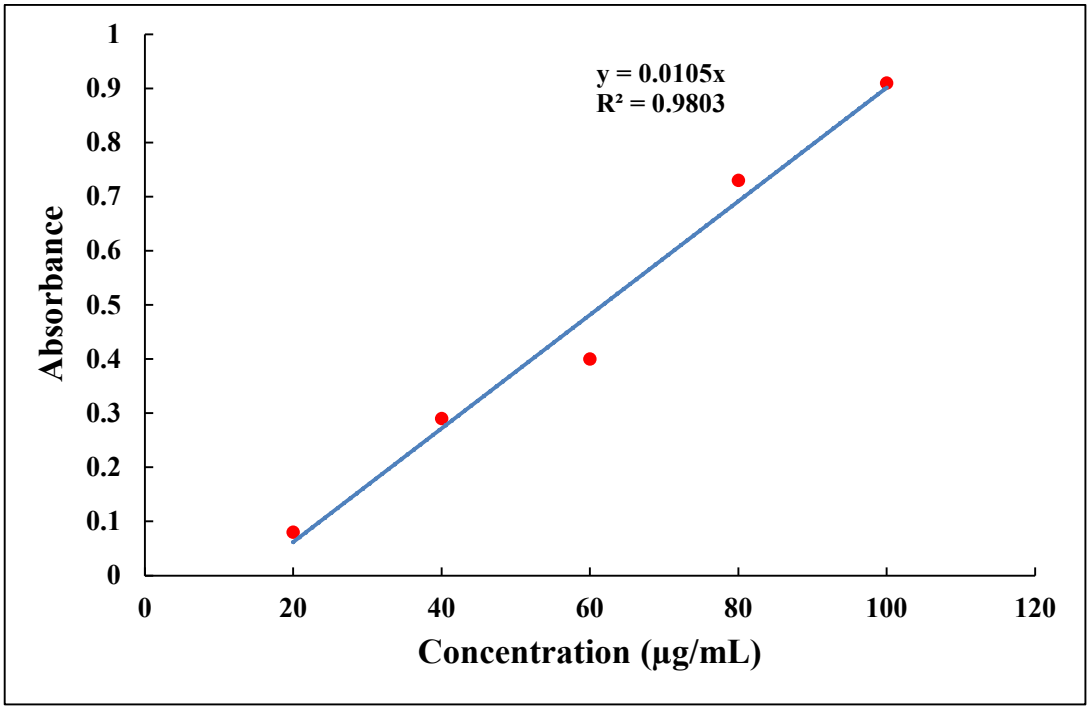



**Figure 7: Standard calibration curve of quercetin for the determination of total flavonoid content**

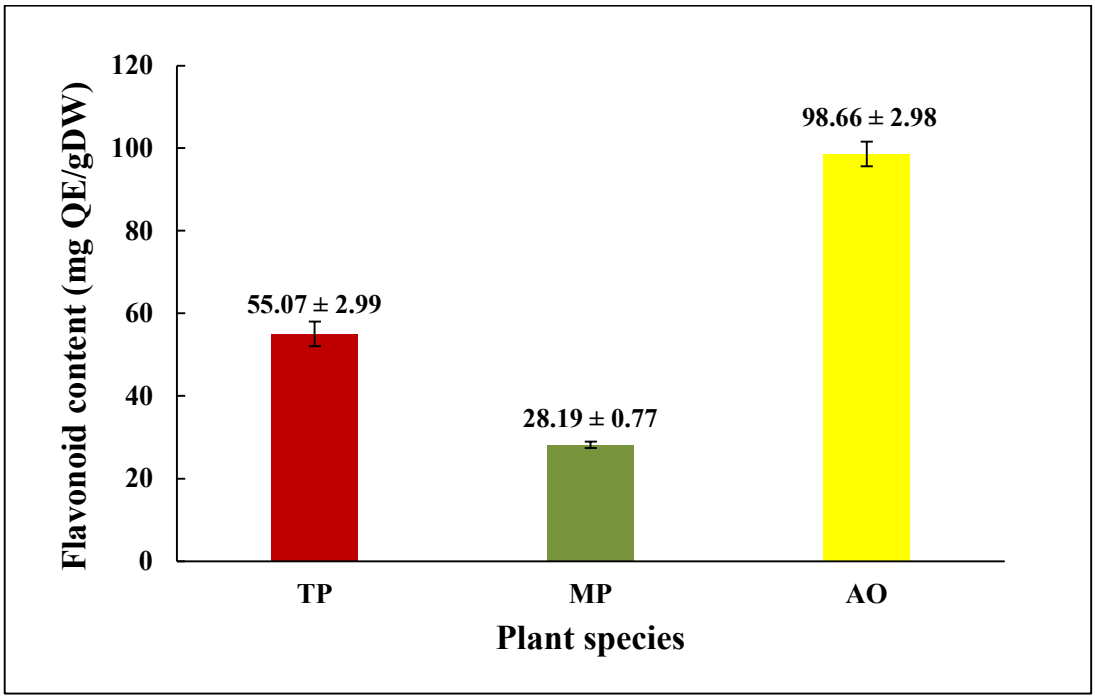



**Figure 8: Quantitative estimation of total flavonoid content of selected dye-yielding plants. TP – *Terminalia paniculata*; MP – *Mallotus philippensis*; AO – *Albizia odoratissima***

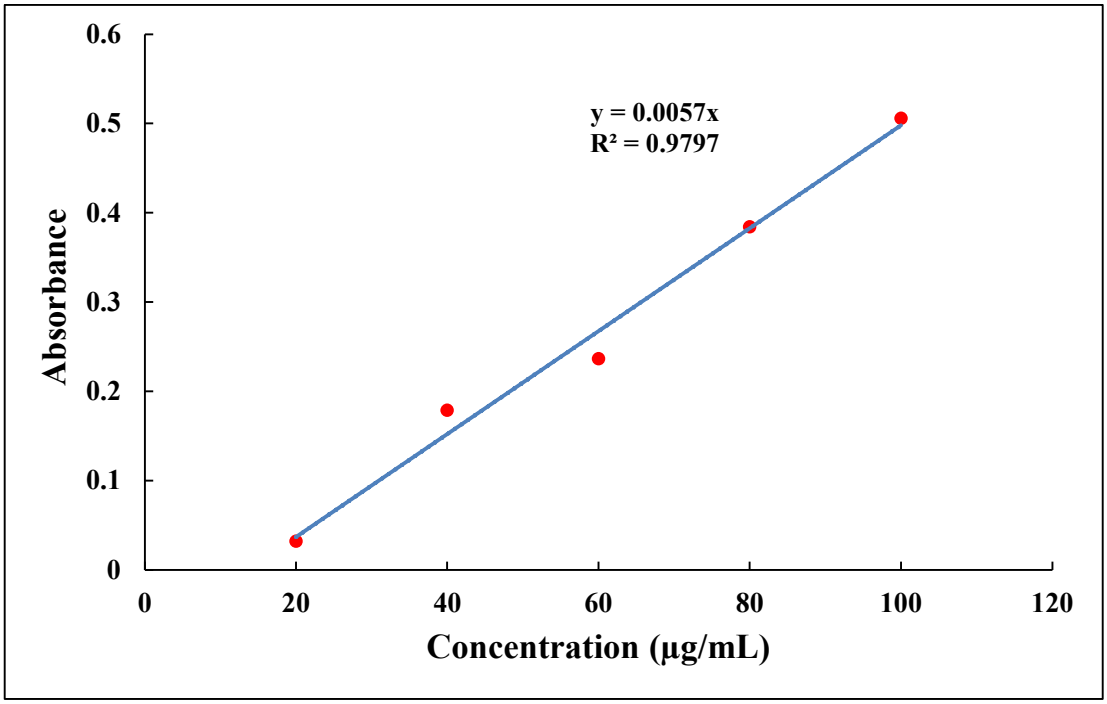



**Figure 9: Standard calibration curve of caffeine for the determination of total alkaloid content**

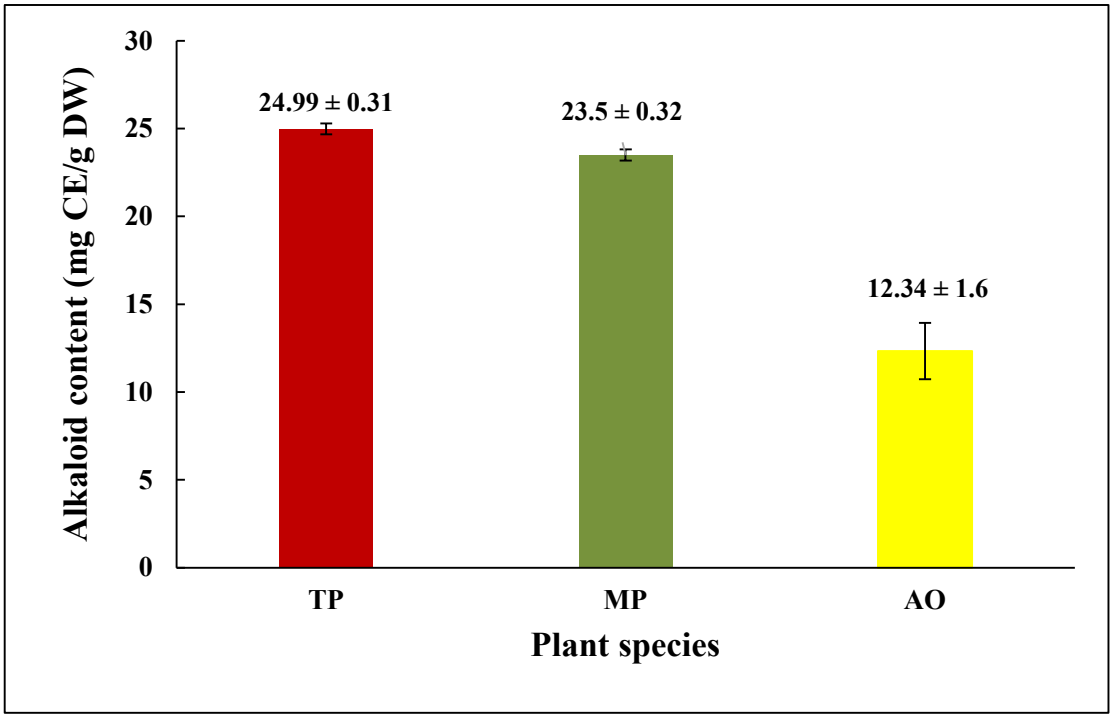



**Figure 10: Quantitative estimation of total alkaloid content of selected dye-yielding plants. TP – *Terminalia paniculata*; MP – *Mallotus philippensis*; AO – *Albizia odoratissima***

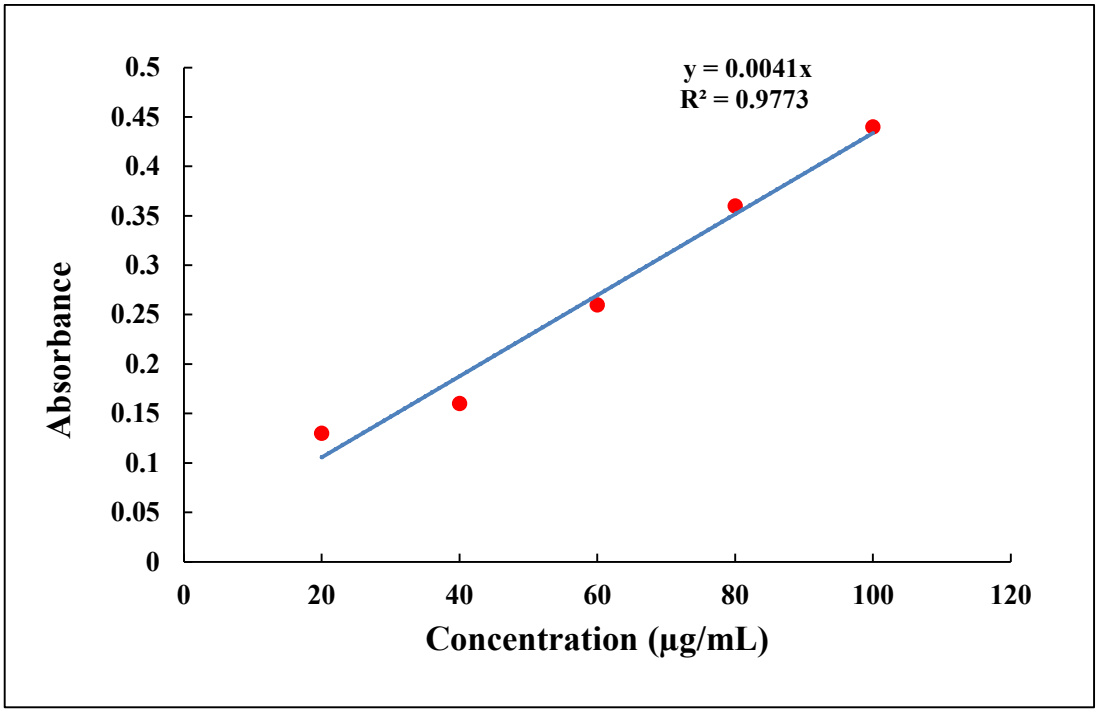



**Figure 11: Standard calibration curve of linalool for the determination of total terpenoid content**

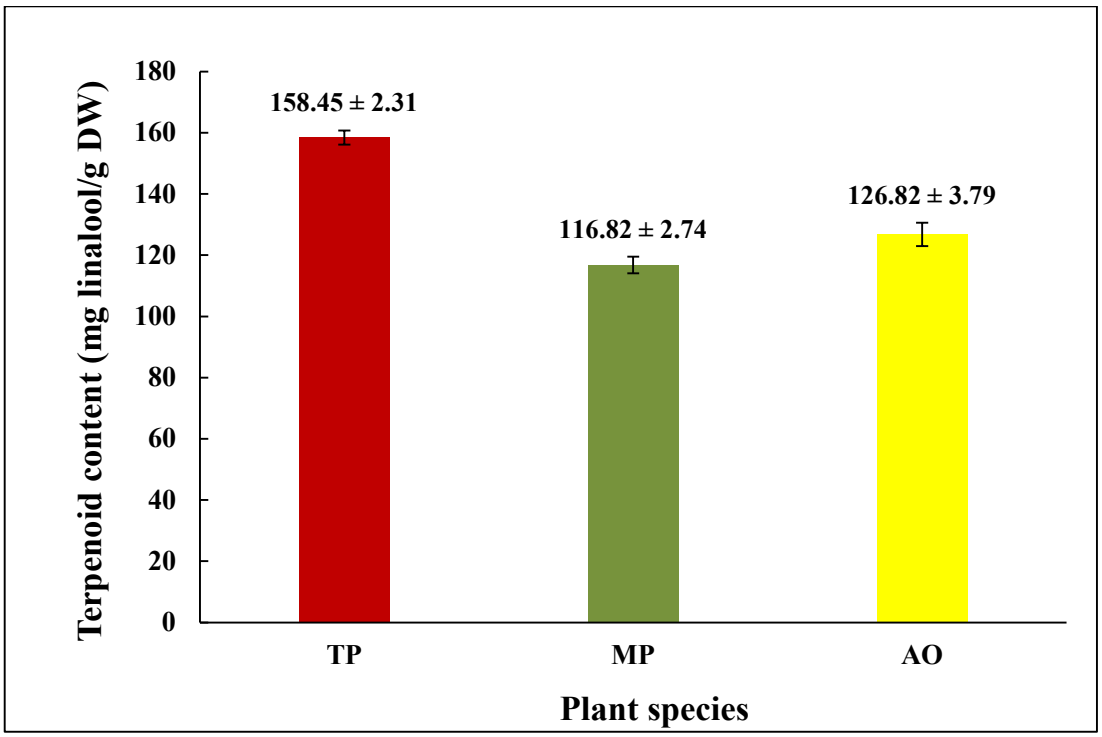



**Figure 12: Quantitative estimation of total terpenoid content of selected dye-yielding plants: TP – *Terminalia paniculata*; MP – *Mallotus philippensis*; AO – *Albizia odoratissima***

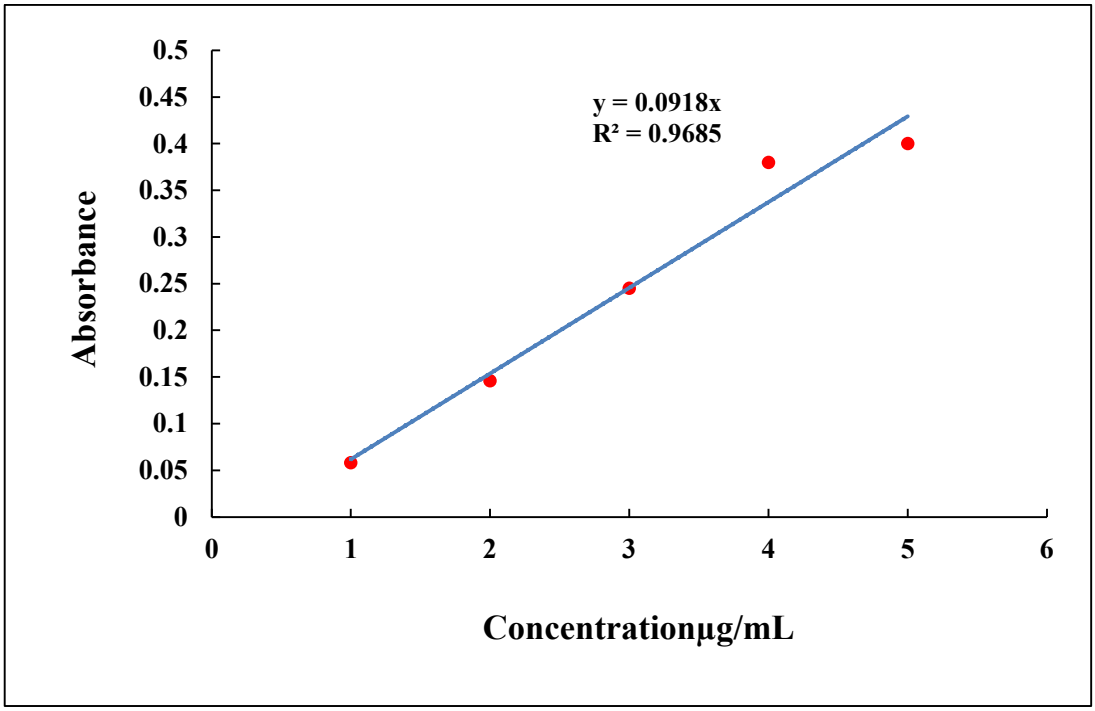



**Figure 13: Standard calibration curve of tannic acid for the determination of total tannin content**

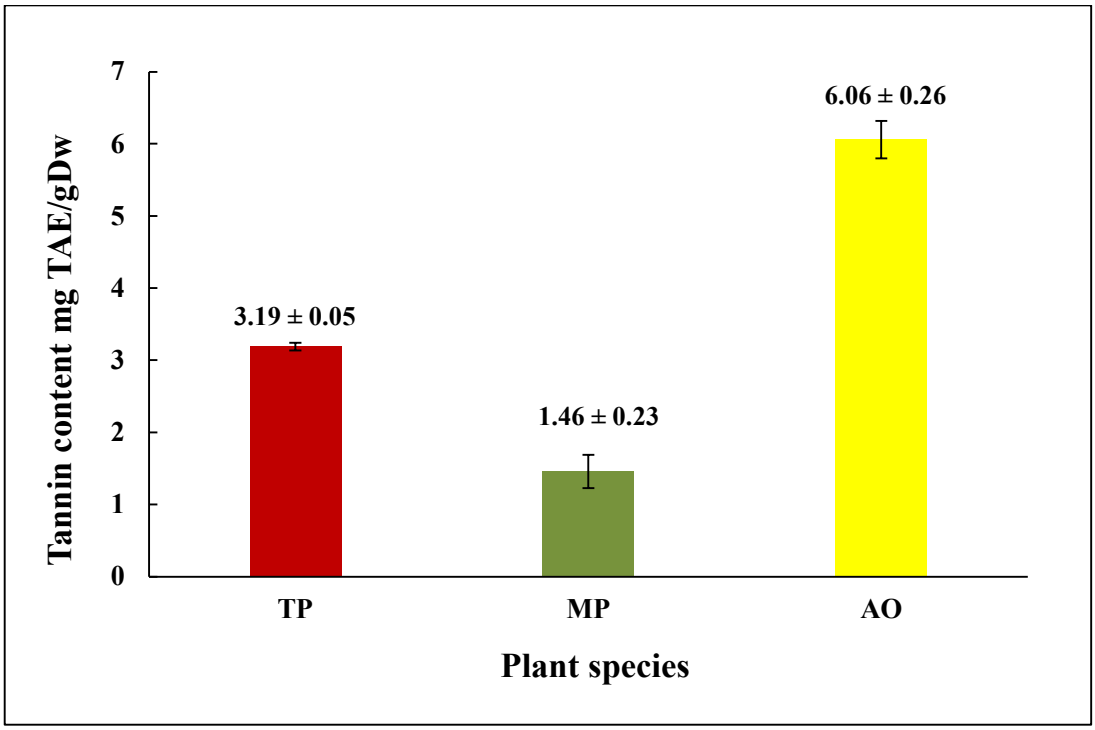



**Figure 14: Quantitative estimation of total tannin content of selected dye-yielding plants. TP - *Terminalia paniculata*; MP - *Mallotus philippensis*; AO - *Albizia odoratissima***

**c) Gas chromatography-Mass Spectrometry (GC-MS) analysis**

The presence of volatile components in a plant extract could be revealed by GC-MS analysis. The phytocompounds were categorised by their retention time and the amount was depicted as the area percentage. By GC-MS analysis, about 17 volatile phytoconstituents were detected in the methanolic extracts of selected dye-yielding plant parts of *T. paniculata*, *M. philippensis*, and *A. odoratissima*. Alkaloids, terpenoids, fatty acids, flavonoids, lipids, etc. are the major class of compounds observed. The compounds identified through the GC-MS analysis were enlisted in **Table 6** and gas chromatograms are given as **Figures 15, 16,** and **17**. The compounds were obtained within the range of 9.895 to 28.761 retention time. The mass spectrum of each compound is shown in **Figure 18 (i-vi)**. From these results, palmitic acid and methyl stearate was found in all three extracts with different peak area percentage. In *T. paniculata* and *M. philippensis*, palmitic acid was found to be the compound with the highest content with 62.73% and 13.7% of peak area respectively. Hordenine (84.19%) was found to be the most prominent one in *A. odoratissima*.

In the case of methyl stearate, the highest content was observed in *T. paniculata* (22.46%) followed by *M. philippensis* (4.69%) and *A. odoratissima* (3.84%). In *T. paniculata*, tetracosamethyl-cyclododecasiloxane was the compound with the least content of 5.87%. Similarly, in *M. philippensis* it was 2-(7 Heptadecynyloxy) tetrahydro-2H-pyran (0.81%) and in *A. odoratissima*, methyl stearate (3.84%) was recorded as the component with least quantity. In *T. paniculata*, only 4 phytoconstituents could be detected. The first and last eluted compounds were palmitic acid (RT = 15.983) and methyl stearate (RT = 19.459) respectively. Whereas in *A. odoratissima*, only 3 compounds were resolved. They were eluted at a range of retention time between 9.895 to 19.459. Apart from these, the highest number of phytoconstituents was resolved in *M. philippensis* and it was about 14 diverse constituents. Hence the results revealed that the volatile

compounds detected through GC-MS analysis is very less in all the three different methanolic extracts.

**Table 6: Compounds detected from the GC-MS analysis of selected dye-yielding plants (TP, MP and AO)**

| Sl. No. | RT | Compound | Class | Peak area (%) | | |
|---|---|---|---|---|---|---|
| | | | | TP | MP | AO |
| 1 | 9.895 | Hordenine | Alkaloid | - | - | **84.19** |
| 2 | 10.276 | α-Selinene | Sesquiterpene | - | 3.42 | - |
| 3 | 11.689 | Neointermedeol | Terpenoid | - | 1.67 | - |
| 4 | 12.489 | 1-Heptadec-1-ynyl-cyclopentanol | Cyclopentanol | - | 1.97 | - |
| 5 | 12.814 | Methyl tetradecanoate | Fatty acid | - | 1.34 | - |
| 6 | 13.095 | 2-(7 Heptadecynyloxy) tetrahydro-2H-pyran | Oxane | - | 0.81 | - |
| 7 | 13.877 | (Z)-7-Hexadecenal | Fatty aldehydes | - | 1.06 | - |
| 8 | 15.983 | **Palmitic acid** | Fatty acid | **62.73** | **13.70** | **11.96** |
| 9 | 17.008 | Tetracosamethyl-cyclododecasiloxane | Organoheterosilane | 5.87 | - | - |
| 10 | 18.921 | 9,12-Octadecadienoic acid, methyl ester | Linoleic acid | 8.92 | - | - |
| 11 | 19.459 | **Methyl stearate** | Fatty acid | **22.46** | **4.69** | **3.84** |
| 12 | 19.584 | Oleic acid | Fatty acid | - | 3.25 | - |
| 13 | 19.990 | Stearic acid | Fatty acid | - | 2.30 | - |
| 14 | 25.504 | Cabreuvin | Flavonoid | - | 1.42 | - |
| 15 | 25.672 | Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester | Fatty acid ester | - | 4.92 | - |
| 16 | 28.679 | 2"-Galloylhyperin | Carboxylic acid | - | 1.90 | - |
| 17 | 28.761 | Octadecanoic acid, 2,3-dihydroxypropyl ester | Lipid | - | 3.66 | - |

**(RT:** retention time**; TP:** *T. paniculata***, MP:** *M. philippensis***, AO:** *A. odoratissima***)**

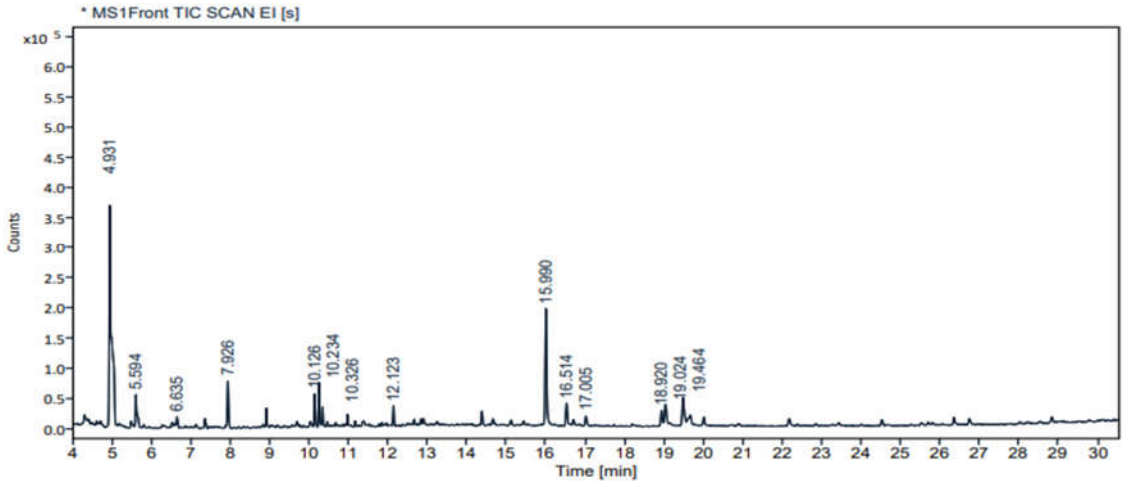



**Figure 15: Gas chromatogram of methanolic fruit extract of *T. paniculata***

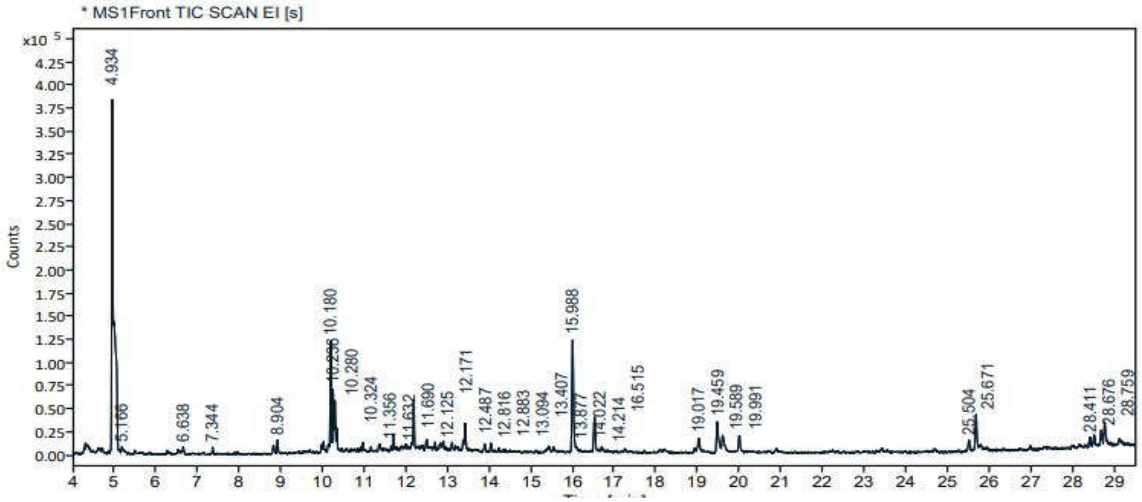



**Figure 16: Gas chromatogram of methanolic fruit extract of *M. philippensis***

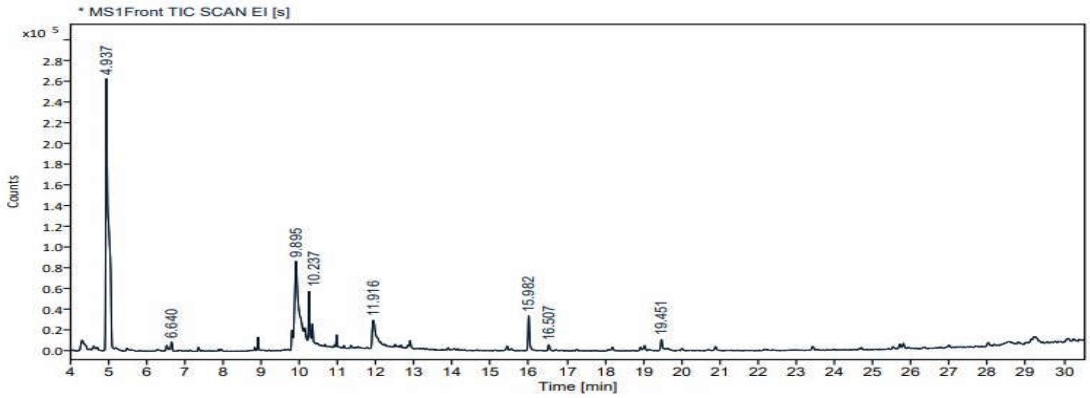



**Figure 17: Gas chromatogram of methanolic bark extract of *A. odoratissima***

**d) High Resolution Liquid Chromatography-Mass Spectrometry (HR LC-MS) analysis**

The methanolic extract of the selected dye-yielding plant parts was subjected to HR LC-MS analysis to identify the non-volatile compounds present in it. During this analysis, about 54 potential phytocompounds were obtained from three different extracts at different retention times that ranged from 0.953 to 20.848 (**Table 7**). The high-resolution liquid chromatogram of both positive and negative ionization modes of each extract is shown in **Figures 19 (a, b), 20 (a, b),** and **21 (a, b)**. The mass spectra of each compound were shown in **Figure 22 (i-xiv).** Table 7 clearly depicted the presence of biopeptides in each extract. Pro-Leu, gallic acid, pantothenic acid, Ile-Arg-Ala, and ellagic acid were common in both *T. paniculata* and *M. philippensis*. The highest number of compounds are found in *M. philippensis* followed by *T. paniculata* and *A. odoratissima*. Hence the results revealed that the number of non-volatile compounds was found to be high in methanolic fruit extracts when compared to the bark extract. The edible compound perseitol was found only in *T. paniculata*. Phenols, flavonoids, terpenoids, etc. are the other major classes of compounds obtained through the HR LC-MS analysis.

**Table 7: Phytochemical constituents detected in the selected dye-yielding plants identified through HR LC-MS**

| Sl. No. | RT | Compounds | Molecular Formula | Molecular Mass | Class | $[M+H]^+$ (m/z) | TP | MP | AO |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.953 | Ser Thr Gln | $C_{12}H_{22}N_4O_7$ | 334.1495 | Biopeptide | 357.1391 | - | + | - |
| 2 | 1.101 | Pro Leu | $C_{11}H_{20}N_2O_3$ | 228.1451 | Biopeptide | 229.1524 | + | + | - |
| 3 | 1.117 | Lys Thr His | $C_{16}H_{28}N_6O_5$ | 384.2128 | Biopeptide | 367.2114 | - | - | + |
| 4 | 1.364 | Cys Pro | $C_8H_{14}N_2O_3S$ | 218.0745 | Biopeptide | 201.0712 | + | - | - |
| 5 | 1.409 | Perseitol | $C_7H_{16}O_7$ | 212.0872 | Monosaccharide | 217.0659 | + | - | - |
| 6 | 1.43 | Gallic acid | $C_7H_6O_5$ | 170.0217 | Phenol | 169.0145 | + | + | - |
| 7 | 1.475 | Apionic acid | $C_5H_{10}O_6$ | 166.0462 | Hydrocarbons | 171.0247 | - | + | - |
| 8 | 1.505 | 3,7-epoxycaryophyllan-6-one | $C_{15}H_{24}O_2$ | 236.1741 | Sesquiterpenoid | 241.1528 | - | + | - |
| 9 | 1.568 | Pantothenic Acid | $C_9H_{17}NO_5$ | 219.1083 | Vitamin | 242.0976 | + | + | - |
| 10 | 3.286 | Pro Gln Pro | $C_{15}H_{24}N_4O_5$ | 340.171 | Biopeptide | 345.1498 | - | + | - |
| 11 | 3.461 | Tyr Asn Thr | $C_{17}H_{24}N_4O_7$ | 396.1614 | Biopeptide | 419.1505 | - | + | - |
| 12 | 3.601 | Ser Gln Tyr | $C_{17}H_{24}N_4O_7$ | 396.1653 | Biopeptide | 395.1581 | - | + | - |
| 13 | 3.618 | Epigallocatechin | C15 H14 O7 | 306.0747 | Polyphenol | 305.0675 | - | - | + |
| 14 | 3.994 | Glu Tyr | $C_{14}H_{18}N_2O_6$ | 310.1169 | Biopeptide | 327.0741 | - | + | - |
| 15 | 4.159 | Quercitrin | $C_{21}H_{20}O_{11}$ | 448.1116 | Flavonoid | 465.07 | - | + | - |
| 16 | 4.232 | Asp Trp Asn | $C_{19}H_{23}N_5O_7$ | 433.1572 | Biopeptide | 438.1358 | - | - | + |
| 17 | 4.269 | Orotidine | $C_{10}H_{12}NO_8$ | 274.0577 | Carbohydrate | 291.0164 | - | + | - |
| 18 | 4.328 | Gln Val Val | $C_{15}H_{28}N_4O_5$ | 344.2043 | Biopeptide | 345.2115 | - | + | - |
| 19 | 4.393 | Trp Cys Trp | $C_{25}H_{27}N_5O_4S$ | 493.1778 | Biopeptide | 498.1564 | - | - | + |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 20 | 4.71 | Catechin | $C_{15}H_{14}O_6$ | 290.0793 | Phenol | 325.0488 | - | - | + |
| 21 | 5.039 | Apiin | $C_{26}H_{28}O_{14}$ | 564.1476 | Flavonoid | 565.1538 | - | + | - |
| 22 | 5.299 | Asp His Phe | $C_{19}H_{23}N_5O_6$ | 417.1625 | Biopeptide | 422.1411 | - | - | + |
| 23 | 5.58 | Ellagic acid | $C_{14}H_6O_8$ | 302.0086 | Polyphenol | 301.0013 | + | + | - |
| 24 | 5.586 | Cosmosin | $C_{21}H_{20}O_{10}$ | 432.1036 | Flavonoid | 433.1109 | - | + | - |
| 25 | 5.697 | Hypericin | $C_{30}H_{16}O_8$ | 504.084 | Anthraquinone | 487.0808 | + | - | - |
| 26 | 5.969 | 7-Epiloganin tetraacetate | $C_{25}H_{34}O_{14}$ | 558.185 | Terpenoid | 593.1541 | + | - | - |
| 27 | 5.988 | Glutathione | $C_{10}H_{17}N_6S$ | 612.1653 | Tripeptide | 595.1614 | + | - | - |
| 28 | 5.998 | Phloridzin | $C_{21}H_{24}O_{10}$ | 436.1332 | Polyphenol | 437.1403 | - | + | - |
| 29 | 6.043 | Ginkgolide C | $C_{20}H_{24}O_{11}$ | 440.1328 | Terpene | 421.1152 | + | - | - |
| 30 | 6.398 | Glu Met Tyr | $C_{19}H_{27}N_3O_7S$ | 441.1506 | Biopeptide | 446.1278 | - | + | - |
| 31 | 6.96 | Picrotin | $C_{15}H_{18}O_7$ | 310.1046 | Terpene | 315.0832 | - | - | + |
| 32 | 7.444 | His Lys Thr | $C_{16}H_{28}N_6O_5$ | 384.2118 | Biopeptide | 367.2077 | - | + | - |
| 33 | 7.514 | Asn Tyr Tyr | $C_{22}H_{26}N_4O_7$ | 458.1851 | Biopeptide | 493.1542 | - | + | - |
| 34 | 7.943 | Lys Asp His | $C_{16}H_{26}N_6O_6$ | 398.1919 | Biopeptide | 403.1707 | - | + | - |
| 35 | 8.044 | Dihydromyricetin | $C_{15}H_{12}O_8$ | 320.0537 | Flavonoid | 301.0359 | + | - | - |
| 36 | 8.662 | Dihydrotricetin | $C_{15}H_{12}O_7$ | 304.0596 | Flavanone | 285.0418 | + | - | - |
| 37 | 8.691 | Asn Arg | $C_{10}H_{20}N_6O_4$ | 288.1562 | Biopeptide | 311.1453 | - | + | - |
| 38 | 9.028 | Swietenine | $C_{32}H_{40}O_9$ | 568.2694 | Triterpene | 573.2484 | + | - | - |
| 39 | 9.995 | Tyr Glu Tyr | $C_{23}H_{27}N_3O_8$ | 473.1798 | Biopeptide | 490.1385 | - | + | - |
| 40 | 10.565 | Prephenic acid | $C_{10}H_{10}O_6$ | 226.0488 | Amino acid | 225.0416 | - | + | - |
| 41 | 12.182 | Ile Arg Ala | $C_{15}H_{30}N_6O_4$ | 358.2306 | Biopeptide | 359.2379 | + | + | - |
| 42 | 12.31 | Trp Tyr Tyr | $C_{29}H_{30}N_4O_6$ | 530.2069 | Biopeptide | 547.1652 | - | + | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 43 | 12.669 | Trp Asp Glu | $C_{20}H_{24}N_4O_8$ | 448.1503 | Biopeptide | 449.1572 | - | + | - |
| 44 | 12.703 | Trp Met Met | $C_{21}H_{30}N_4O_4S_2$ | 466.1627 | Biopeptide | 489.152 | - | + | - |
| 45 | 12.708 | Rhoifolin | $C_{27}H_{30}O_{14}$ | 578.1775 | Flavonoid | 579.1844 | - | + | - |
| 46 | 13.752 | Cys Trp Phe | $C_{23}H_{26}N_4O_4S$ | 454.1634 | Biopeptide | 477.1518 | - | + | - |
| 47 | 13.841 | Met Gln Tyr | $C_{19}H_{28}N_4O_6S$ | 440.1753 | Biopeptide | 475.1432 | - | + | - |
| 48 | 14.342 | Tyr Trp Glu | $C_{25}H_{28}N_4O_7$ | 496.2007 | Biopeptide | 531.1695 | - | + | - |
| 49 | 14.826 | Gibberellin A51-catabolite | $C_{19}H_{22}O_5$ | 330.1465 | Terpenoid | 335.1251 | - | + | - |
| 50 | 17.689 | Harpagoside | $C_{24}H_{30}O_{11}$ | 494.1803 | Glycosides | 475.1631 | - | + | - |
| 51 | 18.182 | Ginkgolide B | $C_{20}H_{24}O_{10}$ | 424.1388 | Diterpenoid | 405.1211 | - | + | - |
| 52 | 18.458 | Tyr Glu Trp | $C_{25}H_{28}N_4O_7$ | 496.1991 | Biopeptide | 531.1684 | - | + | - |
| 53 | 20.275 | Tyr Trp Tyr | $C_{29}H_{30}N_4O_6$ | 530.2106 | Biopeptide | 565.1754 | - | + | - |
| 54 | 20.848 | Tyr Trp Phe | $C_{29}H_{30}N_4O_5$ | 514.211 | Biopeptide | 549.1795 | - | + | - |

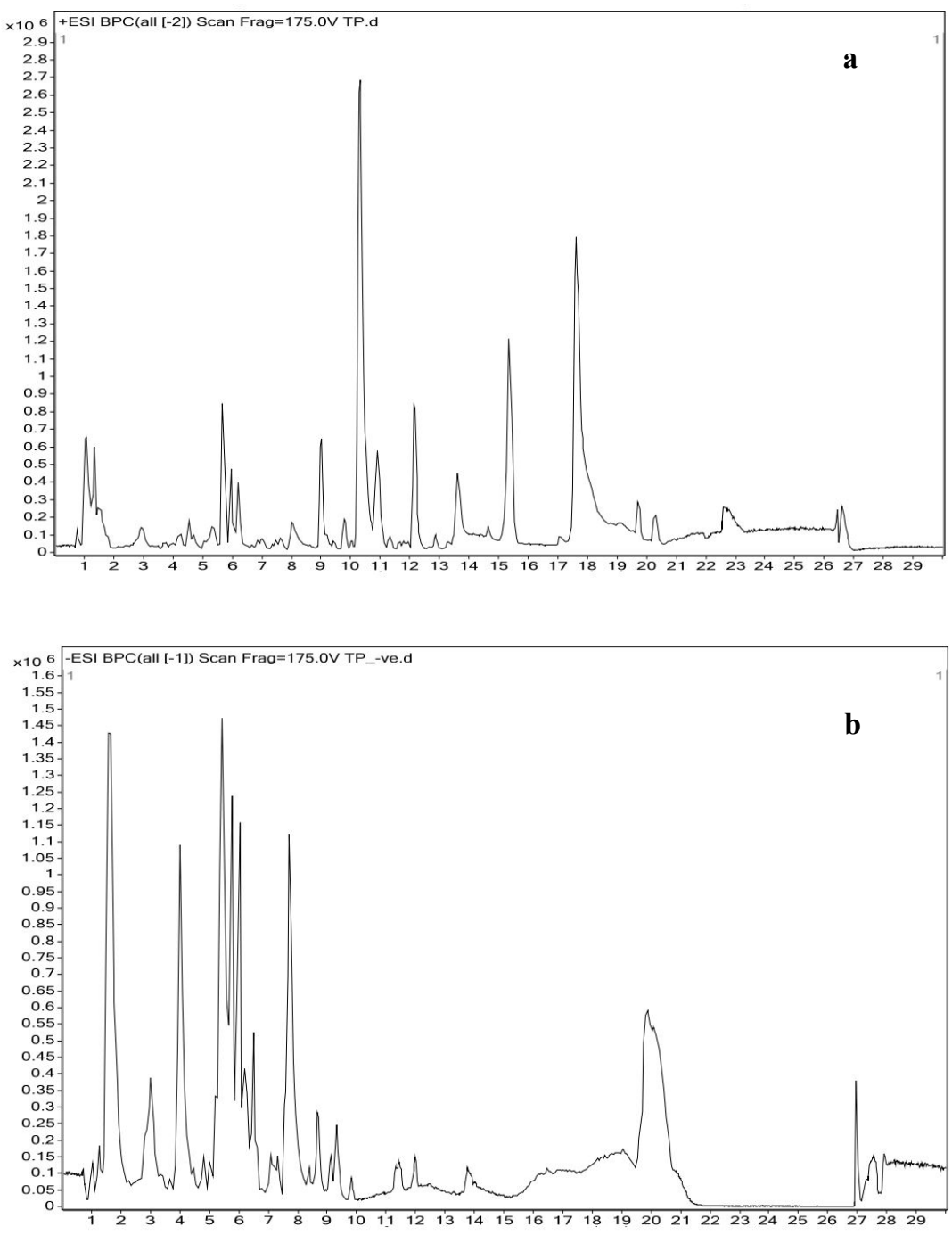



**Figure 19: Liquid chromatogram of methanolic fruit extract of *T. paniculata* - a** Positive compounds; **b** Negative compounds

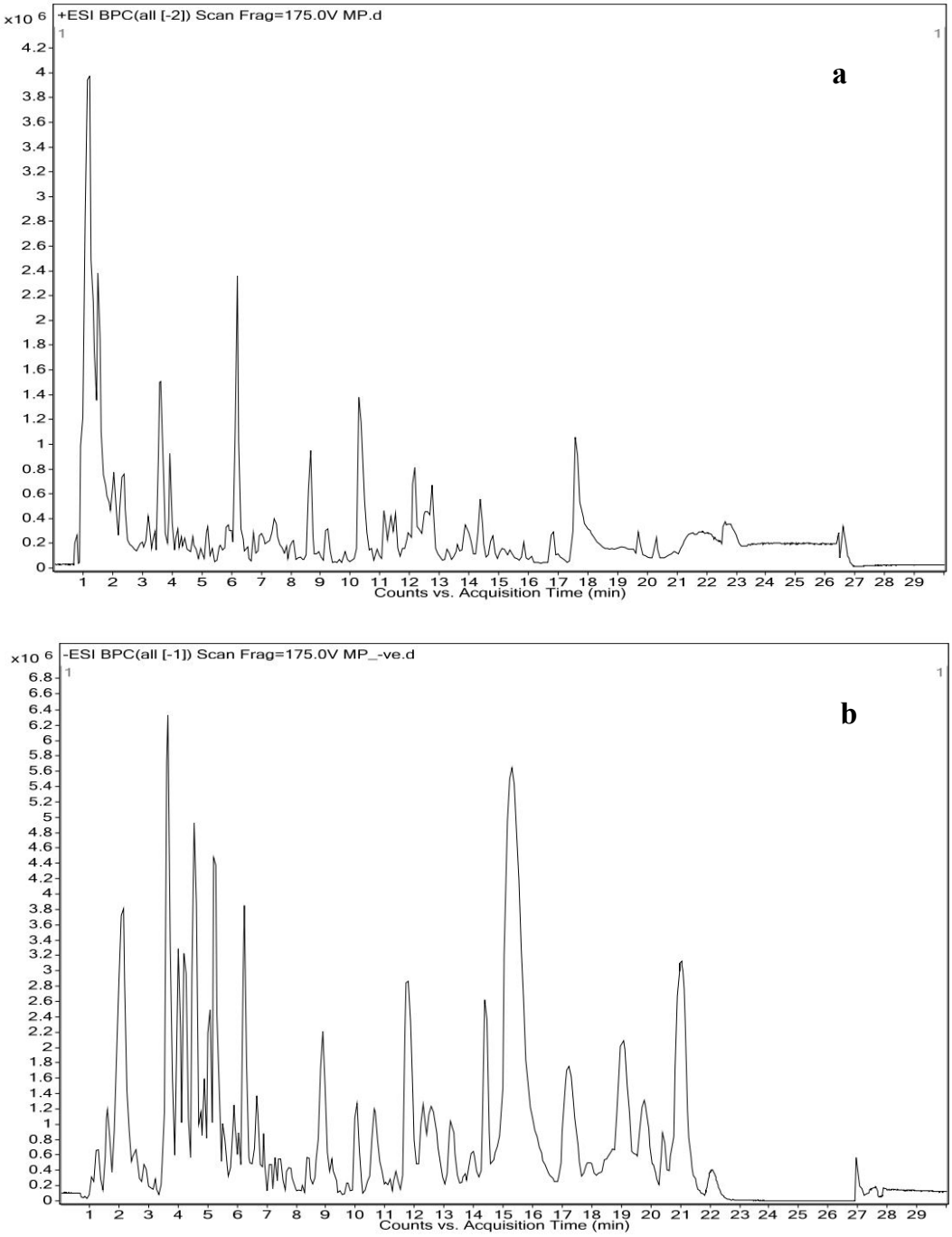



**Figure 20: Liquid chromatogram of methanolic fruit extract of *M. philippensis* - a** Positive compounds; **b** Negative compounds

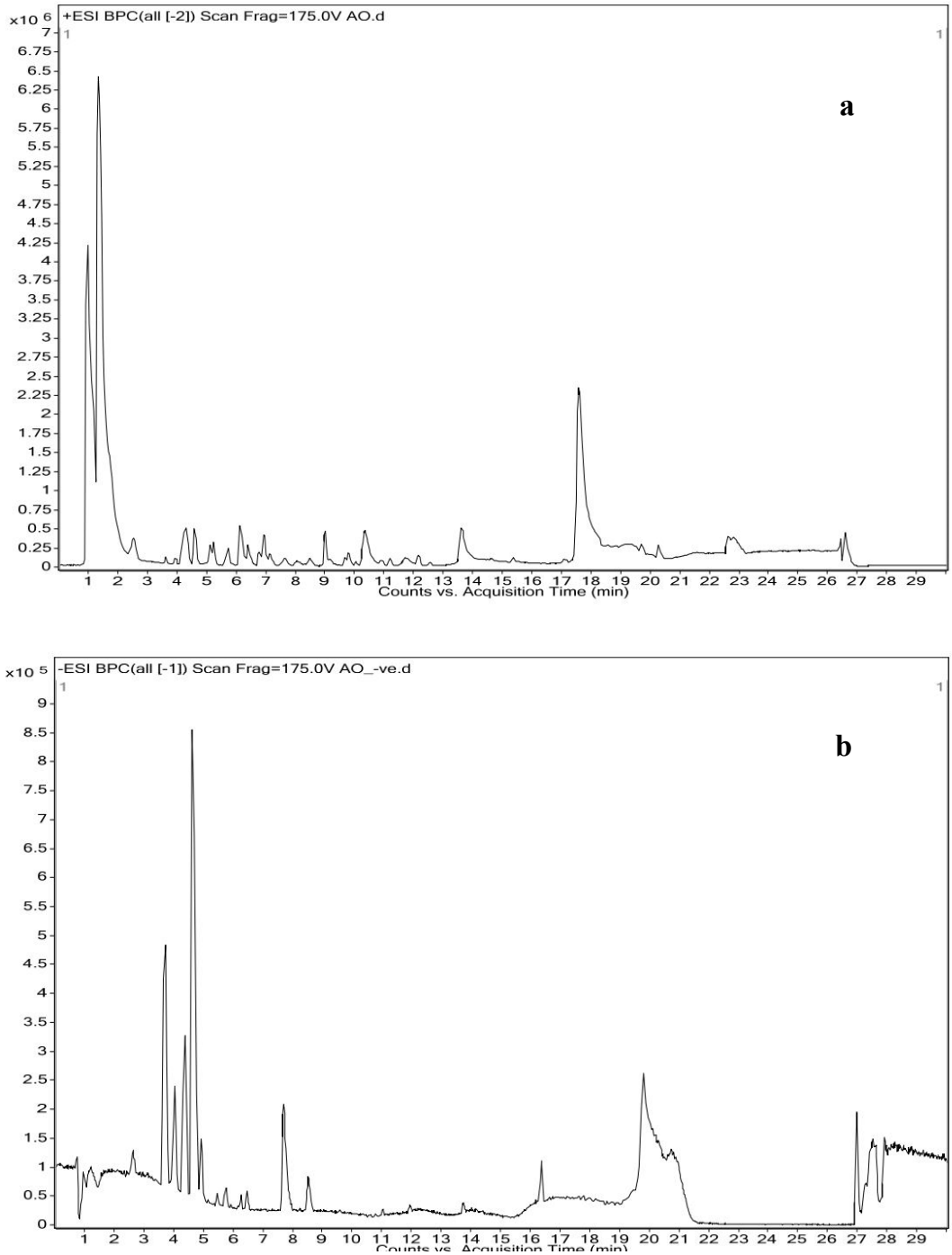



**Figure 21: Liquid chromatogram of methanolic bark extract of *A. odoratissima* – a** Positive compounds; **b** Negative compounds

# PART I I– BIOACTIVITY SCREENING

## A. Free Radical Scavenging Activity

Free radical scavenging activity of three selected dye-yielding plants was carried out by four different assays *viz*., DPPH radical scavenging assay, hydroxyl radical scavenging assay, nitric oxide radical scavenging assay, and superoxide free radical scavenging assay.

### i. DPPH free radical scavenging assay

In DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging assay, all three dye-yielding plants exhibit a dose-dependent antioxidant potential (**Figure 23**). The concentrations of the samples adopted for the study are 12.5, 25, 50, 100, and 200 µg/mL. Ascorbic acid was used as the standard for the assay. DPPH consists of a purple colour which turns to yellow by scavenging activity. Finally, the discoloration indicates the antioxidant capacity of the extract. The concentration at which 50 percent of scavenging activity has been achieved is called $IC_{50}$ (**Table 8**). The $IC_{50}$ value of the standard is 20.399 ± 1.07 µg/mL. Whereas methanolic extracts of *T. paniculata*, *M. philippensis* and *A. odoratissima* showed $IC_{50}$ values of 23.068 ± 1.58, 71.017 ± 1.31 and 121.88 ± 1.20µg/mL respectively. From these results, *T. paniculata* showed a significant antioxidant potential which is almost similar to the $IC_{50}$ value of standard and was found to be better than the other two extracts. The maximum inhibition percentage of *T. paniculata* was 75.93 ± 1.49% observed at 200 µg/mL concentration and it is 71.22 ± 0.61% and 65.2 ± 0.25% in *M. philippensis* and *A. odoratissima* respectively (**Figure 23**).

### ii. Hydroxyl free radical scavenging assay

In the hydroxyl free radical scavenging assay, the quenching ability of hydroxyl free radicals by the antioxidants present in the samples was tested. For this analysis 125, 250, 500, 1000, 2000 µg/mL concentrations of the standard (gallic acid), as well as plant extracts, were selected. Each extract showed a characteristic scavenging capacity with a possible $IC_{50}$ value (**Table**

**8**). The standard (gallic acid) showed 199.23 ± 1.61µg/mL as $IC_{50}$. The maximum percentage of inhibition of *A. odoratissima* (64.52 ± 0.28%) and *T. paniculata* (63.57 ± 0.92%) was almost equal at the highest concentration (2000 µg/mL). *M. philippensis* extract showed maximum hydroxyl scavenging activity at a high concentration (2000µg/mL) and was found to be 53.57 ± 0.92% (**Figure 24**).

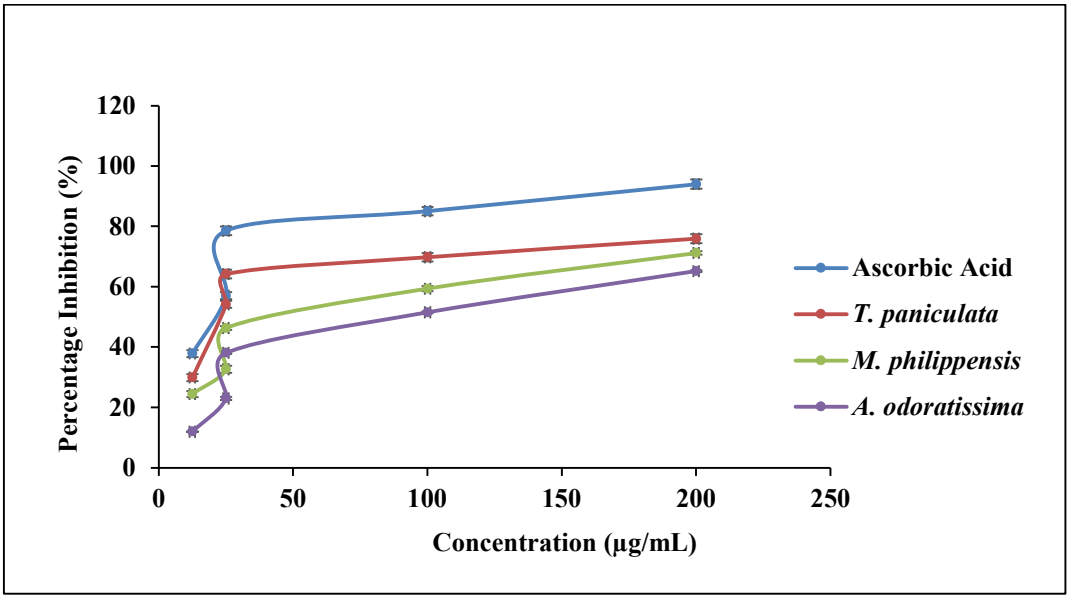



**Figure 23: *In vitro* DPPH radical scavenging activity of the standard ascorbic acid and selected dye-yielding plants**

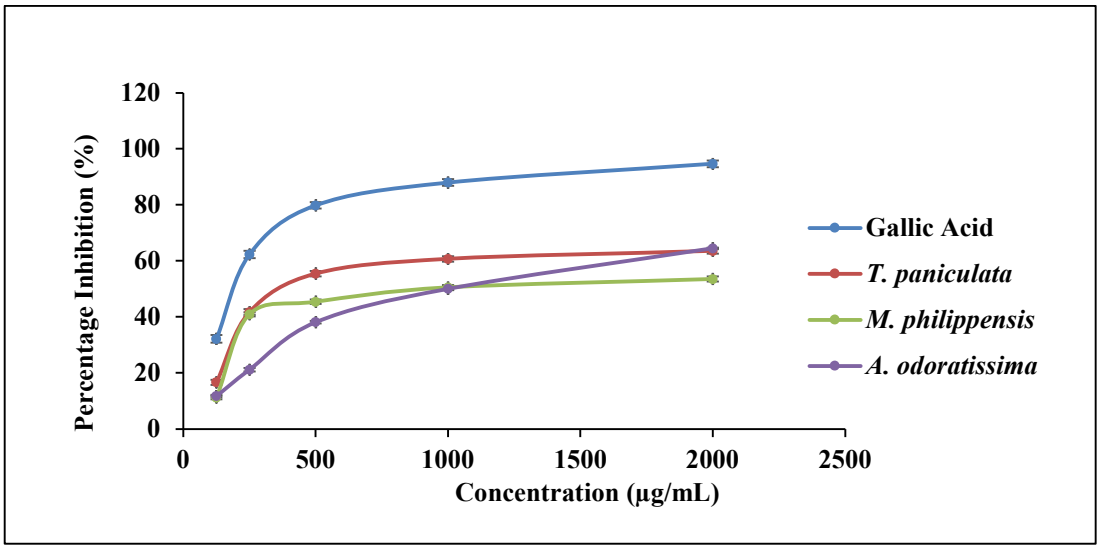



**Figure 24: *In vitro* hydroxyl radical scavenging activity of the standard gallic acid and selected dye-yielding plants**

**iii. Nitric oxide free radical scavenging assay**

For the nitric oxide free radical scavenging assay, the selected concentrations were 125, 250, 500, 1000, 2000 µg/mL. For this assay, gallic acid was used as the standard with 286.64 ± 1.27 µg/mL as the $IC_{50}$ value. The maximum inhibition percentage of each extract was detected at high concentration (2000µg/mL) (**Figure 25**). The inhibition of 75.77 ± 0.94 % was observed in *T. paniculata* at high concentration. Similarly, in *M. philippensis* it was 42.72 ± 0.91% and in *A. odoratissima* 63.2 ± 0.47% percentage of inhibition was obtained. Moreover, the maximum inhibition of gallic acid was 94.93 ± 1.19% at the high concentration (2000 µg/mL), which was much higher than that of the three plant extracts. The observed $IC_{50}$ values for *T. paniculata*, *M. philippensis,* and *A. odoratissima* were 1239.4 ± 1.40, 2281.4 ± 0.98, and 1305.79 ± 1.08 µg/mL respectively (**Table 8**). The values were found to be within the range of concentrations except for *M. philippensis*. The $IC_{50}$ value of the standard (gallic acid) (286.64 ± 1.27µg/mL) was too low when compared with that of the plant extracts. So, only a moderate effect of nitric oxide scavenging activity was observed.

**iv. Superoxide free radical scavenging assay**

In superoxide free radical scavenging activity, a dose-dependent antioxidant effect was observed (**Figure 26**). The selected concentrations for the assay were 125, 250, 500, 1000 and 2000 µg/mL. All three plant extracts including the standard (ascorbic acid) showed the maximum percentage of inhibition at high concentration (2000 µg/mL). *M. philippensis* showed the highest percentage of inhibition (83.8 ± 0.73%), which was almost near to that of the standard (94.63 ± 1.16%). Whereas in *T. paniculata* and *A. odoratissima*, the inhibition was found to be 77.63 ± 0.90% and 66.09 ± 0.47% respectively. The $IC_{50}$ value of standard was 384.205 ± 2.28 µg/mL. When compared to this value, a low $IC_{50}$ value was observed in *T. paniculata* i. e., 47.69 ± 0.52 µg/mL. Besides, the $IC_{50}$ values of all the extracts were found to be comparatively lesser than the standard (**Table 8**).

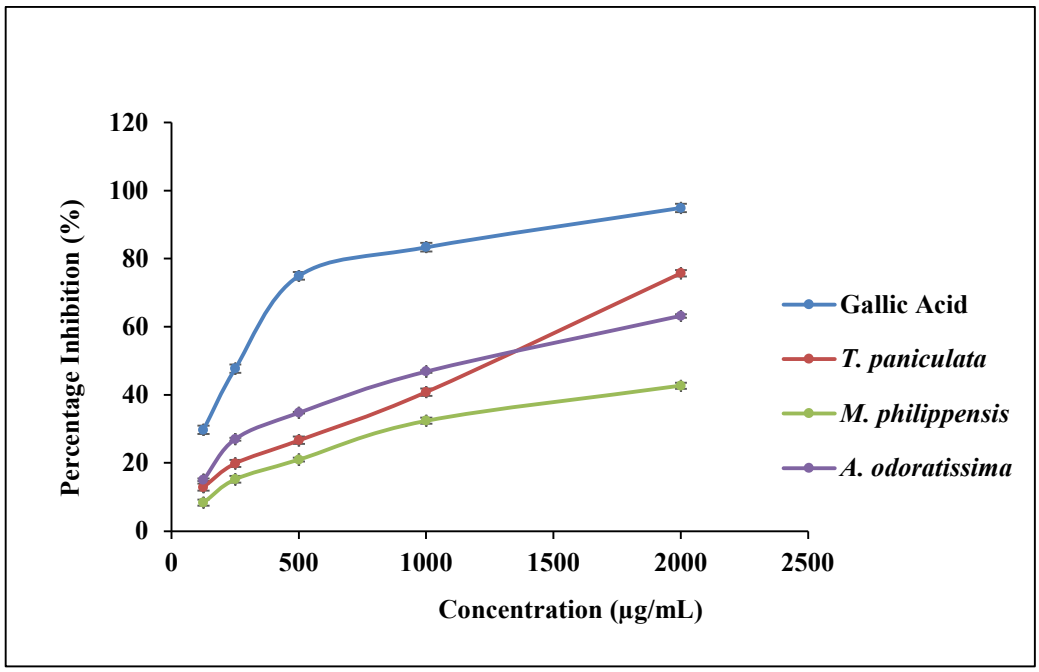



**Figure 25: *In vitro* nitric oxide free radical scavenging activity of the standard gallic acid and selected dye-yielding plants**

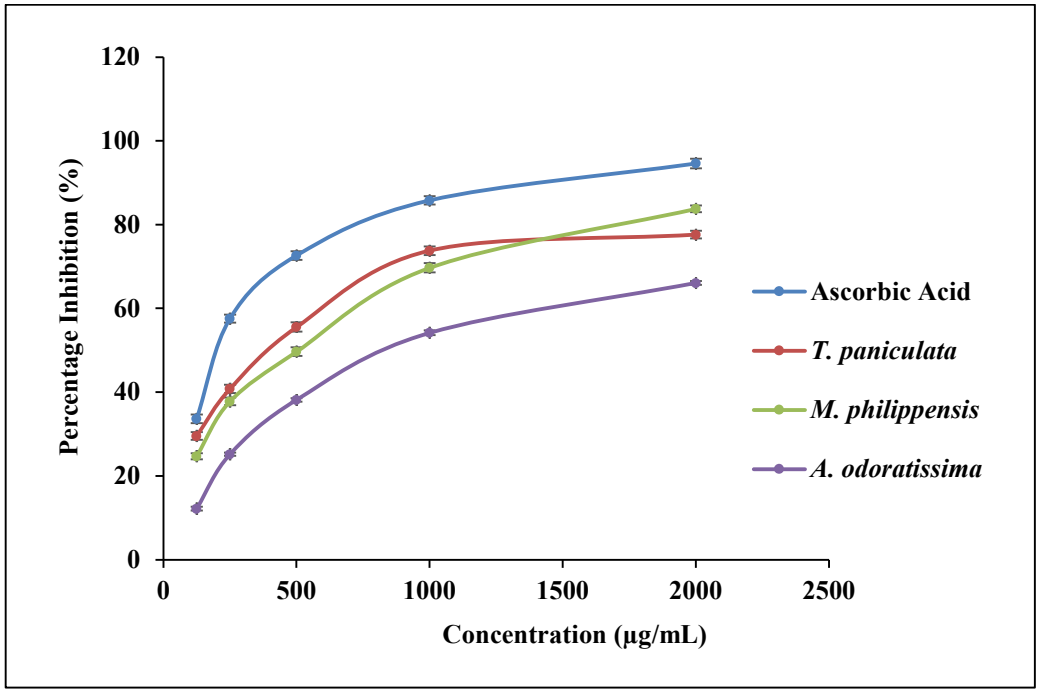



**Figure 26: *In vitro* superoxide free radical scavenging activity of the standard ascorbic acid and selected dye-yielding plants**

**Table 8: Inhibition concentration in antioxidant assays**

| Plant | $IC_{50}$ Value (µg/mL) | | | |
|---|---|---|---|---|
| | **DPPH radical scavenging assay** | **Hydroxyl radical scavenging assay** | **Nitric oxide radical scavenging assay** | **Superoxide radical scavenging assay** |
| **TP** | 23.068 ± 1.58 | 419.64 ± 1.12 | 1239.4 ± 1.40 | 47.69 ± 0.52 |
| **MP** | 71.017 ± 1.31 | 843.65 ± 0.86 | 2281.4 ± 0.98 | 675.62 ± 1.90 |
| **AO** | 121.88 ± 1.20 | 1265.03 ± 1.61 | 1305.79 ± 1.08 | 117.37 ± 1.88 |
| **Standard** | 20.399 ± 1.07 | 199.23 ± 1.61 | 286.64 ± 1.27 | 384.205 ± 2.28 |

(All values are expressed in mean ± SE)

Thus, results concluded that in DPPH, hydroxyl, and superoxide radical scavenging assay *T. paniculata* showed maximum inhibition than *M. philippensis* and *A. odoratissima*. In the case of the nitric oxide free radical scavenging assay, *T. paniculata* and *A. odoratissima* possessed almost equal effects for inhibiting free radicals. Hence, all three dye-yielding plants revealed a characteristic antioxidant potential from four different assays.

**B. Hepatotoxicity Screening**

Plant extract with excellent antioxidant activity was selected for the hepatotoxicity screening. In this study, the methanolic fruit extract of *T. paniculata* showed a promising antioxidant potential. Hence, further hepatic studies were carried out on the same. For an effective comparison, a synthetic colourant (Lemon Yellow – 19140) was also used as the corresponding dye. Cytotoxicity on HepG2 (Liver hepatic cells) was evaluated by the MTT method. Concentrations of both fruit extract and lemon yellow were selected as 6.25, 12.5, 25, 50, and 100 µg/mL. The mean values of the viability percentage were calculated from the absorbance values obtained.

Viability percentage of control cells for lemon yellow: 99.57%

Viability percentage of control cells for *T. paniculata*: 99.93%

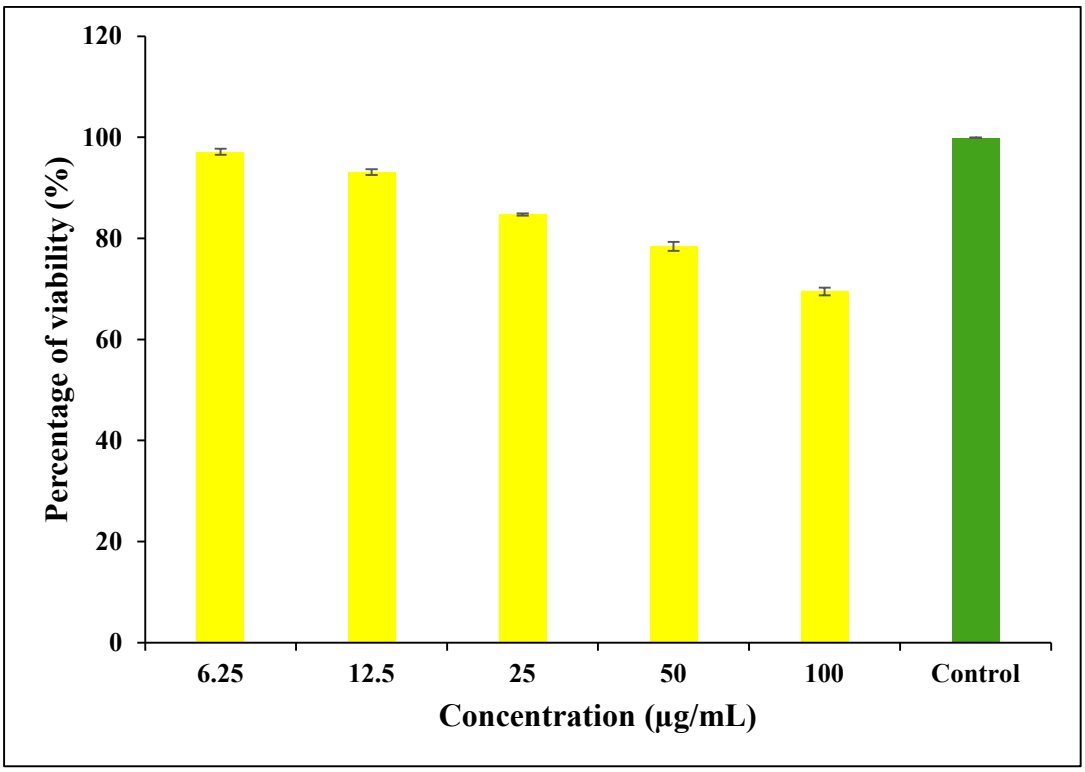



**Figure 27: Hepatotoxicity of lemon yellow on HepG2 liver cells**

The percentage of viable Hep G2 cells at the maximum concentration (100 µg/mL) was lower in lemon yellow (69.51 ± 0.78%) than *T. paniculata* (78.45 ± 0.75%) (**Figure 27 & 28**). The effect of lemon yellow and *T. paniculata* extract against HepG2 liver cells was shown in **Plate 2** and **Plate 3** respectively. The $LC_{50}$ of *T. paniculata* fruit extract is 228.688 µg/mL, whereas in lemon yellow it was 162.269 µg/mL. This means lemon yellow induces more toxicity to the growth of HepG2 cells than *T. paniculata*.

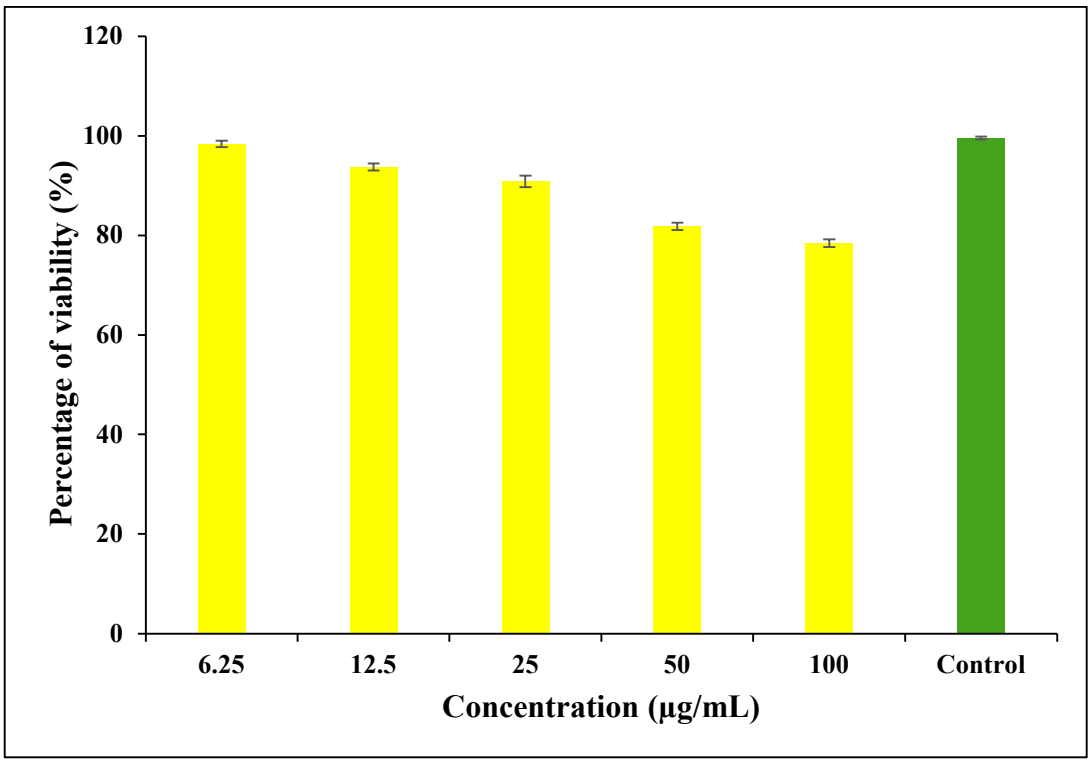



**Figure 28: Hepatotoxicity of *T. paniculata* fruit extract on HepG2 liver cells**

**C. Hepatoprotective Screening**

During the hepatotoxicity screening, methanolic fruit extract of *T. paniculata* revealed less toxicity to HepG2 liver cells. So, the protective ability of fruit extract was determined on human hepatocytes (HepG2 cells). Acetaminophen was used to induce toxicity to HepG2 cells and thereafter treated with methanolic fruit extract of *T. paniculata*. Nuclear disintegration and dead cells are the major aberrations observed in the HepG2 cells. In order to know the protective ability of *T. paniculata*, five different concentrations were used i.e., 6.25, 12.5, 25, 50, and 100 µg/mL.

Percentage viability of control cells: 99.31%

Percentage viability of acetaminophen-induced cells: 51.17%

The protective effect of *T. paniculata* fruit extract was observed through a microscope and images were depicted in **Plate 4**. The percentage viability of fruit extract ranged from 77.85 ± 2.59 to 85.56 ± 1.88% as the concentration was increased from 6.25 to 100 µg/mL (**Figure 29**). After inducing acetaminophen, the viability percentage of liver cells was found to be very less. However, the addition of *T. paniculata* fruit extract increased the viability percentage of HepG2 cells. Hence, these results clearly reveal that the fruit extract possessed a considerable protective effect against acetaminophen-induced toxicity on hepatocytes. So, the synthetic colourant lemon yellow can be replaced by *T. paniculata* fruit extract due to its promising protective ability on human hepatocytes.

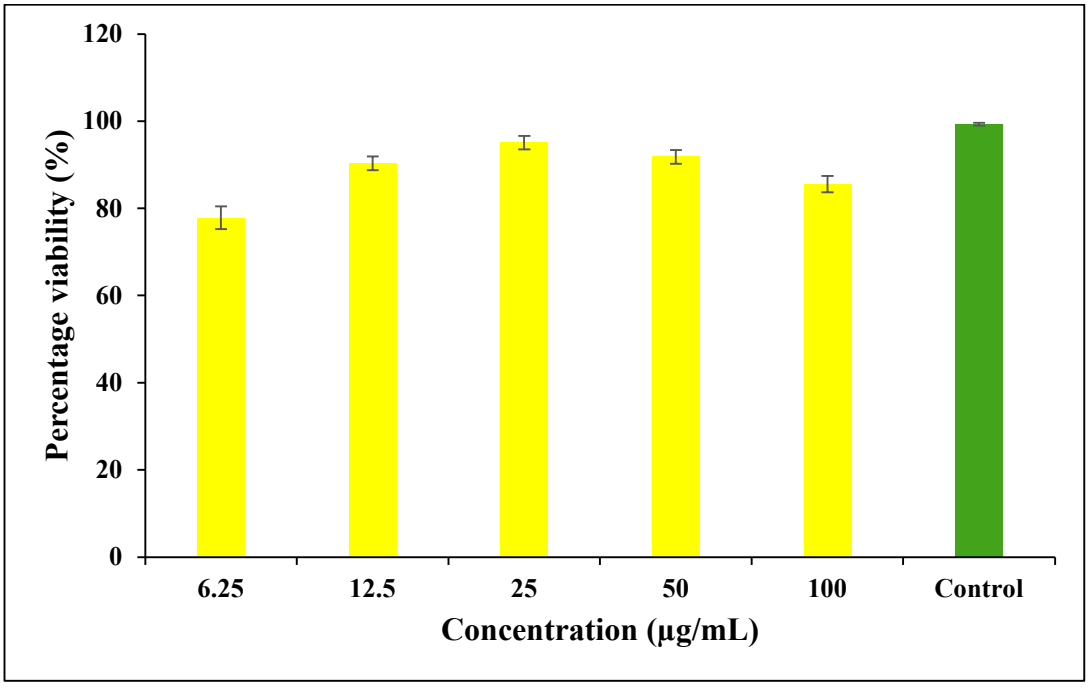



**Figure 29: Hepatoprotective ability of methanolic fruit extract of *T. paniculata* on human hepatocytes - HepG2 cells**

**D. Cytotoxicity Studies on *Allium cepa***

Methanolic extracts of the selected dye-yielding plant parts of *T. paniculata*, *M. philippensis* and *A. odoratissima* were subjected to cytotoxicity by *Allium cepa* assay. For this assay 25, 50, 75 and 100 µg/mL were the selected concentrations of each extract. A 24 h treatment was adopted to identify the cytotoxic effect of each methanolic extract. The normal mitotic stages of *Allium cepa* were shown in **Plate 5**. Mitotic index and abnormality percentage were noticed. The mitotic index was found to be inversely proportional to the selected concentration. When the concentration of the extract increases, the mitotic index decreases (**Figure 30a**). Whereas, the abnormality percentage was found to be increasing with an increase in the concentrations (**Figure 30b**). For negative control, the mitotic index was very high (80.79 ± 0.23%) whereas in positive control it was only 22.78 ± 0.29%. Among the extracts, the highest mitotic index was observed in *A. odoratissima* (75.66 ± 0.62%) at the lowest concentration (25 µg/mL) (**Figure 30a**). However, the lowest mitotic index observed at the highest concentration (100 µg/mL) was 45.43 ± 0.37% in *M. philippensis* (**Figure 30a**). The abnormality percentage was found to be very high in the positive control (77.76 ± 0.75%), whereas for the negative control it was only 9.04 ± 0.58%. In the case of methanolic extracts dose-dependent results were observed (**Figure 30b**). The highest abnormality percentage found in *A. odoratissima* was 64.24 ± 0.71% at the highest concentration (100 µg/mL). Furthermore, *M. philippensis* showed the lowest abnormality percentage (59.6 ± 0.23%) at maximum concentration when compared to other extracts. However, the range of mitotic indices was between 80.79 ± 0.23% to 22.78 ± 0.29%, whereas the abnormality percentage was found to be between 9.04 ± 0.58% to 77.76 ± 0.75%. All three extracts possessed both clastogenic and non-clastogenic aberrations. The major clastogenic aberrations observed include nuclear lesions, nuclear erosion, chromosome stickiness, chromosome bridges, chromosome fragmentation, pulverized chromosomes, coagulated chromosomes, chained chromosomes, etc. Additionally, non-clastogenic

abnormalities involve macro and micro cell formation, C-metaphase, stellate chromosomes, hypoploidy, hyperploidy, polyploidy, disturbed chromosomes, diagonal arrangement of chromosomes, stathmo-anaphase, chromosome vagrants, chromosome laggards, scattered chromosomes, pole to pole arrangement of chromosomes, cytostasis, tropokinesis, ball-shaped chromosomes, *etc*. (**Plates 6, 7, 8, 9, 10** and **11**). In all three extracts the number of aberrations was found to be high in both metaphase and anaphase.

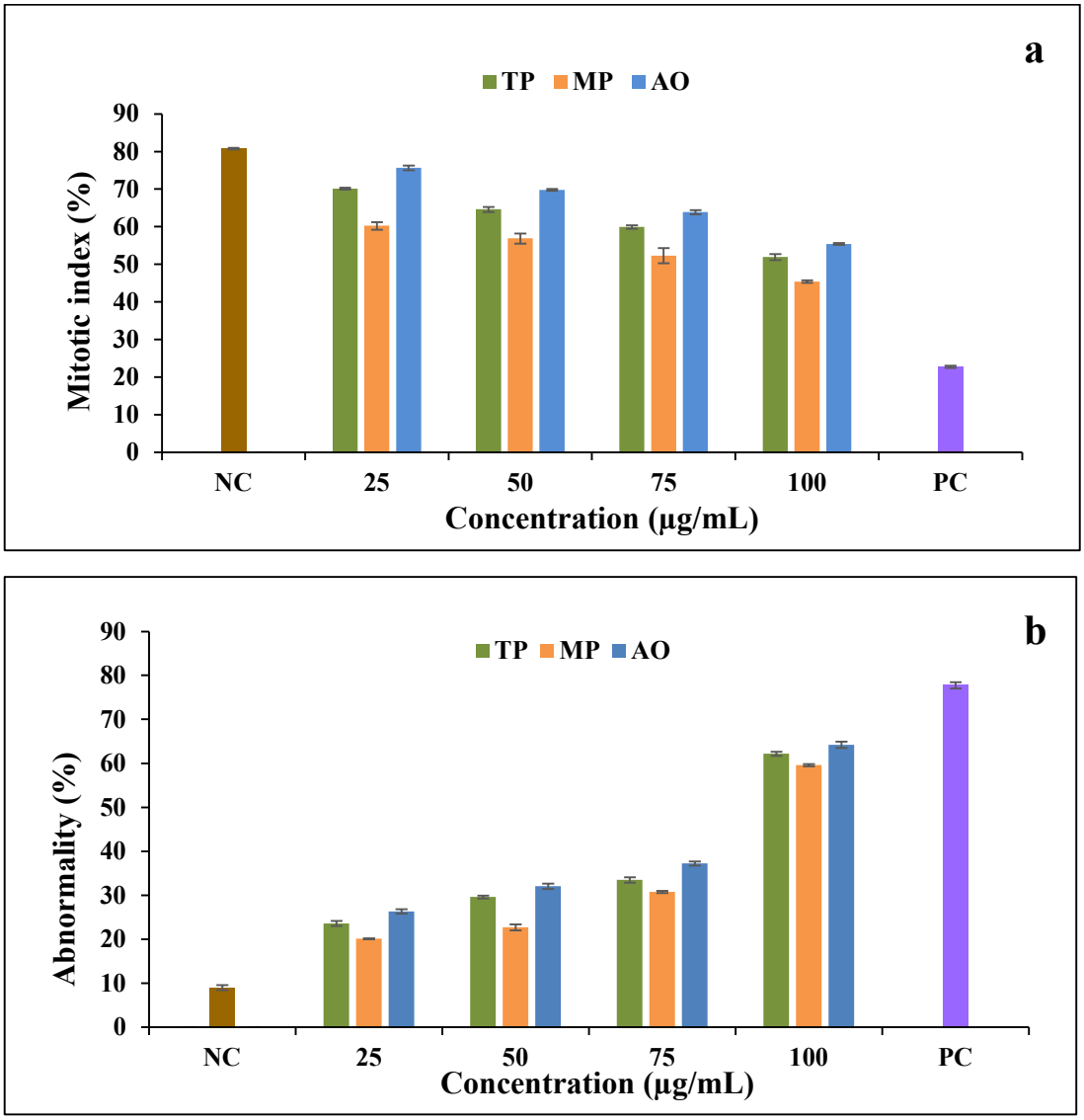



**Figure 30: Cytotoxicity in *Allium cepa* by selected dye-yielding plants: a. Mitotic index; b. Abnormality percentage**, TP - *Terminalia paniculata*; MP - *Mallotus philippensis*; AO - *Albizia odoratissima*; NC - negative control; PC - positive control

## E. Antiproliferative activity

Antiproliferative activity was carried out in the normal cell line (L929) as well as the human breast cancer cell line, *viz*., MDA-MB 231. The cytotoxic effect was evaluated with the help of the MTT assay. The effective plant was selected based on the $LC_{50}$ values obtained in both cell lines, followed by further detailed studies.

### i. *In vitro* cytotoxicity on normal cell line (L929) by MTT assay

The cytotoxic effect of selected dye-yielding plant extracts was examined on L929 cell lines. The adopted concentrations for the study are 6.25, 12.5, 25, 50, and 100 µg/mL. The $LC_{50}$ value for the extracts of *T. paniculata* and *A. odoratissima* was lesser in normal cell lines than in MDA-MB 231 carcinoma cells (**Table 9**). Whereas, *M. philippensis* extract showed a little bit higher $LC_{50}$ value in the normal L929 cell lines when compared with that of MDA-MB 231. The percentage viability of *M. philippensis* extract-treated cells at the highest concentration is very less (38.46 ± 0.59%) and that of *A. odoratissima* and *T. paniculata* is 63.72 ± 0.56% and 48.59 ± 0.79% respectively (**Figure 31**). In the lowest concentration, all three extracts possessed significant viability on L929 cells and ranges between 98.07 ± 0.9% and 98.15 ± 0.7%. There is a considerable decrease in percentage viability observed in *M. philippensis* and *T. paniculata* extract-treated cells. The cytotoxic effect of *T. paniculata*, *M. philippensis* and *A. odoratissima* on L929 cell lines is shown in **Plates 12, 13** and **14** respectively.

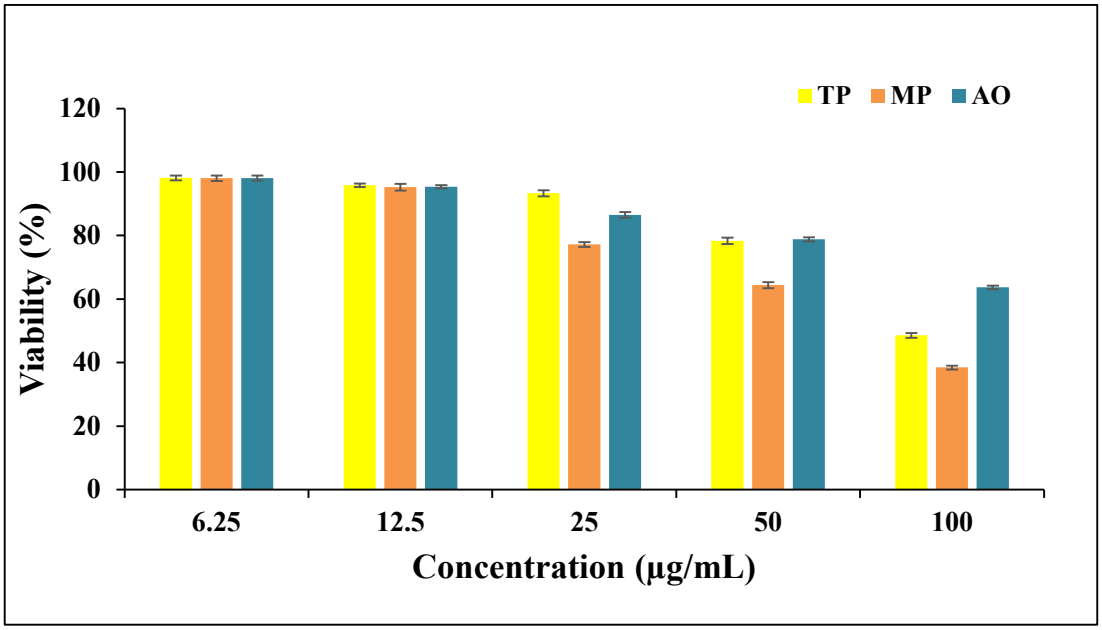



**Figure 31: Evaluation of cytotoxic effect of methanolic extract of selected dye-yielding plants on L929 cells using MTT assay:** TP - *Terminalia paniculata*; MP - *Mallotus philippensis*; AO - *Albizia odoratissima*.

### ii. *In vitro* anticancer activity on MDA-MB 231 cell line using MTT assay

The antiproliferative activity of the methanolic extract of *T. paniculata*, *M. philippensis* and *A. odoratissima* was examined with the help of human breast cancer cell lines (MDA-MB 231). 6.25, 12.5, 25, 50, and 100 µg/mL were the different selected concentrations for the study. The aberrations observed in all the concentrations were nuclear fragmentation, condensed nuclei, cell shrinkage, membrane blebbing, apoptotic bodies, budding, and echinoid spikes (**Plates 15, 16, 17**). Lowest $LC_{50}$ value was obtained in *M. philippensis* (**Table 9**). So, it was considered the most effective plant than the other two. A dose-dependent decrease was found in all three extracts. The lowest viability percentage (30 ± 1.08%) was found at the highest concentration (100 µg/mL) in *M. philippensis* (**Figure 32**). Also, *M. philippensis* exhibited the lowest viability percentage (84.08 ± 0.88%) in the lowest concentration than the other two plants. For all the concentrations, the

highest viability percentage was noticed in *A. odoratissima*. Besides, the highest $LC_{50}$ value (157 ± 1.63µg/mL) was also recorded in the same (**Table 9**). The range of viability percentage from lower to higher concentrations of *T. paniculata* was 89.68 ± 1.05% to 52.59 ± 0.81% respectively. Hence, based on the lowest $LC_{50}$ value as well as the lowest viability percentage, *M. philippensis* was considered a better candidate for further studies.

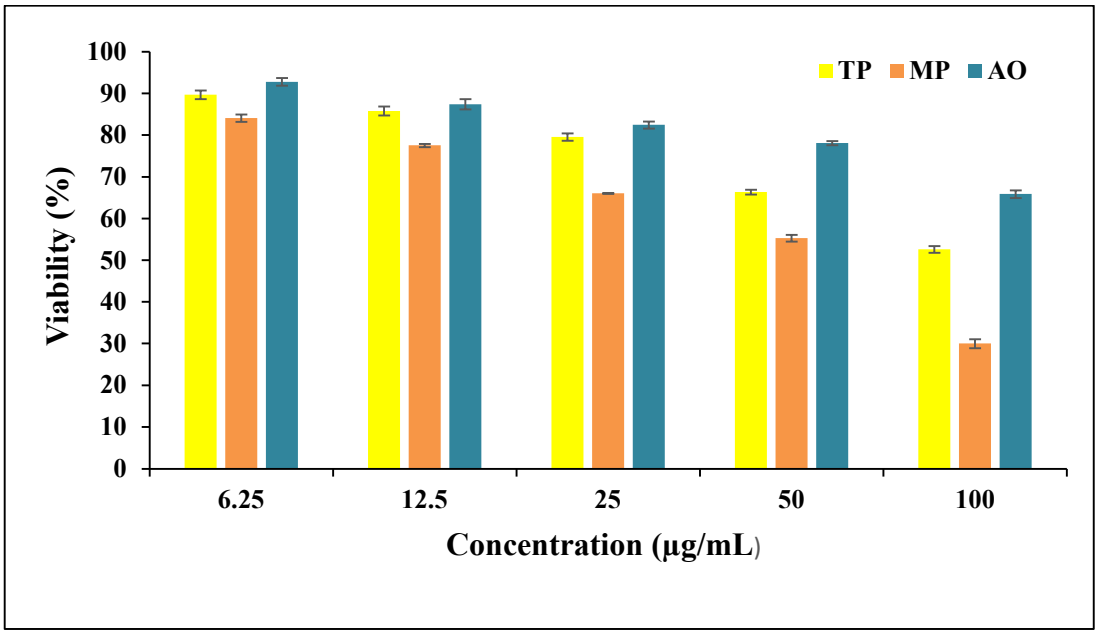



**Figure 32: Evaluation of cytotoxic effect of methanolic extract of selected dye-yielding plants on MDA-MB 231 cells using MTT assay:** TP - *Terminalia paniculata*; MP - *Mallotus philippensis*; AO - *Albizia odoratissima*

**Table 9: Effect of methanolic extracts of dye-yielding plants in L929 and MDA-MB231 cell lines**

| Plant | $LC_{50}$ ± SE (µg/mL) | |
|---|---|---|
| | L929 | MDA-MB 231 |
| ***T. paniculata*** | 99.72 ± 1.1 | 101.6 ± 0.81 |
| ***M. philippensis*** | 77.83 ± 1.77 | 61.5 ± 1.19 |
| ***A. odoratissima*** | 134.6 ± 1.14 | 157 ± 1.63 |

(All values are expressed in mean ± SE)

### iii. Double staining using acridine orange (AO) and ethidium bromide (EtBr)

Double staining is a technique in which the morphological visualization of cell death was examined by acridine orange and ethidium bromide. Cell death can easily be demonstrated by this technique. *In vitro* antiproliferative activity of *M. philippensis* on MDA-MB 231 revealed a promising inhibitory activity. Therefore, it was followed by the double staining method. After treating the methanolic fruit extract of *M. philippensis* with MDA-MB 231 cell lines, they were stained with acridine orange-ethidium bromide. A remarkable representation of live and dead cells could be described by this technique and also have the ability to differentiate apoptotic and necrotic cells. In the control cells, the green nucleus was clearly noticeable, which represents the normal cells. Bright green colour depicted the early apoptotic cells with condensed or fragmented chromatin. Orange-stained nucleus was clearly visible in the treated cells and which portrays the late apoptotic cells with chromatin condensation or fragmentation. Moreover, uniform orange-stained cells represent the necrotic cells (**Plate 18**). These are the four major morphological visualizations happening during the double stain technique. From this study, it is clear that apoptosis is the major cause of cell death caused by the methanolic fruit extract of *M. philippensis*.

### iv. Cell cycle analysis using flow cytometry

The methanolic fruit extract of *M. philippensis* was confirmed as the most effective plant extract through the MTT assay than the other two ones. Due to its least $LC_{50}$ value (61.5 ± 1.19 µg/mL) it was subjected to analyse the DNA content profile and cell cycle distribution in MDA-MB 231 cells. Measurement of DNA content and the population profile can be assessed by flow cytometry. It could recognize the five different phases of the cell cycle i.e., G0 (the resting phase), G1 (normal growth phase), S (DNA replication

phase), G2 (involving growth and preparation for mitosis), and M (mitosis) phase. Population profile seems to be a much-scattered condition of cells in untreated control cells whereas for treated cells it was nearly aggregated. Hence cell cycle arrest might have happened in a particular phase (**Figure 33a & b**).

For the untreated control cells, the population histogram represents the DNA content and was found to be 52.7, 19.7 and 23.7% in G0/G1, S and G2/M phases respectively (**Figure 34a**). But in treated cells, cell count was found to be in a decreasing order from G0/G1 to G2/M phase. 64.2% of cell count was recorded in G0/G1 phase and 15.2%, as well as 14.3%, were noticed in S and G2/M phases respectively (**Figure 34b**). The reduction of cell counts in treated cells designates a significant apoptotic mechanism in cells. This reduction indicates that the cell cycle arrest happened at G0/G1 phase by the action of the methanolic fruit extract of *M. philippensis*.

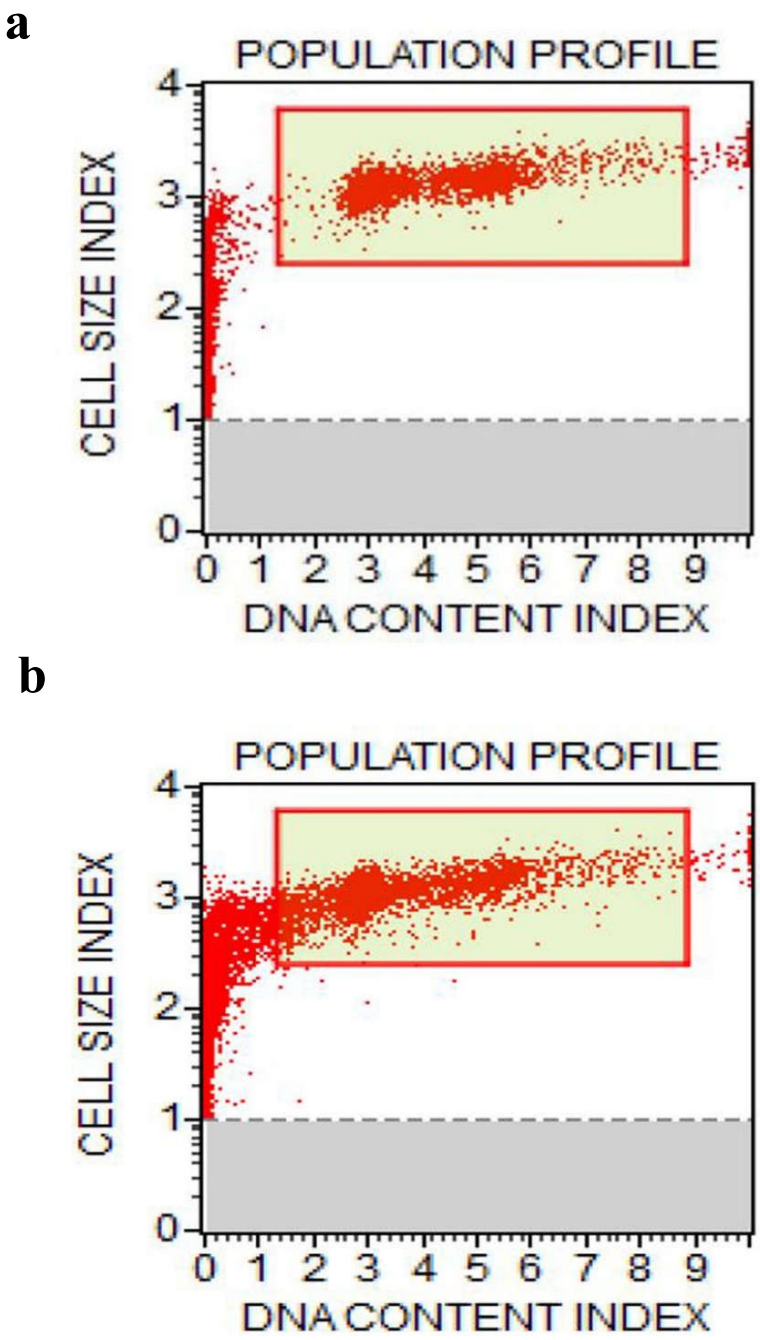



**Figure 33: Population profile of MDA-MB 231 cell lines in determination of cell cycle arrest using flow cytometry**, **a** Control, **b** MDA-MB 231 cells treated with methanolic fruit extract of *M. philippensis*. The rectangle denotes the cells of interest excluding the cellular debris. Apoptotic cells were recognized by their weaker staining intensity with propidium iodide

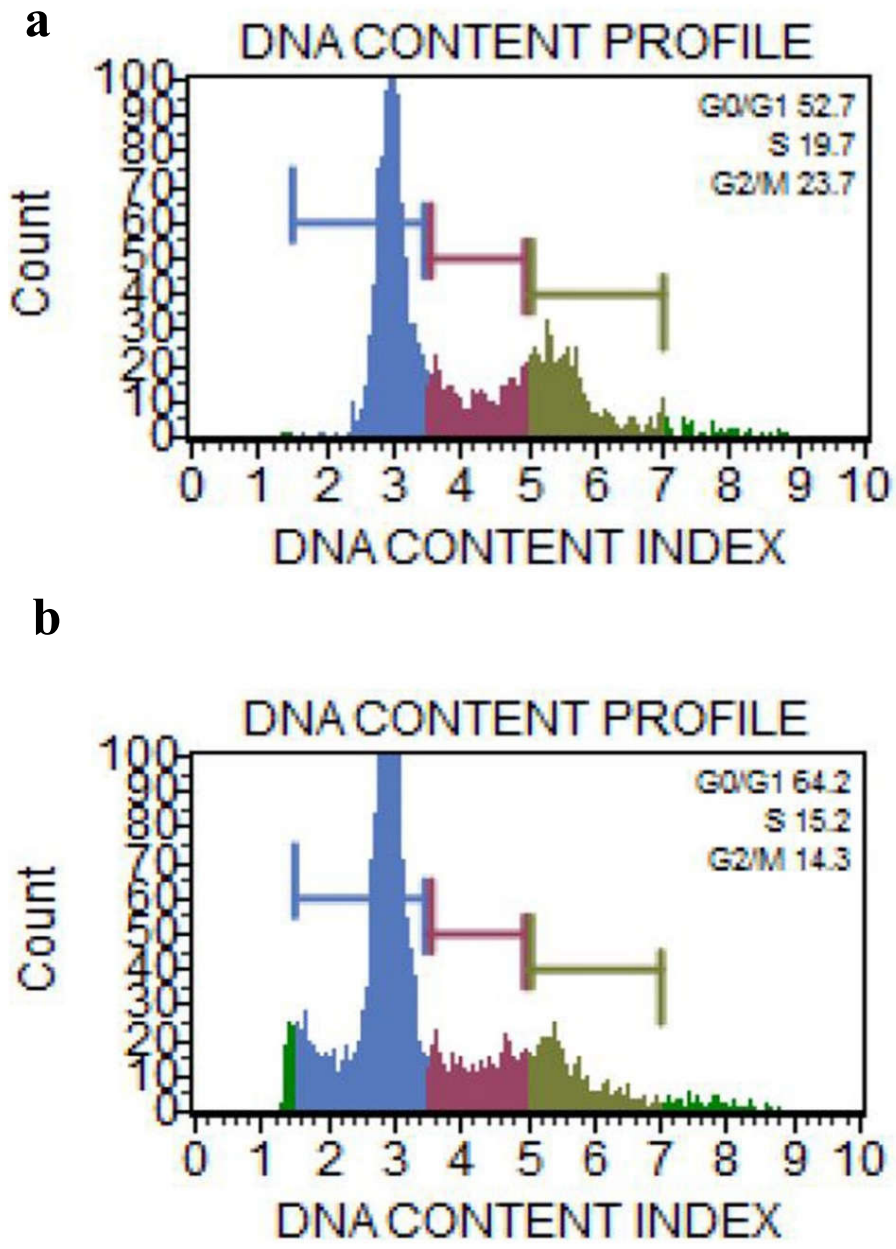



**Figure 34: DNA content profile in determination of cell cycle arrest in MDA-MB 231 cells using flow cytometry, a** Control, **b** MDA-MB 231 cells treated with methanolic fruit extract of *M. philippensis*

## V. Gene expression study using RT-qPCR

The ability of the methanolic fruit extract of *M. philippensis* to induce apoptosis was studied. It was analysed on the expression of two apoptosis-related genes *viz*., p53 and TGF β. Both these genes are involved to mediate growth inhibition as well as apoptosis, which can be correlated with its function as a tumour suppressor. The expression of these genes was analysed by RT-qPCR and followed by gel electrophoresis to know the separation and visualization of DNA fragments. For this, GAPDH (housekeeping gene) was used as the loading control. In the study, MDA-MB 231 cells treated with 61.5 ± 1.19 µg/mL of *M. philippensis* fruit extract for 24h were used and untreated cells were considered as the control (**Figure 35** and **36**). **Figure 35** clearly depicts that treatment with extract had effectively induced upregulation in the expression of both p53 and TGF β. Where the apoptotic efficiency of p53 and TGF β has increased and the change in the cycle threshold ($\Delta\Delta C_t$) value was 1.52 and 1.7 - fold respectively. In both cases, genes had expressed a significant fold change than that of the control. Hence p53 and TGF β leads to growth arrest or apoptosis and the methanolic fruit extract of *M. philippensis* unveiled its potential to induce cell cycle arrest followed by apoptosis.

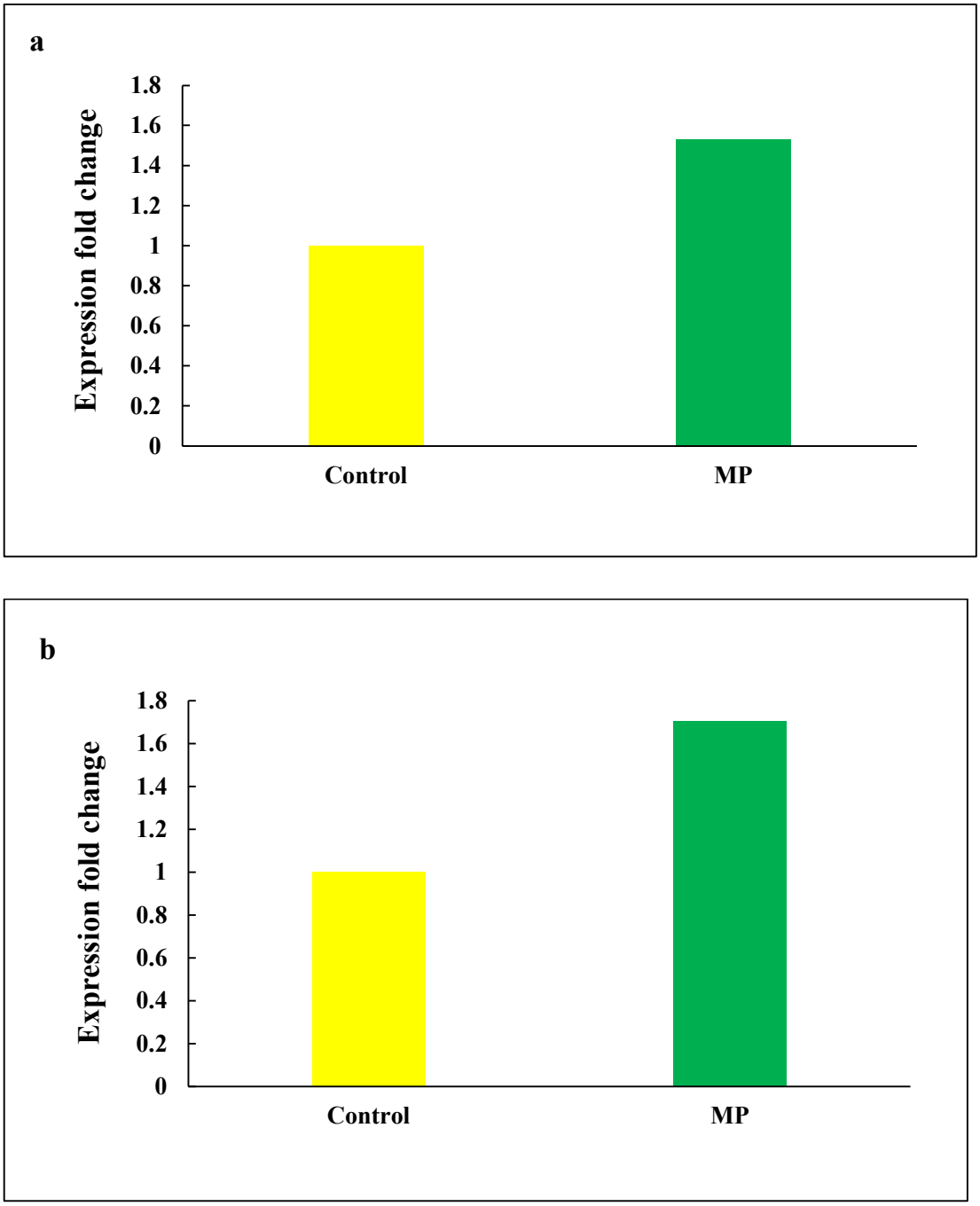



**Figure 35: Expression fold changes of genes in MDA-MB 231 cells treated with methanolic fruit extract of *Mallotus philippensis* (MP): a.** p53; **b.** TGF β

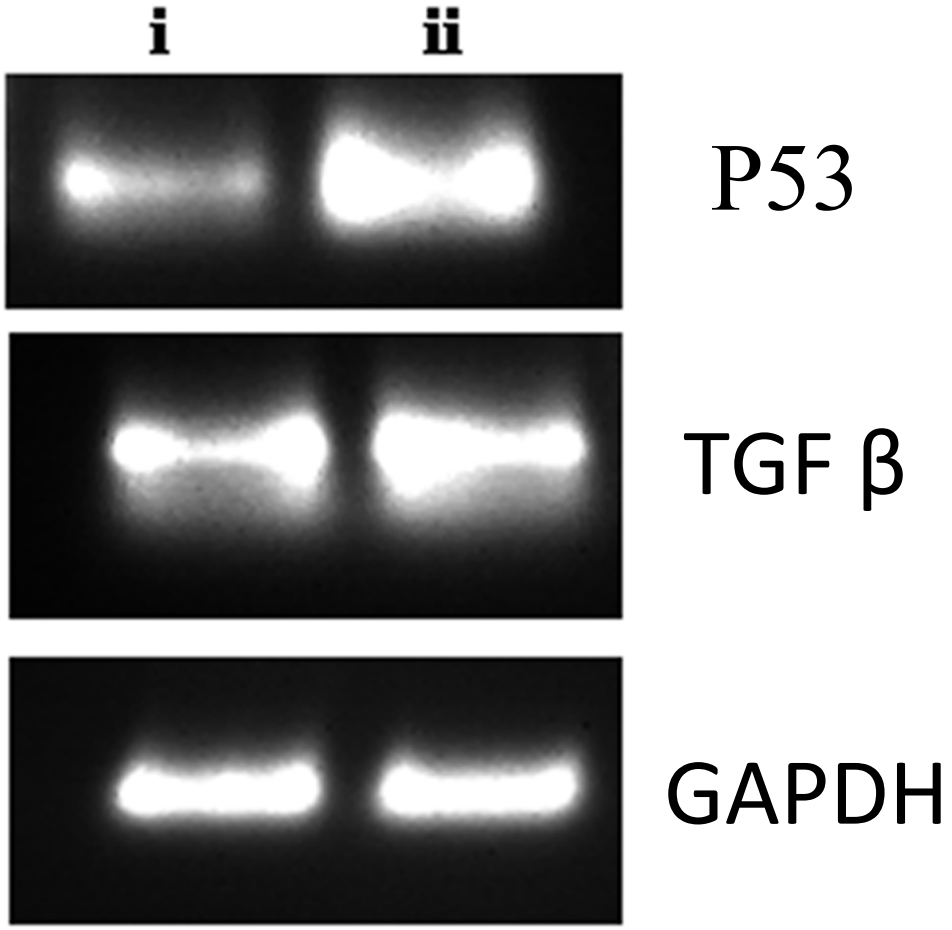



**Figure 36: Expression analysis of genes p53, TGF β and GAPDH in MDA-MB 231 cells treated with methanolic fruit extract of *M philippensis* using Real Time PCR**

## F. Epithelial-Mesenchymal Transition (EMT) Screening

Epithelial-mesenchymal transition (EMT) is a cellular programme in which epithelial cells lose their cell polarity and cell-cell adhesion. It is known to be crucial for embryogenesis, wound healing, and malignant progression.

EMT is analysed using 3 different assays

- Cell Migration assay
- Cell aggregation assay
- Clonogenic assay

### i. Cell migration assay

The effect of *M. philippensis* on cell migration was determined using a cell migration assay. MDA-MB 231 cell lines were used for the assay. For this assay, 0 to 72 hour treatment was performed in both treated and untreated control cells. The wound area was measured by pixel (px) at different time intervals (**Table 10**). At 0 hour, the wound area for control and treated cells were 2858188 and 2751372 px respectively. The size of the wound area had decreased in the control cells from 0 to 72 hours. But in the case of treated cells, the wound area remains with a size of 77956 px at 72 hours. Whereas, at 72 hours no wound area was observed in the control cells (**Plate 19**). The wound area had completely disappeared for the untreated control cells. Finally, the results revealed that the methanolic fruit extract of *M. philippensis* with $LC_{50}$ concentration (61.5 ± 1.19 µg/mL) had led to the restricted migration of MDA-MB 231 breast cancer cells.

**Table 10: Measurements of wound area in control and treated cells by migration assay at different time intervals**

| Time interval (hours) | Untreated control cells (px) | Treated cells (px) |
|---|---|---|
| 0 | 2858188 | 2751372 |
| 24 | 1445428 | 1678736 |
| 48 | 224274 | 440574 |
| 72 | 0 | 77956 |

### ii. Cell aggregation assay

MDA-MB 231 breast cancer cell lines were used for the cell aggregation assay. For this assay, 24 to 72 h treatment was preferred. For an effective comparison untreated control cells were also maintained in the same way. Results revealed that the formation of cell aggregates is very less in MDA-MB 231 cells exposed to *M. philippensis* extract than in the untreated control cells (**Plate 20**). Large compact cell aggregates were found in control cells at 48 and 72 hours. Hence, the results unveiled that methanolic fruit extract of *M. philippensis* with $LC_{50}$ concentration (61.5 ± 1.19 µg/mL) reduces the formation of cell aggregates. After 72 h treatment, both treated and untreated cells were stained with crystal violet. It clearly depicted the inhibition of cell aggregation by *M. philippensis* fruit extract on MDA-MB 231 breast cancer cell lines (**Figure 37**).

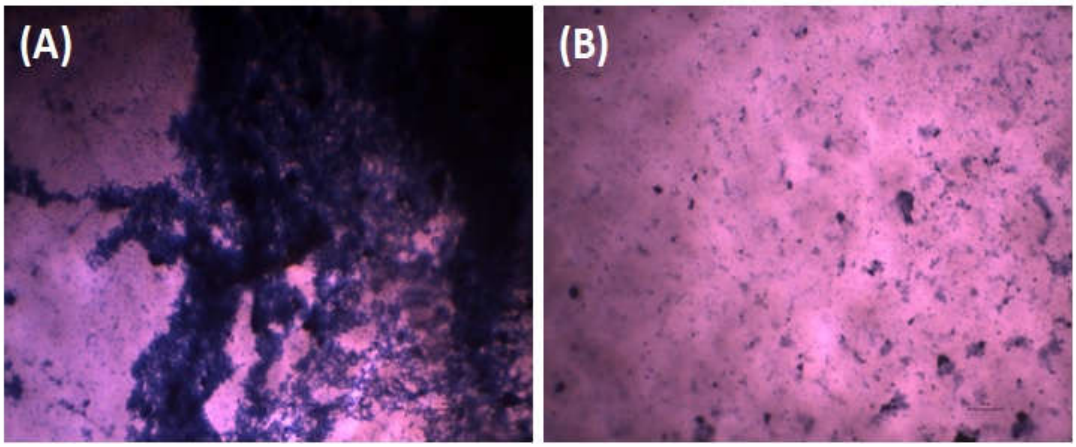



**Figure 37:** Cell aggregation assay by crystal violet staining after 72 hours **(A)** Untreated control cells, **(B)** MDA-MB 231 cells exposed with MP

### iii. Clonogenic assay

It is an *in vitro* cell survival assay revealing the capability of a single cell to turn into a colony. MDA-MB 231 breast cancer cell lines were used to determine the effect of *M. philippensis* fruit extract using clonogenic assay. Untreated control cells were also maintained. The assay was made in triplicate and at least 50 cells should be counted to be considered as colonies. In the present study, the number of colonies is very high in untreated control cells than in the MDA-MB 231 cells exposed to *M. philippensis* extract (**Table 11**). Also, colonies were stained with crystal violet and the number of produced colonies could be clearly noticed in Figure **38**. Therefore, *M. philippensis* fruit extract has the ability to reduce the formation of colonies as evidenced by the clonogenic assay.

**Table 11: Number of colonies produced by clonogenic assay**

| Sample treated | No. of colonies |
|---|---|
| Untreated control cells | 112 |
| MP exposed cells | 29 |

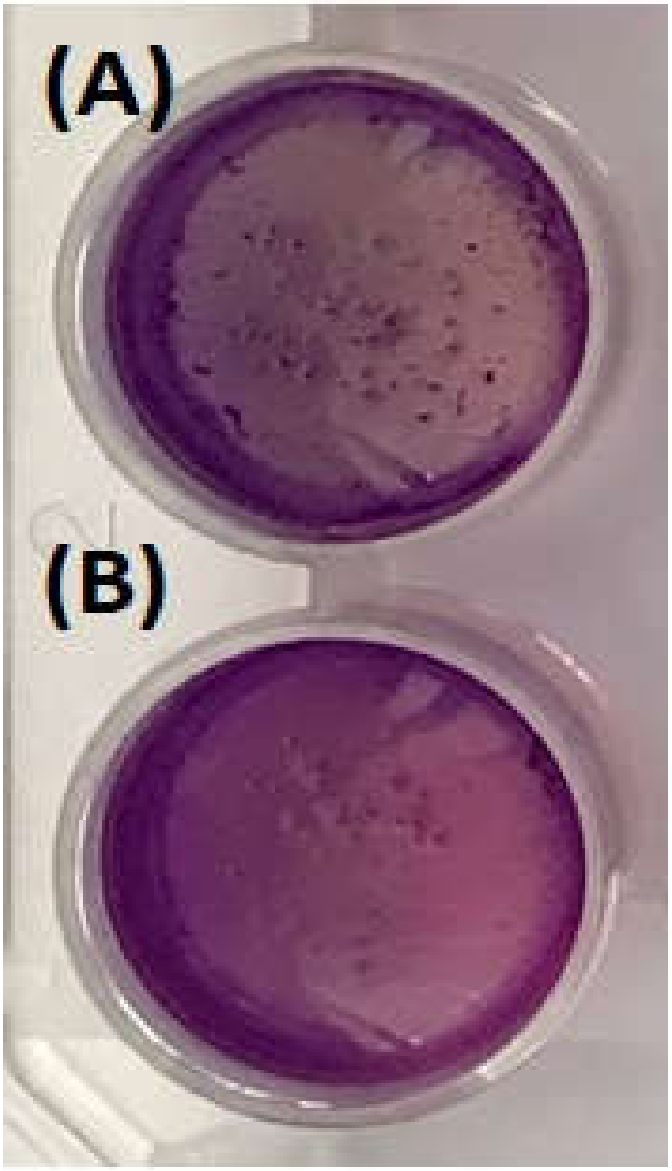



**Figure 38**: Colonies formed by the clonogenic assay and stained with crystal violet. (**A**) Untreated control cells, (**B**) MDA-MB 231 cells exposed with MP

## PART III – GREENSYNTHESIS OF SILVER NANO PARTICLES

### i. Synthesis of Silver Nanoparticles

The selected three dye-yielding plants *viz*., *T. paniculata*, *M. philippensis* and *A. odoratissima* were subjected to the green synthesis of silver nanoparticles. Physicochemical characterizations such as UV-Vis-NIR (Ultraviolet-visible-near infrared) spectral analysis, FE-SEM (Field emission scanning electron microscopy), EDAX (Energy-dispersive-X ray) analysis, and XRD (X-ray diffraction) were carried out to evaluate the detailed description of the synthesized silver nanoparticles. The reduction of silver nitrate into silver nanoparticles occurred by the combined action of selected methanolic extracts and silver nitrate solution. The favourable conditions for the green synthesis of silver nanoparticles were adopted to be 10-minute incubation, 80°C temperature, 2 mM silver nitrate solution, pH of the reaction mixture of 8, and the required proportion for 2 mM silver nitrate and methanolic extract as 9:1. The gradual colour change depicts the formation of silver nanoparticles as well as the reduction of silver nitrate.

In this study, the presence of yellowish-brown colour indicates the reduction of silver nitrate and the synthesis of nanoparticles. For *T. paniculata*, a dark brown colour was obtained after the reaction between their methanolic fruit extract and 2 mM silver nitrate solution (**Plate 21**). Whereas, comparatively a lesser brown colour formation had happened in the case of *M. philippensis* and *A. odoratissima*. A yellowish-brown colour was obtained in both conditions, which stated the appearance of silver nanoparticles at 80°C. A colourless blank solution of silver nitrate was depicted in **Plate 21**.

### ii. AgNP Characterization

### a. UV-Vis-NIR spectral analysis

The formation of silver nanoparticles was confirmed by using UV-Vis-NIR spectral analysis at 200-800 nm. The surface plasmon resonance (SPR)

of silver nanoparticles produced a distinguishable absorption peak at 456 nm, 438 nm and 465 nm in *T. paniculata*, *M. philippensis* and *A. odoratissima* respectively (**Figure 39**). Furthermore, it confirms the presence of silver nanoparticles in the methanolic extracts of all three selected dye-yielding plants. Also, the maximum absorbance was observed at 1.05, 1.04, and 1.47 a. u for *T. paniculata*, *M. philippensis* and *A. odoratissima* respectively. The considered λ max for the silver nanoparticles is in between 380 - 470 nm. In the present study, all three extracts possessed a characteristic peak between these ranges. Hence, UV-Vis-NIR spectra confirm the presence of silver nanoparticles.

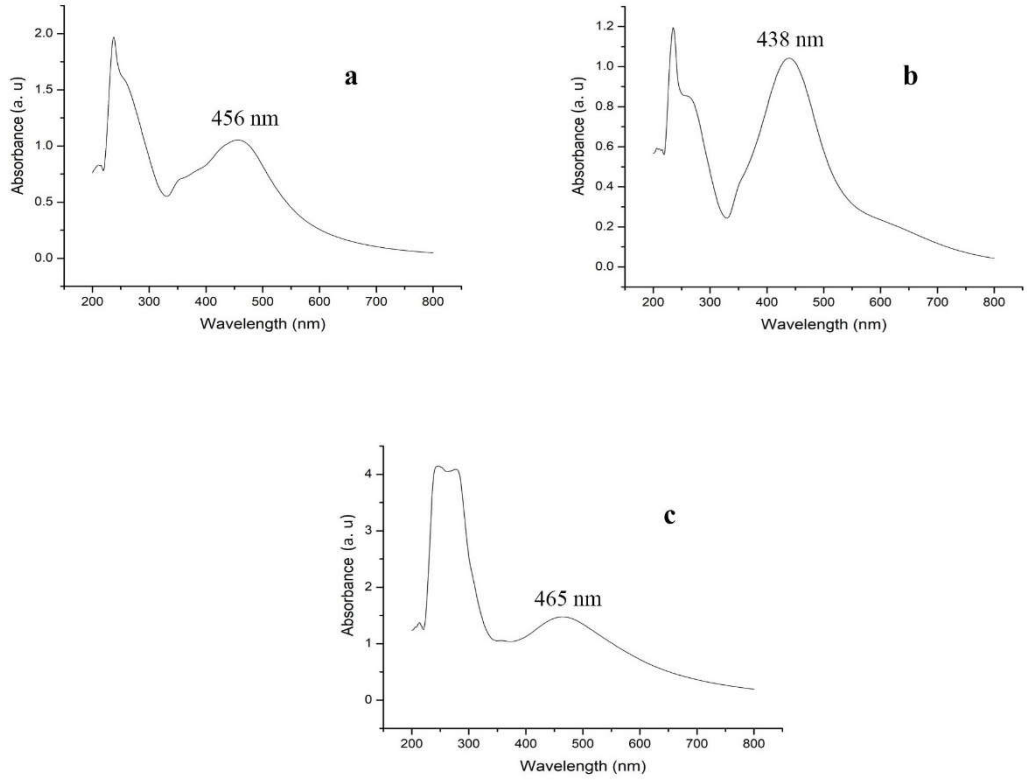



**Figure 39: UV-Vis-NIR spectra of silver nanoparticles synthesized by selected dye-yielding plants** - **a** *T. paniculata* fruit extract, **b** *M. philippensis* fruit extract, **c** *A. odoratissima* bark extract

**b. FE-SEM analysis**

The surface details of the synthesized silver nanoparticles could be specified through SEM images (**Plate 22, 23, 24**). SEM images provide detailed information regarding the size, shape, and diameter of silver nanoparticles. In *T. paniculata* and *A. odoratissima,* relatively spherical nanoparticles are formed whereas for *M. philippensis* it was nearly cubical. The size of the nanoparticle obtained is in the range between 20-60 nm for *T. paniculata*, 21-45 nm for *M. philippensis*, and 30-90 nm for *A. odoratissima*. When the λmax increases, nanoparticle crystalline size increases due to particle aggregation. After a general comparison, the silver nanoparticles produced by the fruit extracts revealed an almost equal range of particle size. Whereas, for bark extract, the size of the nanoparticle is very large than the other two ones. So, this confirms that all three dye-yielding plants exhibited a remarkable synthesis of silver nanoparticles.

**c. EDAX analysis**

Energy Dispersive X-ray Analysis (EDAX), an X-ray assisted technique was used to identify the elemental composition of materials in the study. The data procured by EDAX analysis include a spectrum with definite peaks corresponding to the composition of all the three samples analysed in its truest sense. It is non-destructive and the samples were examined *in situ* with little preparative steps. A characteristic peak was obtained in all the dye-yielding plants analysed which indicated the prominence of Ag **(Plate25).**

**d. XRD analysis**

X-ray diffraction technique is a very useful characterization tool to know the crystalline nature of the silver nanoparticles. The diffraction patterns were obtained by measuring of angles at which the X-ray beam is diffracted by the crystalline phases in the particle. In this study, the diffraction peaks of *T. paniculata* at 2θ values were 38.03°, 44.21°, 64.42° and 77.01° corresponding to (111), (200), (220) and (311), respectively. Whereas, *M. philippensis* showed diffraction peaks at 2θ values of 38.08°, 43.66°, 64.57° and 77.29°. Besides, 2θ values of *A. odoratissima* was 38.15°, 44.21°, 64.55° and 77.27°. The highest peak was obtained at the 2θ values of 38.03° in *T. paniculata*, 38.08° in *M. philippensis* and 38.15° in *A. odoratissima* (**Plate 26**). Based on these results, the obtained Bragg reflections revealed the face-centered cubic structure of silver. The average crystallite size of the silver nanoparticles synthesized from *T. paniculata*, *M. philippensis*, and *A. odoratissima* were 17.7 nm, 4.36 nm and 7.11 nm respectively (**Table 12**). Hence, the diffraction peaks obtained from XRD and the mean crystallite size clearly depict that the synthesized silver nanoparticles were nanocrystalline in nature.

**Table 12: Average crystallite size of silver nanoparticles from XRD data**

| Plant | Peak No. | 2θ (degrees) | FWHM (radians) | Crystallite size (nm) | Average crystallite size (nm) |
|---|---|---|---|---|---|
| **TP** | 1 | 38.03 | 0.869 | 9.661 | 17.70 |
| | 2 | 44.21 | 0.278 | 30.816 | |
| | 3 | 64.42 | 0.472 | 19.864 | |
| | 4 | 77.01 | 0.968 | 10.478 | |
| **MP** | 1 | 38.08 | 2.155 | 3.898 | 4.36 |
| | 2 | 43.66 | 7.711 | 1.109 | |
| | 3 | 64.57 | 1.239 | 7.584 | |
| | 4 | 77.29 | 2.090 | 4.866 | |
| **AO** | 1 | 38.15 | 0.473 | 17.768 | 7.11 |
| | 2 | 44.21 | 0.842 | 10.176 | |
| | 3 | 64.55 | 47.755 | 0.196 | |
| | 4 | 77.27 | 33.640 | 0.302 | |

TP - *Terminalia paniculata*, MP - *Mallotus philippensis*, AO - *Albizia odoratissima*, FWHM - Full width half maximum, nm - nano meter


**Plate 1: Habit of selected dye-yielding plants. a** *Terminalia paniculata* Roth., **a1** fruits; **b** *Mallotus philippensis* (Lam.) Muell. Arg., **b1** fruits; **c** *Albizia odoratissima* (L.f.) Benth., **c1** bark

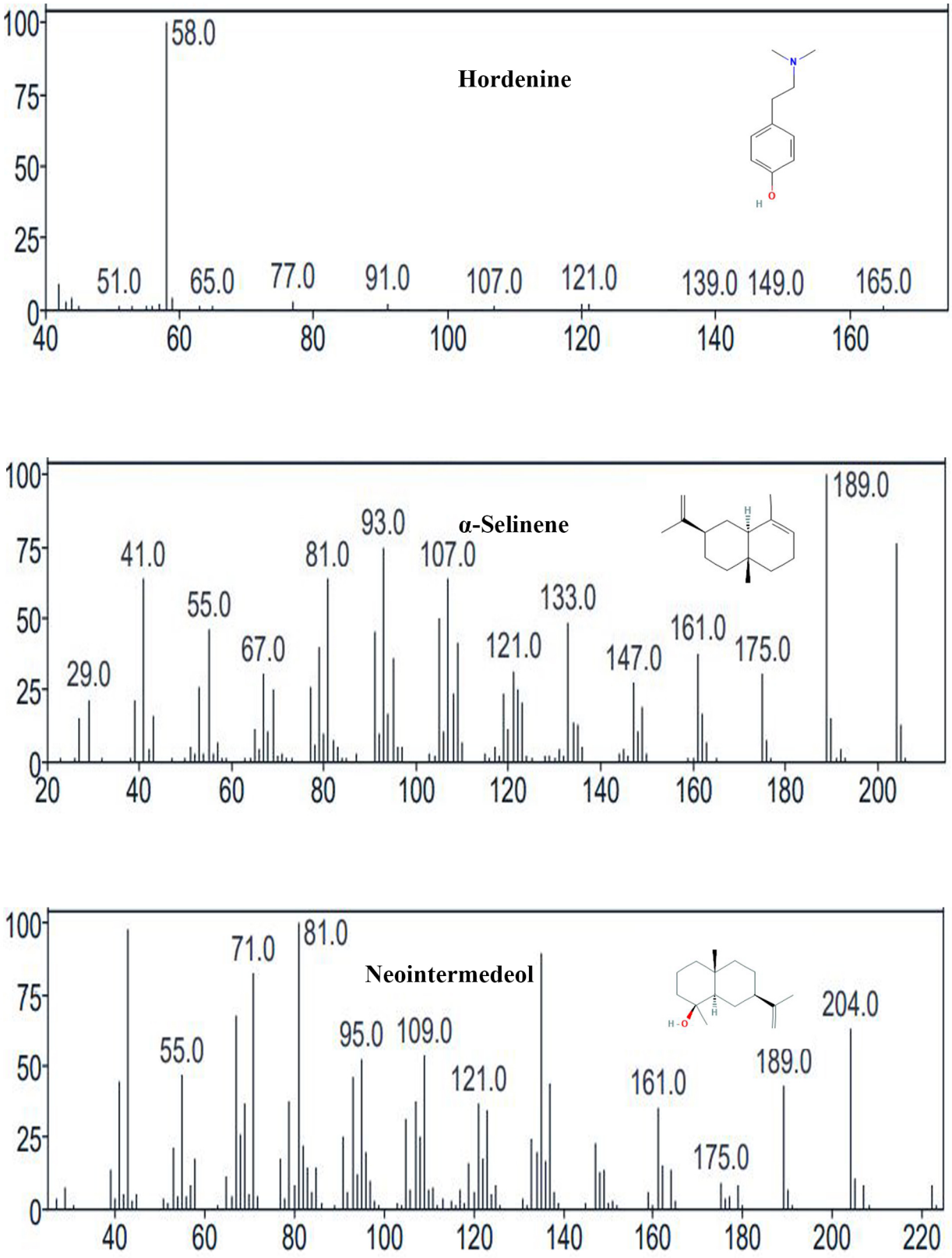



**Figure 18 (i): Mass spectra of compounds detected by GC-MS analysis of methanolic extract of selected dye-yielding plants**

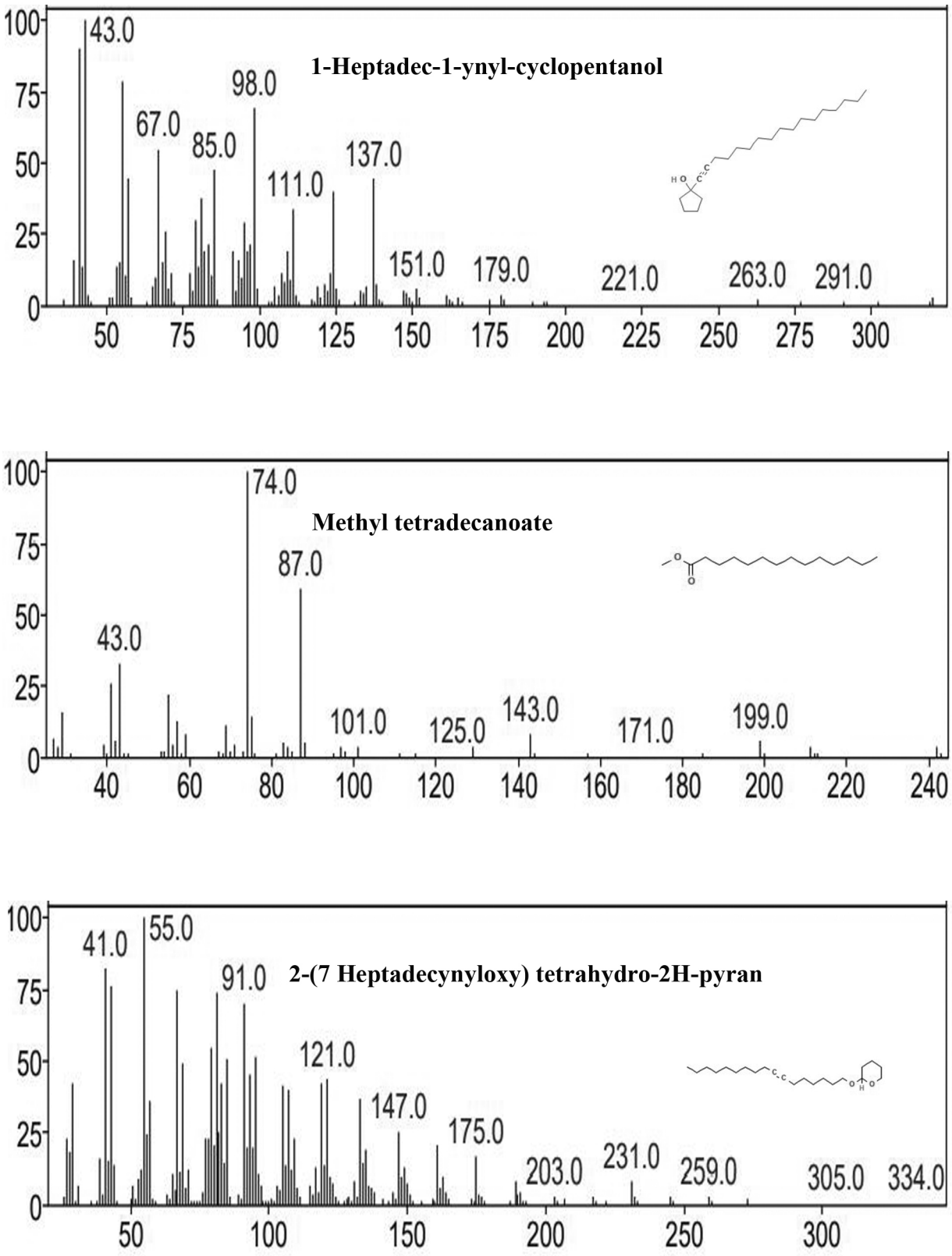



**Figure 18 (ii): Mass spectra of compounds detected by GC-MS analysis of methanolic extract of selected dye-yielding plants**

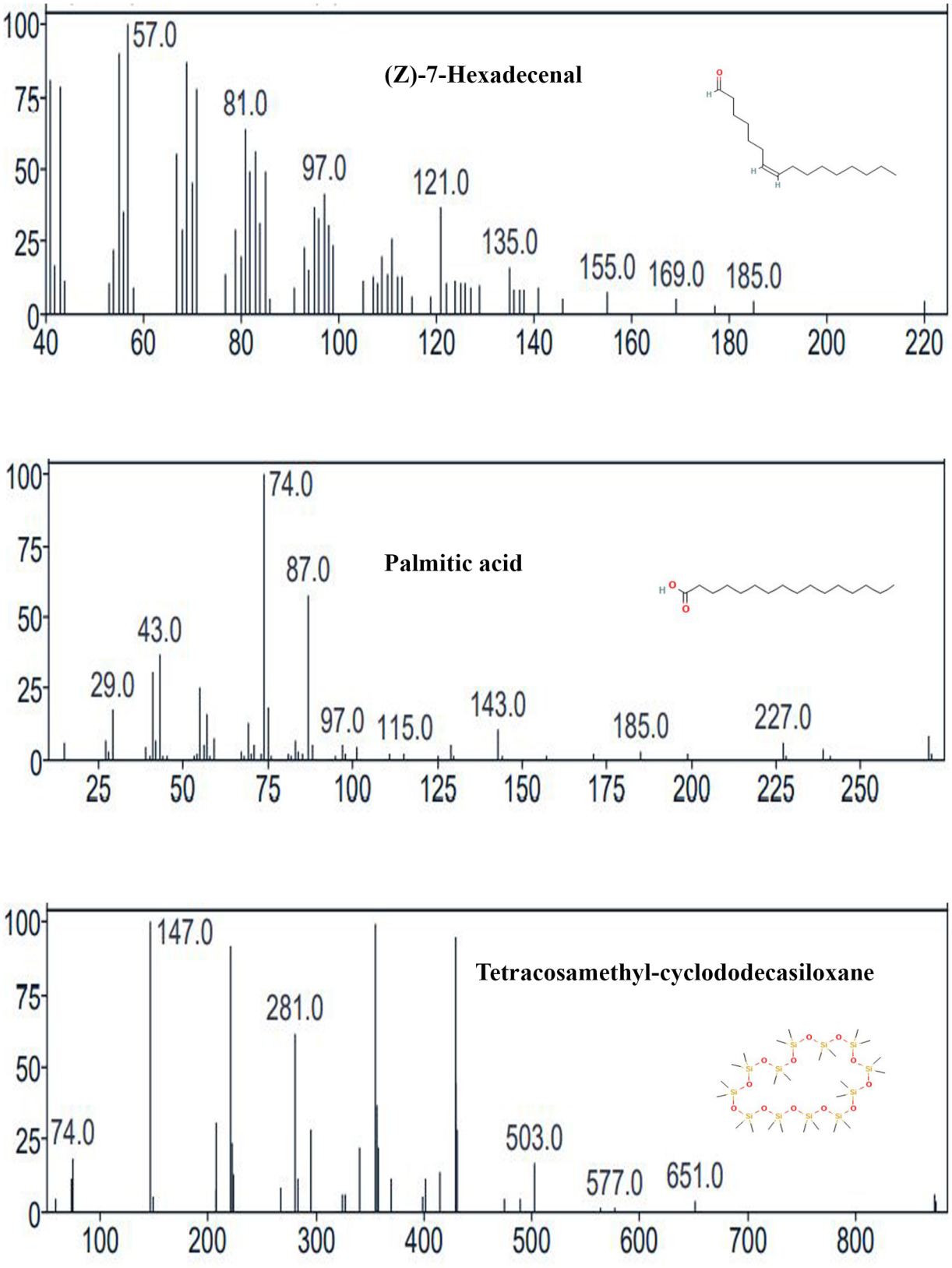



**Figure 18 (iii): Mass spectra of compounds detected by GC-MS analysis of methanolic extract of selected dye-yielding plants**

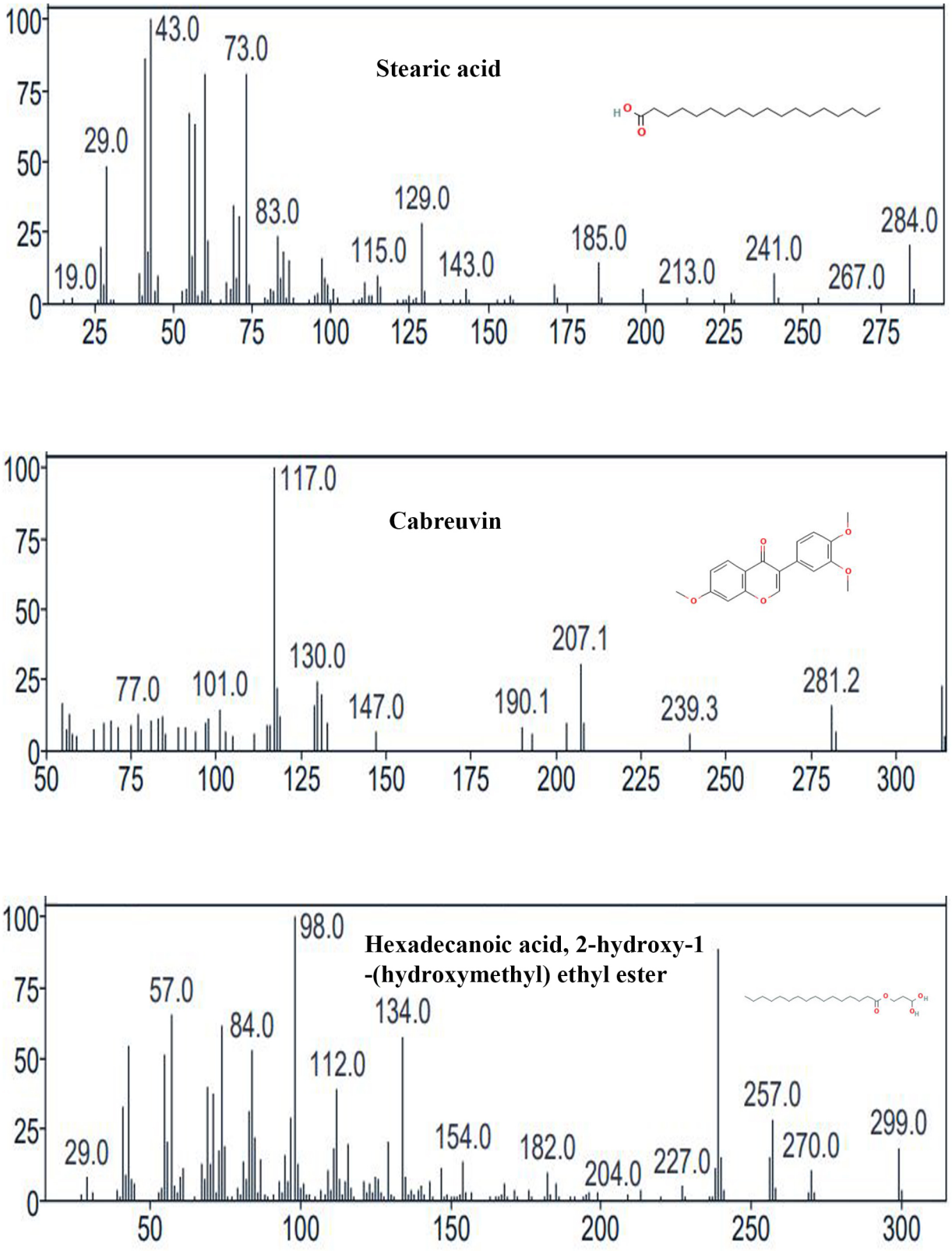



**Figure 18 (v): Mass spectra of compounds detected by GC-MS analysis of methanolic extract of selected dye-yielding plants**

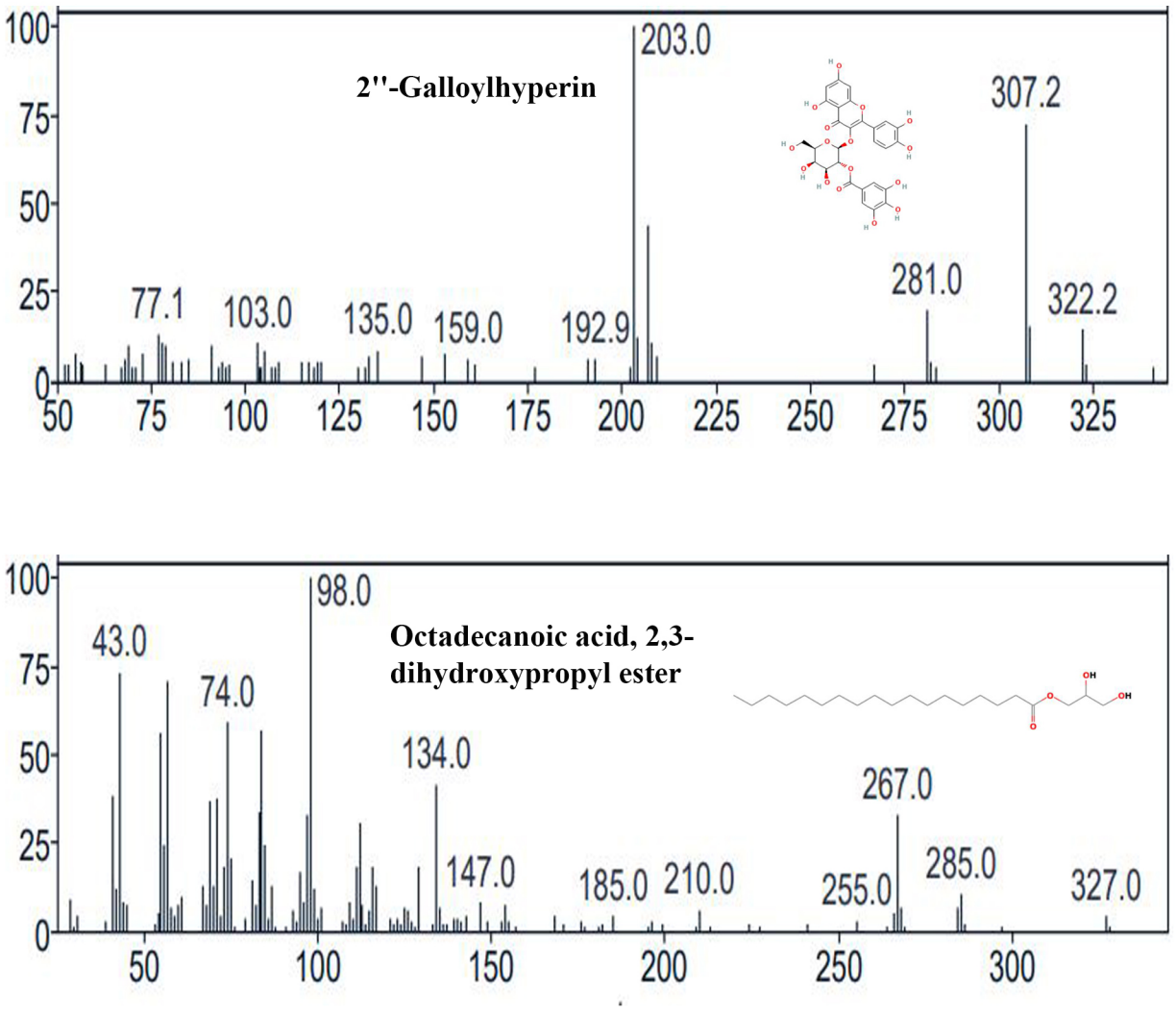



**Figure 18 (vi): Mass spectra of compounds detected by GC-MS analysis of methanolic extract of selected dye-yielding plants**

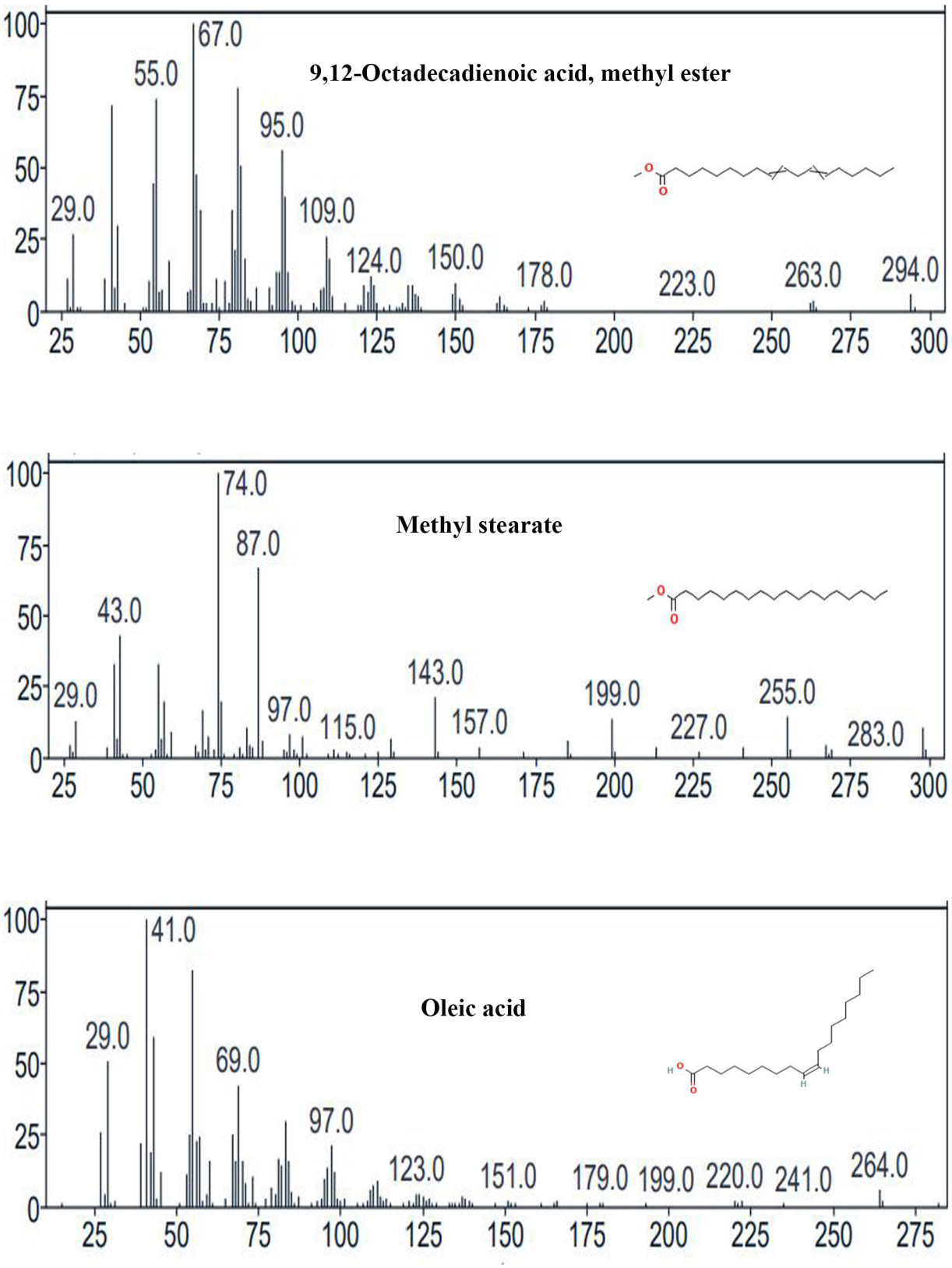



**Figure 18 (iv): Mass spectra of compounds detected by GC-MS analysis of methanolic extract of selected dye-yielding plants**

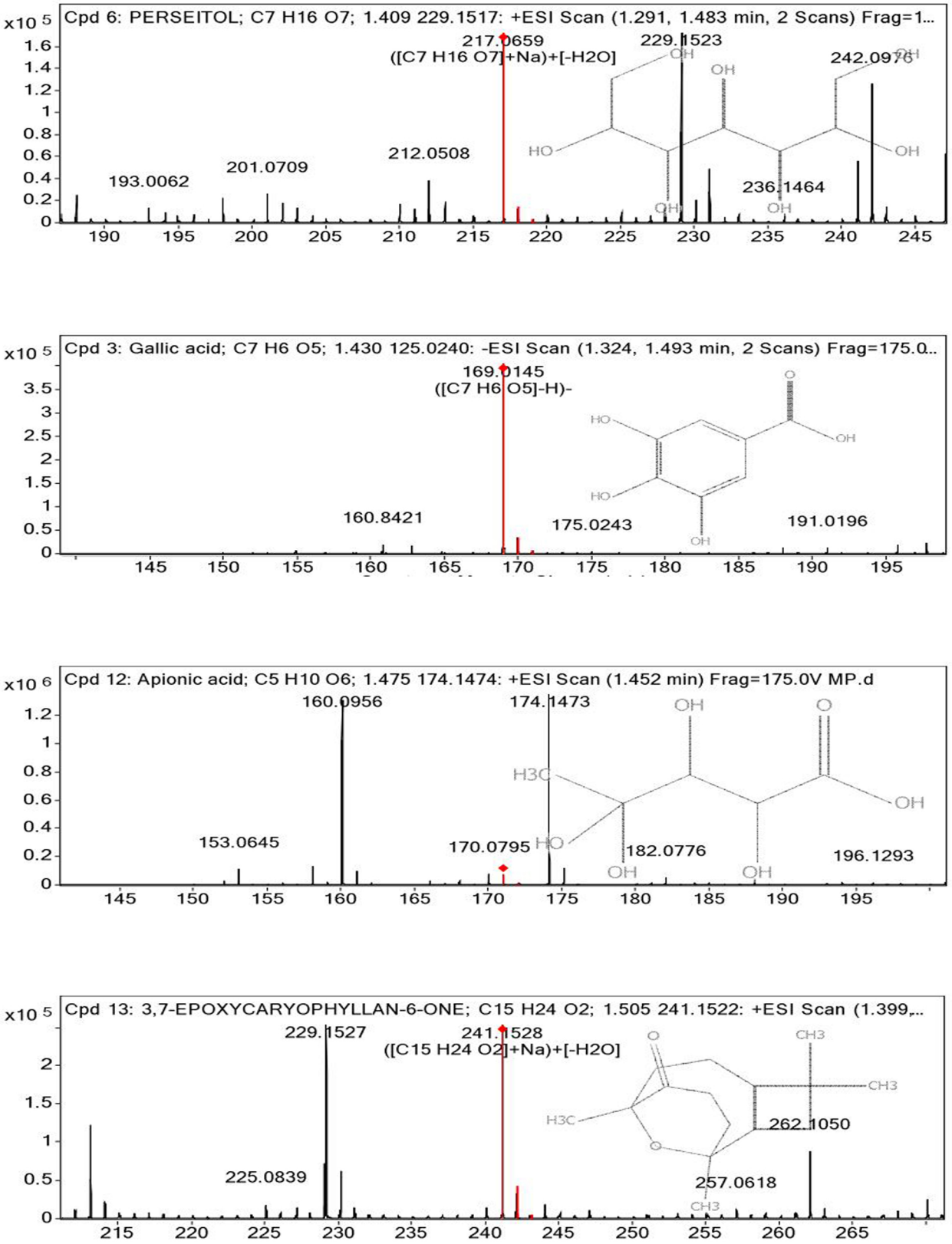



**Figure 22 (ii): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

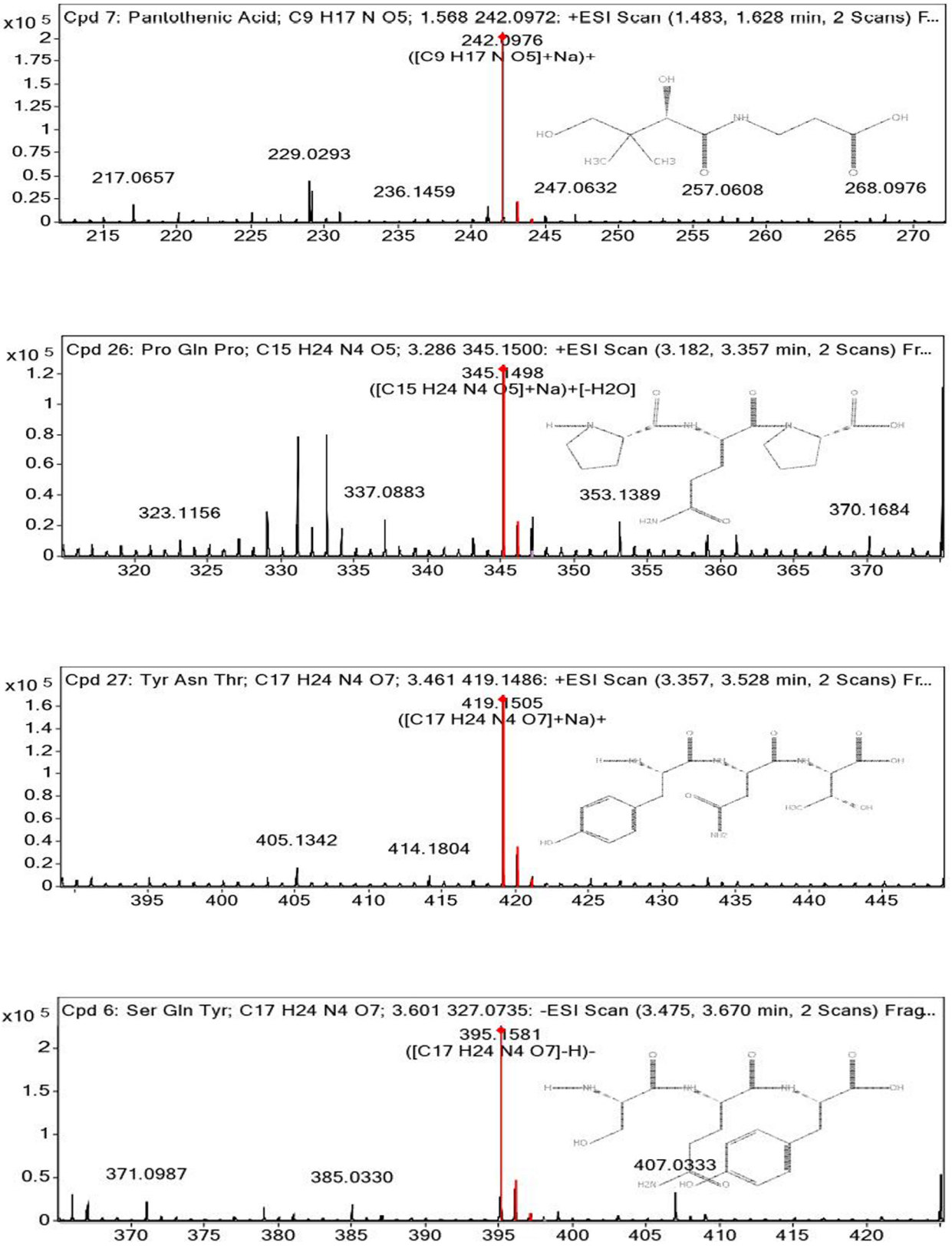



**Figure 22 (iii): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

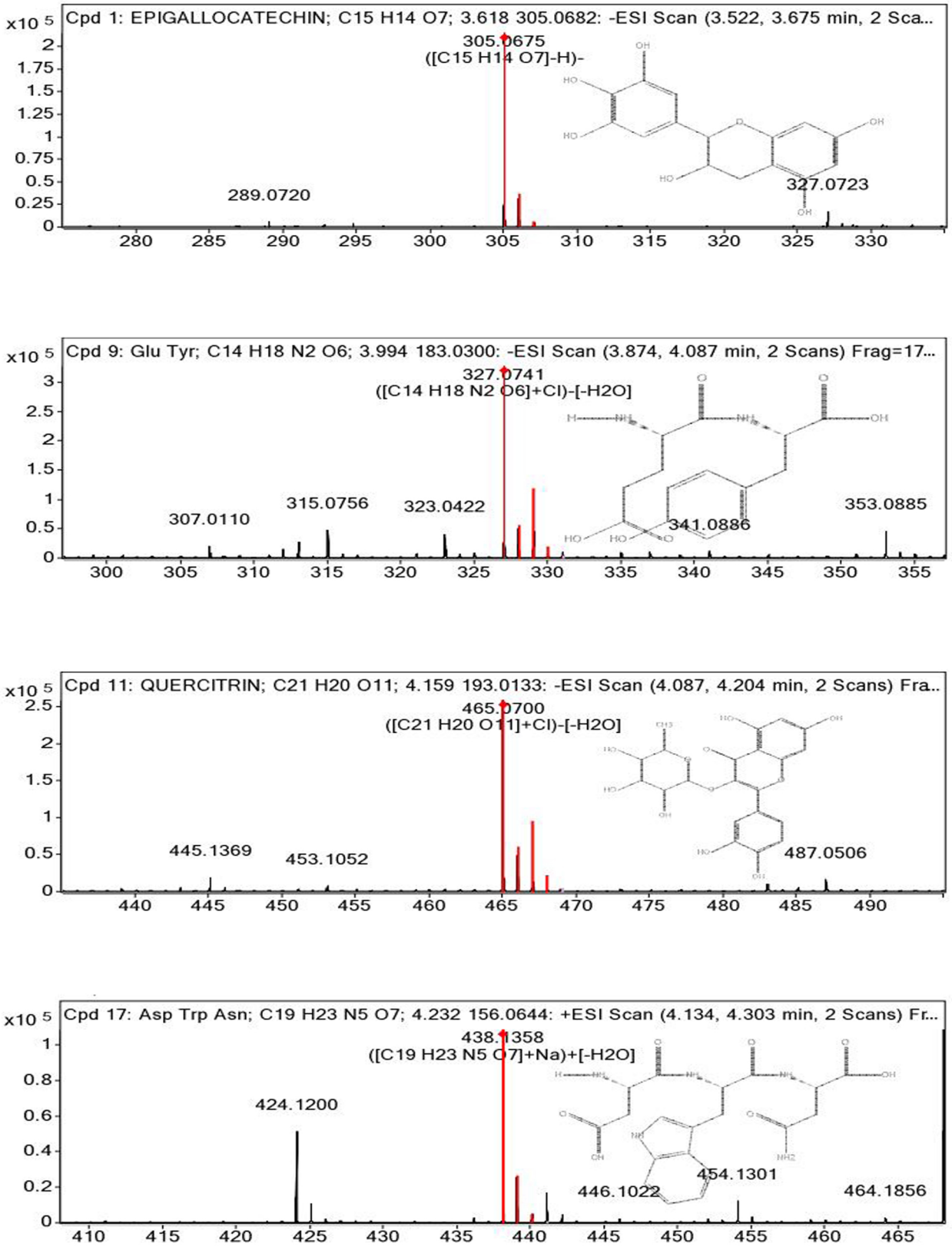



**Figure 22 (iv): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

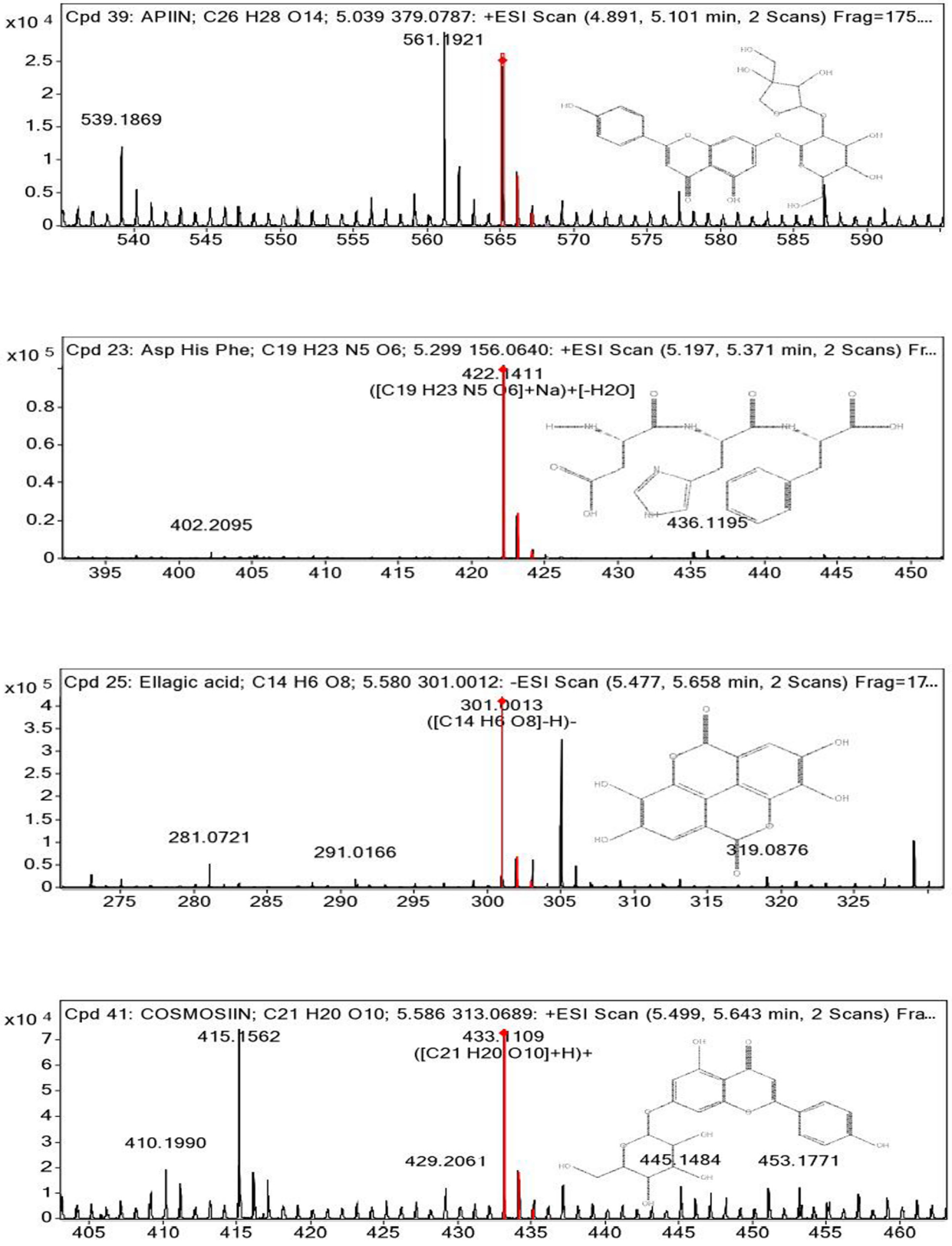



**Figure 22 (vi): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yieding plants**

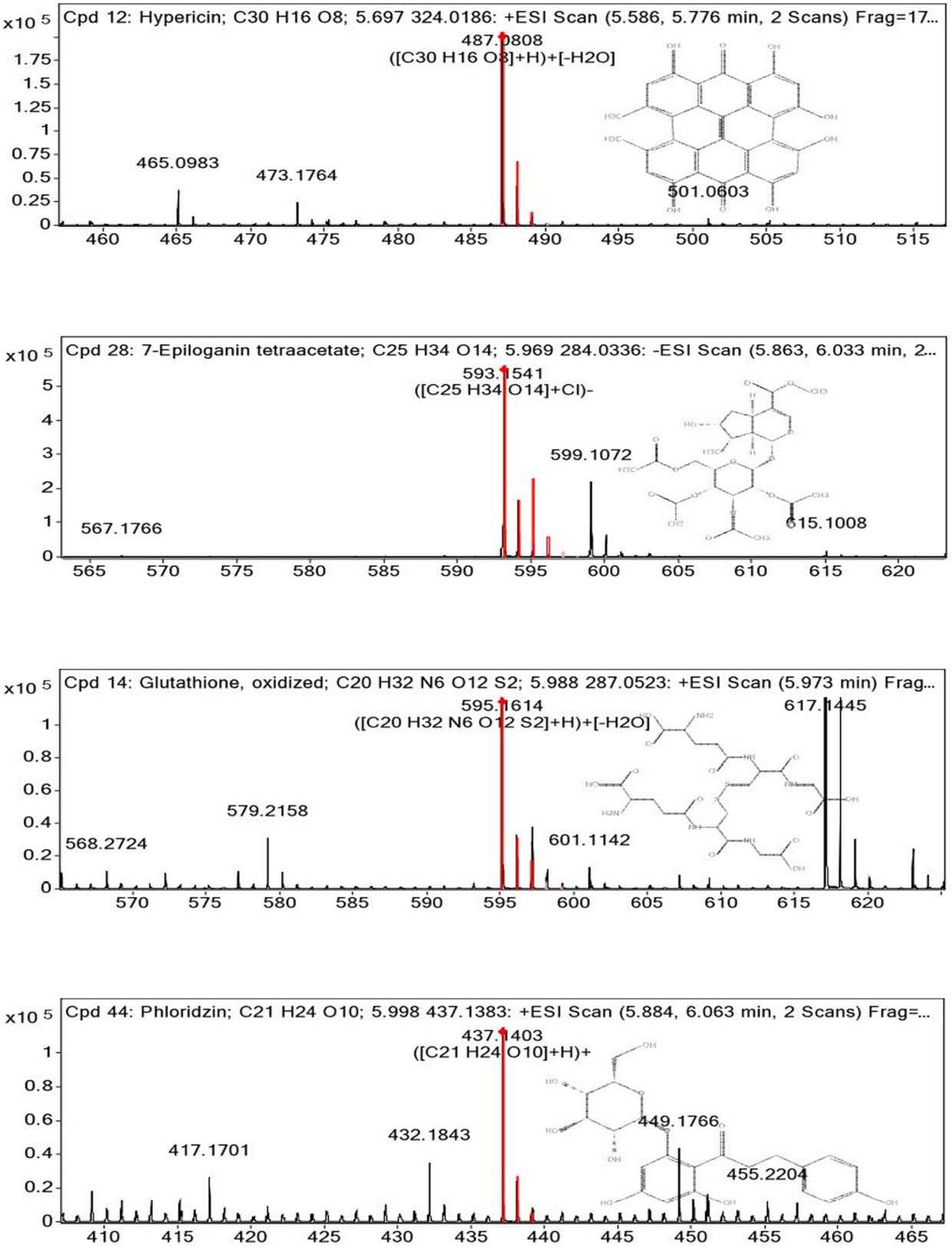



**Figure 22 (vii): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

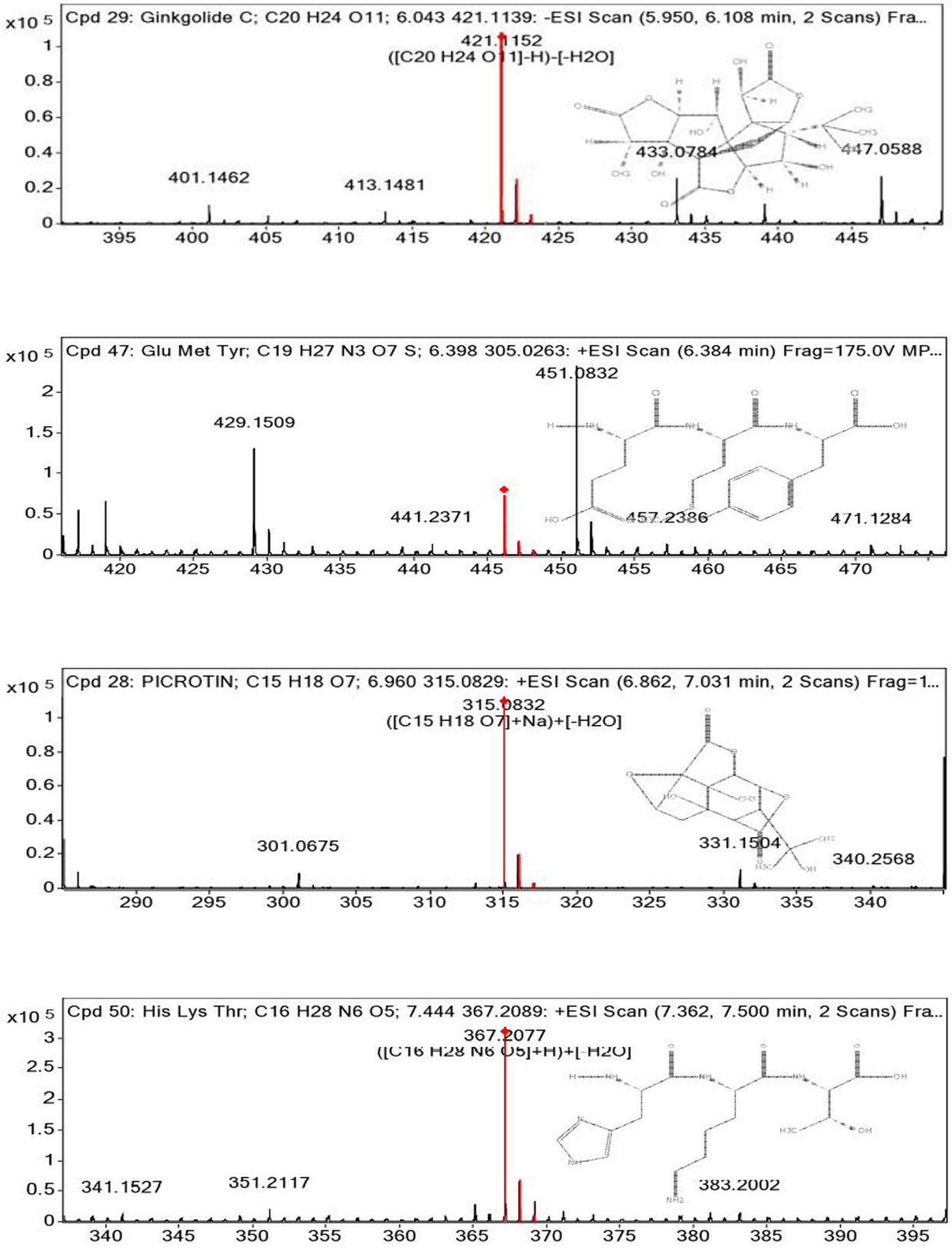



**Figure 22 (viii): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

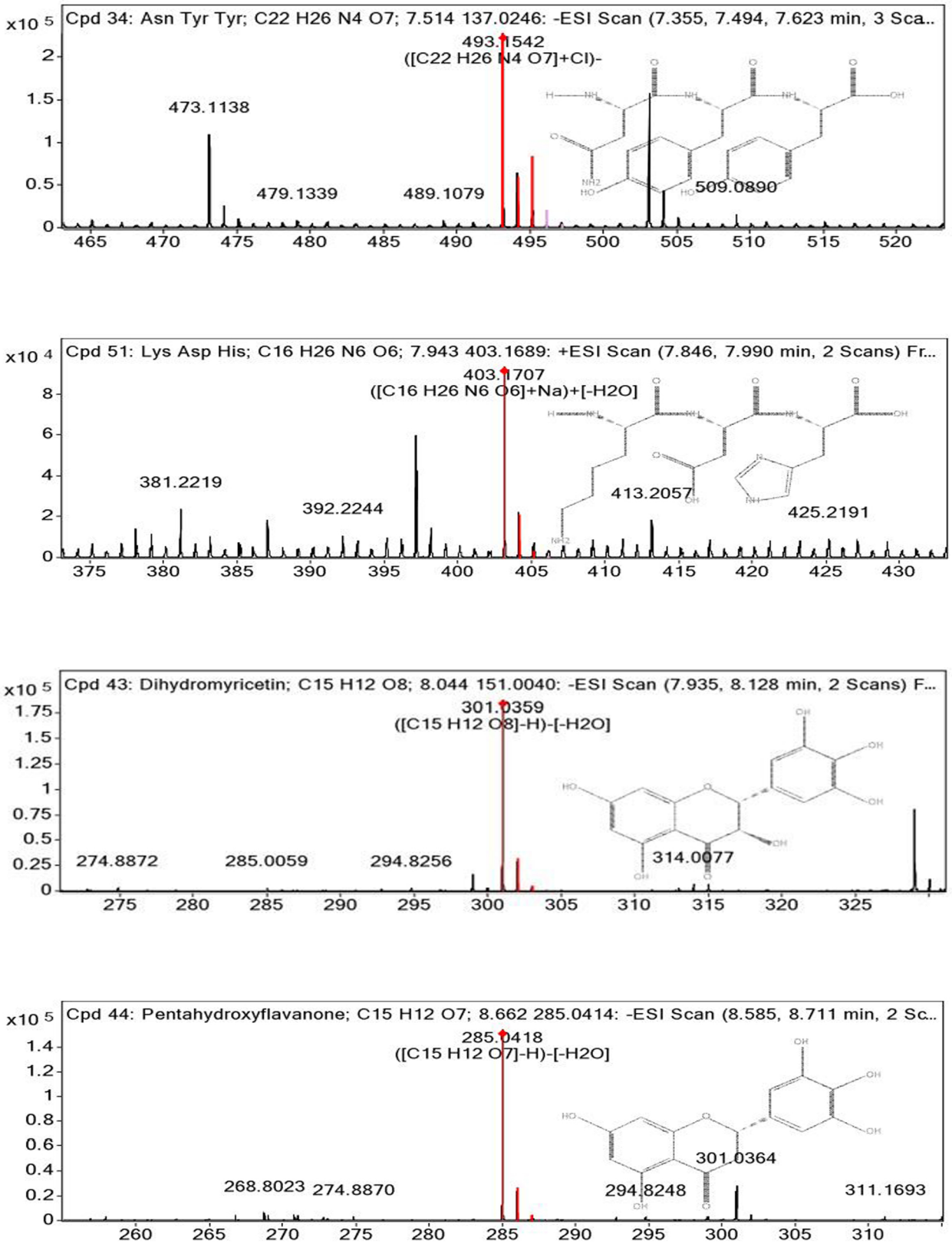



**Figure 22 (ix): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

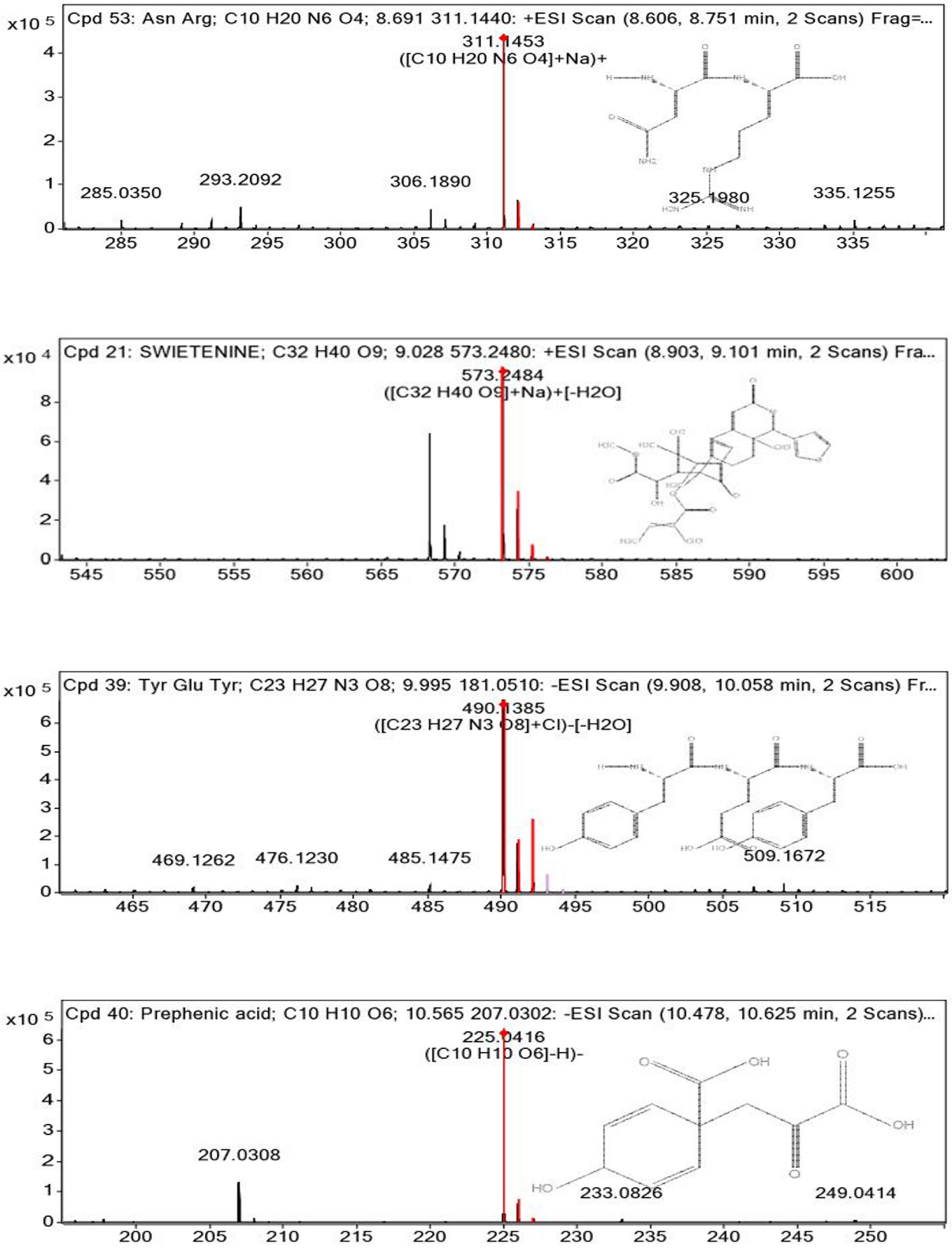



**Figure 22 (x): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

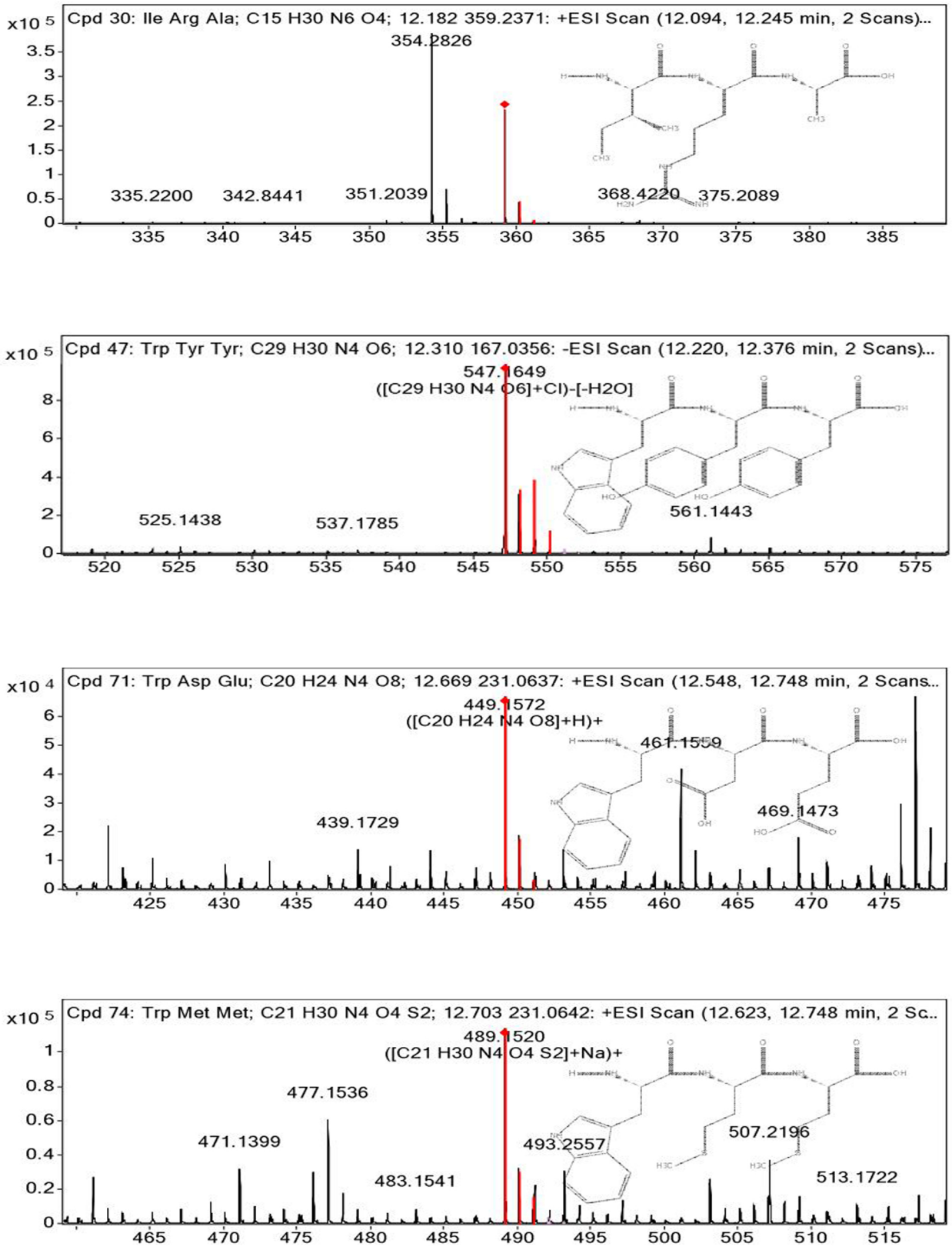



**Figure 22 (xi): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

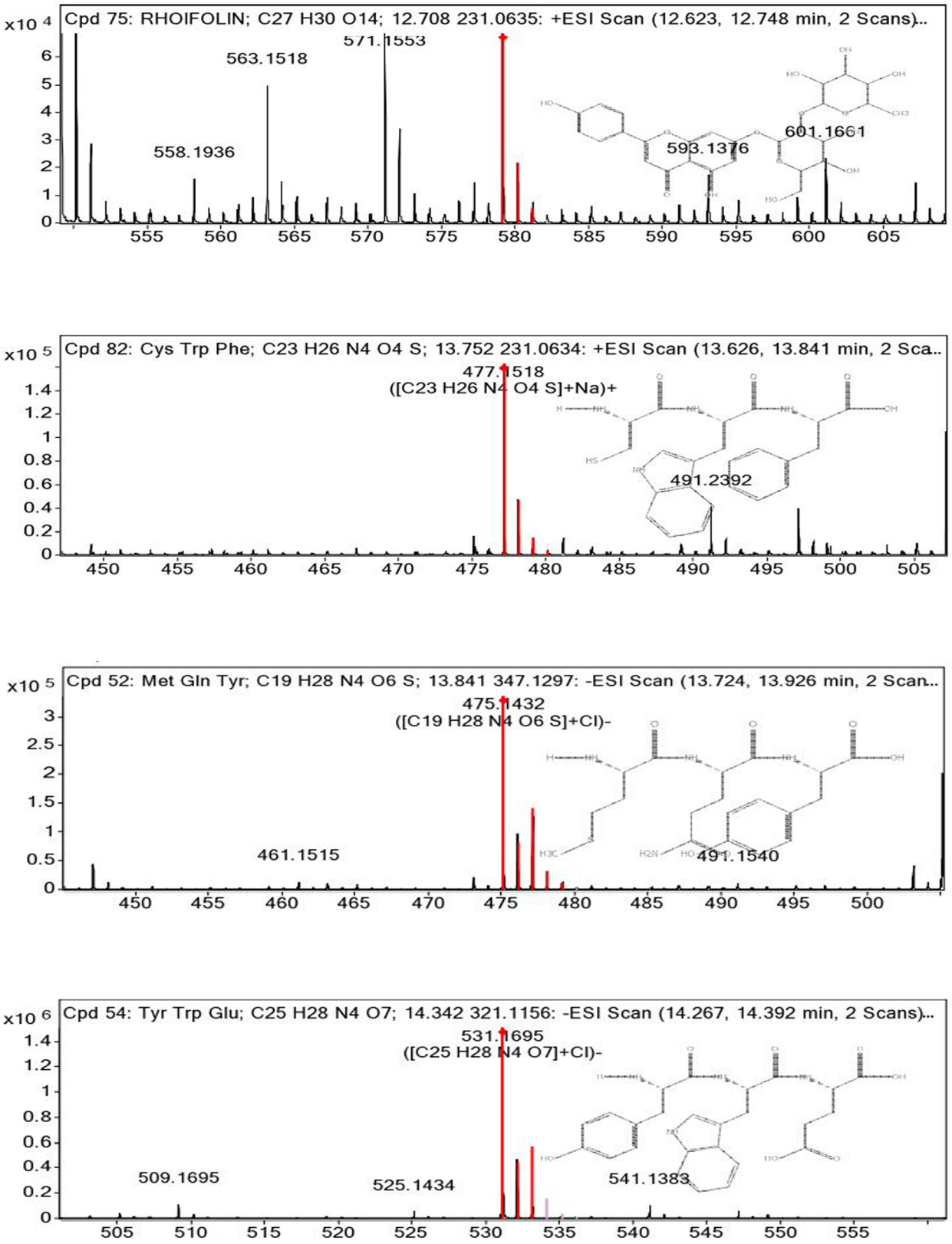



**Figure 22 (xii): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

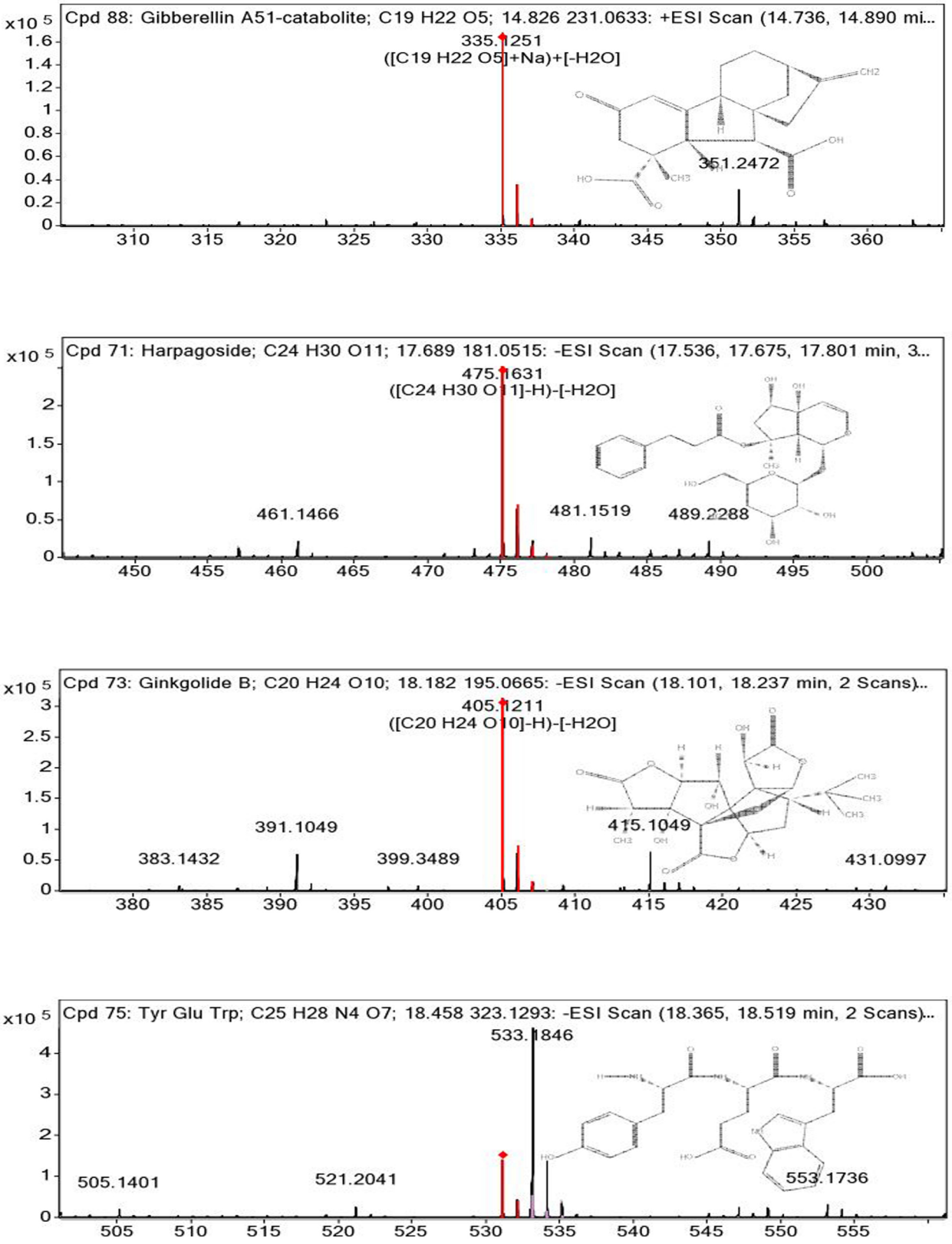



**Figure 22 (xiii): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

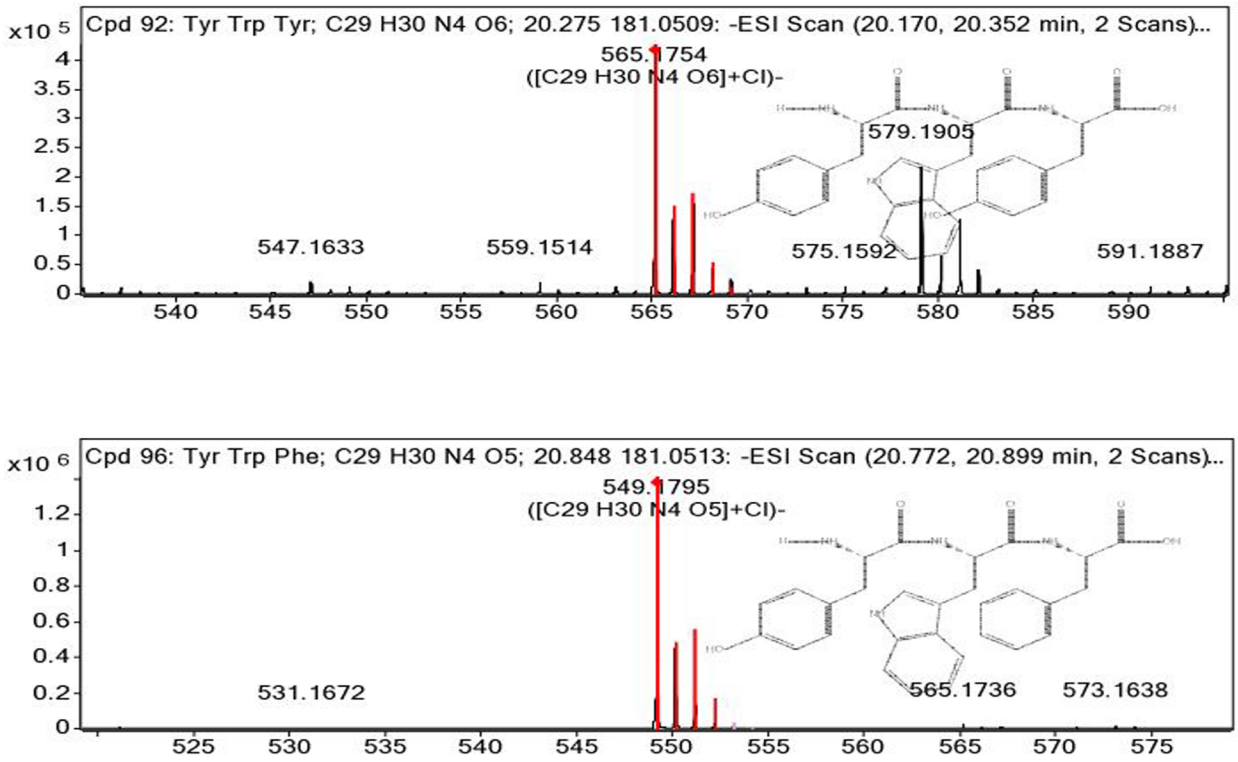



**Figure 22 (xiv): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

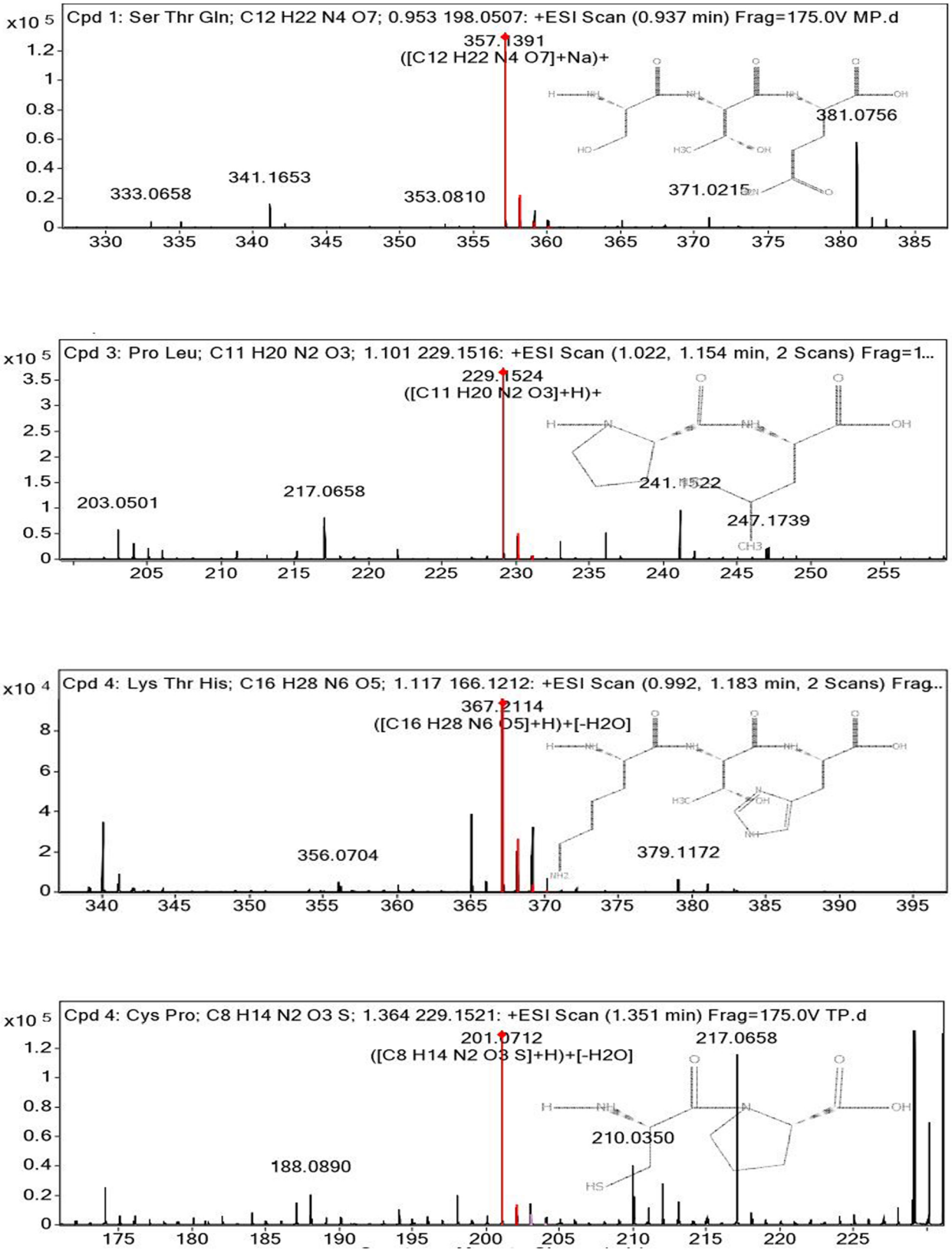



**Figure 22 (i): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

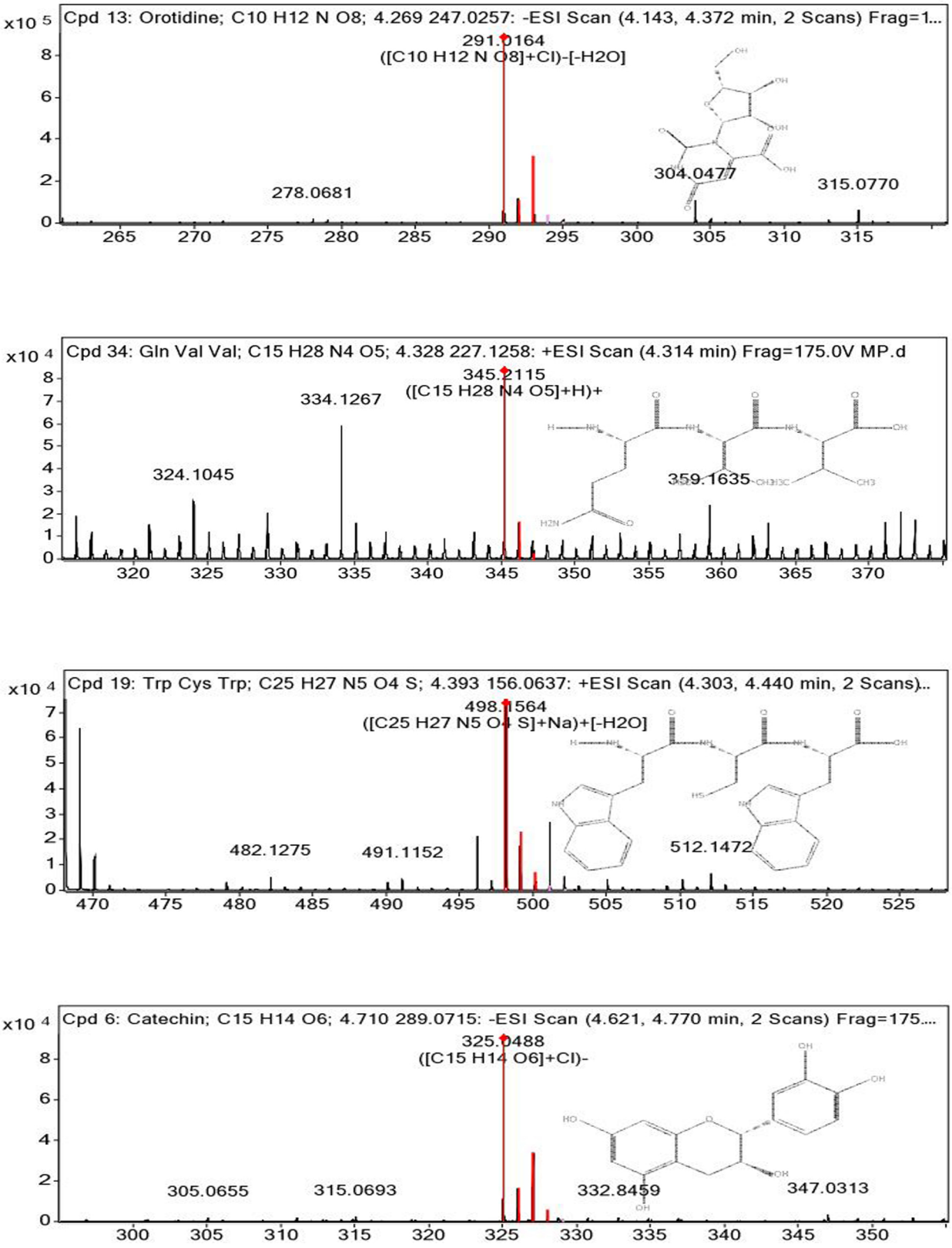



**Figure 22 (v): Mass spectra of compounds detected by HR LC-MS analysis of methanolic extract of selected dye-yielding plants**

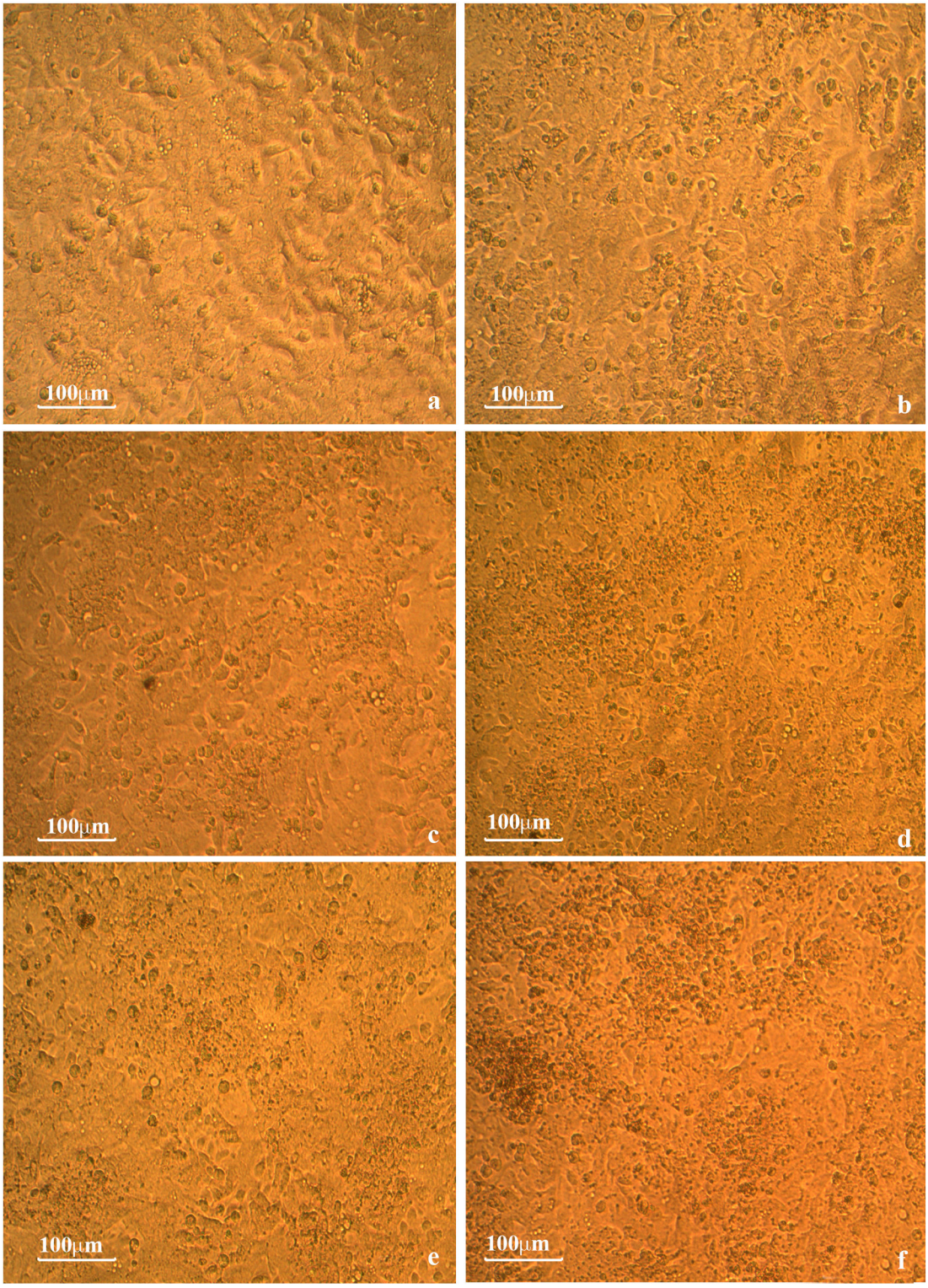



**Plate 2: Hepatotoxic activity of lemon yellow on HepG2 cells - a** Control, **b** 6.25 µg/mL, **c** 12.5 µg/mL, **d** 25 µg/mL, **e 50** µg/mL, **f 100** µg/mL

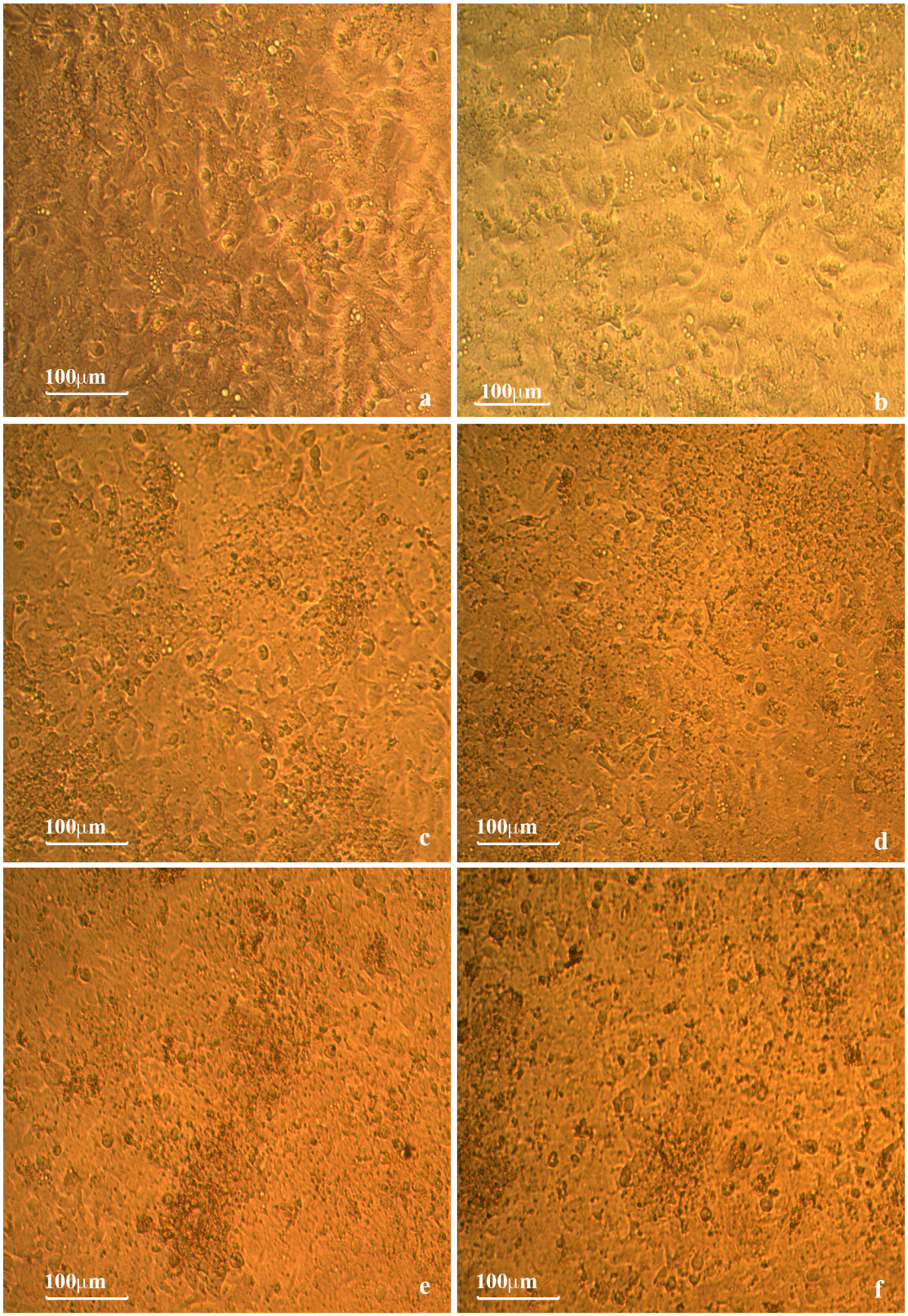



**Plate 3: Hepatotoxic activity of methanolic fruit extract of *T. paniculata* on HepG2 cells - a** Control, **b** 6.25 µg/mL, **c** 12.5 µg/mL , **d** 25 µg/mL, **e** 50 µg/mL, **f** 100 µg/mL

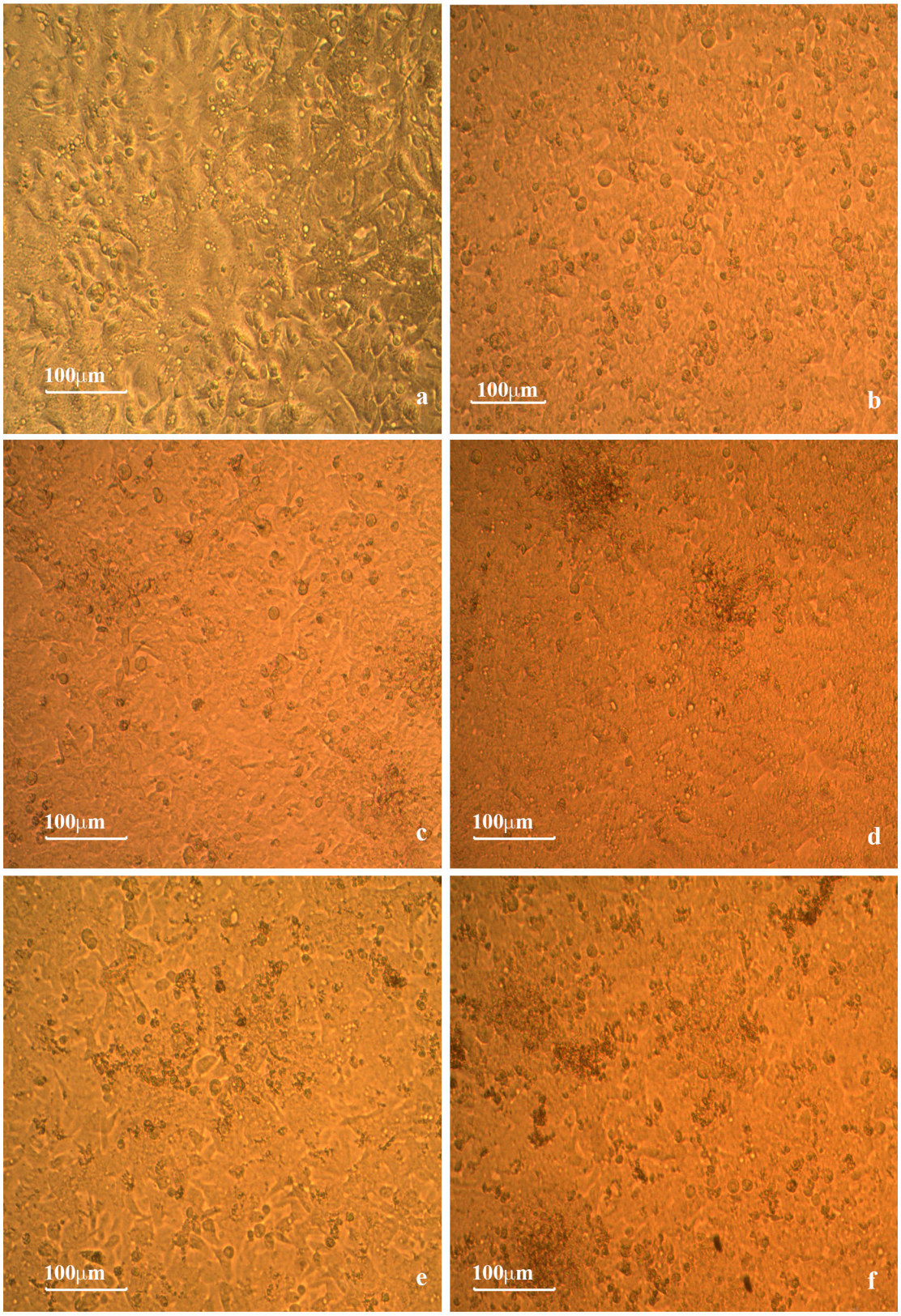



**Plate 4: Hepatoprotective activity exhibited by the methanolic fruit extract of *T. paniculata* on acetaminophen treated HepG2 cells - a** Control, **b** 6.25 µg/mL, **c** 12.5 µg/mL, **d** 25 µg/mL, **e** 50 µg/mL, **f** 100 µg/mL

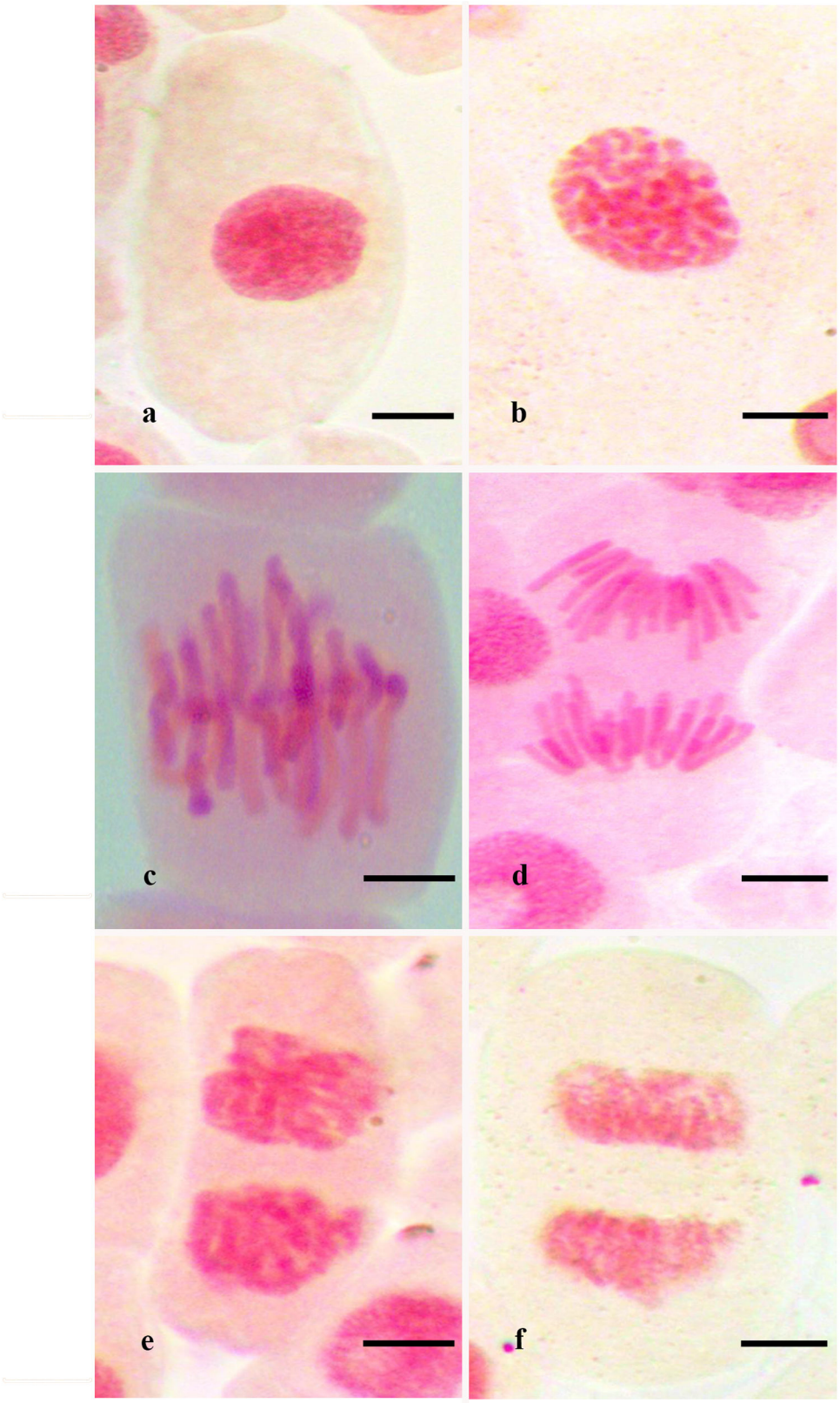



**Plate 5: Normal mitotic stages in *Allium cepa.*** **a** Interphase, **b** Prophase, **c** Metaphase, **d** Anaphase, **e** Telophase, **f** Cytokinesis Bar - 10 µm

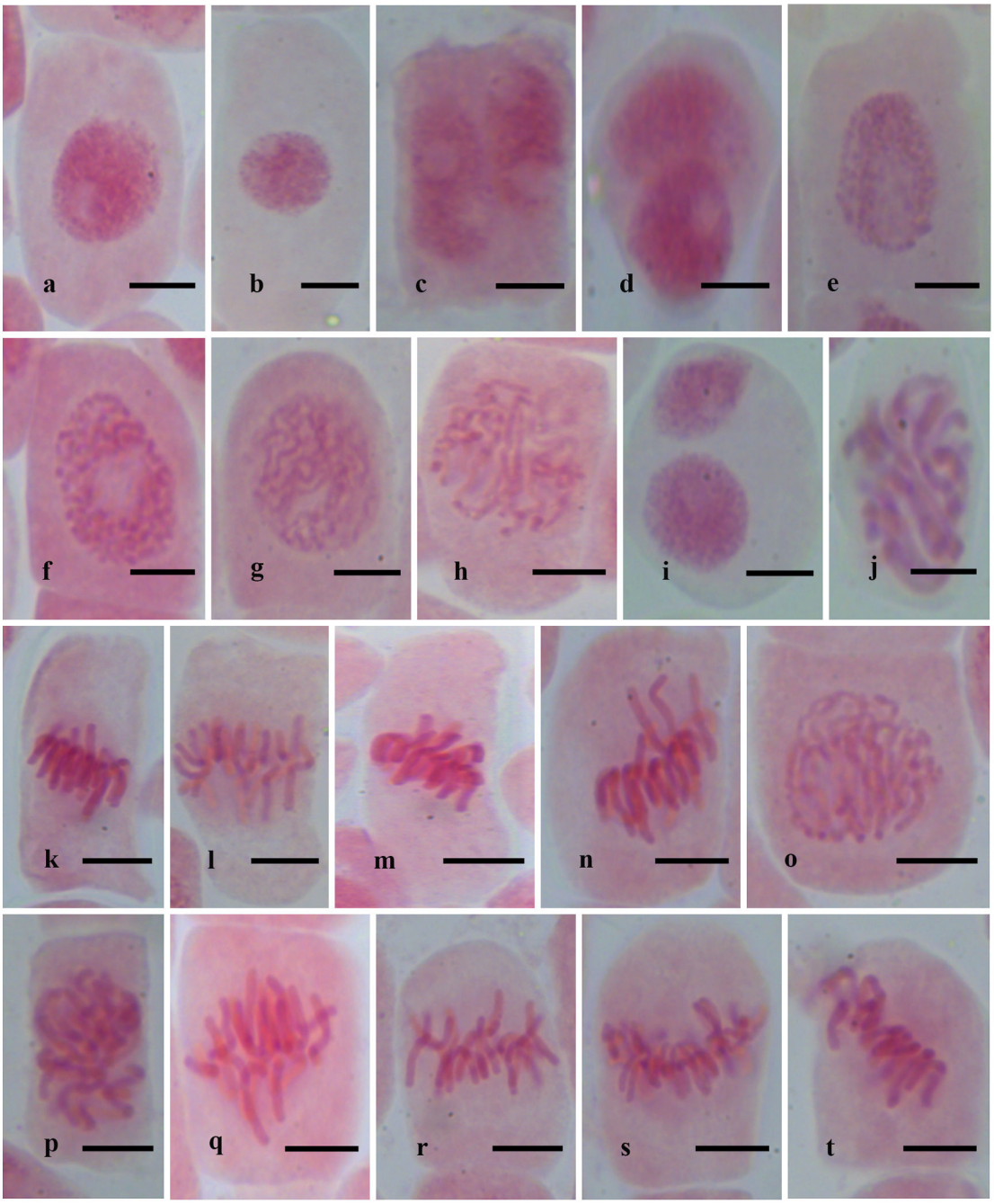



**Plate 6: Chromosomal aberrations induced by the methanolic fruit extract of *T. paniculata* in *A. cepa.* a** Chromatin erosion at interphase, **b** Giant cell showing sticky chromatin at interphase, **c** Pulverized chromatin and nuclear lesion at interphase in a binucleate cell after equitorial separation, **d** Binucleate cell with lesion, **e** Sticky chromatin at prophase, **f** Lesion at prophase, **g** Chromatin erosion at late prophase, **h** Chromatin scaterring at late prophase, **i** Binucleate cell showing chromatin erosion at prophase, **j** Misorientation of chromosomes at metaphase, **k** Chromosome clumping at metaphase, **l** Cytostasis at metaphase, **m** Diagonal metaphase showing clumping, **n** Misorientation at metaphase showing vagrants, **o** Early ball metaphase showing lesions, **p** Pole to pole sticky metaphase, **q** Stellate metaphase, **r** Sticky metaphase, **s** Tropokinesis, **t** Shift in equitorial plate at metaphase Bar 10 µm

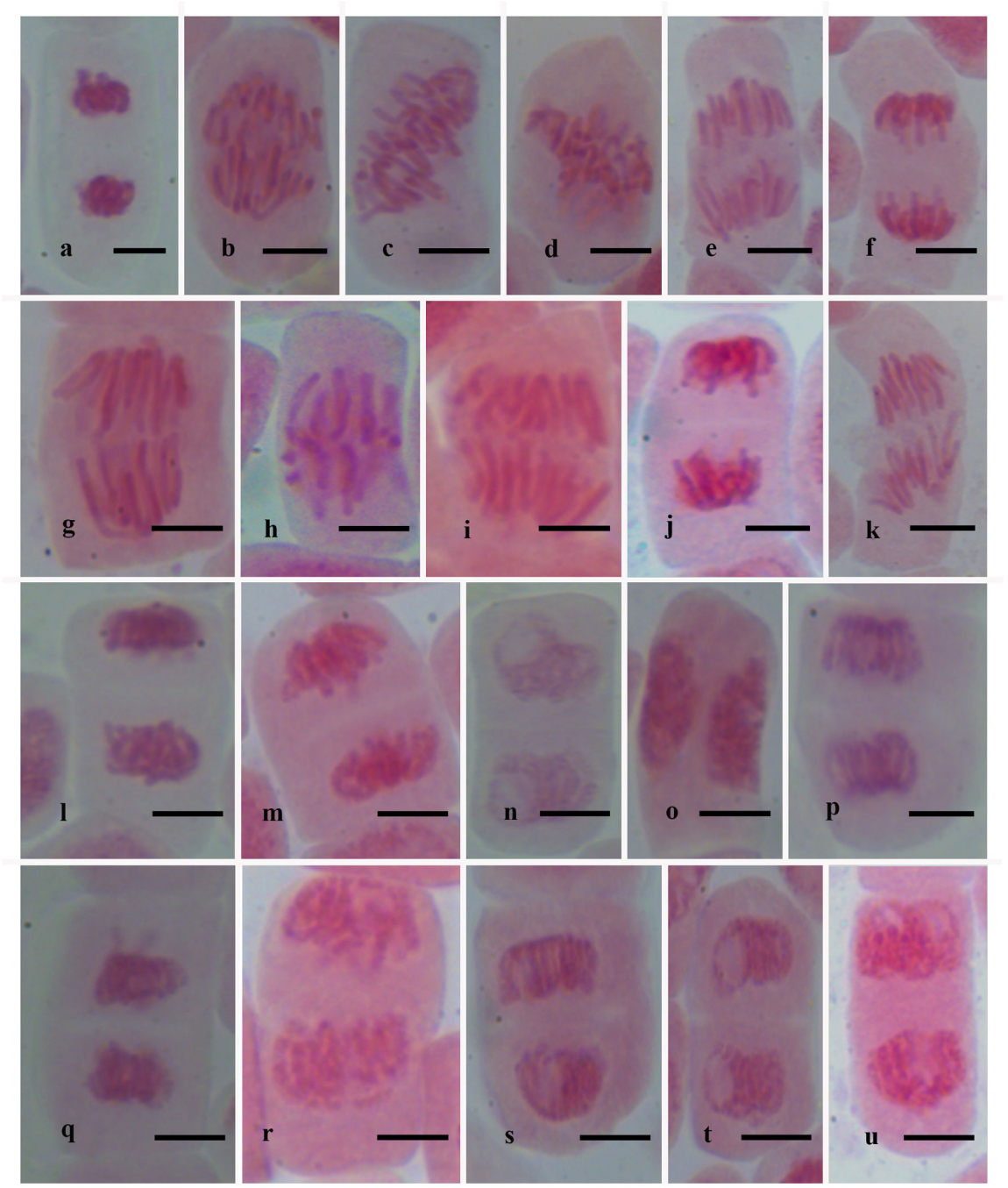



**Plate 7: Chromosomal aberrations induced by the methanolic fruit extract of *T. paniculata* in *A. cepa.*** **a** Coagulated anaphase, **b** Chromosome bridge and lagging chromosome at anaphase, **c** Diagonal early anaphase, **d** Diagonal sticky stathmo anaphase, **e** Disturbed anaphase, **f** Sticky anaphase, **g** Wavy chromosome bridge at anaphase, **h** Shift in MTOC at anaphase, **i** Fragmentation at anaphase, **j** Sticky anaphase showing early cell plate formation, **k** Misorientation of chromosomes showing erosion at anaphase, **l** Sticky telophase, **m** Sticky telophase showing early cell plate formation, **n** Chromosome erosion at early telophase, **o** Pulverized chromosomes at telophase after equatorial separation, **p** Binucleate cell at late telophase showing pulverized chromatin and lesion, **q** Sticky late telophase, **r** Pulverized chromatin at cytokinesis, **s** Chromatin fragmentation at cytokinesis, **t** Cytokinesis showing chromatin erosion, **u** Cytokinesis showing lesions Bar-10 µm

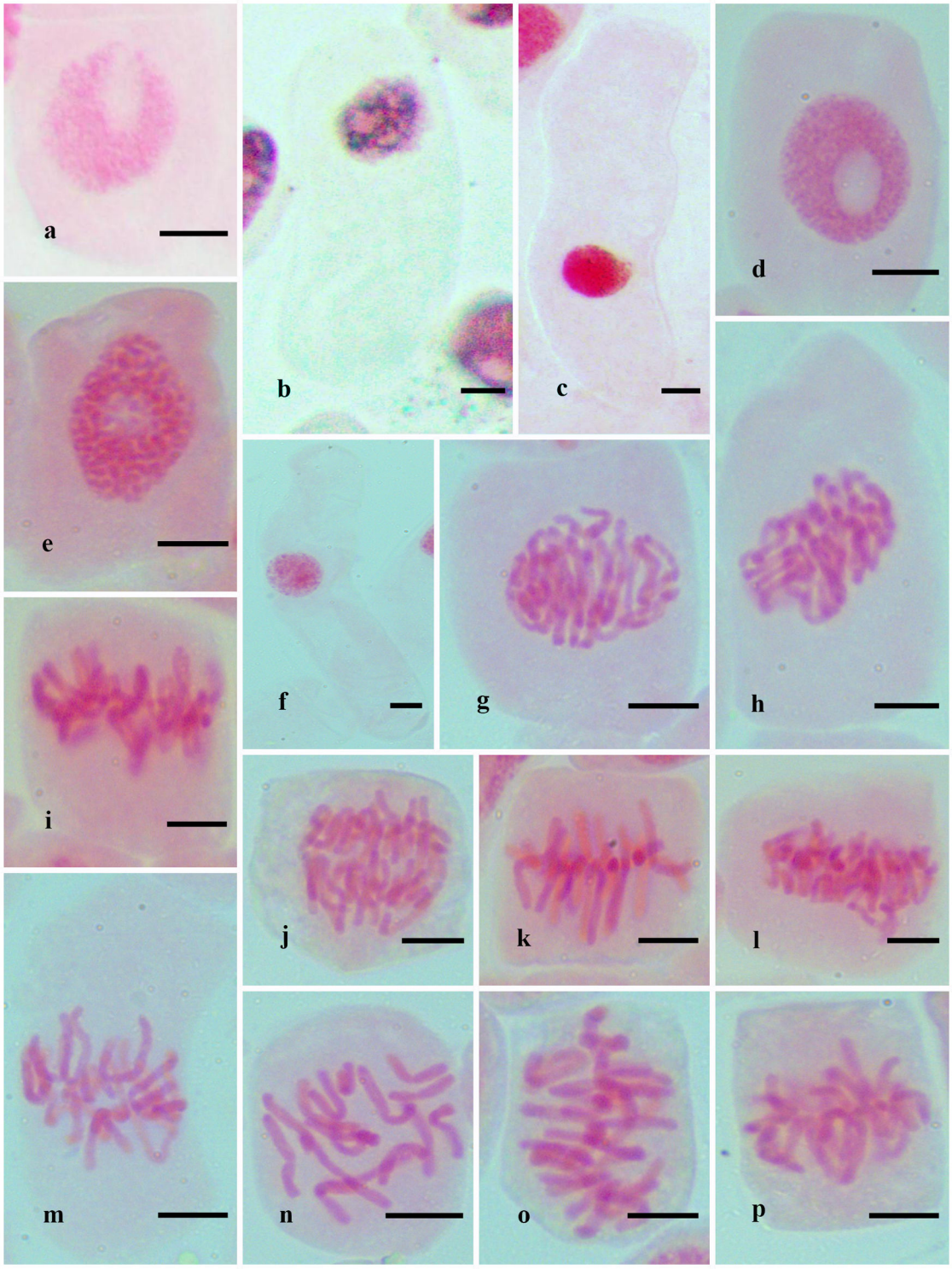



**Plate 8: Chromosomal aberrations induced by the methanolic fruit extract of *M. philippensis* in *A. cepa*. a** Nuclear lesion at interphase, **b** Nuclear erosion at interphase **c** Nuclear distortion at interphase in a giant cell **d** Single nuclear lesion at early prophase **e** Formation of lesion at late prophase **f** Pulverised chromatin at prophase in a giant cell **g** Ball metaphase**, h** Chained metaphase**, i** Chained sticky metaphase, **j** Misorientaion at metaphase, **k** Disturbed metaphase with vagrants, **l** Metaphase in a hypoploid cell, **m** Metaphase showing vagrants in a hyperploid cell, **n** Scattered metaphase, **o** Pole to pole metaphase, **p** Stellate metaphase Bar-10 µm

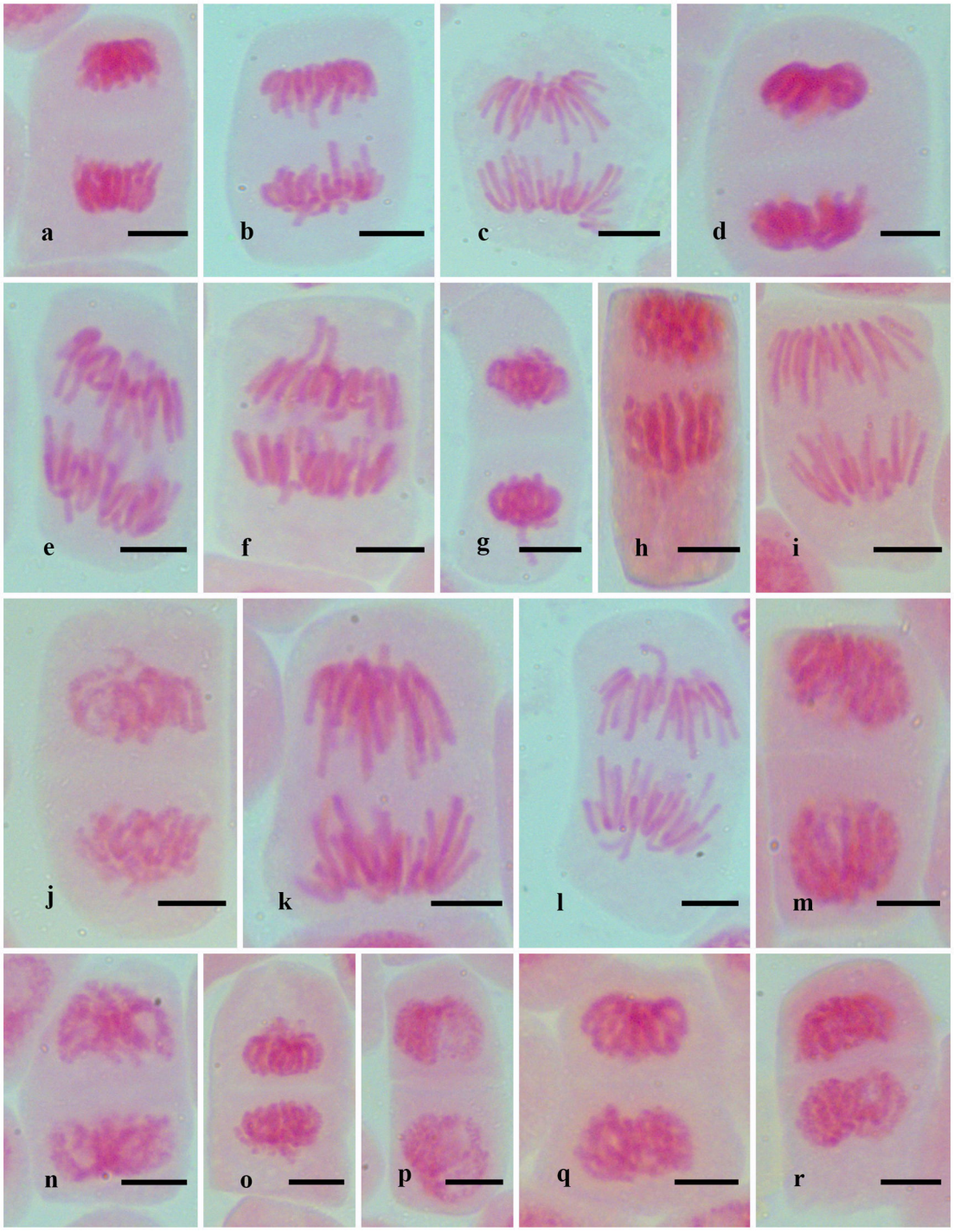



**Plate 9: Chromosomal aberrations induced by the methanolic fruit extract of *M. philippensis* in *A. cepa*. a** Sticky anaphase, **b** Chromosome fragments at anaphase, **c** Chromosome disintegration at anaphase, **d** Coagulated anaphase, **e** Diagonal anaphase showing sticky bridges, **f** Chromosome laggards and vagrants at anaphase, **g** Hemi-lolipop anaphase, **h** Coagulated anaphase and fragmentation in one chromosome group, **i** Scattered anaphase, **j** Pulverised anaphase, **k** Unequal separation at anaphase, **l** Vagrants at anaphase, **m** Chromosome erosion at telophase, **n** Pulverised chromatin at telophase, **o** Chromosome erosion at cytokinesis, **p** Formation of lesion at cytokinesis, **q** Sticky chromatin at cytokinesis, **r** Macro and micro cell at cytokinesis Bar-10 µm

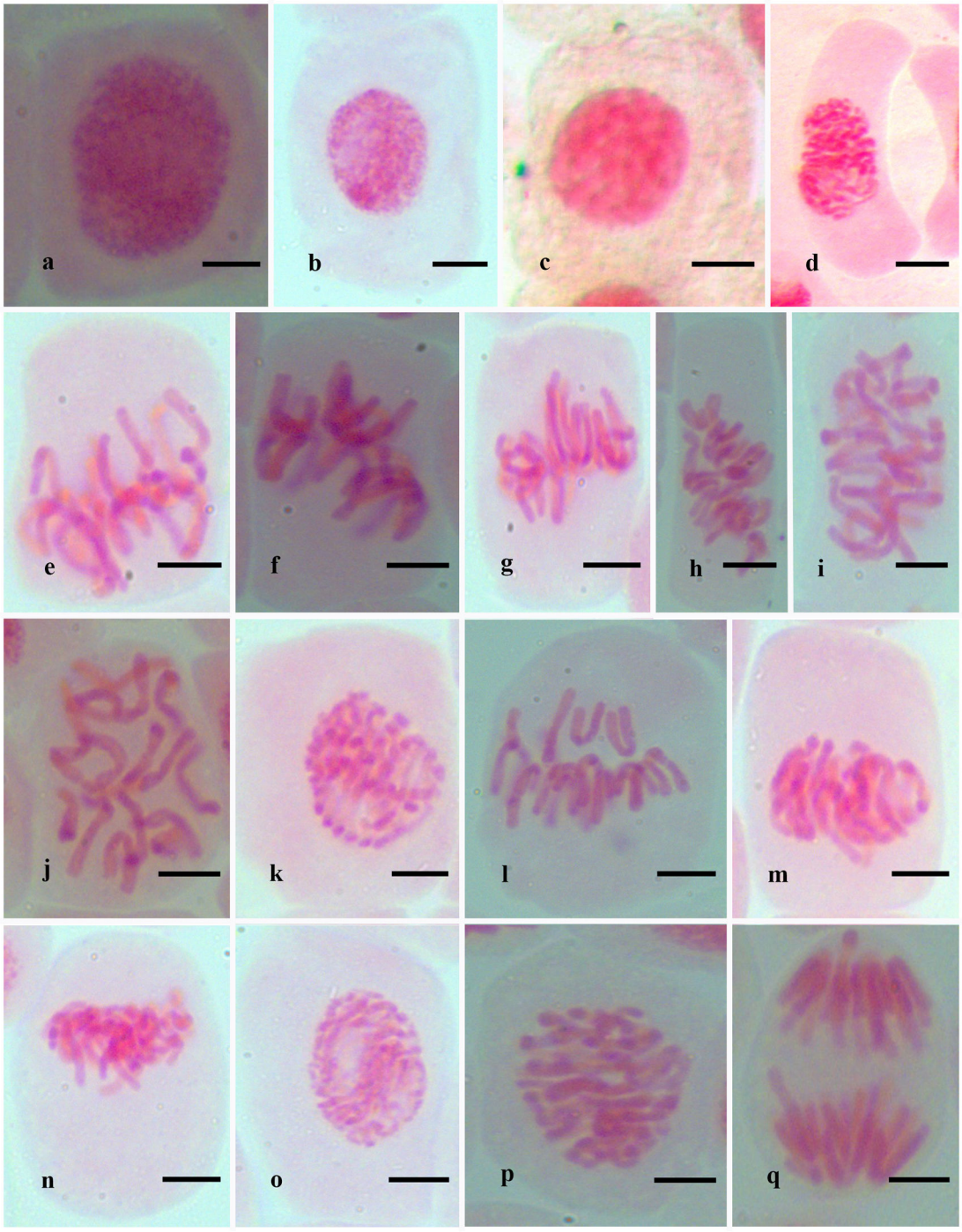



**Plate 10 : Chromosomal aberrations induced by the methanolic bark extract of *A. odoratissima* in *A. cepa.*** **a** Formation of lesion at interphase; **b** Nuclear erosion at late interphase; **c** Coagulated prophase; **d** Abnormal condensation at prophase; **e** Diagonal metaphase; **f** Diagonal disturbed metaphase; **g** Tropokinesis at metaphase; **h** Diagonal partial sticky c - metaphase; **i** Pole-to-pole metaphase; **j** Partial c - metaphase; **k** Ball metaphase showing lesions; **l** Cytostasis in a hypoploid cell; **m** Chained metaphase; **n** Chained metaphase showing vagrants; **o** Ball metaphase in a hyperploid cell showing chromosome erosion; **p** Chained sticky metaphase; **q** Sticky anaphase showing vagrants Bar-10µm

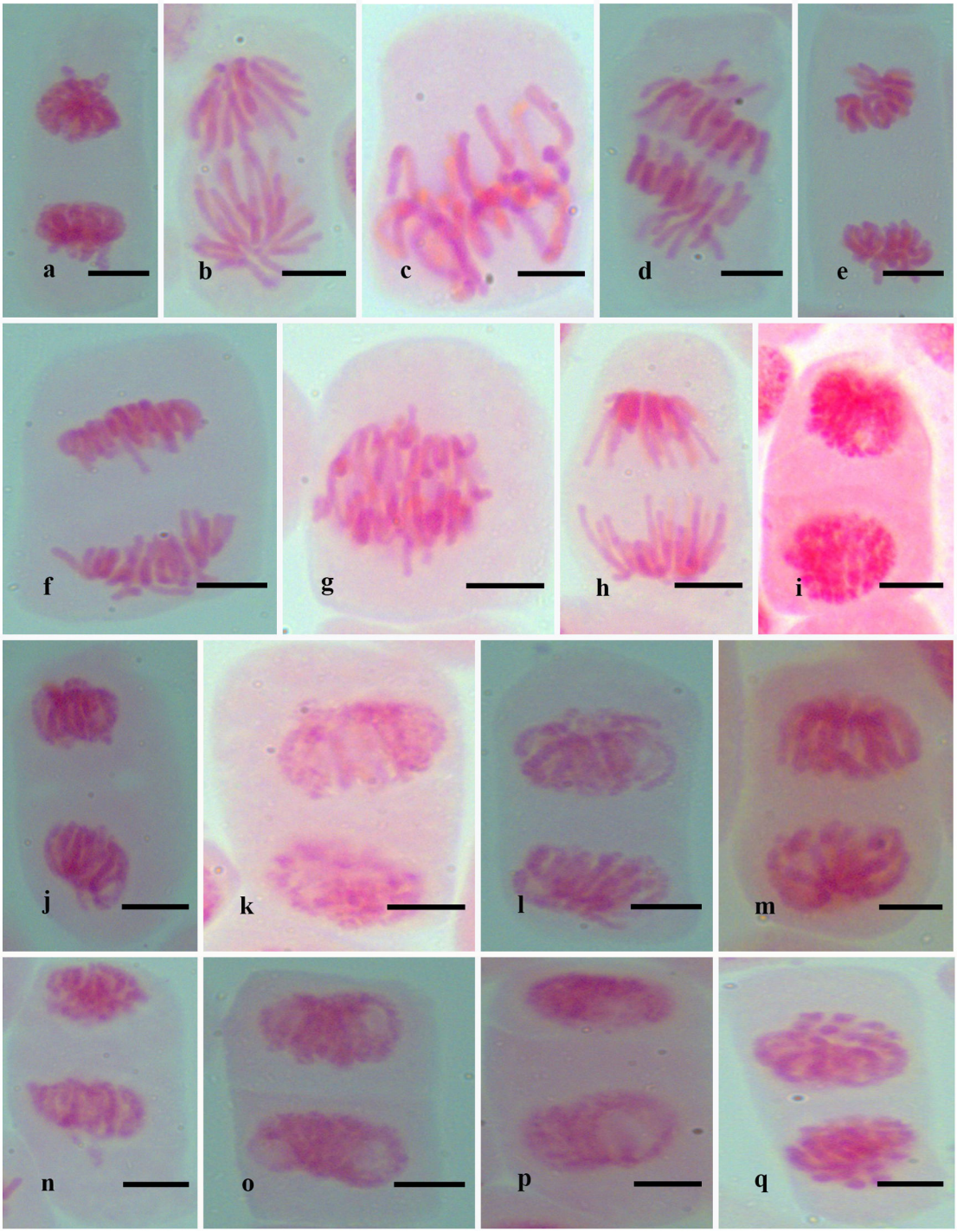



**Plate 11: Chromosomal aberrations induced by the methanolic bark extract of *A. odoratissima* in *A. cepa.*** **a** Ball anaphase showing vagrants; **b** Chromosome bridges at anaphase with one group showing stellate arrangement; **c** Diagonal anaphase; **d** Diagonal anaphase showing vagrants; **e** Disturbed anaphase; **f** Shift in MTOC showing vagrants at anaphase; **g** Sticky stathmo-anaphase; **h** Sticky unequal separation of chromosomes at anaphase; **i** Coagulated chromatin at telophase; **j** Chromosome erosion at late telophase showing shift in MTOC; **k** Chromosome erosion at late telophase; **l** Pulverised chromatin at late telophase; **m** Chromosome erosion at cytokinesis; **n** Pulverised cytokinesis with a chromatin fragment; **o** Chromatin erosion at cytokinesis showing oblique cell plate; **p** Macro and micro cell formation at cytokinesis showing lesion; **q** Sticky chromatin at cytokinesis Bar-10µm

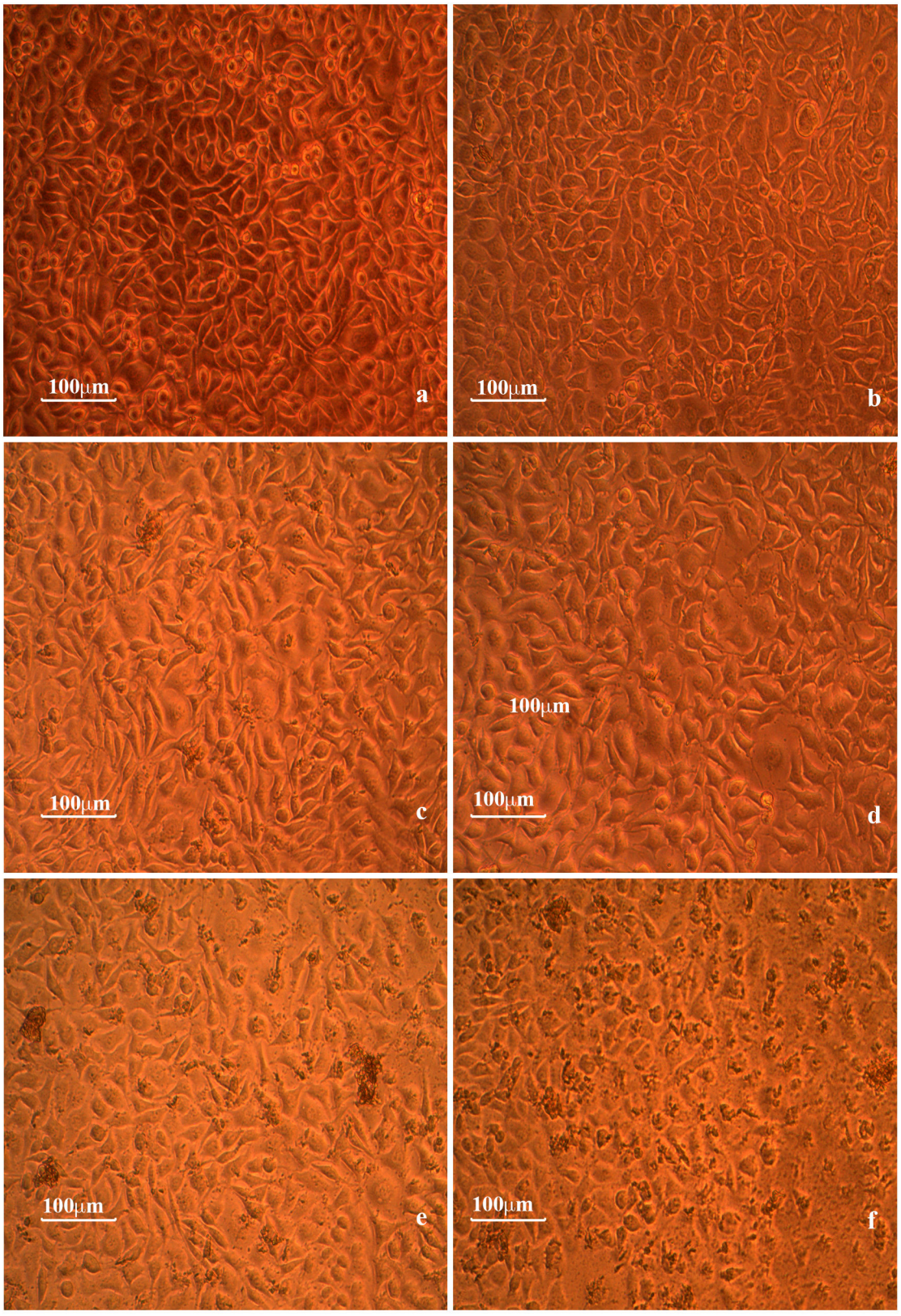



**Plate 12: Effect of methanolic fruit extract of *T. paniculata* on L929 cell lines.**
**a** Control, **b** 6.25 µg/mL, **c** 12.5 µg/mL, **d** 25 µg/mL, **e** 50 µg/mL, **f** 100 µg/mL

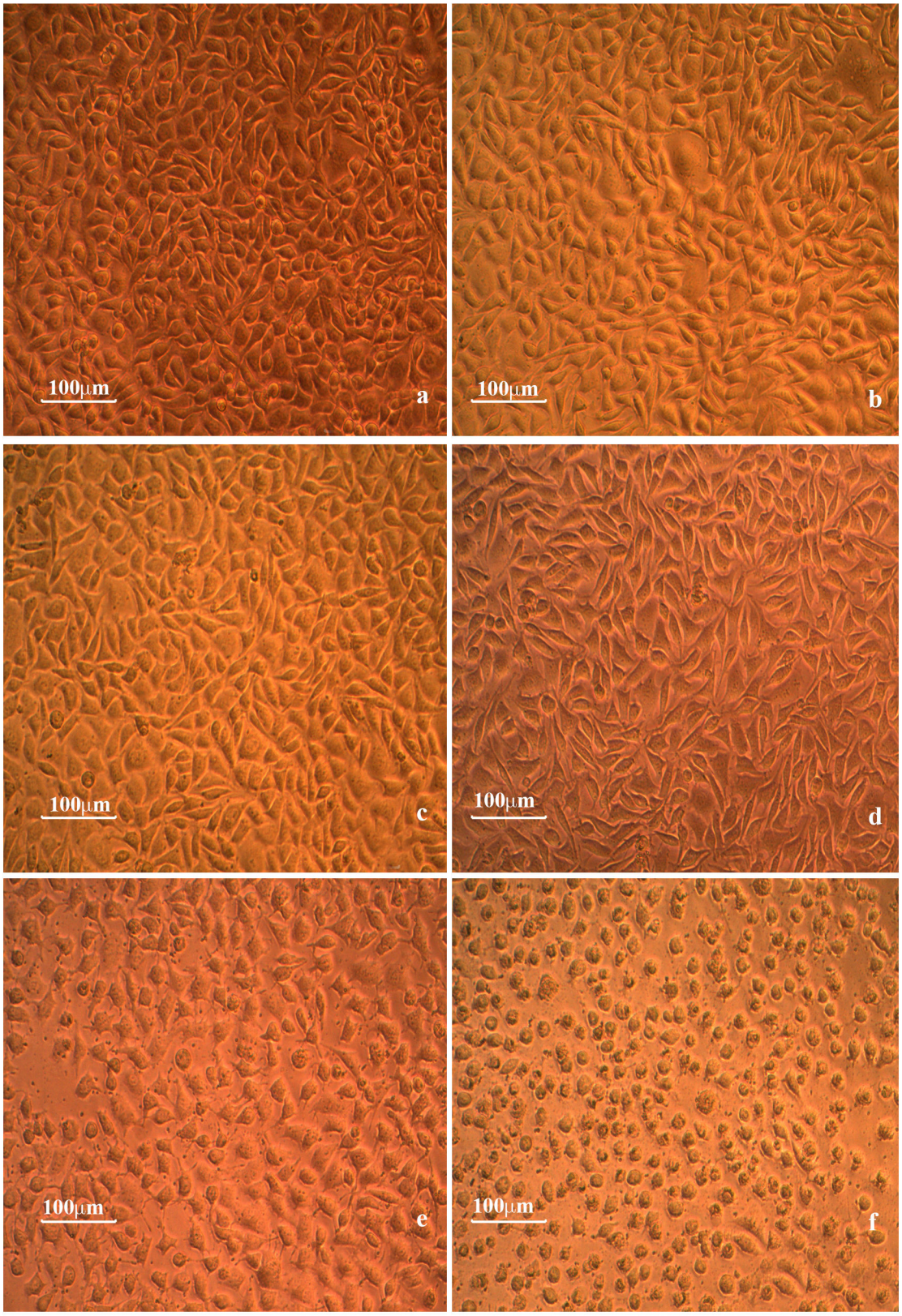



**Plate 13: Effect of methanolic fruit extract of *M. philippensis* on L929 cell lines.**
**a** control, **b** 6.25µg/mL, **c** 12.5 µg/mL, **d** 25 µg/mL, **e** 50 µg/mL, **f** 100 µg/mL

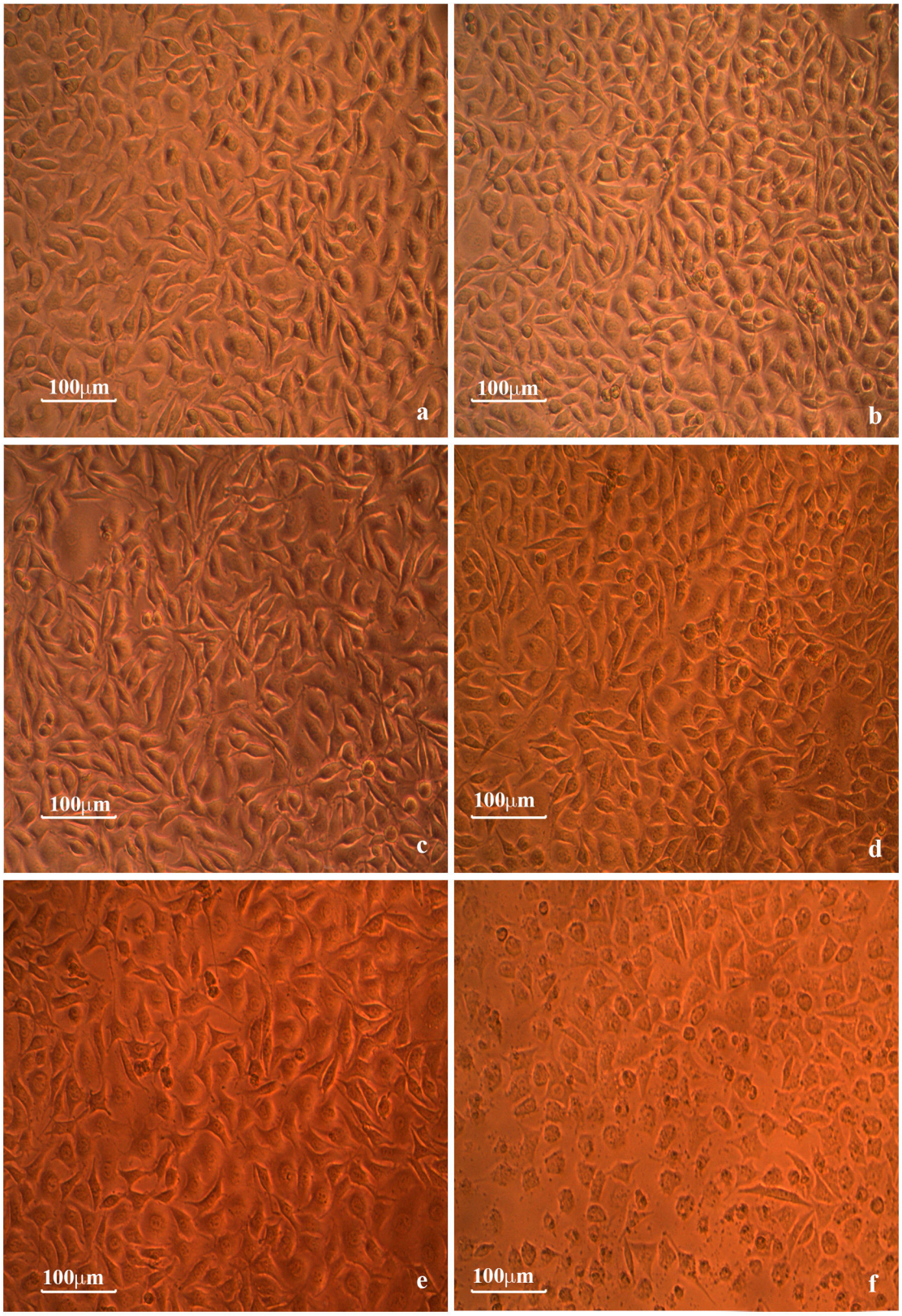



**Plate 14: Effect of methanolic bark extract of *A. odoratissima* on L929 cell lines.**
**a** Control, **b** 6.25 µg/mL, **c** 12.5 µg/mL, **d** 25 µg/mL, **e** 50 µg/mL, **f** 100 µg/mL

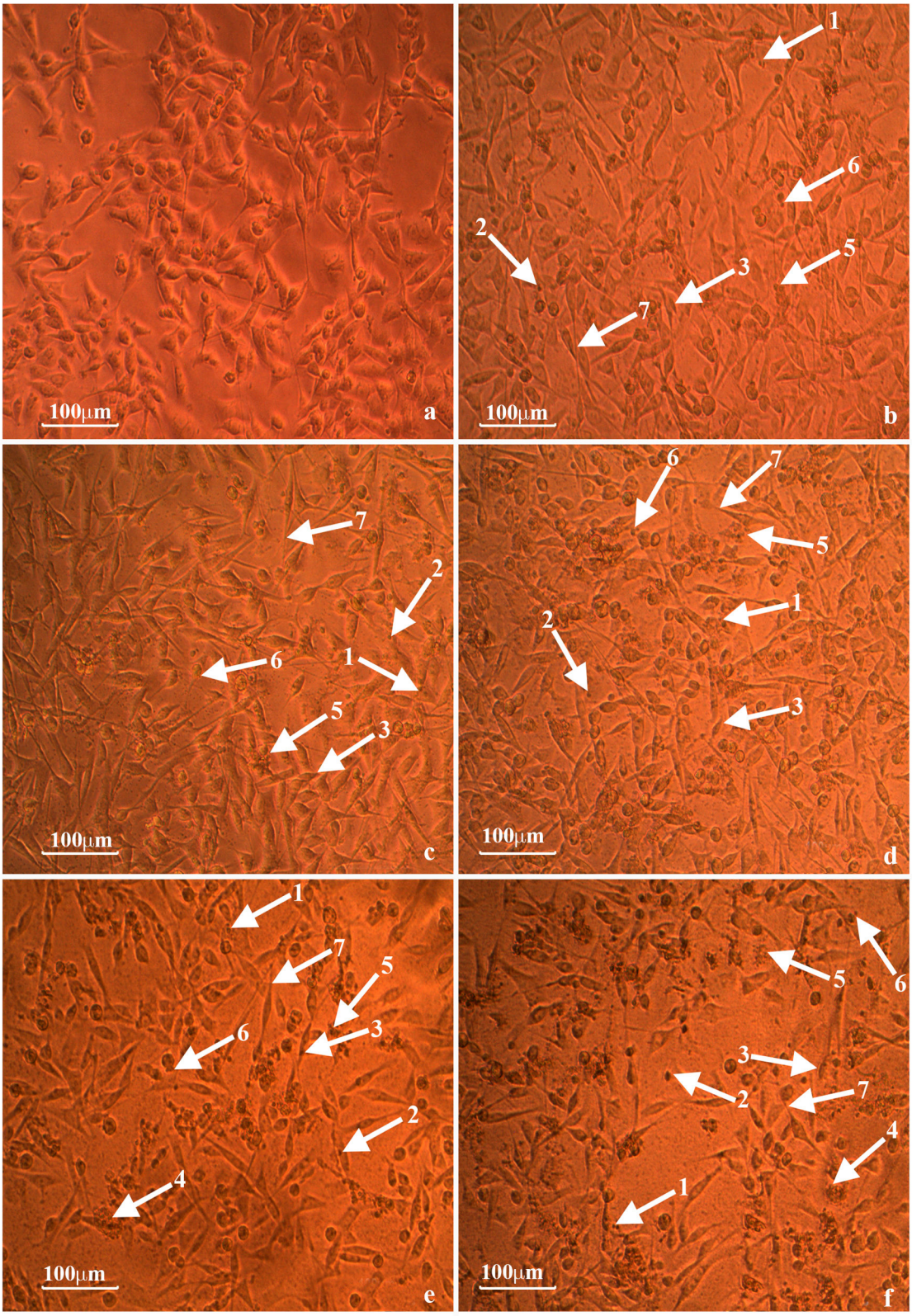



**Plate 15:** ***In vitro*** **cytotoxic effects of diferent concentrations of methanolic fruit extract of** ***T. paniculata*** **on MDA-MB 231 cells. a** Control, **b** 6.25 µg/mL, **c** 12.5 µg/mL, **d** 25 µg/mL, **e** 50 µg/mL, **f** 100 µg/mL; Arrows indicating apoptotic signals **1.** Nuclear fragmentation, **2.** Condensed nuclei, **3.** Cell shrinkage, **4.** Membrane blebbing, **5.** Apoptotic bodies, **6.** Budding, **7.** Echinoid spikes

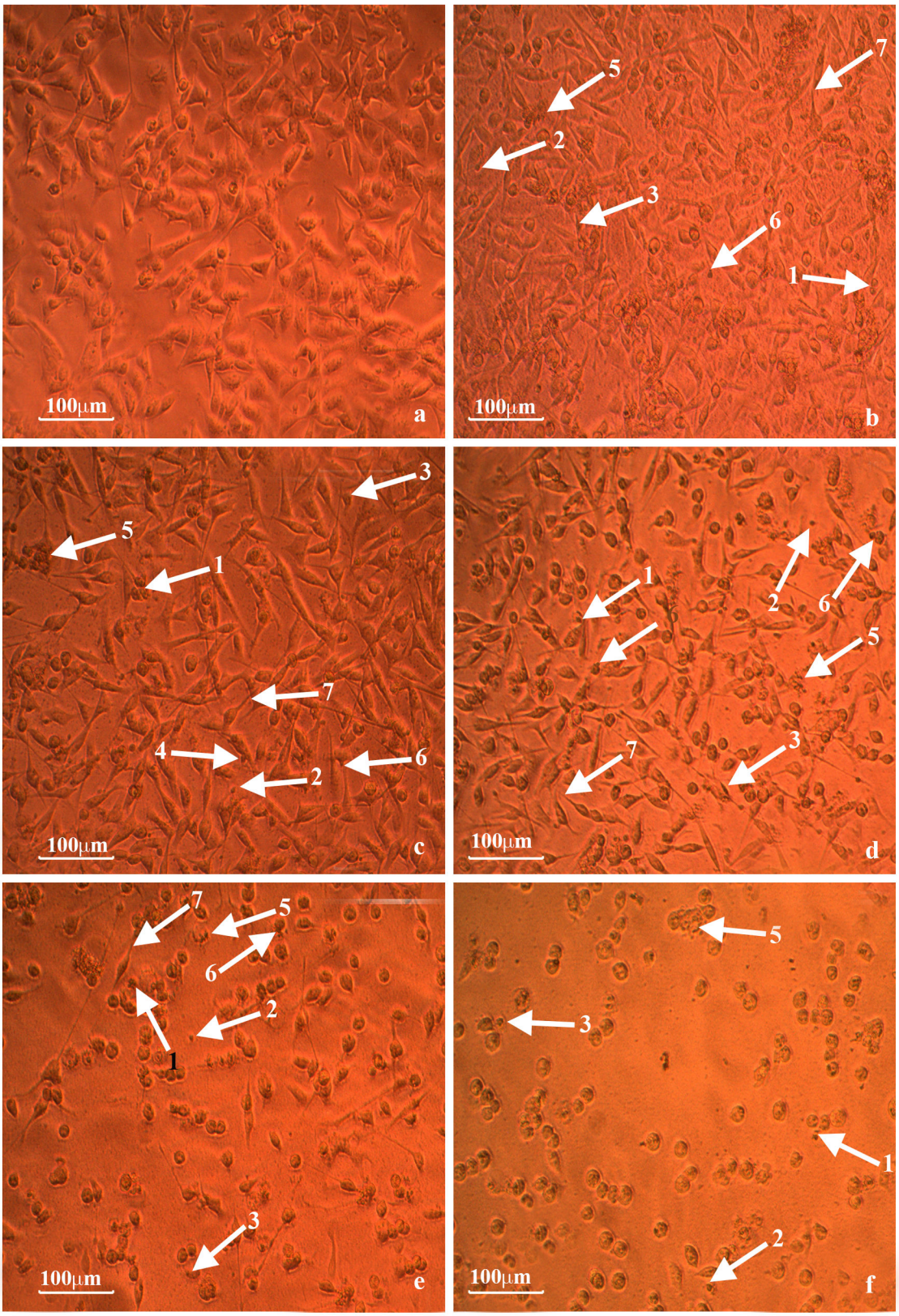



**Plate 16:** ***In vitro*** **cytotoxic effects of different concentrations of methanolic fruit extract of** ***M. philippensis*** **on MDA-MB 231 cells. a** Control, **b** 6.25 µg/mL, **c** 12.5 µg/mL, **d** 25 µg/mL, **e** 50 µg/mL, **f** 100 µg/mL ; Arrows indicating apoptotic signals **1.** Nuclear fragmentation, **2.** Condensed nuclei, **3.** Cell shrinkage, **4.** Membrane blebbing, **5.** Apoptotic bodies, **6.** Budding, **7.** Echinoid spikes

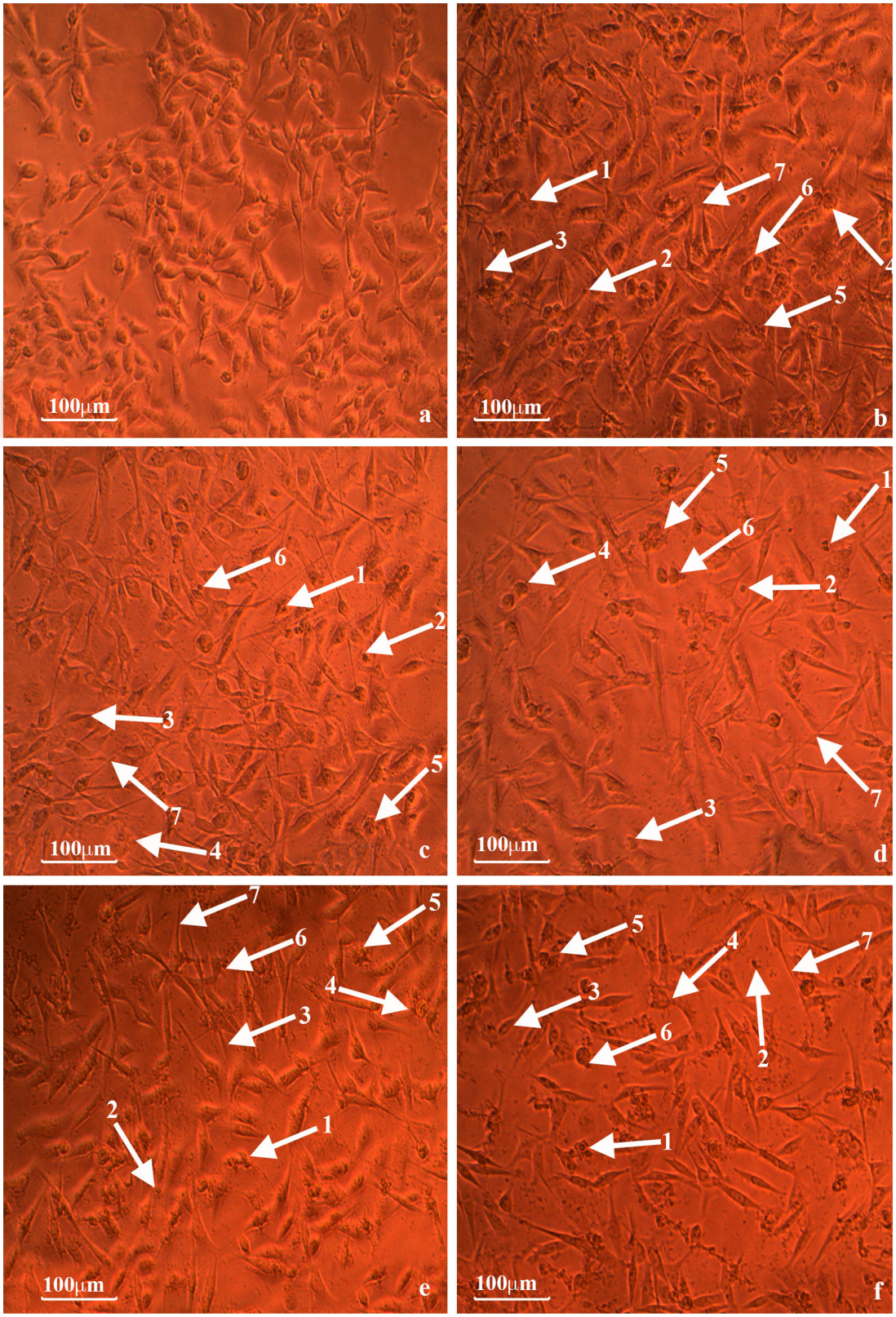



**Plate 17:** ***In vitro*** **cytotoxic effects of different concentrations of methanolic bark extract of** ***A. odoratissima*** **on MDA-MB 231 cells. a** Control, **b** 6.25 µg/mL, **c** 12.5 µg/mL, **d** 25 µg/mL, **e** 50 µg/mL, **f** 100 µg/mL; Arrows indicating apoptotic signals **1.** Nuclear fragmentation, **2.** Condensed nuclei, **3.** Cell shrinkage, **4.** Membrane blebbing, **5.** Apoptotic bodies, **6.** Budding, **7.** Echinoid spikes

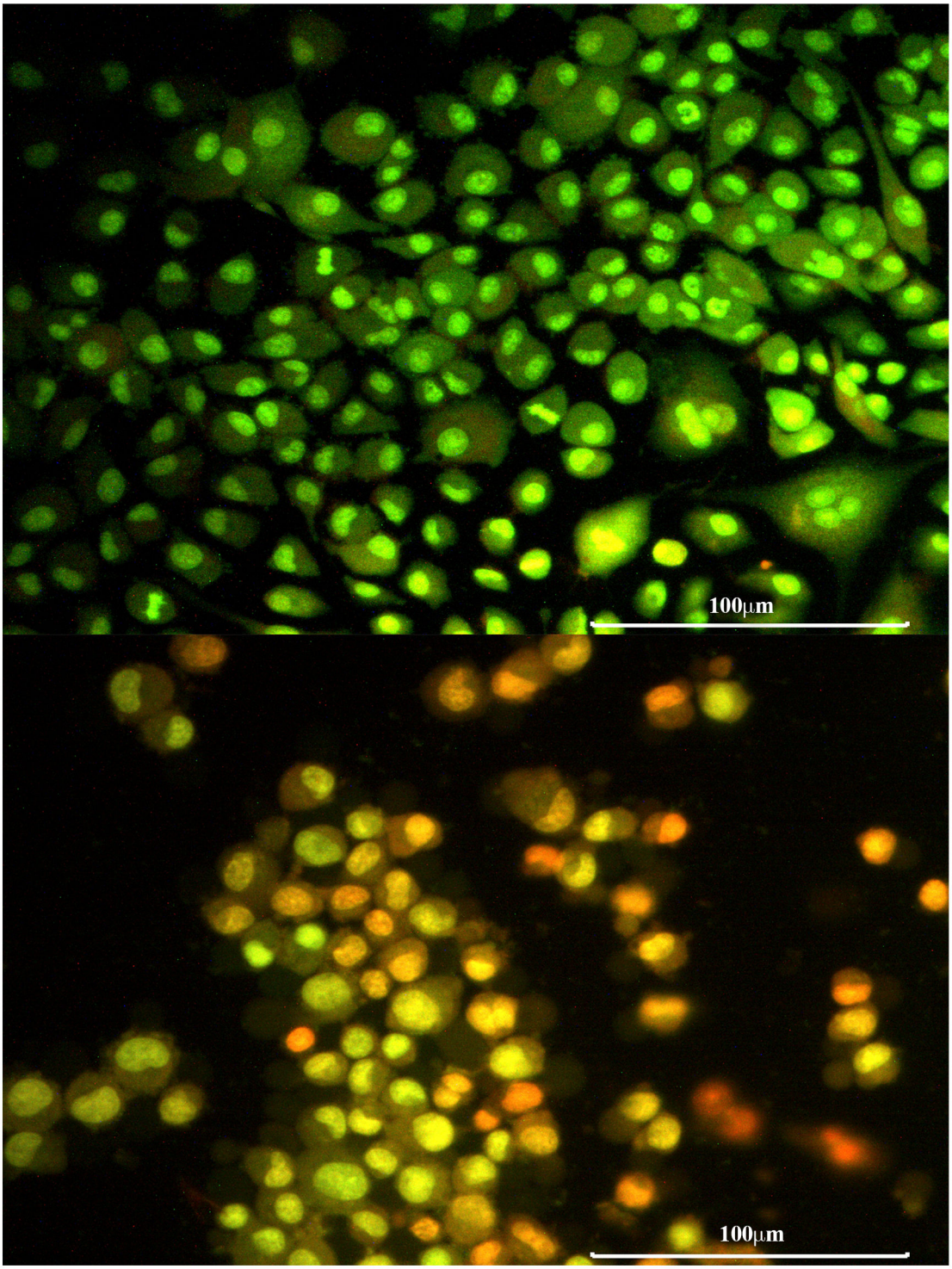



**Plate 18: Detection of apoptosis by AO/EtBr staining on MDA-MB 231 cells. a** Control, **b** Cells treated with methanolic fruit extract of *M. philippensis* (61.5 ± 1.19 μg/mL)

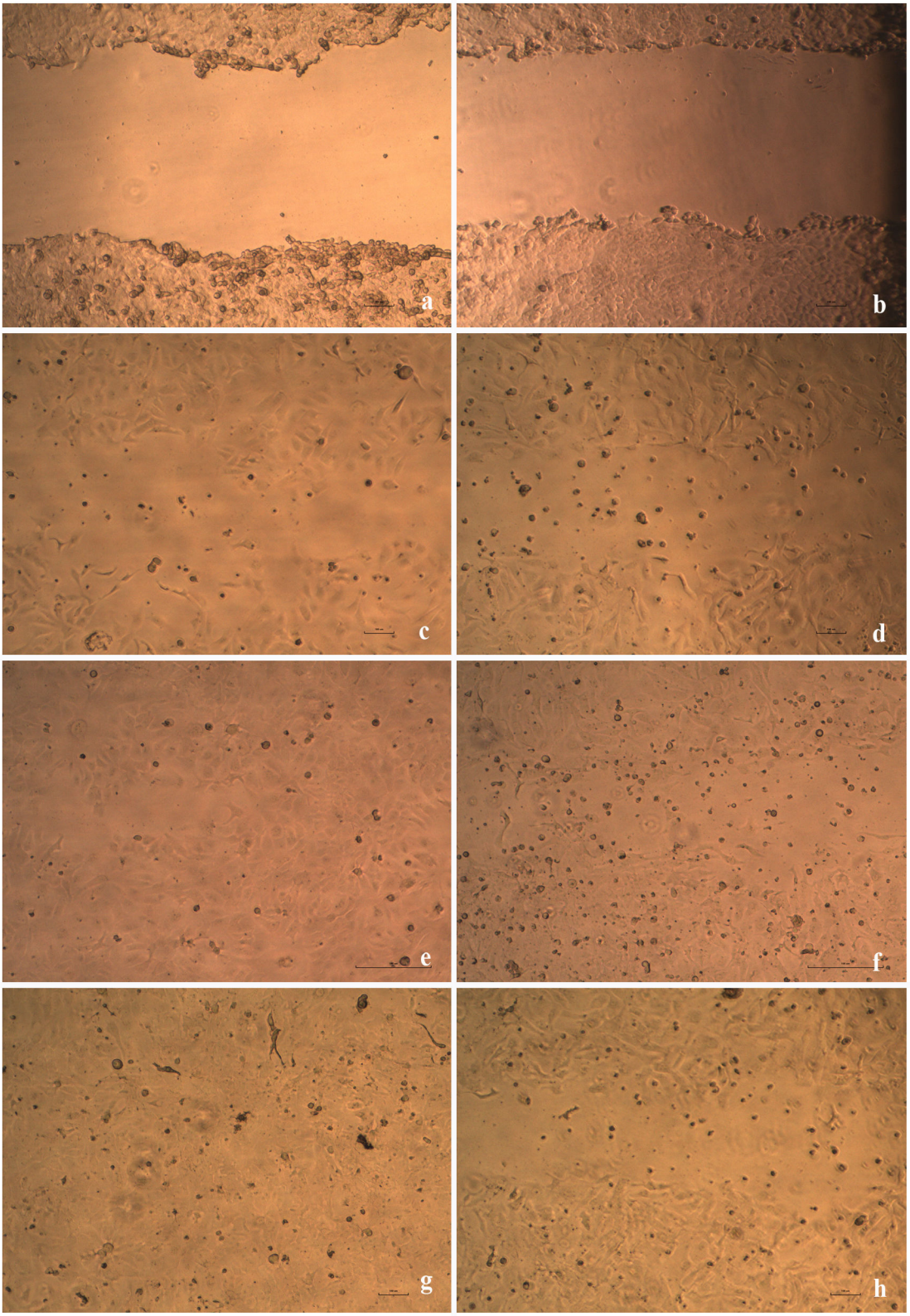



**Plate 19: Cell migration assay - a** Untreated control cells (0 h),**b** MDA-MB 231 cells exposed with MP (0 h), **c** Untreated control cells (24 h), **d** MDA-MB 231 cells exposed with MP (24 h), **e** Untreated control cells (48 h), **f** MDA-MB 231 cells exposed with MP (48 h), **g** Untreated control cells (72 h), **h** MDA-MB 231 cells exposed with MP (72 h)

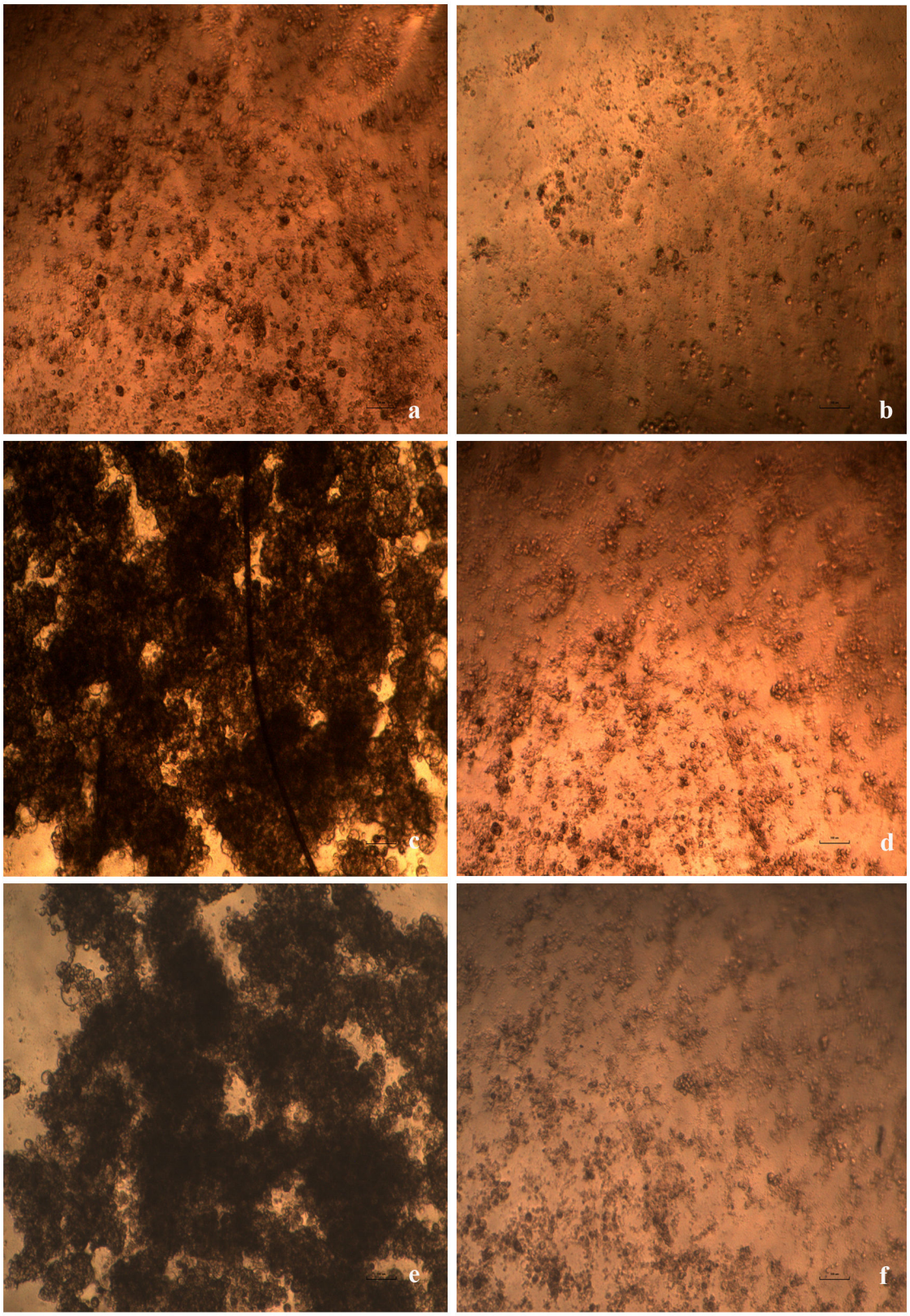
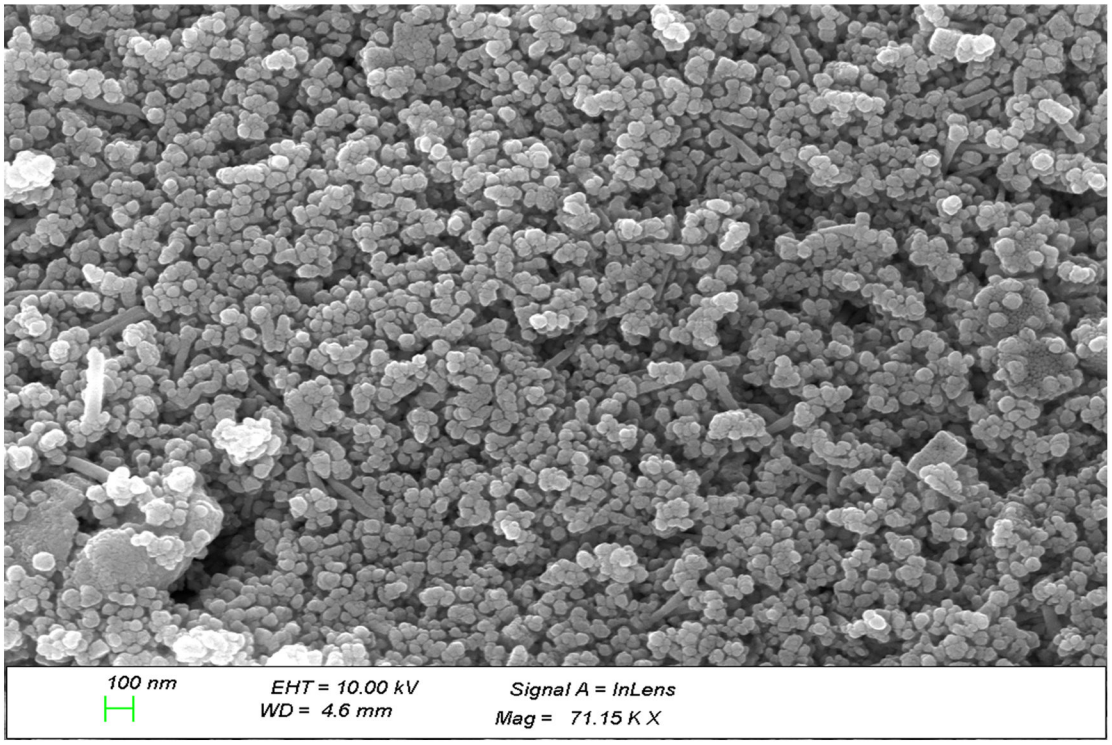



**Plate 20: Cell aggregation assay** - **a** Untreated control cells (0 h), **b** MDA-MB 231 cells exposed with MP (0 h), **c** Untreated control cells (24 h), **d** MDA-MB 231 cells exposed with MP (24 h), **e** Untreated control cells (48 h), **f** MDA-MB 231 cells exposed with MP (48 h)


**Plate 21: Green synthesis of silver nanoparticles from selected dye-yielding plants** - **a, b, c** - TP, MP, AO solutions respectively; **d, e, f** - 2mM silver nitrate solution; **g, h, i** - TP, MP, AO solutions after the synthesis of silver nanoparticles respectively; TP - *Terminalia paniculata*, MP - *Mallotus philippensis*, AO - *Albizia odoratissima*



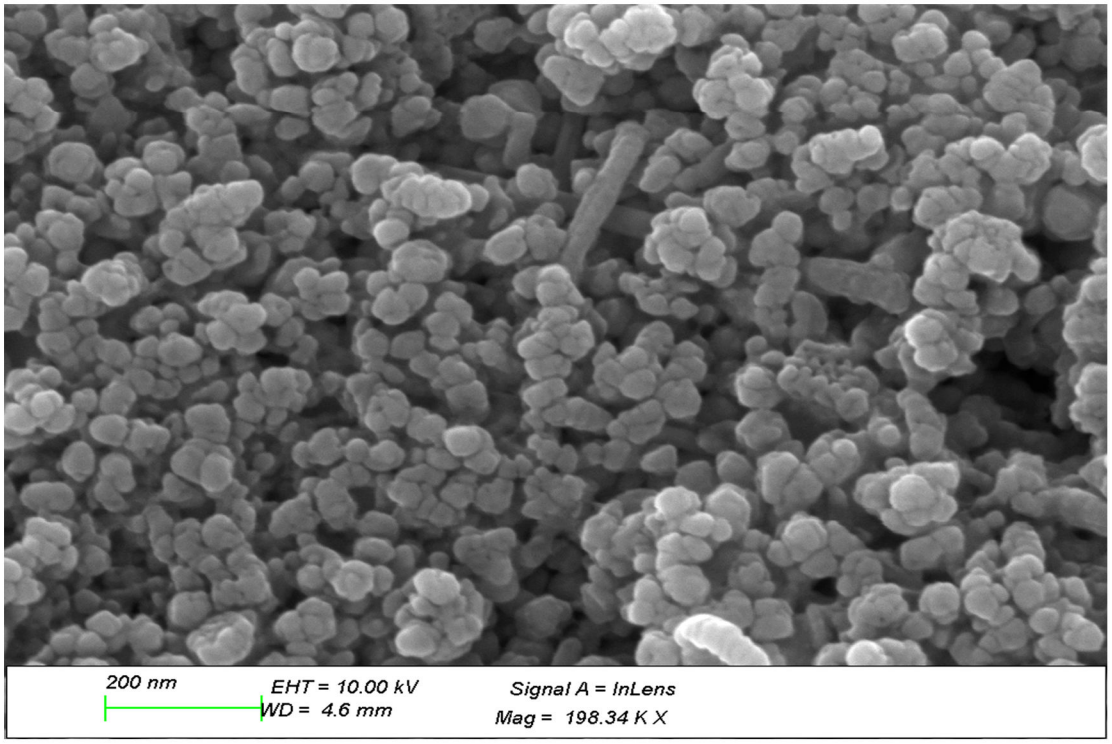
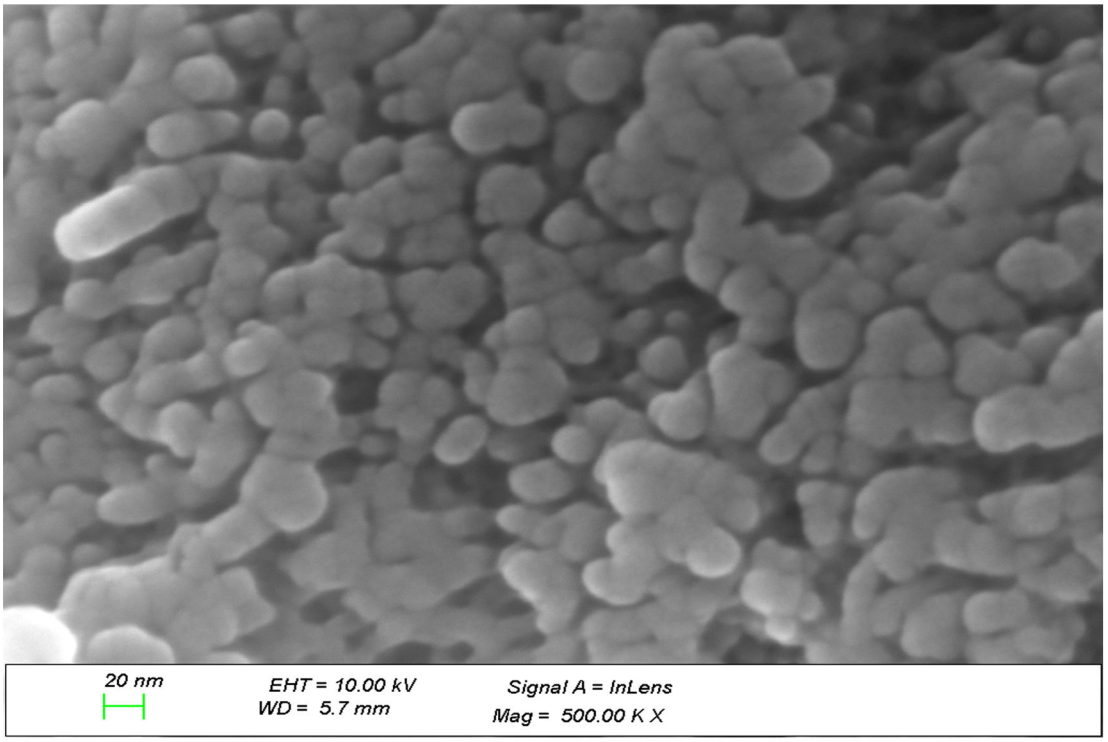




**Plate 22: Scanning electron micrographs of silver nanoparticles synthesized using *T. paniculata* fruit extract. a** Low magnification, **b** Higher magnification



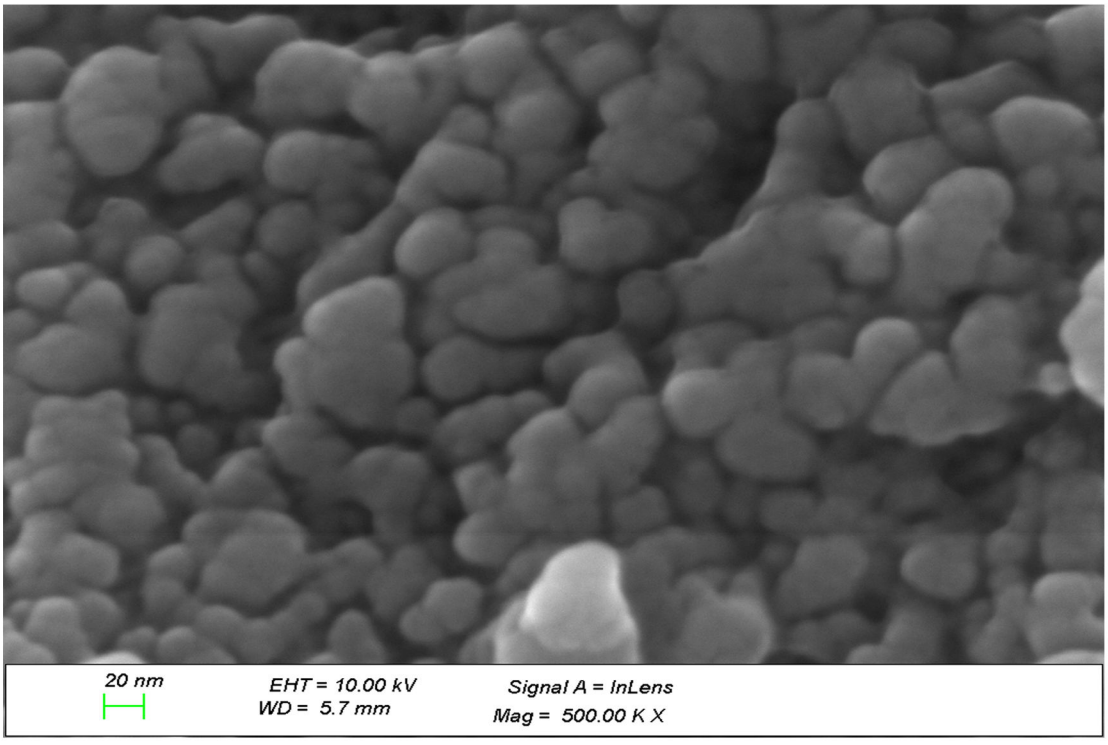
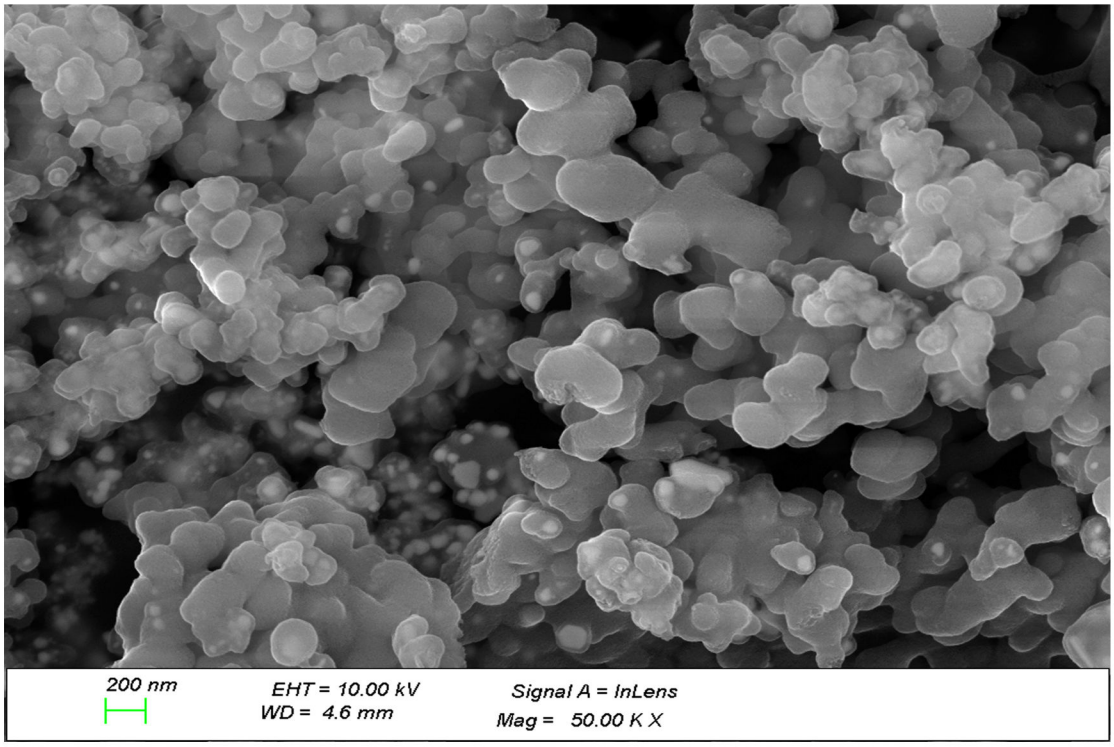




**Plate 23: Scanning electron micrographs of silver nanoparticles synthesized using *M. philippensis* fruit extract. a** Low magnification, **b** Higher magnification



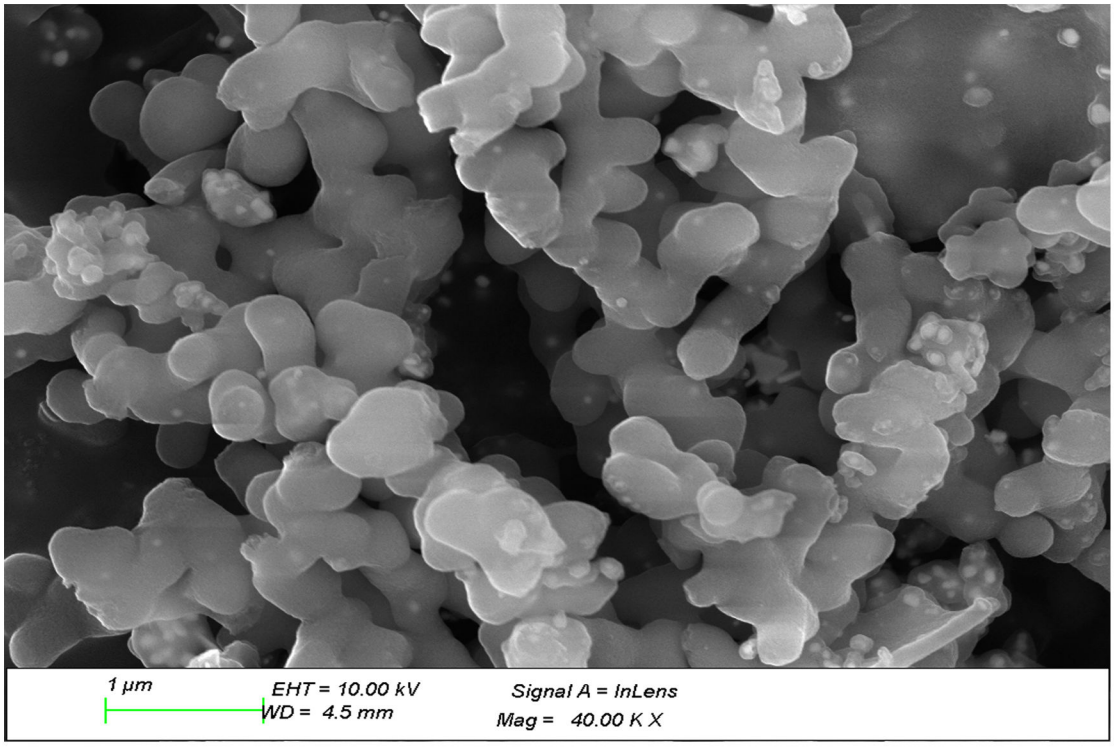
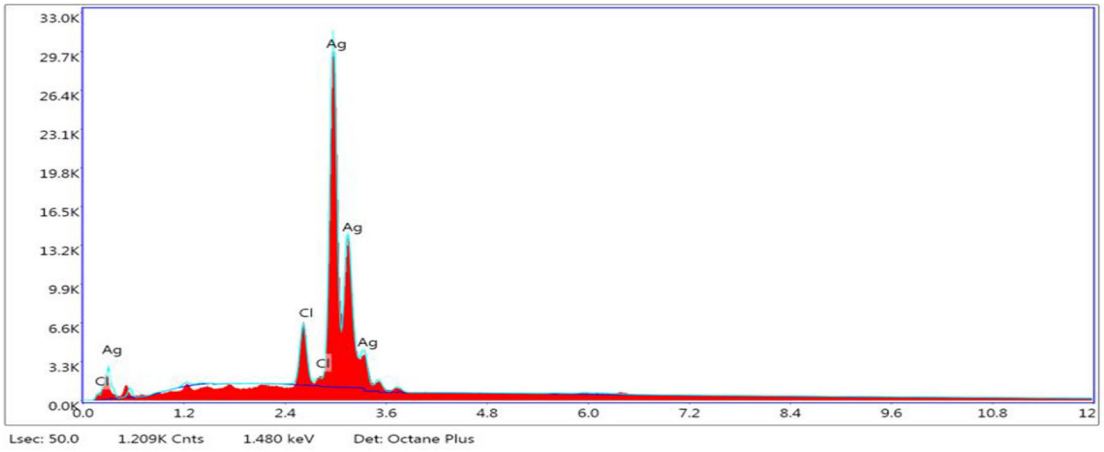
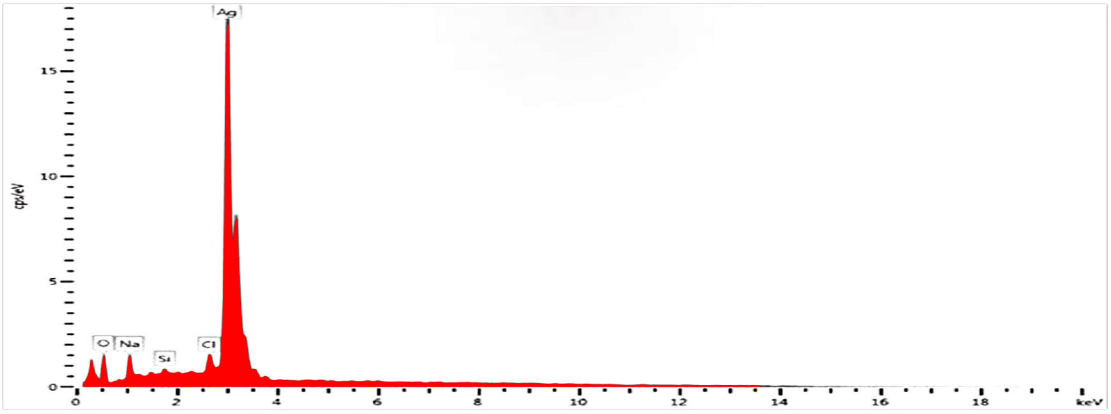




**Plate 24: Scanning electron micrographs of silver nanoparticles synthesized using *A. odoratissima* bark extract. a** Low magnification, **b** Higher magnification



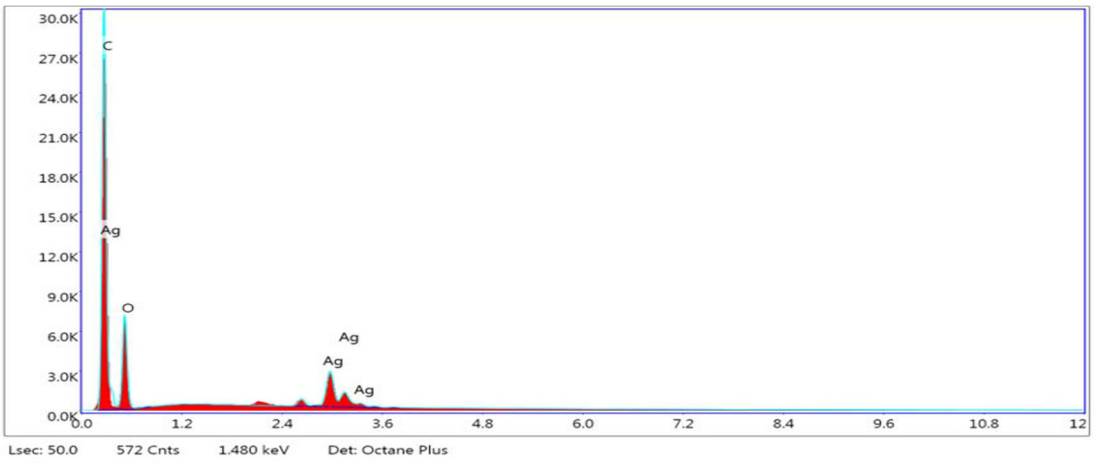




**Plate 25: Elemental composition of silver nanoparticles biosynthesized by selected dye-yielding plants. a** EDAX spectrum of *T. paniculata*, **b** EDAX spectrum of *M. philippensis*, **c** EDAX spectrum of *A. odoratissima*

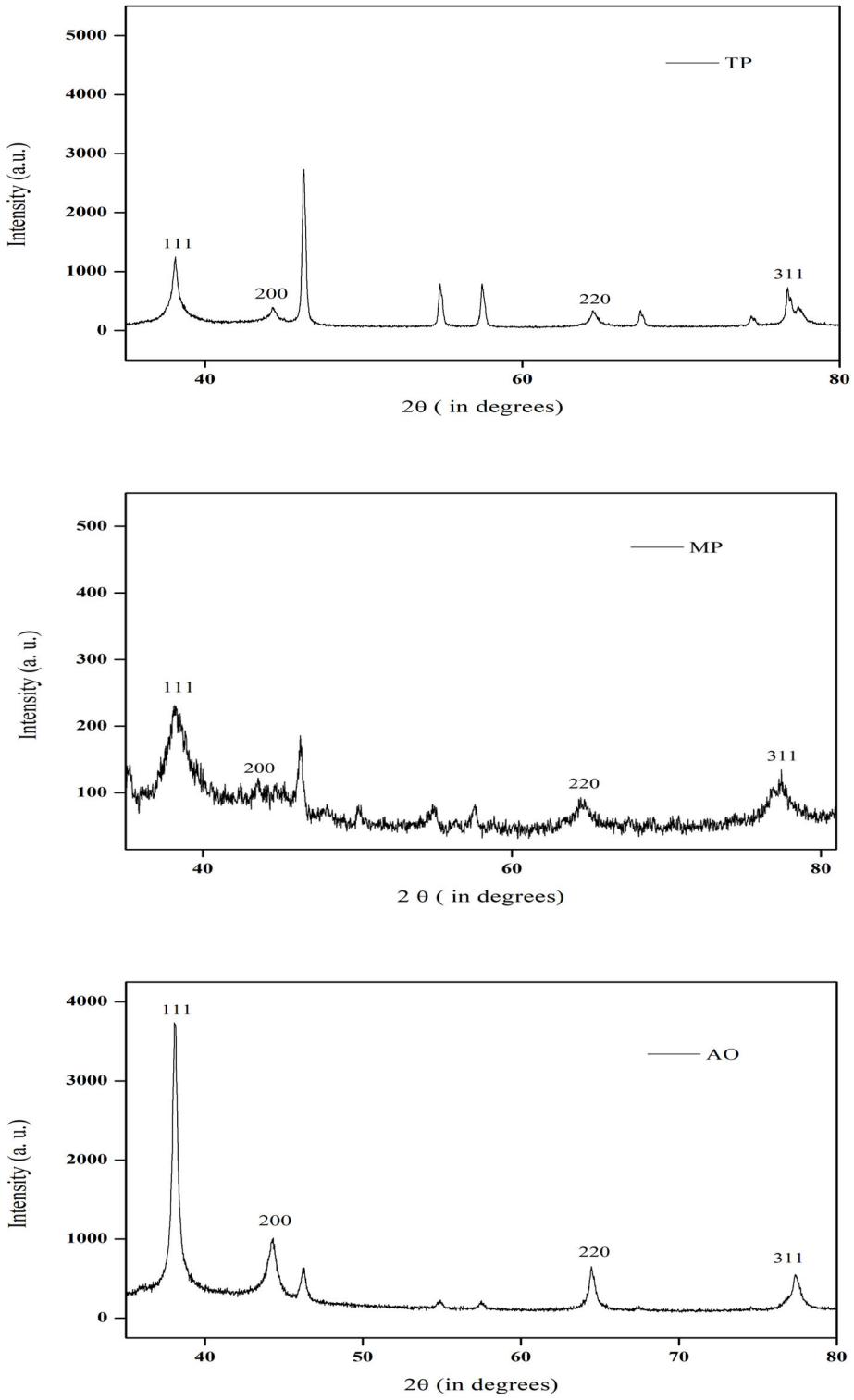



**Plate 26: XRD pattern of silver nanoparticles biosynthesized by treating silver nitrate with the selected methanolic extracts. a** *T. paniculata,* **b** *M. philippensis,* **c** *A. odoratissima*

# DISCUSSION

## PART I – PHYTOCHEMICAL PROFILING

Phytochemical profiling is an excellent method for searching new resources of therapeutically and industrially significant compounds like alkaloids, flavonoids, phenolics, saponins, steroids, tannins, terpenoids, etc. (Akindele & Adeyemi, 2007). Moreover, the exploration of these secondary metabolites can provide immense value in the dye production sector for various purposes due to their protective activity. In the present study, all three extracts of dye-producing parts of the selected plants possessed a characteristic abundance of potentially bioactive compounds.

### a. Qualitative phytochemical estimation

The preliminary phytochemical analysis gives awareness of the presence of secondary metabolites that prevails in the plant extract. The qualitative phytochemical analysis involves preliminary experimental tests, GC-MS and HR LC-MS, which provide the volatile and non-volatile compounds present in the methanolic extracts of selected dye-yielding plants. In *T. paniculata*, all the tested compounds are present which include alkaloids, flavonoids, phenols, tannins, terpenoids, steroids, saponins, glycosides, phlobatannins, anthraquinones, coumarins, resins, proteins and amino acids (**Table 5**). The presence of these compounds from *T. paniculata* by colour change and precipitation reactions had been already published by Savithramma et al. (2013). According to them, leucoanthocyanins and fixed oils are absent in *T. paniculata*. But they carried out their experiments in aqueous leaf extracts and almost all the compounds were similar to the compounds present in the methanolic fruit extract of *T. paniculata*. They also reported that the anti-inflammatory, antispasmodic, analgesic, and diuretic

activities are attributed to their high levels of phenols, tannins, triterpenoids, saponins, and flavonoids. While, the present investigation clearly depicted the antioxidant, hepatoprotective and anticancer activity of the methanolic fruit extract of *T. paniculata*. Hence, based on the previous reports, the current study could prove that medicinal plants are potentially rich in phytochemicals and had been widely used for various ailments. Moreover, the genus *Mallotus* contains major phytochemicals which include phenols, diterpenoids, steroids, flavonoids, cardenolides, triterpenoids, coumarins, isocoumarins, etc. with potent bioactivities and many more to discover (Gangwar et al., 2014). The present phytochemical work on *M. philippensis* could be correlated with these results due to the presence of similar phytochemicals obtained. The major phytochemicals in this plant include alkaloids, phenols, flavonoids, tannins, terpenoids, steroids, glycosides, anthraquinones, coumarins, resins, proteins, and amino acids. Whereas, saponins and phlobatannins are absent in the methanolic fruit extract of *M. philippensis*. Among the 13 classes of phytochemical compounds, resins and coumarins are not detected in the present work on the methanolic bark extract of *A. odoratissima* (**Table 5**). Reports revealed that majority of the *Albizia* species contain triterpenoids, saponins, flavonoids, lignanoids, alkaloids, phenolic glycosides, etc. *Albizia* species have been established to possess various pharmacological activities which include antidiabetic, anti-inflammatory, antifertility, antianxiety, antidepressant, and anti-fever properties, which are consistent with the traditional and local applications of the *Albizia* species (He et al., 2020). The presence of bioactive compounds in various species of *Terminalia, Mallotus*, and *Albizia* is represented in **Table 1**.

**b. Quantitative phytochemical estimation**

After the confirmation of the presence of major phytochemical components *viz*., phenols, flavonoids, alkaloids, terpenoids, and tannins by

preliminary phytochemical tests, the extracts were used for quantitative estimation. Phytochemicals are the chemicals produced by various parts of the plants and these compounds were responsible for many pharmacological activities. Moreover, the evaluation of all the drugs is based on phytochemical and pharmacological outcomes which lead to drug discovery (Foye et al., 2008). So, the quantification of phytochemicals is an essential approach for doing the same.

The term phenolic compound holds a wide range of plant substances which own in common, an aromatic ring bearing one or more hydroxyl substituents. Phenolic constituents tend to be water-soluble since they most frequently remain combined with sugar as glycosides and they are generally located in the cell vacuole. Between the natural phenolics, a thousand structures are known, out of which the flavonoids form the largest group. However, simple monocyclic phenols, phenylpropanoids, and phenolic quinones all exist in considerable numbers (Harborne, 1973). Phenols are commonly present in *T. paniculata*. The reports of Savithramma et al. (2013) disclosed that the aqueous leaf extract of *T. paniculata* contains about 1.290 ± 0.045 mg/gwt of phenol components. Whereas in the present investigation, the methanolic fruit extract of *T. paniculata* exhibited the maximum level of phenols i.e. 133 ± 1.14 mg GAE/g DW. The phenolic content was evaluated with the help of the standard gallic acid (**Figure 5**). In *M. philippensis* and *A. odoratissima* it was 84.65 ± 2.55 and 111.8 ± 3.67 GAE/g DW respectively (**Figure 6**). About 58.8 % of phenolic content was already reported in the aqueous leaf extract of *M. oppositifolius* (Adetunji et al., 2022) and has been reported to exhibit various bioactivities, including antioxidant, antidepressant, anti-inflammatory, antimicrobial, anticancer and cardio-protection attribute (Maroyi, 2019). Whereas, the phenolic content of about 3.980 ± 0.006 mg/g of catechol was found in the ethanolic extract in the areal parts of *A. procera* (Sivakrishnan et al., 2013). Higher phenolic components evidenced their role

in the regulation of plant growth, development, and disease resistance. Also, plant phenols are ubiquitously present in fruits and have the power to affect the cancer process as well as shown to comprise higher levels of antioxidant activities (Sagwan et al., 2010). The health profits of phenolics are directly accompanied by regular intake and their bioavailability. Moreover, regular consumption of fruits exhibited that phenols were found to prevent various diseases associated with oxidative stress (Haminiuk et al., 2012).

Flavonoids are usually known as plant phytochemicals that hold an aromatic ring bearing at least one hydroxyl group like phenolic compounds. The high antioxidant property of flavonoids has been providing benefits to human health, curing many diseases therapeutically. Also, flavonoids had proven their activity as antioxidants, anticancer, anti-bacterial, cardioprotective, promoting the immune system, skin protection from UV rays and can be used as a potential candidate for pharmaceutical and medical applications (Tungmunnithum et al., 2018). According to Savithramma et al. (2013), maximum amount of flavonoid was found in *T. paniculata*, which was 0.077 ± 0.040 mg/gwt and also described the free radical scavenging activity, anti-inflammatory activity, as well as inhibition of hydrolytic and oxidative enzymes. Hence, the potent antioxidant activity of flavonoids is proven to be considered for human health and nutrition. The present study remarkably revealed the flavonoid content in three dye-yielding plants *viz*., *T. paniculata*, *M. philippensis* and *A. odoratissima* against quercetin. All these three species showed considerable flavonoid content but the methanolic bark extract of *A. odoratissima* possessed the maximum amount of flavonoid content (98.66 ± 2.98 mg QE/g DW). Whereas the total flavonoid content of methanolic fruit extract of *T. paniculata* and *M. philippensis* is 55.07 ± 2.99 and 28.19 ± 0.77 mg QE/g DW respectively (**Figure 8**). The standard used for the study was quercetin (**Figure 7**). Based on the aluminium chloride colorimetric method, the determination of the flavonoid content of *M. philippensis* was carried out

in an earlier study (Subedi et al., 2014). They described that total flavonoid content was obtained by precipitating the extract of *M. philippensis* with aluminium chloride in an alkalinized medium. It produced an intense yellow florescence when observed under UV spectrophotometer. About 879.48±24.75 mg QE/g dry extract wt. of flavonoid content was observed in *M. philippensis*. So, the present study clearly depicted the potential of the fruits of *M. philippensis* based on the flavonoid production from the whole plant. The ethanolic extract from the aerial parts of *A. procera* was used to estimate the total flavonoid content by Sivakrishnan et al. (2013). About 2.651 ± 0.077 mg/g of flavonoid content was obtained from the aerial parts. Tremendous availability of flavonoid content in the bark extract of *A. odoratissima* was revealed in the current study. Hence, it is clear that the genus *Albizia* possesses considerable flavonoid content in almost all its parts. The majority, of the action of these flavonoids, is by scavenging process (Cook & Samman, 1996). Also, flavonoids have the capability to inhibit the growth of microbes that are resistant to antibiotics. The other proven action of flavonoids is anticancer (Salah et al., 1995) and anti-inflammatory activity (Okwu, 2001).

Plant alkaloids are one of the largest groups of natural products with a basic nitrogen atom that is located at any position of the molecule. Narcotic analgesics, morphine, codeine, apomorphine (a derivative of morphine used in Parkinson's disease), the muscle relaxant papaverine and the antimicrobial agents sanguinarine and berberine are the important plant alkaloids. Moreover, active anti-cancer drugs have been developed from these plant compounds (Bribi, 2018). The therapeutic activity of alkaloids is well known. They have the ability to prevent the onset of several degenerative diseases by free radical quenching activity as well as binding with the oxidative reaction catalyst. A wide range of pharmaceutical activities of alkaloids has been evaluated in several studies (Roy, 2017). Major pharmacological effects of

alkaloids are antimicrobial, local anesthetic and stimulant, anti-bacteria, anticancer, antiasthma, antimalarial, and antihypertension activities (Verma, 2010). The alkaloid content of the aqueous leaf extract of *T. paniculata* was quantified by Savithramma et al. (2013), which was found to be 0.300 ± 0.011 mg/ gwt. But in the present study, total alkaloid content of the methanolic fruit extract of *T. paniculata* is about 24.99 ± 0.31 mg CE/g DW. The alkaloid content was analysed by comparing it with the reference caffeine (**Figure 9**). Among the three plants, analyzed in the present work, *T. paniculata* showed the maximum level of alkaloid content followed by *M. philippensis* (23.5 ± 0.32 mg CE/g DW) and *A. odoratissima* (12.34 ± 1.6 mg CE/g DW) (**Figure 10**). The alkaloid content of the ethanolic leaf extract of *M. oppositifolius* was estimated by Essiet et al. (2020). They found that only 3% of alkaloid content existed in the extract. This was supported by the report of Barku et al. (2013) in their alkaloid estimation of the methanolic and aqueous leaf extract of *M. oppositifolius*. They observed that the percentage alkaloid content was 8.6% and 4.3% in methanol and aqueous extracts respectively. These results suggest that the total alkaloid content was higher in the methanolic extract when compared to that of the aqueous and ethanol extracts. Similarly, the alkaloid content of leaf, stem, bark, and root extracts in various solvents of *A. chevaleri* was observed by Labaran (2017). Three solvents were used for their study i.e. methanol, ethyl acetate, and hexane. In leaves, 46.94 and 16.30 g/100g of alkaloid were found to be in methanolic and ethyl acetate extracts respectively. In the case of stem bark, ethyl acetate extract (25.72 g/100g) possesses the highest alkaloid content than methanolic extract (10.78 g/100g). However, *A. chevaleri* root methanolic extract (55.08 g/100g) showed the highest alkaloid content than ethyl acetate extract (21.34 g/100g). In all conditions, the hexane extract did not exhibit any alkaloid content.

Terpenoids are structurally varied and the most profuse plant secondary metabolites. They play a significant role in plant life through direct

and indirect plant defense mechanisms. Terpenoids are also prominent because of their diverse applications in the pharmaceutical, food, and cosmetic industries. Besides, terpenoids are essential for plant growth and development (Abbas et al., 2017). The investigations conducted by Malik et al. (2017) on *T. bellerica* revealed that the total terpenoid content was above 60%. In the present investigation, the fruit extract of *T. paniculata* (158.45 ± 2.31 mg linalool/g DW) showed maximum terpenoid content than *A. odoratissima* (126.82 ± 3.79 mg linalool/g DW) and *M. philippensis* (116.82 ± 2.74 mg linalool/g DW) (**Figure 12**). The terpenoid content was calculated based on the equivalents of linalool (**Figure 11**). The methanolic leaf extract of *A. chevalieri* was found to possess a good amount of terpenoids which supports the present work (Labaran et al., 2017). Triterpenoids have the ability to suppress the growth of cancer cells without affecting normal cells. Also, there is extensive evidence that both naturally occurring and synthetic derivatives of triterpenoids show chemopreventive activity and therapeutic effects against colon, breast, prostate, and skin cancer (Sakarkar & Deshmukah, 2011; Carro et al., 2013). Hence, the high percentage of terpenoid content in fruits, seeds, and bark may be used in therapeutic medicine against deadly diseases like cancer and Alzheimer's disease (Malik et al., 2017).

Tannins are polyphenolic compounds and are widely distributed in nature. They can act as antioxidants, and astringent (Okwu, 2001) and have chemo-protective potential. They were often considered anti-nutrients but the availability was acceptable in all the plants studied. In the present study tannin content in the three extracts was evaluated by using vanillin reagent and was expressed as mg tannic acid/g DW (**Figure 13**). The highest content was found in *A. odoratissima* (6.06 ± 0.26 mg TAE/g DW) and the lowest was observed in *M. philippensis* (1.46 ± 0.23 mg TAE/g DW) (**Figure 14**). According to Savithramma et al. (2013), about 0.026 ± 0.030 mg/gwt of

tannin content was observed in *T. paniculata* aqueous leaf extract. But in the present study, the methanolic fruit extract possesses 3.19 ± 0.05 mg TAE/g DW of tannin content. Hence, it is evident that the fruit extract of *T. paniculata* has high tannin content than the leaf extract. The major pharmacological activities of tannins are antimicrobial and antitumor activities (Okuda & Ito, 2011), anti-inflammatory, antioxidant, and anticancer activities as well as their involvement in cardiovascular, neuroprotective, and in general metabolic disease prevention (Maugeri et al., 2022).

**c) Gas Chromatography-Mass Spectrometry (GC-MS) analysis**

The gas chromatography analysis of the selected dye-yielding plants brought out 17 potential phytochemicals with different retention times (**Table 6**). The chromatogram of each extract has been represented in **Figures 15, 16, 17**. Individual mass spectra of each compound obtained from the selected dye-yielding plants (*T. paniculata*, *M. philippensis,* and *A. odoratissima*) are shown in **Figure 18 (i-vi)**. Among these results, palmitic acid and methyl stearate was common in all three methanolic extracts.

Palmitic acid is a well-known saturated fatty acid and it was resolved at a retention time of 15.983. It is the end product of fatty acid synthesis in virtually almost all organisms and is a major component of storage lipids. Although when it is consumed in the diet in high amounts, it tends to elevate levels of circulating cholesterol, and the cause of it is still not known (Puigserver & Spiegelman, 2003). But the excess consumption of palmitic acid causes a high risk of cardiovascular issues (Mancini et al., 2015). Also, palmitic acid in turn is associated with an increased risk of coronary heart disease and some tumours. The evidence regarding cancer caused by palmitic acid is scanty and the reports were not convincing (Fattore & Fanelli, 2013). Reports revealed that the genus *Terminalia* possesses a considerable content of palmitic acid. According to Ladele et al. (2016), about 40% of palmitic

acid has been found in *T. catappa.* Another supporting report for palmitic acid is in *T. catappa* as recorded by Sarkar et al. (2020). They revealed that about 52.4% of palmitic acid was observed in the seeds of *T. paniculata*. These results clearly designated that almond oil is a good source of edible oil for human consumption like other vegetable oils and its high content of polyunsaturated and monounsaturated fatty acids are responsible to reduce the adverse effect of atherosclerosis, high blood pressure, and cardiovascular diseases. Furthermore, they observed that the seed oil prepared from *T. catappa* has been used as a hair oil due to the presence of palmitic acid. Major *Albizia* species including *A. lebbeck* and *A. saman* as well as *M. philippensis* are also rich in palmitic acid (Knothe et al., 2015; Kumar et al., 2020). In the present study, the methanolic extract of *T. paniculata*, *M. Philippensis,* and *A. odoratissima* showed 62.73, 13.7, and 11.96% of palmitic acid respectively. Hence, these dye-yielding plants could be used for various purposes as mentioned above.

Methyl stearate is a bioactive compound and it is reported in several plants. These plants include the species of the selected genus also i.e. *Terminalia*, *Mallotus,* and *Albizia*. In the present study, methyl stearate was observed in all three selected dye-yielding plant parts (**Table 6**). Among them, *T. paniculata* (22.46 %) showed the highest amount of methyl stearate when compared to *M. philippensis* (4.69%) and *A. odoratissima* (3.84%). According to Suryavanshi and Saxena (2022) the ethanolic extract of *T. catappa* revealed a considerable amount of methyl stearate through GC-MS analysis at 59.27 retention time. Also, they inferred that methyl stearate is a bioactive compound and has wide applications in pharmaceutical industries. Moreover, 4.58 % of methyl stearate was observed in the seeds of *T. catappa* and was recorded by Muhammada et al. (2018). But in the present study, the methanolic fruit extract of *T. paniculata* revealed a remarkable amount of methyl stearate with 22.46%. So, these results revealed that the amount of

methyl stearate is very much higher in *T. paniculata.* Similarly, only 1.60% of methyl stearate was found in the green mature leaves of *M. obongifolius* and was reported by Qin et al. (2019). Whereas the present study proved that the amount of methyl stearate in the fruits of *M. philippensis* was 4.69 %. Additionally, in *A. zygia* about 19.13% of methyl stearate was observed by Eddy et al. (2013). But in the present study, the amount of methyl stearate in *A. odoratissima* bark extract was very low when compared to this earlier work. The antioxidant and anti-inflammatory activity of *A. zygia* was already proven. It might be due to the activity of their bioactive compounds like methyl stearate (Olukanni, 2020).

Stearic acid is a fatty acid found in many plant species. It is a long-chain saturated fatty acid. Nevertheless, in contrast with other saturated fatty acids, stearic acid does not raise serum cholesterol concentrations. Moreover, studies revealed that stearic acid is not hypercholesterolemic and will not raise low-density-lipoprotein cholesterol relative to oleic acid, which is neutral in its effects on cholesterol concentrations. In contradiction to palmitic acid, stearic acid might be used in cholesterol-lowering diets due to its low cholesterol concentration effect (Grundy, 1994). In the present study, it is found only in *M. philippensis* (2.30%) (**Table 6**). In *M. philippensis* stearic acid was already reported by Kumar et al. (2020). According to Smith et al. (2013), about 3.2 ± 0.1 percentage of stearic acid was found in the seeds of *M. philippensis*. Besides, there are reports that the fatty acids of kamala oil which are prepared from the seeds of *M. philippensis* consist of stearic acid. A rare feature of the acid composition of kamala oil is the virtual absence of stearic acid in the saturated components, which consist mostly of myristic and palmitic acids while the unsaturated components are almost entirely confined to the $C_{18}$ series (Gupta et al., 1954). Moreover, the oil extracted from the seed kernel of *T. bellerica* by solvent extraction process is a rich source of stearic acid (14.93%), which was reported by Molla et al. (2007). But in the

current investigation, the presence of stearic acid was not found in *T. paniculata* and *A. odoratissima*. However, a small amount is present in *M. philippensis*. The major reported applications of stearic acids include its use in cosmetics technology (Kelm & Wickett, 2017) or in pharmaceutics and biomedicals as a therapeutic adjuvant and drug delivery agent (Katdare et al., 2019).

Hordenine is a simple alkaloid that is found in many plants. It is a nootropic (non-pharmaceutical cognitive enhancers) compound that enhances cognitive ability (Hoffman et al., 2009). It is an MAO-B (Monoamine oxidase type B) inhibitor that increases the level of norepinephrine and is hence considered a norepinephrine and noradrenaline uptake inhibitor (Frank et al., 1990). In the current study, the presence of hordenine was only found in the bark extract of *A. odoratissima*. About 84.19% of hordenine was reported in the methanolic bark extract. In *T. paniculata* and *M. philippensis* the total absence of hordenine was recorded (**Table 6**). There are several reports regarding hordenine, which is a diuretic and affects the central nervous system. Furthermore, reports revealed that in the past, hordenine was used for the treatment of diarrhoea and dysentery (Schweitzer & Wright, 1938). It has a helpful inotropic effect on the heart, and increases the systolic and diastolic blood pressure, peripheral blood volume, and inhibits gut movement (Hapke & Strathmann, 1995). All effects are short and only noticeable at high doses. Also, hordenine showed an inhibitory effect on human lung cancer cells (A549 and H1299) with an admirable $IC_{50}$ value. Based on the molecular docking studies, the affinity of hordenine to the PDK3 (pyruvate dehydrogenase kinase 3) was excellent ($K = 10^6\ M^{-1}$) and binds in the active site pocket of PDK3 and forms a significant number of non-covalent interactions with functionally important residues. So, it clearly depicts the effect of hordenine in drug discovery. In anticancer therapy, targeting PDK3 with developing new therapeutic molecules is an interesting field.

Chemically, synthetic molecules were accompanied by severe adverse effects and which encourage the use of phytochemicals as lead molecules in anticancer therapy. Consequently, targeting PDK3 by these natural phytochemicals can be an attractive therapeutic approach for identifying and developing small molecule inhibitors. Hordenine may be exploited as a novel scaffold to inhibit the kinase activity of PDK3 along with the cytotoxic effects on lung cancer cell lines. All these observations suggested the upshot of hordenine in the therapeutic management of lung cancer and other PDK3-associated diseases (Anwar et al., 2020).

**d) High Resolution Liquid Chromatography - Mass Spectrometry (HR LC-MS) analysis**

For the detection of non-volatile compounds, HR LC-MS analysis was performed. A total of 54 non-volatile phytocompounds were obtained from the three dye-yielding plant extracts (**Table 7**). Out of these, the number of biopeptides was found to be very high in each extract. There is no common compound obtained from the three plant extracts. The first and last compound was resolved at the retention time of 0.953 and 20.848 respectively. The first resolved compound is a biopeptide known as Ser-Thr-Gln with a molecular mass of 334.1495. It was only reported in the methanolic fruit extract of *M. philippensis*. The least number of compounds were found in *A. odoratissima*. Only 7 non-volatile compounds were resolved from the methanolic extract of *A. odoratissima*. The non-volatile phytoconstituents *viz.*, Pro-Leu, gallic acid, pantothenic acid, Ile-Arg-Ala, and ellagic acid were the common ones among *T. paniculata* and *M. philippensis*.

Mass spectrometers convert the compounds into the charged ionized states. It is identified based on their mass-to-charge ratios. The negative ion mode charges the analyte through deprotonation whereas the positive ion mode charges through protonation. Liquid chromatography/mass

spectroscopy is the best method for the analysis of compounds with medium and high polarity. LC-MS analysis is very effective for metabolite profiling (Wilson et al., 2005). In ultra-performance liquid chromatography, sensitivity increases due to the reduction in the broadening of bands. Hence, better chromatographic peak resolution and increased speed sensitivity can be obtained. In, LC-MS, electrospray ionization (ESI) is a better technique to be used. It needs a high electric field and the major advantages of ESI were soft ionization, no need for derivatizations, the ability to ionize compounds of a large mass range, better quantitative analysis, and high sensitivity. For the identification of trace constituents of complex mixtures, quadrupole and oaTOF-MS coupled to UPLC have been proven to be a powerful technique. It provides accurate mass measurement with accuracies of <5 ppm and affords interpretation ambiguities (Lu et al., 2008).

In the present investigation, the availability of biopeptides was appreciable in all three extracts. The biopeptide called Pro-Leu and Ile-Arg-Ala were common among *T. paniculata* and *M. philippensis*. The highest number of biopeptides are found in *M. philippensis* whereas the lowest number was observed in *T. paniculata*. About 4 different biopeptides were obtained in *A. odoratissima*. Biopeptides mostly have 2-20 amino acids with less than 6000 Da molecular mass (Meisel & FitzGerald, 2003; Sun et al., 2004). Based on their structural characteristics as well as their amino acid residues located on N and C terminal, they may be responsible for the natural bio resistance "Shelf-life-predictions" (Bell, 1997). Moreover, these biopeptides exhibit several biological functions like antihypertensive, antimicrobial, mineral binding, antithrombotic, immunomodulatory, hypercholesterolemic, and antioxidant activities. Several biopeptides are appreciably responsible for more than one biological function. Furthermore, biopeptides have an excellent nutritional platform which is helpful in the

dairy, industrial, nutraceutical, therapeutic, and vaccination sectors (Saadi et al., 2015).

In the plant kingdom, gallic acid is undoubtedly one of the most abundant phenolic acids. It has a vast range of applications in the food and pharmaceutical industries. Gallic acid and its various derivatives like lauryl gallate, propyl gallate, octyl gallate, etc., have the ability to inhibit oxidation and can scavenge free radicals. Hence, its antioxidant nature is one of the best characteristic features of gallic acid (Kahkeshani et al., 2019). In the present study, HR LC-MS revealed the presence of gallic acid in the methanolic fruit extract of *T. paniculata* and *M. philippensis*. In *A. odoratissima* no gallic acid was detected. When correlating this, the high antioxidant potential of *T. paniculata* might be due to the effect of gallic acid present in it. Moreover, gallic acid and its derivatives were widely used as flavouring agents and preservatives in the food industry. Antimicrobial, anti-inflammatory, anticancer, cardioprotective, gastroprotective, and neuroprotective effects are the other major biological applications of gallic acid (Choubey et al., 2015). In the present investigation, *in vitro* anticancer activity on human breast cancer cell lines (MDA-MB 231) was proved in the *M. philippensis* fruit extract very effectively followed by *T. paniculata* and *A. odoratissima*. Gallic acid can unveil cytotoxic and antitumor effects based on the modulation of antioxidant/pro-oxidant balance. In some cases, it has the power to control the reactive oxygen species (ROS)-induced carcinogenesis by increasing the activity of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx) and/or by reducing the lipid peroxidation and ROS production. Also, gallic acid can induce cell cycle arrest, autophagy, and apoptosis by activating the caspases pathway and ROS generation (Kahkeshani et al., 2019). In addition, gallic acid controls cell-cycle-related proteins like cyclin A, cyclin D1, and cyclin E, and slows down cell division by the induction of the p27KIP enzyme and inhibition of CDK

activity (Huang et al., 2012). In hepatocellular carcinoma, gallic acid reduces the tumour size and the serum level of tumor marker enzymes like aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) by inhibiting the proliferation of hepatic cells (Jagan et al., 2008). The antifungal activity of gallic acid isolated from *T. nigrovenulosa* bark against *Fusarium solani* was reported by Seo et al. (2013). Bergenin is a C-glucoside of 4-O-methyl gallic acid and was isolated from *Bergenia crassifolia*, *Corylopsis spicata*, *Caesalpinia digyna*, *Mallotus japonicus* and *Sacoglottis gabonensis* by Patel et al. (2017). They observed that bergenin exhibited plenty of pharmacological activities like antihepatotoxic, antiulcerogenic, anti-HIV, antifungal, hepatoprotective, antiarrhythmic, neuroprotective, anti-inflammatory, immunomodulatory, and burn wound healing properties. In the present study, promising content of gallic acid was found in *T. paniculata* which might be a cause of antioxidant potential as well as significant hepatoprotective activity.

Pantothenic acid is an essential vitamin and acts as the metabolic precursor for coenzyme A (CoA). It is a participant in several metabolic reactions including lipids, proteins, and carbohydrates (Miller & Rucker, 2020). The presence of pantothenic acid was proved in both *T. paniculata* and *M. philippensis*. Pantothenic acid is a structural component of an acyl carrier protein (ACP) and fatty acid synthetase complex. It is required for fatty acid synthesis (Tahiliani & Beinlich, 1991). Furthermore, pantothenic acid has broader functions in fatty acid oxidation, ketone body metabolism, the oxidative metabolism of pyruvate through pyruvate dehydrogenase, and the citric acid cycle. In addition, it is responsible for the metabolism of various organic acids, involved in catabolic pathways of amino acid metabolism (Sweetman, 2004). *Euphorbia antisyphilitica* residues are found to be a new source of ellagic acid (Ascacio-Valdés et al., 2010). There are reports on

ellagic acid isolated from *Rhodiola rosea,* which has the ability to inhibit the cell entry of the Anti-Ebola Virus. About twenty commercial compounds were isolated from *R. rosea* in which ellagic acid and gallic acid are two structurally related compounds and the most effective ones (Cui et al., 2018).

Ellagic acid is a phenolic phytochemical resolved from the methanolic fruit extract of both *T. paniculata* and *M. philippensis* by HR LC-MS analysis. Most of the *Terminalia* species including *T. arjuna*, *T. chebula*, *T. laxiflora*, *T. brownii* etc. are very rich sources of ellagic acid (Singh et al., 2016a; Sarabhai et al., 2013; Salih et al., 2018). It is commonly found in fruits and vegetables and has a beneficial role in the health effects against oxidation-linked chronic diseases like cancer and cardiovascular problems. Ellagic acid is believed to reduce oxidative stress while acting as an antioxidant or by activating cellular antioxidant enzyme systems (Vattem & Shetty, 2005). In plants, ellagic acid is present in the form of hydrolysable tannins called ellagitannins. They are the esters of glucose and when hydrolysed they produce ellagic acid (Marwan & Nagel, 1986). Reports revealed that ellagic acid possesses antimutagenic, antioxidant, and anti-inflammatory activity (Vattem & Shetty, 2005). Furthermore, ellagic acid exhibits a potent anticarcinogenic effect and has anticancer benefits by modulating the metabolism of environmental toxins. Thus, it prevents the initiation of carcinogenesis caused by these chemicals. The ability to inhibit the direct binding of these carcinogens to the DNA revealed its antimutagenic activity (Teel et al., 1986). According to Narayanan et al. (1999), ellagic acid causes G1 arrest and inhibited the overall cell growth and leads to apoptosis in tumour cells. In the present investigation, the reduction of cell counts in *M. philippensis* indicates the cell cycle arrest at G0/G1 phase and induces apoptosis, which might be due to the effect of ellagic acid.

Catechin is a phenolic compound mainly found in several foods and herbs. In the present study, it was only reported in *A. odoratissima*. Reports revealed that *Camellia sinensis* is one of the richest sources of catechin as the (-)-epigallocatechin-3-gallate. Many health benefits are reported on catechins, which include anticancer, anti-obesity, anti-diabetic, anti-cardiovascular, anti-infectious, hepatoprotective, and neuroprotective effects. Moreover, it has a dual action on ROS as an antioxidant and a pro-oxidant. Catechins are commonly distributed in tea, apples, persimmons, cacaos, grapes, and berries (Isemura, 2019). In many of the plants, (+) enantiomer of catechin and (-) enantiomer of epicatechin are produced. In cocoa products, (+) catechin along with (-) epicatechin were observed (Donovan et al., 2006). (-) catechin isomer is predominantly found in chocolates (Gotti et al., 2006). In *A. odoratissima* a terpene compound called picrotin was observed. A considerable degree of picrotin with medicinal effects was also found in *Picrorhiza kurroa* by Bohraa et al. (2015). HR LC-MS analysis revealed a vast variety of non-volatile compounds from all three selected dye-yielding plants. All these phytocompounds exhibited a wide range of pharmacological activities. Hence the potentialities of these phytocompounds provide a significant therapeutic value for these natural dye-yielding plants.

# PART II – BIOACTIVITY SCREENING

## A. Antioxidant activities

Free radicals are generated in the body by metabolic processes. The minimum concentration of various free radicals is useful for the functioning of the human body. But the increased generation of free radicals will make various diseases through oxidative stress. Phytochemical compounds present in the plants have the capability to scavenge these free radicals and protect from the adverse effects caused by the same. Oxidative stress is widely used in medical sciences in the past three decades. It takes an active role in the physiology of very common human disorders such as diabetes, high blood pressure, preeclampsia, atherosclerosis, acute renal failure, Alzheimer's, and Parkinson's diseases (Rodrigo & Rodrigo, 2009). Hence, antioxidants are an ever more interesting field due to their protective roles in the body against oxidative stress-mediated pathological processes. Since natural antioxidants and designing new antioxidant compounds need suitable methods of antioxidant activity evaluation (Munteanu & Apetrei, 2021). In the present study antioxidant activity of three dye-yielding plants was evaluated. All three plants revealed a significant antioxidant potential. So, these plants can be used as a source of natural antioxidant agents that can reduce the effects of free radicals produced by synthetic agents. Four different assays such as DPPH radical scavenging assay, hydroxyl free radical scavenging assay, nitric oxide free radical scavenging assay, and superoxide free radical scavenging assays have been used to analyse the scavenging potential of the selected dye-yielding plants.

DPPH is a stable free radical with a purple colour that turns into a yellow product. This occurs when diphenylpicryl hydrazine, in a concentration-dependent manner is scavenged by the antioxidants present in the sample (Olugbami et al., 2015). Usually, the duration of the reaction between DPPH solutions and sample varied from 1 minute (Sroka & Cisowski, 2005) to 240 minutes (Miller et al., 2000; Prakash, 2001). Radical

scavenging activity by DPPH is determined under 517 nm wavelength by Prakash et al. (2001). In the present study similar wavelength was used to determine the absorbance of the reaction. The methanolic extract of selected dye-yielding plants revealed a dose-dependent antioxidant activity. The highest inhibition percentage (75.93 ± 1.49%) was found to be in *T. paniculata* at the maximum concentration (200 µg/mL) (**Figure 23**). The $IC_{50}$ value of *T. paniculata* (23.068 ± 1.58 µg/mL) was almost similar to that of the standard (20.399 ± 1.07 µg/mL) (**Table 8**). The potentiality of the methanolic fruit extract of *T. paniculata* showing significant antioxidant potential was reported by Aswathi and Thoppil (2020). According to Olugbami et al. (2015) the extract contains phytochemicals which brings the capability to act as antioxidants as well as free radical scavengers. *T. chebula* and *T. bellerica* were the related species of the genus *Terminalia* and the study on the 70% methanol extract of the fruits of these plants reveal that they may be useful as potent sources of natural antioxidants (Hazra et al., 2010). Almost all the plant parts of *Terminalia* species are rich in phytochemicals with antioxidant potential. The stem bark of *T. glaucescens* contains numerous phytochemicals with antioxidant properties (Olugbami et al., 2015). Chatha et al. (2014) carried out DPPH free radical scavenging assay on the bark and leaf extracts of *T. arjuna* in different solvents like water and ethanol. When the $IC_{50}$ values of the extract are lower, it will be more effective for inhibition of DPPH free radicals and vice versa. They inferred that the activity of the extract increases with the increase in concentration of phenolic compounds. A similar condition was observed in the present study, where the highest phenolic content was obtained in the methanolic fruit extract of *T. paniculata*. The antioxidant activity of about 33 species of *Mallotus* was evaluated by Hong et al. (2011). According to this report, *M. philippensis* is a valid source of natural antioxidant agents and its free radical scavenging activity seems to be due to the presence of tannins. They recorded about 6.7% of tannin in the ethyl acetate partition of *M. philippensis* and 93.7 ± 3.7 percentage of inhibition was exposed through DPPH assay. But in the present study

1.46 ± 0.23% of tannin was observed in the methanolic fruit extract of *M. philippensis* by quantitative estimation. When correlating this result with radical scavenging activity, the DPPH assay gave about 71.22 ± 0.61% of inhibition. Hence, the presence of tannin could play a role in antioxidant potential. A lower $IC_{50}$ value designates a higher antioxidant activity (Banothu et al., 2017). In the present study, methanolic bark extract of *A. odoratissima* exhibit the highest $IC_{50}$ value (121.88 ± 1.20 µg/mL) *via* DPPH assay. So, the antioxidant potential of the same remains lesser than that of *T. paniculata* and *M. philippensis*.

Hydroxyl radical scavenging activity was measured by the effect of the extracts to scavenge hydroxyl free radicals produced by $Fe^{3+}$ - ascorbate – EDTA – $H_2O_2$ system (Fenton reaction) (Halliwell et al., 1987). These free radicals are very powerful and are directly involved in the irreversible damage caused by oxidative stress. Free radicals mainly cause metagenesis, carcinogenesis, and aging (Halliwell & Gutteridge, 2015). The presence of anti-oxidants in the extracts induced the removal of hydroxyl radicals and thus prevented the degradation of 2-deoxy-D-ribose in a dose-dependent manner (Olugbami et al., 2015). The methanolic extract of selected dye-yielding plant parts was taken as 125, 250, 500, 1000, and 2000 µg/mL of concentration. The antioxidant potential of all these extracts was evaluated against a standard gallic acid (**Figure 24**). All these extracts exhibit a concentration-dependent antioxidant potential as mentioned by Olugbami et al. (2015). At the highest concentration (2000 µg/mL), the maximum percentage of inhibition was evaluated in *A. odoratissima* (64.52 ± 0.28%) and *T. paniculata* (63.57 ± 0.92%). Whereas, the inhibition percentage of *M. philippensis* using 2000 µg/mL concentration of the extract is lesser than that of *T. paniculata* and *A. odoratissima*. The reports of Olugbami et al. (2015) reveal that the presence of potent phytoconstituents have the ability to scavenge hydroxyl radicals, while the activities of such components may have been shielded by the presence of other components in the heterogenous extract. Another important fact is that such constituents might be phenolic in

nature based on the high value of the total phenolic content of the extract in comparison with its total flavonoid content. Based on the quantitative estimation, all the three methanolic extracts possessed a significant amount of phenolic and flavonoid content, which might be a reason for the free radical scavenging potential.

In the nitric oxide free radical scavenging assay, the ability of selected dye-yielding plants to scavenge nitric oxide radicals was evaluated in a reaction with methanolic extracts, 5 mM sodium nitroprusside, and Griess reagent. This showed that all three extracts scavenged NO• in a dose-dependent manner as shown in **Figure 25**. However, *T. paniculata* fruit extract had a higher percentage of inhibition (75.77 ± 0.91%) with an $IC_{50}$ value of 1239 ± 1.40 µg/mL (**Table 8**). Gallic acid was used as the standard for the study. According to Oyeniran et al. (2021) the availability of greater amounts of polyphenolic constituents in the *T. catappa* extracts could display higher free radical inhibition. Similarly, the present investigation proved that the polyphenolic compounds are found to be very high in *T. paniculata*. So, this finding corresponds with the earlier mentioned studies regarding radical scavenging capacities. Besides, the maximum inhibition percentage of *A. odoratissima* and *M. philippensis* was 63.2 ± 0.47% and 42.72 ± 0.91% respectively. When compared to *T. paniculata*, the amount of phenolic content in *A. odoratissima* and *M. philippensis* is less. So, the results conclude that free radical scavenging activity is truly reliant upon polyphenolic constituents as mentioned earlier.

Superoxide free radicals are generated from the riboflavin-NADH system by the oxidation of NADH and evaluated by the reduction of NBT resulting in the formation of blue formazan product (Valentão et al., 2002). The selected concentrations for the study are 125, 250, 500, 1000, and 2000 µg/mL. All the plant extracts showed a concentration-dependent activity (**Figure 26**). Among the selected plants, *M. philippensis* showed the highest inhibition percentage (83.8 ± 0.73%) at the maximum concentration

(2000 µg/mL). This was almost near to that of standard ascorbic acid (94.63 ± 1.16%). Antioxidant properties of flavonoids were effective mainly via scavenging of superoxide free radicals. Also, radical scavenging might be responsible due to the presence of hydrolysable tannins (Robak & Gryglewski, 1988). In the present study, all three methanolic extracts possessed a characteristic amount of flavonoid and tannin content. When an effective comparison of the superoxide free radical scavenging capacity was made, results were expressed as $IC_{50}$ values, which are the 50% scavenging ability that was achieved from interpolation of linear regression analysis (Chyau et al., 2006). $IC_{50}$ values of *T. paniculata*, *M. philippensis* and *A. odoratissima* were 47.69 ± 0.52, 675.62 ± 1.90, and 117.37 ± 1.88 µg/mL respectively (**Table 8**). However, standard ascorbic acid exhibit 384.205 ± 2.28 µg/mL of $IC_{50}$ value. So, it can be concluded that *M. philippensis* displayed the highest $IC_{50}$ value (675.62 ± 1.90 µg/mL) than that of ascorbic acid. Hence the superoxide free radical scavenging ability of *M. philippensis* fruit extract seems to be moderate. Whereas, in *T. paniculata* $IC_{50}$ value is very less (47.69 ± 0.52 µg/mL) than that of the standard. But the inhibition percentage was observed as 77.63 ± 0.90% at the maximum concentration (2000 µg/mL). So based on the $IC_{50}$ values, *T. paniculata* showed a significant antioxidant potential.

Free radical scavenging capacity varies with species and analysis. For all the different assays, the methanolic fruit extract of *T. paniculata* showed excellent antioxidant activity. In liver diseases like hepatocellular carcinoma, viral and alcoholic hepatitis, and non-alcoholic steatosis, reactive oxygen and nitrogen species play a critical role in disease initiation and progression (Morisco et al., 2008; Nagata et al., 2007; Loguercio & Federico, 2003). Foods rich in antioxidants have been proposed as a preventive tool to cure liver damage (Morisco et al., 2008). The high free-radical quenching of the fruit extract of *T. paniculata* was proved by Aswathi and Thoppil (2020). Thus, the fruit extract of *T. paniculata* may be exploited as a promising agent

for the adequate protection of the liver. Hence, a further hepatoprotective study was carried out in *T. paniculata* based on its high antioxidant potential.

**B. Hepatoprotective Screening**

The liver is one of the chief organs in the body, and liver disease is a primary health problem faced by modern men, mainly due to their bad food habits. Even with the invention of new drugs are going on, an active drug to regenerate hepatic cells is not discovered (Adewusi & Afolayan, 2010). The reasons behind the liver disease are hepatotoxic chemicals that induce lipid peroxidation and other oxidative damages (Dianzani et al., 1991). In the last century, acetaminophen is an analgesic and antipyretic drug leading to liver necrosis in humans and experimental animals (Lin et al., 1995). Likewise, alcohol is a severe cause of liver disease and is common for liver transplantation. The continuous consumption of alcohol and fluctuations in the diet system drastically cause morbidity and mortality in addition to viral hepatitis (Mandayam et al., 2004). Liver diseases gradually progress from fatty liver to alcoholic hepatitis and eventually lead to fibrosis and cirrhosis. The usage of medicinal plants and their derivatives is very vital for treating liver diseases. So, the influence of phytochemicals like phenols, coumarins, monoterpenes, glycosides, alkaloids, and xanthenes can cure liver dysfunctions (Bhawna & Kumar, 2009). *T. paniculata* has plenty of therapeutic activities. The high free-radical quenching of fruit extract of *T. paniculata* was already proved (Aswathi & Thoppil, 2020). Hence it can act as a promising agent for the adequate protection of liver.

The present study focused on the *in vitro* hepatoprotective ability of *T. paniculata* fruit extract against acetaminophen-induced hepatotoxicity. For effective comparison, the hepatotoxicity of *T. paniculata* and the corresponding synthetic colourant (lemon yellow) was also carried out. 6.25, 12.5, 25, 50, and 100 µg/mL was the selected concentrations for the study. In

hepatotoxicity, the viability percentage was lower in lemon yellow than *T. paniculata* (**Figure 27**). Based on the $LC_{50}$ values, lemon yellow (162.269 µg/mL) induce more toxicity to the growth of HepG2 cells than *T. paniculata* (228.688 µg/mL) (**Plate 2 & Plate 3**). Hence, the hepatoprotective ability of *T. paniculata* fruit extract was evaluated by MTT assay. Where, characteristic percentage viability by the fruit extract was observed in each concentration (**Figure 29**). The addition of *T. paniculata* fruit extract increases the viability percentage of HepG2 cells induced with acetaminophen (**Plate 4**). So, the present study proved a promising hepatoprotective activity of *T. paniculata* fruit extract.

Tartrazine, commonly known as lemon yellow is a synthetic colourant currently used in foods due to its easy production, cheaper cost, and also it provides better colouration. Mainly it is used in the food products like soft drinks, juices, biscuits, ice creams, sauces, snacks, etc. Reports revealed that synthetic food colours cause a wide range of allergic reactions. The administration of lemon yellow in male Swiss albino mice caused an alarming increase in body weight (Gautam et al., 2010). According to Chatlerjea and Shinde (1995), a body weight increase of over 20% was considered obesity. Moreover, the frequent use of such synthetic dyes will adversely affect the reproductive cells and which was proved by Gautam et al. (2010). Lemon yellow is an azo dye and the usage of azo dyes in the pharmaceutical industry has many purposes. Many of the azo dyes are proven to be carcinogenic. They have mutagenic activity and cause allergic reactions. The carcinogenicity of azo dyes depends upon the structure of the molecule as well as its mechanism of degradation *(*Gičević et al*.,* 2019). In the present study, hepatotoxicity screening of lemon yellow on HepG2 (human hepatocytes) was found to be significant. They reduce more viable cells than *T. paniculata* at the micro level (**Figure 27, 28**). Hence, the frequent use of such synthetic colourants

causes various types of liver damage. Therefore, a replaceable natural colourant is essential to reduce the health issues caused by lemon yellow.

Various studies have reported that the antioxidant properties of medicinal plants are due to the vast number of phenolic compounds (Brown & Rice-Evans, 1998; Krings & Berger, 2001). In the present study, the methanolic fruit extract of *T. paniculata* showed a significant amount of phenolics (**Figure 6**). Also, results revealed that scavenging activity against free radicals was very high in *T. paniculata*. So, it clearly reveals the prominent hepatoprotective ability of *T. paniculata* on Hep G2 cells. Reports revealed that the effect of hepatoprotective activity may be due to substantial antioxidant enzyme activity and the capacity to inhibit lipid peroxidation by the extract (Pareek et al., 2013). A published work from the present study had already disclosed the fact that the methanolic fruit extract of *T. paniculata* had promising high antioxidant capacity (Aswathi & Thoppil, 2020). Moreover, flavonoids have the power to scavenge free radicals and increase antioxidant enzymes. So, it can protect the body from the adverse effect of free oxygen species (Pareek et al., 2013; Pietta, 2000). *T. paniculata* is also very rich in flavonoids and flavanones which was proved by phytochemical studies and they might have the ability to enhance the activity of the antioxidant enzymes. Acetaminophen induces toxicity to Hep G2 cell lines and it decreases the efficacy of serum aspartate transaminase, alanine transaminase, and glutathione peroxidase levels (Kurup & Vijayan, 2021). The results of the present studies are in conjunction with the reports of Kurup and Vijayan (2021), which proved that acetaminophen, induces toxicity to the Hep G2 cells and decreases the serum level. The present study revealed that *T. paniculata* fruit extract possesses significant hepatoprotective activity, and hence it can act as a good hepatoprotectant. For confirming the study, further studies on various cell lines as well as animal models are needed. Due to the excellent hepatoprotective efficacy of *T. paniculata* fruit extract, isolation and

purification are required to find out the specific colour producing compound and after that, it can be used to replace the frequent use of the synthetic colouring agent, lemon yellow. Hence, this study is a stepping stone for the exploration of *T. paniculata* fruit extracts in various industries like food, cosmetics, clothes, pharmaceuticals, etc. as a natural colouring agent.

### C. Cytotoxicity using *Allium cepa* assay

*Allium cepa* has been generally used as a test material to evaluate chromosome aberrations and disturbances in the mitotic cycle (Khanna & Sharma, 2013). Cytotoxicity using *A. cepa* assay was introduced by Levan to evaluate the effect of colchicine on mitotic spindles (Levan, 1938). The first protocol of *A. cepa* test was developed by Fiskesjö by designing it as a test organism for environmental monitoring (Fiskesjö, 1985). According to Nefic et al. (2013), the growing root tip of *A. cepa* is an effective site for testing chemicals on chromosomes. Also, it is useful to evaluate DNA damages that lead to chromosomal aberrations, disturbances in the mitotic cycle, nuclear alterations, and the presence of micronuclei in the meristematic cells of root tips. To verify the mutagenicity of different chemicals, mitotic index and chromosomal abnormalities were used to evaluate genotoxicity and micronucleus analysis also serves similar purposes (Khanna & Sharma, 2013). When the mitotic index was found to be decreasing in the meristematic cells of *A. cepa*, it may be determining the presence of cytotoxic agents in the environment. The reduction of mitotic activity might have happened due to the inhibition of DNA synthesis or blockage of the cell cycle in G2 phase, thereby preventing the cells from entering into mitosis (Sudhakar et al., 2001). Additionally, chromosomal aberrations exhibited the changes in either chromosomal structure or in the total number of chromosomes. Structural chromosomal changes may be due to the DNA breaks, inhibition of DNA synthesis and replication of altered DNA. *A. cepa* test is also used for

monitoring the carcinogenic potential (Nefic et al., 2013). Constantin and Owens (1982) also suggested that *A. cepa* species is very efficient for the evaluation of chromosome aberrations and can be used as a cytogenetic test.

In the present study, methanolic extracts of *T. paniculata*, *M. philippensis* and *A. odoratissima* unveiled cytotoxicity by *A. cepa* assay. In all the extracts, the mitotic index decreases when concentration increases whereas the abnormality percentage increases with increasing concentration. The negative control showed a high mitotic index and lesser abnormality percentage whereas in positive control abnormality percentage was very high (**Figure 30**). Cytotoxic assay displayed numerous cytological anomalies including both clastogenic and non-clastogenic aberrations (**Plates 6, 7, 8, 9,** and **10**). 25, 50, 75, and 100 µg/mL concentrations of each extract were selected for the study. In these experiments, higher mitotic index was observed in *A. odoratissima* (75.66 ± 0.62%) at the lowest concentration (25 µg/mL). The lowest mitotic index was found in *M. philippensis* (45.43 ± 0.37%). The highest and lowest abnormality percentage was observed in *A. odoratissima* (64.24 ± 0.71%) and *M. philippensis* (59.6 ± 0.23%) respectively at maximum concentration. According to Debnath et al. (2016), the cytotoxicity effect of *A. cepa* showed statistically significant inhibition of cell division in the treatment with different concentrations of *T. arjuna* bark extract as compared with the mitotic index value of the control. A highly reduced mitotic index was observed when the concentration increases. Bark extract induced cytological alteration and chromosomal aberration such as sticky metaphase, clumped metaphases, laggard chromosomes, vagrant chromosomes, chromosome bridges at anaphase, and telophase. A wide range of aberrations was observed in the *A. cepa* root tip cells when treated with *T. paniculata* fruit extract. Aswathi and Thoppil (2020) investigated this by the potential activity of extract to modulate the intensity of aberrations due to the exogenous oxidant. The treated root tips showed several aberrations

particularly nuclear lesions, cytoplasmic shrinkage, nuclear disintegration, bizarre nucleus, etc. Micronucleus is one of the important cytogenetic aberrations and is formed by the condensation of entire chromosomes or chromosome fragments that are not incorporated into daughter nuclei at mitosis, due to clastogenic or aneugenic effects (Heddle et al., 1991). Furthermore, Shimizu et al. (1998), reported that the amplified DNA is localised selectively to specific sites at the edges of the nucleus and eliminated through nuclear budding to form micronuclei. For detecting the genotoxic effects, nuclear aberration assay has been extensively used. Among these, the micronuclei test is one of the best methods for nuclear aberration assays and played a significant role in providing early caution about potential genotoxic threats (Tan et al., 2014). Along with the cytological aberrations, genetic material was also disturbed, which involves micronucleus, chromatin globules, nuclear fragmentation, hyperchromasia, pulverised nucleus, and heteropycnosis. Among these, pycnosis is one of the common genetic aberrations and is formed by chromatin condensation, a characteristic feature of apoptosis (Elmore, 2007).

Cytological deformations were accompanied by cell membrane damage and vacuolar disintegrations. The prominent membrane damages were found in the highest concentration of plant extracts (Bhagyanathan & Thoppil, 2016). A cell that is disturbed while affecting the genotoxic stress may select to complete nuclear division or choose apoptosis (Kirsch-Volders et al., 1997). There are various reports on food preservatives that will reduce the mitotic index while increasing the concentration when compared to the respective control. Chromatid break and multiple breaks are the major aberrations detected throughout the experiments. Major mitotic abnormalities induced by soft drinks treated on *A. cepa* were C-mitosis, stickiness, adherent chromosomes, and chromosome laggards (Chandraker et al., 2014). Severe cytotoxicity was observed in *A. cepa* root tips treated with the two synthetic

colorants *viz*., brilliant blue and fast green. Both clastogenic and non-clastogenic abnormalities were induced by different concentrations of these food colorants (Pokkadath et al., 2022). The major aberrations found in the present study are nuclear lesion, nuclear erosion, chromosome stickiness, chromosome bridges, chromosome fragmentation, pulverized chromosomes, coagulated chromosomes, chained chromosomes, macro and micro cell formation, C-metaphase, stellate chromosomes, hypoploidy, hyperploidy, polyploidy, disturbed chromosomes, diagonal arrangement of chromosomes, stathmo-anaphase, chromosome vagrants, chromosome laggards, scattered chromosomes, pole-to-pole arranged chromosomes, cytostasis, tropokinesis, ball shaped chromosomes, etc. (**Plates 6, 7, 8, 9** and **10**).

Nuclear lesions are associated with programmed cell death and their enormous occurrence displayed the significant cytotoxicity of the extract (Pasqualini et al., 2003). Nuclear erosions are the commonly found aberrations in the *A. cepa* root tips treated with the plant extract and which are formed by the partial dissolution of nucleoproteins (Sharma, 1980). In the present study, sticky chromosomes were observed to be very high in all three extracts. Several reports proved that stickiness is caused by various reasons. This might have happened as a result of depolymerisation of DNA, partial dissolution of nucleoproteins, effect of the extract on the protein, etc. (Darlington, 1942; Kaufmann, 1955; El-Sadek, 1972). Chromosome coagulation is a characteristic aberration found in *A. cepa* root tips and it is an after-effect of chromosome stickiness. Depolymerisation of DNA is a significant reason for the occurrence of coagulated chromosomes (Hollaender, 1954). According to Young and Young (1993), chromosome bridges are formed by the fusion of broken chromosomes and chromosome fragmentations might be formed by the stretching of chromosomes at metaphase followed by their breakage (Chauhan & Chauhan, 1999). Furthermore, pulverised chromosomes are commonly found in all three

extracts and which is due to the premature condensation of chromosomes caused by the effect of chemical substances found in the extract (Knuutila et al., 1981).

The present study showed many non-clastogenic aberrations in which the diagonal arrangement of chromosomes was one among them. It may occur due to the slight tilt in the spindle apparatus by the effect of the extract (Das et al., 1968). Ball-shaped chromosomes are commonly found in metaphase and anaphase stages. In the metaphase stage it might have happened due to the complete degeneration of the cell (Barber & Callan, 1943). Whereas, in the anaphase stage, sister chromatids separate into a hollow ball of chromosomes which occurred by the early cleavage divisions in several abnormal cells (Morgan, 2007). In the present study, chromosome laggards were observed and it is formed by the delayed terminalisation, stickiness of the chromosome ends, or by the failure of the proper movement of chromosomes (Kaur, 1985). Stathmo-anaphase is a non-clastogenic chromosomal anomaly where daughter chromosomes will never separate completely but they remain joined by the partial overlapping of the arms. Abnormal functioning of the spindle apparatus is also another reason for the formation of stathmo-anaphase. Any chemical agent present in the extract, which adversely affects the proper functioning of the spindle fibres, is considered as a stathmo-kinetic agent and it was recorded by Shehab (1979). In this study, the occurrence of stathmo-anaphase is significant in all the extracts at varying concentrations, and it might be due to the effect of the extract having stathmo-kinetic agents. Abnormal spindle activity is the major cause of the pole-to-pole arrangement of chromosomes as well as tropokinesis (Ford & Correl, 1992; Ananthakrishnan et al., 2013). Reports by Rank (2003) described that vagrant chromosomes are a significant representation of spindle poisoning. Therefore, these clastogenic and aneugenic aberrations indicate the significant growth inhibition of *A. cepa* root by the extract. If mitotic inhibition was more than

45%, then it means that the agents of the extract are toxic to the studied organism (Konuk et al., 2007). Hence, the characteristic cytotoxicity of each extract depicts the possible antiproliferative activity of the same.

**D. Antiproliferative activity of selected dye-yielding plants**

**i. Cytotoxicity evaluation using MTT assay**

Cancer is one of the deadliest diseases that affect human beings all over the world. The uncontrolled cell division leads to the onset of cancer. Thus, the research on anticancer drugs had increased day by day. The present study also can point out a similar aspect, in which, the effect of dye-yielding plants on inhibiting the proliferation of cancer cell lines was proved. For this, methanolic extracts from the dye-yielding plant parts are selected. Also, this potential can be exploited in the field of natural colour-based industries. The frequent use of artificial colours had proven that it may ultimately lead to cancer. Hence, this study provides a stepping stone to replace the use of artificial colours with natural colours produced from the various dye-yielding plants.

Breast cancer is one of the most frequently spreading cancers among women. It is the major cause of death worldwide. There are so many reasons behind the progression and establishment of breast cancer. It includes oxidative stress, modern lifestyle as well as the use of modern food and liquid refreshments like coloured drinks and fast-food products. These colours will attract human beings, especially children. To reduce the use of such colours, the present study makes an initiative to replace them with the selected dye-yielding plants. The first part of the anticancer study aims to undertake the cytotoxicity evaluation of the methanolic extract on *Allium cepa* root tips. The extracts showed remarkable cytotoxicity on the root meristem of *A. cepa*. The antiproliferative activity of the extract was determined by MTT assay (Li et

al., 2020). The breast cancer cell line, MDA-MB 231 was used to evaluate the anticancer efficacy of the extracts, in which the formation of formazan is directly proportional to the number of viable cells. Among the selected three methanolic extracts, *M. philippensis* fruit extract showed the highest antiproliferative activity (**Figure 32**; **Plate 16**). The lowest $LC_{50}$ value was obtained in *M. philippensis* (61.5 ± 1.19 µg/mL) followed by *T. paniculata* **(**101.6 ± 0.81 µg/mL) and *A. odoratissima* (157 ± 1.63 µg/mL) (**Table 9**). Here all three extracts possessed a significant inhibitory effect on the breast cancer cell line *viz*., MDA-MB 231. But based on the lowest $LC_{50}$ value (61.5 ± 1.19 µg/mL) the most effective plant was found to be *M. philippensis*. So, further studies including double staining technique, cell cycle analysis, gene expression studies, and EMT screening were performed on *M. philippensis*.

Reports revealed that fruits, seeds, leaves, stem bark, roots, flowers, whole plant, and young shoot of *M. philippensis* are used in 67 different disease conditions, either internally or externally. Anticancer activity is one of the major pharmacological effects of *M. philippensis* (Buha & Acharya, 2020). Antiproliferative activity of an isolated compound known as 4 - hydroxyrottlerin from the fruits of *M. philippensis* showed a 54% growth inhibition of the Thp-1 cell line (Kulkarni et al., 2014). *M. macrostachyus*, a closely related plant in Euphorbiaceae had unveiled a remarkable antiproliferative activity against breast cancer MCF-7 and T47D cell lines. Moreover, *M. macrostachyus* and leaves of other *Mallotus* species exhibited characteristic anticancer activity on different cancer cell lines (Nurhanan et al., 2008). *M. paniculatus* is used in the treatment of various diseases in rural areas. Its leaf extracts in different solvents including ethanol, ethyl acetate, and hexane possess anticancer properties. These extracts were cytotoxic against breast cancer (MCF-7), colon cancer (HT-29), and cervical cancer (HeLa) cell lines. The ethanolic extract had proved to possess a potential cytotoxic effect against MCF-7 (230 ± 3.1 µg/mL) than ethyl acetate (700 ±

2.5 µg/mL) and hexane extracts (400 ± 1.5 µg/mL) (Bahaman et al., 2020). Hence, the antiproliferative activity of *M. paniculatus* on breast cancer cell lines explained possibly the existence of flavonoids and other bioactive compounds in the plant extract (Tistaert et al., 2012). Apoptosis-promoting effects against cancer cells are another characteristic feature of flavonoids (Skrovankova et al., 2015). In the present study, the methanolic fruit extract of *M. philippensis* displayed a considerable amount of flavonoids (55.07 ± 2.99%) (**Figure 8**). So, based on a previously mentioned report, it can be stated that the antiproliferative activity of *M. philippensis* against MDA-MB 231 breast cancer cell lines might be due to the effect of flavonoids. There are various flavonoid compounds resolved in the extract of *M. philippensis*. The phytochemicals like 5,7-dihydroxy-8-methyl-6-prenylflavanone, mallopenin D, mallopenin E, and 6,6-dimethylpyrano (2″,3″:7,6)-5-hydroxy-8-methylflavanone were isolated from the fruits of *M. philippensis* (Zaidi et al., 2009; Cheenpracha et al., 2019; Furusawa et al., 2005). Whereas, isovitexin and ampelopsin were isolated from the leaves itself (Mai et al., 2010). Anh et al. (2022) suggested that the effects of these flavonoid compounds are the major reason for the antitumour activity of *M. philippensis* against various cancer cells. Moreover, they described that phenolic compounds, terpenoids, tannins, chalcone derivatives, lignans, *etc*. are the responsible phytoconstituents for anticancer activity. In *M. philippensis* fruits, the number of chalcones is very high, that include kamalachalcone A, B, D, E, rotterin, mallotophilippen C, D, F, 3-prenylrubranine, etc. (Anh et al., 2022). *M. mollissimus* is another plant belonging to Euphorbiaceae, which had displayed anti-cancer activity against various cancer cells including MCF-7 (63 ± 1.32 µg/mL) (Ismail et al., 2021). In the present study, the $LC_{50}$ value of *M. philippensis* (61.5 ± 1.19 µg/mL) fruit extract against MDA-MB 231 cell lines is near to this value. So, this clearly indicates that both these *Mallotus* species have almost equal effects on breast cancer cells. Furthermore, polyphenolic

compounds revealed *via* GC-MS analysis of *M. philippensis* were found to be responsible for the inhibition of cancer growth and induction of apoptosis (Tanaka et al., 2008). The presence of phenolic compounds is prominent in the case of *M. philippensis* fruit extract and it was proven by the GC-MS and HR LC-MS analyses performed. Hence the abundance of polyphenolic compounds in the fruit extract might be another cause for the anticancer activity observed against MDA-MB 231 cell lines.

**ii. Double staining**

Acridine orange - ethidium bromide (AO/EB) double staining method is mainly implemented to determine the ability of the extract to induce apoptosis on MDA-MB 231 cell line. The antiproliferative activity of *M. philippensis* on MDA-MB 231 cell line revealed a promising inhibitory activity, which was revealed by a dual staining technique. Cell death can easily be detected by this method and also have the ability to define whether it is apoptotic or necrotic cell death. Green-coloured nuclei represented the normal cells and bright green colour denoted the apoptotic cells (**Plate 18**). Whereas the orange-stained nucleus describes the late apoptotic cells and the uniform orange-stained nucleus denotes the necrotic cells. Acridine orange and ethidium bromide (AO/EB) are the fluorescent DNA binding dyes and displaying the various colour shades with the help of a fluorescence light microscope is a simple, rapid, and accurate method. Acridine orange has the capacity to permeate into the cells and make the nuclei green in colour. When the ethidium bromide is taken up by the cells, they may affect the cytoplasmic membrane integrity and turns into a red nucleus. Ethidium bromide always dominates over acridine orange (Renvoize et al., 1998). Hence, in the present study, methanolic fruit extract of *M. philippensis* revealed the presence of apoptotic cells with bright green nuclei (**Plate 18**) and leads to cell death.

### iii. Cell cycle analysis

Flow cytometry is a fast and reliable method used for the measurement of nuclear DNA content (Dpooležel et al., 1989). A typical DNA histogram had been obtained after the measurement of nuclear DNA content using a flow cytometer (**Figure 34**). It contains several peaks representing the G0/G1, S, and G2/M phases. The cell cycle is a molecular event responsible for cell multiplication (Pucci et al., 2000). Genetic information can be transmitted from one cell generation to the next by the replication process occurring in the S phase and their segregation will occur to form the new daughter cells during the M phase. These two phases are very crucial events that exhibit a cyclic process and permit the correct duplication of the cell without any genetic abnormalities. Under normal conditions, the M phase always follows the S phase and it will occur only when the S phase is complete.

In the present study, the most effective plant *i.e*., *M. philippensis* was subjected to experiments in order to analyse the DNA and cell cycle distribution in MDA-MB 231 cells by using flow cytometry. After the analysis, the cell cycle arrest was found to occur in a particular phase (**Figures 33a & b**). For untreated cells, DNA content was found to be 52.7, 19.7, and 23.7% in G0/G1, S and G2/M phases respectively (**Figure 34a**). But a decreasing order of cell count was noticed in the case of treated cells (**Figure 34b**). This prominent decrease in the number of treated cells indicated that the cells were blocked in the G1 phase and had led to the accumulation of cells in G0/G1. Hence cells were not progressing to the remaining phases of the cell cycle. These results were closely related to the previous reports put forward by Dogra et al. (2016) in various cell lines. Hence, the population histogram clearly depicts the reduction of cell counts which clearly distinguishes the apoptotic cells. Moreover, the cell cycle arrest

was found to occur at G0/G1 phase by the effect of the methanolic fruit extract of *M. philippensis*.

Cell cycle and apoptosis favour a relationship link in which apoptosis is regulated by genes that are involved in cell cycle progression (Evan et al., 1995). The order and proper timing of cell cycle events are scrutinized during cell cycle checkpoints that occur at the G1/S boundary, in S - phase, and during the G2/ M - phases (MacLachlan et al., 1995). The checkpoints are a series of control systems that allow the proliferation only in the presence of stimulatory signals like growth factors. They also contribute to the transmission of genetic information, which gets passed on from one generation to the next. The checkpoints are triggered by DNA damage and by misaligned chromosomes at the mitotic spindle. Then the growth arrest will occur by checkpoints that permit the cell to repair the damage. After the damage repair, progression *via* the cell cycle continues. When damage cannot be repaired, the cell gets eliminated by apoptosis (Pucci et al., 2000).

Progression of cells through the four phases of cell cycle takes place through the activation and inactivation of CDKs (cyclin-dependent kinases). CDKs belong to the serine or threonine protein kinases. The presence of activating subunits called cyclins helps their kinase activity. The increase of such cyclins occurred during the phases of the cell cycle when they are required. Whereas cyclins decrease if they are not required. During early G1, cyclin D is associated with CDK 4 and CDK 6. The family of proteins called pRb (retinoblastoma protein) was the primary target of the G1 kinases. Its phosphorylation helps the transcription of genes required for the S phase. So, the cyclin D or CDK complexes are very essential to the cell cycle by coupling extracellular signals to the cell cycle (MacLachlan et al., 1995). pRb is a negative regulator of cell growth and is a tumor suppressor. In many

cancers, such as retinoblastoma and carcinomas of the lung, breast, bladder, bone, and prostate it is mutated or deleted (Riley et al., 1994).

Flavonoids also have the ability to block the cell cycle followed by apoptosis (Singh & Agarwal, 2006). They induce cell cycle arrest in G2 and also the mobile phase of the cell cycle. They also inhibit heat-shock proteins, tyrosine kinase, and ras protein as well as downregulated estrogen receptor-binding capacity. The effect of cancer involves genetic abnormalities that result in p53-mutated proteins. The downregulation of these proteins by flavonoid intake also distresses cancer growth (Veeramuthu et al., 2017). Mallotus B is a compound isolated from *M. philippensis* and it was able to arrest the cell cycle at the G1 phase and induce apoptosis among cancer cell lines (Jain et al., 2013). It causes imperfect cell division and also induces apoptosis, as demonstrated by changes in the cell morphology. 75% cells showed G1 arrest in HL-60 cells by Mallotus B. Hence, it may be the reason for the similar condition observed in the present study. Reports on *M. furetianus* unveiled that cell cycle arrest had occurred during the S phase on Ehrlich ascites tumor cells (EATC) and its inhibition of cell proliferation occurs through the activation of p21 (Shimizu et al., 2015). The reports on the cell cycle analysis in the genus *Mallotus* on the breast cancer cell lines are very scanty. So, the current research can make a crucial role in the field of drug development against breast cancer cell lines.

**iv. Gene expression studies**

During the cell cycle analysis, the DNA damage as well as the apoptotic potential of *M. philippensis* was proved. MDA-MB 231 cell lines were treated with $LC_{50}$ concentration (61.5 ± 1.19 µg/mL) of methanolic fruit extract of *M. philippensis* and revealed that the cell cycle arrest occur at G0/G1 stage after 24 hours. The double staining technique unveiled the appreciable apoptotic nature of *M. philippensis*. The quantitative gene

expression changes effected by the *M. philippensis* fruit extract were evaluated with the help of p53 and TGF beta genes using a real-time reverse polymerase chain reaction (RT-qPCR). The results revealed that the fruit extract induced upregulation in the expression of genes p53 and TGF β. The expression fold changes revealed the expression of apoptotic genes. In both genes, the measurement of expressions varied. The expression fold changes of p53 were 1.52 over the control and that of TGF β was 1.7 (**Figures 35a & b**). Hence, the plant extract has the capacity to enhance the expression of both genes almost double when compared to the control. The positive values of the expression fold changes represent the upregulated mechanism of gene expression.

The p53 is a protein that has the ability to act as an essential growth checkpoint that protects cells from cellular transformation. Also, it leads to a growth arrest or apoptosis by the induction of genes. It is a tumour suppressor gene and also plays a significant role in TGF - β induced growth arrest (Dupont et al., 2004). Authors reported that p53 has the ability to act as a transcription factor and its antiproliferative action is caused by inducing the reversible or irreversible cell cycle arrest or apoptosis. It also enhances DNA repair and inhibits angiogenesis (Lacroix et al., 2006). However, in breast cancer cases, the frequency of mutation in p53 is lower than the other solid tumours. The structure and expression of p53 in breast cancer cases have been widely studied. The expression of mutant p53 was already demonstrated in breast cancer cell lines. Moreover, in primary breast cancer carcinomas loss of heterozygosity (LOH) in the p53 gene was considered a common event. In some cases, it may happen by the mutation of the residual allele (Gasco et al., 2002). Cell cycle arrest in G1/S phases has been detected as a p53-dependent process. The cell cycle arrest that happened in the G1 phase is mainly by the activation of cyclin-dependent kinase (CDK) inhibitor p21 (Karimian et al., 2016). In the present study, cell cycle arrest was observed in the G0/G1 phase

and it can be correlated with the p53 gene-dependent process involved in the cell cycle arrest at G1 phase. Gene expression by p53 may vary even between cell lines consequent from the same clone and depends on the nature of the p53 induction signal and on the cell type (Yu et al., 1999). In addition, p53 gene expression disclosed an upregulation of the cell cycle inhibitor and the DNA repair genes and downregulation of cyclins and kinases, which promote cell cycle and cell division. The overall pattern may be responsible for the growth suppressive function and DNA repair activity of p53 (Kannan et al., 2000). Moreover, apoptosis has been induced by the antioxidants and the p53 status of cancer cells, which is formed as a significant factor that can predict the effect of prooxidant drugs on cancer cells (Sandhya & Mishra, 2006).

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine with various activities like cell-cycle control, the regulation of early development, differentiation, extracellular matrix formation, haematopoiesis, angiogenesis, chemotaxis, immune functions, and apoptotic induction. Active participation of TGF-β in growth inhibition as well as apoptosis can be related to its function as a tumour suppressor. In most human tumours, the components of the TGF-β pathway are defective in some cases either by an inactivating mutation within the TGF-β receptors or by the downstream elements of the pathways (SMAD/DPC4) which is essential for the intracellular TGF-β mediated signal transduction (Schuster & Krieglstein, 2002). Reports revealed that there is a connection between p53 and the growth inhibitory family of TGF- β, which suggests an important paracrine mechanism for tumour growth suppression by p53 (Komarova et al., 1998). Furthermore, normal human mammary epithelial cells are very sensitive to TGF-β and human breast cancer lines require 10-fold to 100-fold more TGF-β to produce an antimitogenic response (Fynan & Reiss, 1993). In human cancers, the loss of growth inhibition by TGF-β causes the loss of TGF-β production or it may lead to the mutational inactivation of the TGF-β

receptors and Smad signalling molecules. These defects are not found to arrest resistant cancer cell lines (Donovan & Slingerland, 2000). In human tumours, the continuous appearance of resistance to more than one inhibitory cytokine describes the significance of the cell cycle effectors of growth arrest, induced by TGF-β as a target for inactivation in cancer (Kerbel, 1992). TGF-β can either participate to lengthen the G1 transit time or can be a reason for the arrest in the late G1 phase. This cell cycle arrest is mostly reversible, but in several cases, it is accompanied by terminal differentiation (Donovan & Slingerland, 2000). When the early G1 phase reaches a 'restriction point' 6-10 h after G0 release, cells are very sensitive to TGF-β. After this critical time point, TGF-β is added and cells complete the cell cycle but growth arrest may happen during the subsequent G1 phase. It is suggested that the TGF-β was acting before the transition of G1 to the S phase to inhibit a pRb kinase and causes the investigation of the effects of TGF-β on cell cycle regulators. Also, it prevents the G1 cyclin-CDK activation and leads to pRb dephosphorylation by multiple mechanisms (LaBaer et al., 1997). TGF-β had also impaired the E2F activity through a decline in E2F mRNA levels. The over-expression of this E2F can inhibit TGF-β arrest and it describes the effects of TGF-β on pRb and E2F (Schwarz et al., 1995). In a cell-type-dependent manner, TGF-β causes the loss of G1 cyclins. In many cell types, cyclin A expression is downregulated by TGF-β, and also a cyclin A promoter and a TGF-β-regulated region has been identified. Effects of TGF-β on cyclin E vary in distinct cell lines. Moreover, TGF-β decreases both mRNA and protein levels of cyclin A and cyclin E in HaCaT keratinocytes. Whereas cyclin E mRNA is reduced but protein levels are not reduced as observed in HMECs. While in some cell types cyclin D1 levels are decreased by TGF-β. This generally happens very late as a consequence of cell cycle arrest (Donovan & Slingerland, 2000).

In the present study, the ability of the methanolic fruit extract of *M. philippensis* revealed the upregulation of apoptotic genes in the gel images obtained *via* electrophoresis (**Figure 36**). In addition, the antiproliferative activity of *M. philippensis* was proved by the aberrations detected in the MTT assay, morphological visualization of apoptotic bodies, cell cycle arrest, and the characteristic expression fold changes in the apoptotic genes revealed using gene expression studies.

## E. EMT Screening

EMT is a physiological process in which epithelial cells acquire the motile and invasive characteristics of mesenchymal cells. It is a process in which drastic changes occur in the cellular organization from epithelial to mesenchymal phenotypes, which may lead to functional changes in cell migration and invasion. It occurs in response to the signals from their microenvironment. Also, it is known to be activated during cancer pathogenesis and tissue fibrosis. Moreover, cell biologists are mainly focused on the thoughtful changes in cell-cell interactions, cell motility, cytoskeletal organization, cell proliferation, and resistance to several stressors that occur during EMT (Yang et al., 2020). Metastasis is the spread of cancer cells, where epithelial cancer cells undertake an epithelial-mesenchymal transition (EMT). This may happen due to the migratory and invasive attitude of the mesenchymal-like cells getting cancer stem cell properties and therapy resistance (Jonckheere et al., 2022). Three different assays were used to perform the EMT screening during this study *viz*., cell migration assay, cell aggregation assay, and clonogenic assay. In these assays, the effect of *M. philippensis* fruit extract on human breast cancer cell lines (MDA-MB 231) was carried out. Each assay revealed a characteristic result on MDA-MB 231 cancer cell lines. In the cell migration assay, the size of the wound area was found to be decreased in the control cells during 0 to 72 hours. Whereas in

treated cells, the wound area remains with a size of 77956 px at 72 hours. However, no wound area was observed at the control cells in 72 hours (**Plate 19**). Hence, the results revealed that the methanolic fruit extract of *M. philippensis* with an $LC_{50}$ concentration of **61.5 ± 1.19 µg/mL** inhibit the migration of MDA-MB 231 breast cancer cells. In cell migration assay, the extract manages to delay the growth of cancer cells towards the created wound and prevent the migration of cancer cells (Syed Najmuddin et al., 2016). A similar condition had happened in the present study, where the migration of MDA-MB 231 cells towards the wound was restricted by *M. philippensis* fruit extract at each time duration. Jafari-Oliayi and Asadi, (2019) suggested that the cell migration ssay is a better method to determine the potential role of the extract on the migratory ability of breast cancer cell lines. There is an interesting finding by Isbilen et al. (2018), in which 9-octadecanamide, a fatty acid found in *Allium willeanum* at a high concentration has the potential role in the inhibition of lateral motility of MCF-7 and MDA-MB 231 breast cancer cells. In the present study, fatty acid concentration was found to be very high in *M. philippensis* fruit extract and was proved by GC-MS analysis. Hence, this might be a reason for the effective migration result obtained in the cell migration assay.

MDA-MB 231 is a metastatic breast cancer cell line and is known as a 'basal' or 'triple-negative' cell line with stem cell-like or post-Epithelial-Mesenchymal Transition (EMT) features and fails to express estrogen receptors, progesterone receptors, or human epidermal growth factor receptor 2 (HER2) (Geng et al., 2013). The aggregation of these MDA-MB 231 cell lines was observed when the extract was applied to the culture. In the present study, the formation of cell aggregates is very less when MDA-MB 231 cells were exposed to *M. philippensis* fruit extract (**Plate 20**). The aggregation of breast cancer cell lines is large and compact in control cells at 48 and 72 hours. Whereas, at the same time interval, when the MDA-MB 231 cells were

exposed to *M. philippensis* fruit extract, it showed cell aggregates to a limited extent. Hence, *M. philippensis* fruit extract unveiled its effect to inhibit the cell aggregation of MDA-MB 231 cells. Cell aggregation assay is mainly used to test the functionality of the complex in epithelioid tumour cells. In between the epithelial cells, the functional integrity of the complex is a prerequisite for cell-cell adhesion, and the *in vitro* measuring of cell aggregation has become another elegant tool to study the differences between invasive and non-invasive cell types (Boterberg et al., 2001). MDA-MB 231 cell lines are the invasive cell types (Sapudom et al., 2019). The *in vitro* measuring of cell aggregates was found to be very less while performing the cell aggregation assay with *M. philippensis* fruit extracts.

Clonogenic assay is an *in vitro* cell survival assay in which the ability of a single cell to form a colony is assessed. In the present study, colony formation of MDA-MB 231 cancer cells is very less in cells treated with *M. philippensis* fruit extract. Whereas, the number of colonies was found to be very high in control cells. This means that the fruit extract of *M. philippensis* promisingly restricted the formation of MDA-MB 231 cancer cell colonies (**Figure 38**). According to Franken et al. (2006), an aggregate consisting of at least 50 cells is considered a colony. In the present study, the untreated control cells produced about 112 colonies whereas the treated cells produced only 29 colonies. Hence, the lesser number of colonies in treated cells is not significant and couldn't be considered clonogenic. Therefore, the potential role of *M. philippensis* fruit extract to inhibit the formation of colonies was revealed very successfully. Clonogenic assay serves as a useful tool to detect all cells that have retained the ability for producing numerous progenies after treatments. This may lead to cell reproductive death as a result of damage to chromosomes, apoptosis, etc. (Brown & Attardi, 2005). In a population, all cells are essentially tested for their ability to undergo unlimited division. Also, it is a choice to determine the cell's productive death after treatment

with ionizing radiation and to know the effectiveness of other cytotoxic agents. Only a fraction of the seeded cells can hold the ability to produce colonies. It will take about 1-3 weeks to produce colonies after appropriate dilutions before or after treatment. Colonies fixed with glutaraldehyde and stained with crystal violet are an effective method for counting under a microscope (Franken et al., 2006). Colonies formed by the clonogenic assay and stained with crystal violet in the present study were depicted very clearly in **Figure 38**. Due to the effect of several hormonal agents like tamoxifen (Tam) and medroxyprogesterone (MPA), human breast cancer specimens did not grow well enough into large-sized colonies (Osborne et al., 1985).

Several authors described that EMT is a crucial process involved in the invasion and metastasis of cancer cells. Downregulation of E-cadherin is a main event that occurs in EMT, which leads to the transition of cells from an epithelial to a mesenchymal state. Zeb1 and Snail are several transcription factors that may bind to the E-cadherin promoter and suppress its transcription. As a result, a subsequent loss of apical-basal polarity of the cells, cell-cell adhesion, and the acquisition of a mesenchymal morphology occur (Craene & Berx, 2013; Whiteman et al., 2008).

## PART III – GREENSYNTHESIS OF SILVER NANO PARTICLES

Dye-yielding plants have been proven to be a strong candidate for the synthesis of silver nanoparticles. Most of the plant parts have the ability to synthesize silver nanoparticles, but in the present study, methanolic extracts of the selected dye-yielding plant parts were chosen. All three dye-yielding plant extracts exhibited a considerable potential to synthesize nanoparticles by their own means. The combined action of silver nitrate solution together with methanolic fruit extracts of *T. paniculata* and *M. philippensis* as well as the bark extract of *A. odoratissima* might be responsible for the synthesis of silver nanoparticles. Among the three extracts, *T. paniculata* synthesized more silver nanoparticles followed by *M. philippensis* and *A. odoratissima* (**Plate 21**). The brown colouration indicates the presence of synthesized silver nanoparticles. Reports revealed that the presence of various metabolites, proteins, and chlorophyll in plant extracts are responsible to act as the capping agents for the synthesis of silver nanoparticles (Srikar et al., 2016). For the detailed characterization of synthesized silver nano particles, physicochemical profiling was also performed. It includes ultraviolet-visible-near infrared (UV-Vis-NIR) spectral analysis, field emission scanning electron microscopy (FE-SEM), energy-dispersive X-ray (EDAX) analysis, and X-ray diffraction (XRD) technique.

In the current study, a dark brown colour was formed in the case of *T. paniculata* fruit extract whereas, *M. philippensis* showed a yellowish-brown colour and in *A. odoratissima* the intensity of brown colour formation was less than that of the other two extracts. The previous reports already revealed that the performance of surface plasmon resonance (SPR) is one of the best reasons for identical colour changes (Aswathi & Thoppil, 2022). A typical SPR peak of the synthesized silver nanoparticles is in the range of 400-500 nm (Chahardooli et al., 2014). The formation of silver nanoparticles by the

reduction of silver ions was monitored with the help of UV-Vis-NIR spectroscopy. The obtained UV-Vis spectrum, confirms the synthesis of silver nano particles, in which the significant absorbance peaks were formed at 456, 438, and 465 nm in *T. paniculata*, *M. philippensis* and *A. odoratissima* respectively (**Figure 39**). A significant absorbance peak between 400 and 500 nm confirms the production of silver nanoparticles. According to Christensen et al. (2011) the colour change from light green to dark orange-brown is due to the increased concentration as well as the production of silver nanoparticles. If no colour change occurs after 90 min, it indicates the complete reduction of silver. In the present study, colour change was observed in all three extracts treated with silver nitrate solution after a few minutes when boiled at 80°C. The fruit extracts of *T. paniculata* and *M. philippensis* had the capacity to reduce and accumulate silver ions into silver nanoparticles as proved by Aswathi and Thoppil (2022). The UV-Vis spectral analysis is mainly used to know about the pH dependency, concentration of metal ions, and extract content in the formation of silver nanoparticles. There are various reports on *Terminalia* species in the field of synthesis of silver nanoparticles. The previous reports reveal that *T. chebula* leaf extract was treated with silver nitrate solution, the reaction takes place within a few seconds and the colour change was clearly observed. UV-Vis spectrum unveils the SPR peak of silver at 421 nm (Espenti et al., 2016). There is another report for *T. chebula* in which the aqueous extract of *T. chebula* could synthesize silver nanoparticles with an SPR peak at 452 nm (Kumar et al., 2012). *T. chebula* is very rich in polyphenolic compounds which include ellagitannins, gallotannins, ellagic acid, gallic acid, etc., which might be the reason for the synthesis of silver nanoparticles (Surveswaran et al., 2007). According to Kumar et al. (2012), a reddish-brown colour was obtained within 15 minutes, which is a quite faster time reported than several previous reports. The higher availability of polyphenols in *T. chebula* is the main

reason behind the rapid formation of silver nano particles. Hence, reports suggested that *T. chebula* extracts were not only responsible for the reduction of silver ions to silver nanoparticles but also act as the capping agents for the synthesized silver nanoparticles. Besides, hydrolysable tannins present in *T. chebula* fruits are also responsible for the synthesis of silver nanoparticles. Gallic acid is a very poor stabilising agent which cannot prevent the aggregation of silver nanoparticles. The present study proved the active presence of polyphenolic compounds in *T. paniculata* fruit extracts and their vital participation in silver nanoparticle synthesis. The presence of ellagic acid and gallic acid in the methanolic fruit extract of *T. paniculata* was already proved by HR LC-MS analysis. Therefore, based on these reports it is clearly understood that *Terminalia* is an active genus for the synthesis of silver nanoparticles. The typical peak of *M. philippensis* was observed at 438 nm due to the surface plasmon resonance of silver nanoparticles. Similarly, Kulikarni et al. (2011) observed the presence of silver nanoparticles by obtaining the peak at 438 nm. Previously, the ethanolic fruit extract of *M. philippensis* revealed the synthesis of silver nanoparticles (Velraj et al., 2017). Quercetin is a flavonoid compound reported in *M. philippensis* fruits, which is actively participating in the synthesis of silver nanoparticles (Velraj et al., 2018). The present study displayed that flavonoid compounds are abundant in *M. philippensis* fruit extracts, which include quercitrin, apiin, cosmosin, etc. In this report, the production of silver nanoparticles was found to be prominent in *M. philippensis,* which was less than that of *T. paniculata* fruit extract and greater than that of *A. odoratissima* (**Plate 21**). Compared to *Terminalia*, reports on the synthesis of silver nanoparticles in *Mallotus* are very meager. Hence, the present study revealed that the exploitation of this genus can form a stepping stone for the synthesis of silver nanoparticles. For *A. procera*, the SPR peak was observed between 400 to 500 nm (Rafique et al., 2019). Moreover, for *A. adianthifolia*, it was observed at 448 nm (Gengan

et al., 2013). In *A. saman.* UV-Vis spectroscopic studies presented an SPR absorption peak at 411 nm, which resulted in the blue shift and specifies the smaller size of silver nanoparticles. Besides, there is an absorption peak at 415 nm in the red shift region, which was larger in size (Daphedar & Taranath, 2017). Furthermore, in *A. odoratissima* SPR peak was observed at 465 nm (**Figure 39**), which confirmed the synthesis of silver nanoparticles.

Synthesized silver nanoparticles were characterized with the help of FE-SEM analysis. It produces the images by scanning the surface with a focused beam of electrons (Aswathi & Thoppil, 2022). Detailed information about the size and shape of the synthesized nanoparticles was obtained by the FE-SEM analysis. In the present study, *T. paniculata* and *A. odoratissima* exhibited spherical-shaped nanoparticles, whereas for *M. philippensis* it was nearly cubical (**Plates 22, 23, 24**). The size of nanoparticles varied in all three plants. For *T. paniculata*, the range of the size was between 20 - 60 nm, for *M. philippensis* it was 21 - 45 nm and for *A. odoratissima* it was between 30 - 90 nm. Hence, a considerable variation in the size of nanoparticles was revealed by the SEM images. Spherical-shaped nanoparticles are depending on a single SPR band formed by UV-Vis spectral analysis. The presence of anisotropic nanoparticles always supports the Mie theory. It describes a single SPR band in the absorption spectra of spherical nanoparticles that envisage the size, distribution, dispersibility, and morphology of the nanoparticles, which was controlled by the variations in the concentration of the stabilizer (Rastogi & Arunachalam, 2011). According to González and Noguez, (2007), the size and shape of silver nanoparticles are varying in different plant extracts. Substrate concentration, biocatalyst concentration, pH, temperature, light, reaction time, etc. are some important parameters responsible for the size, morphology, and various properties of the silver nanoparticles (Gardea-Torresdey et al., 2003; Haverkamp et al., 2007). Nanoparticles with smaller size have more penetration power and their biological activity is moreover

size-specific (Wang et al., 2017). The prominence of elemental silver in the sample was confirmed by the presence of a sharp peak obtained by the EDAX analysis (**Plate 25**). All three extracts exhibited a significant presence of silver. According to Gul et al. (2016) and Ismail et al. (2018), individual spherical-shaped elemental silver displayed a peak at 3 keV. In the present EDAX profile, peak for metallic silver was observed at 2 keV. The other peaks displayed were obtained from the plant-based capping agents. Elements like Cl were observed in *T. paniculata*, which might have originated from biomolecules bound to the surface of silver nanoparticles (Prabhu & Poulose, 2012).

X-ray diffraction is a powerful method for the study of nanoparticles. It contributes detailed information regarding the crystalline nature of the particles. X-ray diffractograms of nanomaterials provide scientific information from phase composition to crystallite size and from lattice strain to crystallographic orientation (Aswathi & Thoppil, 2022). In the present study, *T. paniculata*, *M. philippensis* and *A. odoratissima* revealed a characteristic crystalline structure by the X-ray diffraction technique (**Plate 26**). The diffraction patterns were obtained by measuring of angles at which the X-ray beam is diffracted by the crystalline phases in the particle. The crystalline nature of the silver nanoparticles is already proved as the face-centered cubic structure by the X-ray diffraction method (Awwad et al., 2013). They recorded that silver nanoparticles were evidenced by the peaks at 2θ values of 38.28º, 44.04º, 64.34º, and 77.28º corresponding to (111), (200), (220) and (311) Bragg reflections respectively. In the present study, diffraction peaks of *T. paniculata* at 2θ values were found to be 38.08º, 44.21º, 64.42º, and 77.01º corresponding to (111), (200), (220), and (311) Bragg reflections respectively. The 2θ values of *M. philippensis* were 38.08º, 43.66º, 64.57º, and 77.29º, whereas 38.15º, 44.21º, 64.55º, and 77.27º were

detected for *A. odoratissima*. From these results, the highest peak was observed at 38.03º in *T. paniculata*, 38.08º in *M. philippensis*, and 38.15º in *A. odoratissima*. Rajakumar and Rahuman (2011) suggested that a typical XRD pattern of the peaks for silver nanoparticles exhibited FCC structure. Hence these Bragg reflections revealed the face-centered cubic nature of silver. Furthermore, the average crystallite size of the synthesized silver nanoparticles could be calculated with the help of the Scherrer formula. For *T. paniculata* average crystallite size of silver nanoparticles was 17.7 nm and in *M. philippensis* and *A. odoratissima* it was 4.36 nm and 7.11 nm respectively (**Table 12**). Consequently, these results clearly showed that the biosynthesized nanoparticles were composed of highly crystalline silver.

# SUMMARY AND CONCLUSIONS

The immense use of artificial colourants had increased for various purposes to date, especially in the food, textiles, and pharmaceutical industries. In food industries, the colourants will make attractive deliverables like fast foods, soft drinks, etc. Different colours given to various foods and drinks will attract human beings, especially children. Frequent use of these synthetic colours may lead to various health issues including cancer. Instead of artificial colourants, the production of natural colours/dyes will reduce the adverse effect caused by these synthetic ones. Therefore, dye-yielding plants provide a stepping stone in the field of commercial industries. Natural dyes or colours can be derived from plants, invertebrates, minerals, etc. But plants are used as the major source of natural dyes. Such plants are called dye-yielding plants. In the present study, all selected dye-yielding plants *viz*., *Terminalia paniculata*, *Mallotus philippensis,* and *Albizia odoratissima* can produce diverse coloured extracts from their different parts. Using the Soxhlet extraction technique, *T. paniculata*, *M. philippensis,* and *A. odoratissima* produce yellow brown, orange, and brick red coloured extracts respectively. Fruits are the dye-yielding plant parts for *T. paniculata* and *M. philippensis.* Whereas in the case of *A. odoratissima,* the dye-yielding plant part was bark. The present investigation is mainly focused on the phytochemical characterization, bioactivity studies and silver nanoparticle biosynthesis of the methanolic extracts of the selected dye-yielding plants *viz*., *T. paniculata*, *M. philippensis,* and *A. odoratissima*.

- **Phytochemical profiling**

Phytochemistry studies reveal the presence of major bioactive compounds present in the plant extracts. The methanolic extracts of all three dye-yielding plants *viz*., *T. paniculata*, *M. philippensis,* and *A. odoratissima*

exhibit a wide spectrum of various active phytocomponents. The existence of an array of such phytocompounds in a plant seems to be responsible for its pharmacological activities. Preliminary phytochemical analysis revealed the presence of various phytocompounds such as alkaloids, flavonoids, phenols, tannins, terpenoids, steroids, saponins, glycosides, phlobatannins, anthraquinones, coumarins, resins, proteins, and amino acids. All these compounds are found in the methanolic fruit extract of *T. paniculata*. Apart from saponins and phlobatannins all the other compounds were found to be present in *M. philippensis*. Coumarins and resins were not detected in the methanolic bark extract of *A. odoratissima*, whereas all the other compounds seem to be present.

The total content of the phytochemicals present in the extract could be unveiled by the quantitative evaluation. The total content of phenols, flavonoids, alkaloids, terpenoids, and tannins was evaluated. Among all the three dye-yielding plants, the highest phenolic content was found in *T. paniculata* (133.33 ± 1.14 mg GAE/gDW) followed by *A. odoratissima* (111.8 ± 3.67 mg GAE/gDW) and *M. philippensis* (84.65 ± 2.55 mg GAE/gDW). Gallic acid was used as the standard for phenolic content quantification. For the quantitative analysis of flavonoids, the highest amount (98.66 ± 2.98 mgQE/gDW) was found in *A. odoratissima*. The least amount of flavonoids was observed in *M. philippensis* (28.19 ± 0.77 mg QE/gDW). Quercetin was used as the standard for the determination of total flavonoid content. An equal amount of alkaloid content was found in both *T. paniculata* (24.99 ± 0.31 mgQE/gDW) and *M. philippensis* (23.5 ± 0.32 mg QE/gDW). Only 12.34 ± 1.6 mg QE/gDW of alkaloid content was observed in *A. odoratissima*. A promising amount of terpenoids was found in all three selected dye-yielding plants. Linalool was used as the standard for the quantification of terpenoids. A higher amount of tannin content was observed in *A. odoratissima* (6.06 ± 0.26 mg TAE/g DW) followed by *T. paniculata*

(3.19 ± 0.05 mg TAE/g DW) and *M. philippensis* (1.46 ± 0.23 mg TAE/g DW). Tannic acid was used as the standard for the determination of tannin content in all three extracts.

Volatile and non-volatile phytoconstituents could be identified through sophisticated techniques called GC-MS and HR LC-MS analyses respectively. About 17 different compounds were identified using GC-MS analysis with a retention time ranging from 9.895 to 28.761. Palmitic acid and methyl stearate were the common volatile compounds found in all three extracts. About 54 various non-volatile compounds were identified using HR LC-MS analysis. Pro-Leu, gallic acid, pantothenic acid, Ile-Arg-Ala, and ellagic acid are the common compounds found in both *T. paniculata* and *M. philippensis*. The highest number of volatile and non-volatile compounds was observed in *M. philippensis* followed by *T. paniculata* and *A. odoratissima*.

✹ **Bioactivity screening**

❖ **Antioxidant activity**

To reveal the free radical scavenging activity, all three extracts were evaluated using four different assays *viz*., DPPH radical scavenging assay, hydroxyl free radical scavenging assay, nitric oxide free radical scavenging assay, and superoxide free radical scavenging assay. In DPPH, hydroxyl, and superoxide free radical scavenging assays, *T. paniculata* showed the maximum scavenging capacity. Whereas, in the case of the nitric oxide free radical scavenging assay, *T. paniculata* and *A. odoratissima* exhibited an almost equal effect of scavenging ability. Due to the significant antioxidant potential of *T. paniculata* than the other two dye-yielding plants, further hepatoprotective studies were conducted on it.

❖ **Hepatoprotective activity**

To find out the hepatoprotective ability, first of all, hepatotoxicity screening was evaluated in the methanolic fruit extract of *T. paniculata* due to its high antioxidant potential. To know about the cytotoxicity of *T. paniculata* extract on normal liver hepatic cells (Hep G2), an MTT assay was carried out. The yellow-coloured methanolic extract obtained from the fruits of *T. paniculata*, a dye-yielding plant was compared with Lemon Yellow-19140, a synthetic food colourant. 6.25, 12.5, 25, 50, and 100 µg/mL are the selected concentrations for the study. The percentage of growth inhibition of Hep G2 cells is higher in lemon yellow (78.45 ± 0.75%) than *T. paniculata* fruit extract (69.51 ± 0.78%) at the maximum concentration (100 µg/mL). Hence, lemon yellow with a high $LC_{50}$ value (228.688 µg/mL) was found to induce more toxicity to the growth of Hep G2 cells than *T. paniculata* fruit extract. Due to the lesser level of toxicity, *T. paniculata* was further subjected to the screening test for hepatoprotective activity.

Hepatoprotective screening of the methanolic fruit extract of *T. paniculata* was determined on normal human hepatocytes (Hep G2 cells). Acetaminophen was used to induce toxicity to the Hep G2 cells which were then treated with the fruit extract. The protective ability of the fruit extract ranged from 77.85 ± 2.59 to 85.56 ± 1.88% as concentration increased from 6.25 to 100 µg/mL. Hence, the fruit extract was found to increase the viability of Hep G2 cells. Due to the less toxicity and increased viability percentage of *T. paniculata*, on Hep G2 cells, this dye-yielding plant can be used as a better safe food colourant to replace the synthetic one, lemon yellow.

❖ **Cytotoxicity using *Allium cepa***

Cytotoxicity of all three extracts of selected dye-yielding plants *viz*., *T. paniculata*, *M. philippensis,* and *A. odoratissima* were determined by using

*Allium cepa* root tip assay. The selected concentrations for the assay were 25, 50, 75, and 100 µg/mL. The 24h treatment was used to identify the cytotoxic effect of the methanolic extracts. In all the experiments, results unveiled that the mitotic index decreases with increasing concentrations, and aberration percentages were found to increase in a dose-dependent manner. Both clastogenic and non-clastogenic aberrations were observed in all the treatments. After that, cytotoxicity was also analyzed on the human normal cell line (L929).

❖ **Antiproliferative activity**

The antiproliferative activity of all three extracts was examined on both the human normal cell line (L929) as well as the breast cancer cell line (MDA-MB 231). 6.25, 12.5, 25, 50, and 100 µg/mL were the selected concentrations of each extract for the assay. Out of these three extracts, *M. philippensis* showed the least $LC_{50}$ value in both L929 (77.83 ± 1.77 µg/mL) and MDA-MB 231 cell lines (61.5 ± 1.19 µg/mL) compared to the other two dye-yielding plant extracts. In all the treatments, the major cytological aberrations were found to be nuclear fragmentation, condensed nuclei, cell shrinkage, membrane blebbing, apoptotic bodies, budding, and echinoid spikes. All these aberrations indicated the apoptotic nature of action of *M. philippensis* extract. Hence, the characteristic effects on both normal and cancer cell lines reveal that *M. philippensis* could be used as a promising candidate for further studies. *A. odoratissima* showed the highest $LC_{50}$ value in both normal and cancer cell lines and *T. paniculata* exhibited an almost equal value. After that, the morphological visualization of cell death was evaluated by double staining technique using acridine orange and ethidium bromide. *In vitro* antiproliferative activity of *M. philippensis* on MDA-MB 231 cells exposed a significant inhibitory activity as observed in the double

staining method. The apoptotic cells with orange-stained nuclei was obtained due to the effect of the methanolic fruit extract of *M. philippensis.*

Flow cytometry is a method, which was used to measure the DNA content as well as the population profile. In the untreated control cells, the population profile of the apoptotic cells was found to be in a scattered condition. But in the treated cells, it was nearly aggregated. Population histogram represents the DNA content for control cells as 52.7, 19.7 and 23.7% in G0/G1, S, and G2/M phases respectively. A promising decrease in cell count from G0/G1 to G2/M phases was observed in treated cells. For G0/G1 phase it was found to be more with 64.2%. Whereas only 15.2% of cell count was found in the S phase and for the G2/M phase it was only 14.3%. This reveals a drastic decrease in cell count in the treated cells and points out the presence of significant apoptotic cells. Hence, the reduction of cell count designated that the cell cycle arrest had occurred at the G0/G1 phase by the effect of the extract of *M. philippensis*. The mechanism by which the fruit extract of *M. philippensis* induced apoptosis was further substantiated by the gene expression studies on two related genes namely p53 and TGF β using RT-qPCR. Both these genes are tumour suppressor genes and are involved in growth inhibition as well as apoptosis. In the present study, the separation and visualization of DNA fragments were analyzed by using gel electrophoresis. GAPDH (housekeeping gene) was used as a loading control. Here, both the genes p53 and TGF β displayed an upregulated expression with a significant fold change when compared to the control.

❖ **EMT screening**

EMT screening action of *M. philippensis* fruit extract was carried out with the help of MDA-MB 231 human breast cancer cell lines. For this analysis, three different assays were adopted namely cell migration assay, cell aggregation assay, and clonogenic assay. In the cell migration assay, the

wound area was found to be completely vanished by the continuous migration of cancer cells. Whereas, in the case of treated cells, the wound area remains with a size of 77956 px after 72 hours. Hence, it was inferred that methanolic fruit extract of *M. philippensis* ($LC_{50}$ concentration - 61.505 µg/mL) restricted the migration of MDA-MB 231 cancer cells towards the wound area. In the cell aggregation assay, a 24 to 72 hour treatment was adopted. The cell aggregates were found to be very less in treated cells than in the untreated control cells. After 72-hour treatment, large compact cell aggregates were found in control cells but in the treated cells the formation of cell aggregates was found to be reduced. In conclusion, *M. philippensis* fruit extract with $LC_{50}$ concentration inhibits the growth of MDA-MB 231 cells and restricted the formation of cell aggregates. The clonogenic assay is an *in vitro* cell survival assay in which the effect of *M. philippensis* in the colony formation of MDA-MB 231 cancer cells was determined. In the present study, the number of MDA-MB 231 colonies is very high in control cells (112 colonies) than in the MDA-MB 231 cells exposed to *M. philippensis* fruit extract (29 colonies). A reduction in the number of MDA-MB 231 cell colonies formed (less than 50 colonies) in the clonogenic assay, reveals the high potential of the extract used. Hence in the present study, the formation of only 29 MDA-MB 231 cell colonies in the *M. philippensis* fruit extract-treated cells reveals the excellent potential in the clonogenic assay.

✹ **Green synthesis of silver nanoparticles**

Green synthesis of silver nanoparticles is an eco-friendly and cost-effective method compared to other conventional nanoparticle synthesizing methods. The selected three dye-yielding plants were subjected to the biosynthesis of silver nanoparticles and their physicochemical characterization was also done. UV-Vis-NIR spectral analysis, FE-SEM,

EDAX, and XRD analysis are the adopted techniques used for the characterization of biosynthesized silver nanoparticles.

All three plants revealed a significant reduction of silver nitrate leading to the synthesis of nanoparticles. A brown colour was observed after the reaction between methanolic extracts and 2 mM silver nitrate solution which indicates the presence of silver nanoparticles. The surface plasmon resonance (SPR) vibrations of synthesized silver nanoparticles were revealed through the UV-Vis-NIR spectral analysis. Absorption peak was obtained at 456 nm, 438 nm, and 465 nm in *T. paniculata*, *M. philippensis* and *A. odoratissima* respectively. All three extracts exhibited a characteristic absorption peak within the range of 380 – 470 nm. Hence, these peaks confirm the presence of silver nanoparticles in the extracts by UV-Vis-NIR spectral analysis. FE-SEM images depicted the surface details of synthesized silver nanoparticles. The size and shape of silver nanoparticles were also noticed. Relatively spherical-shaped nanoparticles were identified in *T. paniculata* and *A. odoratissima.* Whereas, for *M. philippensis* it was nearly cubical. All the extract-synthesized silver nanoparticles revealed a size range of 20 to 90 nm. EDAX analysis was used to evaluate the elemental composition of the samples. A significant peak for silver was identified in all the extracts through the analysis. Finally, the crystalline nature of silver nanoparticles was disclosed by the X-ray diffraction technique. In the XRD analysis, Bragg reflections revealed the face-centered cubic (FCC) structure of silver. Using the Scherrer formula, the average crystallite size of each synthesized silver nanoparticle was also calculated. It was 17.7 nm, 4.36 nm and 7.11 nm in *T. paniculata*, *M. philippensis,* and *A. odoratissima* respectively. Hence, the diffraction peaks and mean crystallite size of each nanoparticle confirm their nanocrystalline nature.

The present study thus confirmed that the dye-yielding plants *viz*., *T. paniculata*, *M. philippensis,* and *A. odoratissima* are strong candidates in both commercial as well as pharmaceutical fields. The promising availability of a wide spectrum of phytochemicals and their possible bioactivities unveiled their effectiveness as possible drugs. The hepatoprotective activity of *T. paniculata* revealed that its fruit extract can be used as a natural food colourant in place of the synthetic food colourant lemon yellow. Moreover, the extracts of *M. philippensis* and *A. odoratissima* can also be used as natural colourants since they have a wide spectrum of bioactivities. The significant activity of synthesizing silver nanoparticles also points towards their effectiveness which may be exploited for the formation of useful drugs. In conclusion, the synergistic action of phytocompounds present in the extracts is responsible for the potential cytotoxic, antioxidant, and anticancer activities. Out of the three selected plants, *M. philippensis* was considered the most effective plant and the findings thus form a stepping stone towards replenishing the valuable resources of active plant-based drugs and natural colourants.

**Deliverables:**

- ✓ Active phytoconstituents of the three selected plants *viz*., *T. paniculata*, *M. philippensis* and *A. odoratissima* have been detected through various phytochemical analyses
- ✓ Potential antioxidant activity of the three dye-yielding plants has been unveiled
- ✓ Hepatoprotective activity of the methanolic fruit extract of *T. paniculata* was revealed
- ✓ Cytotoxicity using *A. cepa* root tip assay forms a lead towards further antiproliferative studies

- ✓ EMT screening of the methanolic fruit extract of *M. philippensis* revealed its potential activity to restrict the growth of human breast cancer cells
- ✓ An effective, less toxic, and eco-friendly technique for the biosynthesis of silver nanoparticles from plant extracts and their physicochemical details were unveiled

## RECOMMENDATIONS

- ✓ Isolation of specific colour-producing compounds from the selected dye-yielding plant parts of *T. paniculata*, *M. philippensis* and *A. odoratissima*
- ✓ An elaborate investigation of anticancer therapeutics, together with detailed on *in vivo* animal models
- ✓ Exploration of biosynthesized silver nanoparticles towards the fabrication of various high-tech biomedical products having potential applications

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# APPENDICES

**Wagner's reagent** **Appendix 1**

Iodine: 1.27 g

KI: 2 g

Dissolve the above chemicals in 5 ml $H_2SO_4$ and make upto 100 mL.

**Griess reagent** **Appendix 2**

Naphthyl ethylenediamine HCl: 0.1% distilled water

Sulfanilamide: 1% in 5% $H_3PO_4$

Both solutions are mixed in 1:1 ratio

**Phosphate buffer saline (PBS)** **Appendix 3**

NaCl: 8 g

KCl: 0.2 g

$Na_2HPO_4$: 1.44 g

$KH_2PO_4$: 0.24 g

Dissolve in 1 L double distilled water and adjust pH to 7.4.

**DMEM (Dulbecco's Modified Eagle's medium)** **Appendix 4**

Sodium bicarbonate: 1.85 g

HEPES: 2.95 g

DMEM powder: 1 packet

Distilled water: 1 L

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

**Modified Carnoy's fluid** **Appendix 5**

Acetic acid: 10 mL

Ethanol: 30 mL

**Acetocarmine** **Appendix 6**

Carmine: 2 g

Acetic acid: 100 mL of 45% acetic acid

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

**Ethidium bromide** **Appendix 7**

Ethidium bromide: 20 µg/ mL

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.

**TE (Tris-EDTA) buffer** **Appendix 8**

Tris HCl: 10 mM, pH 8

EDTA: 0.1 mM, pH 8

**Ringer's salt solution** **Appendix 9**

NaCl: 8.6 g

$CaCl_2$. $2H_2O$: 330 mg

KCl: 300 mg

pH 8

Dissolve the above chemicals in 900 mL of distilled water

Sterilized the solution by filtration and stored at 4°C

**Moscona solution** **Appendix 10**

NaCl: 8 g

KCl: 0.3 g

$Na_2HPO_4$. $H_2O$: 0.05 g

$KH_2PO_4$: 0.025 g

$NaHCO_3$: 1.0 g

D (+) – glucose: 2 g

pH 7.4

Dissolve the above chemicals in 900 mL of distilled water

Sterilized by filtration and stored at 20°C.

**Appendix 11**

**Calcium- and magnesium-free Hank's balanced salt solution**

NaCl: 8 g

KCl: 0.4 g

$KH_2PO_4$: 0.06 g

$NaHCO_3$: 0.35 g

$Na_2HPO_4$. $12H_2O$: 0.112 g

pH 7.4

Dissolve the above chemicals in 900 mL of distilled water

Sterilized by filtration and stored at 4°C.

# RESEARCH PUBLICATIONS AND PRESENTATIONS

**Research publications from the Ph.D. work**

1. **Aswathi, P**., & Thoppil, J. E. (2022). Synthesis and physicochemical characterization of silver nanoparticles from the dye-yielding plants, *Terminalia paniculata* and *Mallotus philippensis*. *Nanotechnology for Environmental Engineering*, *2022*, 1-10. Springer publishers.

2. **Aswathi, P**., & Thoppil, J. E. (2020). Assessment of antioxidant potential and antigenotoxicity analysis through hydrogen peroxide-induced oxidative stress in *Terminalia paniculata* Roth. *Asian Journal of Pharmaceutical and Clinical Research*. *13*(8), 106-110. Innovare Academic Science Publishers.

**Other publication by the candidate**

1. **Pokkadath, A**., Chembrammal, R., & Thoppil, J. E. (2022). Chromosomal instability and abnormal mitochondrial activity induced by two synthetic food colorants. *Botanica Pacifica*, *11*(2), 1-7.

**Paper presentations**

1. **Aswathi, P**., & Thoppil, J. E. (2019). Cytotoxic studies and membrane stability of two synthetic food colourants in *Allium cepa* root tip cells. In the international conference on recent innovations in bio-sustainability and environmental research, held at Annamalai University, Tamil Nadu (Oral presentation)

2. **Aswathi, P**., & Thoppil, J. E. (2019). Bioactivity evaluation and phytochemical profiling of an important dye-yielding plant – *Terminalia paniculata* Roth. In XLII All India botanical conference of

the Indian botanical society and national symposium on innovations and inventions in plant science research, held at Calicut University Campus (Poster presentation)

3. **Aswathi P**., & Thoppil J. E. (2020). Evaluation of antioxidant efficacy and genoprotective activity of *Terminalia paniculata* Roth. against oxidative stress induced by $H_2O_2$. Current trends and advances in biological sciences (CTAB 2020), held at Bishop Moore College, Mavelikara (Oral presentation)