Cytogenetical studies, phytochemical profiling and bioactivity screening of selected species of *Ophiorrhiza* L. (Rubiaceae)

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by

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CERTIFICATE

This is to certify that the thesis entitled **"Cytogenetical studies, phytochemical profiling and bioactivity screening of selected species of *Ophiorrhiza* L. (Rubiaceae)"** 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 **Preetha Mol S. N.**, during the period of study (2017-2022) at the Cell and Molecular Biology Division, Department of Botany, University of Calicut under my supervision and guidance and that it has not formed the basis for award of any degree or diploma. Also certified that the contents in the thesis are subjected to plagiarism check using the software OURIGINAL, that no text or data is reproduced from other works.

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DECLARATION

I, Preetha Mol S. N., hereby declare that the thesis entitled **"Cytogenetical studies, phytochemical profiling and bioactivity screening of selected species of *Ophiorrhiza* L. (Rubiaceae)"** 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.

Date: **Preetha Mol S. N.**

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ABBREVIATIONS

A	:	Degree of asymmetry of karyotype
A1	:	Intrachromosomal asymmetry index
A2	:	Interchromosomal asymmetry index
ACL	:	Average chromosome length
$AlCl_3$	:	Aluminium chloride
ANOVA	:	Analysis of Variance
As K %	:	Karyotype asymmetry index
BCG	:	Bromocresol green
cDNA	:	Complementary DNA
cm	:	Centimetre
CO_2	:	Carbon dioxide
COX	:	Cyclooxygenase
DI	:	Disparity index
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
EDTA	:	Ethylenediamine tetra acetic acid
ELISA	:	Enzyme linked immunosorbent assay
eV	:	Electron volt
FACS	:	Fluorescence activated cell sorting
$FeCl_3$	:	Ferric chloride
Fig.	:	Figure
FITC	:	Fluorescein isothiocyanate
g	:	Gram
g/l	:	Gram per litre
GC-MS	:	Gas Chromatography-Mass Spectrometry
h	:	Hour
H_2O_2	:	Hydrogen peroxide
H_2SO_4	:	Sulphuric acid

HCl	:	Hydrochloric acid
HEPES	:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR LC-MS	:	High resolution Liquid Chromatography-Mass spectrometry
HRP	:	Horseradish peroxidase
IC_{50}	:	Inhibitory concentration 50%
iNOS	:	Inducible nitric oxide synthase
kb	:	Kilo base pair
KH_2PO_4	:	Potassium dihydrogen phosphate
KOH	:	Potassium hydroxide
l	:	Litre
L929	:	Normal fibroblast cell line
LC_{50}	:	Least concentration 50%
LOX	:	Lipoxygenase
LPS	:	Lipopolysaccharide
M	:	Molar
m/z	:	Mass-to-charge
MDA-MB 231	:	M. D. Anderson- metastatic breast 231(human breast cancer cell line)
mg	:	Milligram
min	:	Minute
ml	:	Millilitre
mM	:	Millimolar
mmol/l	:	Millimoles per litre
MPO	:	Myeloperoxidase
MS-QTOF	:	Mass spectrometry quadrupole time of flight
MTT	:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	:	Normal
NaOH	:	Sodium hydroxide
NBT	:	Nitroblue tetrazolium
NCCS	:	National Centre for Cell Sciences
NIST	:	National Institute of Standards and Technology
nm	:	Nanometre

NO	:	Nitric oxide
NOS	:	Nitric oxide synthase
NSAIDs	:	Non-steroidal anti-inflammatory drugs
OD	:	Optical density
PBS	:	Phosphate buffered saline
PC	:	Primary constriction
PDB	:	Para dichlorobenzene
RAW 264.7	:	Mouse leukemic monocyte macrophage cell line
RCL	:	Range of chromosome length
REC	:	Resemblance between chromosomes
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
SPSS	:	Statistical Package for Social Sciences
TBA	:	Thio barbituric acid
TCA	:	Trichloroacetic acid
TCL	:	Total chromosome length
TE	:	Tris EDTA
TF %	:	Total forma percentage
U/ml	:	Units per millilitre
V	:	Volt
v/v	:	Volume per volume
VC	:	Variation Coefficient
w/v	:	Weight per volume
WHO	:	World Health Organisation
µg	:	Microgram
µg/ml	:	Microgram per millilitre
µl	:	Microlitre
µM	:	Micromolar

INTRODUCTION

The association between human beings and nature has begun along with the emergence of mankind on earth. Humans have explored nature mainly for food for his survival and medicines for curing diseases. Plants are the major sources of natural medicines and the usage of medicinal plants dates back to 60,000 years ago (Srivastava, 2018). Studies on early human civilizations reveal the use of many plant-based drugs for the treatment of several ailments. As per the olden literature, all the plant components are potential medicinal substances. Traditional systems of medicines like folklore medicines, Ayurveda, Siddha, Unani and Homeopathy rely on herbal products for treating diseases and their use is widespread in countries like China, India, Japan, Pakistan, Sri Lanka and Thailand (Manoharachary & Nagaraju, 2016). Though plants have great pharmaceutical importance, the outbreak of various infectious diseases made humans to depend more on synthetic or chemical drugs for the quick curing of diseases. But, this obsession with the modern medical systems has resulted in several adverse effects related to synthetic drugs and people have started to look back at the traditional healing systems. Now, herbal medicines have great demand in both developed and developing nations because of their effective medicinal properties, easy availability, low prices and lesser side effects (Baregama & Goyal, 2019).

India is one amongst the mega biodiversity centres of the world with vast area having wide variation in climate, soil, altitude and latitude (Meena et al., 2009). About 17000-18000 flowering plants are reported from India, out of which, around 7000 plants are used in various medicinal systems. Indian folk medicine makes use of around 25000 effective plant-based formulations and around 10000 formulations are mentioned within the indigenous medical texts, thus making India one of the richest plant medical cultures in the world

(Manoharachary & Nagaraju, 2016). Ancient Indian literature is rich in references of medicinal plants utilised for treatments of various ailments and is estimated that about 15000 plant species are used as medicinal sources (Meena et al., 2009), which makes the country, the biggest repository of medicinal plants in the world and thus a potential centre of herbal medicines and products.

Every year, the percentage of people getting attracted to herbal medicines is doubling due to their minimal side effects and permanent curing properties. While synthetic drugs focus on symptoms of diseases, herbal medicines boost up the immune system of our body to fight the diseases. Though herbal products are generally considered safe, they can be toxic if the plants are misidentified or incorrectly prepared or used. So, the correct identification of the plant materials and their clinical authentication for proper medical application is necessary (Karimi et al., 2015).

Chromosomes form the vehicle of genetic constitution and have great importance in understanding taxonomic problems. Chromosome numbers and morphology serve as useful characters to distinguish between different taxa at least to the genus level (Kuo, 2013). Furthermore, cytological data plays a great role in the taxonomical and evolutionary studies of plants as they help in interpreting inter or intra specific relations within the genus (Saenprom et al., 2018). Karyotype is a very stable character that is unique for each specimen. Thus, karyotype analysis helps in the determination of the taxonomic status of a taxon, especially when the usual guidelines become insufficient to delimit the taxa (Das, 2018). However, same chromosome number can be seen in different species of the same genus and also in different genera, where the karyotype differs in their structural configuration (Venkatesh & Munirajappa, 2015).

Chromosome numbering and karyotyping forms the primary step towards the understanding of the structure, organisation and functioning of the genome. Karyotype of an organism is its genome itself, so, species with more similar karyotypes are likely to be more closely related than those with less similar ones. Changes in the karyotype due to polyploidy, chromosome inversions or reciprocal translocations can have adverse effect on the survival capacity of these individuals, which later can influence the evolutionary trend. Differences in the chromosome structure reflect the variations in the number of gene products or proteins produced by the organism. Thus, cytological data is an excellent and unique source of information for taxonomical and evolutionary studies. It provides us an understanding regarding the relationships between species and genera (Guerra, 2012). Based on the position of the centromere (primary constriction/kinetochore), chromosomes are classified into four groups as metacentric, sub-metacentric, acrocentric and telocentric (Abraham & Prasad, 1983). The chromosomes are also described as median, nearly median, nearly submedian, submedian, nearly subterminal, subterminal, nearly terminal and terminal (Sharma & Sharma, 1962; Adhikary, 1963; Sharma, 1964; Bose & Flory, 1965). As several classification systems can only create confusions, Abraham and Prasad (1983) introduced a system to classify the chromosomes effectively by incorporating both the arm ratios and centromeric indices of both arms. The system also helps in tracing even minor differences in chromosome morphology.

Cytogenetical studies also involve the evaluation of karyotype asymmetry or symmetry by revealing the karyomorphological characters. An asymmetric karyotype represents an evolutionary flux, while the more symmetric ones are considered to be more stable (Stebbins, 1971). Karyotype analysis along with advanced techniques like chromosome morphometry and *in situ* hybridisation methods can reveal minute differences in the

chromosome structure among closely related species thus giving more details about the evolution and karyotypic diversity (Rodrigues et al., 2014).

Plants are great reservoirs of biologically active compounds with several protective as well as disease preventing properties (Peteros & Uy, 2010). These compounds, often called as phytochemicals, are produced by plants as a result of various biochemical pathways which help plants to synthesise a wide array of chemical compounds (Kennedy & Wightman, 2011). Based on the chemical class, biosynthetic origin, and functional groups, phytochemicals can be grouped into primary and secondary metabolites. Primary metabolites are those which are involved in the growth and development of the plants (like chlorophyll, amino acids, nucleotides *etc*.), while secondary metabolites are produced as a result of secondary biochemical pathways (Geetha & Geetha, 2014). Plants synthesise secondary metabolites in response to various environmental stimuli, such as animal attacks, pathogen induced damage or nutrient deficiency (Kennedy & Wightman, 2011). Though secondary metabolites are primarily produced for the protection, research prove that these compounds possess various potential bioactivities. The major secondary metabolites include phenols, flavonoids, terpenoids, alkaloids, sterols, tannins *etc*. and are reported to possess various pharmacological activities like antioxidant, anti-inflammatory, anticancer, antimicrobial, antidiabetic, analgesic, *etc*. (Raghuveer et al., 2015). Thus, the therapeutic potential of the medicinal plants can be ascribed to the presence of various bioactive secondary metabolites (Preethamol & Thoppil, 2020).

Medicinal plants may contain many bioactive phytocomponents, which may act individually as well as can work synergistically with each other in enhancing the bioactivities of other components and thereby produce pharmacological actions (Meena et al., 2009). Phytochemical analysis of plant extracts is very important as it helps to identify compounds with therapeutic

properties and also provides the leads and clues for the development of a new therapeutic agent (Yadav et al., 2014). Thus, indigenous plants which are used in folklore medicines should be screened for potential phytochemicals so that their use in traditional treatment can be scientifically explained (Anyasor et al., 2010).

Secondary metabolites are produced by plants as their response to various extrinsic and intrinsic factors. So, these compounds can be unique to specific taxa. Compared to the primary metabolites, plants produce secondary metabolites in smaller quantities. Moreover, these are produced within specialised cells at particular developmental stages making their extraction and purification complicated (Raghuveer et al., 2015). Furthermore, there are innumerous metabolites ranging from simple to complex that are present within a plant, which makes it difficult to characterise these compounds using a single analytical technique. So, a combination of different biochemical assays, experimentations and instrument platforms are required to analyse the phytoconstituents present in a complex mixture like plant extract (Kasture et al., 2012).

All the secondary metabolites with bioactive properties can exert their activity individually and their therapeutic potential is well established. Even then, their activities seem to be insufficient to achieve the desirable therapeutic effects. This can be due to the fact that they are present in very small quantities in plant parts and are also synthesised in specified cells. Seasonal variations can also affect the quantity of secondary metabolites produced by the plants. For this same reason, scientific studies on the efficiency of herbal products reveal that the plants with medicinal properties when combined together to form a crude drug may theoretically produce better results, as compared to the use of individual plants or isolated compounds and also their cumulative effect. This phenomenon of positive herb-herb interaction, often known as synergism, is very much in practice

in ayurvedic medicines. Studies suggest that certain pharmacological actions of active phytochemicals become significant only when they are potentiated by the phytochemicals present in other plants and not evident why they are used alone in herbal formulations (Parasuraman et al., 2014).

Present day research makes use of various techniques for the analysis of metabolites such as metabolic profiling, metabolic fingerprinting, metabolomics and metabonomics. Metabolic profiling or phytochemical profiling is an inevitable step that forms the basis for drug development. Qualitative phytochemical analyses using different standard protocols reveal the presence of various bioactive compounds which indicates the medicinal potential of the plant. Quantitative analyses help to estimate the amount of phytocompounds within the plants (Geetha & Geetha, 2014). To further explore the types and number of compounds, various complementary analytical techniques like Nuclear magnetic resonance (NMR) spectroscopy and hyphenated techniques like gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) are also employed (Koek et al., 2011).

The complete profiling of phytochemicals within a sample is a cumbersome process making the selection of the most suitable method for phytochemical fingerprinting a challenge. The selection of the most suitable technique for analysis is mainly based on speed, selectivity and sensitivity. Whatever the technique is, the ultimate aim of any metabolic analysis is the determination of the precise concentration of the compounds present within the sample. However, the largest bottleneck in phytochemical profiling is to find out the accurate methodology for quantification (Koek et al., 2011).

As part of metabolic reactions, living organisms generate highly unstable and reactive free radicals. To attain stability, these free radicals attack other stable molecules in the body and capture their electrons converting them to free radicals and initiate a chain of reactions (Sahoo et al.,

2005). These free radicals include reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) like hydrogen peroxide, superoxide anion, peroxynitrite, singlet oxygen, hypochlorous acid, perhydroxyl radical, hydroxyl radical and nitric oxide (Vajragupta et al., 2004). ROS are generated by the mitochondrial respiratory chain as a part of normal cellular metabolism. But under hypoxic conditions, the mitochondrial respiratory chain produces nitric oxide, which further produces RNS. These oxidants react with protein molecules, DNA molecules, lipids, brain cells as well as immune system and cause oxidative stress. Oxidative stress results in serious disorders like cardiovascular diseases, neurodegenerative diseases, premature ageing, diabetes, arthritis, asthma, stroke and even cancer (Al-Jaber et al., 2011). Antioxidants are substances that counteract these free radicals and inhibit the oxidative damage of biological molecules, thereby stopping the chain reaction and protecting cell (Ndhlala et al., 2010). ROS can play dual function of being both beneficial as well as harmful based on their level within the biological system. At lower concentrations they act as secondary messengers in various signalling pathways, but, when the concentration exceeds, they damage all kinds of biomolecules and induce oxidative stress (Rubeena, 2021).

Extensive research has revealed that continuous formation of free radicals can alter the equilibrium of the body and create oxidative stress. Oxidative damage to biological molecules can cause a wide spectrum of inflammatory diseases as well as cancers (Reuter et al., 2010). Continuous exposure to environmental stress results in the formation of ROS. When ROS are produced over a long time, significant damage occurs to the cell and its functions. Furthermore, somatic mutations and neoplastic transformations can also happen (Fang et al., 2009; Khandrika et al., 2009). Oxidative stress can induce DNA mutation, genome instability as well as cell proliferation; thereby initiating cancer (Visconti & Grieco, 2009).

Living organisms have an endogenous system of antioxidants to maintain the balance between the production and scavenging of free radicals. Antioxidants synthesised within the body to repair cell damage caused by free radicals are called endogenous antioxidants (Adebiyi et al., 2017), and it includes enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include the primary enzymes like the superoxide dismutase, catalase and glutathione peroxidase; secondary enzymes include glutathione reductase and glucose-6-phosphate dehydrogenase. Non-enzymatic antioxidants include water soluble molecules like vitamin C and phenolic compounds as well as lipid soluble biomolecules like Vitamin E and carotenoids (Ndhlala et al., 2010). Though our body has efficient defense system; age, diet and health conditions of an individual can weaken the immunity. Thus, exogenous antioxidants are necessary for the proper maintenance of the body equilibrium (Sultana et al., 2007).

Exogenous antioxidants can be classified into synthetic and natural ones. Due to the beneficiary effects of antioxidants, synthetic antioxidants like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroxyquinone (TBHQ) are used in food and pharmaceutical industries to prevent oxidative degeneration (Ndhlala et al., 2010). But these compounds promote DNA damage, toxicity and tumour formation. Thus, exogenous antioxidants from natural resources are preferred, to complement the endogenous system; mostly because they are less expensive, readily available and are safer than their synthetic counterparts (Hajji et al., 2010). Plants are natural resources of a wide variety of antioxidant molecules like phenolics, vitamins, flavonoids and terpenoids (Cai et al., 2003). Among the various natural antioxidants, phenolic compounds are well known for their radical scavenging, metal chelating, reducing and hydrogen donating properties. So, plants with high polyphenol content have great importance as natural antioxidants (Mathew & Abraham, 2006). Fruits and vegetables are the major sources of dietary antioxidants (Saita et al., 2014). Several food items that we use are enriched with antioxidants that protect our cells

from oxidative damage. However, other sources of natural antioxidants have become the need of the day as only dietary supplements are not sufficient to meet the required activity to prevent oxidative damages.

Plants are excellent sources of antioxidants with ample number of phytoconstituents having free radical scavenging potential. Several herbs and spices are reported to have antioxidant compounds to protect every part of the plant from oxidative stress (Paur et al., 2011). The antioxidant potential of any medicinal plant can be the synergistic contribution of the phytocompounds present in the plant (Badarinath et al., 2010). Thus, researchers have now focused on investigating different plant extracts for their free radical scavenging potential. Different antioxidant assays are used for the investigation of the antioxidant activity of plant extracts. However, extensive research is necessary for the exact determination of the antioxidant potential of the herbs considered for studies.

Anti-inflammatory potential is another important bioactivity for which several plants and plant products are screened by researchers. The word "inflammation" comes from the Latin word "inflammare" which means "to set on fire" (Lin & Li, 2018). It is the natural response of the body's defence and restoration mechanism to the harmful stimuli like pathogen attack, tissue injury, infection, toxic chemicals or radiations (Chen et al., 2018). Inflammation is the process for cleaning and removing foreign particles and injured tissue debris, thereby repairing damaged tissues. Thus, inflammation facilitates the body's natural healing processes to restore the healthy state of tissues (Lin & Li, 2018). The most common symptoms of inflammation include redness, pain, swelling and heat (Medzhitov, 2008).

Acute inflammation is a part of innate immunity which can be considered as an adaptive response of the body to reinstate cellular homeostasis. However, prolonged inflammation can have adverse effects on the body. Inflammatory

responses usually end with the removal of the harmful stimuli and the procedure is known as the resolution of inflammation (Serhaan & Savill, 2005). If the acute inflammation does not resolve for any reason, it progresses into a chronic stage. Thus, chronic inflammation develops when the acute inflammatory mechanisms fail to repair the tissue injury and the triggering stimulus persists for a long time (Lintermans et al., 2014). Chronic inflammation causes several diseases such as asthma, cancer, atherosclerosis, type 2 diabetes, obesity, inflammatory bowel diseases, rheumatoid arthritis, autoimmune disorders and neurodegenerative diseases (Sugimoto et al., 2016).

Inflammation follows a common mechanism which involves recognition of harmful stimuli by the cell surface pattern receptors and the activation of inflammatory pathways, followed by the release of inflammatory markers and the recruitment of inflammatory cells (Chen et al., 2018). Specific transmembrane receptors present on the host cells, known as pattern recognition receptors (PRRs) recognise detrimental stimuli and transmit signals to the nucleus where a selective set of genes are activated by transcriptional and posttranscriptional mechanisms (Akira et al., 2006). Activated transcription factors promote the production of pro-inflammatory cytokines and chemokines (Sen & Baltimore, 1986).

Nuclear factor- κB (NF-κB) is one of the most important transcription factors involved in the generation of inflammatory responses, survival and apoptotic processes. The NF-κB family consists of five structurally related members; NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel. Under normal conditions, NF-κB proteins are suppressed by 1κB proteins present in the cytoplasm. PRRs activate NF-κB proteins by two different signalling mechanisms namely, canonical and non-canonical pathways. Though, both the pathways differ in their mechanisms, are very important in regulating immune and inflammatory mechanisms. NF-κB pathway is very important for the production of pro-inflammatory cytokines and inflammatory cell

recruitment. Deregulated NF-κB activation can cause chronic inflammations and therefore therapeutic strategies focused on NF-κB regulation have great significance (Liu et al., 2017).

The major contributors of inflammatory responses are immune cells like macrophages, dendritic cells, mast cells, neutrophils and lymphocytes as well as non-immune cells like epithelial cells, endothelial cells and fibroblasts (Akira et al., 2006). On injury, damaged epithelial and endothelial cells release substances that initiate inflammatory cascade, together with chemokines and growth factors that attract neutrophils and monocytes to the affected area. Neutrophils are the first cells to be drawn to an injury site followed by monocytes, lymphocytes and mast cells. Monocytes can differentiate into macrophages and dendritic cells and are attracted to the sites of injury by chemotaxis (Stramer et al., 2007; Van et al., 2014; Robb et al., 2016). Neutrophils are important players in the inflammatory response because they direct antigen-presenting cells to activate T cells and release localised factors that attract monocytes and dendritic cells (Jabbour et al., 2009). Despite the fact that neutrophils kill pathogens in the body, they can potentially harm host cells and tissues (Nathan, 2006). Macrophages deliver antigens, engage in phagocytosis, and influence the immune response by generating cytokines and growth factors during inflammation. Mast cells are effector cells that induce inflammatory reactions and are found in connective tissue matrices and on epithelial surfaces. A number of inflammatory mediators are released by activated mast cells, including cytokines, chemokines, histamine, proteases, prostaglandins, leukotrienes, *etc*. (Huang et al., 2010).

Cytokines are produced by macrophages and helper T-cells during physiological and pathological processes. They are of two kinds; pro-inflammatory cytokines and anti-inflammatory cytokines. Pro-inflammatory

cytokines like interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) are mainly produced by activated macrophages and are indulged in the up-regulation of inflammatory responses. IL-1β is produced by monocytes and macrophages at the time of cell injury or infection. Non-immune cells like fibroblasts and endothelial cells also produce IL-1β, whereas IL-6 is produced on injury of neuronal tissue. TNF-α, otherwise known as cachectin, is a part of different signalling pathways and is involved in inflammatory responses. Anti-inflammatory cytokines control pro-inflammatory response. They include IL-4, IL-10, IL-11 and IL-13. IL-10 is the most potent anti-inflammatory cytokine, which represses IL-1β, IL-6 and TNF-α. Deregulated production of pro-inflammatory cytokines or improper action of anti-inflammatory cytokines can lead to chronic inflammations (Zhang & An, 2007). Inflammation can affect several organs like the heart, pancreas, liver, kidney, lung, brain, intestinal tract and reproductive system. It is estimated that about 15% of human cancers are also associated with chronic inflammations (He & Karin, 2011).

Anti-inflammatory agents are widely used to overcome the effects of inflammatory responses on the body. Non-steroidal anti-inflammatory medicines (NSAIDs) are among the most often prescribed drugs for treating inflammation around the world, mainly because of their analgesic, anti-inflammatory and antipyretic properties. Though widely recommended, these drugs have several side effects such as muscle dysfunction, renal damage, liver damage, headaches, skin rash, drowsiness, delayed healing, gastric ulcers and gastrointestinal bleeding (Khansari et al., 2009). Due to these negative effects, natural and new anti-inflammatory medications are now focused. Plant secondary metabolites like polyphenols, flavonoids, terpenoids, alkaloids and sterols are reported to possess anti-inflammatory properties (Omoregiel et al., 2014). Thus, plant products with these bioactive compounds can be excellent candidates for anti-inflammatory drug development.

Cytotoxicity studies are preliminary, but a very important initial step in the determination of the toxicity of test substances including plant extracts or bioactive plant compounds (Kumar & Thoppil, 2019). If the test compounds are intended for cosmetic or pharmaceutical use, minimal to no toxicity is preferred. On the other hand, if the compounds are intended to act as anticancer agents, selective cytotoxicity to cancerous cells is important. Thus, preliminary cytotoxic assays are essential to identify and prioritize test substances for further studies (McGaw et al., 2014).

Diverse species are used for cytotoxic studies to evaluate the detrimental effects of various substances at varying concentrations as well as exposure times. *Allium cepa* L. is an excellent genetic model, widely used for the assessment of cytotoxicity. *Allium cepa* bioassay shows high sensitivity and good correlation with mammalian test systems and hence is used for the evaluation of the cytotoxic and genotoxic effects of various substances (Sumitha & Thoppil, 2016). Root meristematic cells of *A. cepa* are analysed for chromosomal aberrations and mitotic index to identify the cytotoxic potential of the plant extract being tested (Bianchi et al., 2016). Cytotoxicity is a bioactivity of medicinal plants that is linked to their anticancer properties. Various secondary metabolites in the plant cause cytotoxicity by disrupting the normal cell cycle and causing chromosomal abnormalities in various ways (Preethamol & Thoppil, 2019). Thus, screening of the plant extracts for cytotoxicity is an obligatory step, which directs towards the anticancer potential of the plant.

Cancer is a major health issue that forms the world's second leading cause of death. It is a multifactorial disease caused by internal (genetically transformed abnormal cells or immune conditions) or external (carcinogens, viruses, chemicals or radiations) factors (Tariq et al., 2017). In addition to these factors, smoking, dietary habits and infection diseases can also induce the formation of cancer cells. According to Global Cancer Statistics in 2018,

about 18.1 million cancer cases and 9.6 million cancer deaths are reported (Bray et al., 2018). In males, the most common cancers detected are prostate, lung, bronchus, and colorectal cancers. Prostate cancer alone accounts for 1 in 5 diagnoses. In females, the most commonly diagnosed cancers are breast, lung, bronchus and colorectal cancers (Neethu, 2017). Cancer cells are characterised by uncontrolled growth and division of cells that invade, spread and migrate to distant tissues.

Cancer cells undergo anaerobic metabolism and thereby resist low oxygen levels. The higher amount of hexokinase in cancer cells, help them during higher glucose consumption. Cancer cells generate enzymes and other proteins that are required for tumour development (Swaya et al., 2017). Like normal cells, cancer cells do not follow a systematic path of growth, division or death. Normal physiological path of cell death called apoptosis or programmed cell death is absent in cancer cells, hence, they continue to grow and divide, producing tumours (Sajitha, 2019). Thus, absence of apoptosis leads to uncontrolled proliferation of cells and finally metastasis. Suppression of apoptosis is the main part in the development and progression of cancers. Many chemo-preventive agents of cancer have shown apoptosis inducing properties.

Primary treatment for cancer includes malign surgery for localised tumours, chemotherapy and radiotherapy for metastasised tumours (Tariq et al., 2017). Chemotherapy medications successfully suppress malignant cells, but they can cause toxicity and other health issues in the patients (Kroschinsky et al., 2017). Majority of the anticancer drugs basically cause DNA damage that affects the adjacent normal cells also (Samet et al., 2014). The common side effects of anticancer drugs include weakness, alopecia (hair loss), loss of appetite, xeroderma, erythema (skin redness), weight loss and numbness (Kroschinsky et al., 2017). Though a wide range of medications are

available for cancer treatment, development of drug resistance, poor absorption and side effects of these chemo-drugs, highlight the need for new anticancer agents with high selectivity against only malignant cells, improved efficacy, affordability and acceptable toxicity (Swaya et al., 2017).

Ethnomedicinal plants have played a major role in the development of anticancer drugs throughout the world (Nisa et al., 2013). Out of the 121 prescription drugs used for the cancer treatment, 90 are plant derived and about 75% of these are adopted from traditional systems of medicines (Shah et al., 2015). Advances in molecular biology and a better knowledge of cancer pharmacology have helped researchers to develop target-based therapies on a molecular level. These are chemicals that have been pre-programmed to block or change a molecular marker (like enzymes) that is thought to be significant in cancer progression (Narang & Desai, 2009). Thus, development of natural anticancer agents with better therapeutic potential to act against cancer cells is the need of the day.

Anticancer medications work as defence or therapeutic agents in cancer, activating apoptotic signalling pathways to prevent cell proliferation and metastasis. Several medicinal plants used in folklore medicines have a wide array of phytochemicals that can be used to treat cancer (Kraft, 2009). Bioactive phytocomponents like phenols, flavonoids, terpenoids, saccharides, steroids, xanthones and alkaloids show strong anticancer activities (Prakash & Maurya, 2018). These components can act individually, but synergistically produce better anticancer potential. Synergistic action of numerous phytochemical compounds on different cancer cell lines has proven to exhibit higher therapeutic efficiency (Cilla et al., 2015). Thus, plant or plant-based products, which can activate the apoptotic (programmed cell death) signalling pathway or can inhibit cancer progression by blocking the factors that promote

tumourigenesis and thereby control cell proliferation can be considered as useful candidates for anticancer drug development (Elmore, 2007).

Plant-based traditional medicines are a great source for the development of novel drugs (Khan et al., 2014). However, lack of proper documentation, standardisation and scientific validation form a major obstacle for the global acceptance of herbal medicines (Baregama & Goyal, 2019). The information regarding many medicinal plants is not documented but passed on to the next generation by oral dissipation only. This causes many aspects of our traditional medicinal culture to get lost. Traditional knowledge when supported by experimental bases can form powerful engines for the discovery of new, safe and affordable drugs (Meena et al., 2009). Herbal and natural medicines are not just drugs, but simultaneously are beneficial dietary supplements that enhance our immunity (Srivastava, 2018). Thus, there is a global increase in the demand for the scientific authentication and substantiation of the therapeutic potential of the traditionally used medicinal plants. Considering all the above mentioned aspects, the present study was set to explore the cytogenetical characters and bioactivities of selected species of *Ophiorrhiza*, which belongs to the family Rubiaceae.

The family Rubiaceae, commonly known as coffee, madder or bedstraw family, is a family of flowering plants consisting of about 13,500 species in 619 genera. It is the fourth largest angiosperm family and the fifth largest with regard to the number of genera. The family has cosmopolitan distribution with the largest number of species distributed in the tropical and subtropical regions. The members are commonly shrubs (*e.g.*, *Coffea, Psychotria*), trees (*e.g., Cinchona, Nauclea*), lianas (*e.g., Psychotria samoritourei*) or herbs (*e.g.*, *Galium*, *Spermacoce*). The most important characters of the family include simple opposite or whorled leaves with interpetiolar stipules, sympetalous actinomorphic flowers with tubular corolla

and inferior ovary. Many members of the family are economically and medicinally useful. *Coffea* (source of coffee) *Cinchona* (source of quinine – an antimalarial alkaloid), dye yielding plants such as *Rubia,* ornamentals like *Ixora*, *Pentas*, *Gardenia*, *Mussaenda, etc.,* are some of the members of the family (http[1]).

Ophiorrhiza L., is a notably species-rich genus consisting of 321 species, 5 varieties and 1 subspecies and found in wet tropical forests of South-East Asia, extending to Australia, New Guinea and Pacific Islands. In India, 51 species and 5 varieties are recorded. Peninsular India, especially the Western Ghats is one of the diversity centres of *Ophiorrhiza* species followed by the north-eastern Himalayas (Hareesh & Sabu, 2018; Hareesh et al., 2018; Hareesh et al., 2020). *Ophiorrhiza* species are small herbaceous or shrubby plants that can be easily distinguished from other genera by the obcordate and compressed capsules that are dehiscent with two valves along a transverse slit at the top. The name originates from two Greek words 'Ophio" means snake and 'Rhiza' means root. They have been used in traditional and folk medicine as snake bite antitussive, analgesic and for the treatment of ulcers, leprosy, gastropathy and amenorrhea (Rajan et al., 2013). Some members of the genus are reported to possess cytotoxic alkaloids and many other bioactive constituents, which gives great importance to the genus in therapeutic research (Yamazaki et al., 2003b; Martin et al., 2007). Reports show that the members are used in traditional and conventional medicines. Though *Ophiorrhiza* is a species-rich genus, very few members are scientifically investigated and many others are still unexplored.

Based on the aforesaid background, the present study focuses on the cytogenetical, phytochemical and bioactivity analyses of four selected species of *Ophiorrhiza* (*O. grandiflora, O. jacobii, O. pectinata* and *O. trichocarpon*).

The study includes the following objectives:

- To analyse the karyomorphological characters of selected species of *Ophiorrhiza*
- To screen the major bioactive phytoconstituents by qualitative and quantitative analyses
- To identify the volatile and non-volatile components by GC-MS and HR LC-MS analyses
- To evaluate the free radical scavenging potential by *in vitro* assays
- To study the anti-inflammatory activity of the plant extracts on RAW 264.7 cells
- To screen the cytotoxic potential of the plant species by *Allium cepa* bioassay
- To analyse the anti-proliferative potential of the selected plants on MDA-MB 231 (Human breast cancer) cell lines and to elucidate the antiproliferative mechanism by gene expression studies

REVIEW OF LITERATURE

The present work focuses on the cytogenetical studies, phytochemical profiling and bioactivity screening of selected species of *Ophiorrhiza.* A brief survey of literature available on the genus relevant to the present study is done and is presented in this chapter.

I. Taxonomical background

Ophiorrhiza L. is a genus belonging to the tribe Ophiorrhizeae of the subfamily Rubioideae (Bremekamp, 1952; Bremer & Manen, 2000). The first taxonomic revision of the genus *Ophiorrhiza* in India was by Deb and Mondal (1997), in which 47 species and 9 varieties were documented. After this study, few more species and taxa were discovered and added to the flora of India. At present, 51 species and 5 varieties are recorded from India, in which majority are distributed in the Northeastern states and Western Ghats (Hareesh et al., 2020). *O. mungos* and *O. rugosa* are the two species distributed all over India (Krishnakumar et al., 2020). Out of the 24 taxa reported in Western Ghats, 20 taxa are reported from Kerala, in which 15 are endemic. Worldwide, the genus comprises of 321 species, 5 varieties and 1 subspecies, mainly distributed in the Asian regions, particularly India, Indonesia and Malaysia (Taher et al., 2020). The genus generally consists of annual or perennial species that exhibits varying range of habit from creeping herbs to small shrubs growing in humid regions at varying altitudes (Pandey et al., 2022). The most important characters of the genus include succulent stems, pentamerous flowers with white tubular funnel shaped corolla, slightly unequal opposite leaves that are elliptic, lanceolate to ovate and compressed fruits with rhomboid shaped capsular seeds (Sibi et al., 2012). Taxonomical identification of the species is very difficult due to their morphological similarities. For this same reason, some species are converged and some are

split to form new species. Only thorough investigation on the vegetative as well as floral parts can authenticate the correct identity of the species in the genus. The taxonomic position of *Ophiorrhiza* is as follows:

Bentham and Hooker classification			**APG IV classification**		
Kingdom	:	Plantae	Kingdom	:	Plantae
Division	:	Phanerogamae	Clade	:	Tracheophytes
Class	:	Dicotyledonae	Clade	:	Angiosperms
Sub-class	:	Gamopetalae	Clade	:	Eudicots
Series	:	Inferae	Clade	:	Asterids
Order	:	Rubiales	Order	:	Gentianales
Family	:	Rubiaceae	Family	:	Rubiaceae

II. Karyomorphological studies

Data on chromosomes helps in the better understanding of systematic relationships in plants and are a great source of information with evolutionary and taxonomic significance. Though the functionality and dependability of karyomorphological data is well known, chromosomal studies on the genus *Ophiorrhiza* are too scanty. The limited data on the karyological aspects of the genus might be due to the restricted distribution of the plants and also the practical problems such as poor stainabilty of chromosomes or excess staining of cytoplasm (Philip & Mathew, 1988). Rubiaceae is a well distributed angiosperm family especially in the southern part of India. However, cytological information on members indigenous to this region, especially *Ophiorrhiza*, is very few.

Raghavan and Rangaswamy (1941) studied the cytotaxonomical features of some genera of plants in Rubiaceae and reported the somatic chromosome number of about 20 species belonging to different taxa. Root tips fixed in Nawaschin's fluid were used to determine the chromosome number. Only one species of *Ophiorrhiza, O. brunonis* was studied, whose

chromosome number (2n) was found to be 22 with the basic chromosome number as 11. In the study conducted by Gajapathy (1961) on the cytology of some Indian medicinal plants, the meiotic chromosome number of *O. mungos* was reported as 11 and the ploidy level was mentioned as diploid.

Selvaraj (1987) conducted karyomorphological studies in south Indian Rubiaceae to understand the cytotaxonomical relationship of different taxa in the family, using freshly excised root tips of each taxon under consideration. In the study, the chromosome number of *O. pectinata* was reported for the first time. According to the report, the chromosome number of the species was 2n = 44.

Cytological studies on south Indian Rubiaceae by Philip and Mathew (1988), revealed the chromosome numbers of several taxa of the family. Among the various taxa selected for the study, 3 species of *Ophiorrhiza,* namely; *O. eriantha, O. harrisiana* and *O. mungos* were also present. Chromosomal studies on the pollen mother cells showed that the three species have same number of bivalents at the diakinesis and metaphase 1 stages. Since the number of bivalents in all the species was 11, the somatic chromosome number of these species of *Ophiorrhiza* was denoted as 22.

Kiehn (1995) did a chromosomal survey of the family Rubiaceae, according to which the range of chromosome number in the family is 2n = 12 to 2n = 220. The range of basic chromosome number is x = 6 to x = 17. However, the most prominent one is x = 11. The ploidy level in the family was found to be in the range of 2x to 22x. The basic chromosome number of the tribe Ophiorrhizeae was found to be x = 11 or 12. The ploidy level was mentioned as 2x or 4x.

III. Traditional usage

The members of the genus *Ophiorrhiza* are widely used in conventional medicines for the treatment of various ailments. The name "ophiorrhiza" comes from the Greek words "*ophis*," which means "snake" and "*rhiza*" which means "root" (Deb & Mondal, 1997). *Ophiorrhiza* species are well known for healing snakebite, ulcers, wounds, gastropathy, leprosy and amenorrhea. The plants are widely used for the treatment of inflammation, pain and microbial infections as well (Taher et al., 2020).

O. communis is used for treating cough and is also used as poultice. *O. tomentosa* is another species which is also used as poultice (Rubeena, 2021). *O. mungos* and *O. japonica* are two species widely used against snake bite. Both the plants have antihelminthic, alexipharmic and anti-venom properties. *O. mungos* has anti-ulcer properties also (Agarwal & Dhar, 1959). *O. japonica* is a widely used medicinal plant in China for the treatment of ulcers, wounds and leprosy (Kong et al., 1976). Different kinds of skin outbreaks are treated by applying the bruised roots of this plant. The flowers are recommended as a stimulant and cardiac tonic in cases of rheumatism and heart disease. The leaves of the plant have alterative qualities (Tafur et al., 1976). The fruits of *O. fasciculata* are consumed by the people of Arunachal Pradesh (Arbain, 1992). In Bangladesh, tea made from *O. harrisiana* leaves is used to treat chest pain in addition to bodily aches (Alam, 1992). The roots of this plant are boiled with oil and used as a hair and wool dye in Nepal and the Trans-Indus region (Watt, 2014). *O. filistipula* is used in traditional West Sumatran medicine for treating scalp infections in children (Arbain et al., 1993). *O. subcapitata* is another traditionally used species, whose leaves and roots are used to treat facial blemishes. The decoction of leaves and roots is used as a wash, and it is used with honey to treat fever, sore throats and tonsils (Maikhuri & Gangwar, 1993). In Indonesia and West Sumatra, the

traditional healers utilise the poultice of fresh aerial portions of *O. major* to cure skin diseases, particularly eczema (Fuji et al., 1995). The tribes of Nicobar Islands use *O. nicobarica* against herpetic lesions and skin infections (Chattopadhyay et al., 2007). *O. rugosa* var. *prostrata* is a commonly used plant by the local tribes, like Chakma, Marma, and Tanchangya, of Bangladesh for the treatment of earache, chest and body pain, skin infections, diarrhea and dysentery. Among the tribes, the plant is known in different names as Pahari mehedi (Marma), Jariphul (Tanchangya) and Jari (Chakma) (Chy et al., 2021).

IV. Phytochemical studies

Though *Ophiorrhiza* is a species rich genus, the literature survey reveals that there are only few reports on the study of the phytochemical compounds in the genus. The major classes of compounds reported from different species include alkaloids, terpenoids, flavonoids, phenols, steroids and anthraquinones (Krishnakumar et al., 2020).

Ophiorrhiza species are known as alkaloid rich plants and contain many therapeutically potential alkaloids. The common alkaloids obtained from the different species of *Ophiorrhiza* include harman, harmine, harmane and harmaline. The compound harmane or harmine is reported to exhibit antiproliferative and antioxidant properties. The extracts of several species of *Ophiorrhiza* like *O. acuminata, O. japonica, O. kuroiwai, O. liukiuensis, O. communis, O. tomentosa, O. rosacea, O. nicobarica, etc.,* have revealed the presence of harman or harmaline compounds (Taher et al., 2020). Harman-2-oxide, is a crystalline alkaloid obtained from the aerial parts of *O. rosacea*. Strictosidinic acid, palicoside, isomalindine, malindine and isomalindine-16-carboxylate are also important alkaloid compounds (Arbain et al., 2000; Arbain et al., 1997b). The most important alkaloid component obtained from *Ophiorrhiza* species is camptothecin, a modified monoterpene

indole alkaloid. According to reports, this compound has high anti-cancer properties and is profoundly seen in *O. mungos* and *O. mungos* var. *angustifolia* (Rajan et al., 2016a) and also in some other species of the genus. Pumiloside and deoxypumiloside are two important precursors for the biosynthesis of camptothecin, obtained from *O. pumila* (Kitajima, 2007). In addition, the ethanolic extract of this plant exhibited an alkaloid compound, aknadinine (Liu et al., 2020). Ophiorrhizine isolated from *O. major*, ophiorrine A and B from *O. japonica,* ophiorrisides A, B, C, D, E and F from *O. trichocarpon* (collected from Thailand) are similar alkaloid compounds (Lindsay & PrimaáPutra, 1992; Feng et al., 2018; Kitajima et al., 2013).

Flavonoid compounds also add to the medicinal value of *Ophiorrhiza* species. Among the various flavonoid compounds obtained from the plants, Luteolin-7-O-glucosides, obtained from *O. mungos* is very important with antioxidant, anti-inflammatory and anticancer properties. Upon being modelled in rats, the compound is reported to protect the gastrointestinal tract against ethanol and indomethacin-induced gastric ulcer (Baskar et al., 2011; Antonisamy et al., 2016). The butanolic extract of the whole plant of *O. liukiuensis* revealed the presence of hyperin, a tetrahydroxyflavone compound, which produce anti-inflammatory effects (Hamzah, 1994). Though flavonoid compounds are not so abundantly found in *Ophiorrhiza*, the reported compounds are with high therapeutic activities.

Plenty of terpenoid compounds are reported from *Ophiorrhiza* species. Ursolic acid, a triterpene compound having multifunctional property was revealed from the chloroform extract of the whole plant of *O. liukiuensis*. The compound selectively induces cell death, as well as inhibits lipoxygenase and cyclooxygenase, inhibiting cell overgrowth in combination. The methanolic extract of *O. nicobarica* has also revealed the presence of this compound (Chattopadhyay et al., 2007; Martins & Nunez, 2015). The hexane extract of

O. shendurunii, exhibited some triterpenoid fatty acid esters like lupan-20-ol-3(β)-yl hexadecanoate, lupan-20-ol-3(β)-yl acetate and and olean-18-en-3(β)-yl hexadecanoate which have antimicrobial, antifungal, and anti-yeast activities (Rajan et al., 2016b). An ursane type triterpene was isolated from the aerial portions of *O. baviensis,* along with 8 other compounds (Viet Cuong et al., 2019). In addition to the mentioned secondary metabolites, *Ophiorrhiza* species are great repositories of fatty acids, steroids, tannins, coumarins, anthraquinones, glycosides *etc*.

Large number of phytosterols is observed in various species of *Ophiorrhiza*. The methanolic extract of *O. nicobarica* has revealed the presence of β-sitosterol, which is said to have anti-inflammatory, antipyretic, antiulcer, and anticancerous properties. Additionally, β-sitosterol has been shown to greatly improve the activity of helper T-cells, cytokines, interleukin 2, γ-interferon, and NK cells, which makes it effective in the treatment of immune dysfunction illnesses (Chattopadhyay et al., 2007). β-stigmasterol is another phytosterol obtained from the ethanolic extract of *O. pumila* (Liu et al., 2020).

The GC-MS analysis of the whole plant ethanolic extract of *O. tirunelvelica* showed 11 phytocompounds and the major ones were 12-methyl-E,E-2,13-octadecadien-1-ol, E-2-tetradecen-1-ol, cyclopropanedecanoic acid, phenol-2,6-bis(1,1-diemthylethyl), 4-methoxy-5,7-dihydroxy isoflavone and ethyl oleate (Prabha & Karuppusamy, 2017).

Analysis of the ethanolic extract of *O. rugosa* var. *prostrata* leaves by GC-MS revealed about 30 compounds belonging to different classes of secondary metabolites and the major ones among them being phytol, squalene, vitamin E, loliolide and neophytadiene (Adnan et al., 2019).

A summary of the major phytocompounds obtained from different types of extracts; such as ethanol, methanol, chloroform and hexane, from various *Ophiorrhiza* species is given in **Table 1.**

Plants contain different types of secondary metabolites which provide them the medicinal value. The abundance of each metabolite differs from plant to plant. The diverse nature of these compounds demands proper technologies and methodologies for their chemical profiling, identification as well as isolation. Different technologies are developed and used for the proper chemical fingerprinting of active compounds in biological samples. Since a single technique is not enough for the characterisation of compounds in a sample, several analytical procedures have to be followed. Chromatographic techniques like GC-MS and LC-MS are very popular as they can be used to analyse a wider variety of biological molecules. The most often used chromatographic method today for screening plant extracts for non-volatile and thermally unstable chemicals is HPLC (Rubeena, 2021). A multitude of integrated techniques and processes, including non-destructive NMR (nuclear magnetic resonance spectroscopy) and mass spectrometry (MS)-based techniques like GC-MS (gas chromatography-MS), LC-MS (liquid chromatography-MS), and CE-MS (capillary electrophoresis-MS), and FT-ICR-MS (Fourier transform ion cyclotron resonance-MS), currently enable large-scale analysis of highly complex mixture of samples (Okazaki and Saito, 2012; Khakimov et al., 2014). However, metabolomics analysis is designed with combination methodologies to produce superior results to get over the inherent constraints that each analytical methodology has.

Table 1: Major phytochemical compounds reported from various species of *Ophiorrhiza*, studied earlier

Sl. No	Plant	Compounds	References
1	*O. acuminata*	Palicoside, lyalosidic acid, harman	Nonato et al., 1995
2	*O. baviensis*	3b,23,24-Trihydroxyurs-12-en-28-oic acid, 3b,19a,23,24–tetrahydroxyurs-12-en-28-oic acid, 3b,6b,23–trihydroxyolean-12-en-28-oic acid, rutundic acid, blumenol A, vincosamide, (5S)-5-carboxystrictosidine, vogeloside, sweroside	Viet Cuong et al., 2019
3	*O. blumeana*	Bracteatine, blumeanine, ophiorrhizine, ophiorrhizine-12- carboxylate	Arbain et al., 1998a
4	*O. bracteata*	Bracteatine, ophiorrhizine	Arbain et al., 1997b
5	*O. communis*	Isomalindine, isomalindine-16-carboxylate, harman	Hamzah et al., 1994; Arbain et al., 1997a
6	*O. discolor*	Tetrahydroalastonine	Arbain et al., 1991
7	*O. ferruginea*	Isomalindine, malindine, dihydrocycloakagerine, 3,14-dihydrodecussine, tetrahydrocycloakagerine	Arbain et al., 1993
8	*O. filistipula*	7-Methoxy camptothecin, normalindine, strictosidinic acid, camptothecin	Arbain et al., 1993; Ohba et al., 2000
9	*O. hayatana*	Ophiohayatone A, B,C and D, norharman, 6- hydroxyharmane, pumiloside, strictosamide, lyalosidic acid, 9H-β-carboline-1- carboxylic acid, maxonine, lyaloside, desoxycordifolinic	Krishnakumar et al., 2020

		acid, vincoside, 2-hydroxymethyl-3-methoxyanthraquinone, methyl-p-hydroxycinnamate, vanillin, ophiorridin-A, B and C, p-hydroxybenzaldehyde, umbelliferone, ursolic acid	
10	*O. japonica*	Harman, 6-hydroxy harman, lyaloside, lyalosidic acid, ophiorrine A and B, pumiloside, deoxypumiloside, strictosamide, camptothecin, hydroxycamptothecin, friedelin	Fujita & Sumi, 1967; Aimi et al., 1986; Wang et al., 2009; Feng et al., 2018
11	*O. kuroiwai*	Camptothecin, 9-methoxy camptothecin, harman, lyalosidic acid, ophiorrine A and B.	Aimi et al., 1985
12	*O. liukiuensis*	Camptothecin, 6-hydroxyharman, pumiloside, deoxypumiloside, strictosamide, demethylsecologanol, sweroside, loganic acid, loganin, swertiaside-A, scopoletin, hyperin, chlorogenic acid, ursolic acid, β-sitosterol, daucosterol, 2-methyl-1,3,7-trihydroxyanthraquinone, 1- hydroxy-3-methyl- anthraquinone, harman, norharman, ophiorrhizine-A and B, strictosamide, lyalosidic acid, soranjidiol-1-methyl ether, ophiorrhizinone- C, D and E, 1,3-dihydroxy-2-hydroxy ethylanthraquinone, 1,3-dihydroxy-2,8-dimethoxy-7-methyl- anthraquinone, ophiorrhizine A, ophiorrhizol A, inamoside, 3-O-caffeoylquinic methyl ester, sodium chlorogenate, quinic acid 3,4-di-O-caffeate, quinic acid 3,5-di-O-caffeate, ursolic acid	Jose & Satheeshkumar, 2004; Kitajima et al., 2005; Krishnakumar et al., 2020
13	*O. longiflora*	Tetrahydroalstonine	Roza, 1992
14	*O. major*	Bracteatine, ophiorrhizine	Fuji et al., 1995

15	*O. marginata*	Decarbomethoxydihydrogambirtannine, strictosidinic acid	Arbain et al., 1998b
16	*O. mungos*	Camptothecin, 10-methoxy camptothecin, 9-methoxy camptothecin, luteolin-7-*O*-glucoside, 5α-ergosterol-8(14) - ene-3β-ol, 5α-ergosterol-7-ene- 3β-ol	Baskar et al., 2011; Krishnakumar et al., 2020;
17	*O. nicobarica*	Harmaline, ursolic acid, β-sitosterol	Chattopadhyay et al., 2007
18	*O. pumila*	Camptothecin, strictosidine, 9-methoxy camptothecin, 10-hydroxy-camptothecin, strictosamide, pumiloside, deoxypumiloside, chaboside, strictosidinic acid, lucidin-3-O-β purimeveroside hydroxy,2 hydroxymethylanthraquinone, 3-O-caffeoylquinic acid, 9-β-glucosyloxycamptothecin, 1-hydroxy-2 methyl-anthraquinine, 3-hydroxyanthraquinone-2-carbaldehyde, 2-n-butoxymethyl-1,3-dihydroxyanthraquinone, tetraacetyl sweroside, inamoside, penta-O-acetyl loganin, β-sitosterol, aknadinine, β-stigmasterol	Aimi et al., 1990; Arbain, 1992; Kitajima et al., 1998; Kitajima et al., 2002; Yamazaki et al., 2003a; Yamazaki et al., 2003b; Liu et al., 2020
19	*O. rosacea*	Harman-2-oxide, harman, lyalosidic acid, tetrahydroalstonine, strictosidinic acid, valesiaschotamine, isovalesiaschotamine	Arbain et al., 2000
20	*O. rugosa* var. *prostrata*	Phytol, γ-sitosterol, stigmasterol, erucamide, squalene, methyl palmitate, methyl linoleate, vitamin E, methyl stearate, ethyl linolenate, loliolide, neophytadiene	Adnan et al., 2019

21	*O. shendurunni*	Hexadecanoate, lupan-20-ol-3(β)-yl acetate, olean-18-en-3(β)-yl hexadecanoate, dotriacontanoic acid, stigmasterol, rubiadin, nonadecanoic acid, palmitic acid, camptothecin	Rajan et al., 2016b
22	*O. teysmaniana*	Tetrahydroalstonine	Arbain, 2012
23	*O. tirunelvelica*	E-2-Tetradecen-1-ol, 12-methyl-E, E-2,13-octadecadien-1-ol, ethyl oleate, cyclopropanedecanoic acid, 9-octadecanoic acid, 2 hydroxy ethyl isopropyl stearate, docosa-pentan-22-al, phenol-2,6-bis (1,1-diemethyethyl), 2,3-dihydroxyprophyl elaidate, eicosanoic acid ethyl ester, 4-methoxy-5,7-dihydroxy isoflavone	
24	*O. tomentosa*	Strictosidinic acid, harman	Hamzah et al., 1994
25	*O. trichocarpon*	Ophiorrisides A, B, C, D, E and F	Kitajima et al., 2013

V. Camptothecin - a promising drug candidate

Though many classes of compounds are obtained from different species of the genus *Ophiorrhiza,* camptothecin (CPT) is the most important one. CPT is a monoterpenoid indole alkaloid first isolated from the bark and stem extract of the Chinese tree *Camptotheca acuminata* (Nyssaceae) in 1958 (Wall et al., 1966). Later on, the compound was reported from *Nothapodytes foetida* (Govindachari et al., 1972). The structure of CPT consists of a fused five ring backbone that begins with a weakly basic quinoline moiety and terminates with a lactone ring (Pu et al., 2019). CPT inhibits the action of DNA topoisomerase 1 and hence is a potential anticancer agent. Though the compound has great antitumour properties, the complex structure made its artificial synthesis difficult and therefore natural plants were used as the raw

materials for its extraction (Rajan et al., 2013). The increased demand and market value of CPT led the researchers to find new natural sources of CPT.

Several plants were screened and reported to possess CPT contents. The major species were *Camptotheca lowreyana, C. yunnanensis, Ervatamia heyneana, Merrilliodendron megacarpum, Mostuea brunonis, Nothapodytes nimmoniana, Pyrenacantha klaineana* and several species of *Ophiorrhiza* (Pandey et al., 2022). Though all these plants were having CPT, the indigenous trees, *Camptotheca acuminata* and *Nothapodytes nimmoniana* were utilized as the major natural source of CPT. As these plants are woody trees with long life span, the extraction process was difficult and the uncontrolled use of these plants pose threat to their survival status (Rajan et al., 2013). Since, continuous availability of CPT is necessary, search for new sources is important.

Though, different plant genera have been reported to possess CPT, *Ophiorrhiza* species are the most important ones. After the isolation of CPT from *O. mungos,* the genus has emerged as a strong candidate for CPT production as majority of the plants are herbaceous in nature which enables their mass production using tissue culture techniques (Roja, 2008). HPTLC based densitometry studies and other biotechnological studies have revealed the presence of CPT and its derivatives in many *Ophiorrhiza* species like *O. alata* (Ya-ut et al., 2011), *O. decumbens* (Vineesh et al., 2007), *O. erianta* (Rani et al., 2010), *O. filistipula* (Arbain et al., 1993), *O. fucosa* (Viraporn et al., 2011), *O. grandiflora* (Ya-ut et al., 2011), *O. harrisiana* (Viraporn et al., 2011), *O. japonica* (Wang et al., 2009), *O. kuroiiwai* (Aimi et al., 1985), *O. mungos*, *O. mungos* var. *angustifolia*, *O. pectinata* (Rajan et al., 2013), *O. plumbea* (Viraporn et al., 2011), *O. prostrata* (Nicholas et al., 1990), *O. pumila*, (Yamazaki et al., 2003a), *O. ridleyana* (Viraporn et al., 2011), *O. rugosa* (Roja, 2006), *O. shendurunii* (Rajan et al., 2013), *O. trichocarpon* (Sibi et al., 2012) *etc.,* in varying quantities. Previous reports have suggested that *in vitro* raised plants have better secondary metabolite content than the

wild plants (Vineesh et al., 2007). Many researches are being carried out to enhance the production of CPT in micropropagated plants of elite species (Pandey et al., 2022).

Though CPT is a potential antitumour compound, its low water solubility and high toxicity in the S phase of the cell cycle (Khazir et al., 2014), prompted the researchers to develop two first generation camptothecin analogues semisynthetically. The two CPT analogues, namely topotecan (hycamtin) and irinotecan (camptosar or CPT-11) are approved for their use in the treatment of ovarian, colon and lung cancers (Oberlies & Kroll, 2004). Semisynthetic CPTs like chimmitecan, exaletacan mesylate, kerenitecin,ositecan, and gimatecan are all under various stages of clinical testing (Rajan et al., 2013). Recent studies indicated that the natural CPT derivatives 9-methoxy camptothecin and 10-hydroxy-camptothecin are water-soluble and have antitumour properties (Lorence & Nessler, 2004). Following this, camptothecin has emerged as a leading contender for the treatment of multi-drug resistant malignancies.

VI.Reported bioactivities

Antioxidant activity

Abiotic stress results in the formation of free radicals within the body, which the endogenous antioxidant system counteracts. Free radical accumulation within cells due to an imbalance in their generation and elimination results in oxidative stress, which in turn can lead to a number of disorders (Choudhury et al., 2013). Enzymatic or non-enzymatic reactions often detoxify the excess radicals produced by various metabolic processes. However, when the concentration rises above the threshold level, free radicals must be neutralised by exogenous antioxidants, especially by natural antioxidants found in foods and medicinal plants. It has been shown that various isolated components and crude plant extracts of medicinal plants have

positive antioxidant activity. The free radical scavenging mechanism of antioxidants is illustrated in **Fig. 1.**

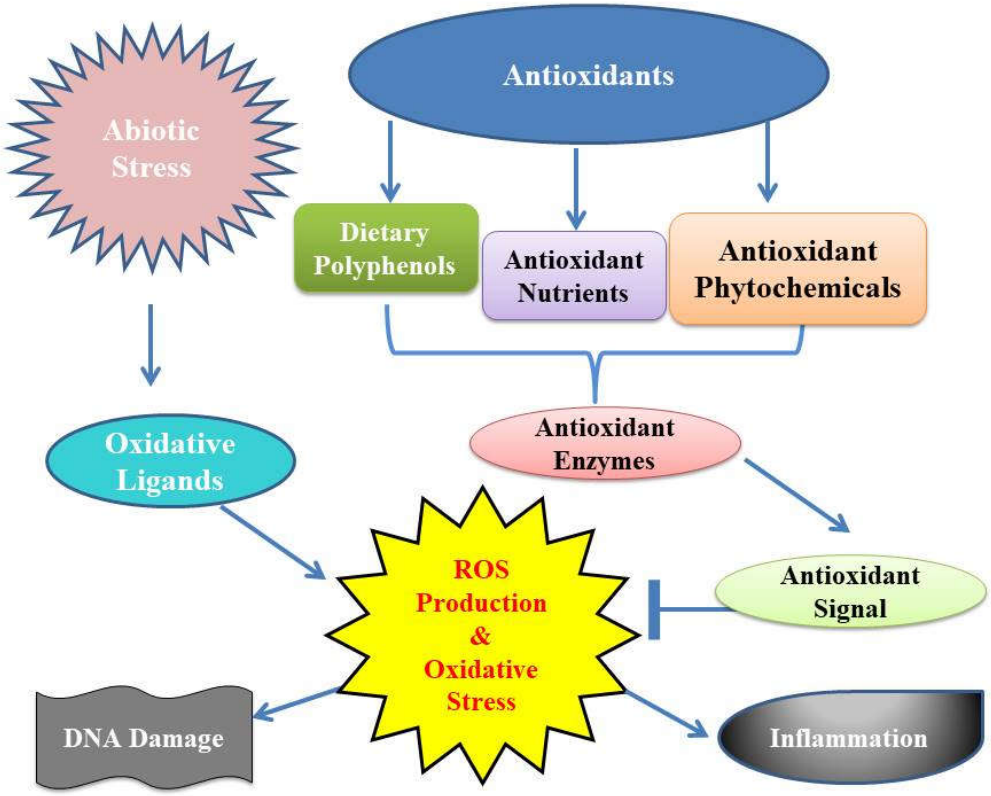


Fig. 1: Free radical scavenging mechanism by antioxidants (Rubeena, 2021).

The antioxidant activity of *Ophiorrhiza* species is not elaborately investigated. Very few reports are available on the radical scavenging potential of these plants, which is reviewed here.

The methanolic root, stem and leaf extracts of *O. mungos* were analysed for their *in vitro* antioxidant capacity using DPPH and reducing power assays. In both experiments, the root extract demonstrated greater antioxidant capacity than the stem and leaf extracts. Different concentrations from 20 to 100 µg/ml were considered for the study. At the highest concentration, the root extract exhibited an inhibition percentage of 65.15 ± 0.002% (IC_{50} value was 0.023 mg/ml). The leaf extract showed

57.57 ± 0.001% of inhibition (IC_{50} value was 0.068 mg/ml). The percentage inhibition exhibited by the stem extract was very less (33.33 ± 0.002%) and thus IC_{50} value was not calculated. The percentages of free radicals that were inhibited in the DPPH assay increased with dosage. The root extract demonstrated greater reducing capacity in the reducing power assay also, followed by leaf and stem extracts (Mohan et al., 2012).

Raveendran et al. (2012), evaluated the ROS scavenging activity of the anthraquinone fraction (AQf) isolated from the *in vitro* cultures of *O. rugosa* var. *decumbens* using superoxide, hydroxyl and nitric oxide radical scavenging assays. The obtained results showed intriguing differences in ROS regulation. AQf facilitated the production of superoxide radicals, but inhibited hydroxyl radicals up to 60% and reduced lipid peroxidation. The IC_{50} value for hydroxyl radical scavenging assay was 190 µg/ml. Despite a statistically significant decrease in NO radical generation, only 15% of NO radicals could be scavenged by the maximum concentration of AQf. The study indicated that the plant species has high scavenging effect on hydroxyl radicals.

The *in vitro* antioxidant activity of the ethanolic extract of *O. tirunelvelica* (whole plant) was screened using DPPH radical scavenging assay. A dose dependent quenching of free radicals with increasing concentration from 3-50 µg/ml was observed in the assay. The IC_{50} value of the standard, ascorbic acid, was 3.2 ± 0.15 µg/ml and that of the plant extract was 4.3 ± 0.88 µg/ml, which represented the moderate radical scavenging capacity of the plant (Prabha & Karuppusamy, 2017).

Baiju et al. (2017) investigated the *in vitro* antioxidant potential of the aqueous leaf extract of *O. mungos* and *O. pectinata* using DPPH radical scavenging assay. The IC_{50} values obtained for O. *mungos* was 78 µg/ml and that for *O. pectinata* was 62 µg/ml. The obtained results for both the species

were not better than the value of the standard, ascorbic acid which was 18 µg/ml. However, the results suggested that the plants have moderate radical scavenging potential.

Anti-inflammatory activity

Administration of NSAIDs often results in various gastrointestinal and renal side effects. Problems like dyspepsia, gastric ulceration, salt and fluid retention, hypertension *etc*., are some of the symptoms observed in patients with rheumatoid arthritis, after the use of NSAIDs (Parasuraman et al., 2014). Thus, herbal drugs and herbal products have gained much attention towards their anti-inflammatory activity. Many *Ophiorrhiza* species have not yet been evaluated for their inflammatory reducing properties. The few reports available on the anti-inflammatory potential of *Ophiorrhiza* species are mentioned herewith.

O. nicobarica is an ethnomedicinal plant commonly used by the Nicobarese tribes to treat hepatic lesions and skin infections. Chattopadhyay et al. (2007) evaluated the analgesic and anti-inflammatory potential of the plant. For the study, the methanolic crude extract of the plant and the isolated fractions (A, B and C) were tested on various models of experiment. The concentrations considered for all the study were 100, 200 and 300 mg/kg of the extract and 50 mg/kg of the fractions (A, B and C).

Swiss albino mice and Wistar albino rats were made to respond to acetic acid-induced writhing, tail flick and tail immersion tests, to assess the analgesic effect. The inhibition of writhing reflexes was highest (55.97%) at 300 mg/kg for the extract and for the fractions were 50.96% (A), 40.36% (B) and 54.91% (C). The standard aspirin gave an inhibition of 75.66%. In the tail flick test, the extract showed highest reaction time (3.87 s) at the highest concentration. For the fractions, the time was 3.27 s (A), 2.01 s (B) and 3.29 s

(C). The reaction time for the control pethidine was 4.21 s. In the tail immersion test, the extract showed highest reaction time (3.54 s) at the highest concentration. For the fractions, the time was 3.30 s (A), 2.02 s (B) and 3.27 s (C). The reaction time for the control pethidine was 4.47 s. The findings showed that the fractions at 50 mg/kg and the crude extract at 300 mg/kg concentrations displayed analgesic efficacy that was substantial when compared to the control groups (Chattopadhyay et al., 2007).

The anti-inflammatory potential of the plant was tested on acute, sub-acute and chronic models of inflammation. Carrageenin induced paw oedema, dextran induced oedema and cotton pellet induced granuloma were the models used respectively. The results showed that the crude extract and the fractions showed a dose-dependent increase in the anti-inflammatory potential in all the models. The maximum potential was revealed at 300 mg/kg for the crude extract and at 50 mg/kg for the fractions at all the models of inflammation. In the acute inflammatory model, the extract showed an inhibition of 57.41% and the fractions showed 59.94% (A), 47.34% (B) and 55.13% (C). The obtained results were comparable with that of indomethacin (67.49%). In the sub-acute model also, the inhibition percentage of the extract (55.36%) and that of the fractions (57.92%, 46.09% and 51.46% respectively) were comparable with that of indomethacin (60.97%). In chronic inflammation model, the inhibition percentage of the extract (52.96%) and that of the fractions (51.0%, 49.23% and 51.23% respectively), were comparable with that of indomethacin (54.07%). (Chattopadhyay et al., 2007).

Anti-inflammatory activity of the anthraquinone fraction (AQf) isolated from the *in vitro* cultures of *O. rugosa* var. *decumbens* was analysed on carrageenin induced oedema on mice. Both the concentrations of the AQf (100 and 200 mg/kg body weight) used had significant inflammation reducing

capacity in the mice, which was found to be better than the activity of the standard, diclofenac used (Raveendran et al., 2012).

Adnan et al. (2019) investigated the anti-inflammatory effect of the ethanolic extract of *O. rugosa* var. *prostrata* leaves (EEOR) using histamine-induced paw oedema. The results showed that the oral administration of EEOR in different concentrations (100-400 mg/kg) exhibited a dose dependent inhibition of inflammatory response. At the highest concentration of 400 mg/kg, a significant decrease in the paw oedema (57.14%) was observed. In the study, diclofenac sodium was used as the standard, which revealed 78.57% inhibition at the highest concentration.

Evaluation of the anti-inflammatory efficacy of the secondary metabolites isolated from the aerial parts of *O. baviensis* was done by Viet Cuong et al. (2019). From the alcoholic extract of the plant, 9 compounds were isolated. These compounds were tested for their inhibition potential on NO in LPS-stimulated RAW 264.7 cells. Out of the 9 compounds tested, the results revealed that 6 of them, namely; 3β, 6β, 23– trihydroxyolean-12-en-28-oic acid, rutundic acid, blumenol A, (5S)-5 carboxystrictosidine, vogeloside and sweroside had good NO inhibiting capacity with IC_{50} values ranging from 58.25 to 93.73 µg/ml.

Chy et al. (2021) studied the anti-inflammatory effect of the crude ethanolic extract of *O. rugosa* leaves on Swiss albino mice using carrageenin induced paw oedema test. The study showed a significant inhibition of inflammation over a period of 4 h at 1 h interval at both the concentration of the extract (200 and 400 mg/kg). The highest concentration at the highest time revealed the maximum inhibition of 45.45%, while that of the standard diclofenac sodium was 70.90%, indicating that the extract has good anti-inflammatory effect.

Cytotoxicity studies on *Allium cepa*

Allium cepa is a commonly used genetic model to assess damages induced by various biotic and abiotic mutagens. The availability and low cost of cultivation are the key benefits of employing *A. cepa*. They enable *in situ* monitoring of cytogenetic changes and are extremely sensitive to environmental factors. It is simple to visualise the chromosomal organisation and the alterations (de Souza et al., 2016). An initial screening test for cytotoxicity or genotoxicity investigations at the cellular level can be performed using the *Allium cepa* bioassay (Bezerra et al., 2016). A variety of genotoxicity markers, including the mitotic index (MI), micronucleus (MN), chromosomal aberrations (CA), and nuclear abnormalities (NA) can be examined using this test system (Alberetini et al., 2000). *A. cepa* has been used as a model organism to examine the genotoxicity effects of various drugs and plant extracts due to its ability to be used to evaluate numerous genetic endpoints, such as point mutations or chromosomal abnormalities in living cells (Grant, 1994).

Cytotoxicity studies on *Allium cepa* in the genus *Ophiorrhiza* is very few. Preethamol and Thoppil (2019) evaluated the cytotoxic potential of the aqueous extract of *O. pectinata* using the *Allium cepa* assay. Various concentrations of the plant extract (0.1%, 0.05%, 0.01% and 0.005%) were considered for the study. The results showed that the extract could induce cytotoxicity at all the tested concentrations, by producing several chromosomal and spindle fibre aberrations, at all the stages of mitosis. The major clastogenic aberrations observed were chromosome coagulation, stickiness, chromosome bridges and fragments, nuclear lesions, and giant cells. The prominent non-clastogenic aberrations formed were polyploidy, C-metaphase, stathmo-anaphase, ball metaphase, vagrants, tropokinesis, *etc*.

The mitotic index and the abnormality percentage showed a dose dependent change in their values on increasing concentration of the plant extract. The mitotic index decreased with increasing concentration of the extract and at the highest concentration (0.1%) exhibited the least value of 28.56 ± 2.93%. The abnormality percentage increased with the increase in the extract concentration and at the highest concentration exhibited a value of 85.86 ± 3.55%.

Anticancer studies

Anticancer medications function by stimulating the apoptotic signalling pathway of the cancer cells and thus preventing them from proliferating (Baskar et al., 2011). The most important apoptotic pathways are extrinsic, intrinsic and perforin or granzyme pathway. All these pathways require particular triggering signals to start an energy-dependent cascade of chemical reactions. Each pathway stimulates its own initiator caspases (8, 9, or 10), which activates caspase-3, the executioner. But the granzyme pathway functions independently of caspase. Some of the distinctive apoptotic features brought on by the execution pathway include cell shrinkage, chromatin condensation, the development of cytoplasmic blebs and apoptotic bodies. Subsequently, the apoptotic bodies undergo phagocytosis by the neighbouring parenchymal cells, neoplastic cells or macrophages (Elmore, 2007). The schematic representation of the main apoptotic pathways is given in **Fig. 2.**

Though, antitumour drugs are widely used in the treatment of cancer, the side effects of these modern allopathic medicines, severely affect the quality of life of the patients. India, alone has about 8% of the global cancer cases and accounts for more than 8% of cancer deaths (Tariq et al., 2017). Thus, traditionally used medicinal plants with proven anticancer activities have become the need of the hour. According to surveys, the highest number of anticancer plants is reported from India (Tariq et al., 2017).

The presence of the cytotoxic alkaloid, CPT, and its water-soluble analogues, make the genus *Ophiorrhiza*, a promising candidate for the development of a herbal as well as cancer specific drug (Preethamol & Thoppil, 2019). Several works have been done to analyse the antiproliferative efficacy of the different species of *Ophiorrhiza*, which are reviewed here.

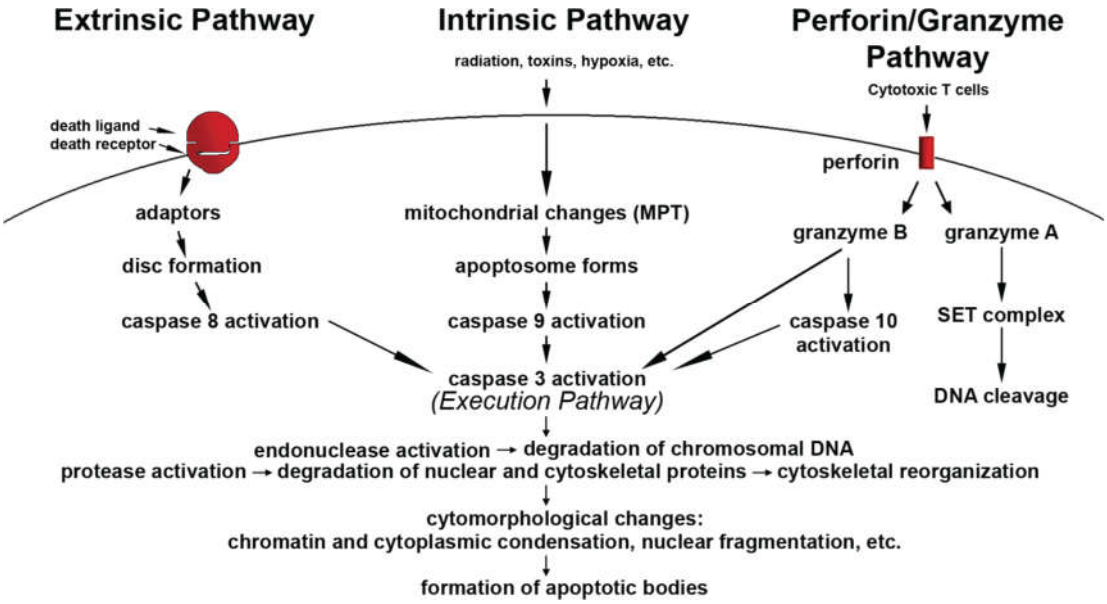


Fig. 2: The major apoptotic pathways (Elmore, 2007)

Baskar et al. (2011) studied the anticancer potential of the phytocompound luteolin-7-O-glucoside (LUT7G) obtained from the leaves of *O. mungos* in both *in vitro* and *in vivo* models.

For the *in vitro* studies, 4 different cancer cell lines namely; COLO 320 DM (human colon adenocarcinoma), AGS (human gastric cancer cell line), MCF-7 (human breast cancer cell line) and A549 (human lung cancer cell lines) and one normal cell line VERO (African green monkey kidney cells) were considered. The antiproliferative effect of LUT7G at different concentrations (31.75 to 1000 µM) at different incubation periods of 6, 12, 24, 48 and 72 hrs were analysed using the MTT assay. The effect was calculated as the survival percent of the 4 different cancer cell lines and was found that

LUT7G significantly inhibited the proliferation of COLO 320 DM with an IC_{50} value of 512.4 µM when compared with the other cell lines. The toxicity on the non-cancerous cells (VERO) was less (IC_{50} value of 1 mM), which indicated that the compound has cancer cell specific toxicity. Further studies were done on the colon cancer cells only. In colon cancer cells, LUT7G could induce apoptosis at 60 µM, DNA fragmentation and tailing at 120 µM. The compound could also downregulate β-catenin in the cancer cells up to 40% (Baskar et al., 2011).

The *in vivo* anticarcinogenic potential of the compound LUT7G was evaluated on male albino Wistar rats aged 5 weeks. Rats administered with 1,2-dimethylhydrazine (DMH-20 mg/kg bw) for 4 consecutive weeks were supplemented with 3 different doses of LUT7G throughout the experimental period of 16 weeks. Animals treated with DMH showed Aberrant Crypt Foci (ACF) with nearly 6 crypts in the colon. The compound could effectively reduce ACF development in the experimental animals. The percentage inhibition of ACF was 33.1%, 47.9% and 67.4% at the 3 different concentrations respectively (Baskar et al., 2011).

Raveendran et al. (2012) studied the antitumour activity of the anthraquinone fraction (AQf) isolated from the *in vitro* cultures of *O. rugosa* var. *decumbens*. The studies were conducted in both *in vitro* and *in vivo* models. The cell lines used were Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC).

For the *in vitro* studies, both the cell lines were propagated in Swiss albino mice. Later the cells were aspirated from mice and incubated with different concentrations of AQf (40-200 µg/ml) for 2 h. Trypan blue exclusion method was used to determine the cytotoxicity of AQf against DLA, which was found to be 130 µg/ml and for EAC it was 60 µg/ml. For the *in vivo* antitumour activity studies, solid tumour induced by DLA cells and ascites

tumour induced by EAC cells were considered. Two concentrations of AQf (100 and 200 mg/kg body weight) were considered. A 200 mg/kg body weight AQf concentration slowed down the growth of solid tumours in mice. In comparison to control mice, it also increased the lifespan of ascites tumour-carrying mice (Raveendran et al., 2012).

Gopalakrishnan and Shankar (2014), evaluated the cytotoxicity of the crude methanolic extracts and the isolated camptothecin (CPT) from the multiple shoot cultures of *O. decumbens* in five cell lines and compared their activity with the commercially available standard CPT. The different cell lines were A549, MCF7, L929, U937 and Jurkat. The IC_{50} value obtained for the standard was less than 1 µg/ml in all the cell lines. The IC_{50} value obtained for the CPT isolated from the plants were different for each cell lines. The values were 1.93 µg/ml (A549), 0.65 µg/ml (MCF7), >10 µg/ml (L929 and U937) and 2.21 µg/ml (Jurkat). The IC_{50} value obtained for the crude extract of the plants was less than 2 µg/ml in all the cell lines tested. In the study, the crude extract of the plant was more effective than the CPT isolated from the tissue cultured plants, which indicated that the crude extract possesses more anticancer potent compounds.

The anticancer potential of alcohol and aqueous leaf extracts of *O. mungos* was assessed on DAL cell line in mice. DAL cells were injected intravenously (106 cells/mouse) into the animals. The dosages of the extracts were 400 and 800 mg/kg. Except the control groups, all the other groups received DAL cells. Following DAL cell inoculation, there was a considerable growth of tumour cells in the animal peritoneal cavity, which the extracts significantly regulated (Madhavan et al., 2015).

Viet Cuong et al. (2019) analysed the cytotoxic effects of the isolated secondary metabolites from the aerial parts of *O. baviensis* on MCF-7, HeLa, KB, A549, and SK-LU-1 cancer cell lines. All the 9 isolated compounds were

tested for their cytotoxicity and 3 compounds namely; 3β, 23,24–trihydroxyurs-12-en-28-oic acid, 3β, 6β, 23– trihydroxyolean-12-en-28-oic acid and rutundic acid showed cytotoxic activity against all tested cell lines with IC_{50} values ranging from 37.89 to 79.6 µg/ml. Ellipticine was the standard used and the IC_{50} values ranging from 0.45 to 0.30 µg/ml were exhibited in the different cell lines used.

On HepG2 and SMMC-7721 cells, Liu et al. (2020), examined the anti-proliferative and anti-migratory activities of the ethanol extract of *O. pumila*. The CCK-8 assay and colony formation assay were used to assess the anti-proliferative effectiveness. Using flow cytometry, it was possible to measure the ROS level, apoptosis, and cell cycle arrest. By using the western blotting method, the expression of associated proteins was estimated. The plant extract was able to cause cell cycle arrest at the G2/M phase, as well as increased apoptosis and amplified ROS generation in the cell lines. The extract could boost the expression of pro-apoptotic Bax and cleaved Caspase-3 while decreasing the expression of the anti-apoptotic protein Bcl-2. The fact that the extract greatly reduced the phosphorylation of P65 suggests that the downregulation of NF-kB signalling may be the cause of the extract's cytotoxic effects.

Fan et al. (2022) studied the *in vitro* and the *in vivo* anti-lymphoma effects of *O. pumila* extract on murine lymphoma cell line (A20). In the *in vitro* studies, CCK-8 assay was done to evaluate the viability of the A20 cells after treatment with different concentrations of the extract. The extract significantly reduced the A20 cell viability in a time-dose-dependent manner. The IC_{50} values were obtained as 223.75 µg/ml (24 h), 27.95 µg/ml (48 h) and 26.4 µg/ml (72 h). The extract caused cell cycle arrest at the S phase of cell division, as seen by the flow cytometry. The Western blot analysis revealed that the extract could lower cyclin A2 expression, a crucial mediator of the

S phase. The extract also increased the expression of apoptosis proteins, Bax and cleaved caspase-3. However, there was no discernible impact on Bcl2 expression. The extract also triggered the apoptosis of A20 cells (Fan et al., 2022).

Studies conducted *in vivo* using xenograft mice model revealed that the extract inhibited the growth of tumours in derivate A20 cells. The outcomes of the western blot analysis matched those of the *in vitro* investigations. The extract also showed the ability to lower the phosphorylation of EGFR in A20 cells. The research therefore demonstrated that the extract facilitates the deactivation of EFGR signalling and exerts anti-proliferative activity by promoting apoptosis both *in vitro* and *in vivo* (Fan et al., 2022).

The major cell lines used to analyse the antiproliferative effects of the extracts of different species of *Ophiorrhiza* are given in **Table 2.**

Table 2: Major cell lines used to reveal the anticancer efficacy of different species of *Ophiorrhiza,* studied earlier

Sl. No.	Plant	Cell lines	References
1	*O. mungos*	COLO 320 DM, AGS, MCF-7, A549, VERO	Baskar et al., 2011
2	*O. rugosa* var. *decumbens*	DLA, EAC	Raveendran et al., 2012
3	*O. decumbens*	A549, MCF-7, L929, U939, Jurkat	Gopalakrishnan & Shankar, 2014
4	*O. mungos*	DAL	Madhavan et al., 2015
5	*O. baviensis*	MCF-7, HeLa, KB, A549, SK-LU-1	Viet Cuong et al., 2019
6	*O. pumila*	HepG2, SMMC-7721	Liu et al., 2020
7	*O. pumila*	A20	Fan et al., 2022

Other bioactivities

Other than the above mentioned bioactivities, studies to evaluate various other activities of the *Ophiorrhiza* species are also carried out by various researchers. The most important ones are mentioned below.

Jayadev et al. (2013), studied the antibacterial effects of the leaf and the stem extracts (hexane, chloroform, ethyl acetate, methanol and ethanol) of *O. mungos* on 9 different bacteria namely; *Bacillus subtilis, Lactococcus lactis, Staphylococcus aureus, Micrococcus luteus, Pseudomonas aeruginosa, Salmonella typhimurium, Klebsiella pneumoniae, Proteus vulgaris* and *Escherichia coli* using disk diffusion assay. In the case of the leaf extracts, ethyl acetate extract showed the inhibition of 8 bacteria (inhibition zone of 19-28 mm), except *Salmonella typhimurium.* In the case of the stem extract, methanolic extract was very potent to inhibit all the 9 bacteria (inhibition zone of 11-26 mm). Harmaline obtained from the ethnomedical plant *O. nicobarica* displayed strong anti-HSV-1 efficacy against both wild-type and clinical isolates of the virus (Bag et al., 2014).

Rajan et al. (2016b) studied the antimicrobial effects of the hexane and chloroform extracts of the plant *O. shendurunni* by agar well diffusion assay. The hexane extract significantly reduced the growth of *Candida albicans* and *Fusarium oxysporum.* But it shows no effect on *Escherichia coli* or *Bacillus subtilis*. However, the chloroform extract showed substantial antibacterial activity against *B. subtilis* and *E. coli* with ampicillin as the control. The extract also demonstrated strong antifungal effects against *C. albicans* and *F. oxysporum* with ketoconazole as the control.

Baiju et al. (2017) evaluated the antibacterial potential of the crude aqueous leaf extract of *O. mungos* and *O. pectinata* using disc diffusion technique. The concentrations of 500, 750 and 1000 µg of the extracts were

used for the study. 50 µg/ml of gentamycin was taken as the control. Higher concentrations of *O. mungos* and *O. pectinata* showed considerable antibacterial activity against *Bacillus subtilis* (inhibition zone of 7.6 and 8.3 mm), *Staphylococcus aureus* (8.3 and 9.3 mm) and *Escherichia coli* (7.6 and 11.7 mm).

Adnan et al. (2019), investigated the antidiarrheal, anti-helminthic and antibacterial potential of the ethanolic extract of *O. rugosa* leaves (EEOR). The antidiarrheal efficacy of EEOR was examined *in vivo* using the gastrointestinal transit, enteropooling, and castor oil-induced diarrhea models. The different EEOR concentrations (100, 200, and 400 mg/kg) caused a reduction in the volume and weight of the intestinal contents as well as a noticeable inhibition of intestinal motility.

The antihelminthic potential of the EEOR was found to be dose dependent from lower to higher concentration (5, 8, 10 mg/ml). The paralysis times were 23.28, 15.30 and 10.67 min respectively. The death times were 57.63, 32.83 and 24.59 min respectively. The paralysis time and the death time for the positive control levamisole (1 mg/ml) were 3.22 and 6.19 min (Adnan et al., 2019).

The antibacterial efficacy of EEOR against Gram positive bacteria (*Staphylococcus aureus, Bacillus subtilis, Bacillus cereus*) and Gram negative bacteria (*Salmonella typhi, Salmonella paratyphi, Escherichia coli, Pseudomonas aeruginosa*) were tested. The extract exhibited inhibitory effects against, *B. subtilis* (14.80 mm), *S. typhi* (12.80 mm) and *E. coli* (16.23 mm) (Adnan et al., 2019).

A summary of the antimicrobial and anticancer potential of the *Ophiorrhiza* species, along with the compounds involved in exhibiting these properties are given in **Fig. 3.**

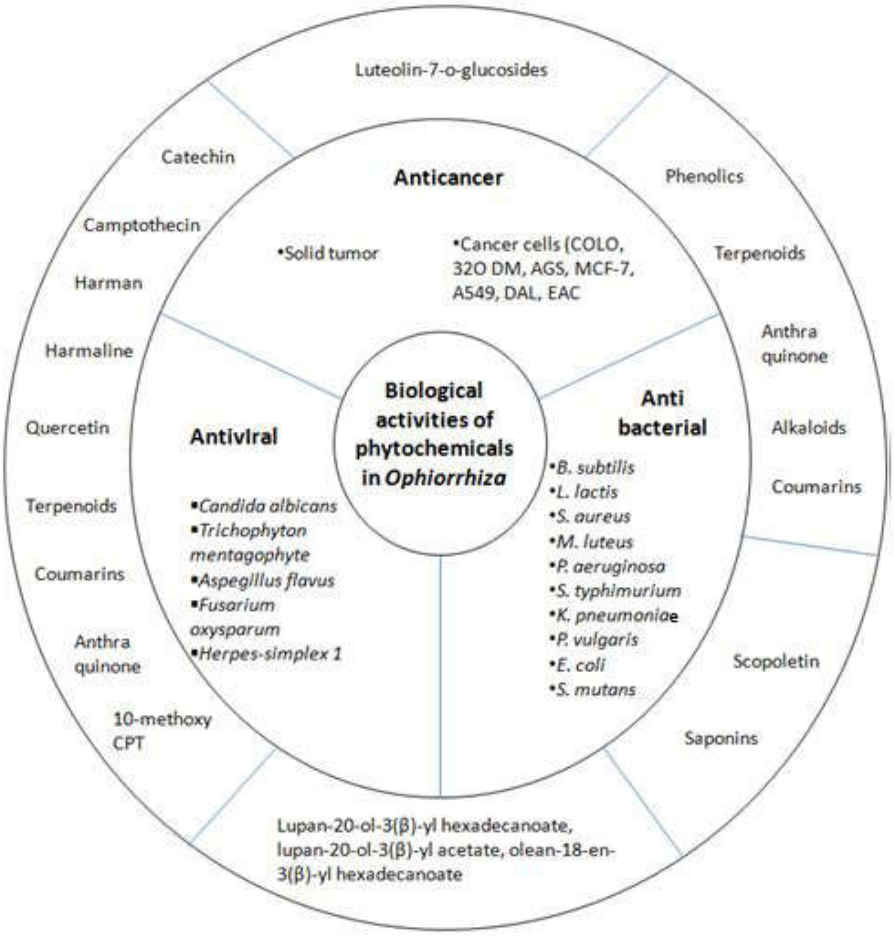


Fig. 3: Bioactivities of phytoconstituents in *Ophiorrhiza* species (Taher et al., 2020)

Thus, a brief survey of literature, that covers all the aspects evaluated for the current study was done. According to the review, there are only limited phytochemical and bioactivity studies on the selected plant species of *Ophiorrhiza*. Though the chromosome number of other species in the genus has been documented in previous cytogenetic research, a full karyomorphometric analysis of the selected plants has never been done. Thus, the current work is significant in all elements of the investigation.

MATERIALS AND METHODS

The major aims of the present work include chromosomal analyses, phytochemical profiling and screening of bioactivities of selected species of *Ophiorrhiza.* The details of the materials selected for the study and the methodology adopted are mentioned in this chapter.

The methodology portion consists of three different parts:

- PART I – Karyomorphological analyses of selected species of *Ophiorrhiza*
- PART II – Phytochemical profiling by GC-MS and HR LC-MS
- PART III – Bioactivity studies of selected species of *Ophiorrhiza,* which includes evaluation of *in vitro* free radical scavenging potential, *in vitro* anti-inflammatory studies using RAW 264.7 macrophage cell line, cytotoxicity studies on *Allium cepa* and evaluation of antiproliferative potential on MDA-MB 231 (human breast cancer) cell line

I. PLANT MATERIALS

Four species of the genus *Ophiorrhiza,* namely, *Ophiorrhiza pectinata, Ophiorrhiza grandiflora*, *Ophiorrhiza jacobii* and *Ophiorrhiza trichocarpon* were selected for the present study. All the plant materials were collected before flowering from different parts of Kerala. *Ophiorrhiza pectinata* and *O. jacobii* were collected from Idukki district of Kerala, while, *O. grandiflora* and *O. trichocarpon* were collected from Thrissur. The taxonomic authentication of the collected plant materials was done by Dr. M. Sabu, Professor (Retd), Angiosperm Taxonomy Division, Department of Botany, University of Calicut, Kerala, India. After identification, the collected plant

materials were planted and maintained in the Botanical Garden of University of Calicut, Kerala and the voucher specimens were deposited in the herbarium of Department of Botany, University of Calicut (CALI). The details of the plant species selected for the study are mentioned below.

❖ ***Ophiorrhiza grandiflora* Wight**

- **Family:** Rubiaceae
- **Habitat:** Evergreen forest
- **Distribution:** India (Kerala & Tamil Nadu) - Endemic to Southern Western Ghats
- **Flowering & Fruiting:** May to December
- **Voucher Specimen No.:** CALI - 143977

Erect herb, 20–60 cm tall; stem woody at base, glabrous. Stipules 3.5–9 mm long, linear-lanceolate, entire or rarely bifid, glabrous, caducous. Petioles 1.5–5 cm long, slender, glabrous; leaf blades 6–17 × 2–6.5 cm, elliptic or elliptic-lanceolate, glabrous on both surfaces, base acute, apex acuminate; secondary veins 7–11 on each side. Inflorescence terminal corymbose cyme; peduncle 1–5 cm long, slender, glabrous; bract and bracteoles 10–24 mm long, linear-lanceolate to filiform, glabrous, sometimes pubescence on the margin, persistent. Pedicels 1.0–3 mm long, glabrous or rarely minutely puberulent. Flowers 22–40 mm long, glabrous, sometimes pubescence on the margin, homostylous. Hypanthium 1.5–2.25 × 1.5–2 mm, obovoid, glabrous. Calyx lobes 2–2.5 mm long, lanceolate, pale green, glabrous. Corolla 17–37 mm long, infundibuliform, white, sometimes pubescence on the margin externally, glabrous internally; lobes 3.5–6 × 3–4.5 mm, ovate, acute. Stamens inserted at the middle of corolla tube and stigma (ovate-lanceolate) positioned below. Capsule obcordate, glabrous, pale green to green.

❖ ***Ophiorrhiza jacobii* Hareesh, Salish, G. Joseph & M. Sabu**

- **Family:** Rubiaceae
- **Habitat:** Evergreen forest
- **Distribution:** India (Kerala) - Endemic to Southern Western Ghats
- **Flowering & Fruiting:** February to December
- **Voucher Specimen No.**: CALI - 143973

Decumbent herb, 20–40 cm tall; stem woody at base, densely pubescent. Stipules 3–10 mm long, ovate to broadly-ovate, acuminate at apex, bifid, puberulous, caducous. Petioles 1.5–4.5 cm long, slender, pubescent; leaf blades 6–12 × 3.0–5.5 cm, ovate to ovate-elliptic, densely pubescent adaxially, glabrous except for pubescent secondary veins abaxially, base acute to attenuate, apex acute to caudate-acuminate; secondary veins 10–14 in each side. Inflorescence terminal corymbose cyme; peduncle 5–9 cm long, slender, densely pubescent; bract and bracteoles 6–9 mm long, linear-lanceolate to filiform, hirsute, persistent. Pedicels 1.0–1.5 mm long, pubescent. Flowers 8–10 mm long, hirsute, heterostylous. Hypanthium 1.0–1.3 × 1.35–1.50 mm, obovoid, villous. Calyx lobes 1.25–1.50 mm long, linear-lanceolate, pink or pale green, villous. Corolla 7.5–8.0 mm long, broadly infundibuliform with constricted throat, pink, villous outside, densely pubescent inside with a ring of villous hairs in the middle; lobes 1.50–1.75 × 1.00–1.25 mm, ovate lanceolate, acute, shortly keeled on back, recurved. Short-styled flowers: stamens inserted at the middle of corolla tube above the villous ring and stigma (ovate-lanceolate) positioned below the villous ring. Long-styled flower: stamens inserted below the villous ring of the corolla tube and stigma (round to obovate) positioned above the villous ring. Capsule obcordate, pubescent, pink to green.

❖ ***Ophiorrhiza pectinata* Arn.**

- **Family:** Rubiaceae
- **Habitat:** Evergreen forest
- **Distribution:** India (Kerala & Tamil Nadu) and Sri Lanka
- **Flowering & Fruiting:** May to December
- **Voucher Specimen No.**: CALI - 143972

Erect herb, 15–45 cm tall; stem woody at base, glabrous or rarely puberulent. Stipules 3–11 mm long, subulate, apex acute, entire or rarely bifid, glabrous, persistent. Petioles 1.5–3.5 cm long, slender, glabrous; leaf blades 3.5–16 × 2.5–5.5 cm, elliptic or ovate lanceolate, glabrous on both sides, base acute to attenuate, apex acute to caudate-acuminate; secondary veins 5–10 on each side. Inflorescence terminal or axillary capitate cyme; peduncle 3.5–9 cm long, slender, glabrous; bract and bracteoles imbricate, 4–10.5 mm long, oblong lanceolate, glabrous, green, persistent. Pedicels 0.75–1.25 mm long, glabrous. Flowers 6–10 mm long, glabrous, heterostylous. Hypanthium 1.0–1.5 × 0.75–1.50 mm, obovoid, glabrous. Calyx lobes 0.75–1mm long, linear-subulate, pale pink or pale green, glabrous. Corolla 5–9 mm long, infundibuliform with constricted throat and base, pink, glabrous externally, inside with a ring of villous hairs in the middle; lobes 1.75–2 × 1.00–1.25 mm, ovate or ovate-lanceolate, acute, keeled on back, recurved. Short-styled flowers: stamens inserted at the middle of corolla tube above the villous ring and stigma (ovate-lanceolate) positioned below the villous ring. Long-styled flowers: stamens inserted below the villous ring of the corolla tube and stigma (round to obovate) positioned above the villous ring. Capsule obcordate, glabrous, rarely puberulent, pale green.



Plate 1: ***Ophiorrhiza grandiflora*****. a:** Habit; **b:** Inflorescence; **c:** Infructescence.



Plate 2: ***Ophiorrhiza jacobii*****. a:** Habit; **b:** Inflorescence; **c:** Infructescence.



Plate 3: ***Ophiorrhiza pectinata*****. a:** Habit; **b:** Inflorescence; **c:** Infructescence with flowers.



Plate 4: ***Ophiorrhiza trichocarpon*****. a & b:** Habit; **c:** Flowers **(**front view).

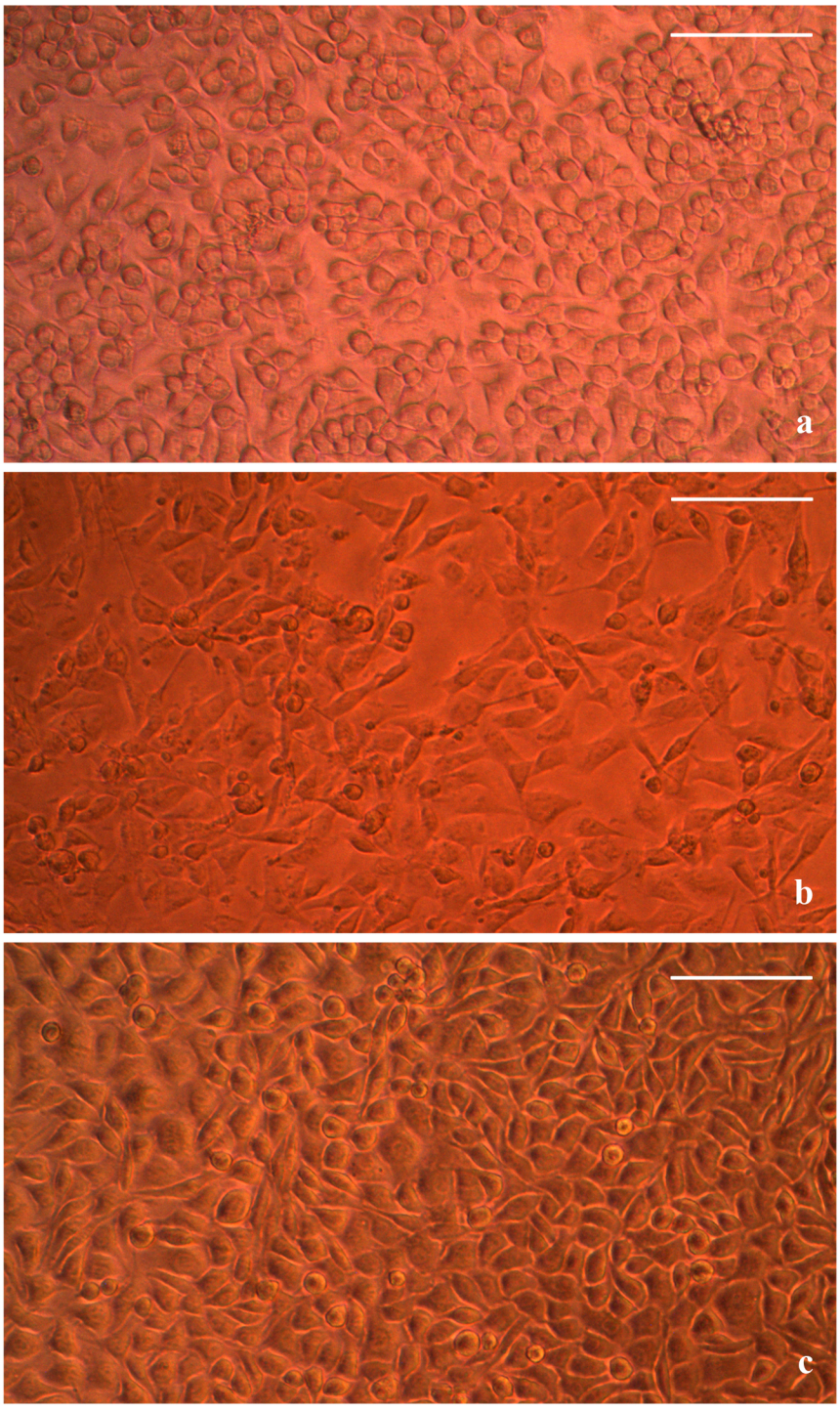


Plate 5: Cell lines used in the study. a: RAW 264.7; **b:** MDA-MB-231; **c:** L929. Scale bars = 100 µm.

❖ ***Ophiorrhiza trichocarpon* Blume**

- **Family:** Rubiaceae
- **Habitat:** Evergreen forest
- **Distribution:** Bangladesh, India (Andaman and Nicobar Islands, Karnataka, Kerala, Orissa and West Bengal), Indonesia, Myanmar, Thailand, Malaysia and Java
- **Flowering & Fruiting:** May to October
- **Voucher Specimen No.**: CALI - 143976

Decumbent/erect herbs, 5–25 cm long, stem woody at the base, branched, pubescent. Stipules 2.5–4.5 mm long, linear with a broadened base, margin, acuminate at apex, entire, pubescent, persistent. Petioles 0.5–5 cm long, slender, pubescent; leaf blades 2–10 × 1–4.5 cm, ovate to ovate-lanceolate, apex obtuse to acute or acuminate, base cuneate, adaxially glabrous, abaxially glabrous except pubescent secondary veins; secondary veins 7–12 in each side. Inflorescence axillary or terminal, corymbose cyme; peduncle up to 1–4 cm long, slender, densely pubescent; bract and bracteoles similar, 0.75–1 mm long, linear, hirsute, persistent. Pedicels *c.* 1 mm long, hirsute. Flowers 4–5.5 mm long, homostylous. Hypanthium 1.75–2 × 1.25–1.25 mm, obovoid. Calyx lobes 0.5–0.75 mm long, triangular, pale green, hirsute. Corolla 4–6.25 mm long, infundibuliform, white, hirsute outside, densely papillose inside with a ring of villous hairs in the middle; lobes 1.5–2 × 1 mm, ovate-lanceolate, slightly curved inwards, shortly keeled on back. Stamens inserted at the middle of corolla tube above the villous ring and stigma (ovate-lanceolate) positioned below the villous ring. Capsule 1.5–2 × 0.5–0.75 mm, obcordate, pale green, hirsute.

II. METHODOLOGY

PART I - KARYOMORPHOLOGICAL STUDIES

Cytogenetical studies were conducted on the selected plants to analyse the chromosomal structure and architecture thereby to explore the taxonomical identity and evolutionary trends. Chromosome numbers of the species along with other parameters like chromosomal length, disparity index, total forma percentage, *etc*., were investigated.

A. Mitotic squash preparation

Chromosomal studies on the selected species of *Ophiorrhiza* were conducted following the methodology of Sharma and Sharma (1990), with slight modifications. Mitotic squash experiments were carried out on very young and healthy meristematic root tips collected at the time of peak mitotic activity. The collected roots were properly washed in distilled water and then pretreated in chilled saturated aqueous paradichlorobenzene (PDB) solution for 3 h at 4ºC. After the pretreatment, the roots were thoroughly washed in distilled water and fixed in modified Carnoy's fluid **(Appendix 1)** for 3 h at room temperature. The fixed roots were again washed in distilled water and then subjected to hydrolysis in 1N hydrochloric acid (HCl) for 10–15 min. To remove the acid content, the hydrolysed roots were thoroughly washed in distilled water and then treated with aceto-orcein stain **(Appendix 2)** for 3–4 h. After destaining with 45% acetic acid, the roots were mounted in the same solution for micro slide preparation. The prepared slides were then screened for well spread metaphase stages under the 100X oil immersion objective of the binocular microscope (Olympus CX21, Japan). Clear metaphase chromosomes were scored and their images were taken using camera (AmScope, USA) attached to the microscope.

B. Karyomorphological analysis

Clearly visible and uniformly focused photomicrographs were used for karyogram and idiogram preparation using computer-aided softwares like Adobe Photoshop, AutoCAD and data based analysing system such as Microsoft Excel. Inorder to obtain the correct numerical data, 3 well-spread mitotic metaphase images were considered. For identification, each chromosome was given a specific number according to the descending order of the chromosome lengths and position of the centromere was noted. The images with falsely numbered chromosomes were then loaded to AutoCAD for karyomorphometrical analysis. Short arm length and long arm length of each chromosome were measured and centromeric indices were calculated. Based on the chromosome length, arm ratio and centromeric indices, homologous chromosomes were identified and correctly numbered. The identified chromosomes were then classified according to Abraham and Prasad (1983) **(Table 3).**

Table 3: Chromosome nomenclature in relation to the centromeric position based on arm ratios and centromeric indices

Nomenclature	Notation	R_1 = s/l	R_2 = l/s	I_1 = 100 s/c	I_2 = 100 l/c
Median	M	1.00	1.00	50.00	50.00
Nearly median	nm	0.99 - 0.61	1.01 - 1.63	49.99 - 38.01	50.01 - 61.99
Nearly submedian	nsm(-)	0.60 - 0.34	1.64 - 2.99	38.00 - 25.01	62.00 - 74.99
Submedian	SM	0.33	3.00	25.00	75.00
Nearly submedian	nsm(+)	0.32 - 0.23	3.01 - 4.26	24.99 - 18.20	75.01 - 81.80
Nearly subterminal	nst(-)	0.22 - 0.15	4.27 - 6.99	18.19 - 12.51	81.81 - 87.49
Subterminal	ST	0.14	7.00	12.50	87.50
Nearly subterminal	nst(+)	0.13 - 0.07	7.01 - 14.38	12.49 - 5.01	87.51 - 94.99
Nearly terminal	nt	0.06 - 0.01	14.39 - 19.99	5.00 - 0.01	95.00 - 99.99
Terminal	T	0.00	α	0.00	100.00

R_1 = arm ratio 1**, R_2** = arm ratio 2**, I_1** = centromeric index 1**, I_2** = centromeric index 2**, s** = short arm length**, l** = long arm length**, c** = total length of chromosome

Karyotype formula was calculated from the data obtained from karyomorphometric analysis, such as length of the chromosome, the position of centromere and the presence or absence of secondary constriction. The chromosome images were exported to Photoshop program and karyogram was prepared by arranging the chromosomes in decreasing order of their size. The idiogram was prepared by arranging chromosomes in descending order using Microsoft Excel. Different karyomorphometric and asymmetry indices are calculated as follows:

Disparity index (DI) of the chromosomes were calculated by following the formula as proposed by Mohanty et al. (1991).

$$\text{DI} = \frac{\text{Longest chromosome} - \text{Shortest chromosome}}{\text{Longest chromosome} + \text{Shortest chromosome}} \times 100$$

The variation coefficient (VC) among chromosome complements was determined using the formula proposed by Verma (1980).

$$\text{VC} = \frac{\text{Standard deviation of total length of chromosomes}}{\text{Mean length of chromosomes}} \times 100$$

The total forma percentage (TF%) or mean centromeric index value was determined following Huziwara (1962).

$$\text{TF\%} = \frac{\text{Total sum of short arm length}}{\text{Total sum of chromosome length}} \times 100$$

The karyotype asymmetry index (As K%) was calculated using the formula developed by Arano (1963).

$$\text{As K\%} = = \frac{\text{Total sum of long arm length}}{\text{Total sum of chromosome length}} \times 100$$

To evaluate karyotype asymmetry, Greilhuber and Speta (1976), developed two indices, namely, the index of karyotype symmetry and the

index of chromosomal resemblance. In 2002, Venora et al. renamed them as Syi index and Rec index respectively (Paszko, 2006). The formulae are given below:

$$\text{Syi} = \frac{\text{Mean length of short arms}}{\text{Mean length of long arms}} \times 100$$

$$\text{Rec} = \frac{\sum_{n}^{i} = 1 \frac{\text{CLi}}{\text{LC}}}{\text{n}} 1 \times 100$$

where; CLi is the length of each chromosome and LC is the longest chromosome and n is the number of chromosomes analysed.

The intrachromosomal asymmetry index (A1) and interchromosomal asymmetry index (A2) was calculated using the formula described by Zarco (1986).

$$\text{A1} = 1 - \left(\frac{\sum_{i}^{n} = 1 \frac{\text{bi}}{\text{Bi}}}{\text{n}}\right)$$

Here, bi is the average length of short arms in every homologous chromosome pair or group, Bi is the average length of long arms in every homologous chromosome pair or group and n is the number of homologous chromosome pairs or groups.

$$\text{A2} = \frac{\text{Mean of chromosome length}}{\text{Standard deviation of chromosome length}}$$

The degree of asymmetry of karyotypes (A) was calculated using the formula developed by Watanabe et al. (1999).

$$A = \frac{\sum_{i}^{n} = 1 \frac{pi - qi}{pi + qi}}{n}$$

where, p and q are the lengths of the long arm and short arm of the chromosome respectively and n is the haploid chromosome number of the species.

PART II – PHYTOCHEMICAL PROFILING

The collected plants (whole plants) were thoroughly washed and were shade dried. After drying, they were chopped and powdered in a blender and stored in moist free sealed containers for further use. For the preliminary phytochemical screening, 3 different extracts of each plant were prepared using the solvents n-hexane, methanol and distilled water. For this, 10 g of the powdered material of each plant was thoroughly mixed with 100 ml of each solvent separately and were allowed to stand overnight. These extracts were then filtered through Whatman filter paper No.1 and the filtrates thus obtained were used for qualitative phytochemical analyses.

A. Qualitative phytochemical analyses

The three different extracts of each plant were subjected to preliminary phytochemical analyses to detect the presence of bioactive secondary phytocompounds using standard protocols (Sofowara, 1993; Trease & Evans, 2002; Kumar et al., 2007; Siddiqui & Ali, 1997; Edeoga et al., 2005; Evans & Evans, 2002).

i. Test for phenolic compounds

- **Ferric chloride test**

2 ml of the extract was treated with a few drops of 5% ferric chloride solution. Formation of deep blue or black colour precipitate indicates the presence of phenols.

- **Spot test**

1 ml of the extract was spotted on a filter paper and a drop of phoshomolybdic acid reagent was added to the spot and was exposed to ammonia vapours. Blue colouration of the spot indicates the presence of phenols.

ii. Test for flavonoids

- **Alkaline reagent test**

About 2 ml of the extract was treated with a few drops of 20% sodium hydroxide solution. Formation of deep yellow colour which disappears on the addition of dilute hydrochloric acid indicates the presence of flavonoids.

- **Lead acetate test**

About 2 ml of the extract was treated with a few drops of 10% lead acetate solution. The formation of a yellow coloured precipitate indicates flavonoid content.

iii. Test for alkaloids

- **Wagner's test**

Few drops of Wagner's reagent (**Appendix 3**) were added to 2 ml of the extract. Formation of a reddish-brown precipitate confirms the presence of alkaloids.

- **Hager's test**

2 ml of the extract was treated with a few drops of Hager's reagent (saturated picric acid solution). Appearance of orange-yellow coloured precipitate indicates the presence of alkaloids.

iv. Test for phytosterols

- **Liebermann-Burchard test**

About 2 ml of the extract was treated with 2 ml of chloroform and was filtered. 2 ml of the filtrate was treated with 2 ml of acetic anhydride and 2 ml of concentrated sulphuric acid. Change of colour from violet to blue indicates the presence of phytosterols.

- **Sulphuric acid test**

2 ml of the extract was mixed with 2 ml of chloroform and a few drops of concentrated sulphuric acid were added to the solution by the sides of the test tube. Red upper layer with the sulphuric acid layer having a yellow-green fluorescence is an indication of the presence of sterols.

v. Test for terpenoids

- **Salkowski test**

To 2 ml of the test solution, 2 ml of chloroform was added and mixed well. Few drops of concentrated sulphuric acid were added to form a layer. A reddish-brown colouration at the interface of the solutions shows the presence of terpenoids.

vi. Test for tannins

- **Breymer's test**

2 ml of the test solution was treated with a few drops of 10% alcoholic ferric chloride solution. Dark green precipitate indicates the presence of tannins.

- **Lead acetate test**

To 5 ml of the test solution, a few drops of 1% lead acetate solution were added. Yellow precipitate indicates the presence of tannins.

vii. Test for saponins

- **Foam test**

About 5 ml of the extract was diluted with 5 ml of distilled water in a test tube. The suspension was shaken vigorously. A layer of foam which persists for sometime indicates the presence of saponins.

viii. Test for phlobatannins

- **Precipitation test**

2 ml of the extract was boiled with 1 ml of 1% hydrochloric acid. The occurrence of phlobatannins is indicated by a red coloured precipitate.

ix. Test for anthraquinones

- **Borntrager's test**

2 ml of the extract was treated with 1 ml of 10% ferric chloride solution and 1 ml of concentrated hydrochloric acid and then heated. The test solution was then cooled and filtered. The filtrate was then added with equal volume of diethyl ether. This solution was then extorted with strong

ammonia. Development of pink or deep red colour in the solution indicates the presence of anthraquinones.

x. Test for coumarins

- **Ferric chloride test**

1 ml of the sample solution was treated with 1 ml of ferric chloride solution. Appearance of deep yellow colour is an indication of the presence of coumarins.

xi. Test for glycosides

- **Legal's test**

2 ml of the sample solution is mixed with pyridine. To this solution, a few drops of sodium nitroprusside and 10% sodium hydroxide were added. Deep red colour indicates glycoside contents.

xii. Test for fixed oils and fats

- **Spot test**

About 1 ml of the sample solution was poured onto a filter paper and pressed against another filter paper and checked for oily appearance on the filter papers which indicates the presence of oils and fats.

B. Quantification of major phytoconstituents

- **Extract preparation**

Among the three different plant extracts prepared from each plant species, methanolic extract showed higher amount of precipitation as well as colour intensity in most of the preliminary phytochemical tests, indicating the greater quantity of secondary metabolites and also exhibited the presence of the maximum number of bioactive phytoconstituents. Thus, methanol was

selected as the solvent to be used for the preparation of the plant extracts for all the further studies.

For the preparation of the plant extract, 20 g was extracted with 200 ml of methanol using Soxhlet extractor for 6–8 h. The extract thus obtained was allowed to cool, then filtered through Whatman filter paper No.1 and the filtrate was concentrated by removing the complete content of solvent. The final extract was then stored in dark at 4ºC for further experiments.

- **Quantification assays**

The major bioactive phytoconstituents revealed from the qualitative phytochemical screening of the selected species, *i.e.,* phenols, flavonoids, terpenoids and alkaloids were estimated using standard protocols as described below. In all the assays, different concentrations of the standard (20, 40, 60, 80 and 100 µg/ml) were considered for obtaining the standard calibration curve.

i. Total phenolic content

The total phenolic content of the extract was determined by the Folin-Ciocalteu assay (Oueslati et al., 2012). The standard used was gallic acid. 0.1 ml of the extract taken from a stock concentration of 1 mg/ml and different concentrations of the standard were mixed with 0.5 ml of distilled water and 1 N 0.125 ml of Folin-Ciocalteu reagent. The solution is then shaken well. After incubation for 5 min, 1.25 ml of 7% of sodium carbonate (w/v) was added and the final volume of the solution was made up to 3 ml with distilled water. The reaction mixture was shaken well and incubated in dark for 90 min and the absorbance was measured against a blank at 760 nm using a spectrophotometer (Elico SL 218, India). The total phenolic content of

the methanolic extract was calculated from the calibration curve of gallic acid and using regression equation, the results were expressed in milligram of gallic acid equivalent per gram dry weight (mg GAE/g DW). The samples were analysed in triplicates.

ii. Total flavonoid content

The total flavonoid content of the extract was determined using aluminium chloride ($AlCl_3$) colorimetric method (Oueslati et al., 2012), with slight modifications. Quercetin was used as the standard. 0.5 ml of the extract (1 mg/ml) and different concentrations of the standard were mixed with 0.5 ml of distilled water and 0.3 ml of 5% sodium nitrate (w/v) and incubated for 5 min at 25ºC. Incubation was followed by the immediate addition of 0.3 ml of 10% $AlCl_3$ (w/v). The reaction mixture was shaken well and 2 ml of 1 M sodium hydroxide was added. Then absorbance was measured against blank at 510 nm. The total flavonoid content of the methanolic extract was calculated from the calibration curve of quercetin using regression equation and the results were expressed in milligram of quercetin equivalent per gram dry weight (mg QE/g DW). The samples were analysed in triplicates.

iii. Total terpenoid content

The total terpenoid content of the extract was determined using the methodology of Ghorai et al. (2012), with slight modifications. Linalool was used as the standard. 0.2 ml of the extract (1 mg/ml) and different concentrations of the standard were mixed with 1.5 ml of chloroform and 1 ml of concentrated sulphuric acid (H_2SO_4). A yellow-green coloured solution appeared and the chloroform layer was seen as a separate layer. After separating the chloroform layer, 2 ml of methanol was added to the remaining mixture. The yellow-green solution was found to be dissolved and the

absorbance of this solution was measured against a blank at 538 nm. The total terpenoid content of the methanolic extract was calculated from the calibration curve of linalool using regression equation and the results were expressed in milligram of linalool equivalent per gram dry weight (mg LE/g DW). The samples were analysed in triplicates.

iv. Total alkaloid content

The total alkaloid content of the extract was determined using the protocol suggested by Shamsa et al. (2008), with slight modifications. Caffeine was used as the standard. To 1 ml of the extract (1 mg/ml) and different concentrations of the standard, 5 ml phosphate buffer (pH 4.7) **(Appendix 4)** and 5 ml bromocresol green (BCG) solution **(Appendix 5)** were added. To this reaction mixture, 1, 2, 3 and 4 ml of chloroform were added sequentially and shaken well. The BCG layer was removed and the chloroform layer containing the alkaloid was considered. The absorbance of the complex in chloroform solution was measured against a blank at 470 nm. Caffeine was used as the standard. The total alkaloid content of the methanolic extract was calculated from the calibration curve of caffeine using regression equation and the results were expressed in milligram of caffeine equivalent per gram dry weight (mg CE/g DW). The samples were analysed in triplicates.

C. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The volatile components in the plant extract were determined by GC-MS analysis performed on Shimadzu GCMS-QP-2010 plus system. Thermal Desorption system TD 20, fitted with 60 m × 0.25 mm × 0.25 m WCOT column coated with diethylene glycol was used for the analysis. Helium was the carrier gas used with a flow rate of 1.21 ml/min, column pressure was

69 - 81.9 kPa. Injector and detector temperatures were maintained at 260ºC, the volume of sample injected was 8 µl and the split ratio of the column was 10:0. The conditions set for component separation were: linear temperature program of 50–280ºC with a total run time of 39.98–50.24 min. The MS parameters used were: electron ionization (EI) voltage of 70 eV, peak width of 2 s, mass range of 40–650 *m/z* and detector voltage of 1.5 V. The resulting compounds were identified by comparing their linear retention indices. The MS fragmentation patterns of the compounds were compared with other compounds of known composition, with patterns in the National Institute of Standards and Technology (NIST) spectrum database, and with data from the literature. The compounds' quantitative estimation was based on their distinct GC peak regions.

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

The non-volatile chemical compounds of the extract were analysed using HR LC-MS Q-TOF (Agilent G6550A, USA) equipped with an electro spray ionization source (DUAL AJS ESI) maintained at 35ºC. The column used was CAPCELL, C18, MG II type with 4.6 mm × 250 mm dimensions and particle size of 5 µm. Elution was performed using a mobile phase consisting of 95% A (water) and 5% B (acetonitrile) at a flow rate of 0.5 ml/min. Injection volume of 5 µl and total run time of 30 min was employed. The eluted compounds were detected using MS Q-TOF equipped with an electro spray ion source using nebulizer gas (nitrogen) 13 l/min at a gas temperature of 250ºC and nozzle voltage of 1000 V.

PART III – BIOACTIVITY SCREENING

A. Free radical scavenging activity of selected species of *Ophiorrhiza*

Free radicals are highly unstable molecules produced by metabolic reactions in our body. They cause oxidative stress, which leads to serious conditions such as premature ageing, cardiovascular disease, neurological disease, asthma, arthritis, diabetes, and stroke. In the present study, four different *in vitro* assays have been performed to evaluate the scavenging potential of the methanolic plant extracts on free radicals produced by various substrates like DPPH, Fe^{3+} - ascorbate - EDTA - H_2O_2 system, sodium nitroprusside and finally potassium ferricyanide.

i. 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging activity

The ability of the plant extract to scavenge DPPH free radicals was determined using the method of Chang et al. (2001). 0.1 mM DPPH solution was prepared by dissolving 4 mg of DPPH in 100 ml of methanol. Different concentrations of the sample (12.5. 25, 50, 100, 200 µg/ml) from stock solution of concentration 10 mg/ml DMSO were made up to a final volume of 20 µl with dimethyl sulphoxide (DMSO) and 1.48 ml DPPH (0.1 mM) solution was added. The reaction solution was then incubated at room temperature in the dark for 20 min. After incubation, the absorbance of the combination was measured at 517 nm against a blank. A control was taken without the test substance but with an equal quantity of distilled water. Ascorbic acid was used as the standard. The ability to scavenge the DPPH radical was expressed as percentage inhibition and calculated using the equation

$$\text{Percentage inhibition} = \frac{Ac-As}{Ac} \times 100$$

here, Ac shows the absorbance of the control; As shows the absorbance of the sample. All samples were evaluated in triplicate, and mean results were computed.

ii. Hydroxyl free radical scavenging activity

The potential of the plant extract to scavenge hydroxyl radicals generated by the Fe^{3+} - ascorbate- EDTA - H_2O_2 system (Fenton reaction) was determined using the method of Kunchandy & Rao (1990). Different concentrations of the sample (125, 250, 500, 1000 and 2000 µg/ml) were taken from a stock concentration (10 mg/ml DMSO) and were mixed with 500 µl of reaction mixture to a final volume of 1 ml. The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbate (0.1 mM) and $KH_2PO_4 - KOH$ buffer (20 mM, pH 7.4). After incubation for 1 h at 37ºC, 1 ml of 2.8% trichloro acetic acid (TCA) was added. Then, 1 ml of 1% aqueous thiobarbituric acid (TBA) was added and the solution was incubated at 90ºC for 15 min. After cooling, the absorbance of the solution was measured at 532 nm against a suitable blank. A control without the test compound, but an equivalent amount of distilled water was taken. Gallic acid was used as the standard. The potential to scavenge hydroxyl radicals was expressed as a percentage inhibition and determined in triplicates using the equation and the mean values were calculated.

$$\text{Percentage inhibition} = \frac{Ac-As}{Ac} \times 100$$

where, Ac is the absorbance of the control; As is the absorbance of the sample. All the samples were tested in triplicates and the mean values were calculated.

iii. Nitric oxide free radical scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide that interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent **(Appendix 6).** The ability of the plant extract to scavenge nitric oxide radicals was determined following the methodology of Kumaran & Karunakaran (2006), with sight modifications.

Sodium nitroprusside (5 mmol/l) in phosphate buffer saline (PBS) **(Appendix 7)** of pH 7.4 was mixed with different concentrations of the sample (125, 250, 500, 1000, 2000 µg/ml) taken from a stock concentration of 10 mg/ml DMSO. The solution was incubated at 25°C for 30 min. After incubation, 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 diaminedihydrochloride). Absorbance of the chromophore formed during diazotisation of the nitrate with sulphanilamide and subsequent coupling with N-1-naphthyl ethylene diamine dihydrochloride was measured against the blank at 546 nm. A control without the test compound, but an equivalent amount of distilled water was taken. Gallic acid was used as the standard. The potential to scavenge nitric oxide radicals was given as the percentage of inhibition and was computed using the equation.

$$\text{Percentage inhibition} = \frac{Ac - As}{Ac} \times 100$$

where, Ac is the absorbance of the control; As is the absorbance of the sample. All the samples were tested in triplicates and the mean values were calculated.

iv. Superoxide free radical scavenging activity

The ability of the plant extracts to scavenge super oxide anions was analysed following the methodology of Valentao et al. (2002).

The sample was obtained at various concentrations (125, 250, 500, 1000, and 2000 g/ml) from a stock solution of 10 mg/ml DMSO and combined with 0.05 ml of riboflavin solution (0.12 mM), 0.2 ml of EDTA solution (0.1 M), and 0.1 ml NBT solution (1.5 mM). The reaction mixture was diluted with phosphate buffer to a volume of 2.64 ml (0.067 M). After 5 min of fluorescent light illumination, the absorbance at 560 nm was measured. The solution was irradiated again for 30 min, and absorbance at 560 nm was measured with a UV visible spectrophotometer. The OD difference was computed. Ascorbic acid was utilised as the standard. The ability to scavenge super oxide radical was expressed as percentage inhibition and calculated using the equation

$$\text{Percentage inhibition} = \frac{Ac-As}{Ac} \times 100$$

where, Ac is the absorbance of the control; As is the absorbance of the sample. All the samples were tested in triplicates and the mean values were calculated.

B. Anti-inflammatory activity

The anti-inflammatory activity of the selected species of *Ophiorrhiza* was primarily screened using different biochemical assays. The most effective plant extract was then scrutinised for its activity on lipopolysaccharide (LPS) induced RAW 264.7 macrophage cell line. Inorder to estimate the major inflammatory mediator involved in the activity, indirect ELISA (Enzyme-linked Immunosorbent Assay) was also carried out.

i. Evaluation of anti-inflammatory activity using biochemical assays

The methanolic plant extracts of the four selected species of *Ophiorrhiza* were first analysed for their anti-inflammatory potential with *in vitro* biochemical assays. After calculating the percentage of inhibition, the most effective plant extract was identified.

a) Inhibition of protein denaturation

The potential of the plant extracts to inhibit protein denaturation was analysed using the methodology of Mizushima and Kobayashi (1968). Different concentrations of the sample (62.5, 125, 250 and 500 µg/ml) from a stock solution of 10 mg/ml DMSO were used for the study. The test solution consisted of different concentrations of the sample which was made up to 0.5 ml with 1% aqueous bovine serum albumin solution. Diclofenac sodium was used as the standard solution. The pH of all the above solutions was adjusted to 6.3 using 1 N HCl. All the samples were then incubated at 37^0C for 20 min and later the temperature was increased to 57^0C for 3 min. After cooling, 2.5 ml of phosphate buffer was added to the solutions. The absorbance was measured spectrophotometrically at 416 nm. The percentage inhibition was calculated using the formula,

$$\% \text{ Inhibition} = 100 - \frac{(\text{OD of test solution} - \text{OD of product control})}{\text{OD of test control}} \times 100$$

b) Proteinase inhibitory activity

Proteinase inhibitory activity of the methanolic extracts of the plants was analysed following the methodology of Oyedepo and Femurewa (1995), with slight modifications. Different concentrations of sample (62.5, 125, 250 and 500 µg/ml) from a stock solution of 10 mg/ml DMSO were used. Diclofenac sodium was used as the standard solution. The samples were then

made up to 1 ml using phosphate buffer (pH 7.4). 1 ml of Tris-HCl buffer (20 mM, pH 7.4) **(Appendix 8)**, which contain trypsin (0.06 mg), was added to all the samples and were incubated for 5 min at 37ºC. Then 1 ml of 0.8% (w/v) casein was added. The reaction mixture was then incubated for another 20 min. Further, 2 ml of perchloric acid (70%) was added to all the test tubes to stop the reaction. Cloudy suspension formed was centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 200 nm against buffer as blank. The percentage inhibition was calculated using the formula,

$$\% \text{ Inhibition} = 100 - \frac{(\text{OD of test solution} - \text{OD of product control})}{\text{OD of test control}} \times 100$$

ii. Anti-inflammatory activity on RAW 264.7 macrophage cell line

The effective extract was further evaluated for its anti-inflammatory activity on RAW 264.7 macrophage cell line. The effect of the extract on cyclooxygenase (COX) activity, lipoxygenase (LOX) activity, myeloperoxidase (MPO) activity, cellular nitrite level (NO) and inducible nitric oxide synthase (iNOS) activity was also analysed.

- **Cell culture**

RAW 264.7 cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco's modified Eagles medium (DMEM) **(Appendix 9)**. The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% fetal bovine serum (FBS), L-glutamine, sodium bicarbonate and antibiotic solution containing penicillin (100 µg/ml /ml), streptomycin (100 µg/ml), and amphoteracin B (2.5 µg/ml). Cultured cell lines were maintained at 37ºC in a humidified 5% CO2 incubator (NBS Eppendorf, Germany).

- **Extract preparation and treatment**

A stock of the extract is prepared by dissolving 1 mg of the plant extract in 1 ml DMEM using a cyclomixer. The solution after filtration was used for further experiments. The cultured cells were grown to 60% confluency followed by activation with 1 µl LPS (1 µg/ml). LPS stimulated RAW cells were treated with different concentrations (25, 50, 100 µg/ml) of the extract and incubated for 24 h. The anti-inflammatory experiments were carried out utilising the cell lysate after incubation. Diclofenac sodium, a standard anti-inflammatory drug in different concentrations, corresponding to that of the sample solution, was used as the positive control.

a) Cyclooxygenase (COX) activity

The effect of the plant extract on COX activity was assayed following the method of Walker and Gierse (2010). 100 µl of the cell lysate was incubated with Tris-HCl buffer (pH 8), glutathione (5 mM/l) and haemoglobin (5 mM/l) for 1 min at 25°C. The reaction began with the addition of arachidonic acid (200 mM/l). After 20 min incubation at 37°C, the reaction was terminated by the addition of 200 µl of 10% trichloroacetic acid in 1 N hydrochloric acid. After the centrifugal separation, 200 µl of thiobarbiturate (1%) was added and the tubes were boiled for 20 min. After cooling, the tubes were centrifuged again for 3 min. COX activity was determined by reading the absorbance at 632 nm. The percentage inhibition of COX was calculated using the formula,

$$\text{Percentage inhibition} = \frac{Ac - As}{Ac} \times 100$$

here, Ac shows the absorbance of the control, As shows the absorbance of the sample. Triplicates of the sample were considered and the mean values were determined.

b) Lipoxygenase (LOX) activity

The effect of the plant extract on LOX activity was evaluated using the methodology of Axelrod et al. (1981). The reaction mixture (2 ml) consisted of Tris-HCl buffer (pH 7.4), 50 µl of cell lysate and 200 µl of sodium linoleate. The LOX activity was measured as an increase in the absorbance at 234 nm which reflects the formation of 5-hydroxyeicosatetraenoic acid. The percentage inhibition of the enzyme was calculated using the formula,

$$\text{Percentage inhibition} = \frac{Ac-As}{Ac} \times 100$$

where, Ac is the absorbance of the control; As is the absorbance of the sample. The sample was tested in triplicates and the mean values were calculated.

c) Myeloperoxidase (MPO) activity

The effect of the plant extract on MPO activity was calculated as per the method of Bradley et al. (1982). Cell lysate was homogenised in a solution containing 50 mM potassium phosphate buffer **(Appendix 10)** and 0.57% hexadecyltrimethyl ammonium bromide (HTAB). The reaction mixture was frozen in liquid nitrogen and then thawed. The samples were further centrifuged at 2000 rpm for 30 min at 4°C. The supernatant was then tested for MPO activity. MPO in the sample was activated by the addition of phosphate buffer (50 mM) (pH 6) containing guaiacol (1.67 mg/ml) and hydrogen peroxide (0.0005%). The change in absorbance was measured at 460 nm. MPO activity was shown as units per ml of cell lysate. One unit of MPO activity can be defined as that quantity which degrades 1 µM of peroxide per minute at 25°C.

$$U = \frac{(\Delta OD \times 4 \times Vt \times \text{dilution factor})}{(L \times £470 \times \Delta t \times Vs)} \times 100$$

here,

ΔOD = density change

Vt = total volume (1.1 ml)

L = light path (1 cm)

£470 = extinction coefficient for tetraguaiacol (26.6 mM-1·cm-1,)

Vs = sample volume

d) Inducible nitric oxide synthase (iNOS) activity

The effect of the plant extract on iNOS activity was determined by the method described by Salter et al. (1996). Cell lysate was homogenised in 2 ml of HEPES buffer **(Appendix 11).** The reaction mixture contained 0.1 ml of L-Arginine (2 µmol/L), 0.1 ml of manganese chloride (4 µmol/l), 0.1 ml of 30 µg dithiothreitol (DTT) (10 mmol/l), 0.1 ml of NADPH (1 mmol/l), 0.1 ml of tetrahydropterin (4 µmol/l), 0.1 ml of oxygenated haemoglobin (10 µmol/l) and 0.1 ml of the cell lysate. Increase in absorbance was recorded at 401 nm. The percentage inhibition of the enzyme was calculated using the formula,

$$\text{Percentage inhibition} = \frac{Ac-As}{Ac} \times 100$$

where, Ac is the absorbance of the control, As is the absorbance of the sample. The sample was tested in triplicates and the mean values were calculated.

e) Cellular nitrite level

The level of cellular nitrite was analysed by the methodology of Lepoivre et al. (1990). To 0.5 ml of the cell lysate, 0.1 ml of sulphosalicylic acid (3%) was added and vortexed well for 30 min. Then, centrifuged for 15 min at 5000 rpm. Further, the protein-free supernatant was used for the

determination of nitrite levels. To 200 µl of the supernatant, 30 µl of NaOH (10%) and 300 µl of Tris-HCl buffer were added and mixed well. To this solution, 530 µl of Griess reagent was added and incubated in dark for 10–15 min. After incubation, the absorbance was read at 540 nm against Griess reagent as the blank. From the standard curves obtained, the amount of nitrite present in the sample was estimated. The sample was tested in triplicates.

iii. Cytotoxic evaluation using MTT assay on RAW 264.7 cells

To determine the suitable concentration of the plant extract for further analysis and also to nullify the anticipated cytotoxicity of the plant extract and its effect on the anti-inflammatory activity of the plant, MTT assay was carried out on RAW 264.7 cells. RAW 264.7 cell line maintained in DMEM medium with supplements was used for the study.

- **Cells seeding in 96 well plate**

Two days old confluent monolayer of cells were trypsinised and suspended in 10% growth medium. 100 µl of cell suspension was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO2 incubator.

- **Stock preparation**

A stock of the extract was prepared by dissolving 1 mg of the plant extract in 1 ml DMEM using a cyclomixer. The solution was then filtered through 0.22 µm millipore syringe filter to ensure the sterility and used for further analysis.

Evaluation of cytotoxicity

After 24 h, the growth medium was removed and freshly prepared stock in DMEM was five times serially diluted by two-fold dilution (100 µg,

50 µg, 25 µg, 12.5 µg, 6.25 µg in 500 µl of DMEM). 100 µl of each concentration was added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO2 incubator. Cells that are not treated were maintained as the control.

Cytotoxicity of the sample was analysed by evaluating the viability of the cells on the basis of the following:

- Inverted Phase Contrast Microscopic observation of cells
- MTT assay method.

Cytotoxicity assay by direct microscopic observation

After 24 h of treatment, the entire plate of cells was observed in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and the observations were recorded as images. Morphological changes within the cells like shrinking or rounding, as well as cytoplasmic granulation and vacuolisation are marked as signs of cytotoxicity.

Cytotoxicity assay by MTT method

MTT (15 mg) was reconstituted in 3 ml PBS until completely dissolved and filter sterilised. After 24 h treatment, the sample contents in wells were removed and reconstituted MTT solution (30 µl) was added to all the test and cell control wells. The plate was gently shaken and further incubated for 4 h at 37ºC in a humidified 5% CO2 incubator. After incubation, the supernatant was removed and 100 µl of DMSO (which acts as MTT solubilisation solution) was added. Gentle mixing of the contents was done by pipetting up and down so as to solubilise the formazan crystals. The absorbance values were measured using a microplate reader at 540 nm (Chow et al., 2004). The percentage of cell viability was calculated using the following formula and the lethal concentration 50% (LC50) was determined.

$$\text{Cell viability \%} = \frac{\text{Mean OD of the sample}}{\text{Mean OD of the control}} \times 100$$

iv. Estimation of inflammatory mediators using ELISA

Estimation of the inflammatory mediator COX-2 in LPS stimulated RAW 264.7 cells was done using ELISA. After attaining the sufficient confluency, LPS stimulated cells were treated with LC_{50} concentration of the sample and incubated for 24 h at 37ºC in a humidified 5% CO_2 incubator. LPS stimulated cells without any treatment were also maintained as control. After incubation, 100 µl of the supernatant was added to the 96 well-plate and incubated at 37^0C overnight. Afterwards, the wells were drained and washed twice with PBS. 200 µl of freshly prepared blocking buffer **(Appendix 12)** was added and incubated for 1 h at room temperature. After incubation, the blocking buffer was removed and the wells were washed twice with PBS Tween **(Appendix 13)**. Then, 100 µl of primary antibody (COX-2) was added and kept for 2 h at room temperature. After incubation, the primary antibody was removed by washing the wells twice with PBS Tween. 100 µl of secondary antibody (anti-human HRP conjugated secondary antibody) was added and kept for 1 h at room temperature. It was then washed with PBS Tween (two times). 200 µl of O-dianizdine hydrochloride was then added and incubated in dark for 30 min at room temperature. Following this, the reaction was stopped by adding 50 µl of HCl (5 N). OD was read at 415 nm in an ELISA reader and the activity of the antibody was calculated using the following equation.

Activity of Antibody = OD Value / Protein Concentration

C. Screening for cytotoxicity with *Allium cepa* L.

All the four selected species of *Ophiorrhiza* were evaluated for their antiproliferative activity with the help of various assays. The preliminary

screening of the cytotoxic potential of the plant extracts was done on *Allium cepa*.

Cytotoxicity studies using *Allium cepa* bioassay

Allium cepa root tip assay was done for analysing the cytotoxic potential of the plant extract. For this, fresh and healthy, uniformly sized onion bulbs were selected and the outer dry scales were removed, without destroying the root primordia and grown on sterilised, moist sand. On germination, onion bulbs with roots of length 1-2 cm were collected at the time of peak mitotic activity (9 to 10 am), standardised by repeated trial and error, and washed in distilled water. After thorough washing, the bulbs were suspended in different concentrations of the plant extract (12.5, 25, 50 and 100 µg/ml) for treatment in four different time periods (1, 12, 24 and 48 h). Care was taken that only roots of the bulbs get immersed in extracts. Distilled water and hydrogen peroxide (0.1%) were used as the negative control (NC) and positive control (PC), respectively. After treatment, roots were cut from the bulbs and washed in distilled water, followed by fixation in modified Carnoy's fluid for 1 h. Mitotic squash preparation was done following the methodology of Sharma and Sharma (1990) with slight modifications. The fixed root tips were washed with distilled water and hydrolysed in 1 N HCl for 5 min. The roots were then washed and stained with acetocarmine **(Appendix 14)** for 3 h. After destaining using 45% acetic acid, the roots were squashed and mounted in 45% acetic acid on clean glass slides. The mounted slides were observed under 40X objective of light microscope (Leica DM 2000 LED, Germany) and photomicrographs were taken. Six different fields were randomly selected to calculate the number of mitotic cells, aberrated cells and total number of cells. Mitotic index (%) and abnormality percentage (%) were calculated using the following formulae, and the results were expressed as mean ± SE.

Mitotic index (%) = Number of dividing cells/ Total number of cells × 100

Abnormality percentage (%) = Number of aberrant cells/ Total number of cells × 100

D. Antiproliferative activity

The antiproliferative activity of the selected species of *Ophiorrhiza* was first screened using *in vitro* cytotoxicity assay by MTT method on both the normal fibroblast cells (L929) and the selected cancer cell line (MDA-MB-231 - Human breast cancer cell line) **(Plate 5-b & c).** The most effective plant extract was then selected and evaluated for its apoptotic activity and finally the gene expression studies were carried out.

i. *In vitro* cytotoxicity determination by MTT assay on L929 cells

- **Culture of the cell line**

L929 cells procured from National Centre for Cell Sciences (NCCS) were maintained in Dulbecco's modified Eagles medium (DMEM). The cell line was cultured in tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing Penicillin (100 µg/ml), Streptomycin (100 µg/ml) and Amphoteracin B (2.5 µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO2 incubator.

- **Cells seeding in 96 well plate**

Two days old confluent monolayer of cells were trypsinised and suspended in 10% growth medium. 100 µl of cell suspension was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO2 incubator.

- **Extract preparation**

A stock of the extract is prepared by dissolving 1 mg of the individual plant extract in 1 ml DMEM using a cyclomixer. The solution was then filtered through 0.22 µm millipore syringe filter to ensure the sterility. From the stock solutions, different concentrations of the extracts (100, 50, 25, 12.5, 6.25 µg in 500 µl of DMEM) were prepared.

Evaluation of cytotoxicity

After 24 h, the growth medium was removed and 100 µl of each concentration of the extracts were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO2 incubator. Cells that are not treated were also maintained as the control.

Cytotoxicity of the extracts were analysed by evaluating the viability of the cells on the basis of the following:

- Inverted Phase Contrast Microscopic observation of cells
- MTT assay method

Cytotoxicity determination by direct microscopic observation

After 24 h of treatment, the entire plate of cells was observed in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and the observations were recorded as images. Morphological changes within the cells like shrinking or rounding, as well as cytoplasmic granulation and vacuolisation can be marked for cytotoxicity.

Cytotoxicity determination by MTT method

MTT (15 mg) was reconstituted in 3 ml PBS until completely dissolved and filter sterilised. After 24 h treatment, the sample contents in

wells were removed and reconstituted MTT solution (30 µl) was added to all the test and cell control wells. The plate was gently shaken and further incubated for 4 h at 37ºC in a humidified 5% CO2 incubator. After incubation, the supernatant was removed and 100 µl of DMSO (which acts as MTT solubilisation solution) was added. Gentle mixing of the contents was done by pipetting up and down so as to solubilise the formazan crystals. The absorbance values were measured using a microplate reader at 540 nm (Chow et al., 2004). The percentage of cell viability was calculated using the following formula and the lethal concentration 50% (LC50) was determined.

$$\text{Cell viability \%} = \frac{\text{Mean OD of the sample}}{\text{Mean OD of the control}} \times 100$$

ii. *In vitro* cytotoxicity determination by MTT assay on MDA-MB-231 cells

MDA-MB-231 cell line procured from NCCS was maintained according to the same procedure followed for the normal fibroblast cells. The cells were seeded in 96 well plate and different concentrations of the extracts were prepared as described previously. The different concentrations of the extracts were tested for their cytotoxicity as mentioned for normal fibroblast cells. On analysing the cytotoxicity, on both the cell lines, the most effective extract and the LC_{50} value was determined.

iii. Detection of apoptosis using fluorescence activated cell sorting (FACS)

MDA-MB-231 cell line was cultured as per standard procedures described earlier. The cells were treated with LC_{50} value of the effective extract, from a stock of 1mg/ml and incubated for 24 h. Untreated wells were also maintained. After the incubation, the cells were trypsinised and 100 µl of cells in suspension was transferred to separate tubes. 100 µl of the Muse™

Annexin V & Dead Cell Reagent were added to each tube. The tubes were mixed thoroughly by pipetting up and down or vortexing at a medium speed for 3 to 5s followed by incubation for 20 min at room temperature in the dark. Cells were gated against untreated control cells and analysed in a flow cytometer for apoptosis using Muse FACS 3.0 software.

iv. Cell cycle analysis using flow cytometry

MDA-MB 231 cells were cultured as per standard procedures described earlier. The cells were then treated with LC_{50} concentration of the most effective sample and incubated for 24 h. The cell sample was collected in a polystyrene tube (12 × 75mm) or a conical flask (50 ml). The sample was then centrifuged at 3000 rpm for 5 min. After centrifugation, the supernatant was removed without disturbing the pellet which would be visible as a white film on the bottom of the tube.

1 ml of PBS was added to the tube and the contents were mixed by pipetting several times or gently vortexing and then centrifuged at 3000 rpm (5 min). The supernatant was discarded without disturbing the cell pellet. The pellets were resuspended in the residual PBS gently by vortexing. The cells were then added drop wise into the tube containing 1ml of ice cold 70% ethanol while vortexing at medium speed and stored at -20ºC overnight.

After the incubation, the sample was centrifuged at 3000 rpm for 5 min at room temperature. After the removal of the supernatant, 250 µl PBS was added to the pellet and again centrifuged at the same rpm and time. After discarding the supernatant, 250 µl of cell cycle reagent was added and incubated at dark for 30 min (which is light sensitive). After this it was analysed using a Flow Cytometer (Merck-Millipore, Germany). Keeping untreated cells as control, gating was performed.

v. Gene expression studies

Regulation of the genes AKT and ERK2 in the human breast cancer cell line MDA-MB-231 by the most effective extract was analysed by the following methodology.

a) Isolation of total RNA (TRIzol method)

The total RNA was isolated using the total RNA isolation kit (Invitrogen, USA). MDA-MB-231 cells were cultured as per standard procedures described earlier. LD_{50} concentration of the sample from a stock of 1mg/ml was added to cells and incubated for 24 h. Untreated cells were also maintained as the control. After incubation, DMEM media was removed aseptically and 1 ml of TRIzol reagent was added to culture well plate and incubated for 5 min. After this, the contents were transferred to a fresh sterile eppendorf tube. 200 µl of chloroform was added and vigorously shaken for 15 s and incubated for 2-3 min at room temperature. After the incubation, centrifugation was done at 14000 rpm for 15 min at 4°C. The aqueous layer was collected and 500 µl of 100% isopropanol was added, followed by incubation for 10 min at room temperature. After this, centrifugation was done at 14000 rpm for 15 min at 4°C. The supernatant was discarded and pellet was washed with 200 µl of 75% ethanol. Centrifugation was done again at 14000 rpm for 5 min at 4°C in a cooling centrifuge (Remi CMI2). The RNA pellet was dried and suspended in TE buffer **(Appendix 15).**

b) Synthesis of cDNA

Template complementary DNA was synthesized using the cDNA preparation kit (G BIOSCIENCES cDNA synthesis kit). 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. Then the total reaction volume was made up to 10 µl by adding sterile distilled water. The solution was then gently

mixed by pipetting up and down. The thermal cycler (Eppendorf Master Cycler) was programmed to undergo cDNA synthesis. The following cycling conditions were employed:

Step	Temperature (°C)	Time (min)	Number of cycles
cDNA synthesis	42	20	1
Inactivation	85	5	1

c) Gene expression analysis by RT-qPCR

Real-Time qRT-PCR analysis was carried out using SYBR Green Master Mix (G BIOSCIENCES) using Light cycler 96 (Roche). All reactions were performed in triplicates and data were analysed according to $\Delta\Delta C_t$ method using Light Cycler 96 SW 1.1 Software. The time and temperature for each step involved in the process are given below:

Steps	Time required	Temperature
Initial activation step	2 min	95°C
3 step cycling		
Denaturation	10 s	95°C
Annealing	1 minu	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 are:

Oligo Name	Sequences
Human GAPDH Forward	5'-ACTCAGAAGACTGTGGATGG -3'
Human GAPDH Reverse	5'- GTCATCATACTTGGCAGGTT-3'
Human ERK2 Forward	5'- GAGCACCAGACCTACTGCCAG-3'
Human ERK2 Reverse	5'- AATTTCTGGAGCCCTGTACCA-3'
Human AKT Forward	5'- CTTAATGTGCCCGTCCTTGT-3'
Human AKT Reverse	5'- TCTATGGCGCTGAGATTGTG-3'

d) Agarose gel electrophoresis

The PCR product was further subjected to Agarose gel electrophoresis. Agarose gel (1.5%) was prepared in 1x TE buffer and melted at 90ºC in a hot water bath. The melted agarose was then cooled down to 45ºC and 6 µl of 10 mg/ml of ethidium bromide was added. The melted agar was then poured into a gel casting apparatus with the gel comb. After setting, the comb was removed from the gel. The electrophoresis buffer was poured in the gel tank and the platform with the gel was placed in it so as to immerse the gel. The samples were loaded into the gel and run at for 30 min at 50 V. The stained gel was visualised using a gel documentation system (E gel imager, Invitrogen).

Statistical analysis

The results obtained in all the phases were analysed statistically by IBM SPSS version 20 (SPSS Inc., Chicago, IL, USA). The data obtained were subjected to one-way ANOVA followed by DMRT (Duncan's multiple range test) to confirm the variability of the data and validity of the results. Each value is represented as the mean ± standard error (SE). Results with $P < 0.05$ were considered statistically significant.

PART I – KARYOMORPHOLOGICAL STUDIES

Cytogenetical studies on the selected species of *Ophiorrhiza* revealed the karyological identity and evolutionary status of the species. The karyomorphological analysis of the species disclosed the somatic chromosome number, ploidy level and many other karyomorphometric parameters which helped to establish their cytogenetical behaviour. Various asymmetry indices were also calculated to understand the symmetry or asymmetry of chromosomes in each species. Details of the karyomorphometric data obtained from each species are given in **Tables 4– 11.** The karyotype, karyogram and idiogram of the somatic chromosomes of the selected species are represented in **Plates 6–13.**

In *O. grandiflora,* the somatic chromosome number was found to be 2n = 22 with 11 pairs of chromosomes **(Plate 6).** The range of chromosomal length was observed as 1.15 µm to 2.03 µm. The total chromosome length was 32.88 µm and the average chromosome length was 1.49 µm. The summary of the karyomorphometrical parameters of the species is given in **Table 4** and a detailed karyomorphometric data is given in **Table 5.** Secondary constrictions were observed in 2 pairs of chromosomes and were nearly submedian in nature. The remaining 9 pairs of chromosomes exhibited nearly median type of primary constriction. The karyogram and idiogram of the chromosome complement were prepared according to the decreasing order of the chromosome size **(Plate 7).**

The somatic chromosome number of *O. jacobii* was observed as 2n = 24 consisting of 12 pairs of chromosomes **(Plate 8).** The range of chromosomal length was 1.15 µm to 2.15 µm. The total chromosome length

and the average chromosome length were 37.62 µm and 1.56 µm respectively. The summary of the karyomorphometric parameters of the species is given in **Table 6** and a detailed karyomorphometric data is given in **Table 7.** Secondary constrictions were observed in 2 pairs of chromosomes; however, all the 12 pairs of chromosomes exhibited nearly median type of primary constriction. The karyogram and idiogram of the chromosome complement were prepared according to the decreasing order of the chromosome size **(Plate 9).**

The somatic chromosome number of *O. pectinata* was found to be 2n = 22 with 11 pairs of chromosomes **(Plate 10).** The range of chromosomal length was observed as 1.10 µm to 2.02 µm. The total chromosome length was 32.96 µm and the average chromosome length was 1.49 µm. The summary of the karyomorphometric parameters of the species is given in **Table 8** and a detailed karyomorphometric data is given in **Table 9.** Similar to *O. jacobii*, only 2 pairs of chromosomes had secondary constrictions and all the chromosome pairs exhibited nearly median type of primary constriction. The karyogram and idiogram of the chromosome complement were prepared according to the decreasing order of the chromosome size **(Plate 11).**

In *O. trichocarpon*, the somatic chromosome number was found to be 2n = 22 with 11 pairs of chromosomes **(Plate 12).** The range of chromosomal length was observed as 1.10 µm to 1.84 µm. The total chromosome length was 30.86 µm and the average chromosome length was 1.40 µm. The summary of the karyomorphometric parameters of the species is given in **Table 10** and a detailed karyomorphometric data is given in **Table 11.** Secondary constrictions were observed in 2 pairs of chromosomes and were

nearly submedian in nature and the other 9 pairs of chromosomes exhibited nearly median type of primary constriction. The karyogram and idiogram of the chromosome complement were prepared according to the decreasing order of the chromosome size **(Plate 13).**

The karyomorphological analysis of all the selected species revealed that these plants possess small sized chromosomes within a range of 1.10 μm (*O. pectinata, O. trichocarpon*) to 2.15 μm (*O. jacobii*). The somatic chromosome number for *O. grandiflora, O. pectinata* and *O. trichocarpon* was 2n = 22, but in *O. jacobii* it was 2n = 24. The diploid nature of chromosomes was observed in all the species and the number of chromosomes with secondary constrictions was also found to be same in all the species. In *O. jacobii* and *O. pectinata*, all the chromosomes exhibited nearly median type of primary constriction whereas in both *O. grandiflora* and *O. trichocarpon,* 2 pairs of chromosomes had nearly submedian nature of primary constriction. Thus, the karyotype of the studied species discloses the predominance of nearly median type of primary constriction. A scatter plot representing the intrachromosomal and interchromosomal asymmetry of chromosomes is given in **Fig. 4. Fig. 5** represents the scatter plot showing the relation between the Rec index and Syi index of chromosomes.

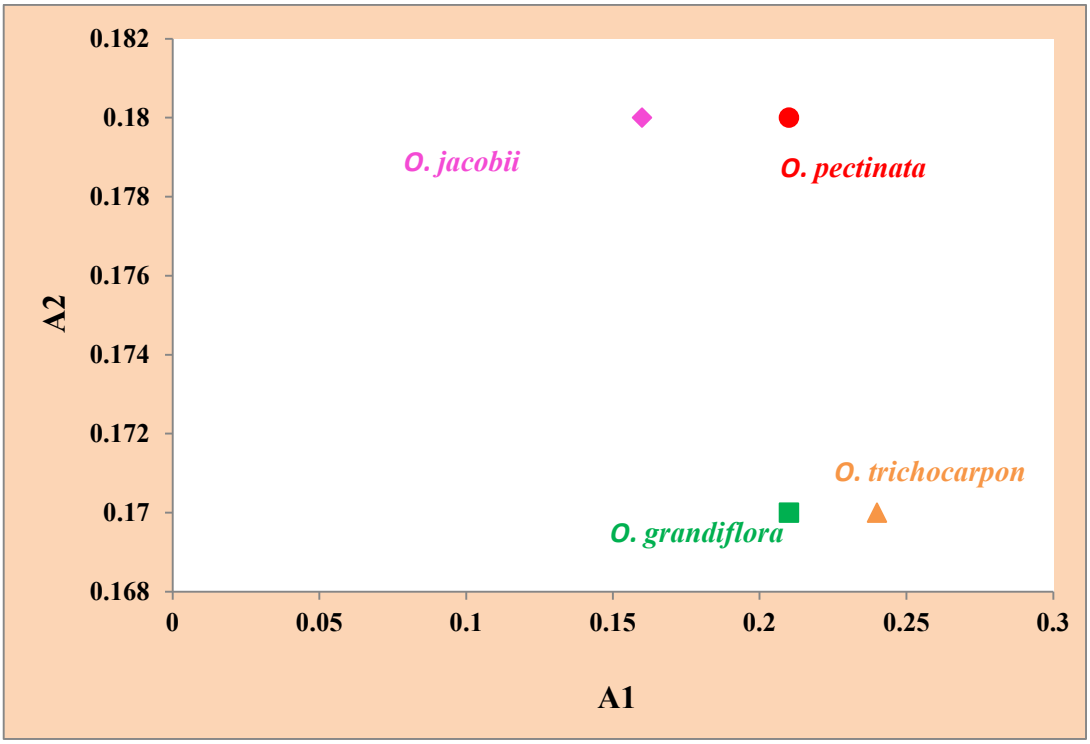


Fig. 4: Scatter plot of intrachromosomal (A1) versus interchromosomal (A2) asymmetry indices of the selected species of *Ophiorrhiza*

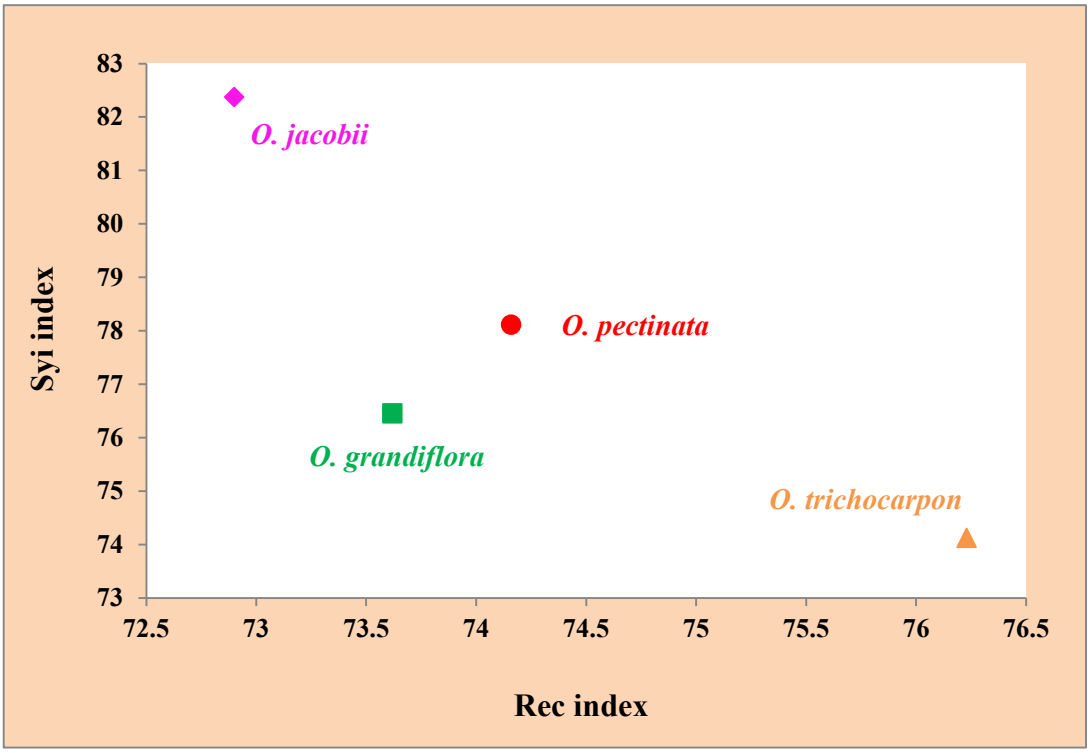


Fig. 5: Scatter plot of Rec index versus Syi index of the selected species of *Ophiorrhiza*

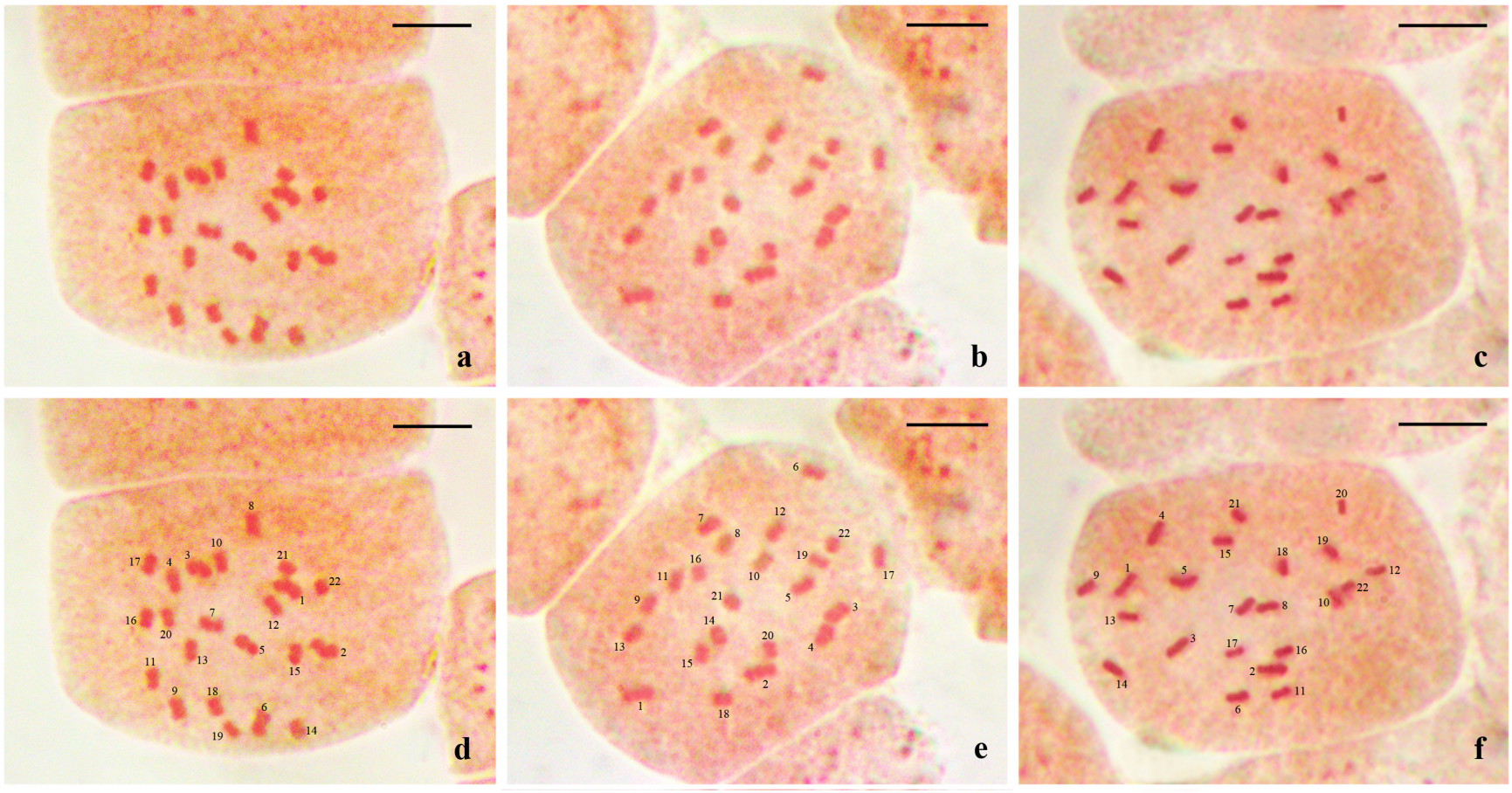


Plate 6: Mitotic chromosome complement images of *Ophiorrhiza grandiflora* (2n = 22). a–c: Cells at mitotic metaphase; **d–f:** Karyotpe with 22 chromosomes enumerated. Scale bars = 5 μm.

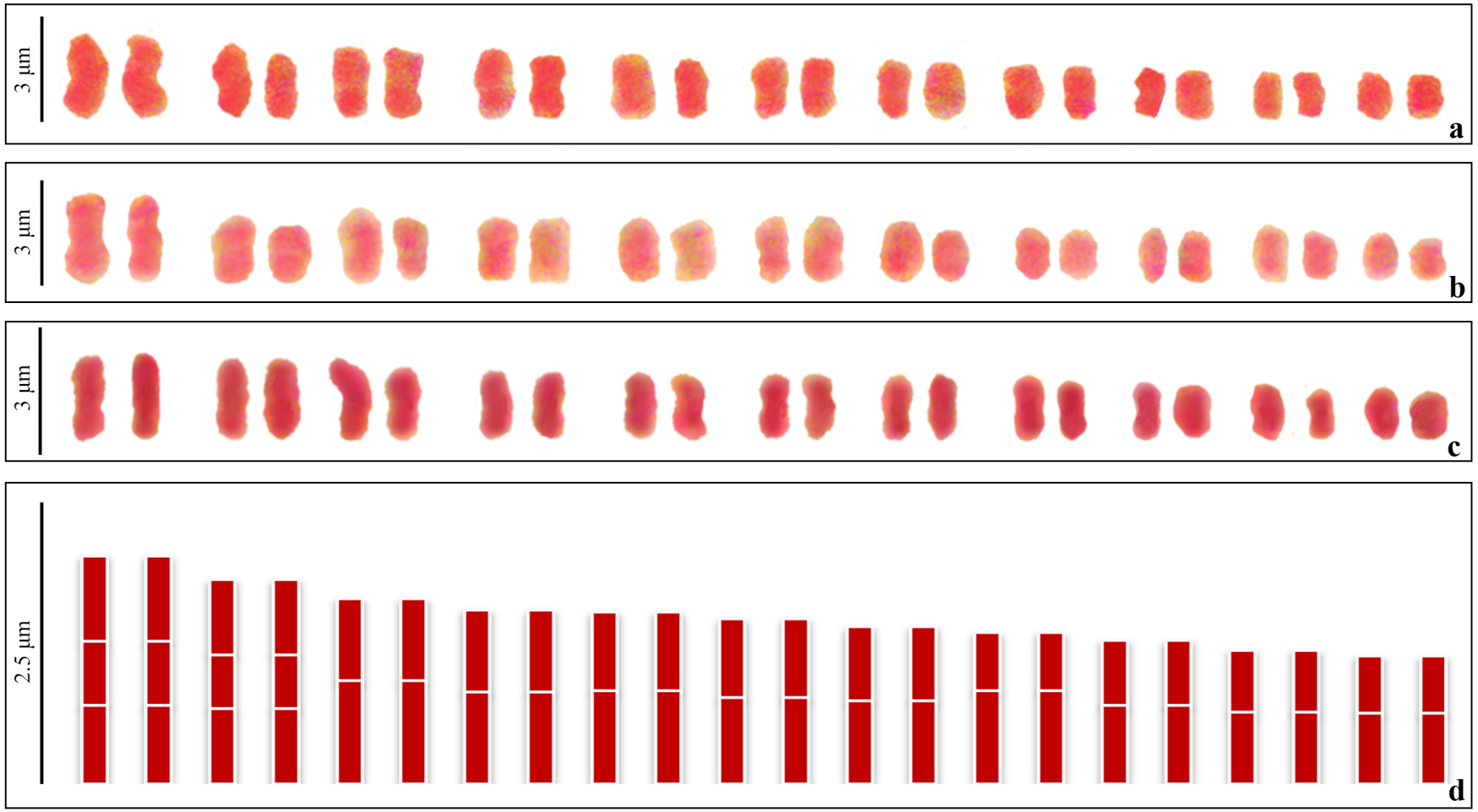


Plate 7: Mitotic chromosome complement images of *Ophiorrhiza grandiflora* (2n = 22). a–c: Karyogram; **d:** Idiogram

Table 4: Summary of mitotic chromosome complement details of *Ophiorrhiza grandiflora* with 2n = 22 chromosomes

Somatic chromosome number	:	22
Ploidy level	:	Diploid; 2n = 2x = 22
Karyotype formula	:	18 nm + 4 nsm(-)
Chromosomes with secondary constrictions	:	4
Range of chromosome length (RCL)	:	1.15 μm to 2.03 μm
Total chromosome length (TCL)	:	32.88 μm
Average chromosome length (ACL)	:	1.49 μm
Disparity index (DI)	:	27.67
Variation coefficient (VC)	:	17.88
TF Value (%)	:	43.24
Karyotype asymmetry index (As K%)	:	56.56
Syi Index	:	76.45
Rec Index	:	73.62
Intrachromosomal asymmetry index (A1)	:	0.21
Interchromosomal asymmetry index (A2)	:	0.17
Degree of asymmetry of karyotypes (A)	:	0.12

Table 5: Detailed karyomorphometric data of *Ophiorrhiza grandiflora* with 2n = 22 chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
1*	2.03 ± 0.08	0.76 ± 0.09	1.27 ± 0.02	0.60 ± 0.07	1.73 ± 0.25	37.20 ± 3.17	62.79 ± 3.17	nsm(-)
2*	1.82 ± 0.06	0.67 ± 0.06	1.15 ± 0.01	0.58 ± 0.06	1.74 ± 0.19	36.76 ± 2.61	63.23 ± 2.61	nsm(-)
3	1.65 ± 0.07	0.73 ± 0.04	0.92 ± 0.07	0.80 ± 0.09	1.27 ± 0.15	44.40 ± 2.97	55.59 ± 2.97	nm
4	1.56 ± 0.05	0.73 ± 0.03	0.82 ± 0.03	0.88 ± 0.03	1.12 ± 0.04	46.99 ± 1.09	53.00± 1.09	nm
5	1.53 ± 0.04	0.70 ± 0.02	0.83 ± 0.04	0.84 ± 0.05	1.19 ± 0.07	45.57 ± 1.62	54.42 ± 1.62	nm
6	1.48 ± 0.04	0.70 ± 0.01	0.77 ± 0.05	0.91 ± 0.02	1.09 ± 0.02	47.78 ± 0.66	52.21 ± 0.66	nm
7	1.40 ± 0.03	0.66 ± 0.01	0.74 ± 0.02	0.88 ± 0.02	1.12 ± 0.02	47.05± 0.58	52.94 ± 0.58	nm
8	1.35 ± 0.02	0.52 ± 0.06	0.83 ± 0.08	0.65 ± 0.14	1.68 ± 0.38	38.79 ± 5.26	61.20 ± 5.26	nm
9	1.28 ± 0.03	0.58 ± 0.03	0.70 ± 0.03	0.84 ± 0.07	1.20 ± 0.09	45.58 ± 2.01	54.41 ± 2.01	nm
10	1.19 ± 0.03	0.55 ± 0.02	0.64 ± 0.01	0.86 ± 0.02	1.16 ± 0.03	46.31 ± 0.83	53.68 ± 0.83	nm
11	1.15 ± 0.05	0.51 ± 0.04	0.63 ± 0.02	0.80 ± 0.07	1.26 ± 0.12	44.48 ± 2.40	55.51 ± 2.40	nm

No. - Number of chromosome pair; **c** - total length of chromosome; **s** - short arm length; **l** - long arm length; **R_1** – arm ratio 1; **R_2** – arm ratio 2; **I_1** – centromeric index 1; **I_2** – centromeric index 2; **PC** – primary constriction; * - chromosome with secondary constriction; **nsm** – nearly submedian; **nm** – nearly median

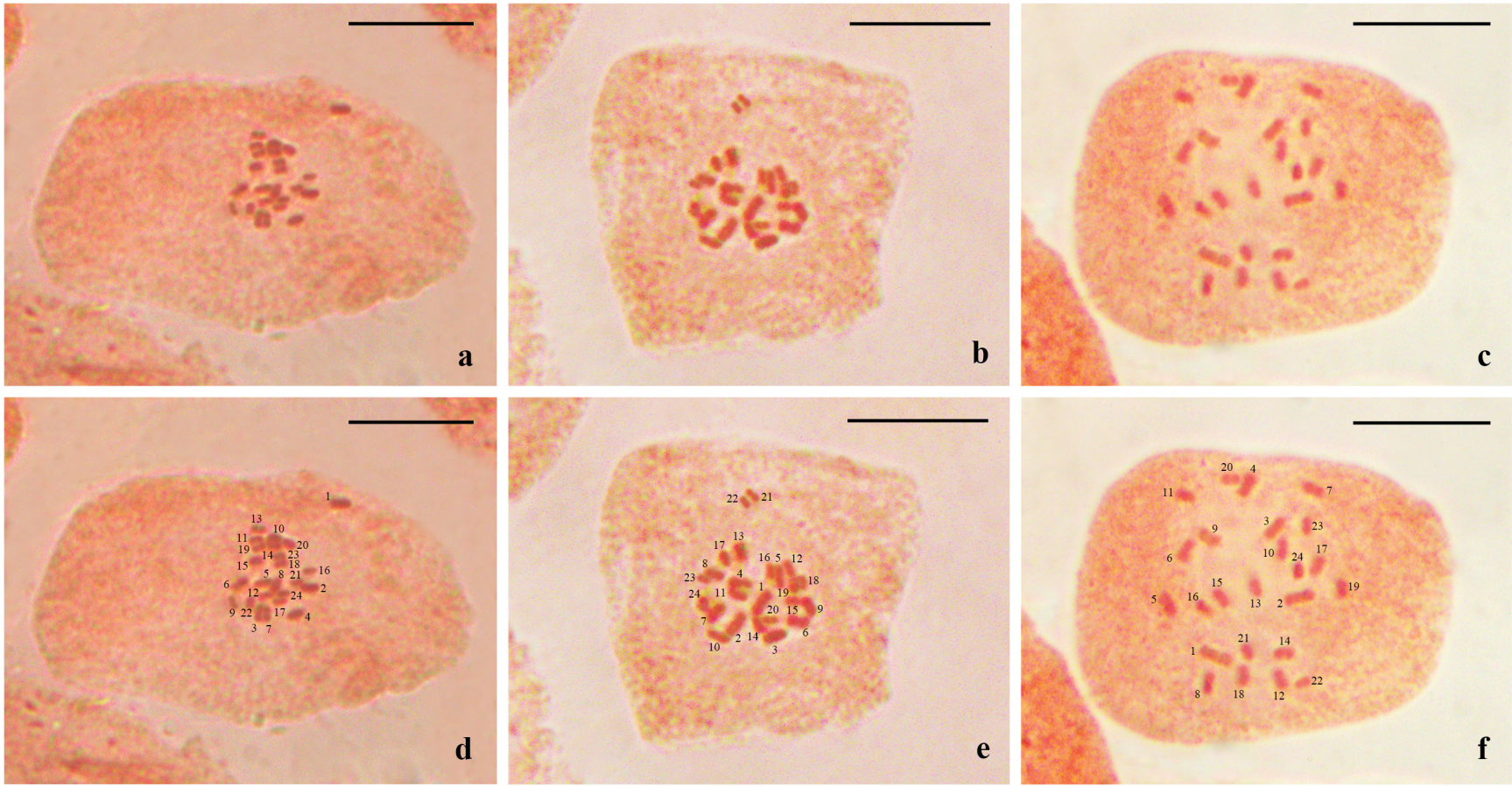


Plate 8: Mitotic chromosome complement images of *Ophiorrhiza jacobii* (2n = 24). a–c: Cells at mitotic metaphase; **d–f:** Karyotpe with 24 chromosomes enumerated. Scale bars = 5 μm.

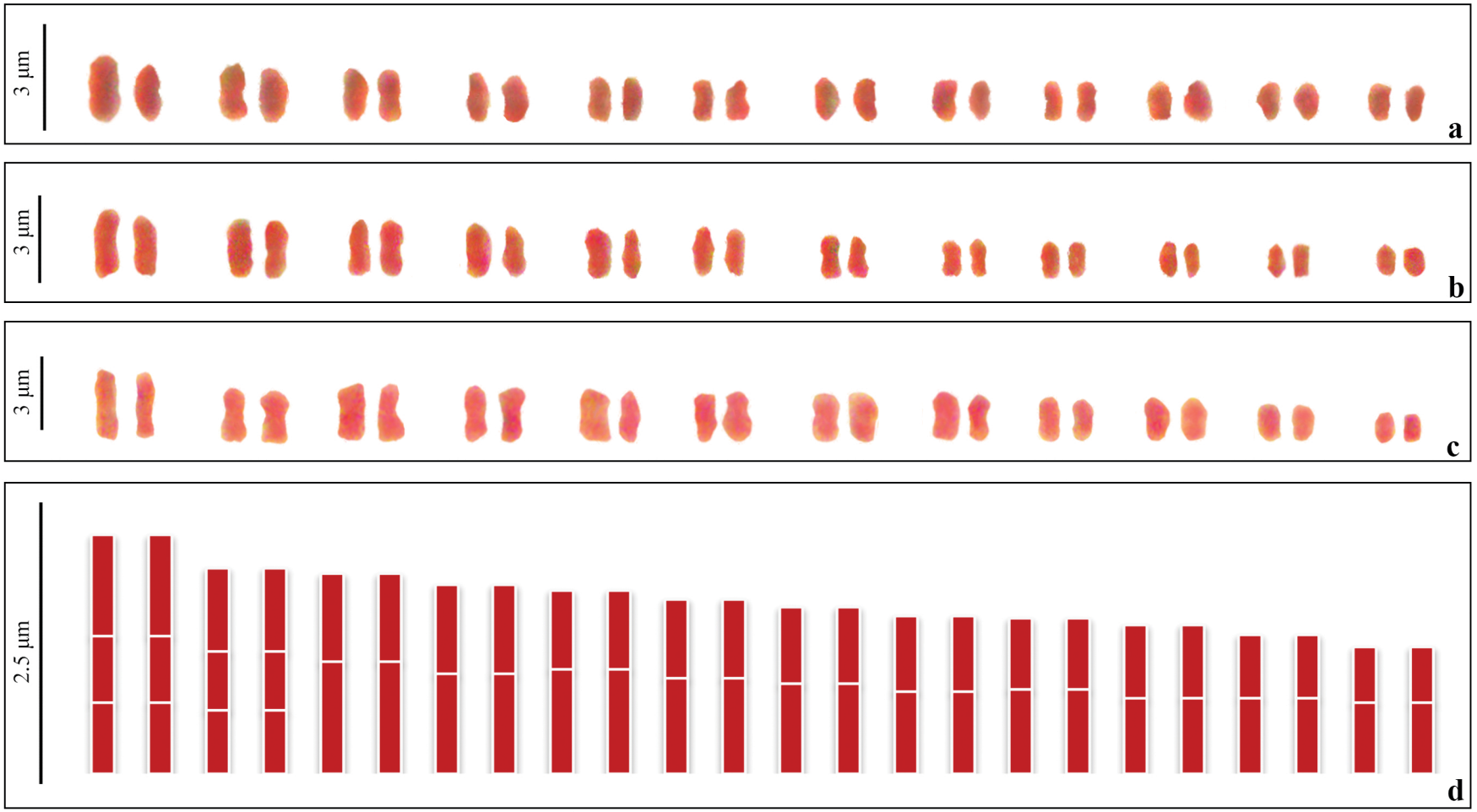


Plate 9: Mitotic chromosome complement images of *Ophiorrhiza jacobii* (2n = 24). a–c: Karyogram; **d:** Idiogram

Table 6: Summary of mitotic chromosome complement details of *Ophiorrhiza jacobii* with 2n = 24 chromosomes

Somatic chromosome number	:	24
Ploidy level	:	Diploid; 2n = 2x = 24
Karyotype formula	:	24 nm
Chromosomes with secondary constrictions	:	4
Range of chromosome length (RCL)	:	1.15 µm to 2.15 µm
Total chromosome length (TCL)	:	37.62 µm
Average chromosome length (ACL)	:	1.56 µm
Disparity index (DI)	:	30.30
Variation coefficient (VC)	:	18.02
TF Value (%)	:	44.71
Karyotype asymmetry index (As K%)	:	54.27
Syi Index	:	82.37
Rec Index	:	72.90
Intrachromosomal asymmetry index (A1)	:	0.16
Interchromosomal asymmetry index (A2)	:	0.18
Degree of asymmetry of karyotypes (A)	:	0.09

Table 7: Detailed karyomorphometric data of *Ophiorrhiza jacobii* with 2n = 24 chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
1*	2.15 ± 0.20	0.91 ± 0.15	1.24 ± 0.09	0.73 ± 0.10	1.42 ± 0.20	41.81 ± 3.46	58.18 ± 3.46	nm
2*	1.85 ± 0.10	0.75 ± 0.45	1.10 ± 0.07	0.68 ± 0.10	1.47 ± 0.05	40.52 ± 0.87	59.47 ± 0.87	nm
3	1.80 ± 0.12	0.79 ± 0.05	1.01 ± 0.07	0.78 ± 0.03	1.27 ± 0.05	43.98 ± 1.06	56.01 ± 1.06	nm
4	1.70 ± 0.16	0.80 ± 0.06	0.90 ± 0.10	0.90 ± 0.03	1.11 ± 0.04	47.38 ± 1.00	52.61 ± 1.00	nm
5	1.65 ± 0.16	0.71 ± 0.10	0.94 ± 0.06	0.74 ± 0.06	1.35 ± 0.11	42.64 ± 2.04	57.35 ± 2.04	nm
6	1.57 ± 0.13	0.71 ± 0.05	0.71 ± 0.08	0.84 ± 0.01	1.19 ± 0.02	45.64 ± 0.54	54.35 ± 0.54	nm
7	1.51 ± 0.14	0.69 ± 0.04	0.81 ± 0.10	0.86 ± 0.04	1.16 ± 0.07	46.20 ± 1.46	53.79 ± 1.46	nm
8	1.43 ± 0.16	0.68 ± 0.07	0.74 ± 0.08	0.92 ± 0.03	1.08 ± 0.04	48.04 ± 1.05	51.95 ± 1.05	nm
9	1.40 ± 0.16	0.64 ± 0.06	0.76 ± 0.09	0.85 ± 0.02	1.17 ± 0.02	45.94 ± 0.62	54.05 ± 0.62	nm
10	1.35 ± 0.13	0.66 ± 0.05	0.68 ± 0.07	0.97 ± 0.02	1.03 ± 0.02	49.24 ± 0.56	50.75 ± 0.56	nm
11	1.25 ± 0.11	0.57 ± 0.06	0.68 ± 0.06	0.84 ± 0.08	1.20 ± 0.12	45.58 ± 2.53	54.41 ± 2.53	nm
12	1.15 ± 0.10	0.50 ± 0.06	0.64 ± 0.04	0.76 ± 0.04	1.31 ± 0.08	43.29 ± 1.61	56.70 ± 1.61	nm

No. - Number of chromosome pair; **c** - total length of chromosome; **s** - short arm length; **l** - long arm length; **R_1** – arm ratio 1; **R_2** – arm ratio 2; **I_1** – centromeric index 1; **I_2** – centromeric index 2; **PC** – primary constriction; * - chromosome with secondary constriction; **nm** – nearly median

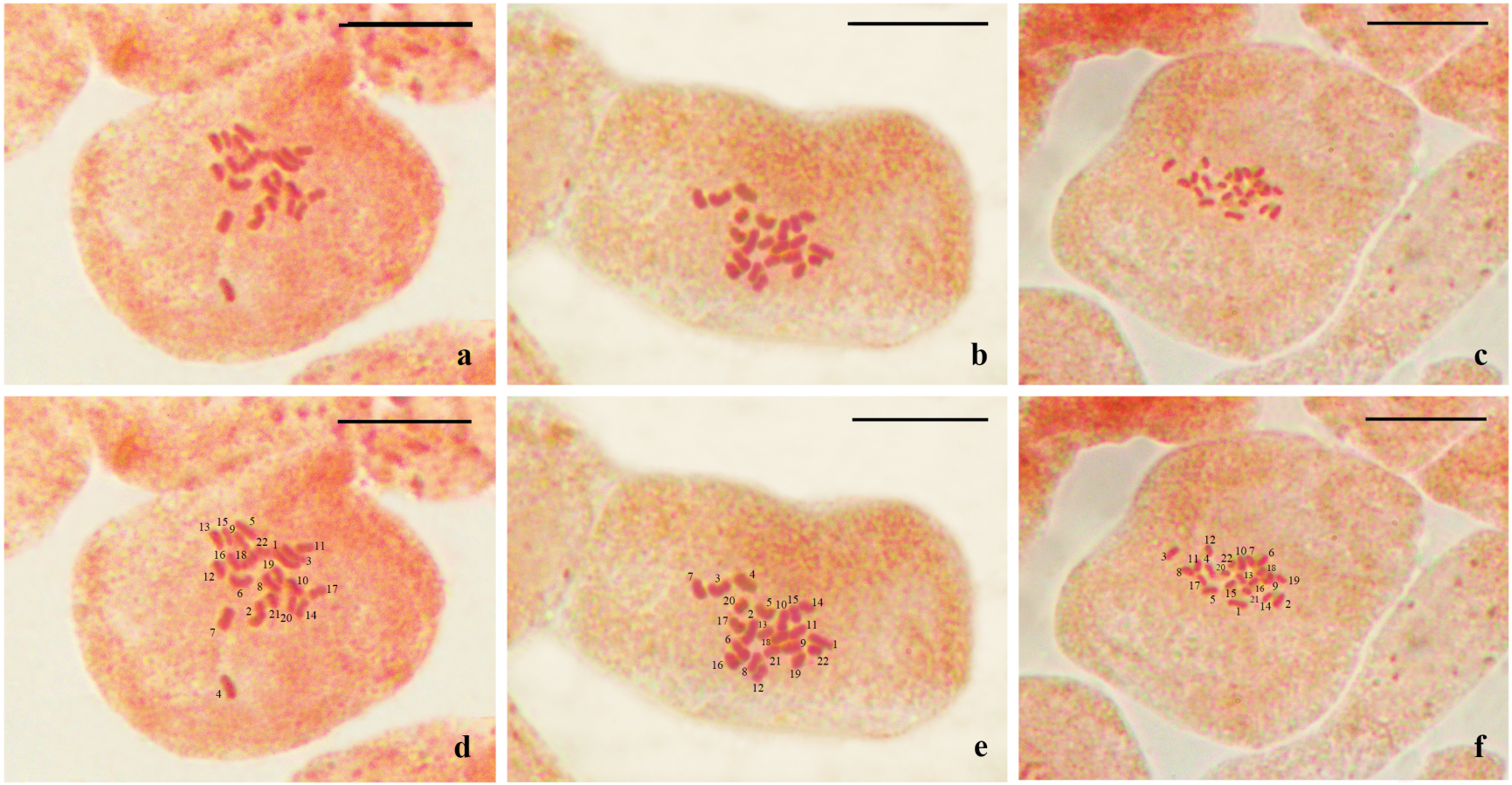


Plate 10: Mitotic chromosome complement images of *Ophiorrhiza pectinata* (2n = 22). a–c: Cells at mitotic metaphase; **d–f:** Karyotpe with 22 chromosomes enumerated. Scale bars = 5 µm.

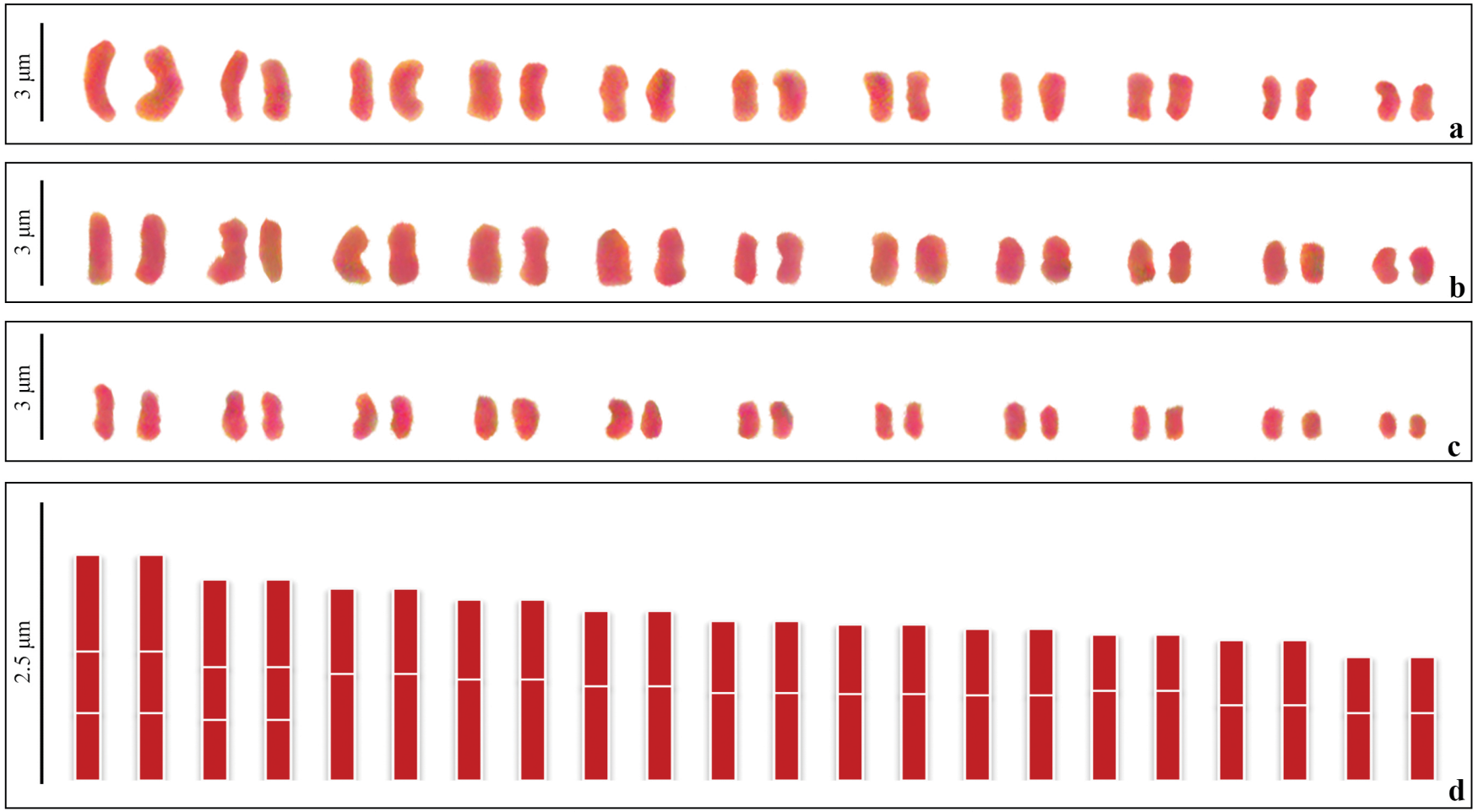


Plate 11: Mitotic chromosome complement images of *Ophiorrhiza pectinata* (2n = 22). a–c: Karyogram; **d:** Idiogram

Table 8: Summary of mitotic chromosome complement details of *Ophiorrhiza pectinata* with 2n = 22 chromosomes

Somatic chromosome number	:	22
Ploidy level	:	Diploid; 2n = 2x = 22
Karyotype formula	:	22 nm
Chromosomes with secondary constrictions	:	4
Range of chromosome length (RCL)	:	1.10 µm to 2.02 µm
Total chromosome length (TCL)	:	32.96 µm
Average chromosome length (ACL)	:	1.49 µm
Disparity index (DI)	:	29.48
Variation coefficient (VC)	:	18.02
TF Value (%)	:	43.75
Karyotype asymmetry index (As K%)	:	56.01
Syi Index	:	78.11
Rec Index	:	74.16
Intrachromosomal asymmetry index (A1)	:	0.21
Interchromosomal asymmetry index (A2)	:	0.18
Degree of asymmetry of karyotypes (A)	:	0.12

Table 9: Detailed karyomorphometric data of *Ophiorrhiza pectinata* with 2n = 22 chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
1*	2.02 ± 0.22	0.86 ± 0.12	1.15 ± 0.11	0.74 ± 0.07	1.36 ± 0.13	42.52 ± 2.34	57.47 ± 2.34	nm
2*	1.80 ± 0.17	0.78 ± 0.07	1.01 ± 0.10	0.77 ± 0.04	1.29 ± 0.07	43.59 ± 1.30	56.40 ± 1.30	nm
3	1.71 ± 0.17	0.76 ± 0.10	0.95 ± 0.17	0.79 ± 0.06	1.27 ± 0.10	44.15 ± 2.01	55.84 ± 2.01	nm
4	1.62 ± 0.17	0.71 ± 0.06	0.90 ± 0.13	0.81 ± 0.09	1.25 ± 0.15	44.69 ± 2.98	55.30 ± 2.98	nm
5	1.51 ± 0.14	0.67 ± 0.09	0.84 ± 0.06	0.79 ± 0.08	1.28 ± 0.12	44.08 ± 2.53	55.91 ± 2.53	nm
6	1.43 ± 0.17	0.64 ± 0.06	0.78 ± 0.11	0.84 ± 0.06	1.20 ± 0.09	45.57 ± 1.81	54.42 ± 1.81	nm
7	1.39 ± 0.17	0.62 ± 0.07	0.77 ± 0.10	0.82 ± 0.08	1.23 ± 0.13	44.99 ± 2.59	55.00 ± 2.59	nm
8	1.35 ± 0.16	0.59 ± 0.06	0.76 ± 0.10	0.78 ± 0.06	1.28 ± 0.10	43.86 ± 2.04	56.13 ± 2.04	nm
9	1.30 ± 0.16	0.50 ± 0.05	0.80 ± 0.16	0.68 ± 0.15	1.65 ± 0.44	39.55 ± 5.78	60.44 ± 5.78	nm
10	1.25 ± 0.16	0.58 ± 0.07	0.67 ± 0.09	0.87 ± 0.05	1.15 ± 0.07	46.54 ± 1.49	53.45 ± 1.49	nm
11	1.10 ± 0.16	0.50 ± 0.06	0.60 ± 0.10	0.85 ± 0.06	1.18 ± 0.08	45.83 ± 1.79	54.16 ± 1.79	nm

No. - Number of chromosome pair; **c** - total length of chromosome; **s** - short arm length; **l** - long arm length; **R_1** – arm ratio 1; **R_2** – arm ratio 2; **I_1** – centromeric index 1; **I_2** – centromeric index 2; **PC** – primary constriction; * - chromosome with secondary constriction; **nm** – nearly median

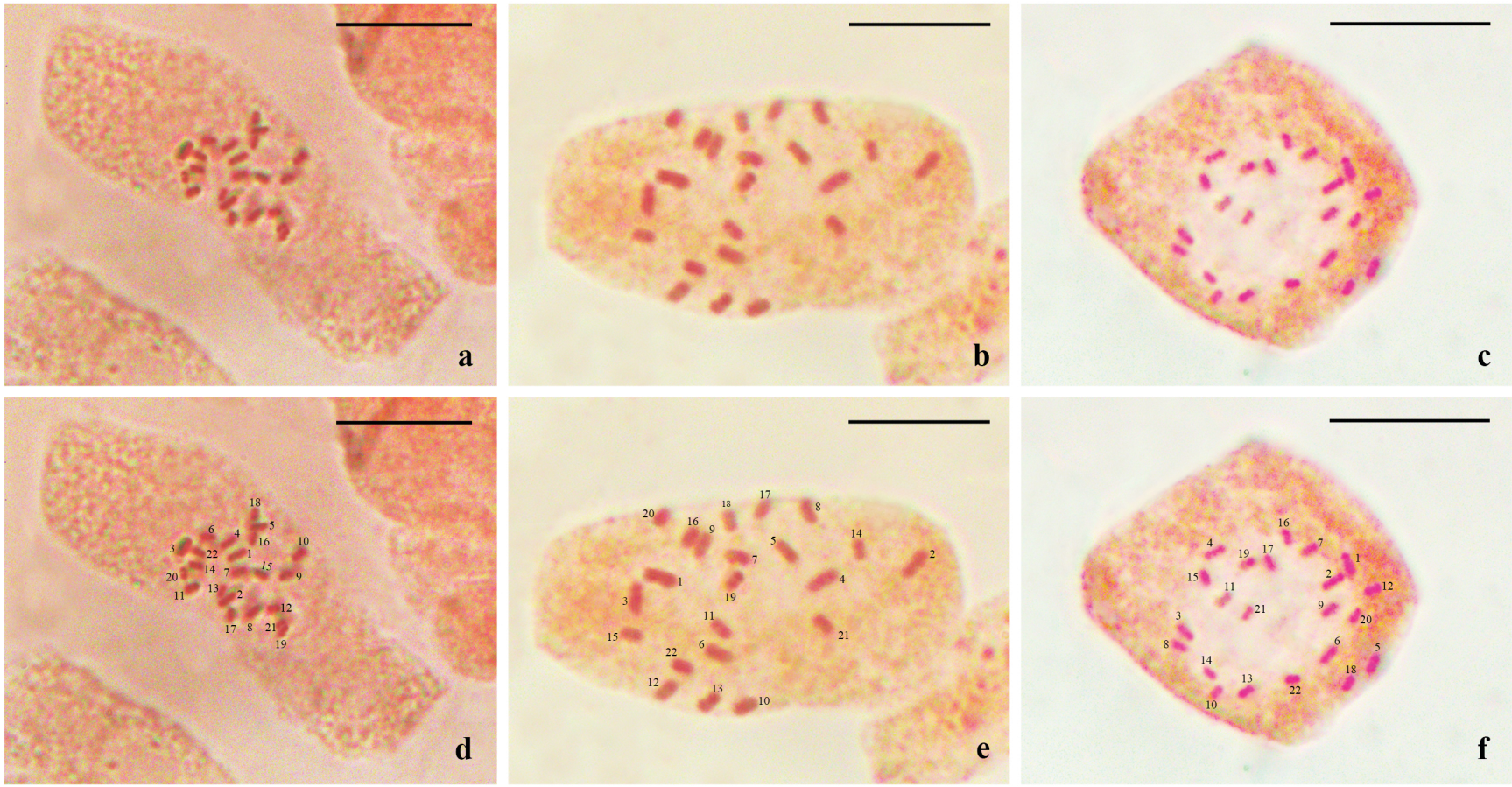


Plate 12: Mitotic chromosome complement images of *Ophiorrhiza trichocarpon* (2n = 22). a–c: Cells at mitotic metaphase; **d–f:** Karyotpe with 22 chromosomes enumerated. Scale bars = 5 μm.

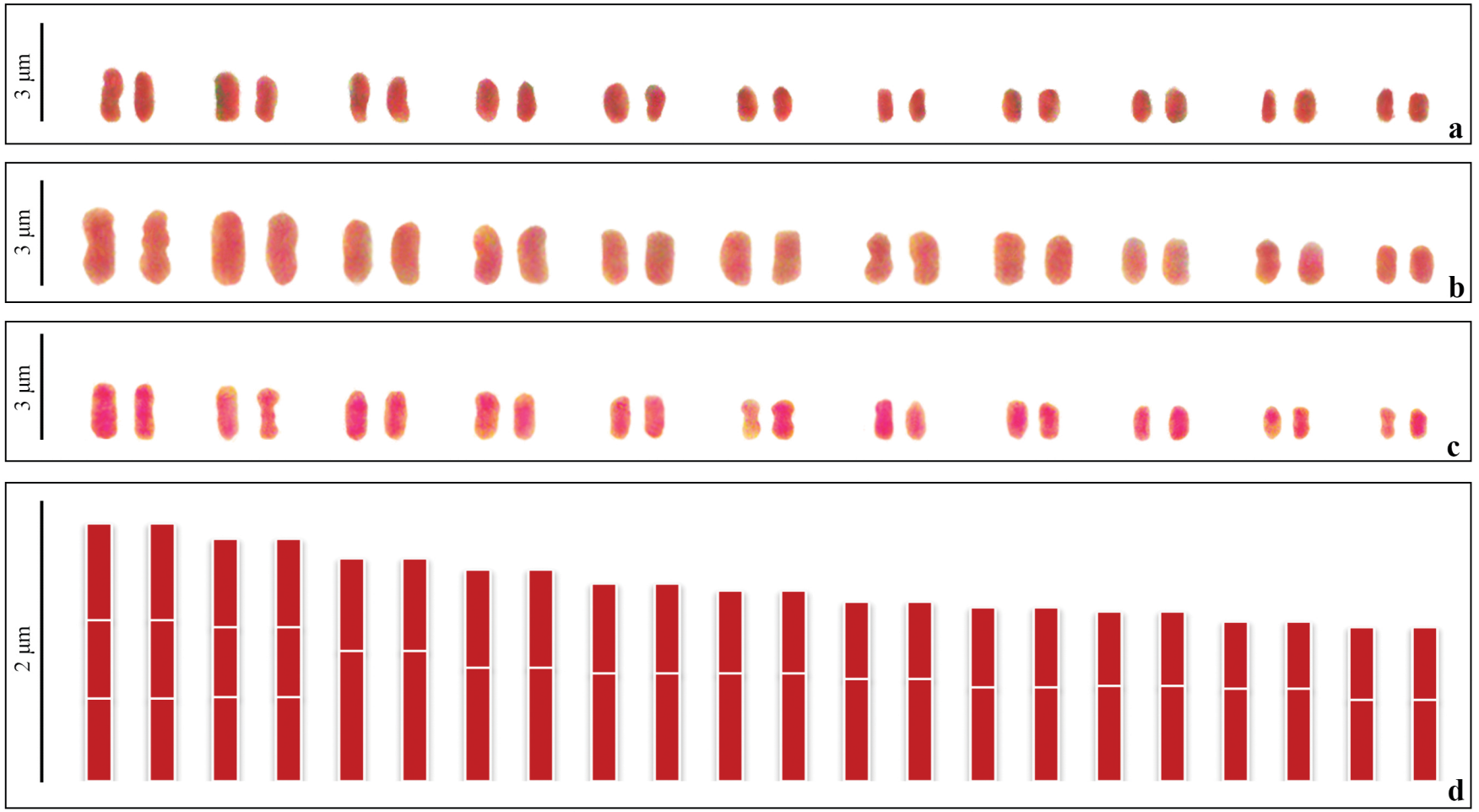


Plate 13: Mitotic chromosome complement images of *Ophiorrhiza trichocarpon* (2n = 22). a–c: Karyogram; **d:** Idiogram

Table 10: Summary of mitotic chromosome complement details of *Ophiorrhiza trichocarpon* with 2n = 22 chromosomes

Somatic chromosome number	:	22
Ploidy level	:	Diploid; 2n = 2x = 22
Karyotype formula	:	18 nm + 4 nsm(-)
Chromosomes with secondary constrictions	:	4
Range of chromosome length (RCL)	:	1.10 µm to 1.84 µm
Total chromosome length (TCL)	:	30.86 µm
Average chromosome length (ACL)	:	1.40 µm
Disparity index (DI)	:	25.17
Variation coefficient (VC)	:	17.16
TF Value (%)	:	42.51
Karyotype asymmetry index (As K%)	:	57.35
Syi Index	:	74.12
Rec Index	:	76.23
Intrachromosomal asymmetry index (A1)	:	0.24
Interchromosomal asymmetry index (A2)	:	0.17
Degree of asymmetry of karyotypes (A)	:	0.14

Table 11: Detailed karyomorphometric data of *Ophiorrhiza trichocarpon* with 2n = 22 chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
1*	1.84 ± 0.18	0.69 ± 0.14	1.15 ± 0.08	0.60 ± 0.11	1.82 ± 0.43	36.98 ± 5.00	63.01 ± 5.00	nsm(-)
2*	1.73 ± 0.20	0.63 ± 0.07	1.10 ± 0.17	0.59 ± 0.11	1.78 ± 0.28	36.72 ± 4.23	63.27 ± 4.23	nsm(-)
3	1.59 ± 0.10	0.66 ± 0.07	0.93 ± 0.06	0.71 ± 0.09	1.46 ± 0.21	41.21 ± 3.42	58.78 ± 3.42	nm
4	1.51 ± 0.10	0.70 ± 0.02	0.81 ± 0.08	0.87 ± 0.06	1.15 ± 0.08	46.59 ± 1.76	53.40 ± 1.76	nm
5	1.41 ± 0.10	0.64 ± 0.03	0.77 ± 0.07	0.83 ± 0.04	1.21 ± 0.06	45.30 ± 1.40	54.69 ± 1.40	nm
6	1.36 ± 0.12	0.59 ± 0.05	0.77 ± 0.07	0.77 ± 0.04	1.30 ± 0.06	43.47 ± 1.29	56.52 ± 1.29	nm
7	1.28 ± 0.11	0.55 ± 0.06	0.73 ± 0.08	0.76 ± 0.10	1.35 ± 0.20	43.03 ± 3.59	56.96 ± 3.59	nm
8	1.24 ± 0.11	0.57 ± 0.03	0.67 ± 0.07	0.84 ± 0.03	1.18 ± 0.05	45.89 ± 1.03	54.10 ± 1.03	nm
9	1.22 ± 0.10	0.53 ± 0.05	0.68 ± 0.04	0.78 ± 0.03	1.28 ± 0.06	43.79 ± 1.20	56.20 ± 1.20	nm
10	1.15 ± 0.07	0.48 ± 0.03	0.66 ± 0.04	0.73 ± 0.01	1.36 ± 0.01	42.20 ± 0.32	57.79 ± 0.32	nm
11	1.10 ± 0.09	0.52 ± 0.07	0.58 ± 0.02	0.89 ± 0.08	1.14 ± 0.10	46.89 ± 2.33	53.10 ± 2.33	nm

No. - Number of chromosome pair; **c** - total length of chromosome; **s** - short arm length; **l** - long arm length; **R_1** – arm ratio 1; **R_2** – arm ratio 2; **I_1** – centromeric index 1; **I_2** – centromeric index 2; **PC** – primary constriction; * - chromosome with secondary constriction; **nsm** – nearly submedian; **nm** – nearly median

PART II – PHYTOCHEMICAL PROFILING

The phytoconstituents present in the selected species of *Ophiorrhiza* were determined by qualitative phytochemical analyses, quantitative estimation of major phytoconstituents, GC-MS and HR LC-MS analyses.

A. Qualitative phytochemical analyses

To determine the presence or absence of different classes of bioactive phytochemicals, standard protocols were followed and their existence was confirmed on the basis of the colour and the precipitation formed in specific reactions. Three extracts of each plant; *viz*., hexane extract, methanolic extract and aqueous extract were considered for the tests. All the four species of *Ophiorrhiza* selected for the study, showed the presence of various classes of compounds. The summary of the results obtained in the preliminary phytochemical screening is given in **Table 12.**

In *O. grandiflora*; phenols, flavonoids, alkaloids, phytosterols, coumarins and glycosides were detected in the methanolic and the aqueous extracts of the plant. The presence of tannins was observed only in the methanolic extract, whereas; fixed oils and fats were absent in all the three extracts. In *O. jacobii*; phenols, flavonoids, alkaloids, phytosterols, tannins, coumarins, glycosides, fixed oils and fats were observed in the methanolic and the aqueous extracts. In *O. pectinata*; phenols, flavonoids, alkaloids, phytosterols, tannins and coumarins were noticed in the methanolic and the aqueous extracts, while glycosides, fixed oils and fats were seen only in the methanolic extract. In *O. trichocarpon*; phenols, flavonoids, alkaloids, tannins, coumarins and glycosides showed up in the methanolic and the aqueous extracts, while phytosterols, fixed oils and fats were present only in the methanolic extract. Terpenoids and anthraquinones were the classes of compounds seen in all the three extracts of each plant, while; saponins and phlobatannins were found to be absent in all the extracts of the plants.

Table 12: Qualitative phytochemical analyses of selected species of *Ophiorrhiza*

Sl No.	Class of compounds	Tests	OG			OJ			OP			OT		
			H	M	DW	H	M	DW	H	M	DW	H	M	DW
1.	Phenols	Ferric chloride test	-	+	+	-	+	+	-	+	+	-	+	+
		Spot test	-	+	+	-	+	+	-	+	+	-	+	+
2.	Flavonoids	Alkaline reagent test	-	+	+	-	+	+	-	+	+	-	+	+
		Lead acetate test	-	+	+	-	+	+	-	+	+	-	+	+
3.	Alkaloids	Wagner's test	-	+	+	-	+	+	-	+	+	-	+	+
		Hager's test	-	+	+	-	+	+	-	+	+	-	+	+
4.	Phytosterols	Liebermann-Burchard test	-	-	-	-	-	-	-	-	-	-	-	-
		Sulphuric acid test	-	+	+	-	+	+	-	+	+	-	+	-
5.	Terpenoids	Salkowski test	+	+	+	+	+	+	+	+	+	+	+	+
6.	Tannins	Breymer's test	-	-	-	-	-	-	-	-	-	-	-	-
		Lead acetate test	-	+	-	-	+	+	-	+	+	-	+	+
7.	Saponins	Foam test	-	-	-	-	-	-	-	-	-	-	-	-
8.	Phlobatannins	Precipitation test	-	-	-	-	-	-	-	-	-	-	-	-
9.	Anthraquinones	Borntrager's test	+	+	+	+	+	+	+	+	+	+	+	+
10.	Coumarins	Ferric chloride test	-	+	+	-	+	+	-	+	+	-	+	+
11.	Glycosides	Legal's test	-	+	+	-	+	+	-	+	-	-	+	+
12.	Fixed oils and fats	Spot test	-	-	-	-	+	+	-	+	-	-	+	-

OG - *O. grandiflora*; **OJ -** *O. jacobii*; **OP -** *O. pectinata*; **OT** - *O. trichocarpon*; **H -** Hexane; **M -** Methanol; **DW -** Distilled water; **(+) -** Presence; (-) – Absence

B. Quantification of major phytoconstituents

The preliminary phytochemical analyses showed that the maximum number of compounds are detected in the methanolic extract of each plant and hence all the further studies are carried out on the methanolic extract of the selected species.

Quantitative estimation of the major class of compounds like phenols, flavonoids, terpenoids and alkaloids showed considerable amounts of these phytoconstituents in all the plants. The total phenolic content of the methanolic extracts of the plants was calculated from the calibration curve of gallic acid **(Fig. 6).** The highest quantity of phenolic content was observed in *O. jacobii* with 454.12 ± 2.13 mg GAE/g DW followed by *O. grandiflora*, *O. pectinata* and O. *trichocarpon* **(Fig. 7).** The total flavonoid content was calculated from the calibration curve of quercetin **(Fig. 8).** *O. trichocarpon* exhibited the highest content of flavonoids *ie.,* 80.33 ± 0.40 mg QE/g DW of the extract. All the other plant extracts also contained flavonoids in considerable amounts **(Fig. 9).** Amount of terpenoids present in the methanolic extracts of the selected plants was determined using the calibration curve of linalool **(Fig. 10).** Among the selected plants *O. pectinata* showed the highest terpenoid content of 451.90 ± 0.96 mg LE/g DW, while *O. jacobii* had the lowest terpenoid content of 89.22 ± 1.74 mg LE/g DW **(Fig. 11).** Alkaloids were present in comparatively smaller quantities in all the extracts of the plants, and are determined using the calibration curve of caffeine **(Fig. 12).** Out of the four species, the methanolic extract of *O. trichocarpon* showed the highest alkaloid content of 14.50 ± 3.882 mg CE/g DW, followed by *O. jacobii, O. grandiflora* and *O. pectinata* **(Fig. 13).** In the present study, all the estimated secondary metabolites were found to be present in considerable amounts and these can contribute to the bioactivity potential of the selected plants.

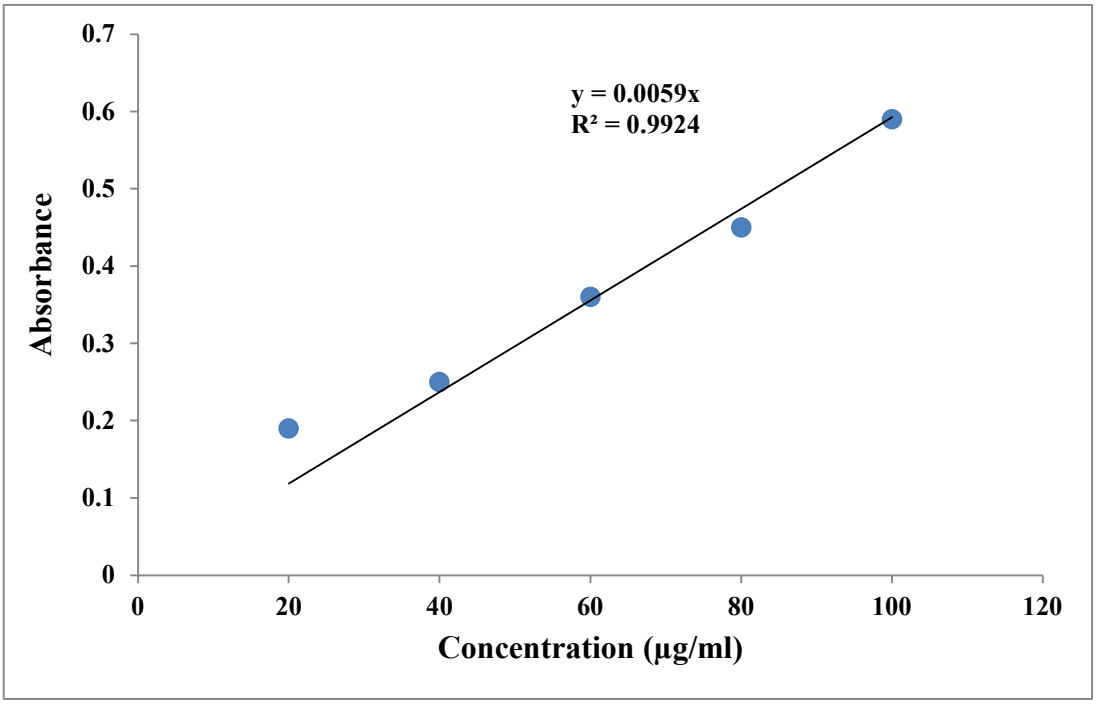


Fig. 6: Standard calibration curve of gallic acid for the determination of total phenolic content

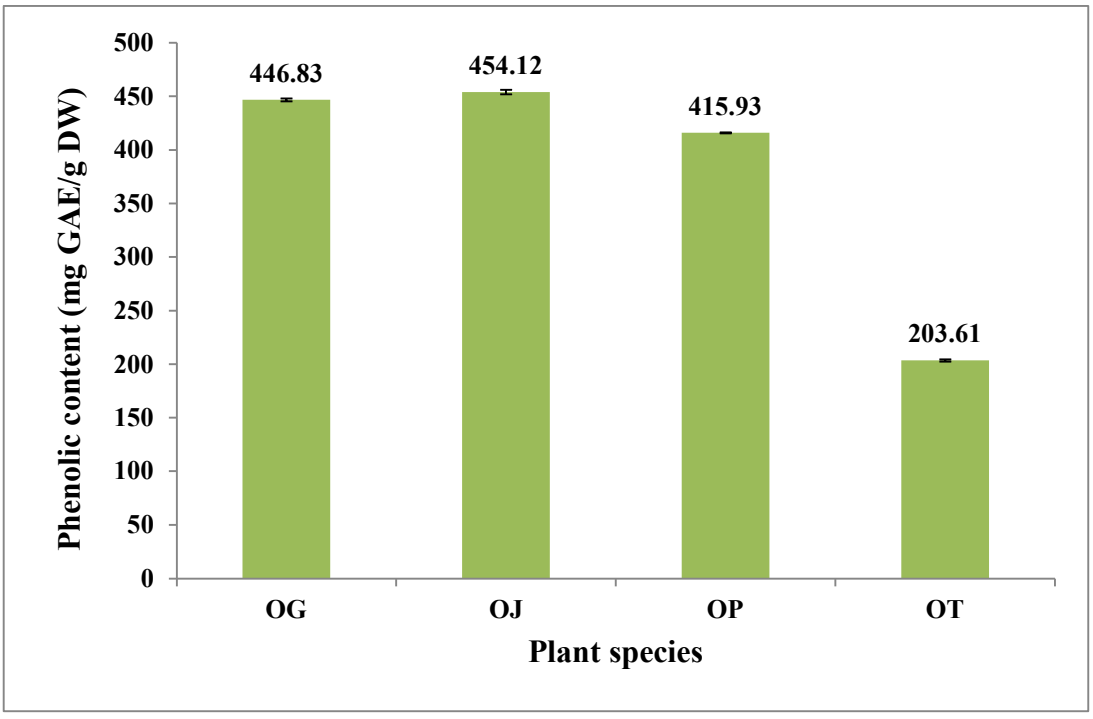


Fig. 7: Quantitative estimation of total phenolic content in selected species of *Ophiorrhiza*. OG - *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon*

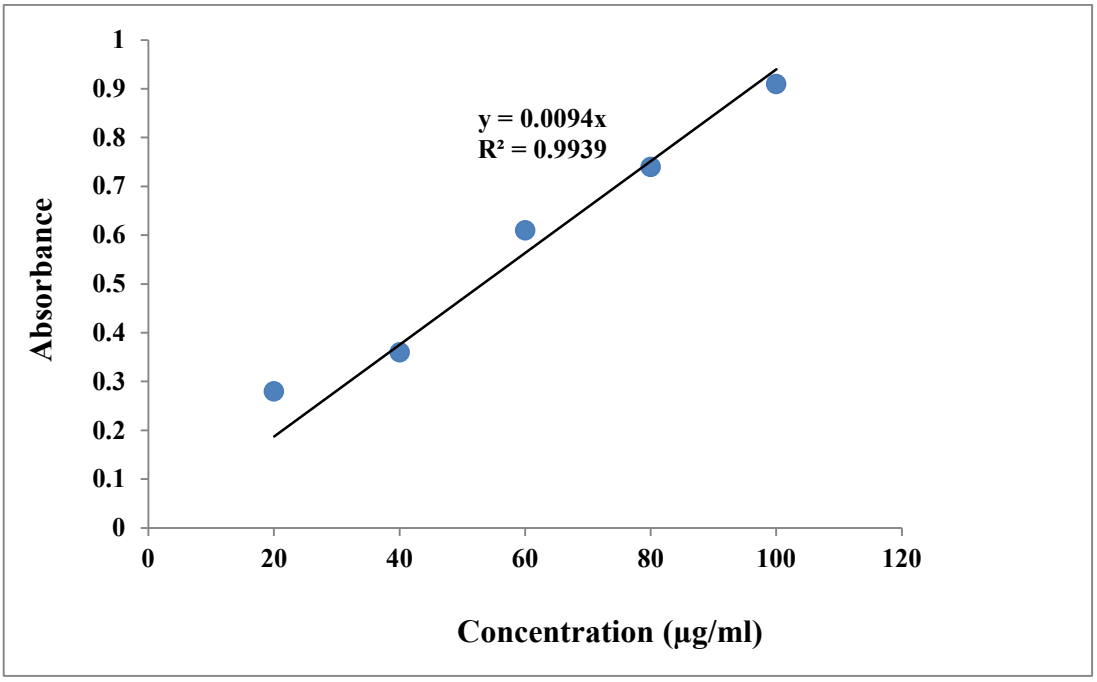


Fig. 8: Standard calibration curve of quercetin for the determination of total flavonoid content

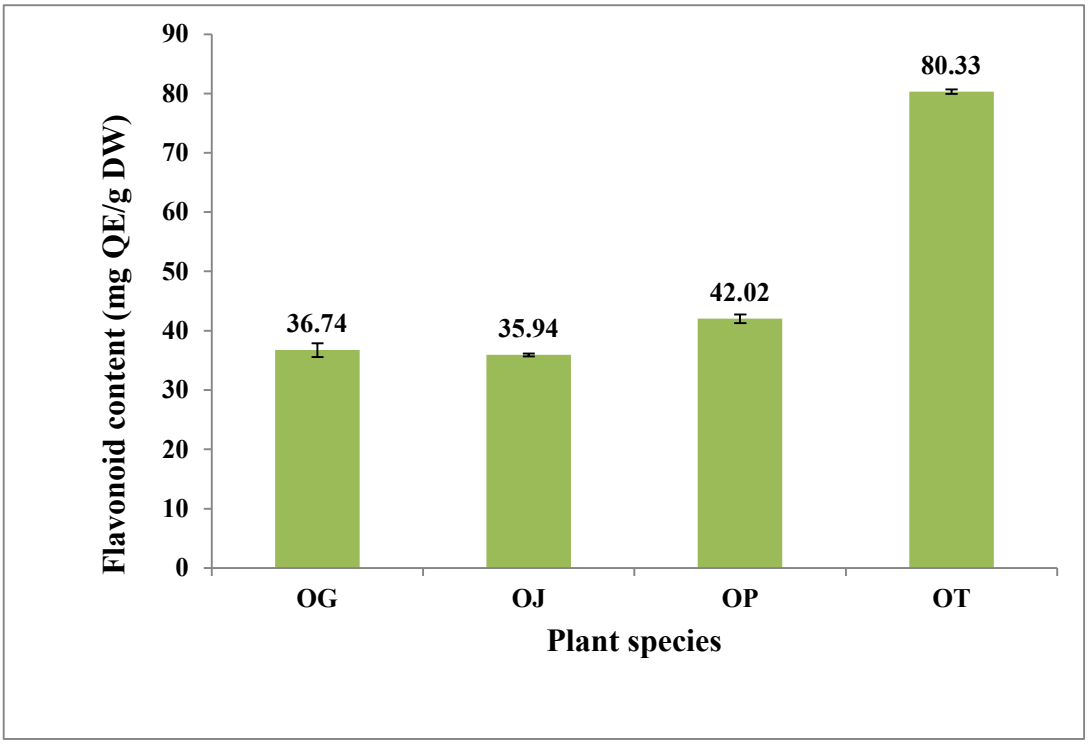


Fig. 9: Quantitative estimation of total flavonoid content in selected species of *Ophiorrhiza.* OG - *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon*

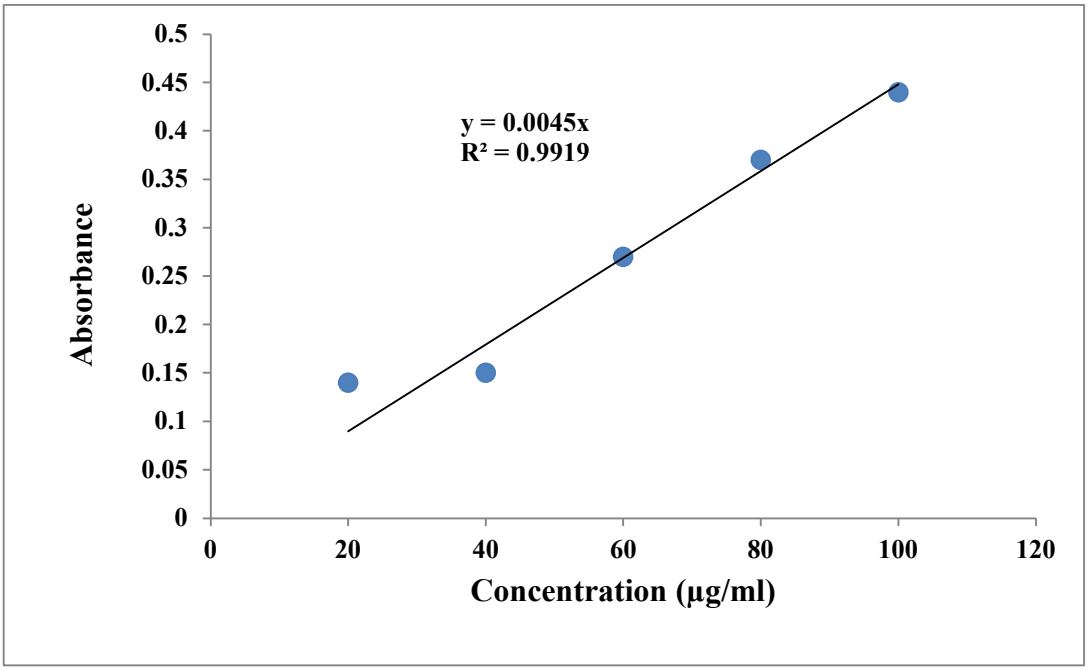


Fig. 10: Standard calibration curve of linalool for the determination of total terpenoid content

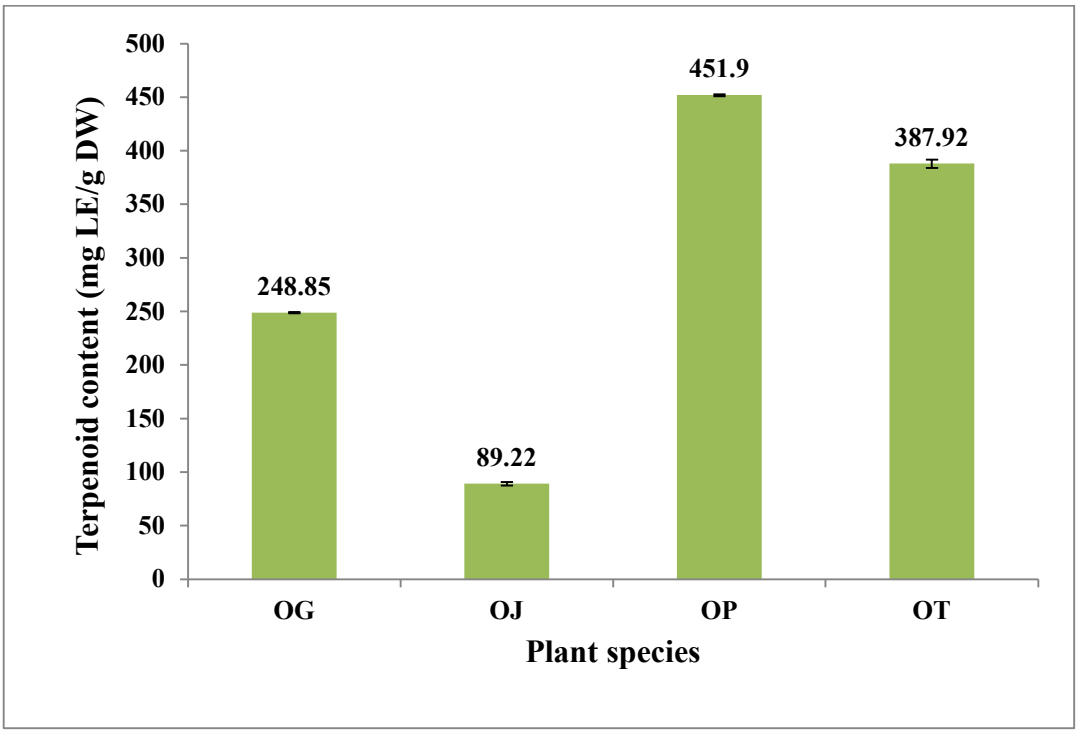


Fig. 11: Quantitative estimation of total terpenoid content in selected species of *Ophiorrhiza.* OG - *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon*

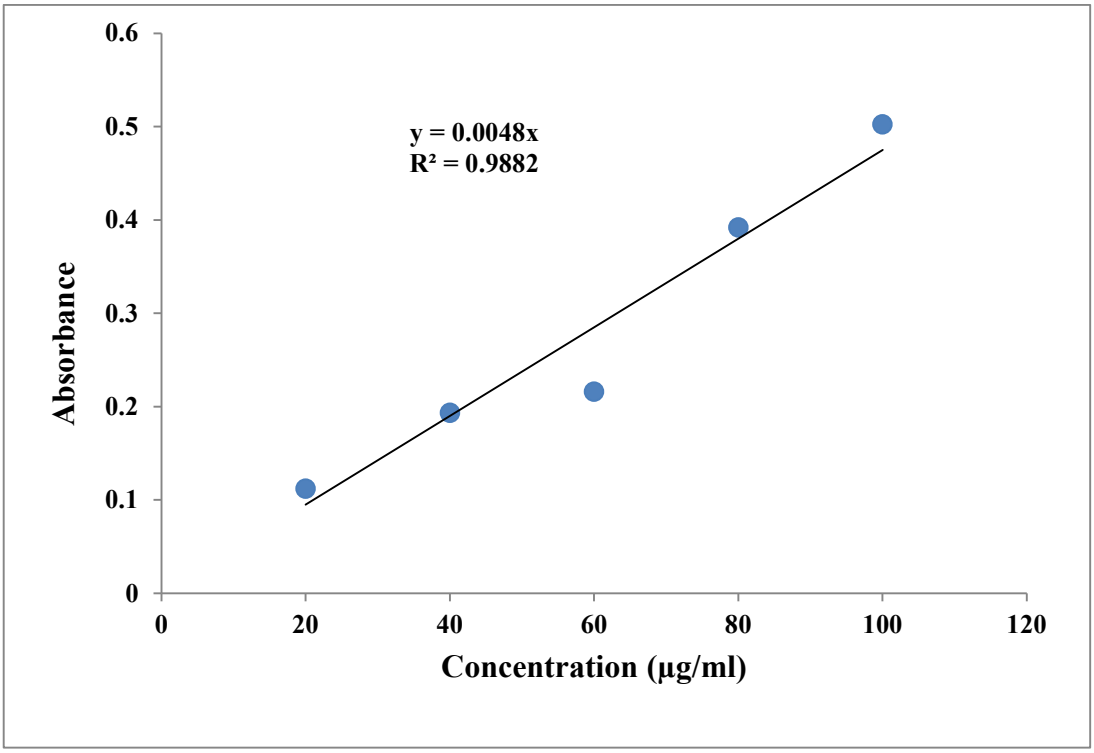


Fig. 12: Standard calibration curve of caffeine for the determination of total alkaloid content

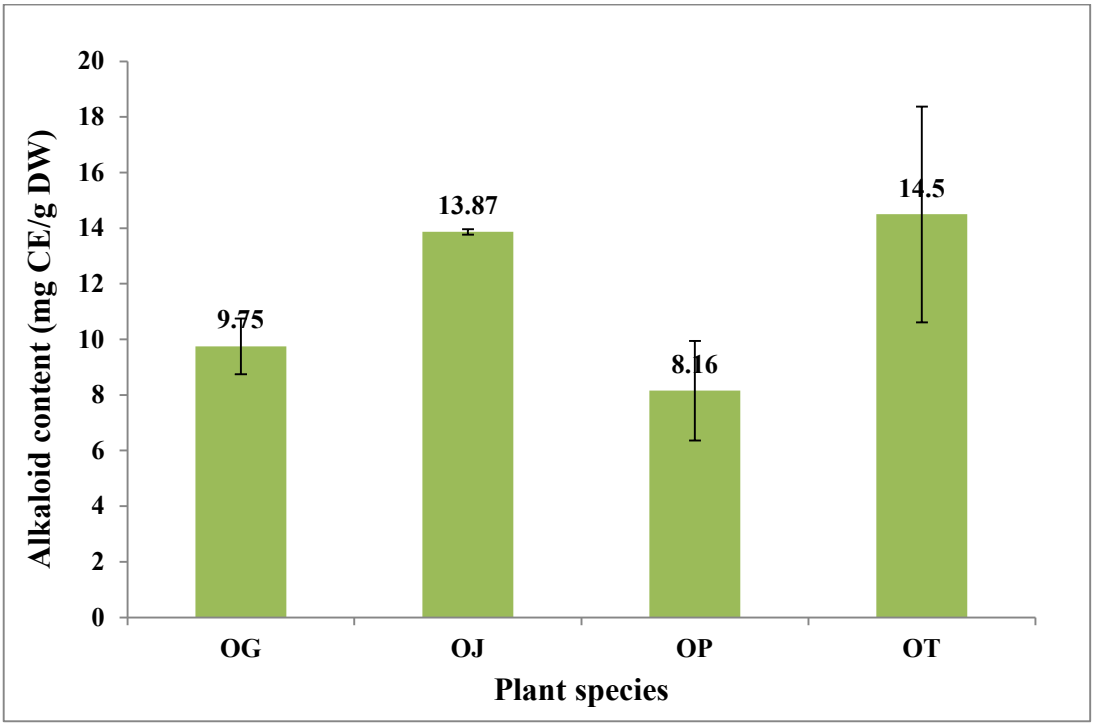


Fig. 13: Quantitative estimation of total alkaloid content in selected species of *Ophiorrhiza*. OG - *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon*

C. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The volatile phytocompounds present in the methanolic extract of the selected species of *Ophiorrhiza* were identified by the technique of GC-MS analysis. About 82 compounds belonging to various classes were revealed from the analysis. The major classes of compounds include terpenoids, flavonoids, phenols, alkanes, fatty acids and their esters, alkaloids, anthraquinones, sterols *etc*. The list of compounds revealed by GC-MS analysis is given in **Table 13.**

In *O. grandiflora*, a total of 27 compounds were identified by GC-MS analysis. The major compounds identified were heneicosane (15.88%), n-hexadecanoic acid (12.92%), γ-sitosterol (11.49%), phytol (7.32%), neophytadiene (7.15%), stigmasterol (6.17%), cyclopentanol (5.21%), nonadecane (4.21%), campesterol (3.96%) and squalene (2.09%). The most prominent class of compounds seen was the alkanes like dodecane, tetradecane, pentadecane, nonadecane and heneicosane. Among the sterols, campesterol, stigmasterol and γ-sitosterol were recognised. n-Hexadecanoic acid was the only fatty acid revealed in the analysis. Neophytadiene and squalene represented the class of terpenoids while phytol was the terpene alcohol found. In alkaloids, 1-anliinoisoquinoline, 1-decanoyl piperidine and aspidofractinine-3,8-dione were identified. Several compounds belonging to fatty acid esters, monosaccharides and fatty aldehydes were also seen in notable quantities. The gas chromatogram of *O. grandiflora* is given in **Fig. 14.**

In *O. jacobii,* 27 compounds were revealed from the GC-MS analysis. The most important compounds were found to be humulane-1,6-dien-3-ol (58.19%), lupan-3-ol acetate (16.49%), γ-sitosterol (4.12%), stigmasterol (3.88%), n-hexadecanoic acid (2.84%), lupenyl acetate (2.08%), campesterol (1.76%), 3-epipapyriferic acid (1.35%), squalene (1.31%) and solanesol (1.09%). Terpenoids were found to be the most predominant class of bioactive compounds in the species with neophytadiene, squalene, lupenyl acetate, incanin, humulane-1,6-dien-3-ol, lupan-3-ol acetate and 3-

epipapyriferic acid in considerable percentages. A good percentage of sterol compounds like, campesterol, stigmasterol, γ-sitosterol and γ-sitostenone were also identified. Vitamin E was the only phenolic compound found and other compounds like alkanes, fatty acids, esters, aldehydes, terpene alcohols were also identified. The gas chromatogram of *O. jacobii* is given in **Fig. 15.**

A total of 33 compounds were revealed from the GC-MS analysis of the methanolic extract of *O. pectinata.* The compounds found in highest percentages were n-hexadecanoic acid (18.77%), γ-sitosterol (15.28%), cycloartenol (12.59%), octadecanoic acid (6.73%), neophytadiene (5.86%), chrysophanic acid (4.53%), methyl pentafuranoside (4.26%), pyranone (4.15%), squalene (3.37%) and linoleic acid (2.55%). Amongst the major bioactive class of compounds, fatty acids were prominent followed by terpenoids, sterols, anthraquinones and alkaloids. Phenols and flavonoids were represented by 4-vinyl-2-methoxy phenol and 2,3-dihydrobenzofuran respectively. Various other compounds like esters and amides were also identified. The gas chromatogram of *O. pectinata* is given in **Fig. 16.**

GC-MS analysis of *O. trichocarpon* showed up 34 compounds. The major compounds obtained were humulane-1,6-dien-3-ol (54.89%), 2,4-bis (1-phenylethyl)-phenol (6.78%), 2,4,6-Tris- (1-phenylethyl)-phenol (4.93%), lupenone (4.43%), linoleic acid (3.66%), γ-sitosterol (3.26%), n-hexadecanoic acid (2.92%), lupenyl acetate (2.71%), stigmasta-5,22-dien-3-ol, (3 beta., 22E)- (2.05%) and campesterol (1.59%). The major class of compounds eluted were terpenoids that included loliolide, neophytadiene, squalene, germanicen, lupenone, 4,22-cholestadien-3-one, methyl ursolate, lupenyl acetate and humulane-1,6-dien-3-ol. Phenols were represented by 2-2-methylenebis-phenol and 2,4-bis (1-phenylethyl)-phenol and further sterols, fatty acids, esters, alcohols and coumarins were also found in considerable amounts. The gas chromatogram of *O. trichocarpon* is given in **Fig. 17.** The mass spectra of individual compounds identified by GC-MS analysis of the methanolic extracts of the selected species of *Ophiorrhiza* are given in **Figs 18 (i-xiv).**

Table 13: Phytocompounds revealed by GC-MS analysis of the selected species of *Ophiorrhiza*

Sl No.	RT (min)	Compounds	Class	Peak area %			
				OG	OJ	OP	OT
1.	5.704	Maltol	Pyran compound	-	-	0.64	-
2.	6.917	Pyranone	Pyran compound	-	-	4.15	-
3.	8.308	2,3-Dihydrobenzofuran	Flavonoid	-	-	0.56	-
4.	9.841	4-Vinyl-2-methoxy phenol	Phenol	-	-	0.49	-
5.	10.463	Hexadecane	Alkane	-	-	-	0.15
6.	11.163	Dodecane	Alkane	1.10	-	-	-
7.	11.246	Tetradecane	Alkane	1.71	0.63	0.31	-
8.	12.962	Methyl pentafuranoside	Organic compound	-	-	4.26	-
9.	13.542	Neophytadiene	Sesquiterpenoid	7.15	0.24	5.86	0.36
10.	13.566	Octadecanoic acid	Fatty acid	-	0.35	6.73	0.26
11.	13.813	4-Ethoxy-2-hydroxy-octadecanophenone	Ketone	-	-	0.33	-
12.	14.348	Octadecane	Alkane	-	-	0.70	-
13.	14.660	4,6,8-Trimethyl-1-azulenecarbaldehyde	Aldehyde	-	-	-	0.28
14.	14.715	Cyclopentanol	Alcohol	5.21	-	-	-
15.	15.121	Loliolide	Terpenoid	-	-	-	0.26
16.	15.262	Pluchidiol	Alcohol	-	-	-	0.18
17.	15.456	d-Gluco-heptulosan	Monosaccharide	2.21	-	-	-
18.	15.832	Nerolidol-epoxyacetate	Sesquiterpene	-	-	0.61	-

19.	16.494	Hexadecanoic acid, methyl ester	Fatty acid ester	-	0.44	1.27	0.57
20.	16.532	Pentadecane	Alkane	1.92	-	-	-
21.	16.922	n-Hexadecanoic acid	Fatty acid	12.92	2.84	18.77	2.92
22.	16.944	3,7,11,15-Tetramethylhexadecyl acetate	Ester	-	-	1.92	-
23.	17.142	Heneicosane	Alkane	15.88	0.15	0.37	-
24.	17.464	Seselin	Coumarin	-	-	-	0.44
25.	17.539	Phytene-2	Diterpenoid	-	-	1.37	-
26.	17.642	2-2-Methylenebis-phenol	Phenol	-	-	-	0.26
27.	17.797	4-Oxazolecarboxylic acid, 4,5-dihydro-2-phenyl-1-methylethyl ester	Carboxylic acid ester	-	-	-	0.25
28.	18.124	Linoleic acid, methyl ester	Fatty acid ester	0.98	0.36	0.79	0.48
29.	18.184	3,6- Octadecadienoic acid, methyl ester	Fatty acid ester	-	-	-	0.99
30.	18.295	Phytol	Diterpene alcohol	7.32	0.80	1.66	1.46
31.	18.363	Tetradecanoic acid	Fatty acid	0.97	-	-	-
32.	18.426	17-Octadecynoic acid	Fatty acid	1.08	-	-	-
33.	18.604	Linoleic acid	Fatty acid	1.14	0.31	2.55	3.66
34.	18.778	Nonadecane	Alkane	4.21	0.08	-	0.19
35.	19.133	8,11,14-Docosatrienoic acid, methyl ester	Fatty acid ester	-	0.34	-	-
36.	19.223	(2E)-3,7,11,15-Tetramethyl-2-hexadecene	Diterpenoid	1.14	-	-	-

37.	19.452	Braylin	Coumarin	-	-	-	0.99
38.	19.555	7-Tetradecenal, (Z)	Fatty aldehyde	-	0.43	-	-
39.	19.951	2-Bromotetradecane	Alkane	-	-	-	0.19
40.	20.3066	Octadecanoic acid, methyl ester	Fatty acid ester	1.99	-	0.25	-
41.	20.568	Stearic acid, allyl ester	Fatty acid ester	-	-	1.37	-
42.	20.730	Tetradecanoic acid, ethyl ester	Fatty acid ester	0.62	-	-	-
43.	20.849	Petroselinic acid, methyl ester	Fatty acid ester	-	-	1.09	-
44.	21.067	2,4-Bis (1-phenylethyl)-phenol	Phenol	-	-	-	6.78
45.	21.804	Hexacosane	Alkane	-	0.93	-	-
46.	21.990	Eicosanoic acid, methyl ester	Fatty acid ester	0.29	-	-	-
47.	22.142	9-Octadecenal, (Z)-	Fatty aldehyde	2.65	-	-	-
48.	22.221	1-Hydroxy-4-methylanthraquinone	Anthraquinone	-	-	0.42	-
49.	22.891	Cypionic acid, 2-dimethylaminoethyl ester	Carboxylic acid ester	-	-	0.30	-
50.	23.078	3-Phenyl-1,2-dihydroquinoxalin-2-one	Alkaloid	-	-	1.30	-
51.	23.293	1-Anliinoisoquinoline	Alkaloid	1.53	-	-	-
52.	24.190	Phytane	Diterpenoid	-	-	0.22	-
53.	24.939	Brompheniramine	Alkaloid	-	-	0.36	-
54.	25.238	4-Benzyloxy-4-[2,2-dimethyl-4-dioxolanyl]- butylaldehyde	Aldehyde	-	-	-	0.35
55.	25.880	Nonacosane	Alkane	-	0.56	-	-
56.	26.219	1-Decanoyl piperidine	Alkaloid	2.52	-	-	-

57.	26.622	Squalene	Triterpene	2.09	1.31	3.37	0.88
58.	26.750	Palmitic acid, vinyl ester	Fatty acid ester	0.78	-	-	-
59.	26.987	Chrysophanic acid	Anthraquinone	-	-	4.53	-
60.	27.974	4-Quinoline carboxylic acid, 6-methyl-2-phenyl, methyl ester	Carboxylic acid ester	-	-	1.04	-
61.	28.040	Aspidofractinine-3,8-dione	Alkaloid	0.97	-	-	-
62.	28.736	9-Octadecanamide	Fatty acid amide	-	-	2.05	-
63.	29.035	2,4,6-Tris- (1-phenylethyl)-phenol	Phenol	-	-	-	4.93
64.	29.516	Solanesol	Terpene alcohol		1.09		0.19
65.	30.832	14-Oxatricyclo [9..2.1.0(1,10)] tetradecane, 2,6,6,10,11-pentamethyl	Alkane	-	0.30	-	-
66.	31.563	Vitamin E	Phenol	-	0.20	-	-
67.	32.405	Campesterol	Sterol	3.96	1.76		1.59
68.	32.925	Stigmasta-5,22-dien-3-ol, (3 beta., 22E)-	Sterol	-	-	-	2.05
69.	33.740	Stigmasterol	Sterol	6.17	3.88	-	-
70.	34.223	γ-sitosterol	Sterol	11.49	4.12	15.28	3.26
71.	34.526	Germanicen	Triterpenoid	-	-	-	0.83
72.	35.117	4-Campestene-3-one	Sterol	-	-	-	0.76
73.	35.489	Lupenone	Triterpenoid	-	-	-	4.43
74.	35.758	4,22-Cholestadien-3-one	Triterpene	-	-	-	0.59
75.	36.232	Methyl ursolate	Triterpene	-	-	-	0.76

76.	36.852	Lupenyl acetate	Triterpenoid	-	2.08	2.49	2.71
77.	37.441	γ-Sitostenone	Sterol	-	0.13	-	1.11
78.	39.001	Incanin	Sesquiterpenoid	-	0.64	-	-
79.	40.466	Humulane-1,6-dien-3-ol	Sesquiterpenoid	-	58.19	-	54.89
80.	41.919	Cycloartenol	Triterpenoid	-	-	12.59	-
81.	43.238	Lupan-3-ol, acetate	Triterpenoid	-	16.49	-	-
82.	44.438	3-Epipapyriferic acid	Triterpene	-	1.35	-	-

RT – Retention time; **OG –** *O. grandiflora*; **OJ –** *O. jacobii*; **OP –** *O. pectinata*; **OT –** *O. trichocarpon*

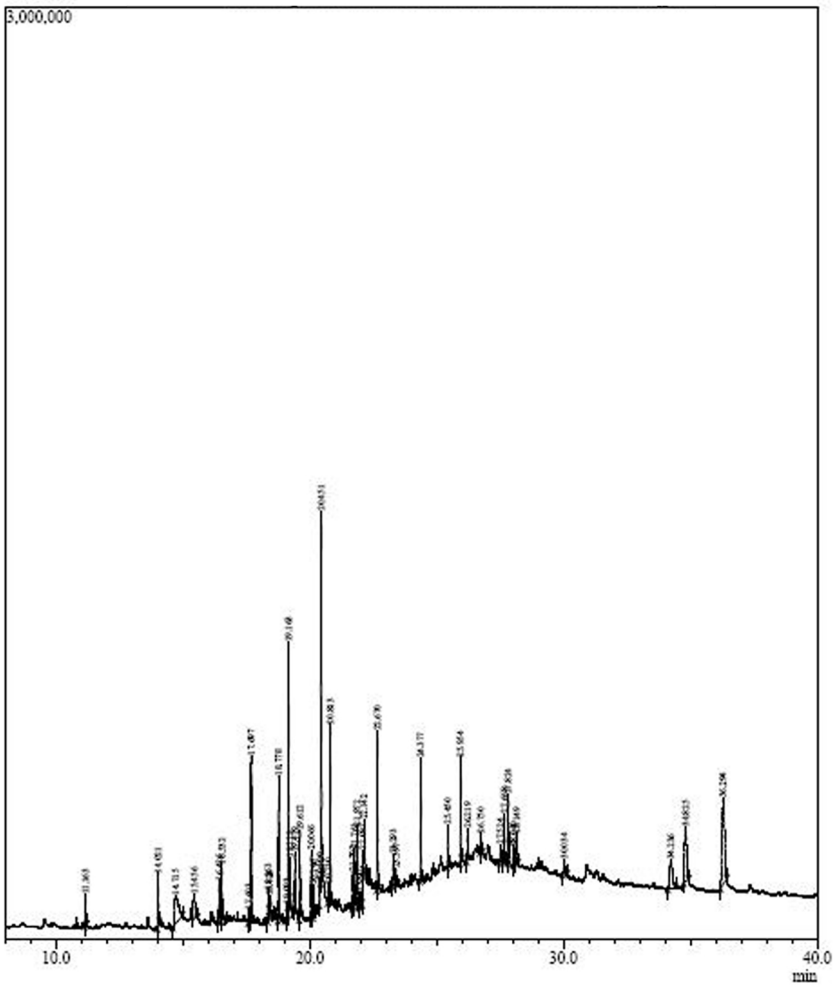


Fig. 14: Gas chromatogram of methanolic extract of *Ophiorrhiza grandiflora*

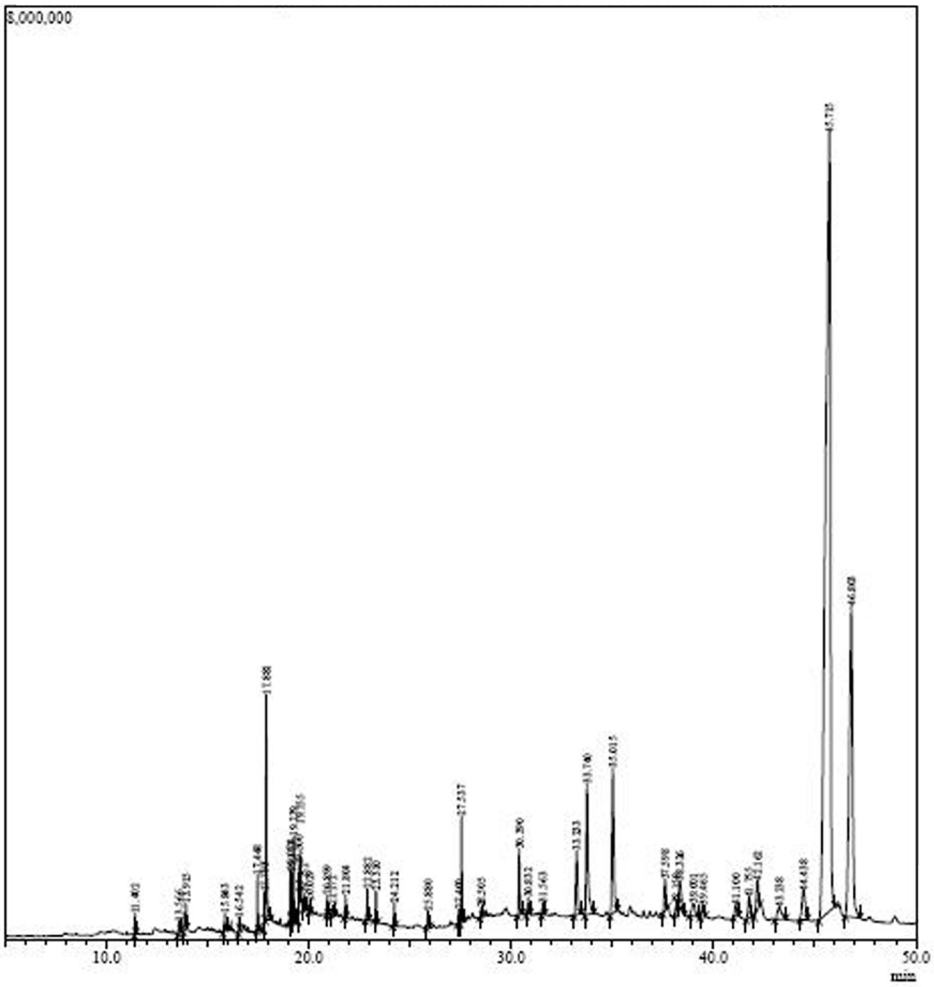


Fig. 15: Gas chromatogram of methanolic extract of *Ophiorrhiza jacobii*

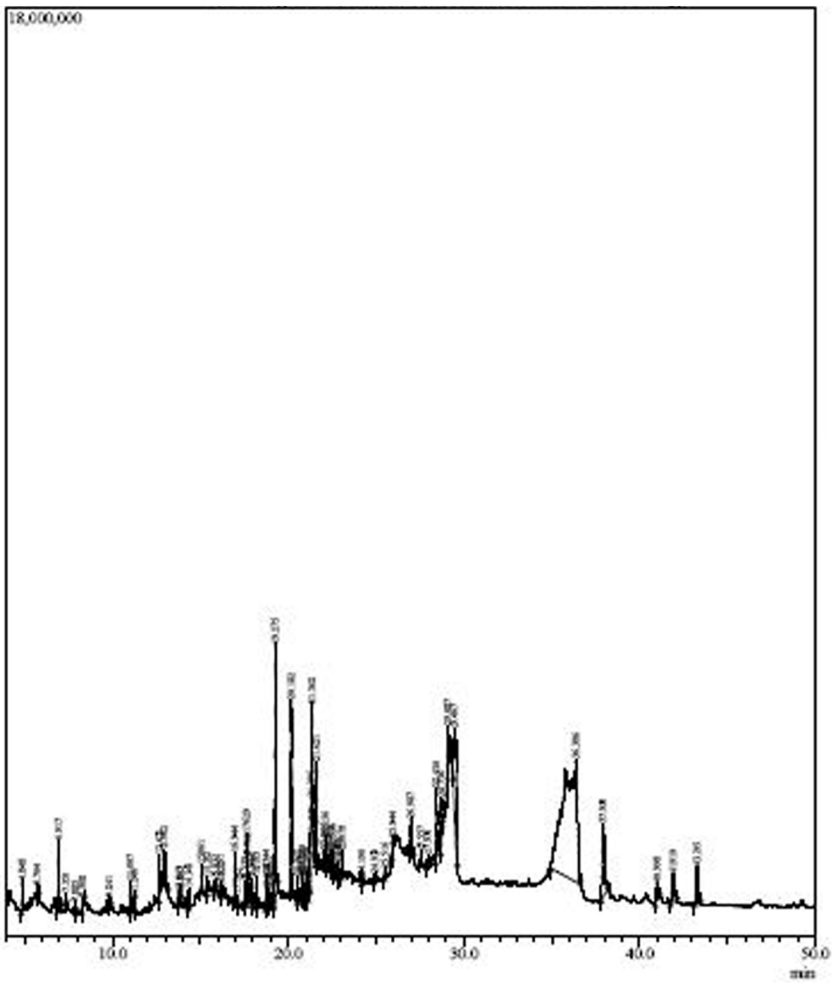


Fig. 16: Gas chromatogram of methanolic extract of *Ophiorrhiza pectinata*

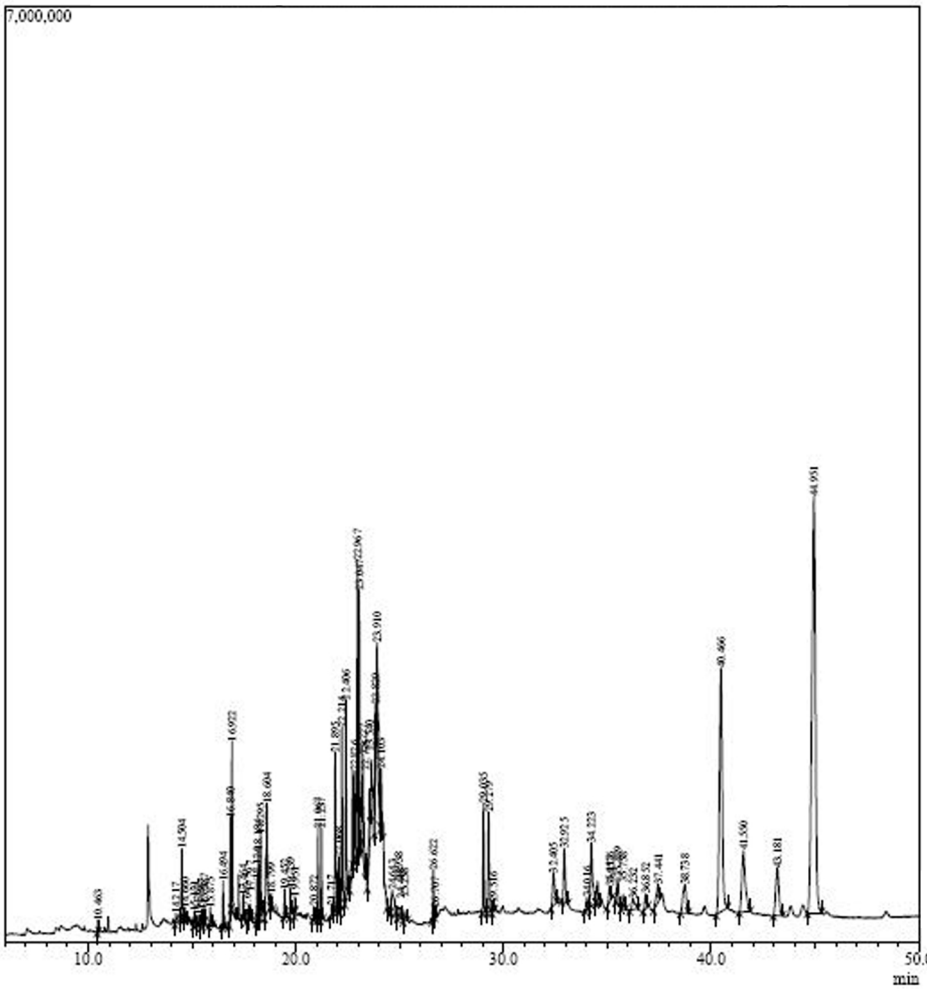


Fig. 17: Gas chromatogram of methanolic extract of *Ophiorrhiza trichocarpon*

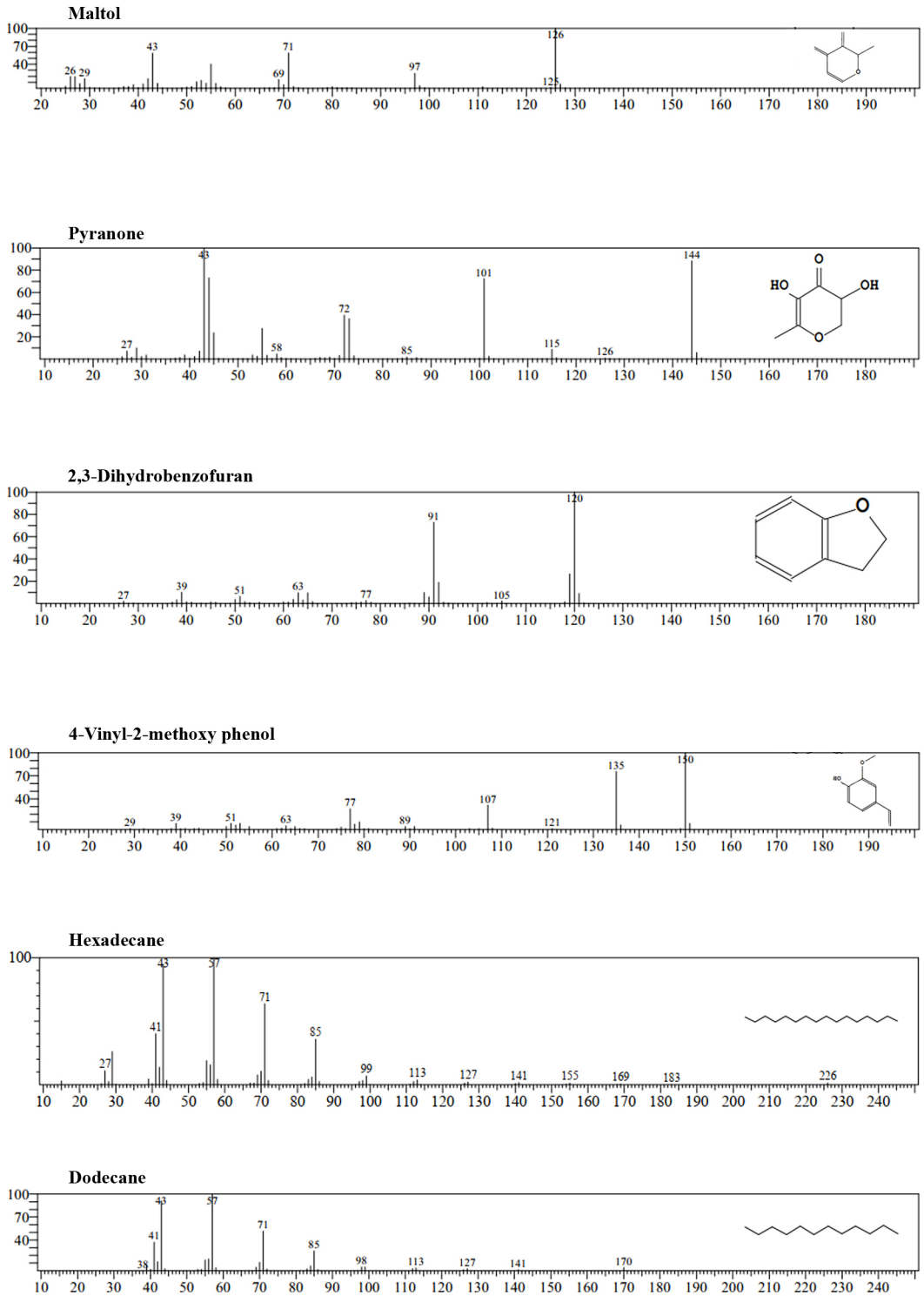


Fig. 18 (i): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

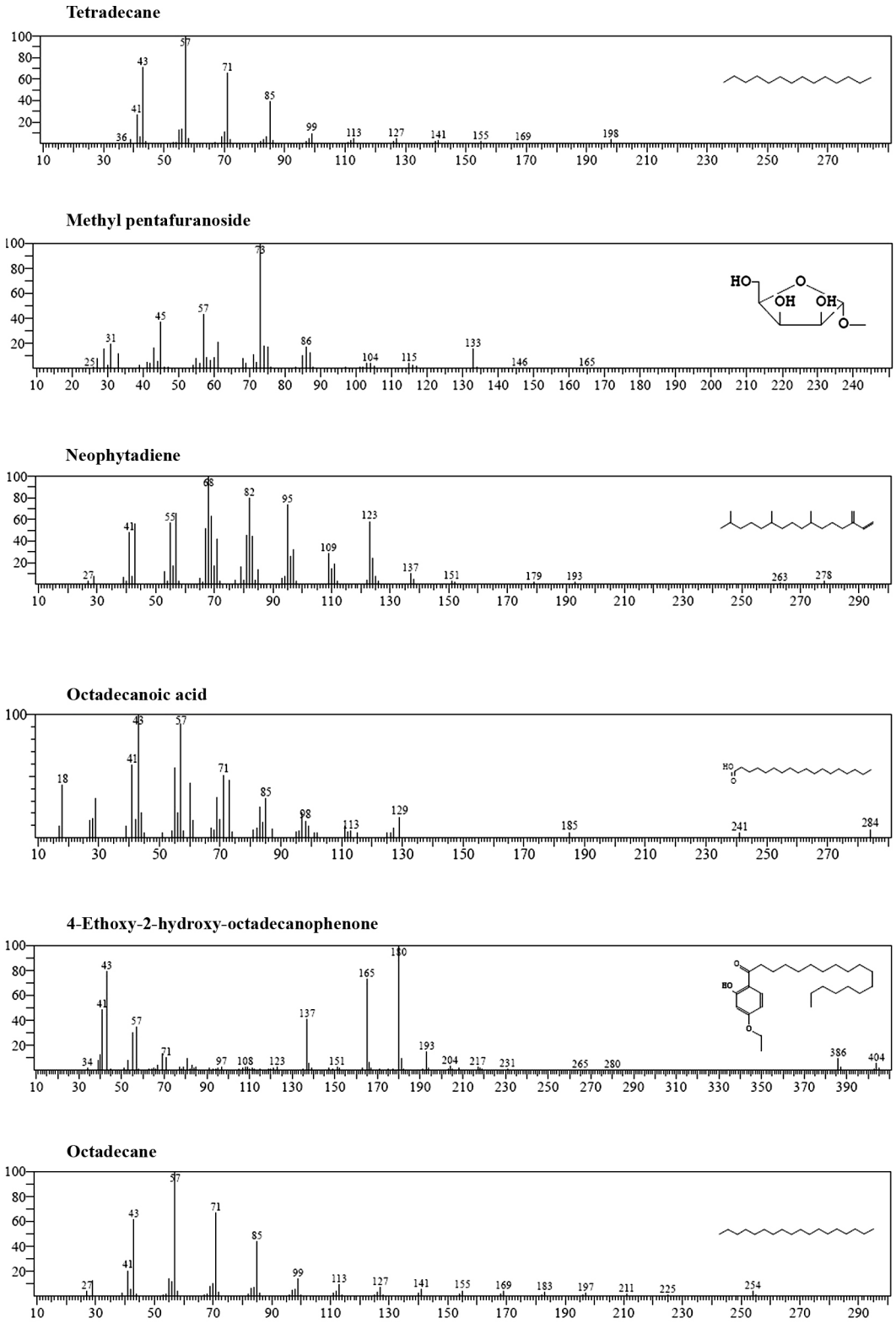


Fig. 18 (ii): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

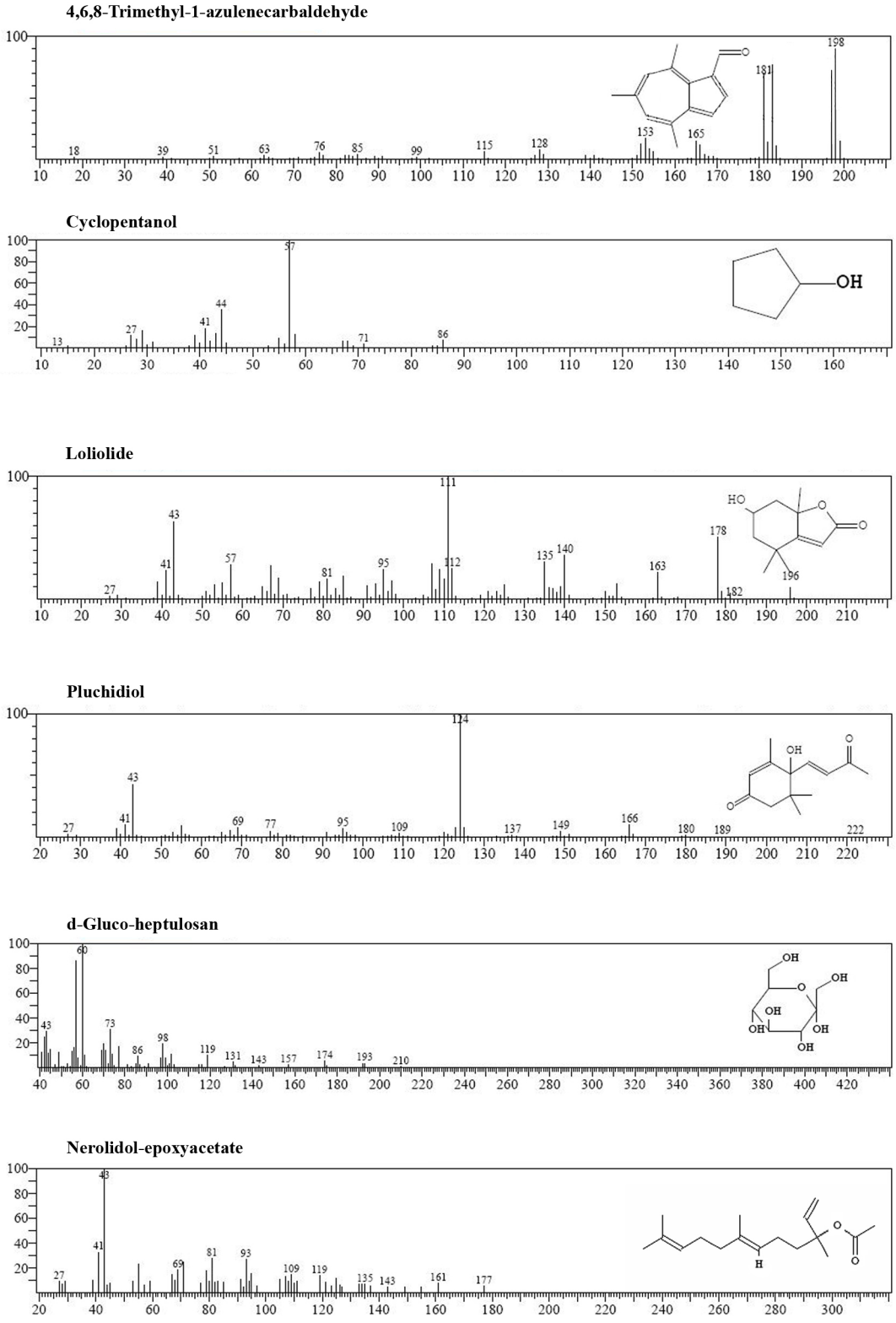


Fig. 18 (iii): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

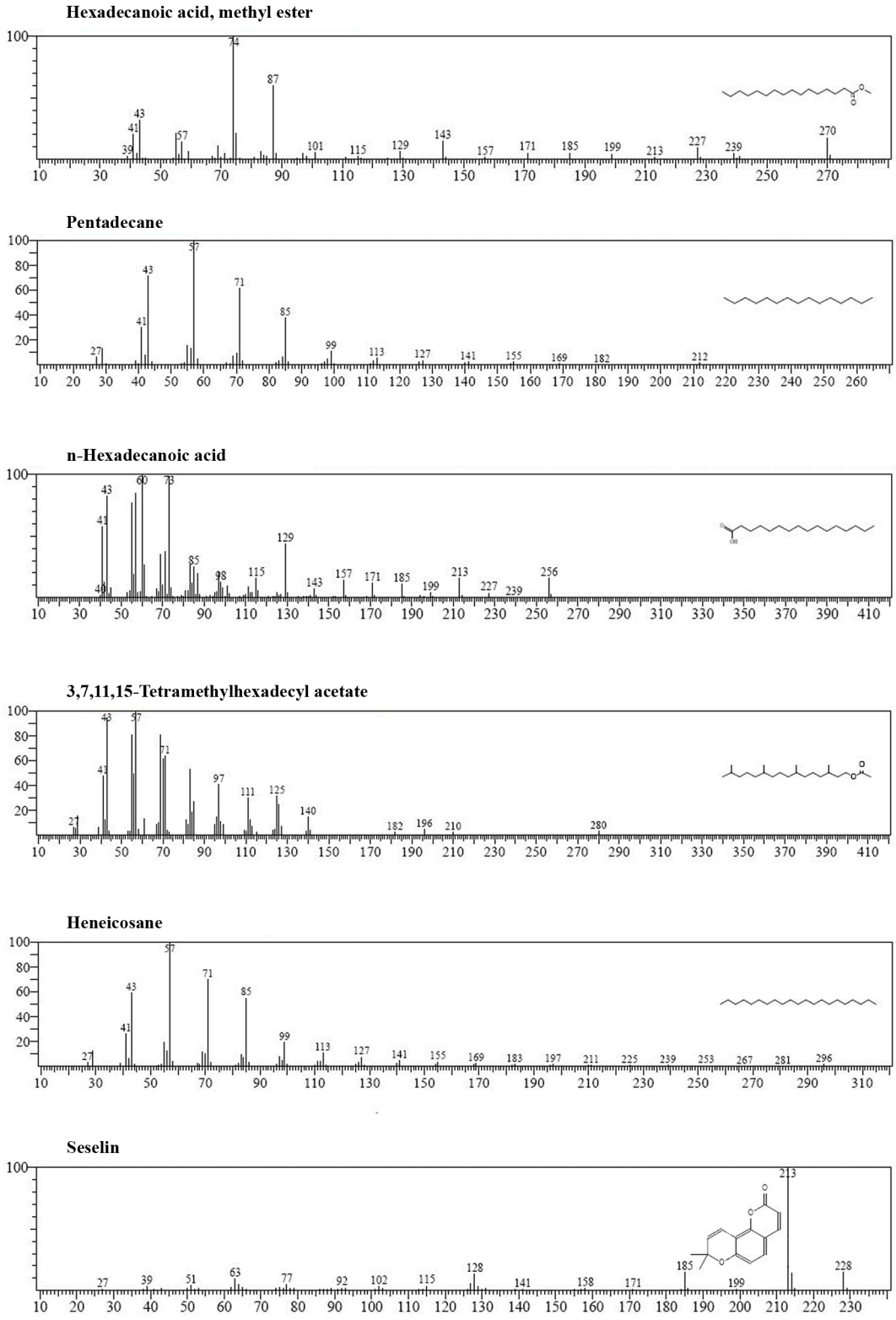


Fig. 18 (iv): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

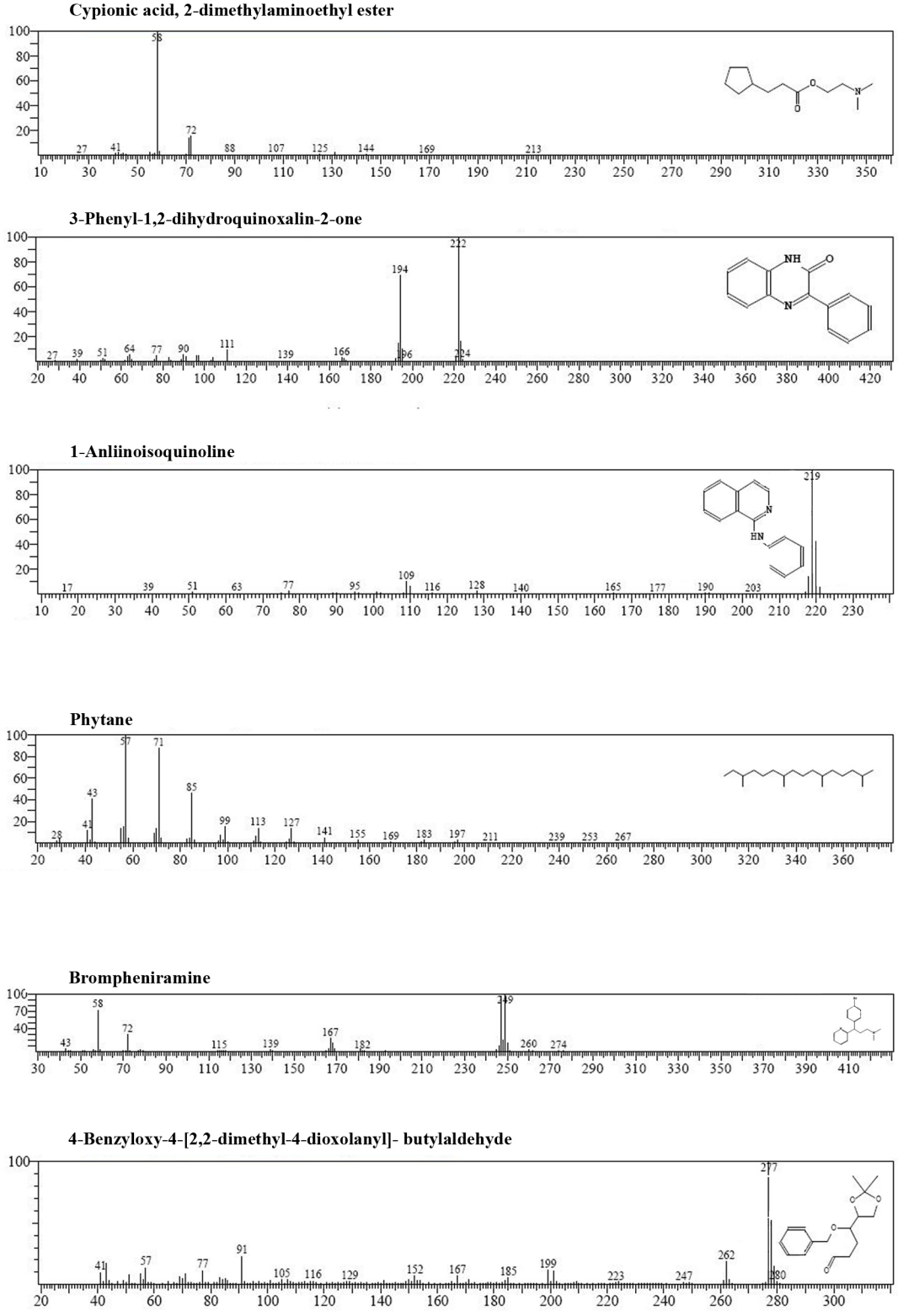


Fig. 18 (ix): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

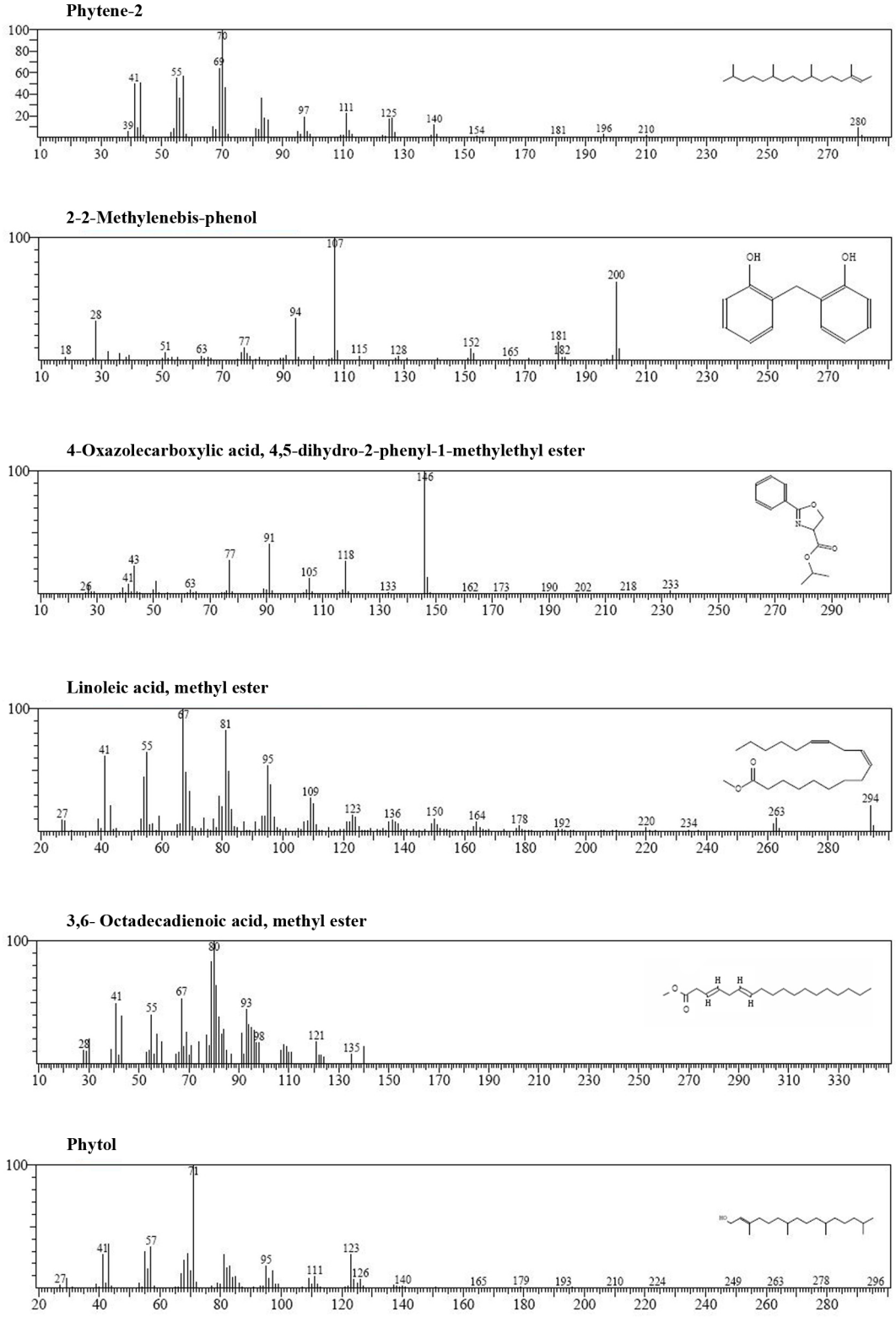


Fig. 18 (v): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

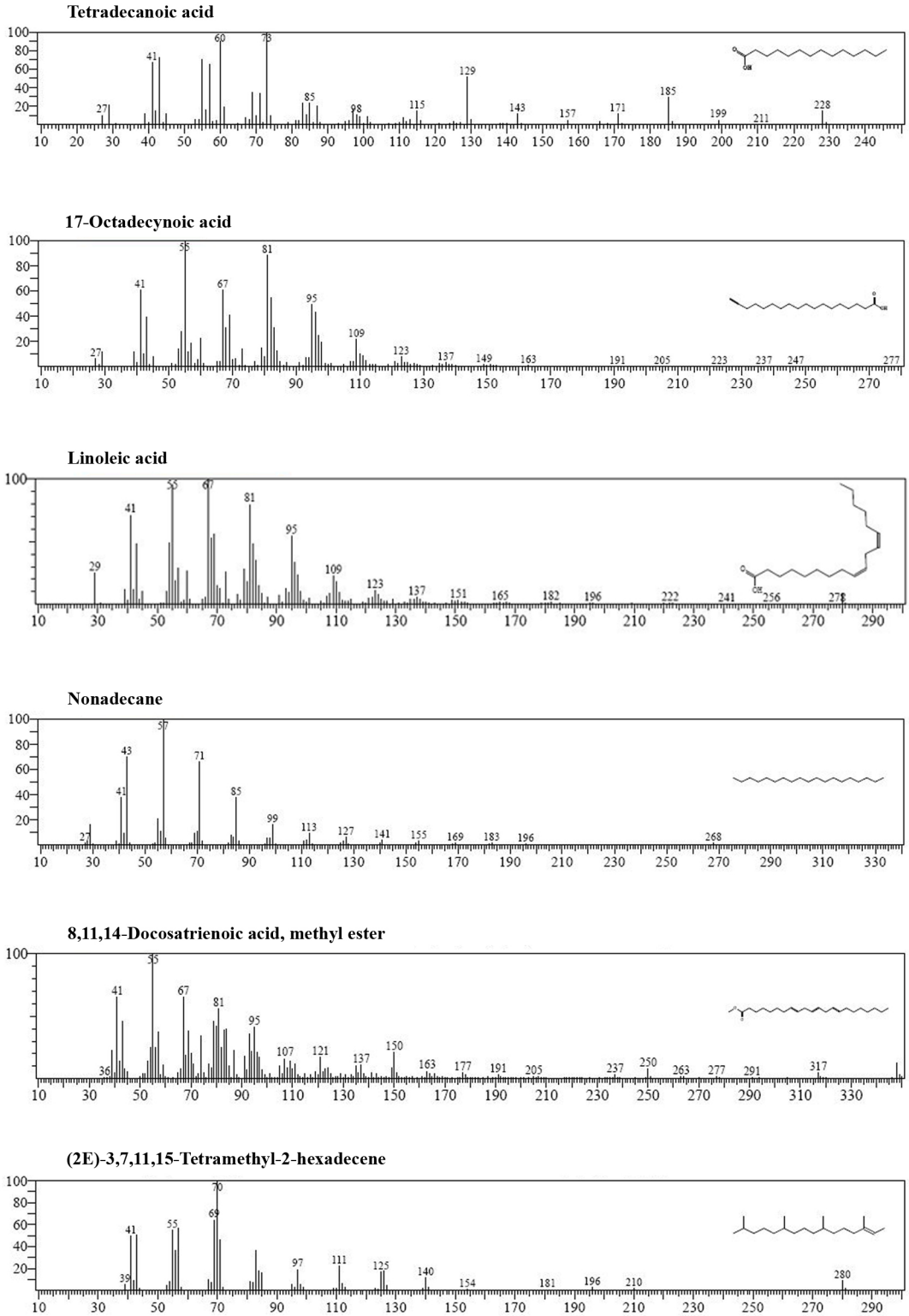


Fig. 18 (vi): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

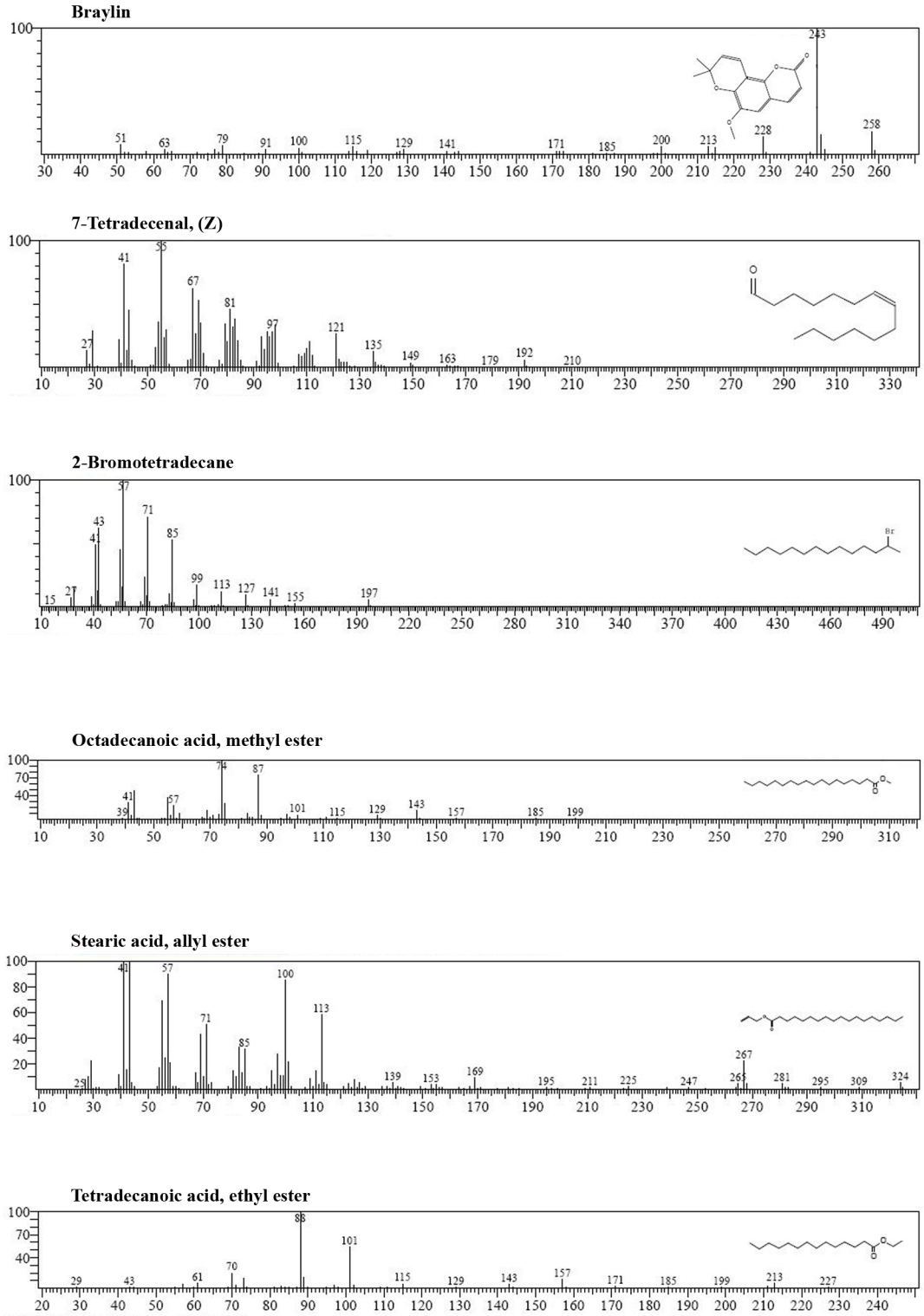
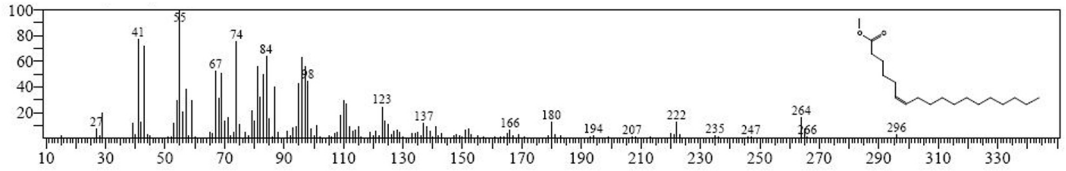
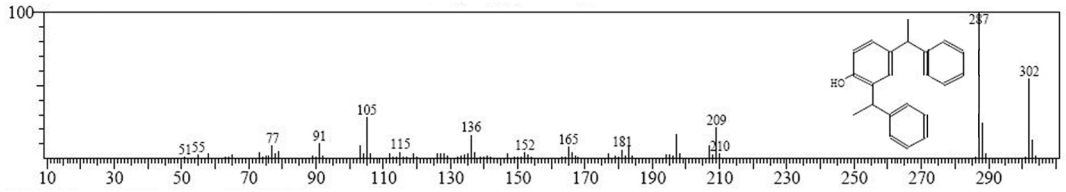


Fig. 18 (vii): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

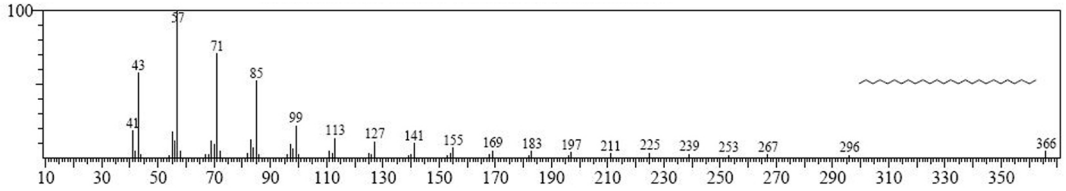
Petroselinic acid, methyl ester



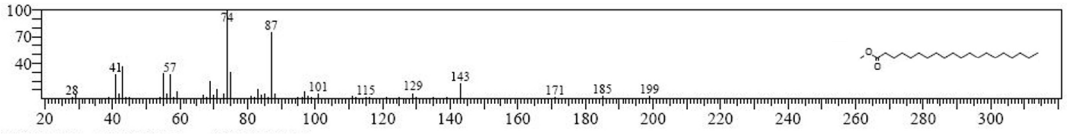
2,4-Bis (1-phenylethyl)-phenol



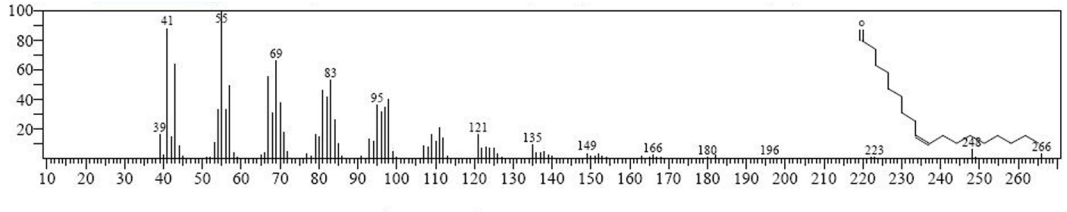
Hexacosane



Eicosanoic acid , methyl ester



9-Octadecenal, (Z)-



1-Hydroxy-4-methylanthraquinone

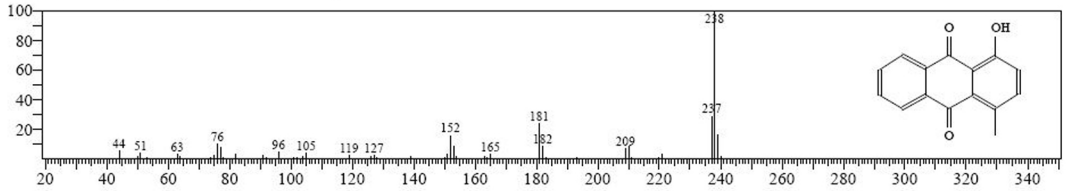


Fig. 18 (viii): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

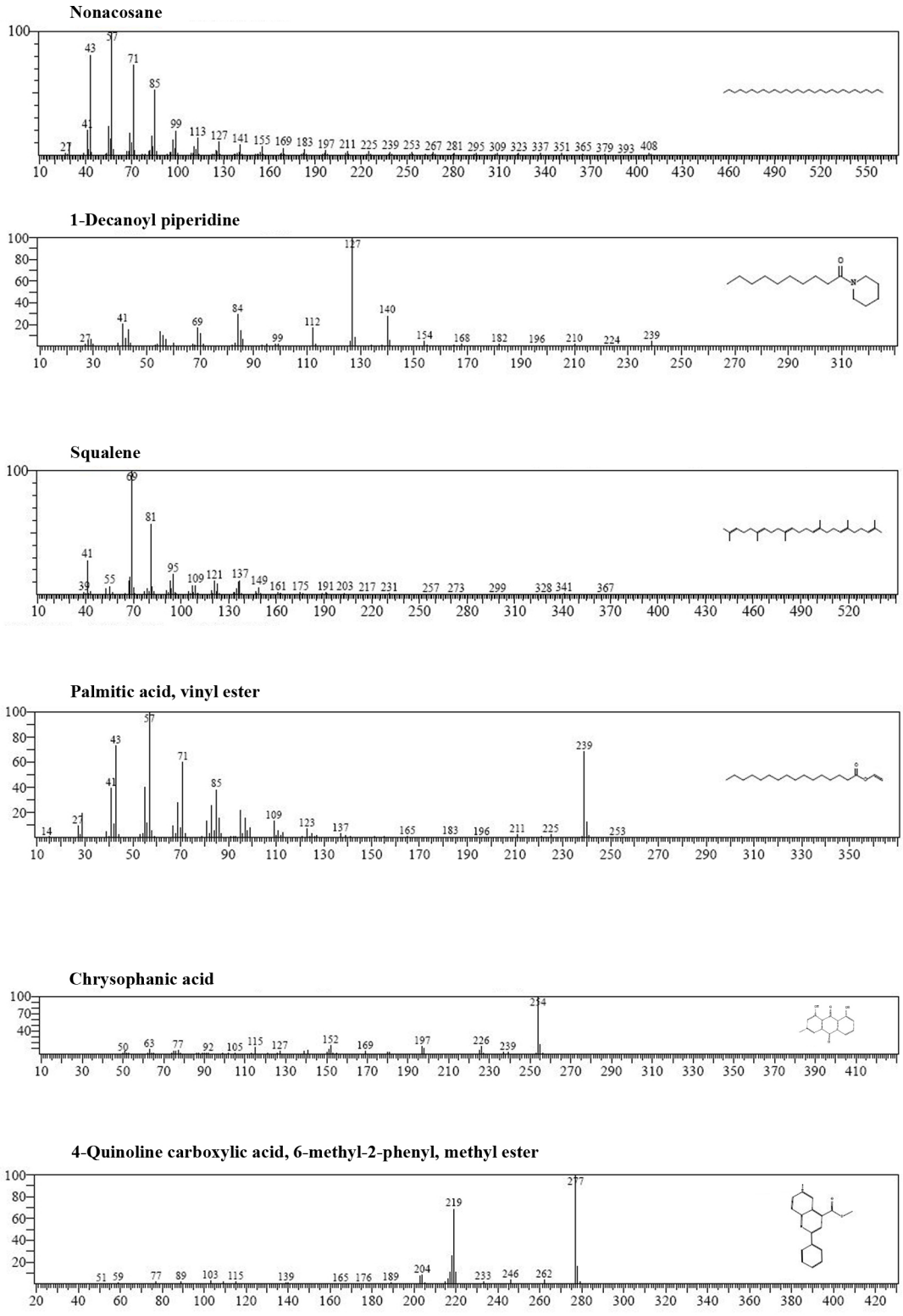


Fig. 18 (x): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

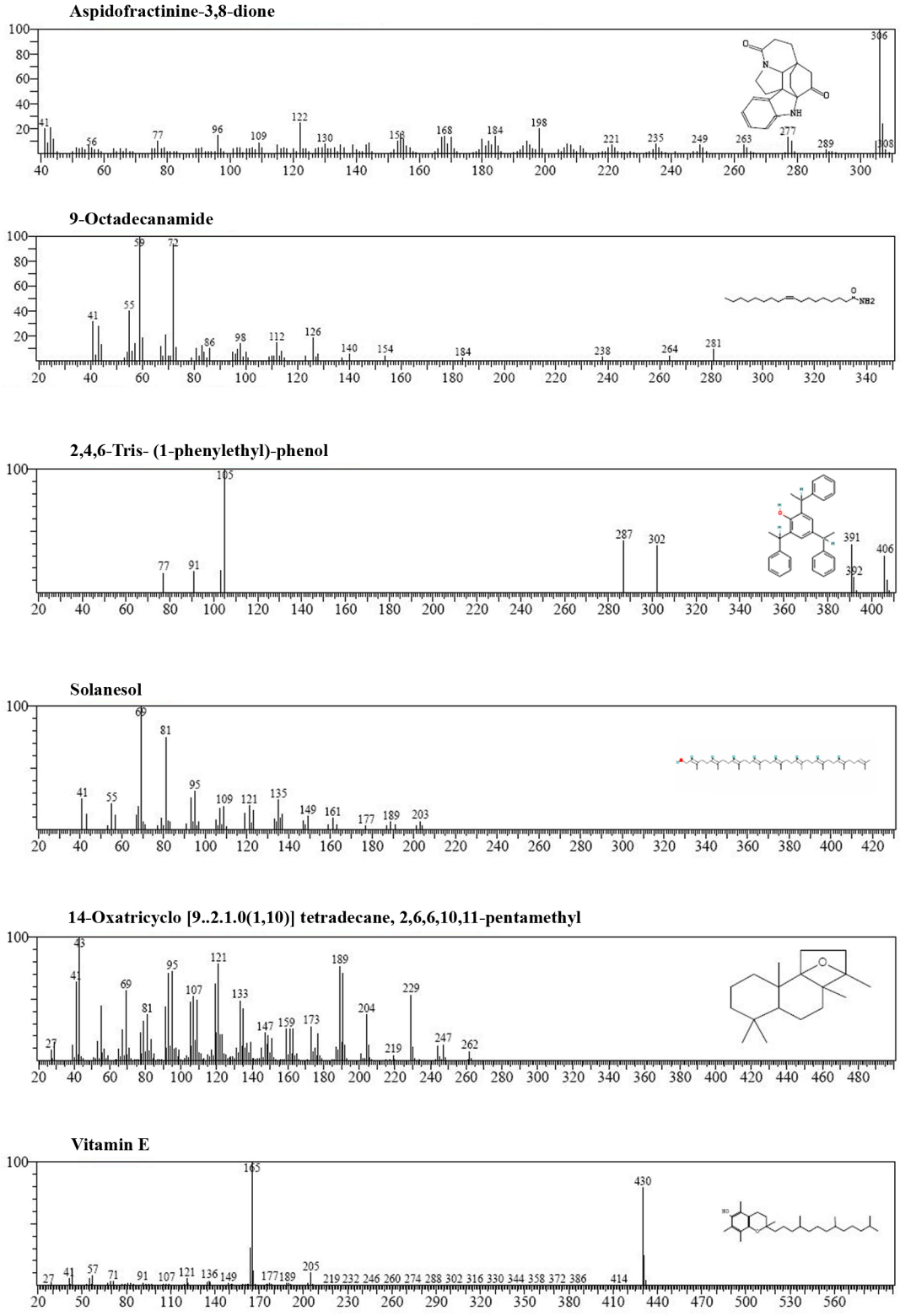


Fig. 18 (xi): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

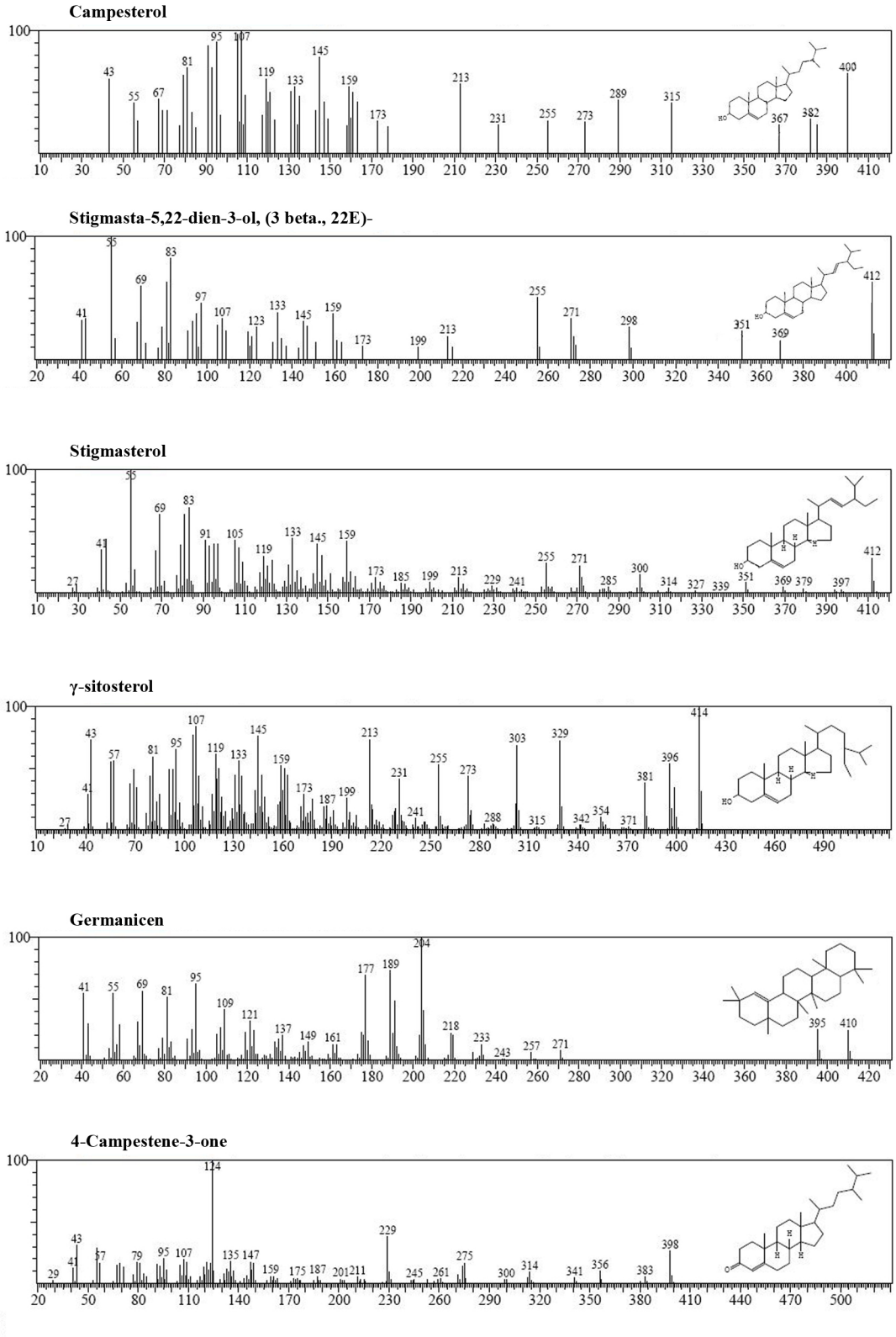


Fig. 18 (xii): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

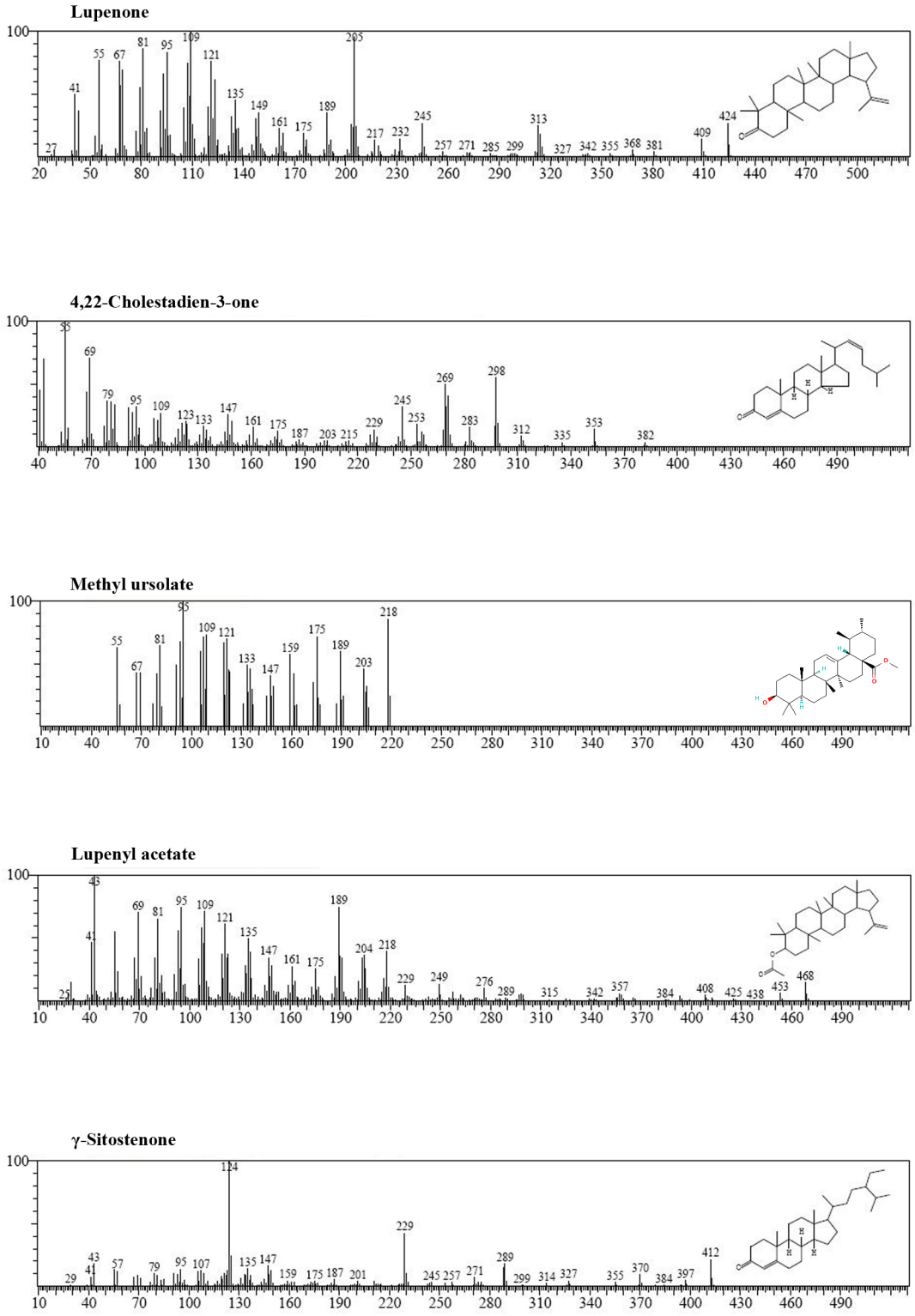


Fig. 18 (xiii): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

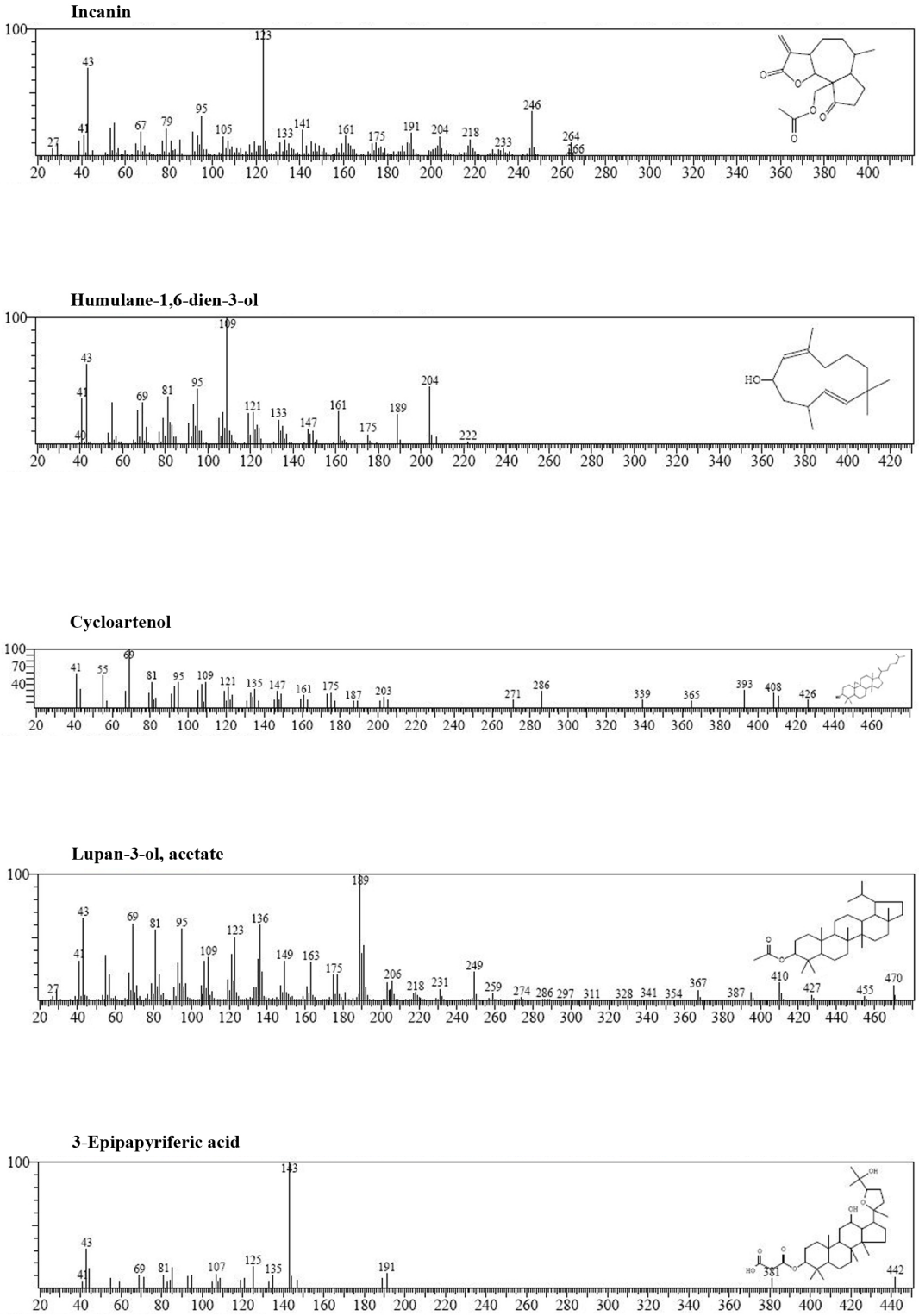


Fig. 18 (xiv): Mass spectra of compounds revealed by GC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

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

The non-volatile components in the methanolic extract of the selected *Ophiorrhiza* species were recognised by HR LC-MS analysis. A total of 108 compounds belonging to different classes were identified. Punaglandin 5 was the compound eluted at the lowest retention time of 0.991 min. The highest retention time was 26.962 min at which a biopeptide was eluted. The major classes of compounds identified were saccharides, biopeptides, fatty acids, terpenoids, phenols, flavonoids, glycosides, alkaloids and sterols. The non-volatile compounds identified by HR LC-MS analysis is given in **Table 14.**

A total of 24 compounds were revealed from the methanolic extract of *O. grandiflora,* in which the most important bioactive compounds were acarbose, 9,12,13-trihydroxy-10-octadecenoic acid, isoreserpine, sedoheptulose, loganic acid, monotropein, hesperidin and apiin. The liquid chromatogram of the methanolic extract of *O. grandiflora* is given in **Fig. 19.** In *O. jacobii,* 41 compounds were identified which included anatabine, 3-α(S)-strictosidine, lappaol B, caribine, swietenine, euphornin, acarbose, amarogentin, rutin, chrysin, rubiadin *etc*. The liquid chromatogram of the methanolic extract of *O. jacobii* is given in **Fig. 20.** Out of the 30 non-volatile compounds observed in *O. pectinata,* 8 compounds with reported bioactivities were identified. They were retinoid, 9,12,13-trihydroxy-10-octadecenoic acid, acarbose, naringenin, isoreserpine, apiin, hesperidin and lupanine. **Fig. 21** shows the liquid chromatogram of *O. pectinata.* In *O. trichocarpon,* 47 compounds were revealed from the HR LC-MS analysis of the methanolic extract. Among the four selected species, the highest number of compounds was eluted in this species. Many compounds with reported bioactivities were found and the major ones were pumiloside, stachyoside A, anatabine, caribine, N-caffeoyltryptophan, physalin O, limonin, swietenine, euphornin,

quinic acids, amarogentin, hesperidin, lespenefril, manniflavanone, tejedine, piperolactam-C, mosloflavone, 1-hydroxy-4-methylanthraquinone and chrysin. The liquid chromatogram of the methanolic extract of *O. trichocarpon* is given in **Fig. 22.** The mass spectra of individual compounds identified by HR LC-MS analysis of the methanolic extracts of the selected species of *Ophiorrhiza* are given in **Figs 23 (i-xviii).**

Table 14: Phytocompounds revealed by HR LC-MS analysis of the selected species of *Ophiorrhiza*

Sl. No.	RT (min)	Compound	Class	Molecular mass	OG	OJ	OP	OT
1.	0.991	Punaglandin 5	Fatty acyl	498.203	+	-	-	-
2.	1.042	Maltose	Disaccharide	342.116	-	-	+	-
3.	1.051	α,α-Trehalose	Disaccharide	342.116	+	-	+	-
4.	1.071	Sedoheptulose	Monosaccharide	210.073	+	-	-	-
5.	1.075	Quinic acid	Carboxylic acid	192.062	-	-	-	+
6.	1.361	His Gly Val	Biopeptide	311.161	-	-	+	-
7.	2.642	Tyr Tyr Ser	Biopeptide	431.170	-	-	+	-
8.	2.774	Cys Cys Lys	Biopeptide	352.124	+	-	+	-
9.	2.852	3-Iodo-hexadecanoic acid	Fatty acid	382.136	-	-	+	-
10.	3.211	2-Iodo-hexadecanoic acid	Fatty acid	382.136	-	-	+	-
11.	3.73	Asn Phe Asp	Biopeptide	394.149	+	-	+	-
12.	3.758	Loganic acid	Monoterpene	376.137	+	-	-	-
13.	3.826	Veranisatin C	Sesquiterpenoid	372.102	-	+	-	+
14.	4.332	Caffeic acid-3-glucoside	Phenol	342.092	-	-	-	+
15.	4.408	Monotropein	Monoterpenoid	390.116	+	-	-	-
16.	4.776	7-Epiloganin tetraacetate	Terpenoid	558.184	-	-	+	-
17.	5.022	Apiin	Flavonoid	564.150	+	-	+	-
18.	5.149	Pumiloside	Glycoside	512.174	-	-	-	+

19.	5.29	Stachyoside A	Alkaloid	526.190	-	-	-	+
20.	5.461	Asp Trp Trp	Biopeptide	505.199	+	-	-	-
21.	5.478	Arg Glu	Biopeptide	303.152	+	-	-	-
22.	5.504	Retinoid	Diterpenoid	296.215	-	-	+	-
23.	5.519	Uridine monophosphate	Nucleotide	324.039	+	-	-	-
24.	5.595	Anatabine	Alkaloid	160.100	-	+	-	+
25.	5.603	Trp Tyr Tyr	Biopeptide	530.229	+	-	-	-
26.	5.623	Tyr Ile Trp	Biopeptide	480.236	+	-	-	-
27.	5.639	Phe Met	Biopeptide	296.119	-	-	+	-
28.	5.790	9,12,13-Trihydroxy-10-octadecenoic acid	Fatty acid	330.234	+	-	+	-
29.	5.83	Acarbose	Trisaccharide	552.240	+	+	+	-
30.	6.024	Hesperidin	Flavonoid	610.193	+	-	+	+
31.	6.066	Gln Asn Gln	Biopeptide	388.170	+	-	+	-
32.	6.077	6-Methoxyluteolin 7-[6"-(2-methylbutyl) glucoside]	Flavonoid	562.168	-	+	-	-
33.	6.087	3-Amino-L-tyrosine	Amino acid	196.085	-	+	-	-
34.	6.574	2,3,4,5,6-Penta-O-acetyl-D-glucose	Carboxylic acid	390.113	-	+	-	-
35.	6.705	Tyr Trp His	Biopeptide	504.212	-	+	-	-
36.	7.254	Deoxyloganin tetraacetate	Monoterpene	542.193	+	-	-	-
37.	7.328	Pro Pro Lys	Biopeptide	340.212	-	+	-	-

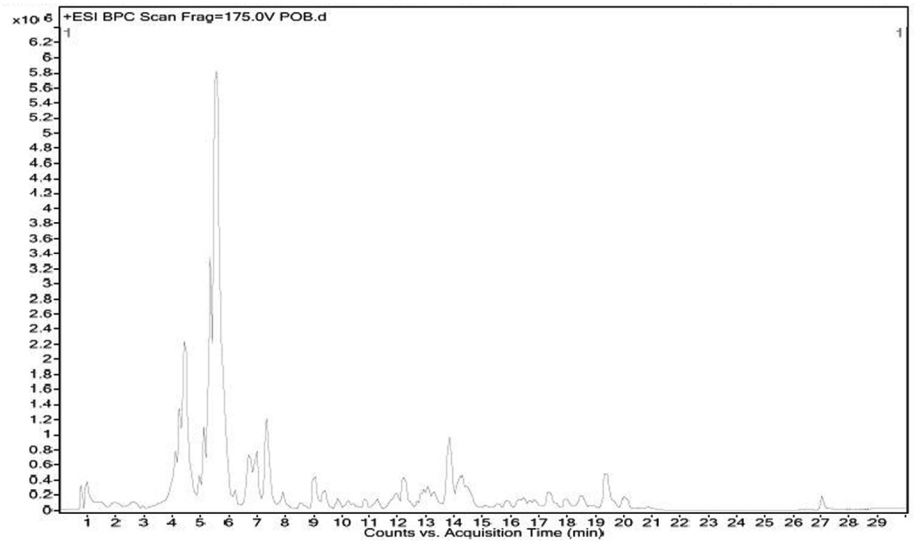
38.	7.71	Ancymidol	Alcohol	256.121	-	-	-	+
39.	7.78	Ile Met Tyr	Biopeptide	425.201	-	-	+	-
40.	7.887	Naringenin	Flavonoid	272.071	-	-	+	-
41.	8.024	Trp Asn Phe	Biopeptide	465.199	-	+	-	-
42.	8.133	3- α(S)-strictosidine	Alkaloid	530.224	-	+	-	-
43.	8.22	Sagecoumarin	Phenol	536.091	-	-	-	+
44.	8.225	(-)-Pinoresinol glucoside	Phenol	520.193	-	+	-	-
45.	8.403	Phe Phe	Biopeptide	312.147	-	+	-	+
46.	8.555	Lappaol B	Flavonoid	550.220	-	+	-	-
47.	8.794	Amorphigenin O-glucoside	Flavonoid	572.195	-	-	-	+
48.	8.885	Lys Pro Ala	Biopeptide	314.196	-	-	-	+
49.	8.939	Amarogentin	Glycoside	586.165	-	+	-	+
50.	8.975	Lespenefril	Flavonoid	578.163	-	-	-	+
51.	8.977	Gln Met Ala	Biopeptide	348.146	-	-	-	+
52.	8.984	Clematin	Flavonoid	610.187	-	+	-	-
53.	9.056	Manniflavanone	Flavonoid	590.113	-	-	-	+
54.	9.113	Phloretin 3',5'-Di-C-glucoside	Flavonoid	598.188	-	-	-	+
55.	9.262	Rutin	Flavonoid	610.152	-	+	-	-
56.	9.66	Caribine	Alkaloid	326.163	-	+	-	+
57.	10.091	8-Hydroxy-9,11-octadecadiynoic acid	Fatty acid	292.203	-	-	+	-
58.	10.152	Tejedine	Alkaloid	668.274	-	-	-	+

59.	10.262	8-Oxo-9,11- octadecadiynoic acid	Fatty acid	290.191	-	-	+	-
60.	11.312	3-Methyl-octanoic acid	Fatty acid	158.131	-	-	-	+
61.	11.398	2"-O-α-L-Rhamnosyl-6-C-fucosyl-luteolin	Flavonoid	578.163	-	+	-	-
62.	11.4	Piperolactam C	Alkaloid	309.098	-	-	-	+
63.	11.834	9,14-Dihydroxy-10,12-octadecadienoic acid	Fatty acid	312.233	+	-	+	-
64.	11.843	Val His Arg	Biopeptide	410.237	+	-	+	-
65.	12.857	1-Hexadecanoyl-sn-glycerol	Monoacylglycerol	330.282	+	-	-	-
66.	13.04	N-Caffeoyltryptophan	Amino acid	366.120	-	-	-	+
67.	13.713	13R-Hydroxy-9E,11Z-octadecadienoic acid	Fatty acid	296.238	+	-	+	-
68.	13.784	Mosloflavone	Flavonoid	298.082	-	-	-	+
69.	13.974	9,13-Dihydroxy-11-octadecenoic acid	Fatty acid	314.247	+	-	-	-
70.	14.252	Pinellic acid	Fatty acid	330.239	-	+	-	+
71.	14.65	1-Hydroxy-4-methylanthraquinone	Anthraquinone	238.062	-	-	-	+
72.	15.403	Leu Arg Ala	Biopeptide	358.231	-	+	-	+
73.	15.783	Cucurbitacin A	Triterpene	574.307	-	-	+	-
74.	15.864	Physalin O	Sterol	528.198	-	-	-	+
75.	15.868	Limonin	Triterpenoid	506.216	-	-	-	+
76.	16.032	Punaglandin 3	Fatty acyl	496.190	-	-	-	+

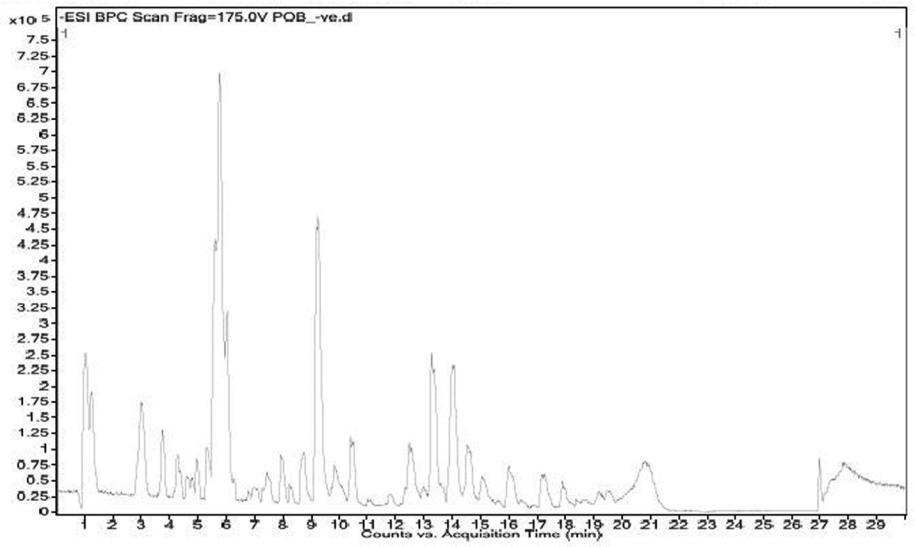
77.	16.033	Chrysin	Flavonoid	254.056	-	+	-	+
78.	16.262	Santin	Flavonoid	344.088	-	-	-	+
79.	16.506	8-Methoxy-13-hydroxy-9,11-octadecadienoic acid	Fatty acid	326.248	-	-	+	-
80.	16.739	Rubiadin	Anthraquinone	254.056	-	+	-	-
81.	16.844	8E-Tetradecenyl acetate	Fatty ester	254.225	-	+	-	+
82.	16.854	Isoreserpine	Alkaloid	608.270	+	-	+	-
83.	17.096	(9S,13S)-10-Oxo-11-phytoenoic acid	Fatty acyl	294.218	-	+	-	+
84.	17.153	11-Hydroxy palmitic acid	Fatty acid	272.237	-	-	+	-
85.	17.329	3S-Hydroxypalmitic acid	Fatty acid	272.236	-	+	-	-
86.	17.493	Palmitic acid	Fatty acid	256.240	-	+	-	+
87.	17.625	Hydroxyoctadecadienoic acid	Fatty acid	296.234	-	+	-	+
88.	17.813	12E-Pentadecenyl acetate	Fatty ester	268.240	-	+	-	-
89.	18.109	Thalidasine	Alkaloid	652.318	-	-	-	+
90.	19.098	8-Hydroxy octadecanoic acid	Fatty acid	300.269	-	-	+	-
91.	19.294	16-Hydroxy hexadecanoic acid	Fatty acid	272.233	-	+	-	-
92.	19.701	Ergostane	Triterpene	386.390	-	+	-	+
93.	19.798	Swietenine	Triterpene	568.267	-	+	-	-
94.	20.001	4-Methyl-cholest-5-en-3β-ol	Sterol	400.369	-	-	-	+
95.	20.033	12R-Hydroxy octadecanoic acid	Fatty acid	300.265	-	+	-	+
96.	20.084	Ergostanol	Sterol	402.385	-	+	-	+

97.	20.18	Aralionine A	Alkaloid	582.287	-	+	-	+
98.	20.27	Euphornin	Diterpene	584.298	-	+	-	+
99.	20.603	Diepomuricanin A	Ether	546.465	-	+	-	+
100.	20.616	Anhydrorhodovibrin	Tetraterpene	566.453	-	+	-	+
101.	20.659	Spheroidene	Tetraterpene	568.469	-	+	-	+
102.	20.834	14-Hydroxy-heneicosanoic acid	Fatty acid	342.313	-	+	-	-
103.	20.862	Lupanine	Alkaloid	248.190	-	-	+	-
104.	20.995	Nervonoylacetone	Ketone	406.378	-	+	-	-
105.	21.276	Sulfated dihydromenaquinone-9	Quinone	882.583	-	+	-	-
106.	21.672	18Z, 21Z-Heptacosadien-10-one	Fatty acyl	390.385	-	-	-	+
107.	24.783	Artemoin A	Acetogenin	550.492	-	-	-	+
108.	26.962	Lys Lys	Biopeptide	274.202	-	-	+	-

RT – Retention time; **OG –** *O. grandiflora*; **OJ –** *O. jacobii*; **OP –** *O. pectinata*; **OT –** *O. trichocarpon*

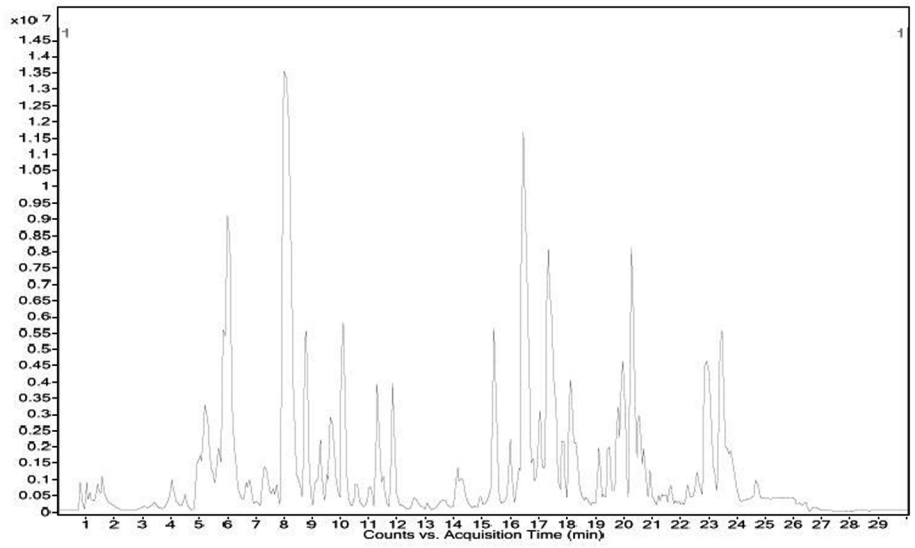


a

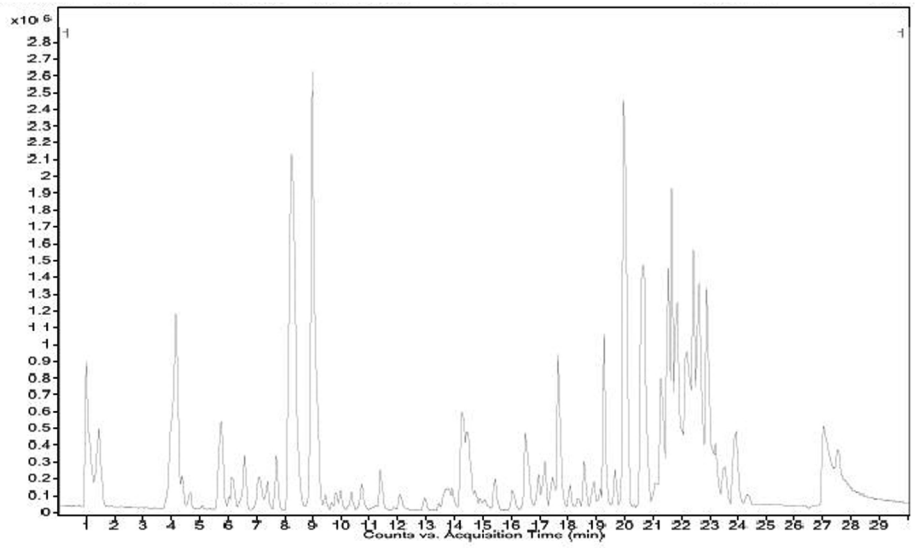


b

Fig. 19: Liquid chromatogram of methanolic extract of *Ophiorrhiza grandiflora.*
a: Positive ionisation mode**; b.** Negative ionisation mode.

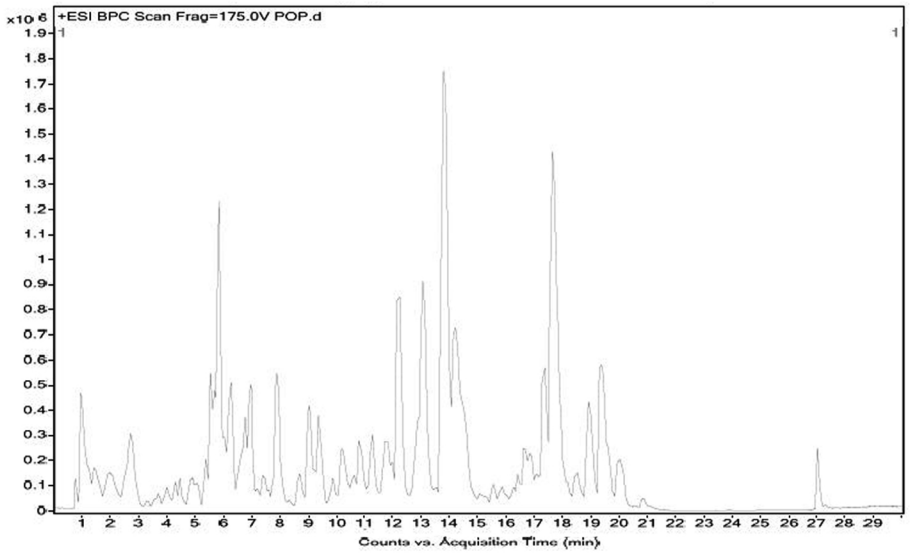


a

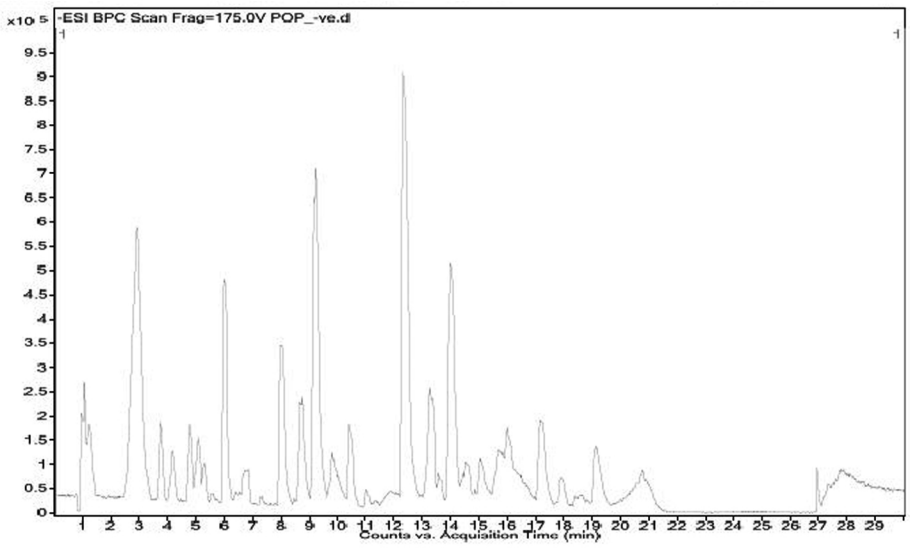


b

Fig. 20: Liquid chromatogram of methanolic extract of *Ophiorrhiza jacobii.*
a: Positive ionisation mode**; b.** Negative ionisation mode.

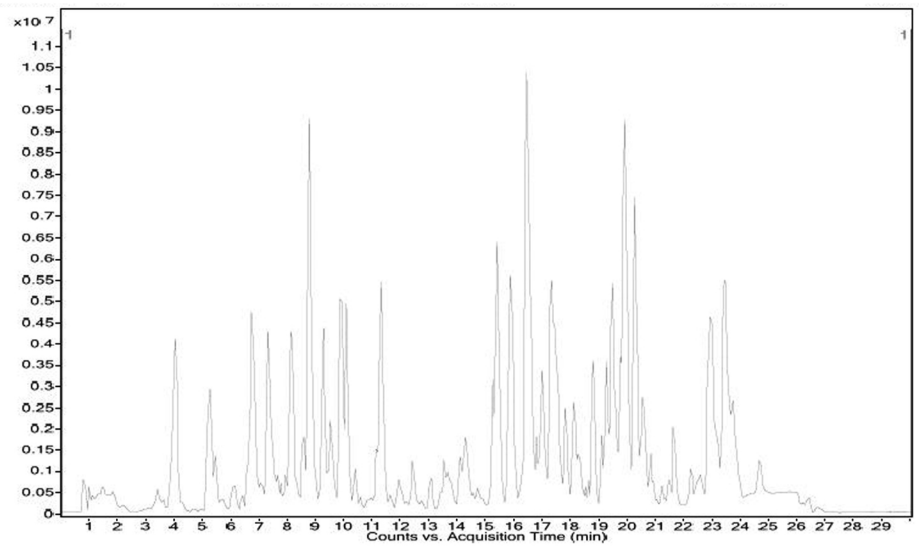


a

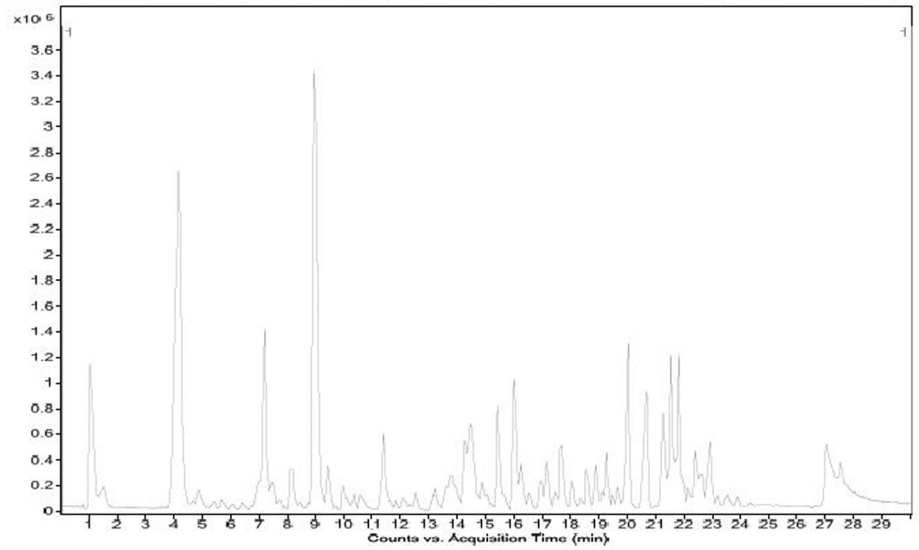


b

Fig. 21: Liquid chromatogram of methanolic extract of *Ophiorrhiza pectinata.*
a: Positive ionisation mode**; b.** Negative ionisation mode.



a



b

Fig. 22: Liquid chromatogram of methanolic extract of *Ophiorrhiza trichocarpon.*
a: Positive ionisation mode**; b.** Negative ionisation mode.

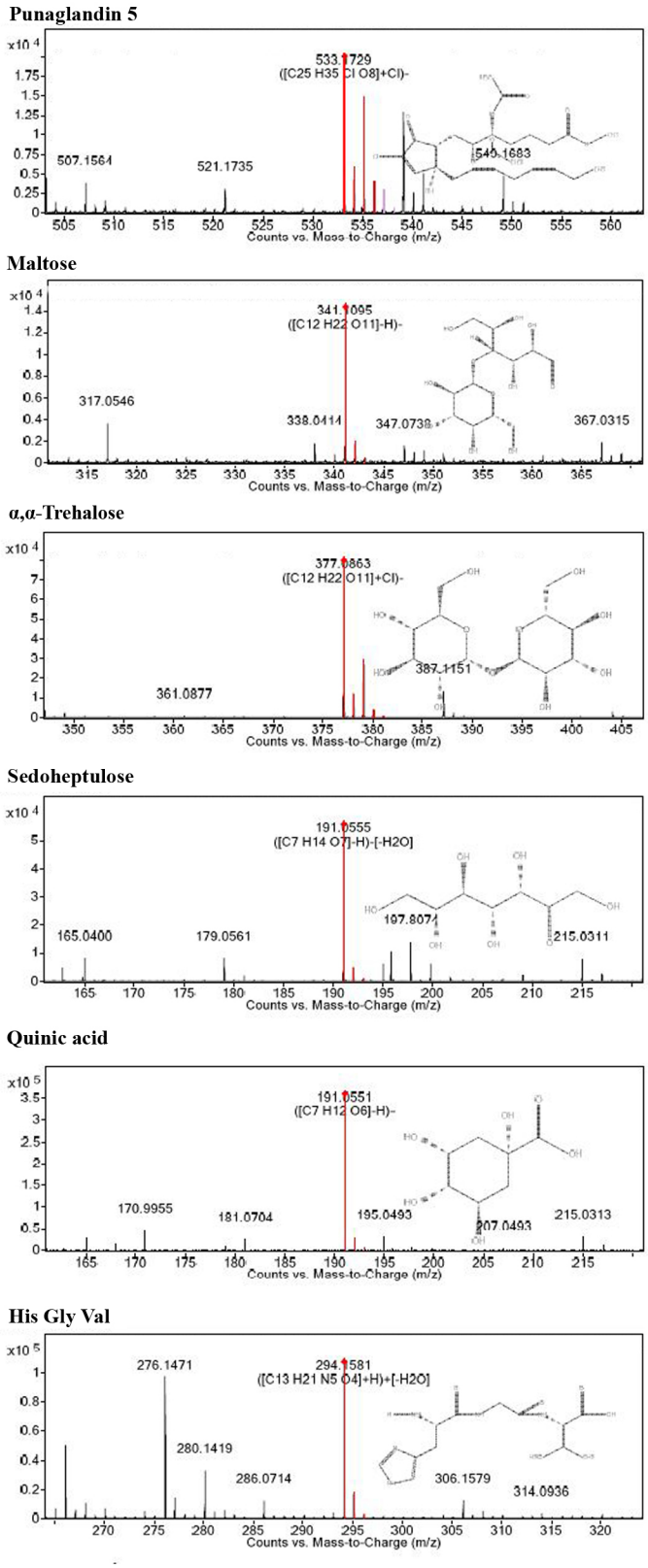


Fig. 23 (i): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

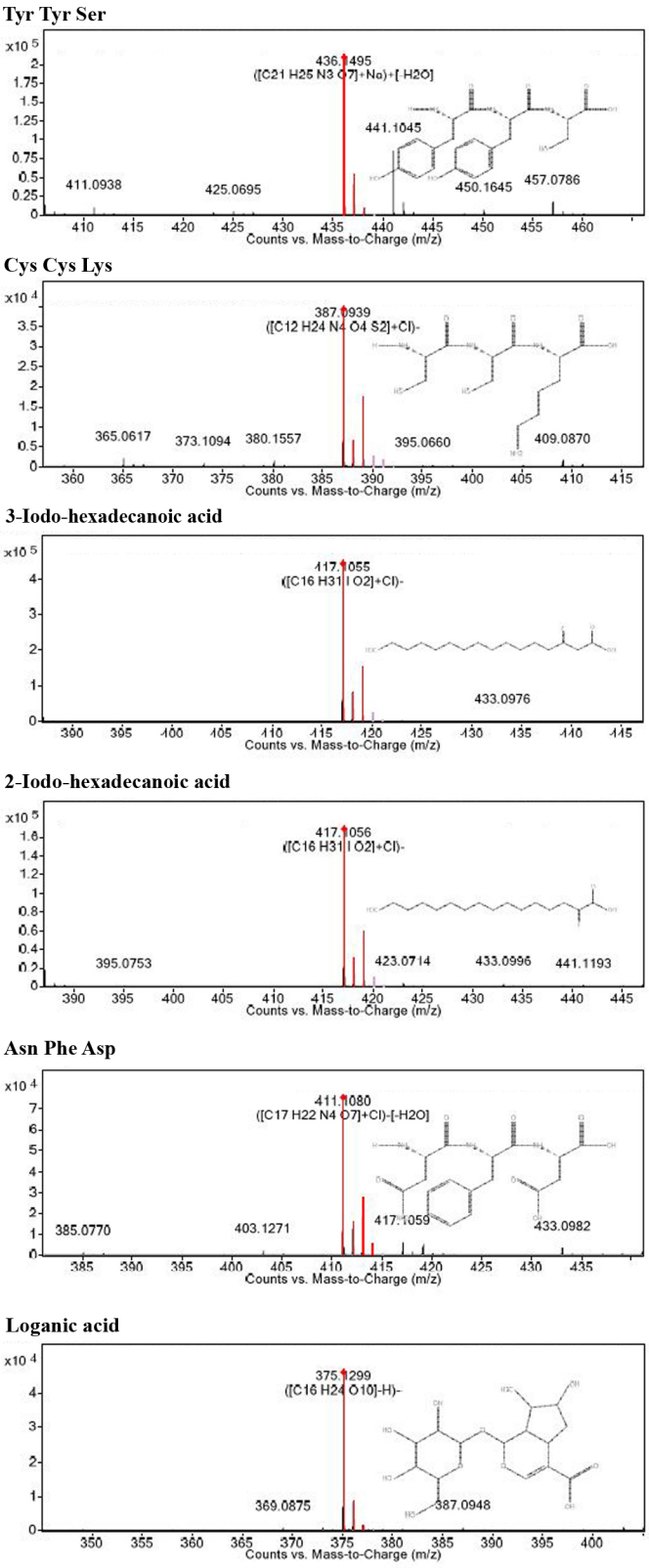
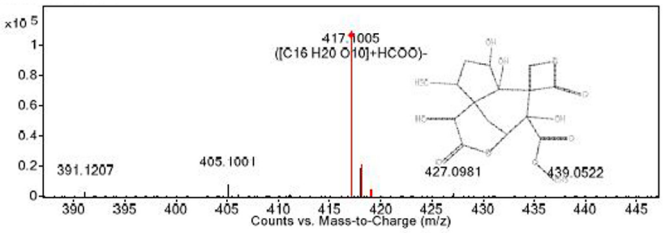
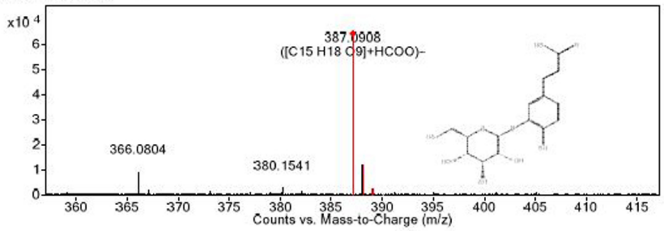


Fig. 23 (ii): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

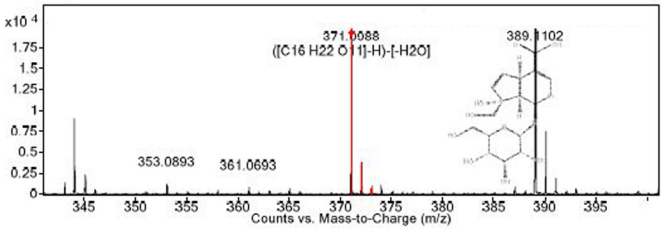
Veranisatin C



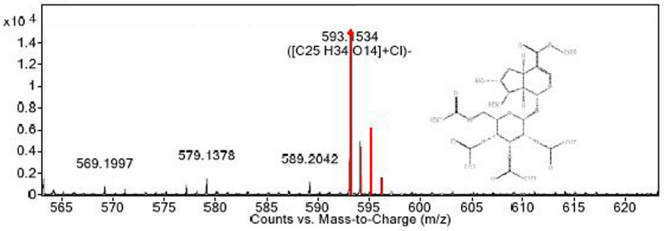
Caffeic acid-3-glucoside



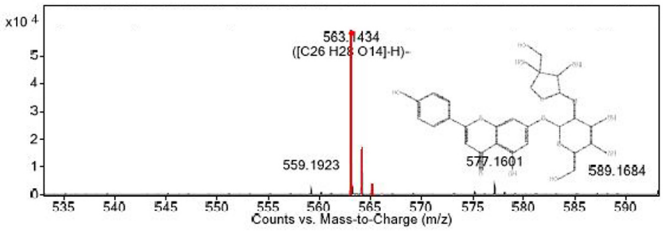
Monotropein



7-Epiloganin tetraacetate



Apiin



Pumiloside

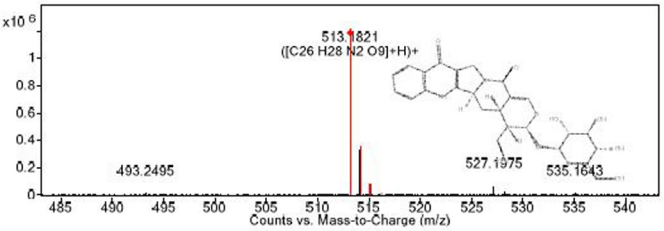


Fig. 23 (iii): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

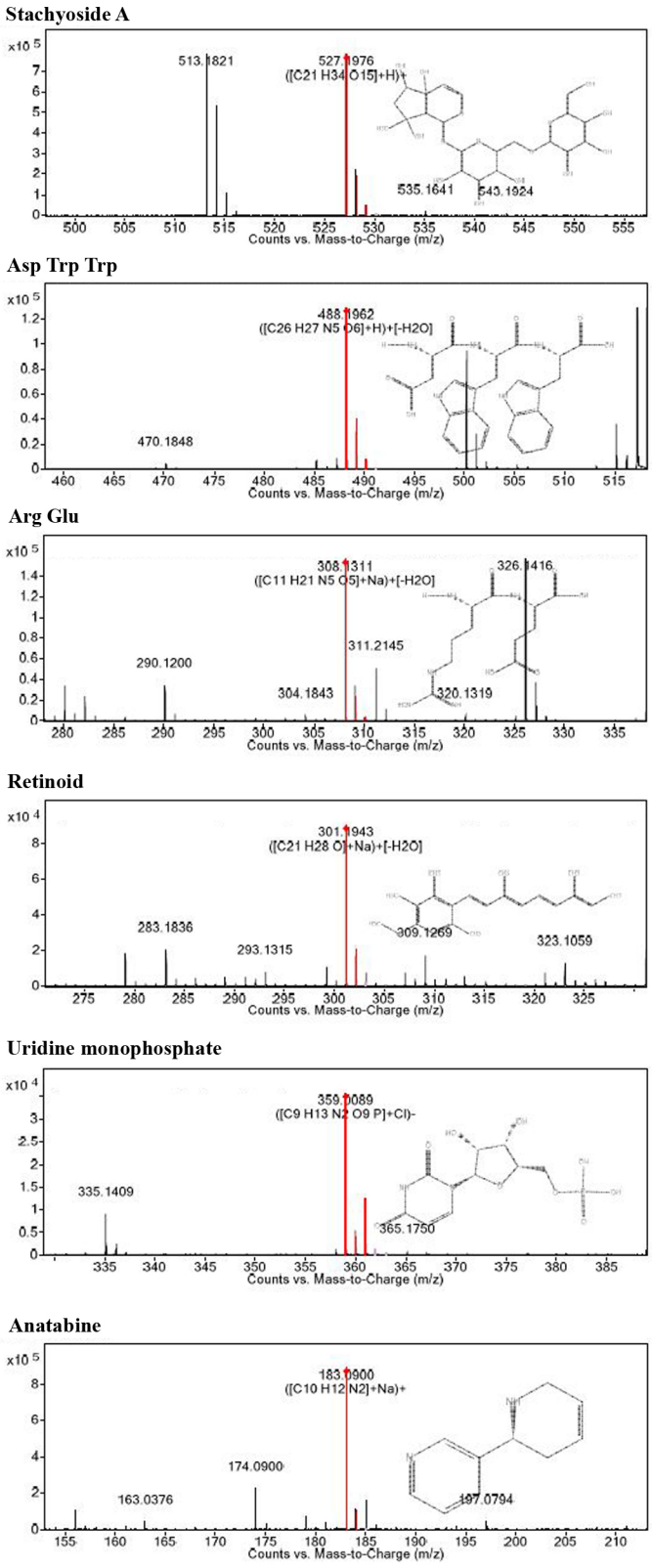


Fig. 23 (iv): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

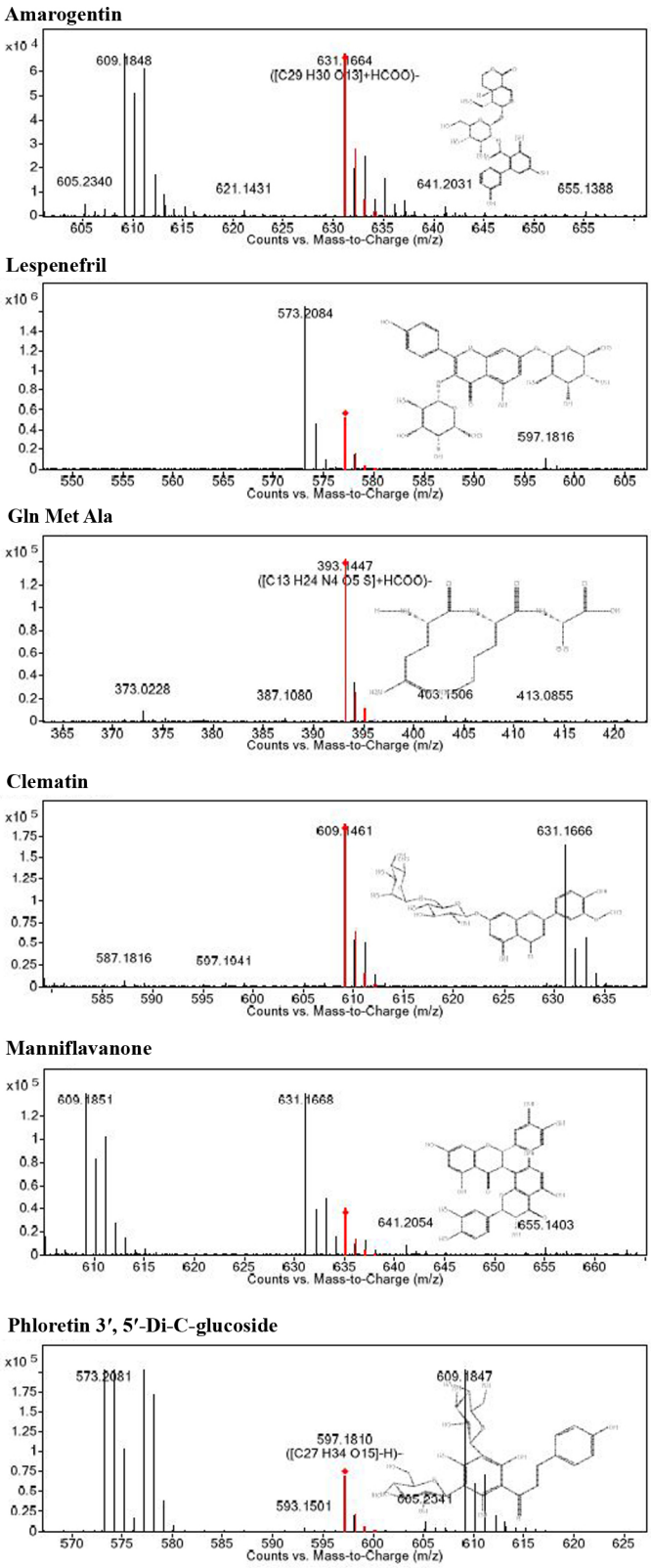


Fig. 23 (ix): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

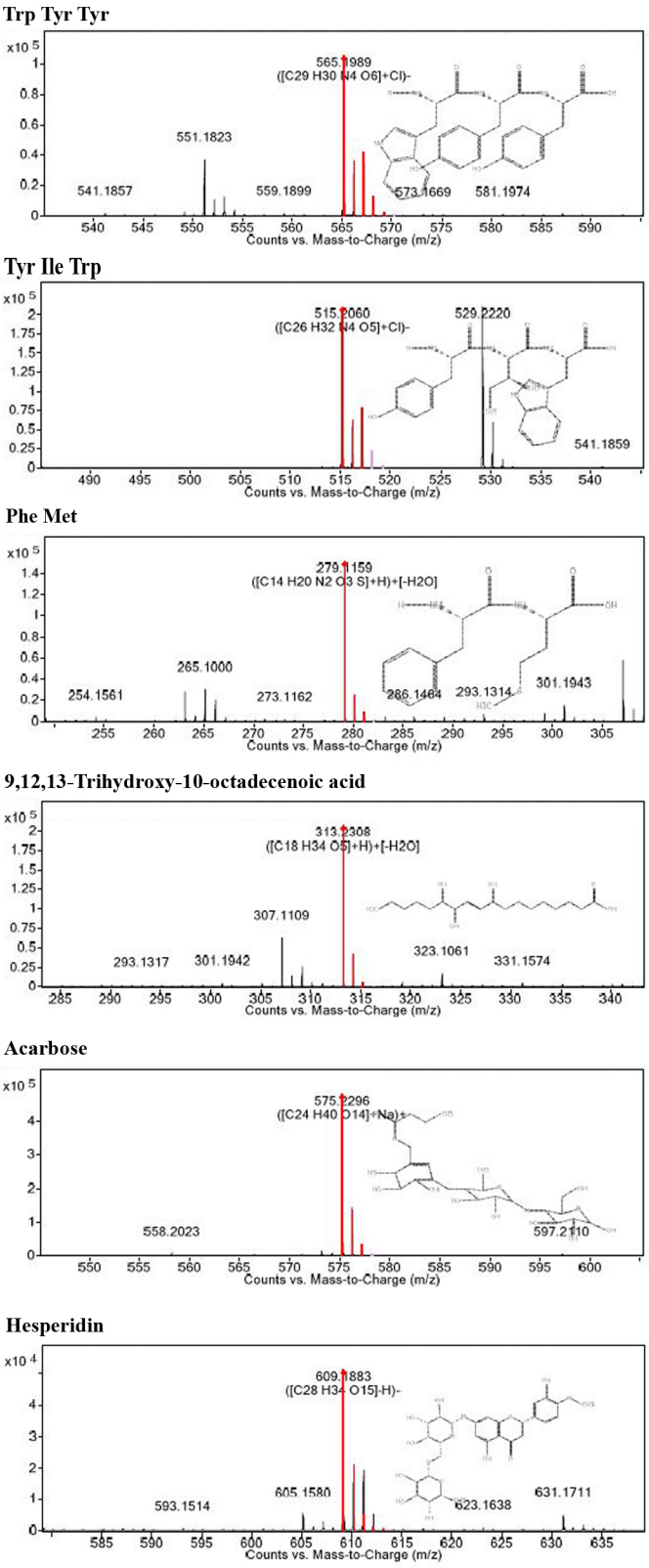


Fig. 23 (v): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

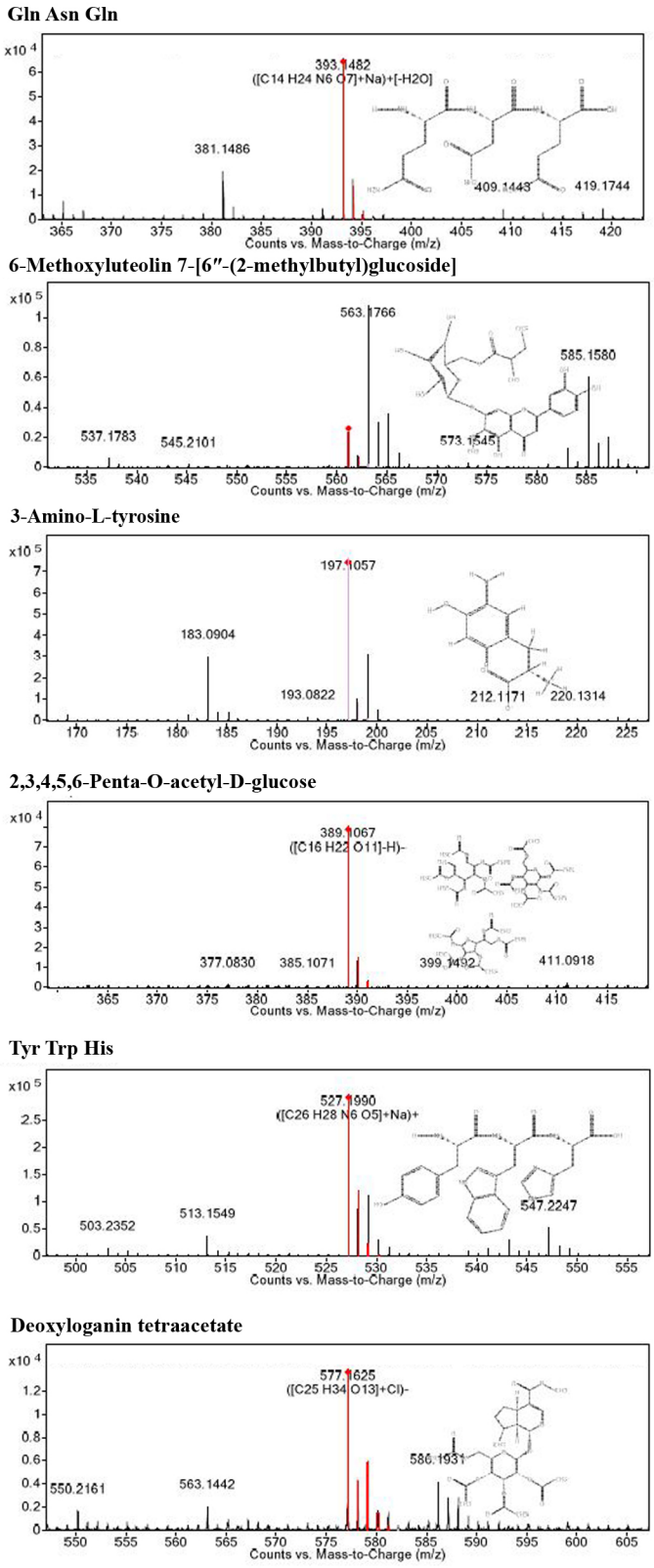


Fig. 23 (vi): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

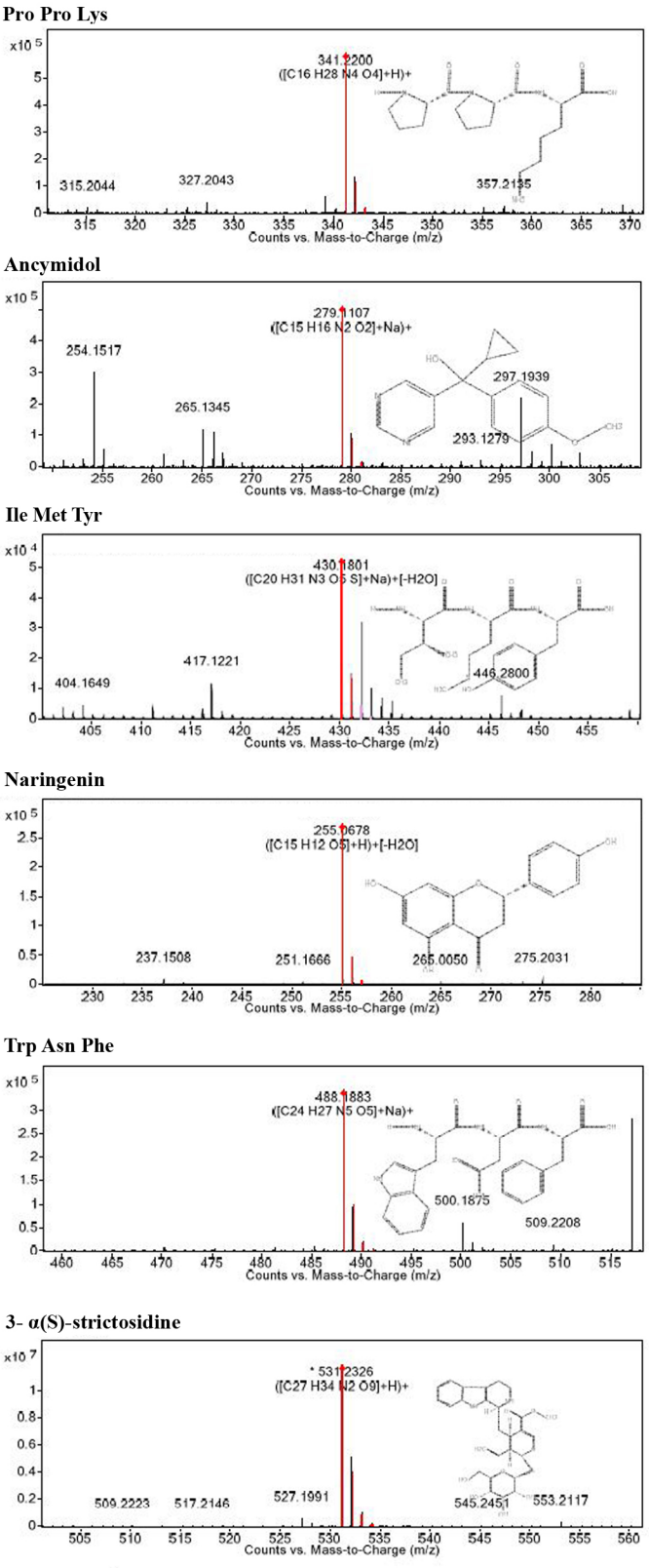


Fig. 23 (vii): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

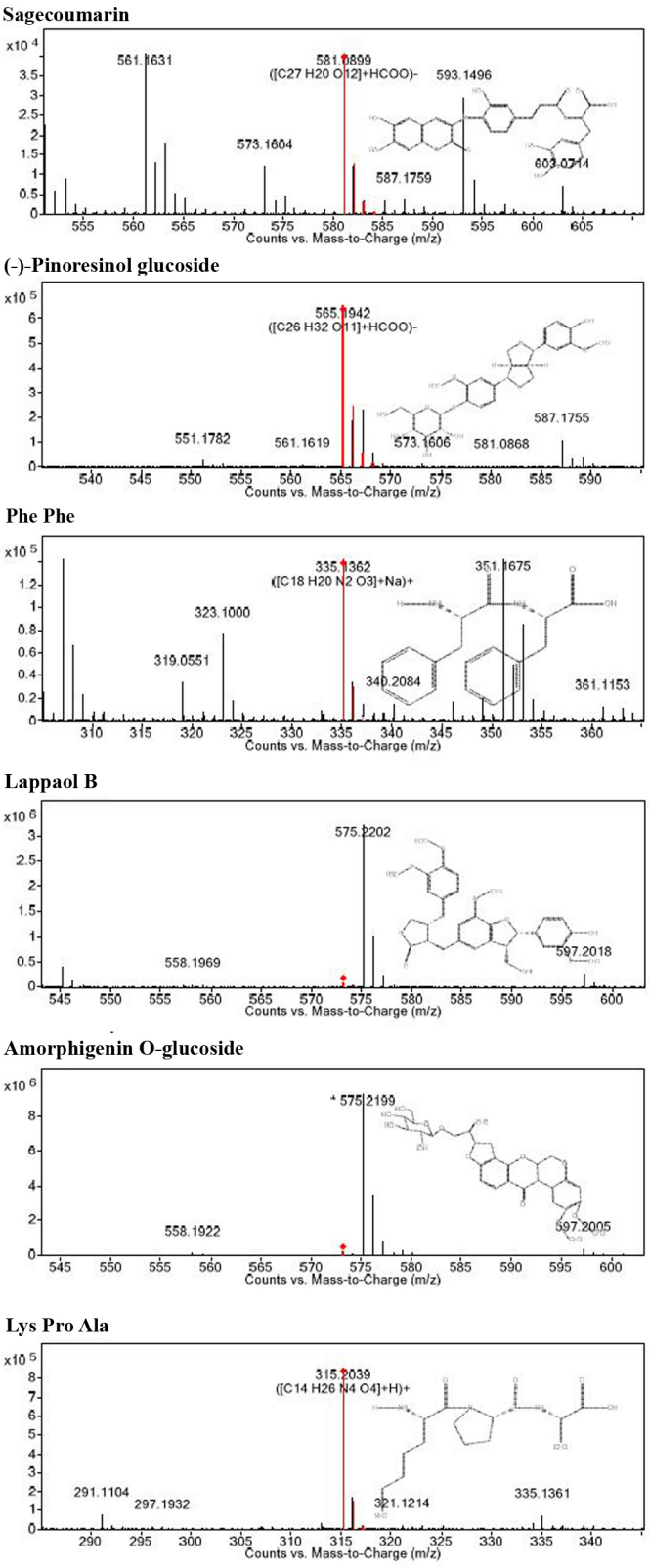
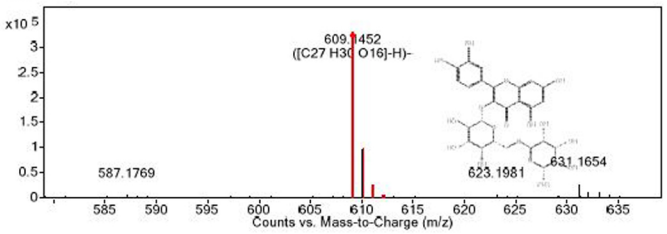
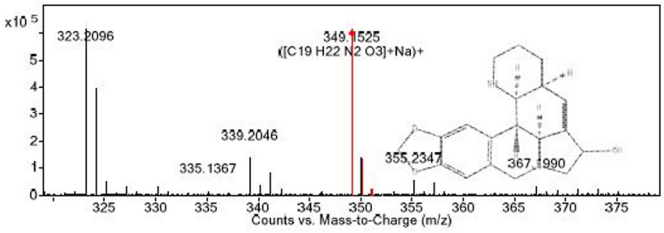


Fig. 23 (viii): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

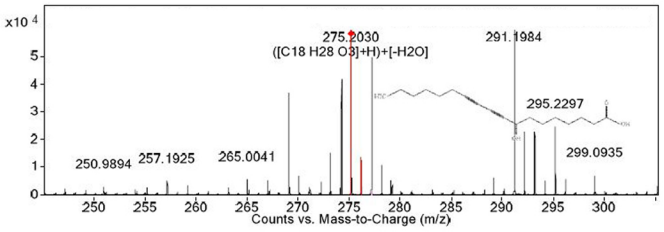
Rutin



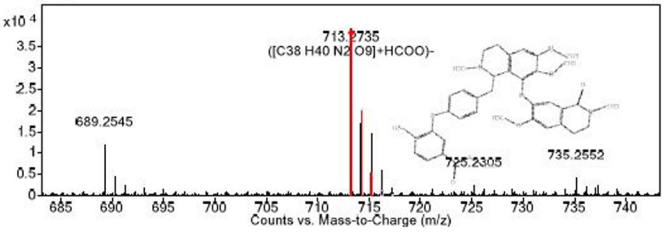
Caribine



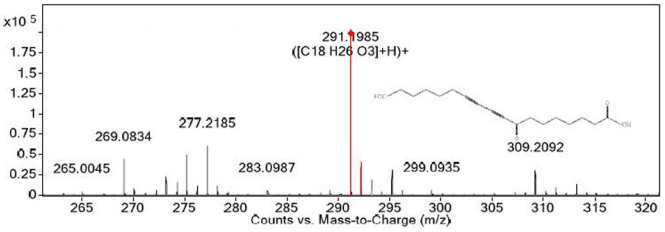
8-Hydroxy-9,11-octadecadiynoic acid



Tejedine



8-Oxo-9,11- octadecadiynoic acid



3-Methyl-octanoic acid

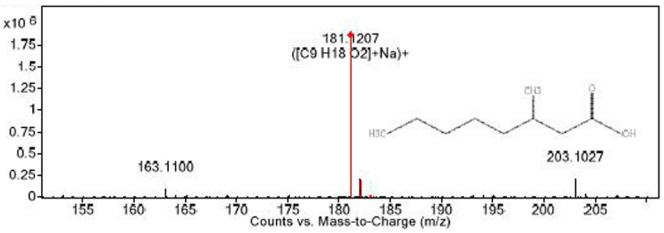
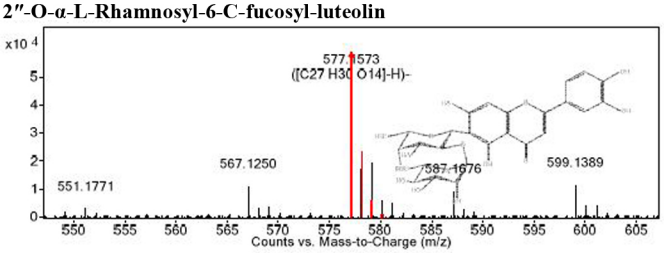
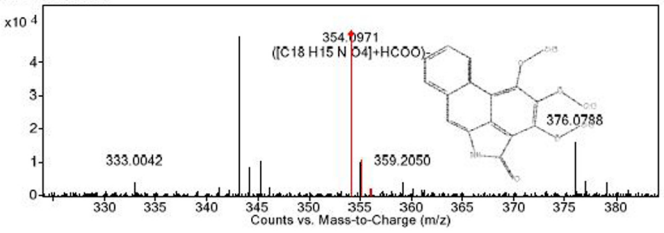


Fig. 23 (x): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

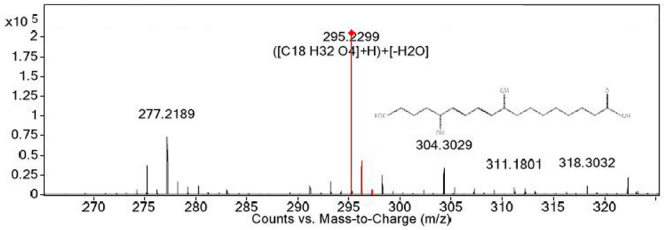
2″-O-α-L-Rhamnosyl-6-C-fucosyl-luteolin



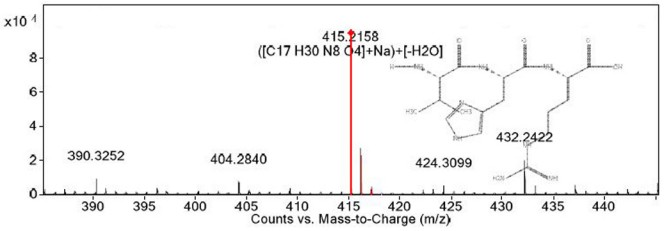
Piperolactam C



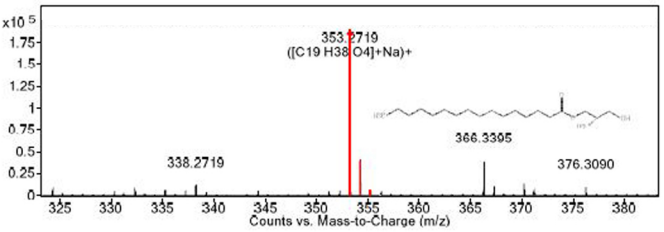
9,14-Dihydroxy-10,12-octadecadienoic acid



Val His Arg



1-Hexadecanoyl-sn-glycerol



N-Caffeoyltryptophan

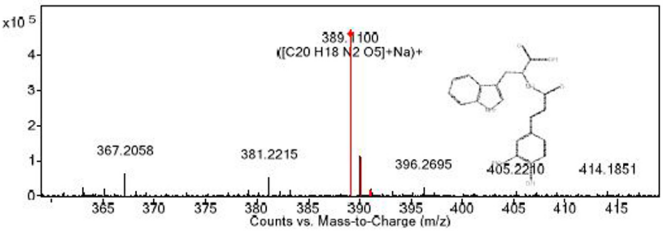
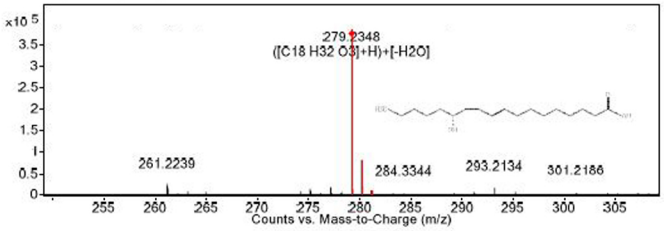
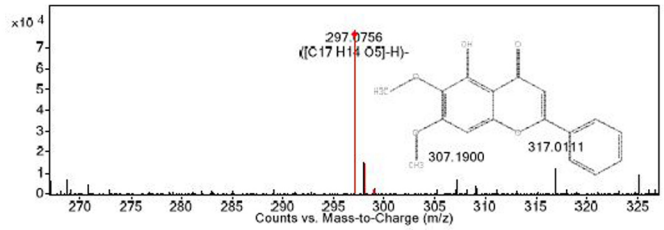


Fig. 23 (xi): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

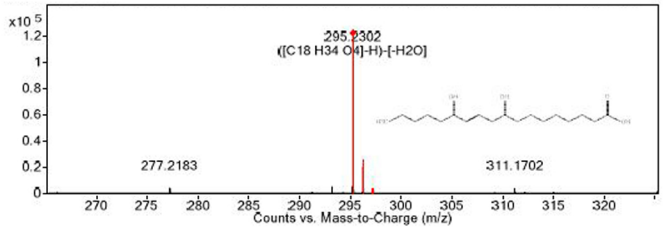
13R-Hydroxy-9E,11Z-octadecadienoic acid



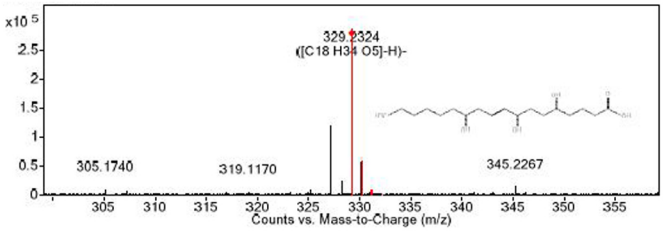
Mosloflavone



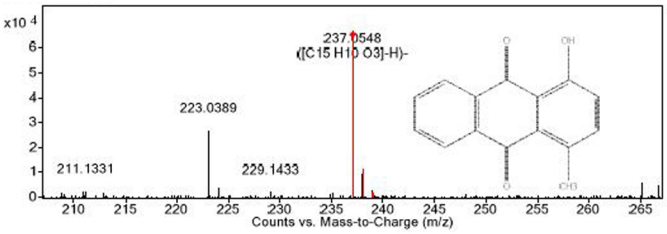
9,13-Dihydroxy-11-octadecenoic acid



Pinellic acid



1-Hydroxy-4-methylanthraquinone



Leu Arg Ala

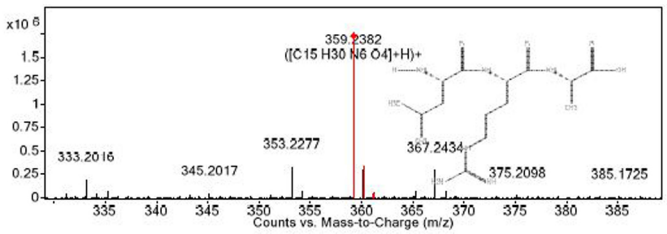


Fig. 23 (xii): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

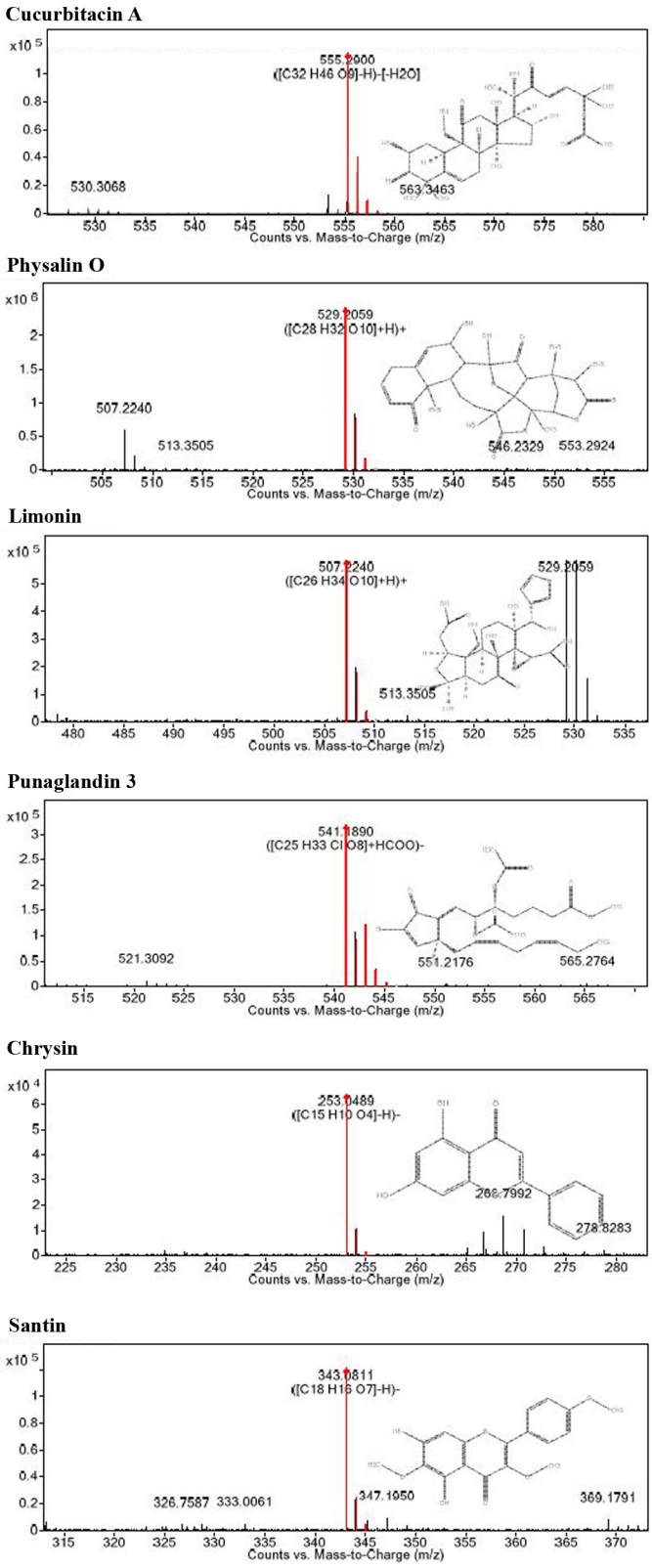
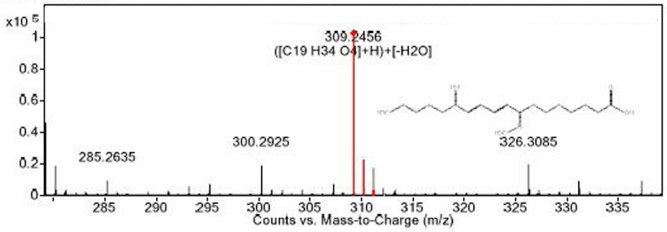
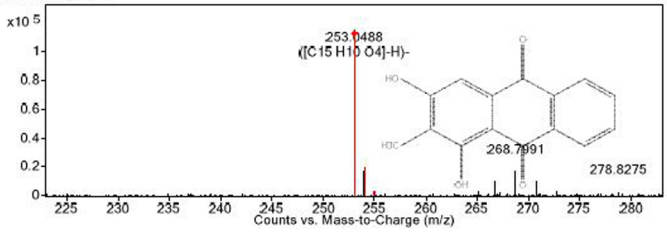


Fig. 23 (xiii): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

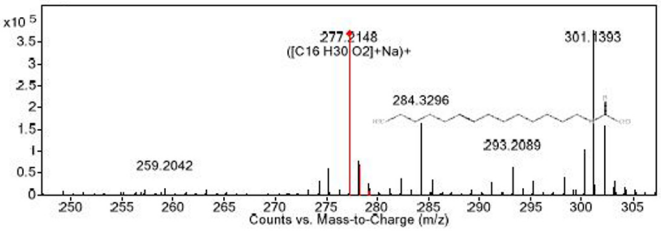
8-Methoxy-13-hydroxy-9,11-octadecadienoic acid



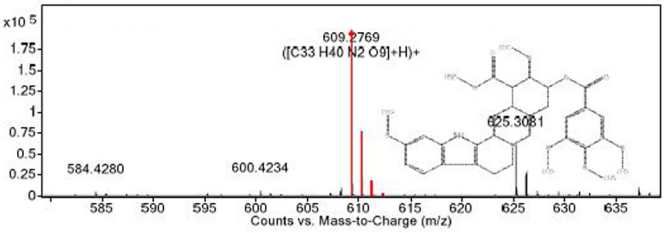
Rubiadin



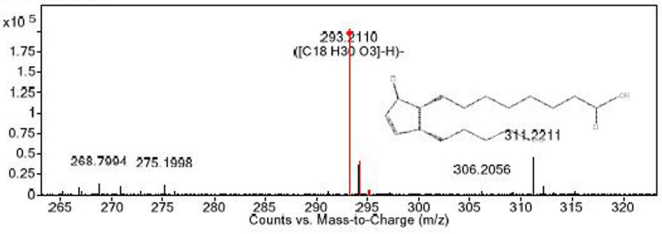
8E-Tetradecenyl acetate



Isoreserpine



(9S,13S)-10-Oxo-11-phytoenoic acid



11-Hydroxy palmitic acid

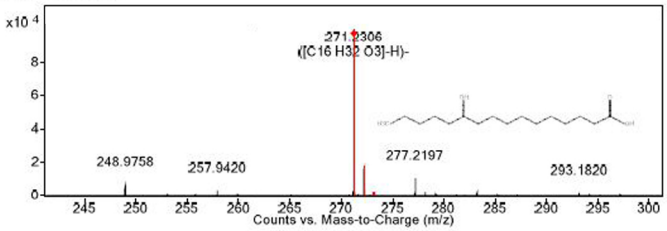
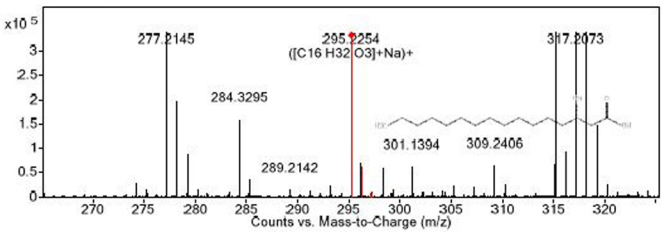
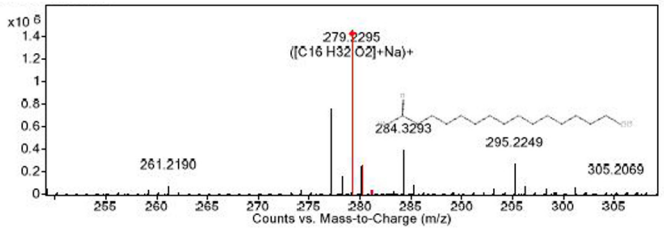


Fig. 23 (xiv): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

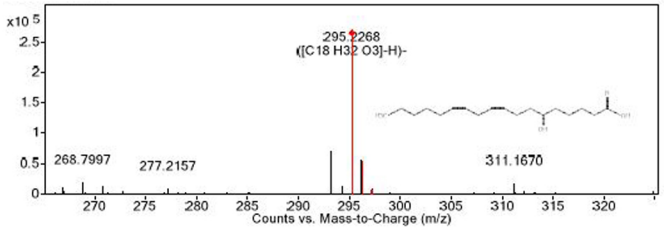
3S-Hydroxypalmitic acid



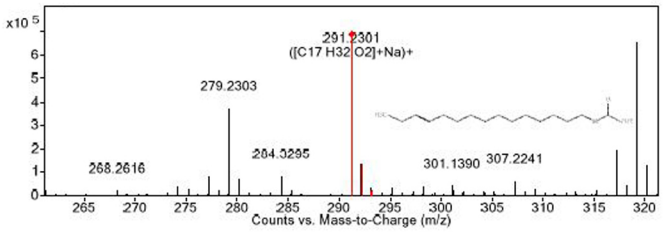
Palmitic acid



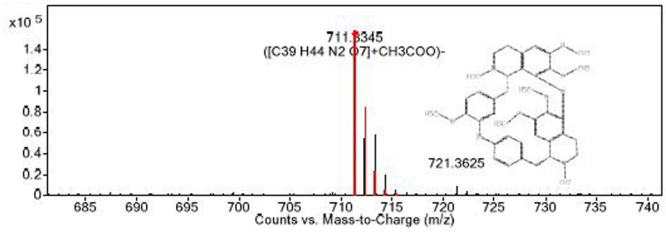
Hydroxyoctadecadienoic acid



12E-Pentadecenyl acetate



Thalidasine



8-Hydroxy octadecanoic acid

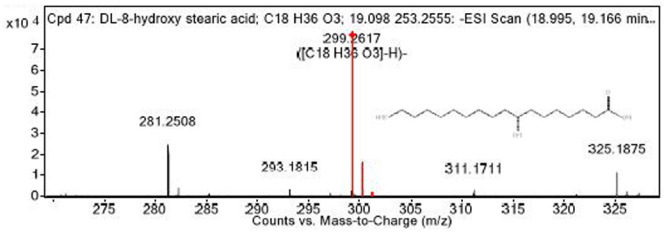


Fig. 23 (xv): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

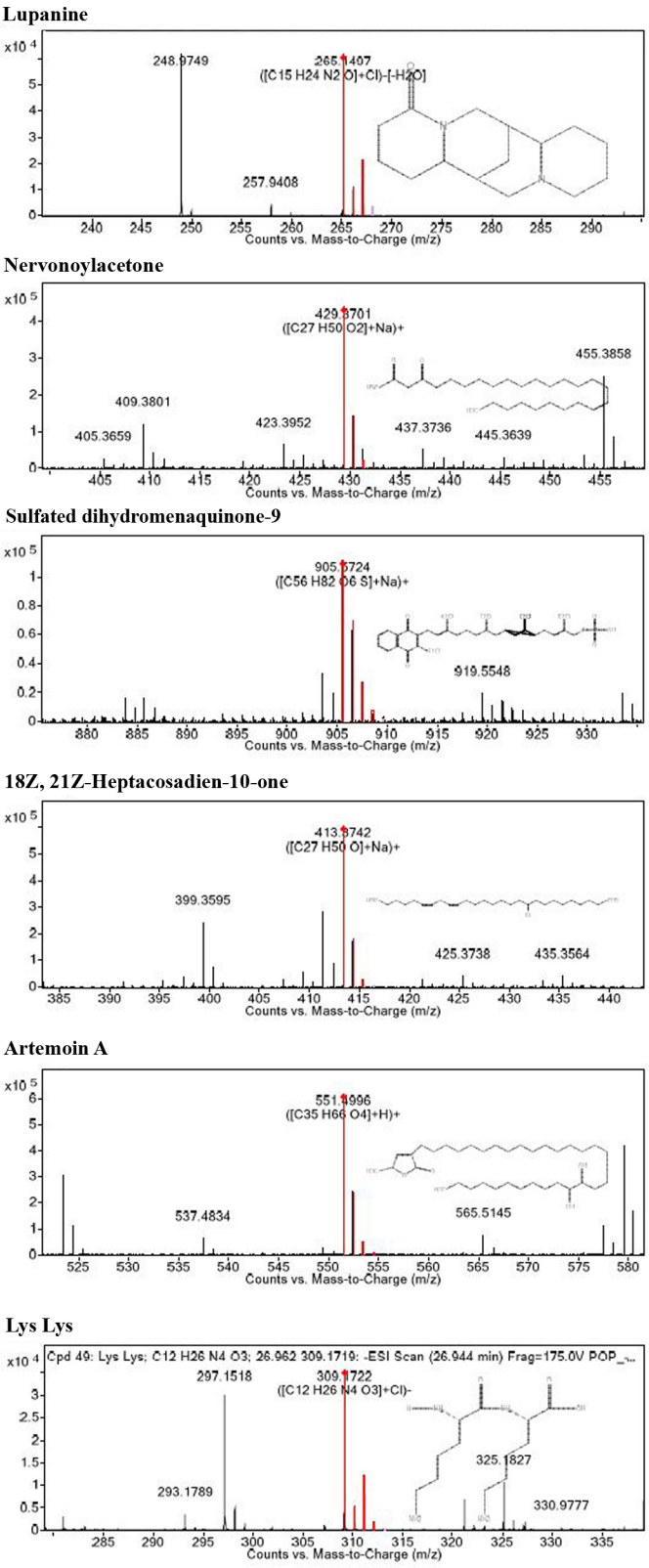
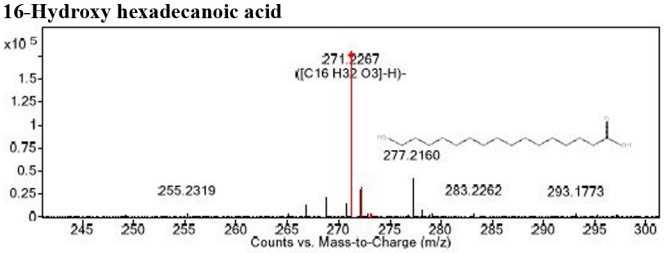
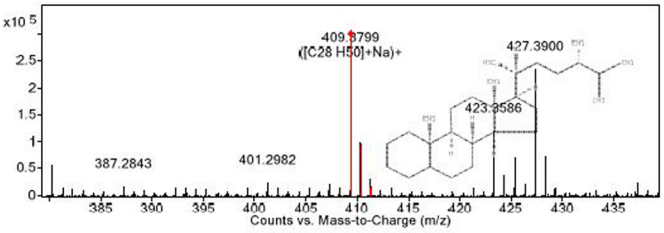


Fig. 23 (xviii): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

16-Hydroxy hexadecanoic acid



Ergostane



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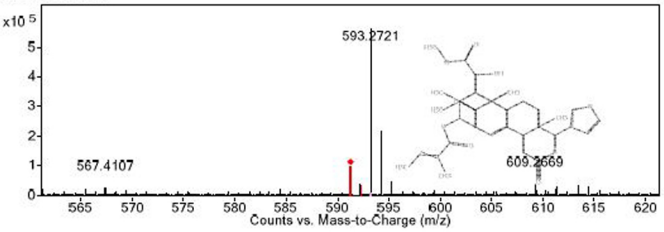
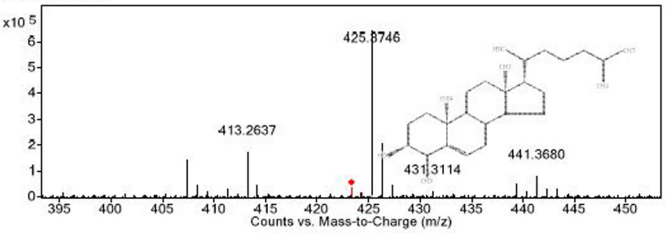
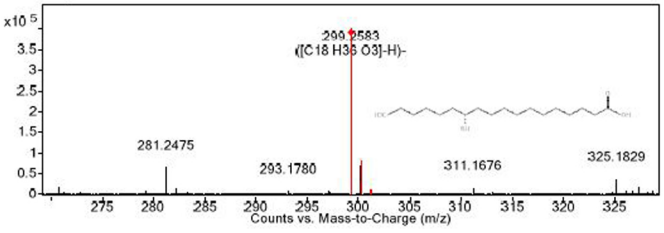


4-Methyl-cholest-5-en-3β-ol



12R-Hydroxy octadecanoic acid



Ergostanol

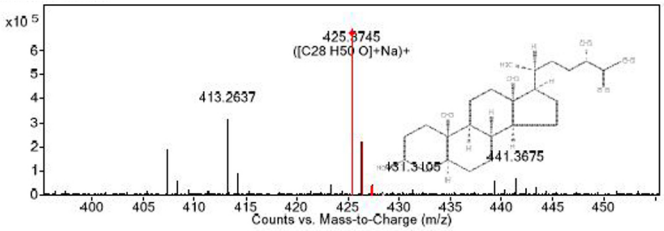
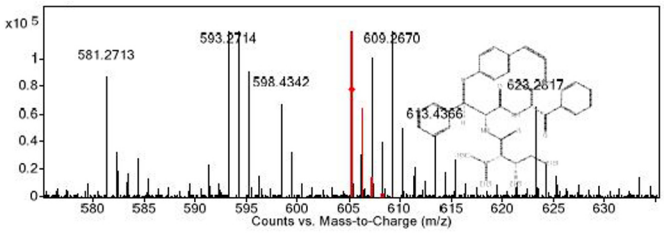


Fig. 23 (xvi): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

Aralionine A



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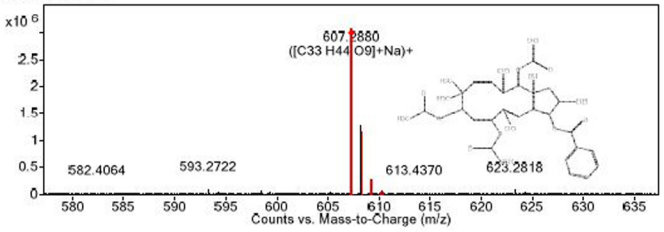
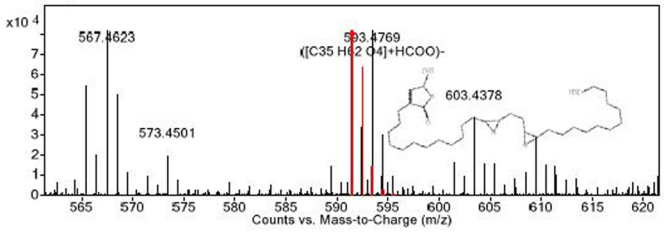
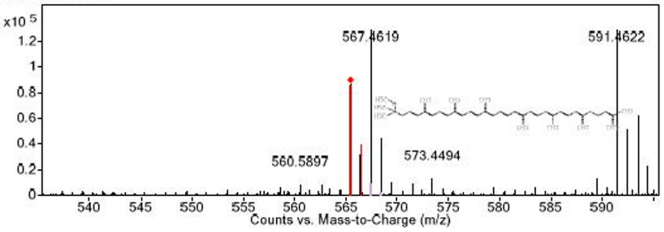


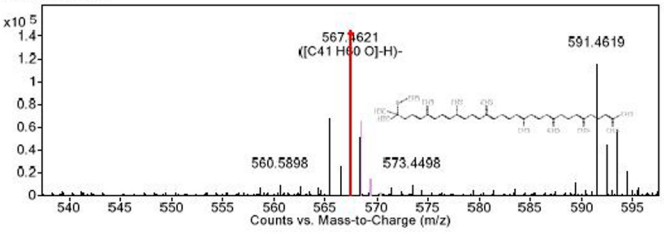
Diepomuricanin A



Anhydrorhodovibrin



Spheroidene



14-Hydroxy-heneicosanoic acid

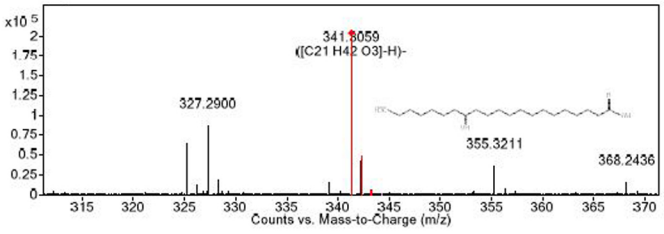


Fig. 23 (xvii): Mass spectra of compounds revealed by HR LC-MS analysis of methanolic extract of selected species of *Ophiorrhiza*

PART III – BIOACTIVITY SCREENING

A. Free radical scavenging activity of selected species of *Ophiorrhiza*

The potential of the selected species of *Ophiorrhiza* to scavenge free radicals was analysed by DPPH, hydroxyl, nitric oxide and superoxide radical scavenging assays.

i. DPPH free radical scavenging activity

In DPPH free radical scavenging assay, the methanolic extracts of all the selected species exhibited a dose dependent increase in their potential to quench the radicals. The concentrations used for the assay were 12.5, 25, 50, 100 and 200 µg/ml of the plant extract. The percentage inhibition of the radicals was found to increase with an increase in the concentration of the extracts from 12.5 to 200 µg/ml. Among the four species, *O. jacobii* exhibited the highest inhibition percentage ranging from 21.04 ± 1.03 to 72.01 ± 0.79 with an IC_{50} value of 92.64 ± 2.16 µg/ml. *O. pectinata* and *O. trichocarpon* showed very close percentages of inhibition from 16.45 ± 1.15 to 64.06 ± 1.12 and 12.14 ± 0.86 to 63.57 ± 0.9. The least inhibition percentage was exhibited by *O. grandiflora* with a range of 1.97 ± 0.91 to 60.5 ± 0.89. Ascorbic acid was used as the standard which showed a percentage inhibition of 21.9 ± 0.52 to 96.15 ± 0.67 with an IC_{50} value of 38.42 ± 1.57 µg/ml. Among all the plant extracts, the lowest IC_{50} value was observed for *O. trichocarpon* (85.65 ± 2.48 µg/ml). The results of DPPH radical scavenging assay of all the selected species are given in **Fig. 24.** IC_{50} values of all the extracts for DPPH radical scavenging assay are given in **Table 15.** Comparing to the standard, the activity of the plant extracts seems to be less; however, the IC_{50} values of all the extracts come within the range of selected concentrations, and hence, all the plant extracts have considerable scavenging effects on the DPPH radical.

ii. Hydroxyl free radical scavenging activity

The ability of the plant extracts to scavenge hydroxyl radicals was tested using different concentrations of the plant extract *i.e*., 125, 250, 500, 1000 and 2000 µg/ml. The methanolic extracts showed a dose dependent increase in the percentage of inhibition of free radicals with increasing concentration of the plant extracts from 125 to 2000 µg/ml. *O. jacobii* exhibited the highest inhibition percentage ranging from 15.48 ± 0.61 to 70.24 ± 0.9 with an IC_{50} value of 767.11 ± 2.33 µg/ml. *O. pectinata* showed the next highest inhibition percentage range of 14.29 ± 0.82 to 66.67 ± 1.01. *O. trichocarpon* showed an inhibition percentage of 13.69 ± 0.89 to 63.39 ± 1.11. *O. grandiflora* showed the least inhibition percentage range of 9.52 ± 0.91 to 59.52 ± 1.41. Gallic acid was used as the standard, which showed an inhibition percentage range of 13.99 ± 1.03 to 90.96 ± 0.63 with an IC_{50} value of 477.49 ± 2.05 µg/ml. The results of hydroxyl radical scavenging assay of all the selected species are given in **Fig. 25.** IC_{50} values of all the extracts for hydroxyl radical scavenging assay are given in **Table 15.** In the assay, the activity shown by *O. jacobii* was comparable to that of the standard. Other plant extracts were not better than that of the standard and were found to have the potential to scavenge hydroxyl radicals from a relatively higher level to a moderate level.

iii. Nitric oxide free radical scavenging activity

In nitric oxide radical scavenging assay, the concentrations of the plant extracts tested were 125, 250, 500, 1000 and 2000 µg/ml. The percentage inhibition of the radicals was found to increase with an increase in the concentration of the extracts from lower to higher. The results of nitric oxide radical scavenging assay of all the selected species are given in **Fig. 26.** IC_{50} values of all the extracts for nitric oxide radical scavenging assay are given in **Table 15.** From the results, it was observed that *O. jacobii, O. pectinata* and *O. trichocarpon* have almost comparable ability to scavenge the free radicals with an inhibition percentage range of 19.49 ± 0.63 to 67.37 ± 1.2, 18.64 ±

1.02 to 67.37 ± 1.1 and 16.1 ± 1.14 to 66.31 ± 1.04 respectively. In this assay also, *O. grandiflora* exhibited the least inhibition percentage range of 15.25 ± 0.89 to 57.42 ± 0.94, which was slightly lesser than the other three. However, the least IC_{50} value among the plant extracts was shown by *O. jacobii* (783.35 ± 2.88 µg/ml). Gallic acid was the standard used and its IC_{50} value was 108.80 ± 1.55 µg/ml. The inhibition percentage range was found to be 57.44 ± 0.77 to 92.9 ± 1.04 for the selected concentrations of the standard from 125 to 2000 µg/ml. The inhibition percentages and IC_{50} values of the plant extracts were not better than the results for standard. Thus, for all the species, the activity seems to be moderate.

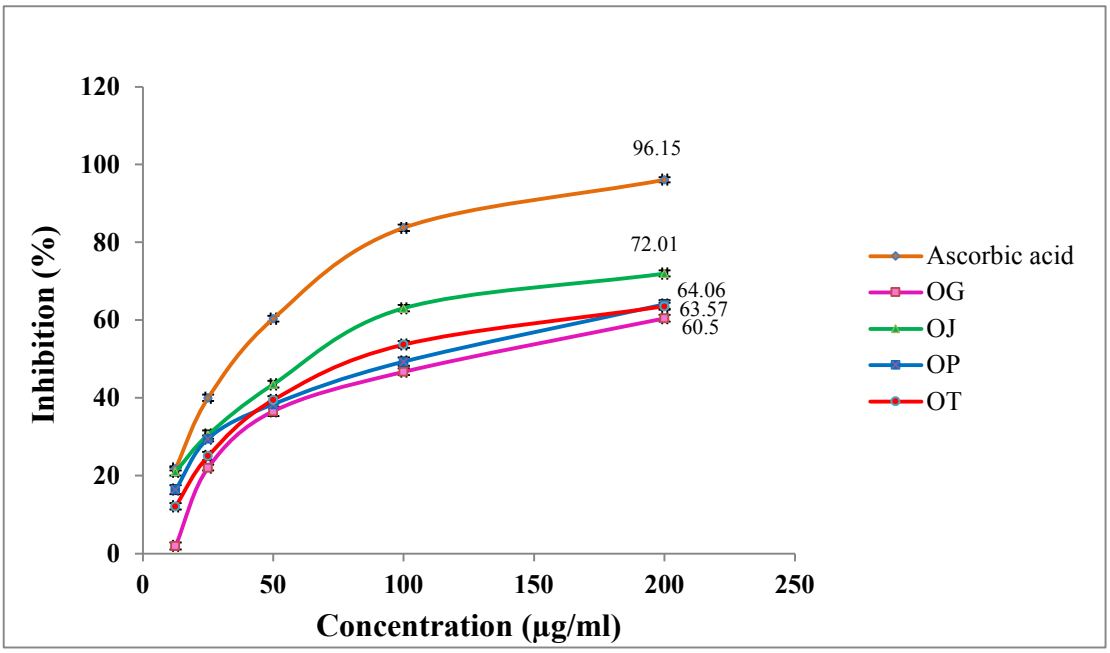


Fig. 24: *In vitro* DPPH radical scavenging activity of selected species of *Ophiorrhiza*. OG – *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon*

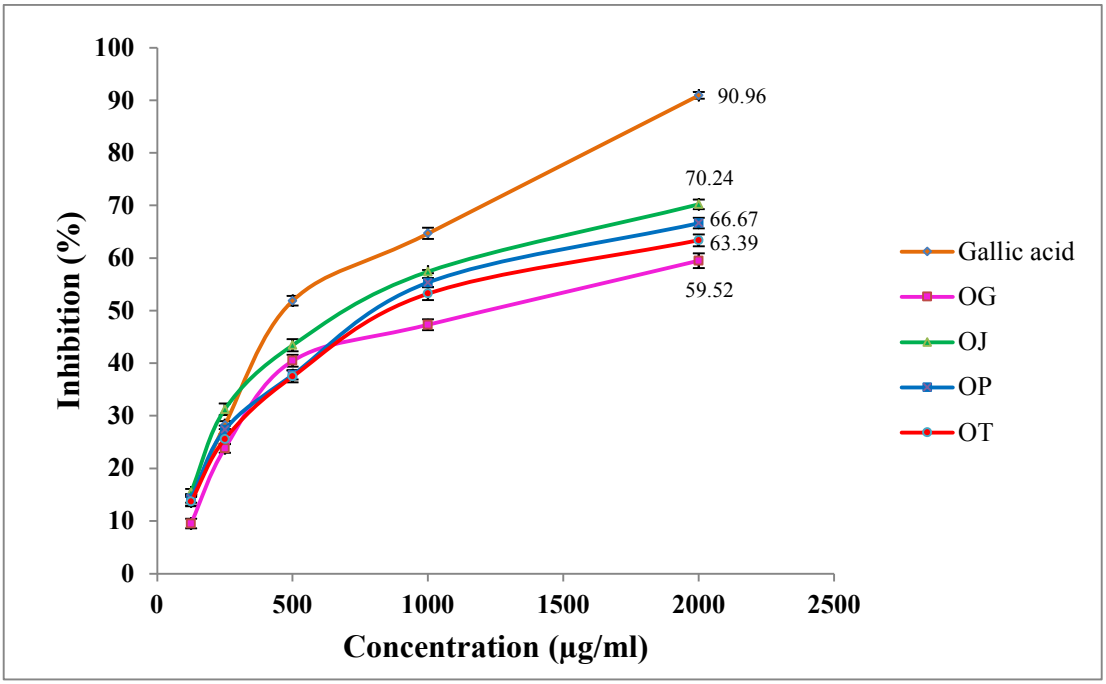


Fig. 25: *In vitro* hydroxyl radical scavenging activity of selected species of *Ophiorrhiza.* OG – *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon*

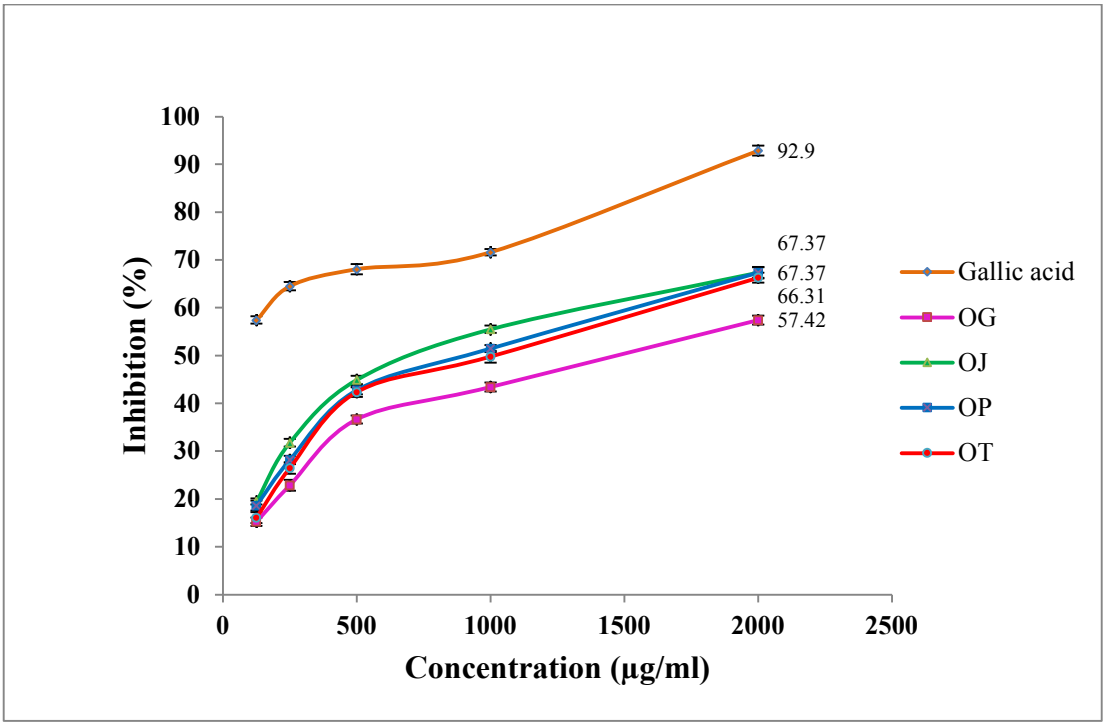


Fig. 26: *In vitro* nitric oxide radical scavenging activity of selected species of *Ophiorrhiza.* OG – *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon*

i. Superoxide free radical scavenging activity

In superoxide radical scavenging assay, the methanolic extracts of all the selected species showed a dose dependent increase in the percentage inhibition of free radicals. The concentrations of the plant extracts used for the study were 125, 250, 500, 1000 and 2000 µg/ml. The maximum inhibition percentage was observed at the highest concentration of 2000 µg/ml. *O. jacobii* exhibited a percentage inhibition range of 9.14 ± 0.63 to 76 ± 1.07, which was the highest percentage of scavenged free radicals by the selected species. *O. pectinata* provided an inhibition percentage range of 7.43 ± 1.1 to 74.29 ± 1.06. *O. trichocarpon* and *O. grandiflora* had almost the same range of percentage inhibition from 5.71 ± 0.82 to 72.8 ± 1.0 and 3.43 ± 0.92 to 72.06 ± 0.72 respectively.

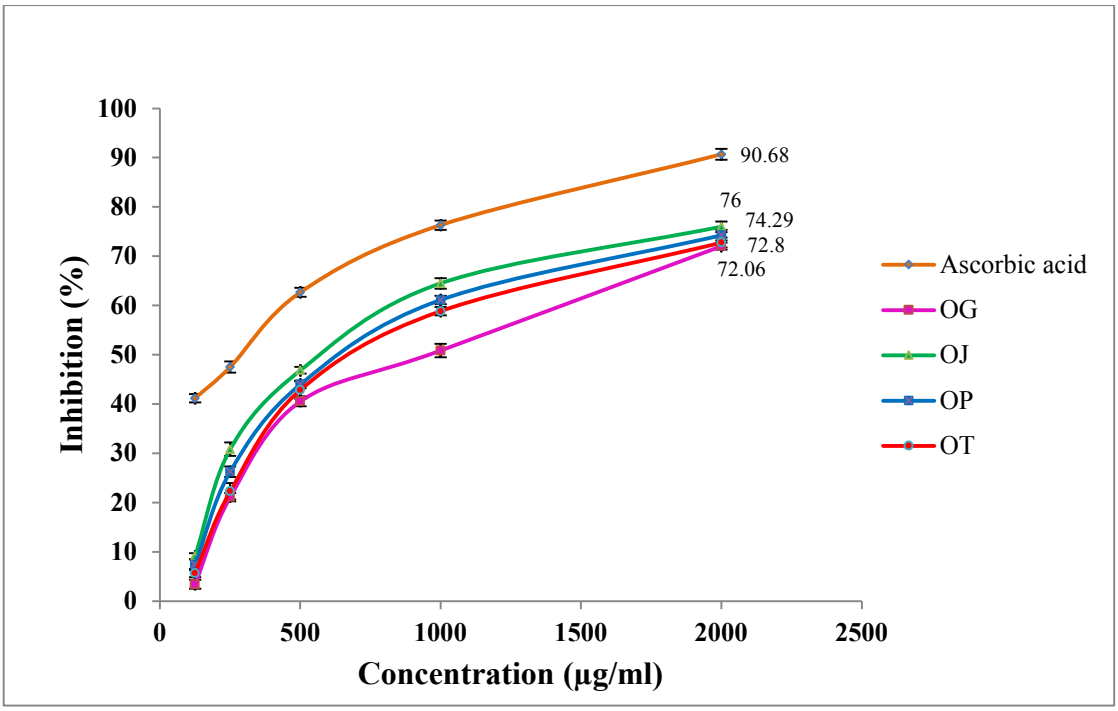


Fig. 27: *In vitro* superoxide radical scavenging activity of selected species of *Ophiorrhiza*. OG – *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon*

The standard used for the study was ascorbic acid, which showed percentage inhibition of 41.19 ± 0.84 to 90.68 ± 1.11 with an IC_{50} value of

238.35 ± 2.17 µg/ml. The extract of *O. pectinata* exhibited the lowest IC_{50} value (737.48 ± 2.66 µg/ml) among the selected species. The results of superoxide radical scavenging assay of all the selected species are given in **Fig. 27.** IC_{50} values of all the extracts for the assay are given in **Table 15.** IC_{50} value obtained for the standard was too low, when compared to that of the plant extracts investigated. The high percentage inhibition values of the standard also indicated that the superoxide radical scavenging ability of the plant extracts was moderate.

Thus, the results of free radical scavenging activity of the selected species of *Ophiorrhiza* showed that the four species have moderate antioxidant potential. Among the four species, *O. jacobii* had the maximum scavenging ability of the free radicals. In all the assays, the standards used were having better IC_{50} values than the plant extracts. But the extracts used were in their crude form and that might be the reason for the lesser activity of the extracts in the assays. Though in crude form, the plant extracts have the ability to scavenge the free radicals from a relatively higher to a moderate level.

Table 15: Inhibition concentrations of selected species of *Ophiorrhiza* in different free radical scavenging assays

	IC_{50} (µg/ml)			
Plant species	**DPPH radical scavenging**	**Hydroxyl radical scavenging**	**Nitric oxide radical scavenging**	**Superoxide radical scavenging**
OG	139.4 ± 2.05	1375.08 ± 2.65	1493.84 ± 3.72	1157.84 ± 3.22
OJ	92.64 ± 2.16	767.11 ± 2.33	783.35 ± 2.88	919.97 ± 3.10
OP	123.43 ± 1.98	840.26 ± 2.46	879.23 ± 2.12	737.48 ± 2.66
OT	85.65 ± 2.48	878.57 ± 2.26	1178.48 ± 3.95	775.02 ± 1.32
Standard	38.42 ± 1.57	477.49 ± 2.05	108.80 ± 1.55	238.35 ± 2.17

OG - *O. grandiflora*; **OJ -** *O. jacobii*; **OP -** *O. pectinata*; **OT** - *O. trichocarpon*;
IC_{50} - concentration that causes 50% inhibition

B. Anti-inflammatory activity

The anti-inflammatory potential of the selected species of *Ophiorrhiza* was first analysed by *in vitro* biochemical assays and the most effective plant extract was selected for further analysis of its activity on LPS induced RAW 264.7 macrophage cell line.

i. Evaluation of anti-inflammatory activity using biochemical assays

a) Inhibition of protein denaturation

In the study, the methanolic extracts of all the selected species exhibited a dose dependent increase in the percentage of inhibition of protein denaturation. The concentrations used for the assay were 62.5, 125, 250 and 500 µg/ml of the plant extract. Among the four species, *O. trichocarpon* exhibited the highest inhibition percentage ranging from 13.83 ± 1.80 to 64.25 ± 2.81. *O. jacobii* also showed very close percentages of inhibition from 12.45 ± 0.97 to 60.48 ± 2.32. *O. pectinata* and *O. grandiflora* showed almost similar inhibition percentages of 58.7 ± 1.87 and 57.23 ± 2.16 respectively, at the highest concentration. Diclofenac sodium was used as the standard which showed a percentage inhibition of 49.25 ± 1.85 to 87.15 ± 2.16. The summary of the results is given in **Fig. 28.** Though all the selected species exhibited comparatively same range of inhibition percentages, better results were obtained in *O. trichocarpon* with the lowest IC_{50} value (376.89 ± 3.92 µg/ml).

b) Proteinase inhibitory activity

Proteinase inhibitory potential of the methanolic extracts of all the selected species were found to be in a concentration-dependent manner. The proteinase inhibitory activity was found to increase gradually from the lowest concentration of 62.5 µg/ml to the highest concentration of 500 µg/ml. *O. trichocarpon* and *O. jacobii* exhibited almost the same efficacy to inhibit proteinase. For *O. trichocarpon,* the inhibition percentage ranged from

17.22 ± 0.44 to 60.81 ± 1.89. *O. jacobii* showed a percentage of inhibition from 13.34 ± 1.55 to 60.05 ± 1.73. At the initial concentrations, *O. pectinata* showed better results than *O. grandiflora.* But at the highest concentration, *O. grandiflora* exhibited higher inhibition percentage than the former. The inhibition percentage range of *O. pectinata* was 10.05 ± 0.62 to 50.84 ± 1.82, whereas the value for *O. grandiflora* was 6.08 ± 0.67 to 53.37 ± 1.61. Diclofenac sodium was used as the standard which showed a percentage inhibition of 71.38 ± 2.56 to 88.37 ± 2.61. Among the selected species, *O. trichocarpon* exhibited the lowest IC_{50} value of 367.153 ± 4.57 µg/ml. The results of proteinase inhibitory activity of the selected plant species are given in **Fig. 29.**

From both the *in vitro* assays, *O. trichocarpon* was found to exhibit better anti-inflammatory potential among the selected species of *Ophiorrhiza*. So, for further analysis, *O. trichocarpon* was selected.

ii. Anti-inflammatory activity on RAW 264.7 macrophage cell line

Inflammatory responses are usually related with the activation and release of several inflammatory enzymes. The effect of the methanolic extract of *O. trichocarpon* on the activity of inflammatory enzymes like cyclooxygenase, lipoxygenase, myeloperoxidase and inducible nitric oxide synthase and also on the cellular nitrite level was analysed on LPS induced RAW 264.7 macrophage cell line.

a) Cyclooxygenase (COX) activity

The potential of the methanolic extract of *O. trichocarpon* to reduce the level of COX enzyme was analysed using three different concentrations of the sample (25, 50 and 100 µg/ml). The efficacy of the plant extract was calculated as the percentage inhibition of the enzyme. At the lowest

concentration of 25 µg/ml, the sample evinced an inhibition percentage of 26.84 ± 1.82; 46.90 ± 1.33 % at 50 µg/ml and 62.41 ± 1.72% at 100 µg/ml.

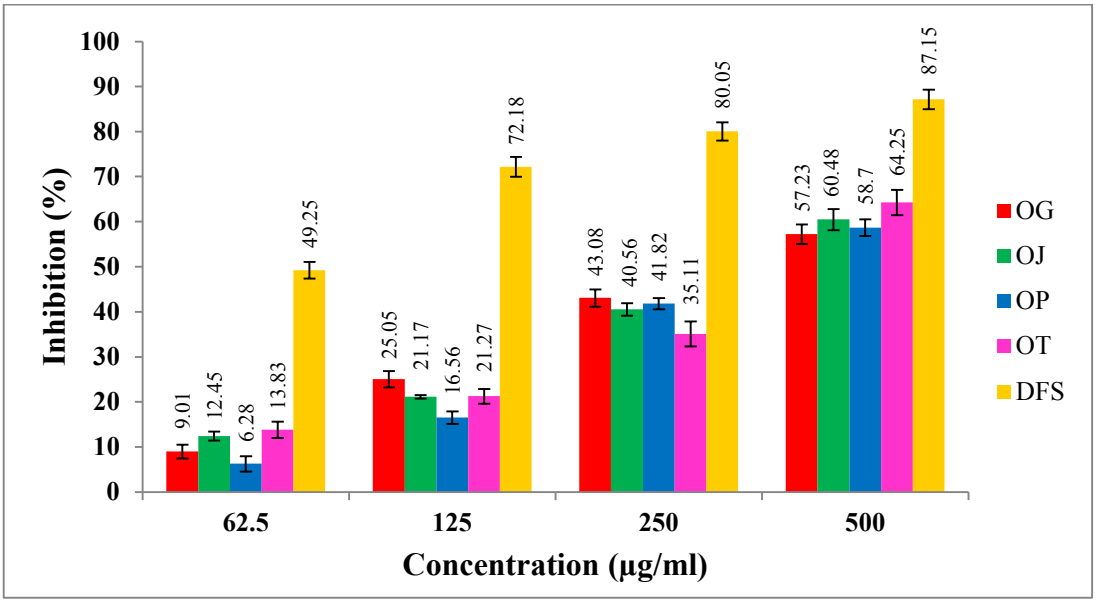


Fig. 28: Inhibition of protein denaturation by methanolic extracts of selected species of *Ophiorrhiza.* OG - *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon*; DFS - Diclofenac sodium

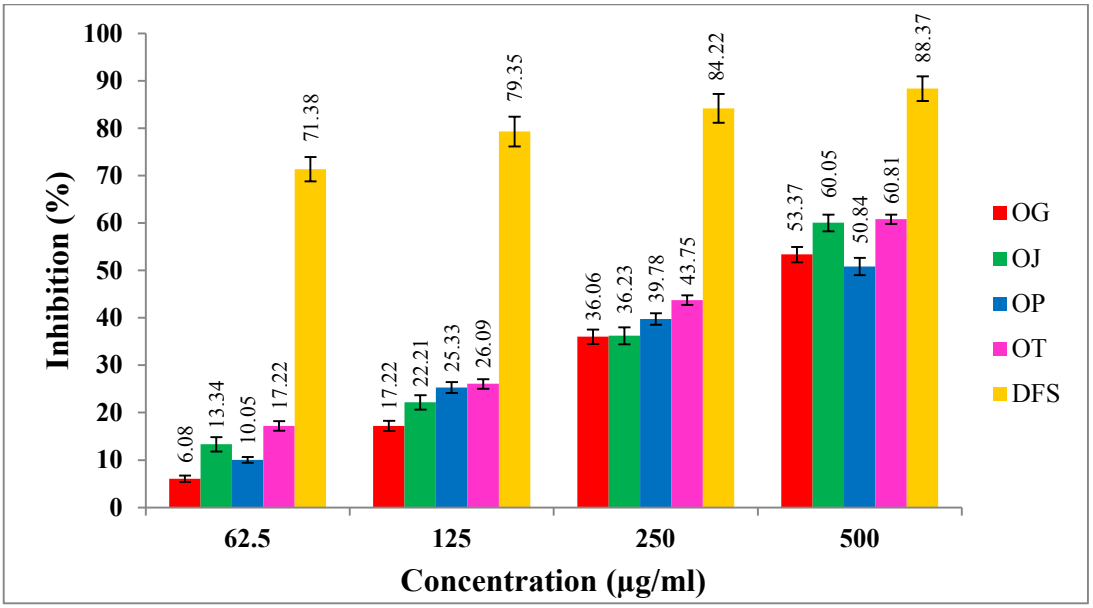


Fig. 29: Proteinase inhibitory activity of methanolic extracts of selected species of *Ophiorrhiza.* OG - *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon*; DFS - Diclofenac sodium

Diclofenac sodium was the standard used in the assay and the inhibition percentage range was observed as 31.83 ± 1.37 to 91.51 ± 1.47. Thus, the extract displayed a concentration-dependent increase in the inhibition percentage of the inflammatory enzyme. The IC_{50} value of *O. trichocarpon* was found to be 56.09 ± 3.25 µg/ml and that of the standard was 33.15 ± 2.05 µg/ml. The result of COX activity inhibition is given in **Fig. 30.** From the graph, it can be inferred that the plant extract has significant ability to suppress COX activity.

b) Lipoxygenase (LOX) activity

The ability of the plant extract to decrease the level of LOX enzyme was analysed using three different concentrations of the sample (25, 50 and 100 µg/ml) and calculated as the percentage inhibition of the enzyme in the assay system. At the concentration of 25 µg/ml, the sample showed an inhibition percentage of 24.41 ± 1.65. At 50 µg/ml and 100 µg/ml, the inhibition percentages were 35.15 ± 2.56 and 60.53 ± 2.23 respectively. The inhibition percentage range shown by diclofenac sodium was observed as 33.90 ± 1.37 to 90.77 ± 1.47. In this assay also, the extract showed a dose-dependent increase in the inhibition percentage of the inflammatory enzyme. The IC_{50} value of *O. trichocarpon* was 80.15 ± 2.92 µg/ml and that of the standard was 34.52 ± 2.35 µg/ml. The results of this assay are given in **Fig. 31.** When compared to the standard values, the plant extract showed moderate ability to repress LOX activity.

c) Myeloperoxidase (MPO) activity

Myeloperoxidase is an important biomarker of inflammation as well as potent target for anti-inflammatory agents. The effect of the plant extract of *O. trichocarpon* to reduce the level of MPO enzyme activity was analysed using three different concentrations of the sample (25, 50 and 100 µg/ml). The

activity of the enzyme was found to be reduced in a dose-dependent manner and is depicted in **Fig. 32.** At the highest concentration of 100 µg/ml, the plant extract reduced the activity of the enzyme to 0.013167 ± 0.00069 U/ml of cell lysate, which is almost the same value obtained for the standard at the initial concentration. Thus, the plant extract can be considered to have significant effect on MPO activity.

d) Inducible nitric oxide synthase (iNOS) activity

The potential of the methanolic extract of *O. trichocarpon* to inhibit iNOS activity was analysed using three different concentrations of the sample (25, 50 and 100 µg/ml). The efficacy of the plant extract was calculated as the percentage inhibition of the enzyme. The sample revealed an inhibition percentage of 20.50 ± 1.85, 35.78 ± 1.19 and 59.26 ± 2.58 at the concentrations of 25, 50 and 100 µg/ml respectively. The standard, diclofenac sodium showed an inhibition percentage range of 29.32 ± 2.13 to 87.73 ± 1.23. Here, the sample showed a concentration-dependent increase in the inhibition percentage of the inflammatory enzyme. The IC_{50} value of *O. trichocarpon* was 80.32 ± 4.15 µg/ml and that of the standard was 38.45 ± 3.45 µg/ml. The result of the assay is given in **Fig. 33.** On analysis, the activity of the plant extract on suppressing the iNOS activity can be considered to be moderate.

e) Cellular nitrite level

The level of cellular nitrite is an indication of increased inflammatory responses which can have adverse effect on the health. In the assay, the ability of the plant extract to decrease the level of nitrite in the cellular system was analysed with three concentrations of the sample (25, 50 and 100 µg/ml). The result was analysed as the ability of the sample to reduce the level of nitrite accumulation within the testing system. From the lowest to the highest

concentration used, the plant extract reduced the level of cellular nitrite from 290.56 ± 1.17 to 191.07 ± 1.56 µg/ml of cell lysate. Within the same concentration range, the standard diclofenac sodium repressed the level of cellular nitrite from 284.62 ± 0.97 to 96.52 ± 1.68 µg/ml of cell lysate. On considering the level of nitrite at the initial and final concentration of the sample, the sample has prominent ability to reduce nitrite level to about half. The activity of the plant extract in reducing nitrite level is depicted in **Fig. 34.**

The evaluation of anti-inflammatory potential of the methanolic extract of *O. trichocarpon* on LPS induced RAW 264.7 macrophage cell line showed varying results in the five assays employed. The plant extract showed noteworthy results in the cyclooxygenase, myeloperoxidase and cellular nitrite level assays. But the results were found to be moderate in lipoxygenase and inducible nitric oxide synthase assay.

iii. Cytotoxic evaluation using MTT assay on RAW 264.7 cells

MTT assay was carried out to evaluate the cytotoxic nature of the plant extract using various concentrations of 6.25, 12.5, 25, 50 and 100 µg/ml. The percentage of cell viability at each concentration was calculated and is given in **Fig. 35.** From the assay, LC_{50} value of the extract was found to be 110.58 ± 1.92 µg/ml and this LC_{50} value was considered for further analysis. The cytotoxic effect of the methanolic extract of *O. trichocarpon* on RAW 264.7 cells is given in **Plate 14.**

iv. Estimation of inflammatory mediators using ELISA

Effect of the plant extract of *O. trichocarpon* on the production of an important inflammatory mediator, COX-2 was analysed on LPS treated RAW 264.7 cells. RAW 264.7 cells, treated only with LPS were considered to be the control. 110.58 µg/ml of the extract was treated with the RAW cells and the level of COX-2 was evaluated. It was found that the extract reduced the level

of COX-2 from 0.46 ± 0.02 to 0.36 ±0.05 U/ml of cell lysate. The result of the experiment is given in **Fig. 36.**

Thus, the anti-inflammatory potential of the plant extract of *O. trichocarpon* can be attributed to the ability of the plant extract to inhibit protein denaturation and inflammatory enzymes and to reduce the levels of cellular nitrite and inflammatory mediators. From the results of all the anti-inflammatory assays done, the plant extract of *O. trichocarpon* proves to be a strong candidate for anti-inflammatory studies.

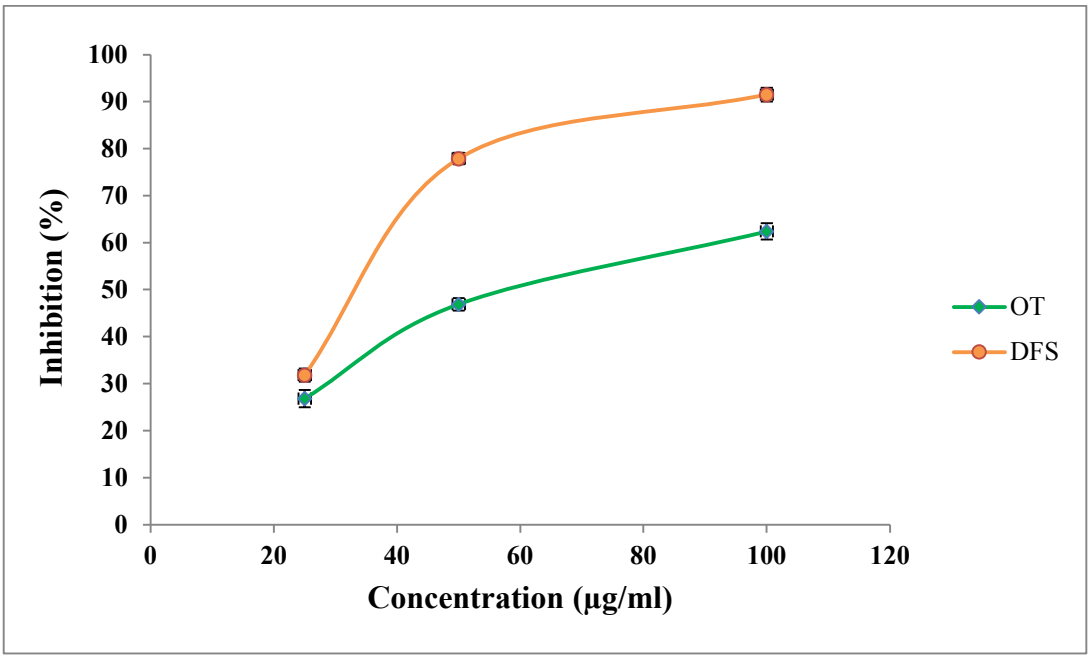


Fig. 30: Effect of methanolic extract of *Ophiorrhiza trichocarpon* on cyclooxygenase enzyme activity. OT - *O. trichocarpon;* **DFS** -Diclofenac sodium

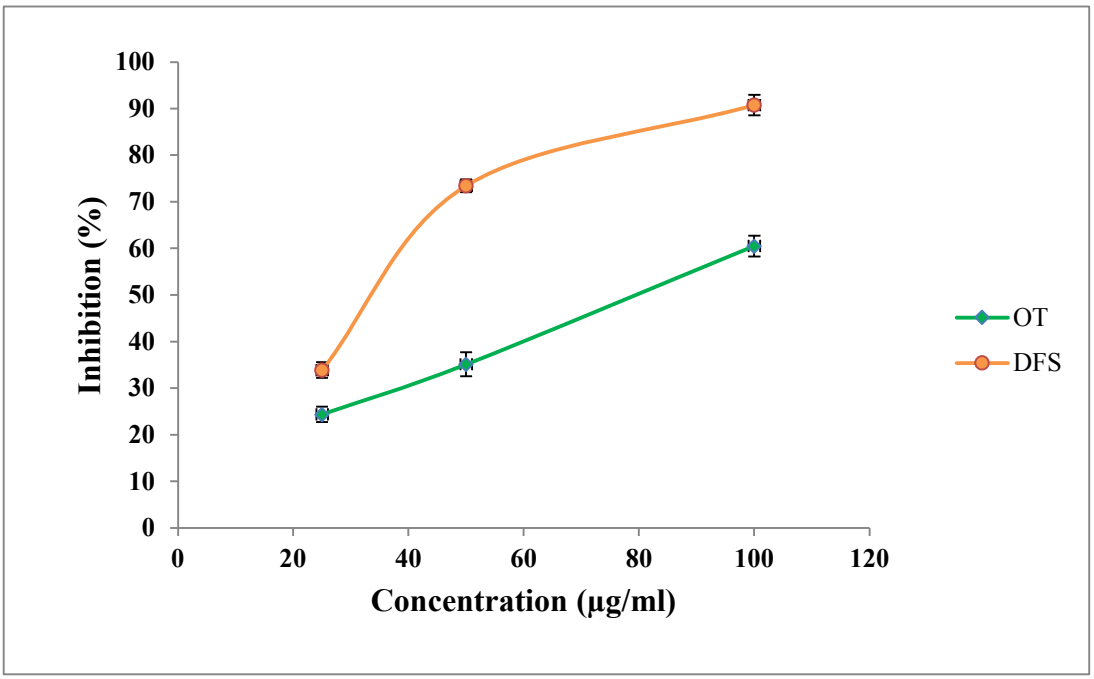


Fig. 31: Effect of methanolic extract of *Ophiorrhiza trichocarpon* on lipoxygenase enzyme activity. OT - *O. trichocarpon;* **DFS** -Diclofenac sodium

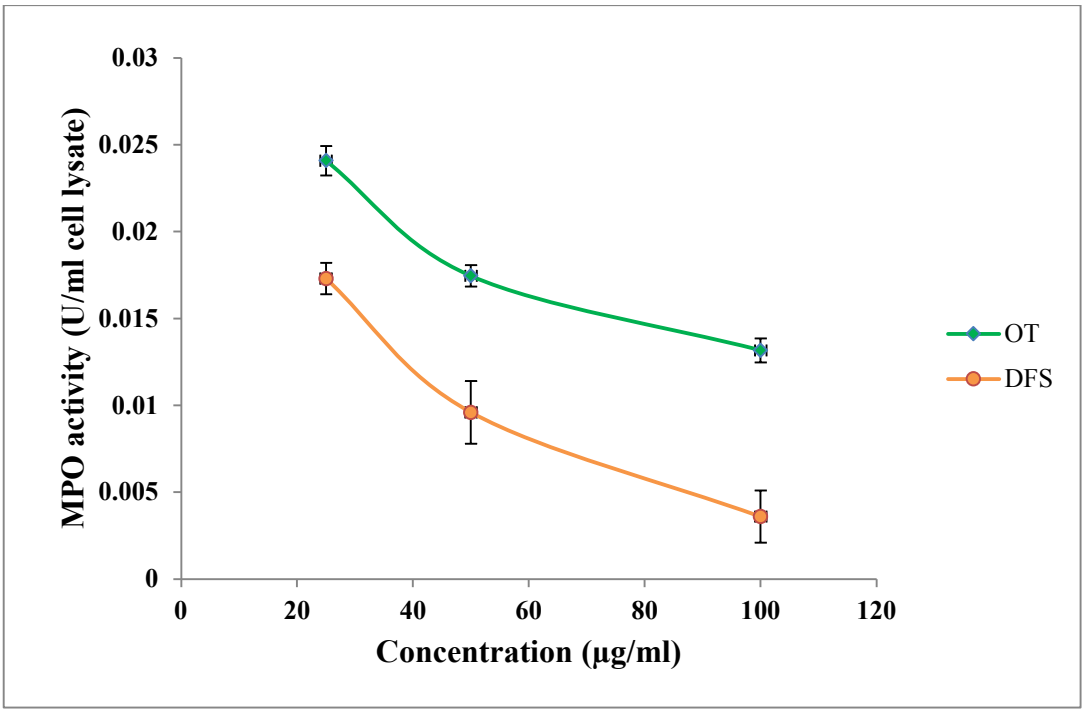


Fig. 32: Effect of methanolic extract of *Ophiorrhiza trichocarpon* on myeloperoxidase enzyme activity. OT - *O. trichocarpon;* **DFS** -Diclofenac sodium

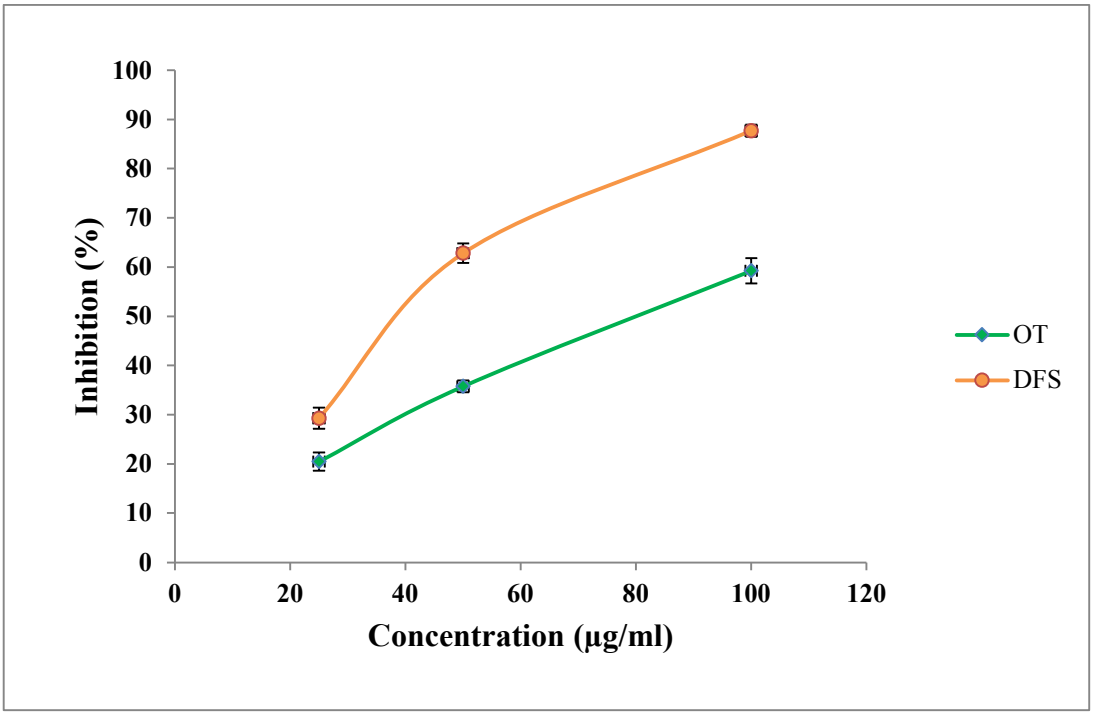


Fig. 33: Effect of methanolic extract of *Ophiorrhiza trichocarpon* on iNOS activity. OT - *O. trichocarpon;* **DFS** - Diclofenac sodium

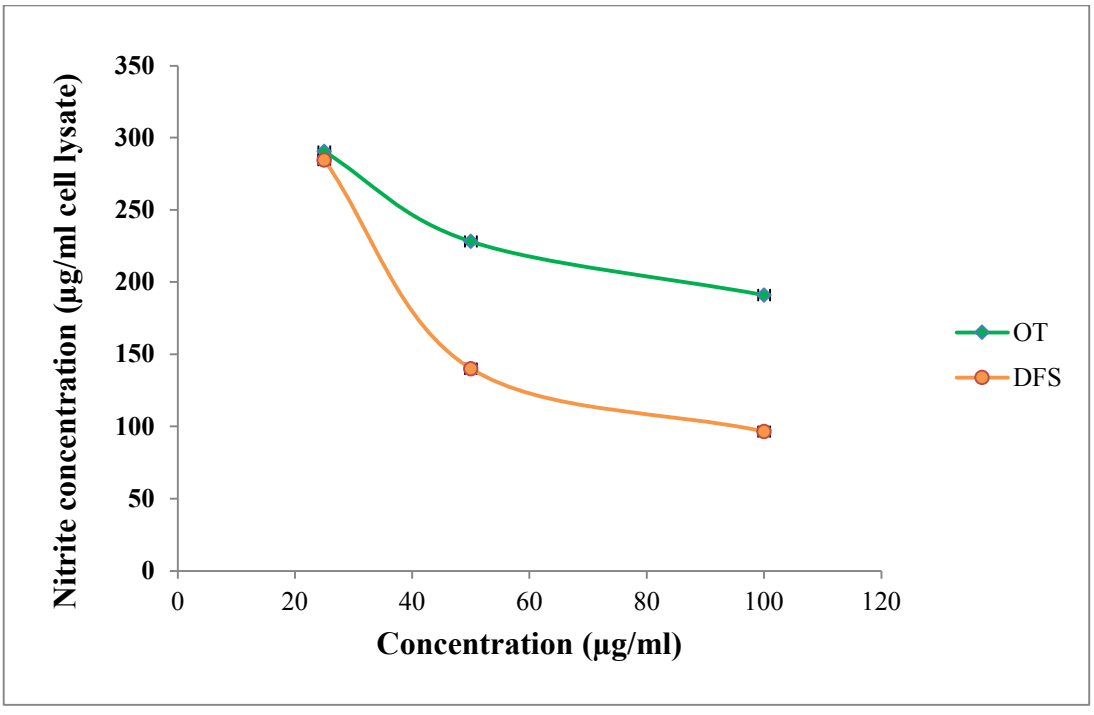


Fig. 34: Effect of methanolic extract of *Ophiorrhiza trichocarpon* on cellular nitrite level. OT - *O. trichocarpon;* **DFS** - Diclofenac sodium

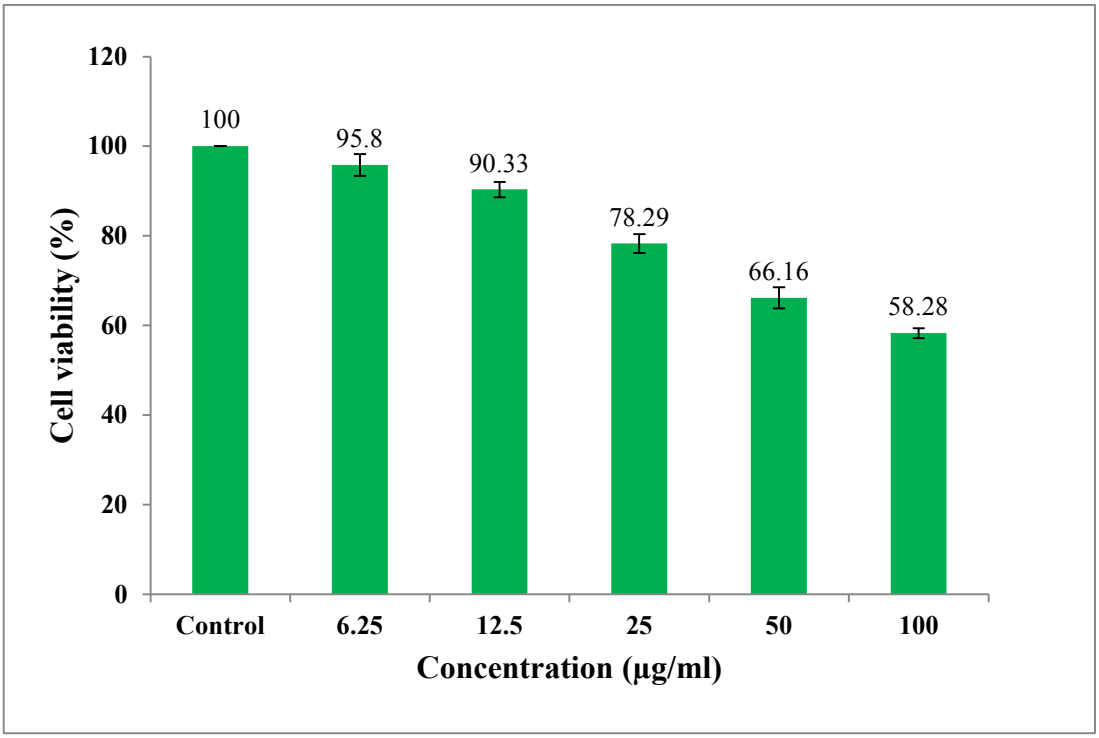


Fig. 35: Cytotoxic effect of methanolic extract of *Ophiorrhiza trichocarpon* on RAW 264.7 cells

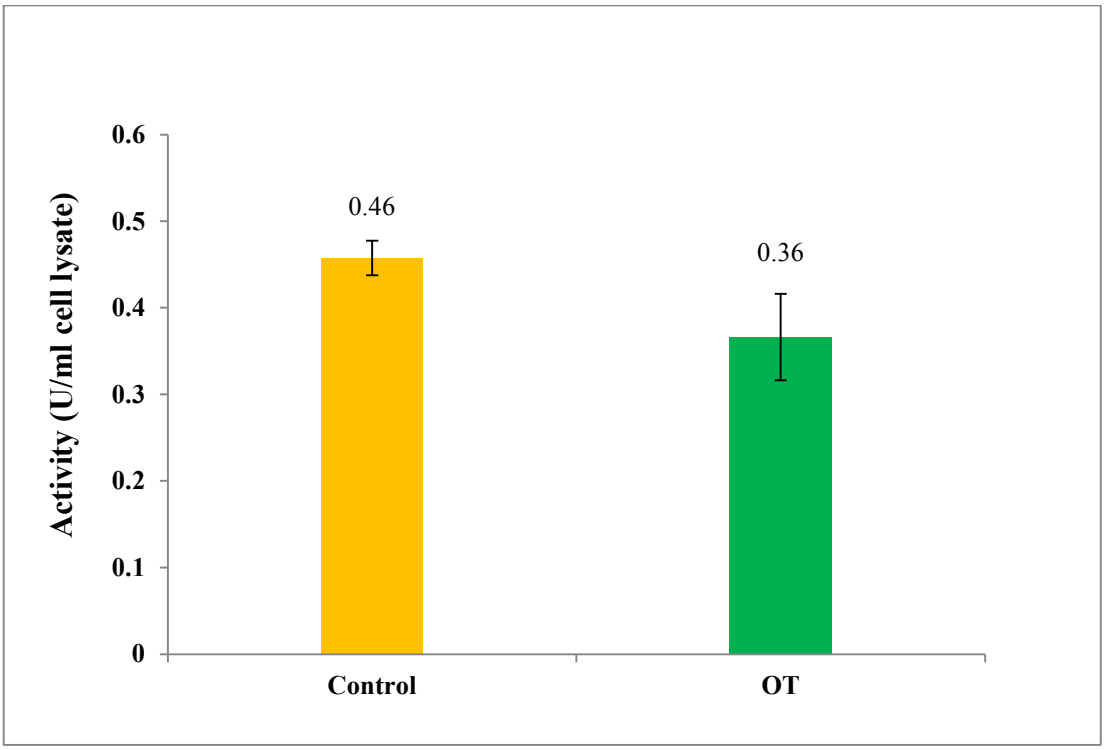


Fig. 36: Effect of methanolic extract of *Ophiorrhiza trichocarpon* on the production of COX-2

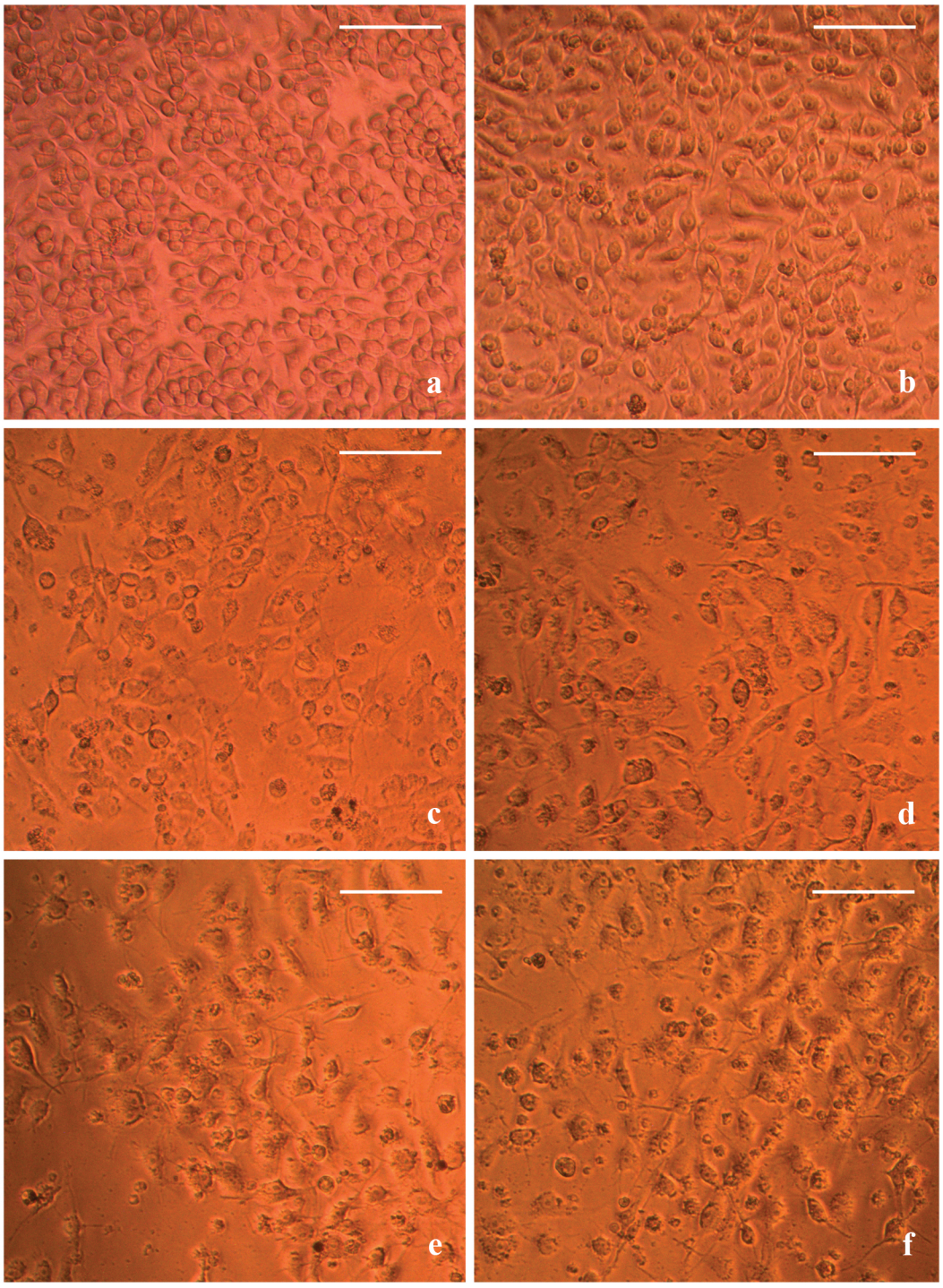


Plate 14: Cytotoxic effect of methanolic extract of *Ophiorrhiza trichocarpon* on RAW 264.7 cells. a: Control; **b:** 6.25 µg/ml; **c:** 12.5 µg/ml; **d:** 25 µg/ml; **e:** 50 µg/ml; **f:** 100 µg/ml. Scale bars = 100 µm.

C. Screening for cytotoxicity with *Allium cepa* L.

The cytotoxic potential of the selected species of *Ophiorrhiza* on *Allium cepa* root tip cells were analysed using different concentrations of the plant extracts (12.5, 25, 50 and 100 µg/ml) which were treated on the root tips at different time periods (1, 12, 24 and 48 h). Distilled water was taken as the negative control and hydrogen peroxide (100 µl/ml) as the positive control. Two different parameters, namely; mitotic index and abnormality percentage were considered to evaluate the mitodepressive effect of the plant extracts on the normal cells. Lowering of the mitotic index and increase in the abnormality percentage are regarded as the indicators of cytotoxicity potential. The statistical data obtained from the cytotoxicity studies are represented in **Figs 37– 40.** In all the selected plant species, mitotic index was found to exhibit a dose dependent reduction from lower to higher concentrations, whereas, abnormality percentage showed a dose dependent increase from lower to higher concentrations. However, an increase in the time period was found to have no effect on these parameters.

After 1 h treatment of the root tips, gradual reduction of mitotic index from the lower concentration of 12.5 µg/ml to the higher concentration of 100 µg/ml was observed in all the selected species. In *O. grandiflora,* the mitotic index was found to be reduced from 72.79 ± 2.40% to 46.15 ± 3.90%. In *O. jacobii,* the range was 86.05 ± 3.55% to 57.99 ± 3.80%. In *O. pectinata,* the mitotic index at the lowest concentration was 66.69 ± 6.55% and that at the highest concentration was 36.22 ± 3.33%. In *O. trichocarpon* also, the gradual reduction in mitotic index was observed from 83.97 ± 3.35% to 44.66 ± 4.35%. An increase in the abnormality percentage indicates the cytotoxic potential of the plant extracts. In the 1 h treatment, all the plant extracts exhibited a gradually increasing percentage of chromosomal abnormality. *O. grandiflora* showed an increasing abnormality percentage from 45.64 ± 7.93% to 84.43 ± 2.09%. Similarly, *O. jacobii* exhibited increasing

abnormality percentage of 49.16 ± 8.54% to 85.68 ± 2.56%. *O. pectinata* showed an abnormality percentage range of 31.02 ± 5.94% to 78.26 ± 4.51%. In *O. trichocarpon* the abnormality percentage was from 62.22 ± 3.86% to 87.21 ± 1.19%. Graphs representing the results of 1 h treatment are given in **Fig. 37.** In this study, the extract of *O. pectinata* showed the lowest mitotic index of 36.22 ± 3.33% at the highest concentration of 100 µg/ml. The value obtained for the positive control was 12.34 ± 2.24%. On the other hand, *O. trichocarpon* exhibited the greater abnormality percentage of 87.21 ± 1.19%, a value very close to that of the positive control value of 88.51 ± 3.43%.

After 12 h treatment of the root tips in the extracts of *O. grandiflora,* the mitotic index was found to be reduced from 82.56 ± 3.00% to 27.34 ± 2.38%. In *O. jacobii,* the range was 89.40 ± 2.11% to 55.73 ± 2.52%. In *O. pectinata,* the mitotic index at the lowest concentration was 86.11 ± 4.84% and that at the highest concentration was 25.34 ± 6.63%. In *O. trichocarpon* also, the gradual reduction in mitotic index was observed from 83.78 ± 2.08% to 49.50 ± 1.78%. In the 12 h treatment, all the plant extracts exhibited a gradually increasing percentage of chromosomal abnormality. *O. grandiflora* showed an increasing abnormality percentage of 51.45 ± 6.04% to 83.33 ± 5.63%. *O. jacobii* exhibited increasing abnormality percentage from 50.21 ± 13.09% to 82.14 ± 2.35%. *O. pectinata* also showed an abnormality percentage range of 17.61 ± 3.84% to 74.54 ± 2.83%. In *O. trichocarpon* the abnormality percentage was 32.96 ± 4.61% to 78.92 ± 2.15%. Graphs representing the results of 12 h treatment are given in **Fig. 38.** Amongst all the extracts, *O. pectinata* showed the lowest mitotic index of 25.34 ± 6.63% at the highest concentration of 100 µg/ml.

Similar to the above experiments, mitotic index gradually declined and abnormality percentage gradually increased in 24 h treatment also. In *O. grandiflora,* the mitotic index reduced from 79.02 ± 2.96% to

45.41 ± 3.08%. In *O. jacobii,* the range was 84.01 ± 2.16% to 62.57 ± 2.06%. In *O. pectinata,* the mitotic index at the lowest concentration was 73.68 ± 6.88% and that at the highest concentration was 28.56 ± 2.94%. In *O. trichocarpon*, the gradual reduction in mitotic index was observed from 81.60 ± 4.90% to 44.10 ± 3.45%. In the case of chromosomal aberrations, *O. grandiflora* showed an increasing abnormality percentage of 35.89 ± 2.69% to 76.80%. *O. jacobii* exhibited increasing abnormality percentage from 51.75 ± 5.02% to 80.72 ± 2.38%. *O. pectinata* also showed an abnormality percentage range of 29.95 ± 4.45% to 85.86 ± 3.57%. In *O. trichocarpon* the abnormality percentage was 57.69 ± 5.14% to 79.56 ± 3.29%. Graphs representing the results of 24 h treatment are given in **Fig. 39.** In 24 hour treatment, the lowest mitotic index (28.56 ± 2.94%) as well as the highest abnormality percentage (85.86 ± 3.57%) was observed in root cells treated with the extract of *O. pectinata.* The values of both the parameters were in close proximity with the values obtained for the positive control.

After 48 h treatment, similar results of decreasing mitotic index and increasing abnormality percentage was observed for all the plant species. In *O. grandiflora,* the mitotic index reduced from 83.50 ± 1.62% to 66.66 ± 6.67%. In *O. jacobii,* the range was 83.28 ± 2.27% to 35.97 ± 3.76%. In *O. pectinata,* the mitotic index at the lowest concentration was 86.49 ± 2.12% and that at the highest concentration was 63.06 ± 10.08%. In *O. trichocarpon* also, the gradual reduction in mitotic index was observed from 81.67 ± 2.46% to 33.13 ± 2.58%. *O. grandiflora* showed an increasing abnormality percentage of 45.15 ± 3.17% to 82.22 ± 2.24%. *O. jacobii* exhibited increasing abnormality percentage from 59.30 ± 1.30% to 84.46 ± 5.25%. *O. pectinata* showed an abnormality percentage range of 26.78 ± 4.45% to 86.32 ± 1.79%. In *O. trichocarpon* the abnormality percentage was 63.74 ± 2.96% to 85.96 ± 2.09%. Graphs representing the

results of 48 h treatment are given in **Fig. 40.** In 48 h treatment, the lowest mitotic index (33.13 ± 2.58%) was exhibited by *O. trichocarpon* but the highest abnormality percentage (86.32 ± 1.79%) was observed in root cells treated with the extract of *O. pectinata.*

While considering the time periods of treatment, a time dependent effect on the mitotic index or abnormality percentage was not observed in the selected species. In the case of *O. grandiflora,* after 1 h treatment, the mitotic index at the highest concentration was 46.15 ± 3.90%. After 12 h treatment, the mitotic index was observed as 27.34 ± 2.38%, but after the 24 h treatment the value was 45.41 ± 3.08%. The chromosomal abnormality percentage was also not time dependent. For the highest concentration, the value was 76.80 ± 2.00% at the 24 h treatment, whereas it was 82.22 ± 2.24% at the 48 h treatment. Similar results were disclosed by all the other species while considering the effect of time on the cytotoxicity parameters.

Microscopic observations of the treated root cells revealed several chromosomal aberrations at each mitotic stage. The normal mitotic stages of cell division are depicted in **Plate 15.** Chromosomal aberrations include both clastogenic and non-clastogenic aberrations. Clastogenic aberrations occur when the genetic material is affected, while non-clastogenic aberrations or aneugenic aberrations happen when the mitotic spindle formation is disturbed. Some of the major types of chromosomal aberrations observed in each of the mitotic stages are given in **Plates 16–20.** Clastogenic aberrations like chromosome coagulation, pulverisation, stickiness, fragmentations, chromosome bridges, chromosome clumping, giant cell formation, nuclear lesions, nuclear erosions, *etc*., were observed. Non-clastogenic aberrations like tropokinesis, vagrants, C-mitosis, ball or star shaped or pole to pole arrangement of chromosomes, *etc.,* were also observed.

Thus, the study suggested that all the plant extracts offer dose dependent cytotoxicity on the treated root tip cells. As concentration

increased, reduction in mitotic index and rise in the abnormality percentage was observed. However, time duration was found to have no significant role in determining the cytotoxic nature of the extracts. From the cytotoxicity assay, all the selected plant species were found to possess significant mitodepressive potential. The induction of chromosomal aberrations can be inferred as the cytotoxicity potential of the plant extract and its potential to interfere with the cell cycle.

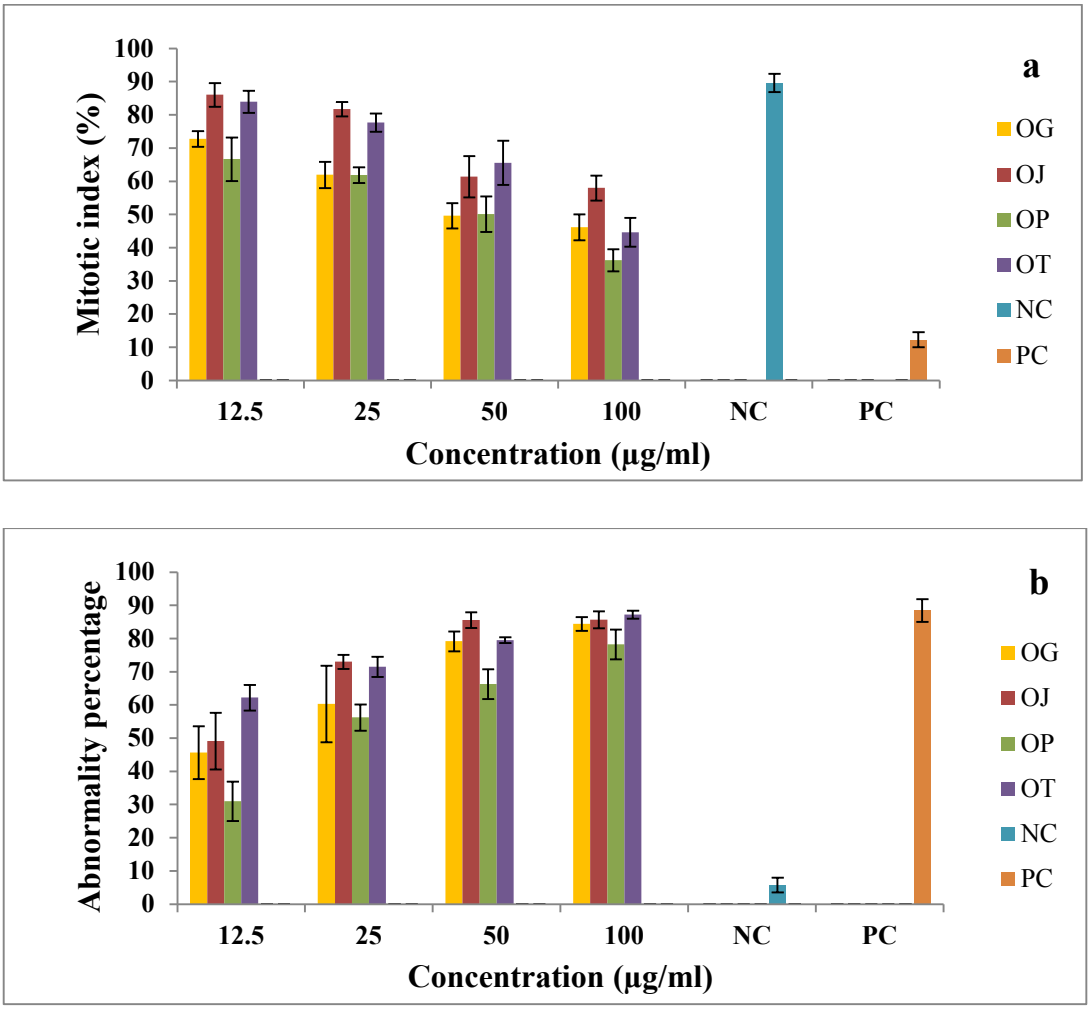


Fig. 37: The effects of different concentrations of methanolic extract of selected species of *Ophiorrhiza* on *Allium cepa* meristematic root tip cells after treatment for 1 h. a: Mitotic index; b: Abnormality percentage. **OG** - *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon;* **NC** - Negative control; **PC** - Positive control

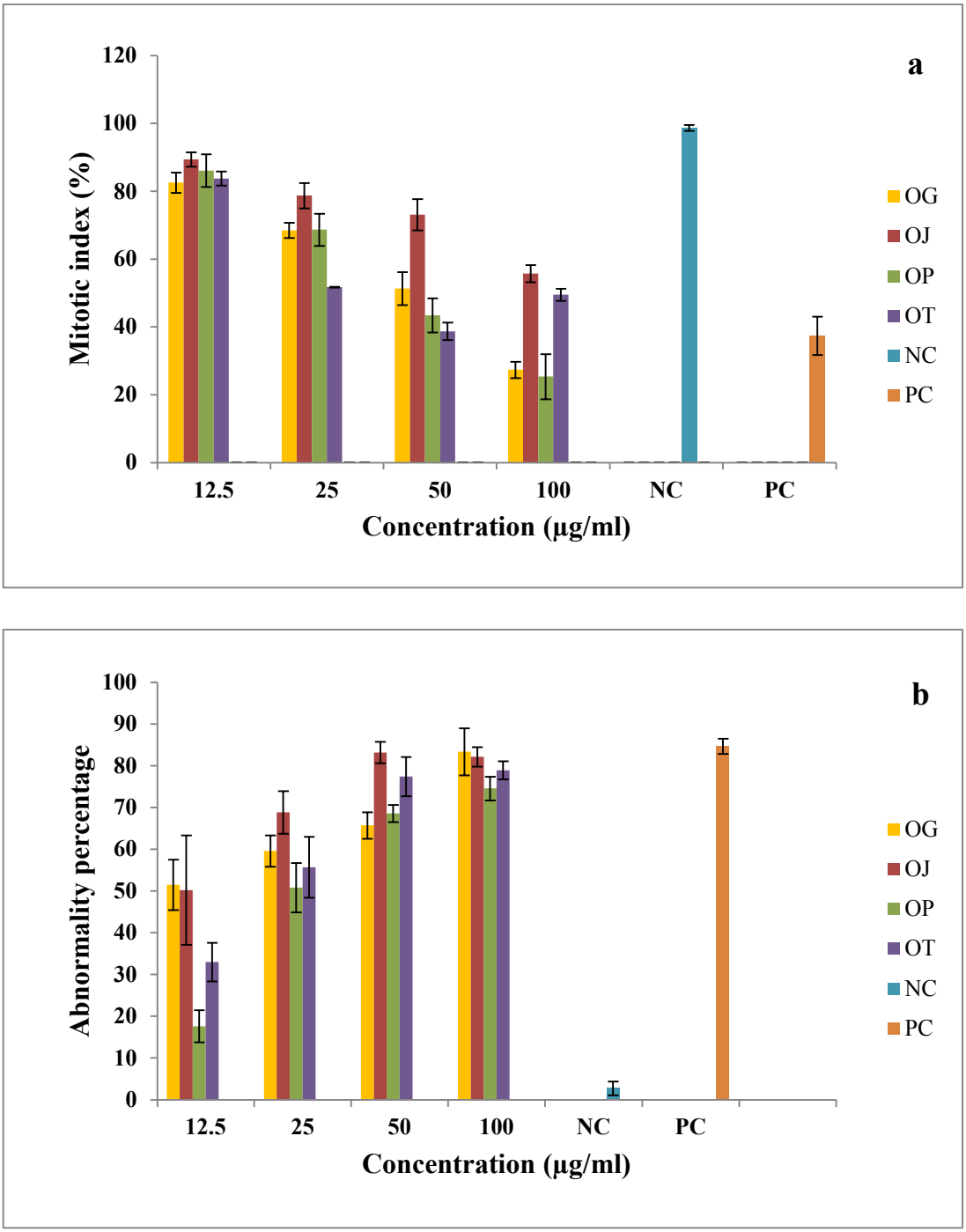


Fig. 38: The effects of different concentrations of methanolic extract of selected species of *Ophiorrhiza* on *Allium cepa* meristematic root tip cells after treatment for 12 h. a: Mitotic index; b: Abnormality percentage. OG - *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon;* **NC** - Negative control; **PC** - Positive control.

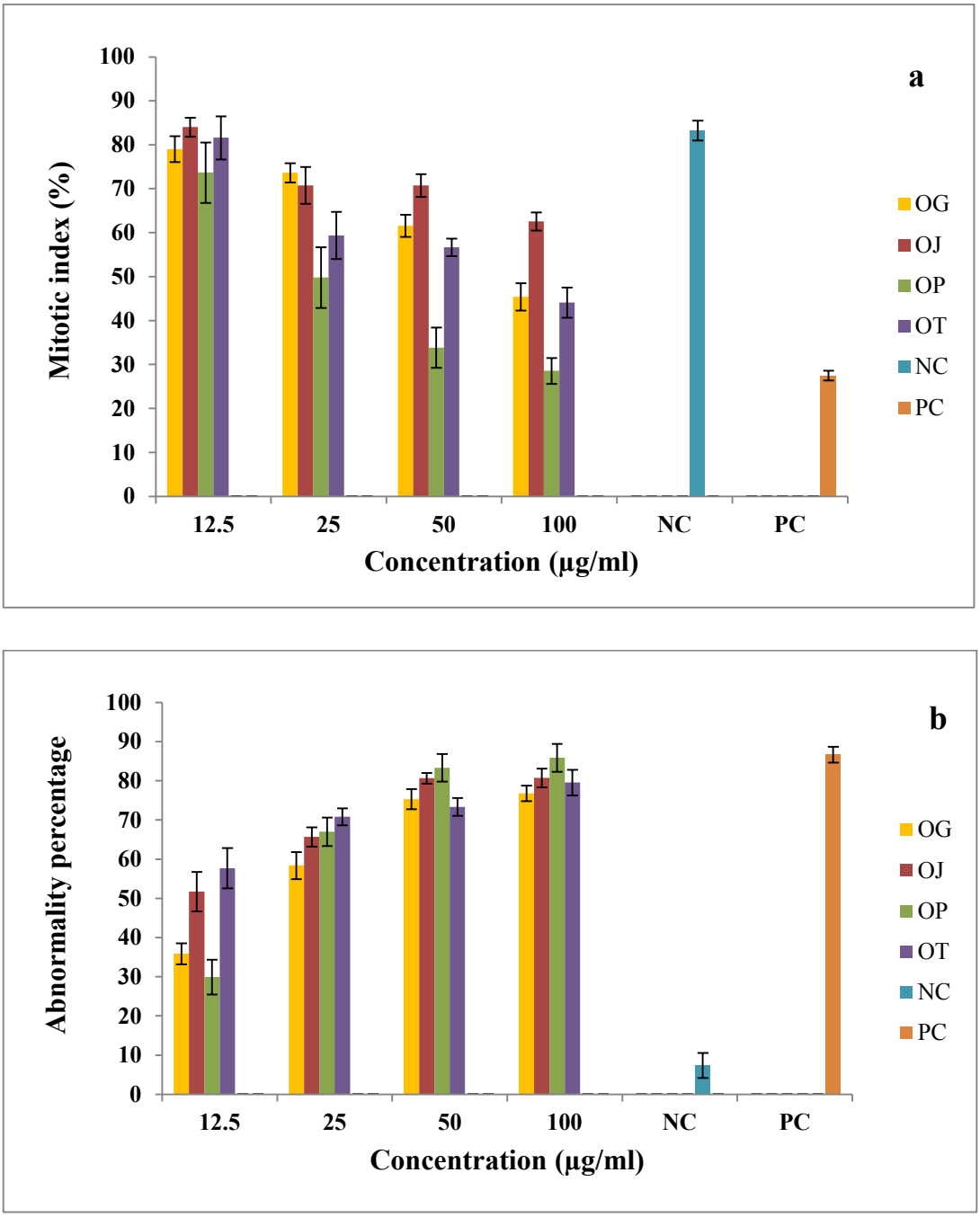


Fig. 39: The effects of different concentrations of methanolic extract of selected species of *Ophiorrhiza* on *Allium cepa* meristematic root tip cells after treatment for 24 h. a: Mitotic index; b: Abnormality percentage. OG - *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon;* **NC** - Negative control; **PC** - Positive control

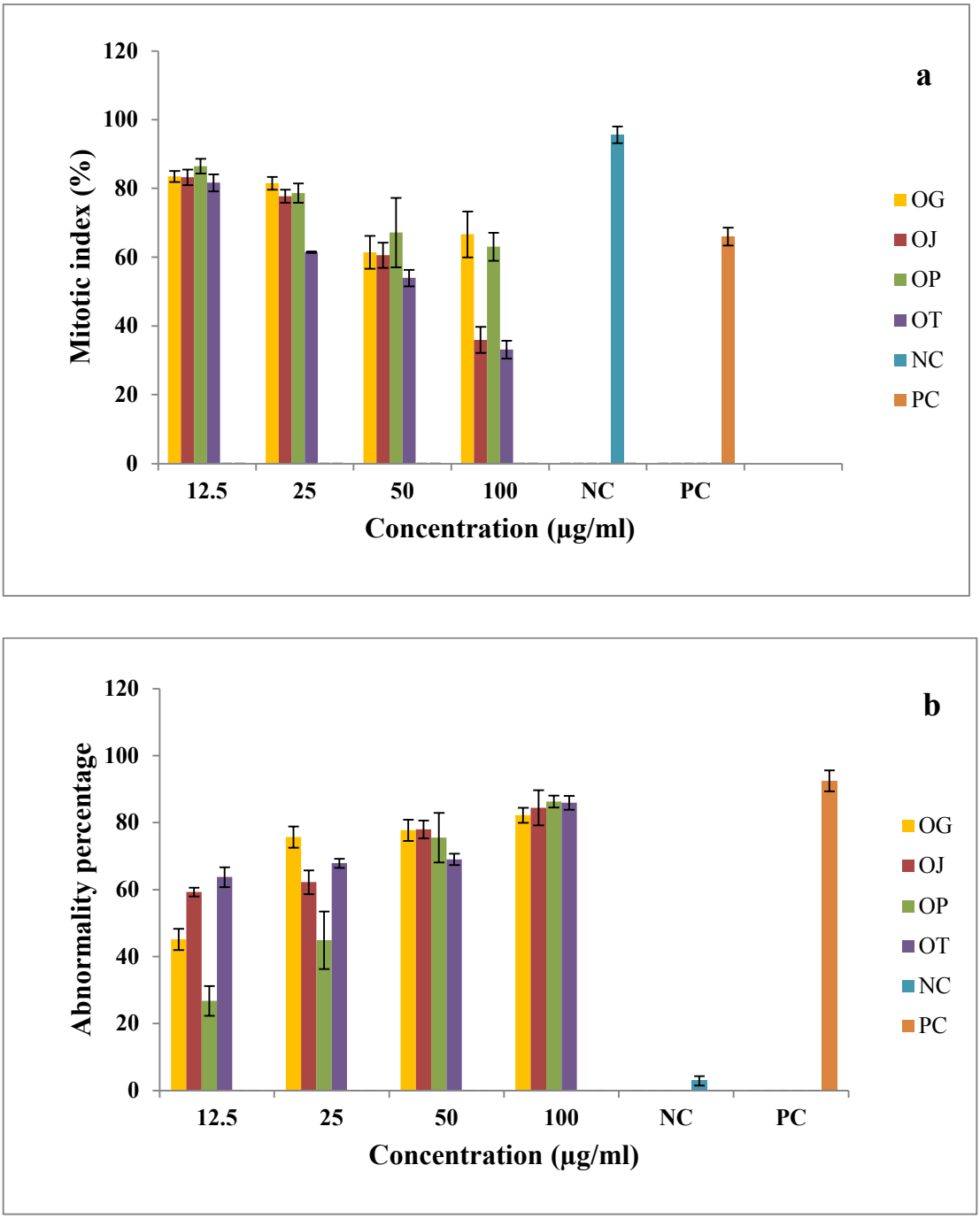


Fig. 40: The effects of different concentrations of methanolic extract of selected species of *Ophiorrhiza* on *Allium cepa* meristematic root tip cells after treatment for 48 h. a: Mitotic index; b: Abnormality percentage. OG - *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** - *O. trichocarpon;* **NC** -Negative control; **PC** - Positive control

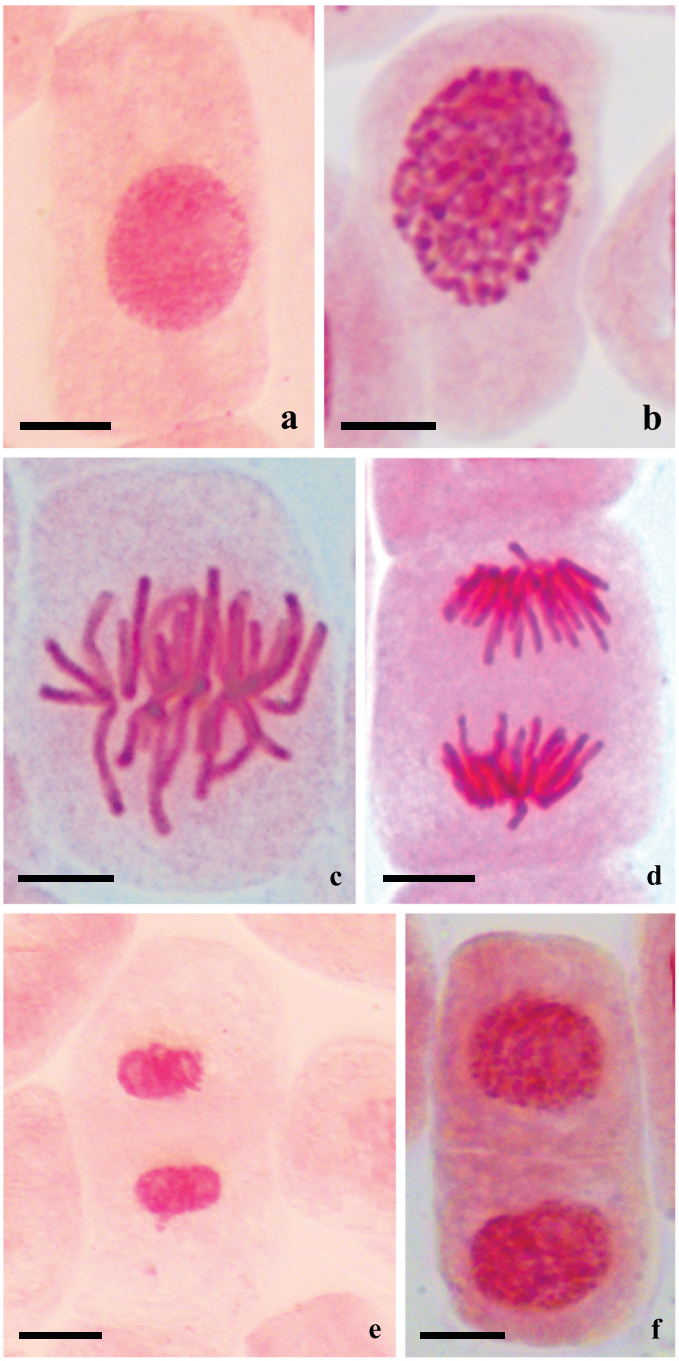


Plate 15: Normal stages of mitotic cell division in *Allium cepa* root tip cells. a: Interphase; **b:** Prophase; **c:** Metaphase; **d:** Anaphase; **e:** Telophase; **f:** Cytokinesis. Scale bars = 10 µm.

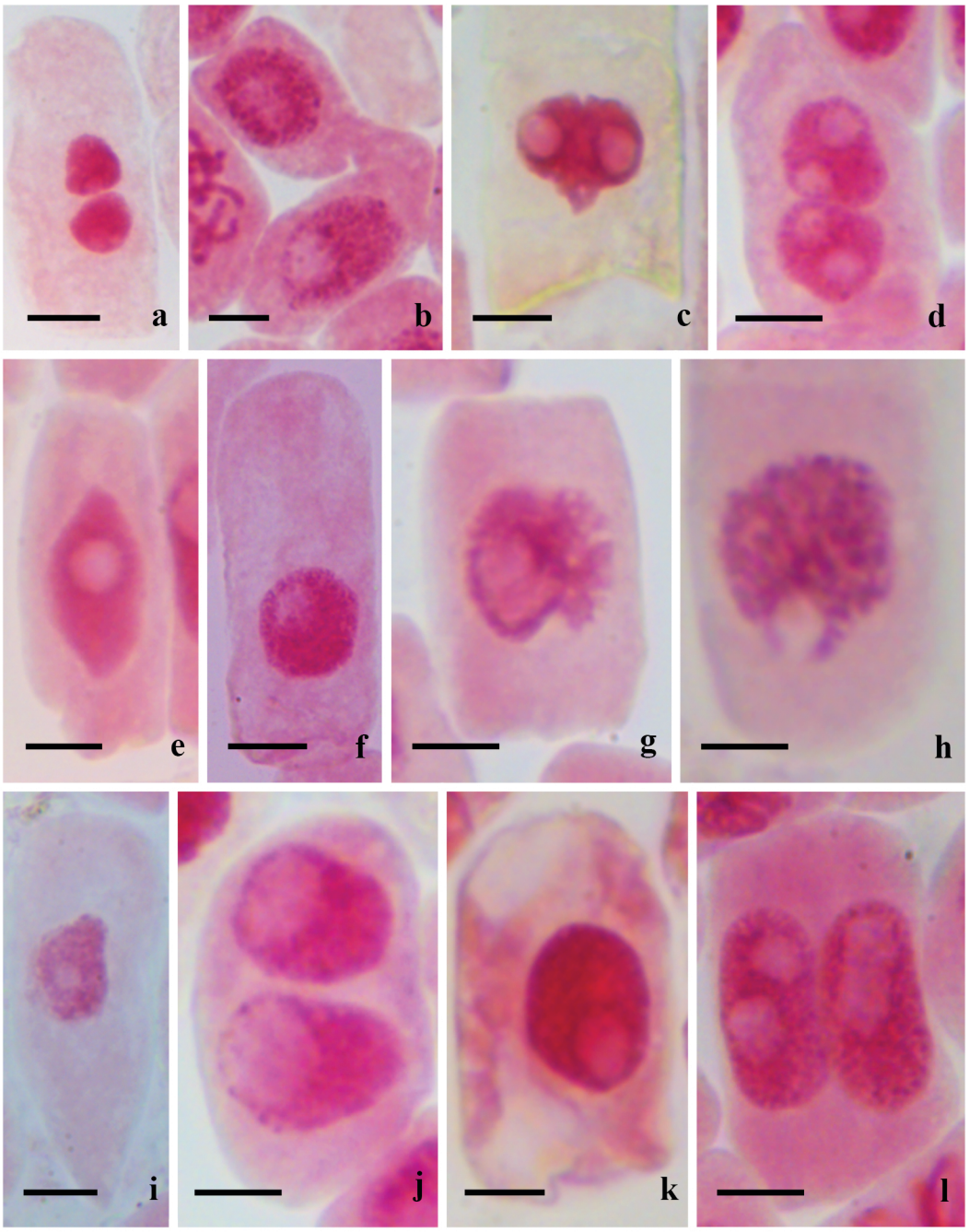


Plate 16: Chromosomal aberrations induced by the extracts of selected species of *Ophiorrhiza* in *Allium cepa* at Interphase. a: Binucleate cell; **b:** Cytomictic channel formation and nuclear lesions at late interphase; **c:** Bizzare nucleus showing lesions; **d:** Binucleate cell showing double nuclear lesions; **e:** Cell shrinkage with nuclear peaks and lesion; **f:** Giant cell showing single nuclear lesion; **g:** Nuclear lesion and erosion**; h:** Nuclear erosion at late interphase**; i:** Nuclear lesion and peak formation**; j:** Binucleate cell showing single nuclear lesions**; k:** Cell showing shrinkage, nuclear and cytoplasmic lesions; **l:** Binucleate cell showing single and double nuclear lesions. Scale bars = 10 µm.

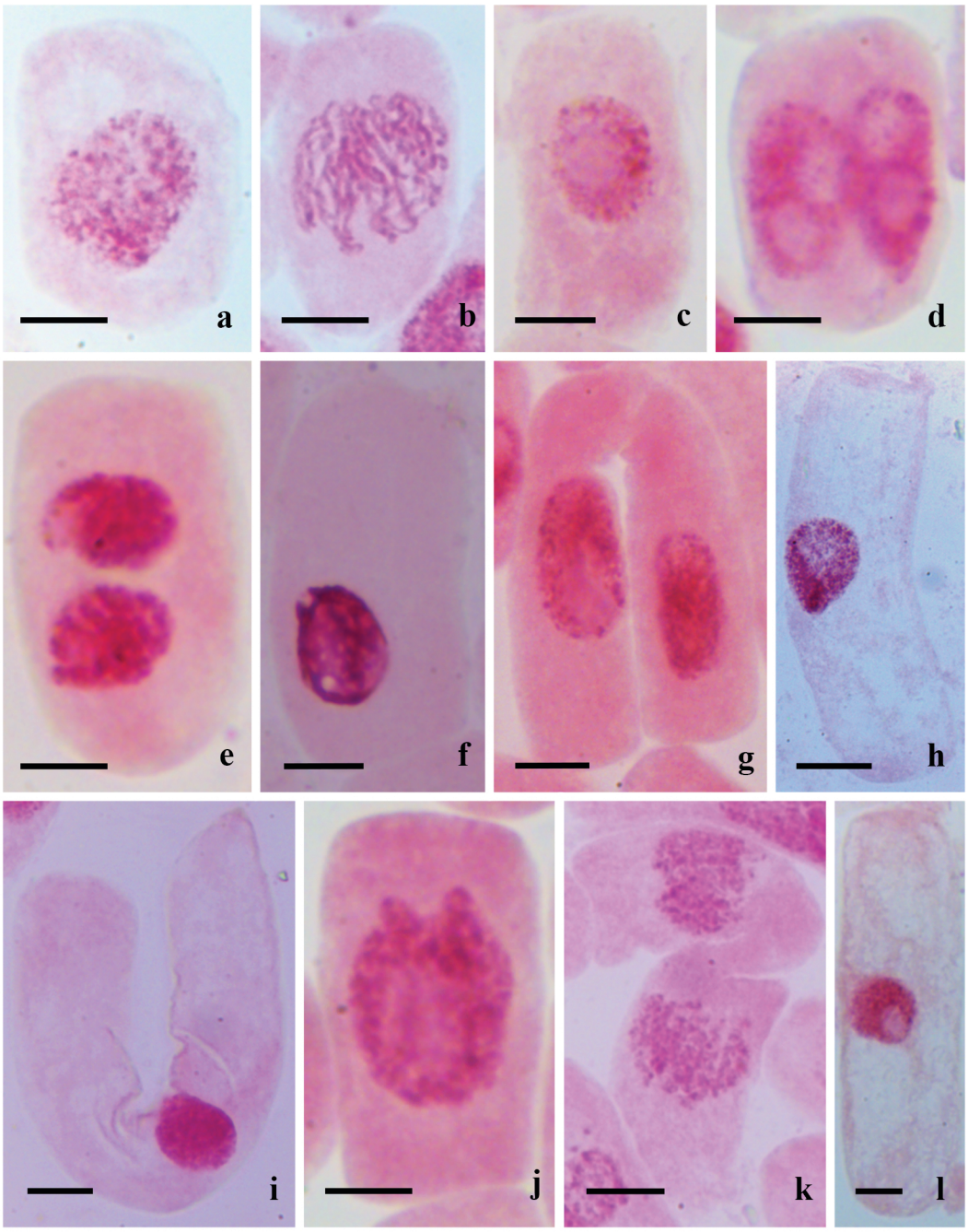


Plate 17: Chromosomal aberrations induced by the extracts of selected species of *Ophiorrhiza* in *Allium cepa* at Prophase. a: Chromatin erosion; **b:** Chromatin disintegration at late prophase; **c:** Nuclear disintegration and lesion; **d:** Binucleate cell at sticky prophase showing double nuclear lesions; **e:** Binucleate cell showing nuclear erosion; **f:** Bizarre nucleus with multiple lesions and double nuclear peaks; **g:** Cytomictic connection between cells**; h:** Nuclear budding**; i:** Giant cell showing shrinkage; **j:** Bizarre nucleus with double nuclear budding**; k:** Cytomictic channel formation showing nuclear disintegration; **l:** Nuclear and cytoplasmic lesions in a giant cell. Scale bars = 10 μm.

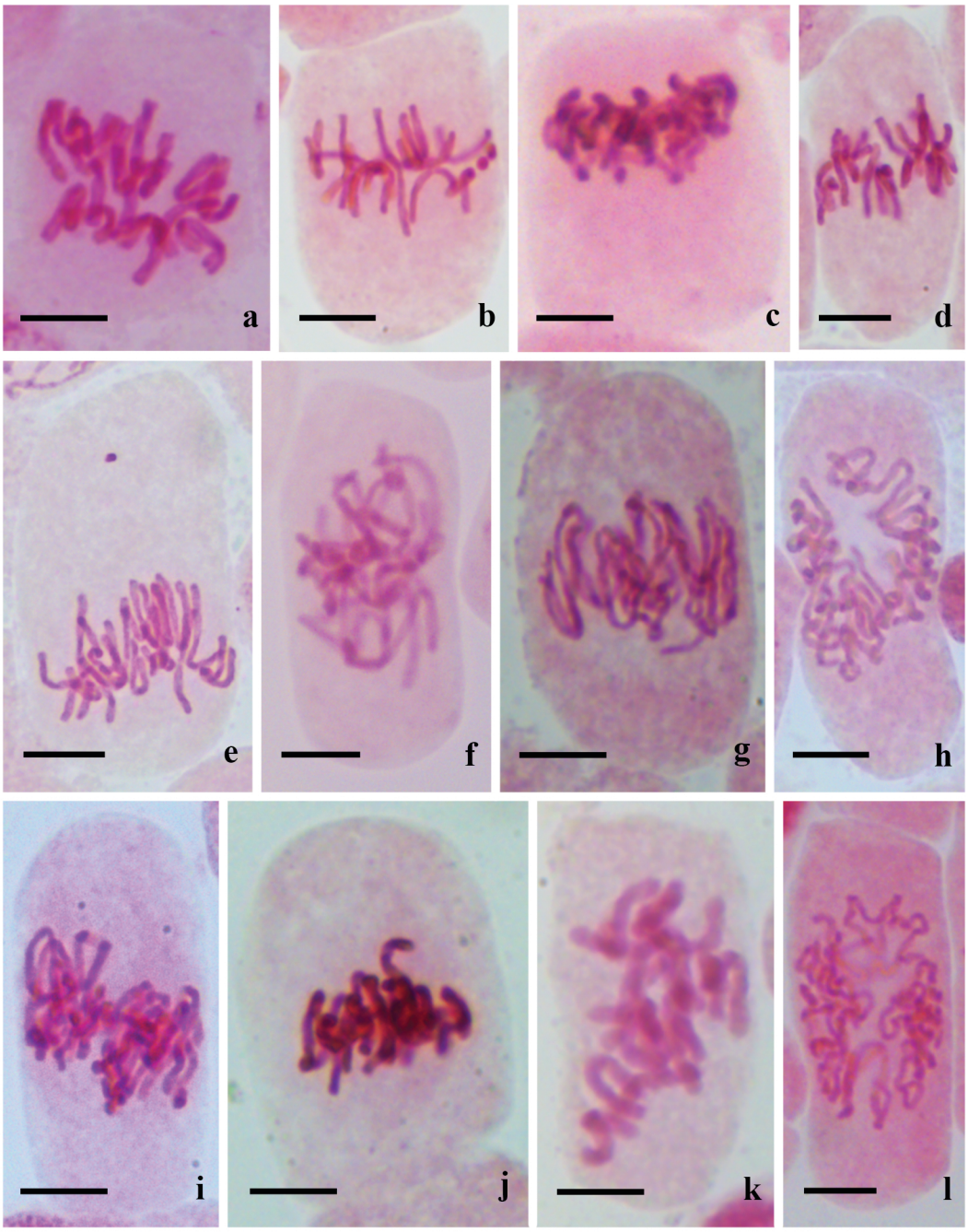


Plate 18 (I): Chromosomal aberrations induced by the extracts of selected species of *Ophiorrhiza* in *Allium cepa* at Metaphase. a: C-metaphase; **b:** Chromosome granulation; **c:** Chained sticky metaphase showing misorientation; **d:** Chromosome erosion; **e:** Chromosome fragment at metaphase showing misorientation; **f:** Chained chromosomes showing abnormal condensation at early metaphase; **g:** Chained early metaphase**; h:** Chained metaphase in a hyperploid cell; **i:** Tilt in equatorial plate at metaphase showing disturbed chromosomes in a hyperploid cell; **j:** Chromosome shrinkage in a hypoploid cell**; k:** Diagonal disturbed metaphase showing hypercondensed chromosomes; **l:** Chained early metaphase showing chromosome fragment. Scale bars = 10 μm.

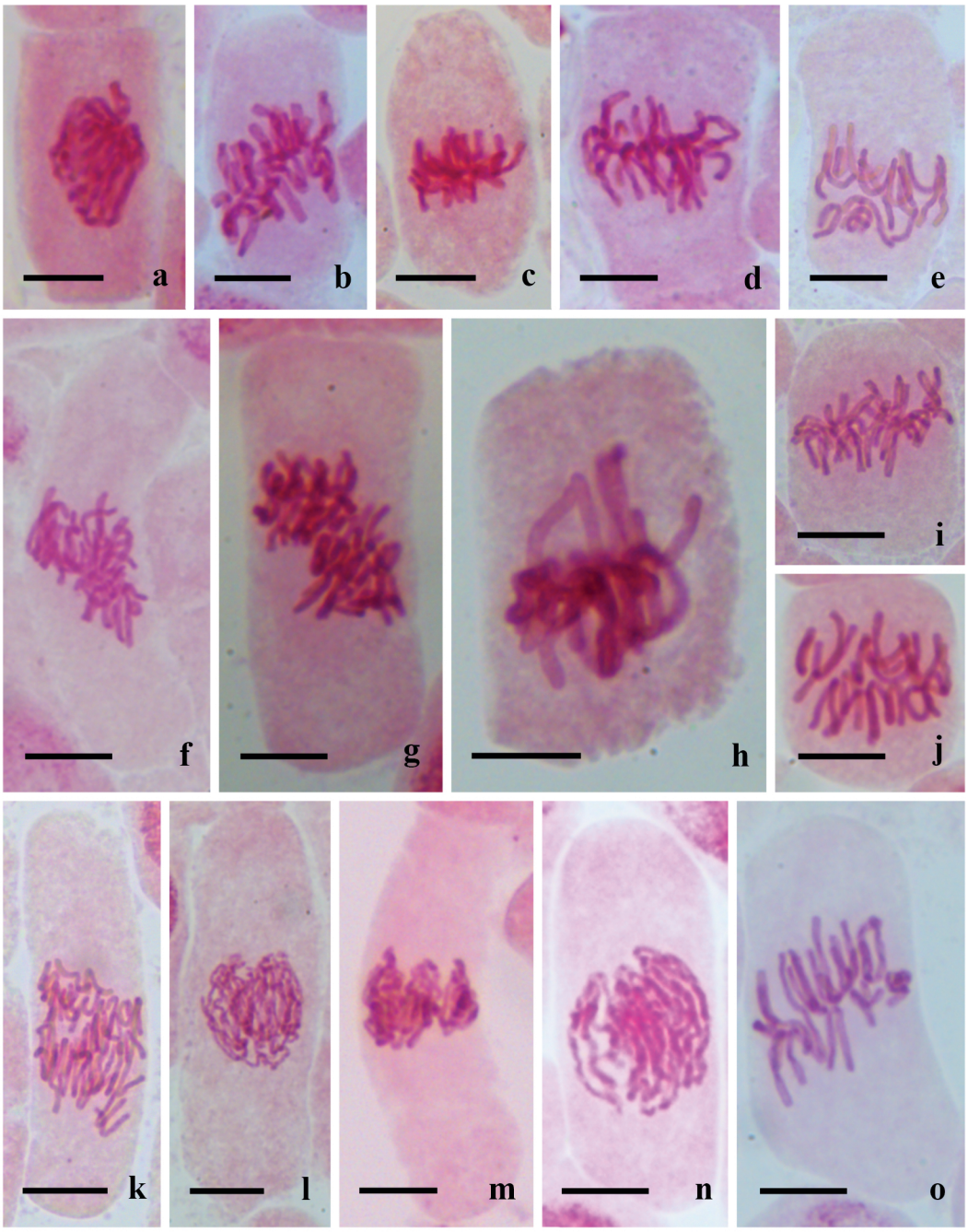


Plate 18 (II): Chromosomal aberrations induced by the extracts of selected species of *Ophiorrhiza* in *Allium cepa* at Metaphase. a: Sticky chained chromosomes; **b:** Diagonal C-metaphase; **c:** Stellate sticky partial C-metaphase; **d:** Disturbed metaphase showing vagrants; **e:** Disturbed metaphase; **f:** Giant hyperploid cell ; **g:** Diagonal metaphase in a polyploid giant cell**; h:** Chromosome clumping; **i:** Misoriented C-metaphase; **j:** Cytostasis at metaphase**; k:** Disturbed metaphase in a polyploid cell; **l:** Ghost cell showing chained early ball metaphase; **m:** Coagulated chromatin at early metaphase; **n:** Early ball metaphase; **o:** Diagonal metaphase showing vagrants. Scale bars = 10 μm.

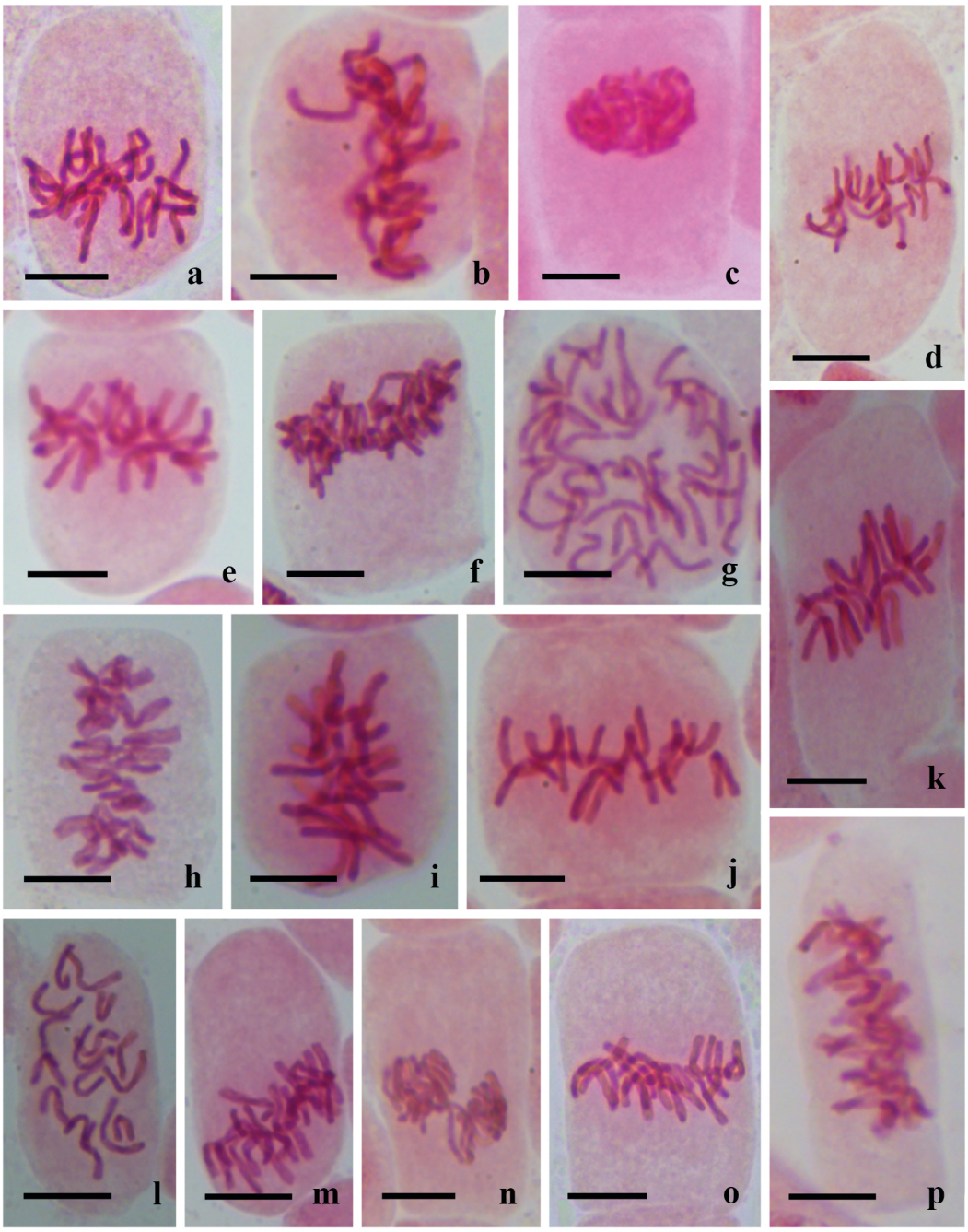


Plate 18 (III): Chromosomal aberrations induced by the extracts of selected species of *Ophiorrhiza* in *Allium cepa* at Metaphase. a: Misorientation of chromosomes; **b:** Pole to pole sticky metaphase; **c:** Sticky ball metaphase; **d:** Sticky metaphase; **e:** Stellate metaphase; **f:** Shift in metaphase plate in a polyploid cell; **g:** Scattered metaphase in a polyploid cell**; h:** Pole to pole metaphase; **i:** Pole to pole stellate metaphase; **j:** Metaphase in a hypoploid cell**; k:** Tropokinesis; **l:** Scattered partial C-metaphase; **m:** Diagonal C-metaphase showing misorientation; **n:** Misoriented chromosomal group formation; **o:** C-metaphase; **p:** Pole to pole sticky C- metaphase. Scale bars = 10 µm.

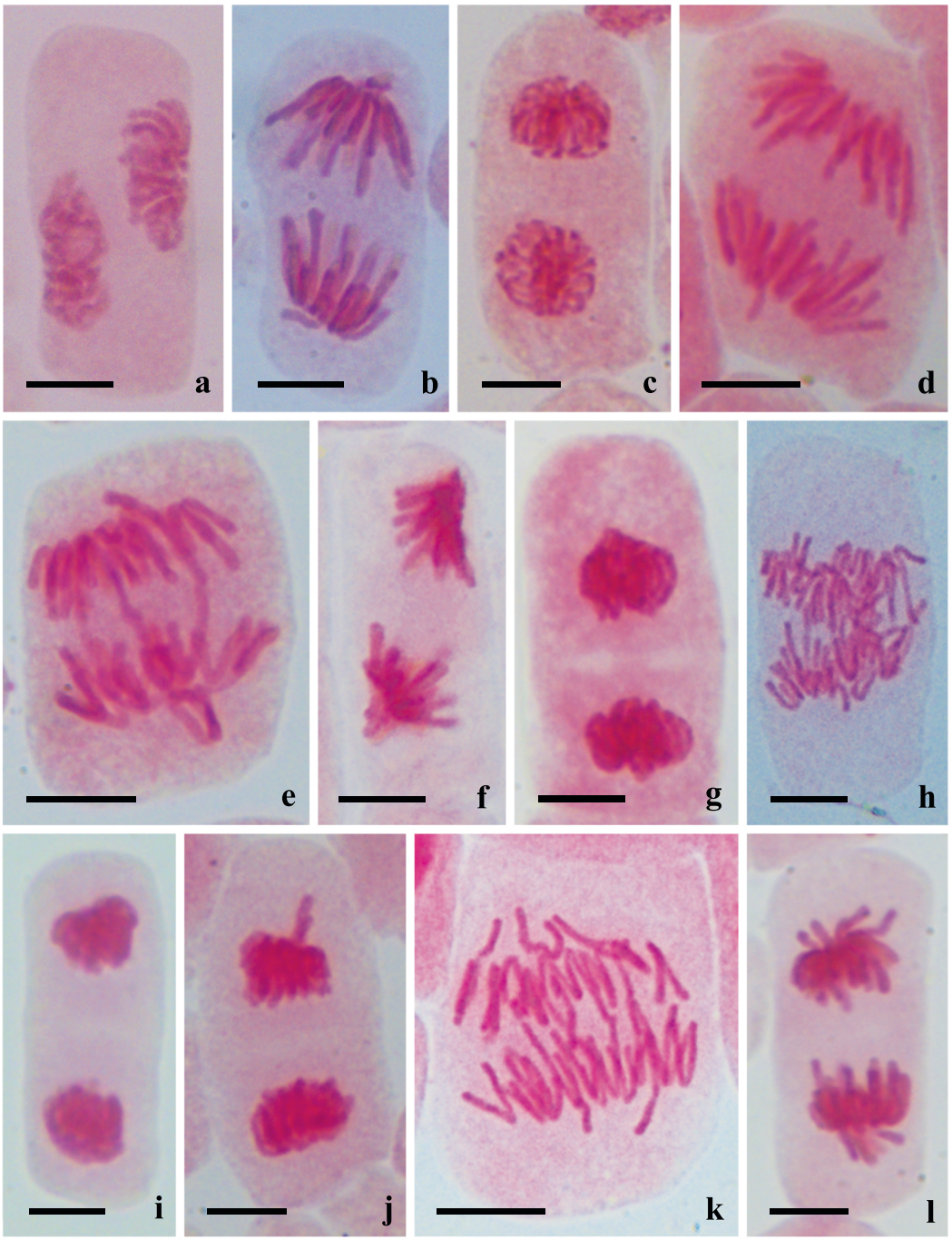


Plate 19 (I): Chromosomal aberrations induced by the extracts of selected species of *Ophiorrhiza* in *Allium cepa* at Anaphase. a: Equatorial separation of chromosomes showing erosion; **b:** Diagonal anaphase showing early movement of chromosomes; **c:** Ball anaphase; **d:** Diagonal anaphase; **e:** Diagonal anaphase showing chromosome bridges and vagrants; **f:** Diagonal coagulated sticky stellate anaphase; **g:** Coagulated sticky stellate anaphase showing early cell plate formation; **h:** Disturbed anaphase showing chromosome bridges and vagrants in a hyperploid cell; **i:** Coagulated anaphase; **j:** Coagulated anaphase showing vagrant chromosome; **k:** Chromosome laggards and vagrants at anaphase; **l:** Hemi-stellate sticky anaphase showing slightly diagonal arrangement. Scale bars = 10 μm.

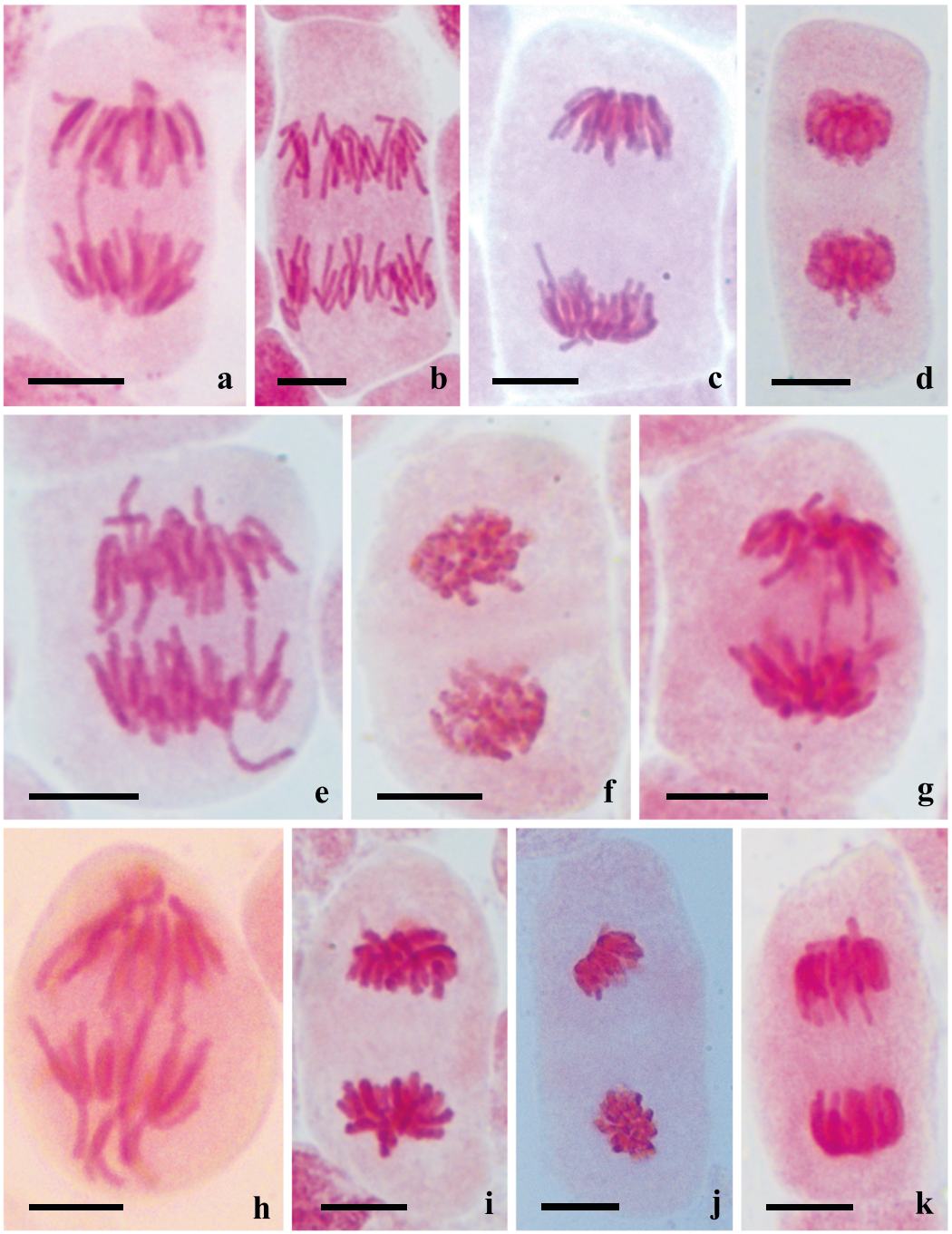


Plate 19 (II): Chromosomal aberrations induced by the extracts of selected species of *Ophiorrhiza* in *Allium cepa* at Anaphase. a: Sticky chromosome bridge and chromosome erosion; **b:** Multipolar separation of chromosomes; **c:** Vagrants and abnormal decondensation of daughter chromosomes; **d:** Sticky late anaphase showing vagrants; **e:** Early movement of chromosomes and vagrants; **f:** Sticky chained anaphase; **g:** Sticky multiple bridges; **h:** Chromosome bridges and vagrants at anaphase showing chromosome erosion; **i:** Sticky hemistellate anaphase; **j:** Hemi-stellate anaphase showing shift in MTOC in one daughter chromosome group; **k:** Sticky anaphase. Scale bars = 10 µm.

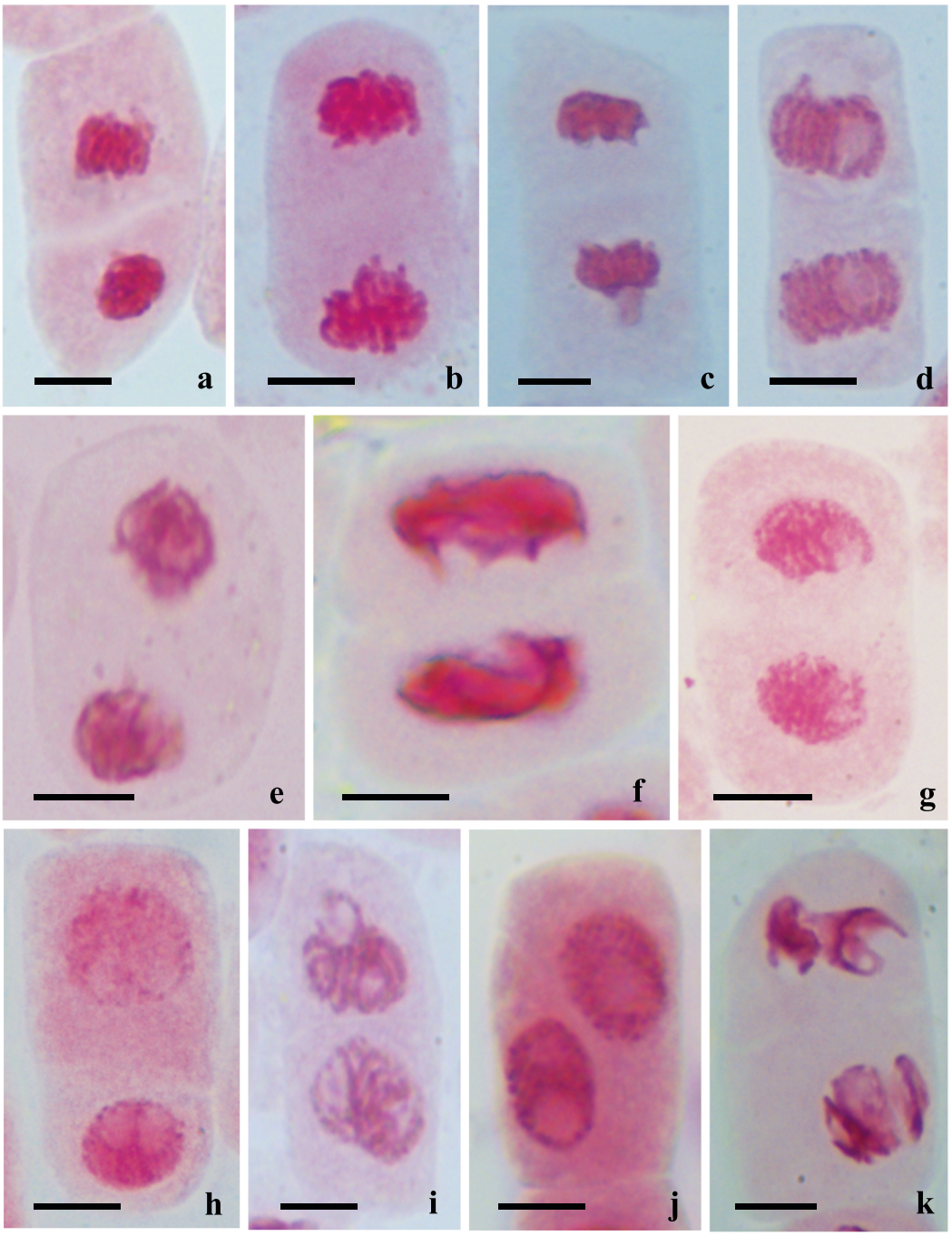


Plate 20: Chromosomal aberrations induced by the extracts of selected species of *Ophiorrhiza* in *Allium cepa* at Telophase (a-e) and Cytokinesis (f-k). a: Coagulated chromatin at telophase showing oblique cell plate; **b:** Sticky telophase; **c:** Coagulated early telophase showing early cell plate formation and vagrant chromosomes; **d:** Chromosome erosion at late telophase; **e:** Chromosome erosion at diagonal telophase; **f:** Bizarre nuclei with coagulated chromatin; **g:** Chromatin erosion; **h**: Nuclear lesions and disintegration; **i:** Pulverised chromatin; **j:** Diagonal cell plate formation showing nuclear lesion; : Bizarre nuclei showing fragmentation and coagulated chromatin. Scale bars = 10 µm.

D. Antiproliferative activity

i. *In vitro* cytotoxicity determination by MTT assay on L929 cells

The cytotoxicity of the selected species of *Ophiorrhiza* was first analysed on the normal L929 cells by MTT assay. Different concentrations of the extracts (6.25, 12.5, 25, 50 and 100 µg/ml) of all the species were treated on the cells for 24 h and the LC_{50} value for each extract was determined. The results are represented in **Plates 21-24** and the cytotoxicity effect of each species on the cell viability is given in **Fig. 41.** The results indicated that all the extracts exhibited a dose dependent cytotoxicity effect on the cells. Cell viability decreased with increase in the concentration. The LC_{50} value obtained for each species is given in **Table 16.** The higher values of LC_{50} showed that all the extracts have less cytotoxic effect on the normal cells. Among the selected species, the highest LC_{50} value was for *O. grandiflora* (269.81 ± 2.54 µg/ml) and the lowest value was obtained for *O. trichocarpon* (107.26 ± 2.55 µg/ml).

ii. *In vitro* cytotoxicity determination by MTT assay on MDA-MB-231 cells

The cytotoxicity activity of the selected species of *Ophiorrhiza* on the MDA-MB 231 cancer cells was evaluated using different concentrations of the extracts (6.25, 12.5, 25, 50 and 100 µg/ml) of all the species and the LC_{50} value for each extract was determined. The results are represented in **Plates 25-28** and the cytotoxicity effect of each species on the cell viability is given in **Fig. 42.** The LC_{50} value obtained for each species is given in **Table 16.** From the microscopic observations, several apoptotic features like nuclear fragmentation, condensed nucleus, cell shrinkage, membrane blebbing, apoptotic bodies, budding and echinoid spikes were clearly visible. The results indicated that all the extracts exhibited a dose dependent cytotoxicity effect on the cancer cells. From the lowest concentration of 6.25 µg/ml to the highest concentration of 100 µg/ml, all the plant extracts showed a gradually decreasing cell viability percentage. The highest cytotoxicity range was

expressed by *O. trichocarpon*, ranging from 91.28 ± 1.34% to 44.86 ± 1.46% with a LC_{50} value of 84.08 ± 2.74 µg/ml. Though, *O. grandiflora* acted as

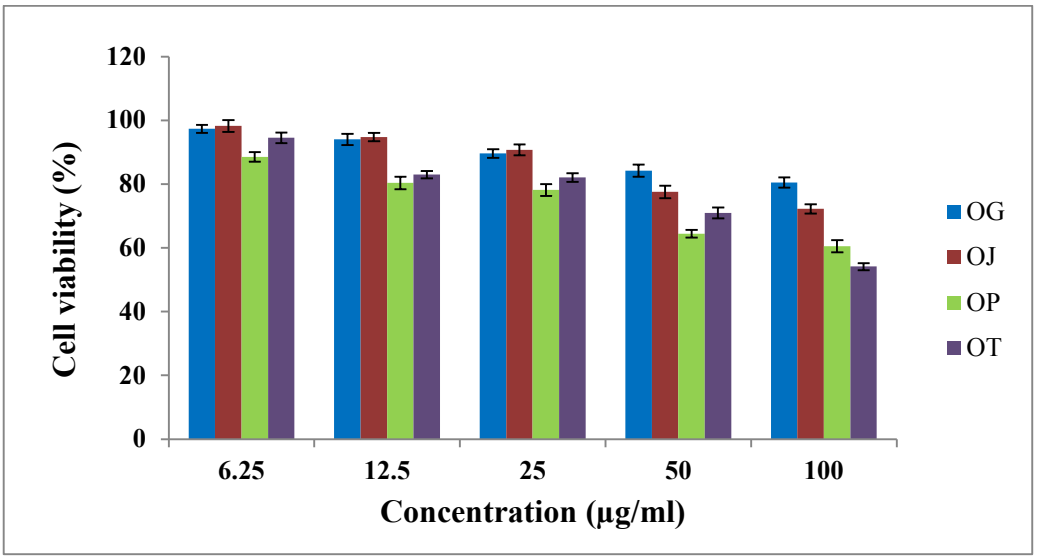


Fig. 41: Cytotoxic effect of selected species of *Ophiorrhiza* on L929 cells using MTT assay. OG - *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** – *O. trichocarpon*

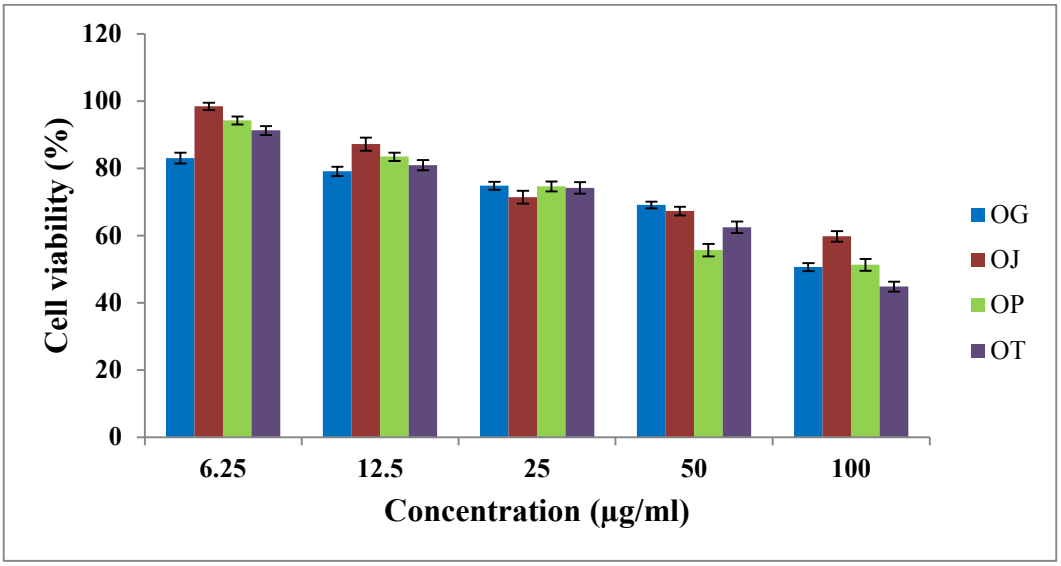


Fig. 42: Cytotoxic effect of selected species of *Ophiorrhiza* on MDA-MB 231 cells using MTT assay. OG - *O. grandiflora*; **OJ** - *O. jacobii*; **OP** - *O. pectinata*; **OT** – *O. trichocarpon*

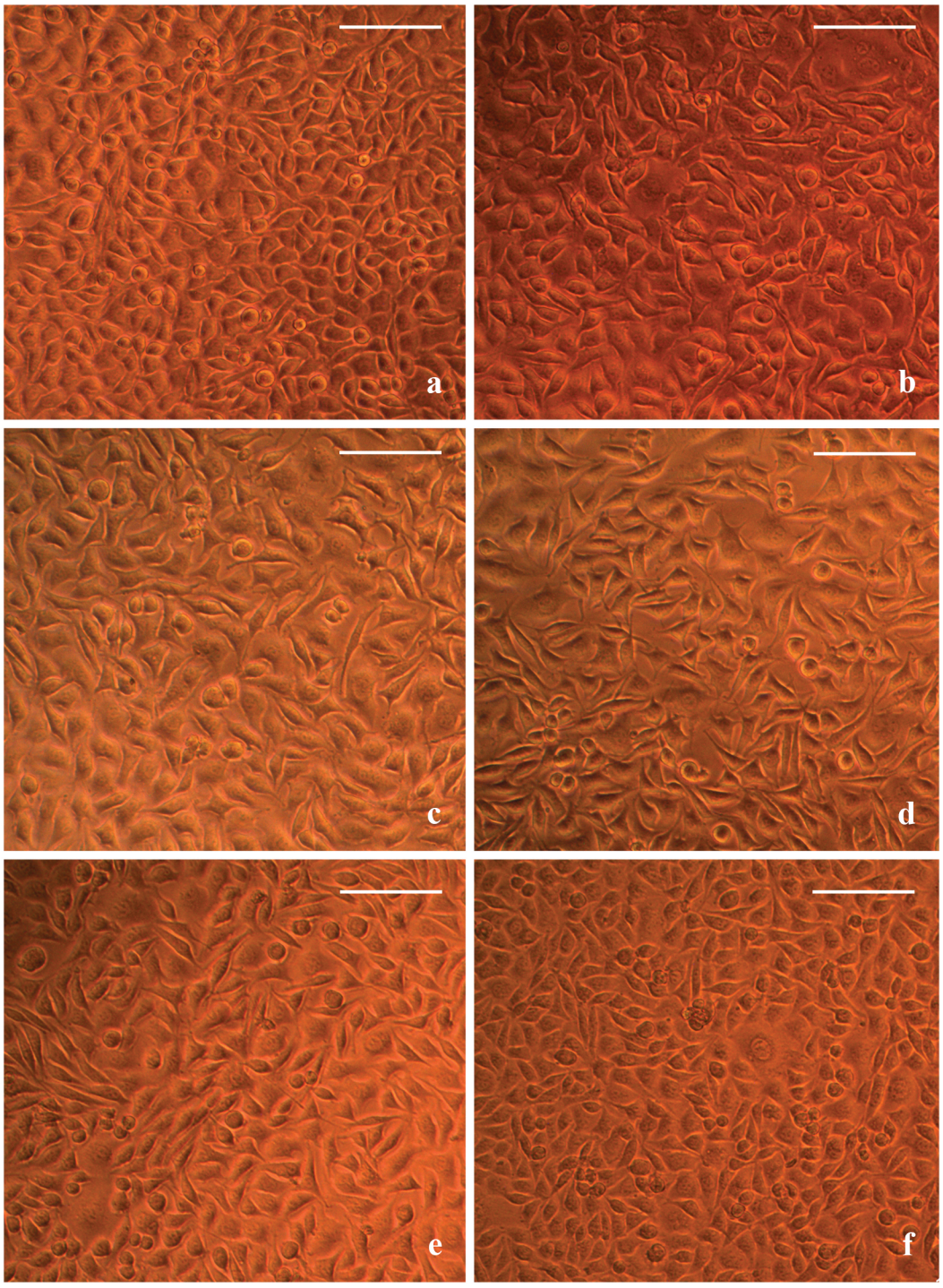


Plate 21: Cytotoxic effect of methanolic extract of *Ophiorrhiza grandiflora* on L929 cells. a: Control; **b:** 6.25 µg/ml; **c:** 12.5 µg/ml; **d:** 25 µg/ml; **e:** 50 µg/ml; **f:** 100 µg/ml. Scale bars = 100 µm.

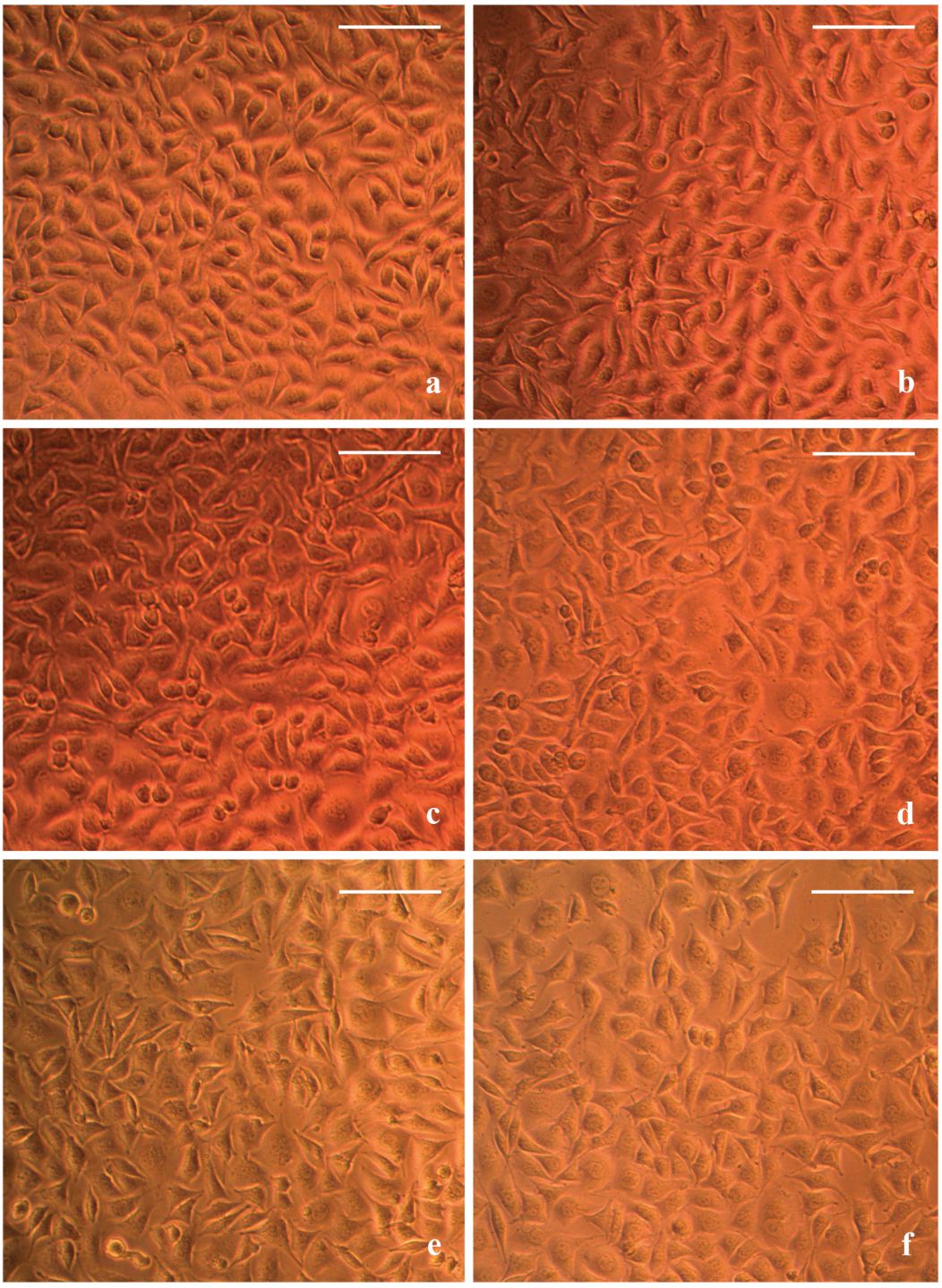


Plate 22: Cytotoxic effect of methanolic extract of *Ophiorrhiza jacobii* on L929 cells. a: Control; **b:** 6.25 µg/ml; **c:** 12.5 µg/ml; **d:** 25 µg/ml; **e:** 50 µg/ml; **f:** 100 µg/ml. Scale bars = 100 µm.

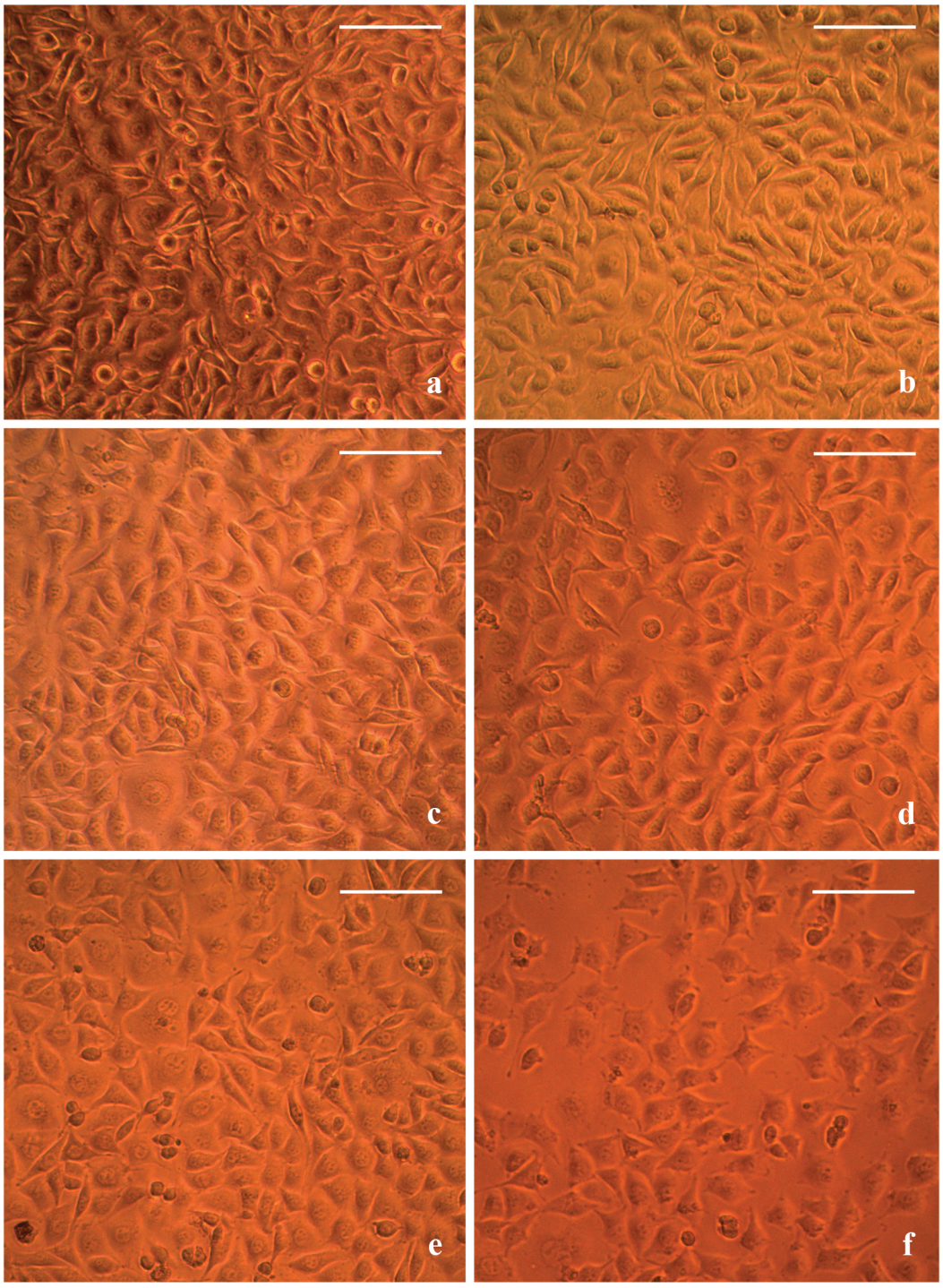


Plate 23: Cytotoxic effect of methanolic extract of *Ophiorrhiza pectinata* on L929 cells. a: Control; **b:** 6.25 µg/ml; **c:** 12.5 µg/ml; **d:** 25 µg/ml; **e:** 50 µg/ml; **f:** 100 µg/ml. Scale bars = 100 µm.

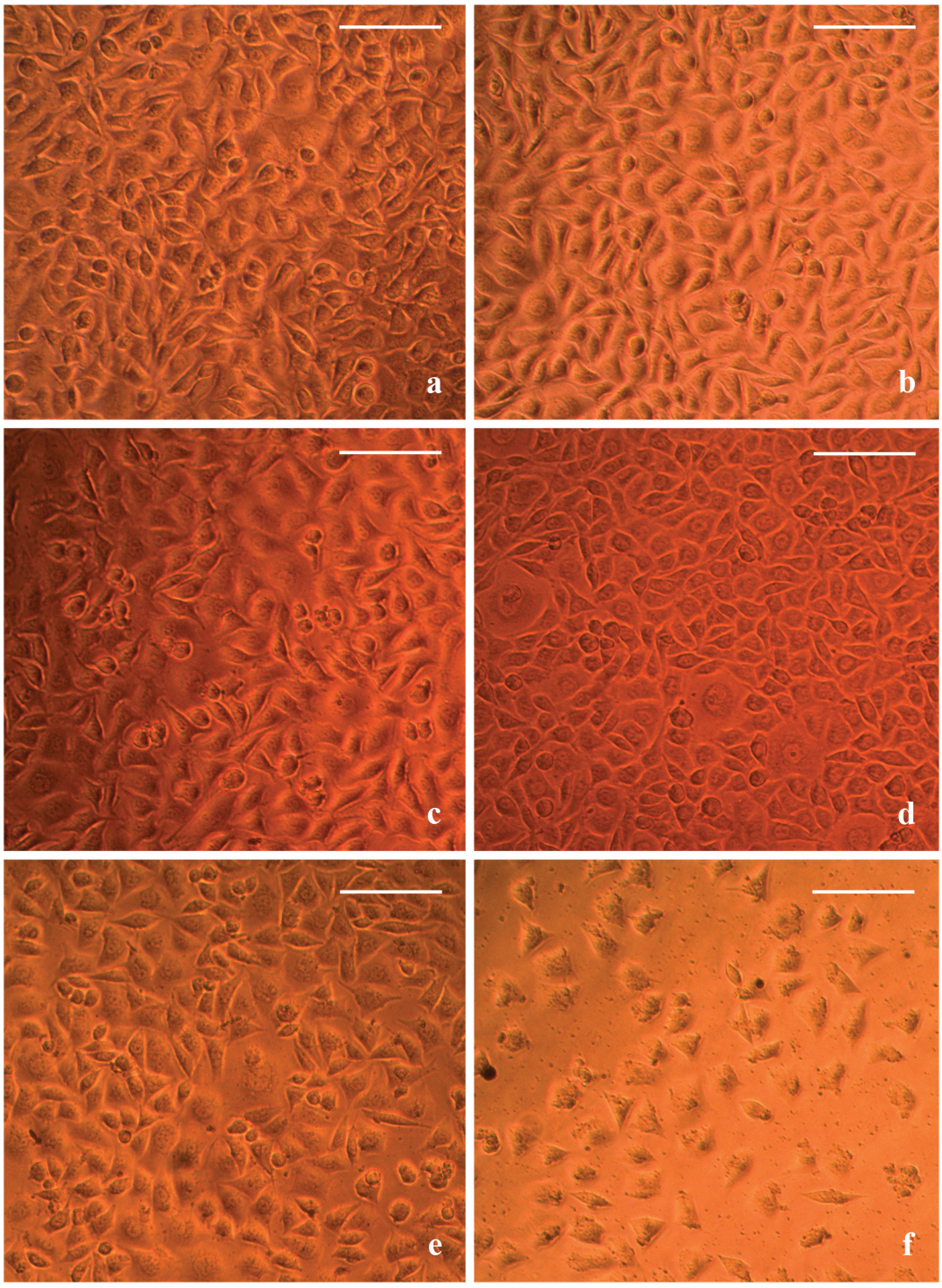


Plate 24: Cytotoxic effect of methanolic extract of *Ophiorrhiza trichocarpon* on L929 cells. a: Control; **b:** 6.25 µg/ml; **c:** 12.5 µg/ml; **d:** 25 µg/ml; **e:** 50 µg/ml; **f:** 100 µg/ml. Scale bars = 100 µm.

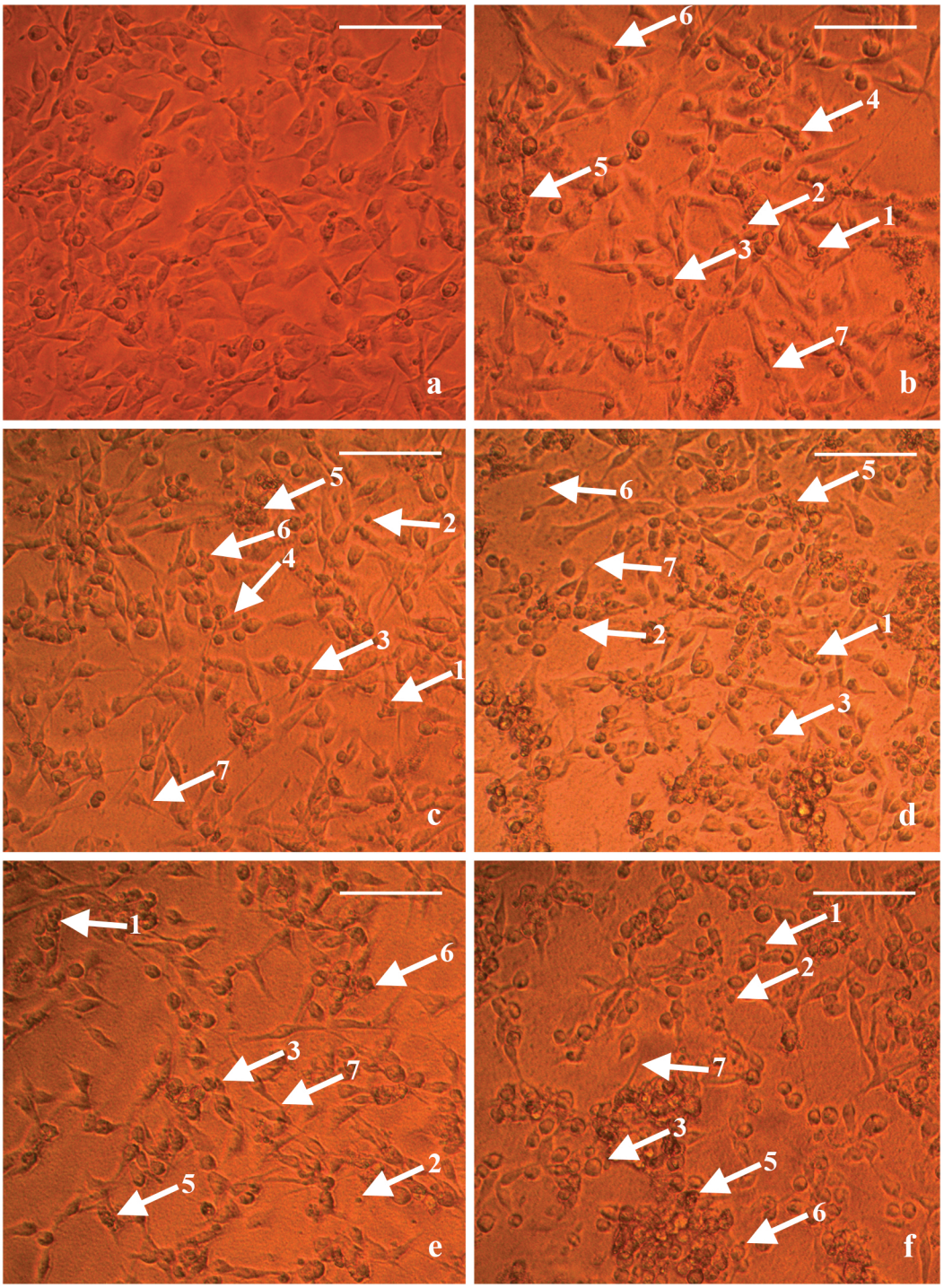


Plate 25: Cytotoxic effect of methanolic extract of *Ophiorrhiza grandiflora* on MDA-MB 231 cells. a: Control; **b:** 6.25 µg/ml; **c:** 12.5 µg/ml; **d:** 25 µg/ml; **e:** 50 µg/ml; **f:** 100 µg/ml. Arrows indicate apoptotic signals **1:** Nuclear fragmentation; **2:** Condensed nucleus; **3:** Cell shrinkage; **4:** Membrane blebbing; **5:** Apoptotic bodies; **6:** Budding; **7:** Echinoid spike. Scale bars = 100 µm.

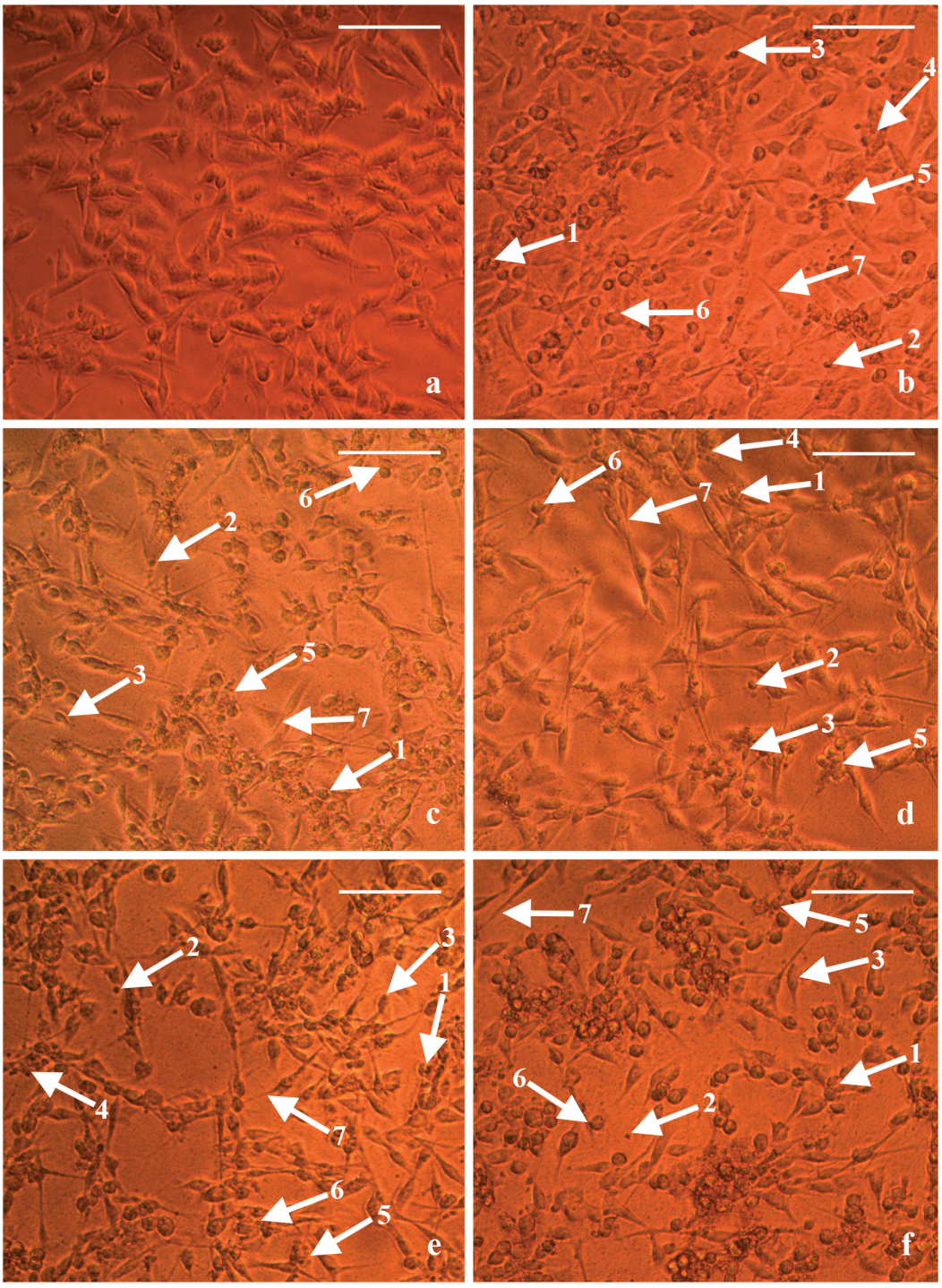


Plate 26: Cytotoxic effect of methanolic extract of *Ophiorrhiza jacobii* on MDA-MB 231 cells. a: Control; **b:** 6.25 µg/ml; **c:** 12.5 µg/ml; **d:** 25 µg/ml; **e:** 50 µg/ml; **f:** 100 µg/ml. Arrows indicate apoptotic signals **1:** Nuclear fragmentation; **2:** Condensed nucleus; **3:** Cell shrinkage; **4:** Membrane blebbing; **5:** Apoptotic bodies; **6:** Budding; **7:** Echinoid spike. Scale bars = 100 µm.

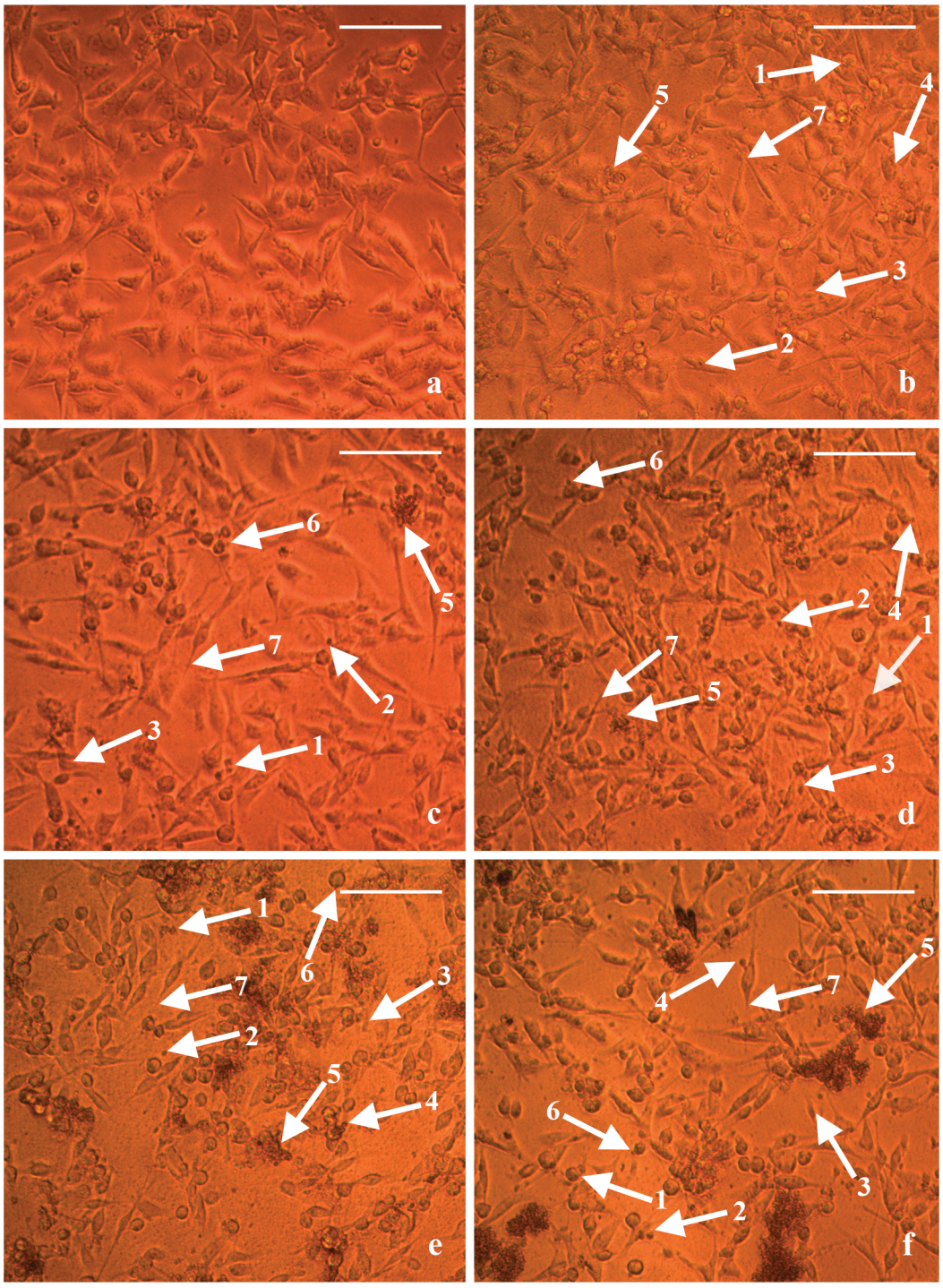


Plate 27: Cytotoxic effect of methanolic extract of *Ophiorrhiza pectinata* on MDA-MB 231 cells. a: Control; **b:** 6.25 µg/ml; **c:** 12.5 µg/ml; **d:** 25 µg/ml; **e:** 50 µg/ml; **f:** 100 µg/ml. Arrows indicate apoptotic signals **1:** Nuclear fragmentation; **2:** Condensed nucleus; **3:** Cell shrinkage; **4:** Membrane blebbing; **5:** Apoptotic bodies; **6:** Budding; **7:** Echinoid spike. Scale bars = 100 µm.

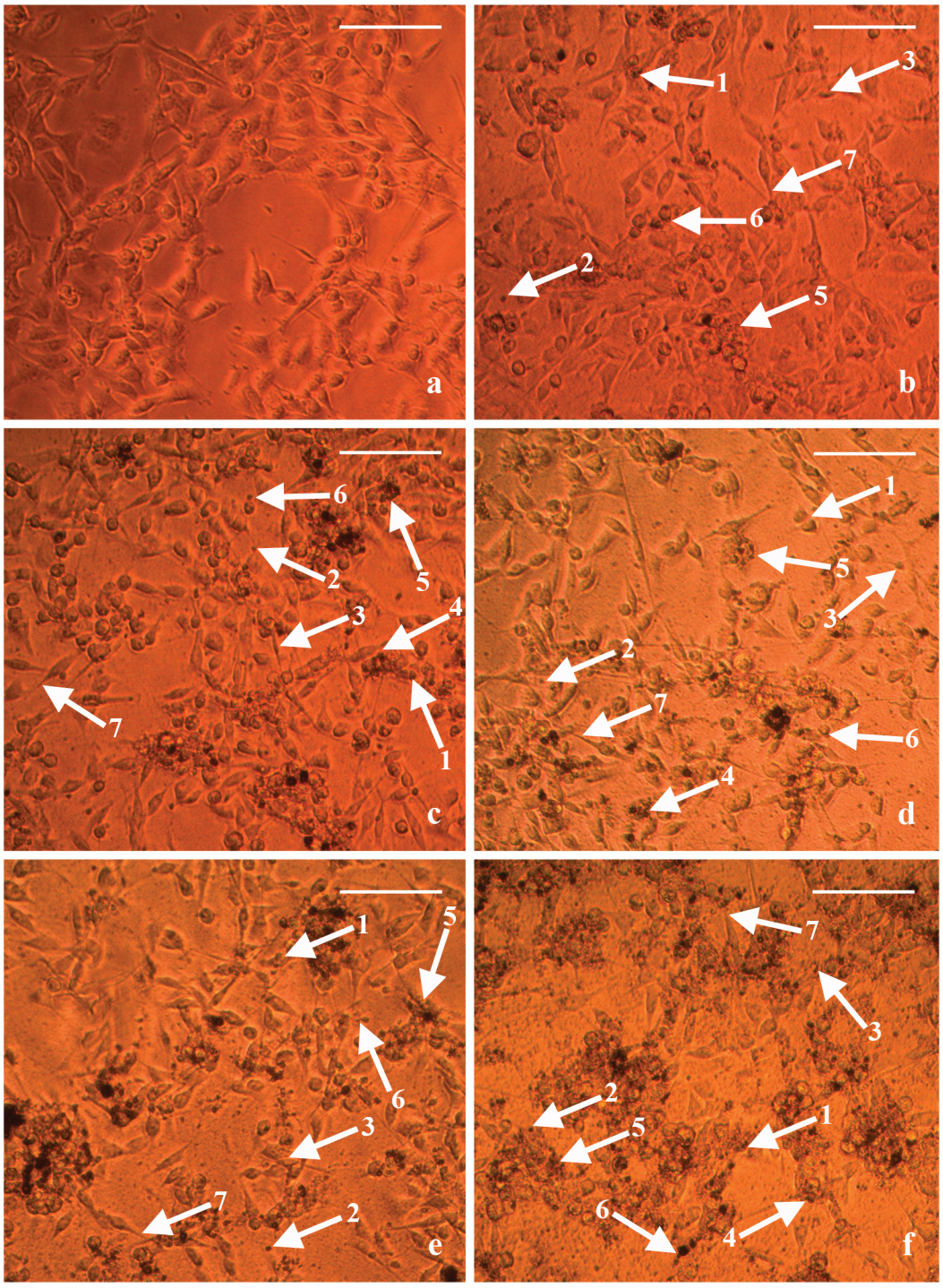


Plate 28: Cytotoxic effect of methanolic extract of *Ophiorrhiza trichocarpon* on MDA-MB 231 cells. a: Control; **b:** 6.25 µg/ml; **c:** 12.5 µg/ml; **d:** 25 µg/ml; **e:** 50 µg/ml; **f:** 100 µg/ml. Arrows indicate apoptotic signals **1:** Nuclear fragmentation; **2:** Condensed nucleus; **3:** Cell shrinkage; **4:** Membrane blebbing; **5:** Apoptotic bodies; **6:** Budding; **7:** Echinoid spike. Scale bars = 100 µm.

Table 16: Lethal concentrations of selected species of *Ophiorrhiza* in L929 and MDA-MB 231cell lines

Plant species	LC_{50} (µg/ml)	
	L929	**MDA-MB 231**
O. grandiflora	269.81 ± 2.54	103.21 ± 3.84
O. jacobii	169.76 ± 3.21	114.78 ± 2.69
O. pectinata	125.76 ± 3.42	114.47 ± 3.88
O. trichocarpon	107.26 ± 2.55	84.08 ± 2.74

a good candidate at the lowest concentration, the subsequent concentrations displayed cell viability almost equal to that expressed by *O. jacobii* and *O. pectinata*. Furthermore, the LC_{50} value was also high (103.21 ± 3.84 µg/ml). As per the results, the highest LC_{50} value was revealed by *O. jacobii* (114.78 ± 2.69 µg/ml) and *O. pectinata* (114.47 ± 3.88 µg/ml) and the lowest value was exhibited by *O. trichocarpon* (84.08 ± 2.74 µg/ml).

On analysing the cell viability percentages in both the cell lines and the LC_{50} values of all the extracts, it was found that *O. trichocarpon* significantly inhibited the proliferation of MDA-MB 231 cancer cell lines (LC_{50} = 84.08 ± 2.74 µg/ml) when compared to other extracts and the extent of toxicity was minimum against the normal L929 cells (LC_{50} = 107.26 ± 2.55 µg/ml). This indicated the cancer cell-specific toxic effect of the extract. Thus, for further analysis, the methanolic extract of *O. trichocarpon* at 84.08 ± 2.74 µg/ml was selected.

iii. Detection of apoptosis using FACS

To determine whether the cell death induced by the plant extract of *O. trichocarpon* on the cell lines was attributable to apoptosis, flow cytometry using Fluorescein isothiocyanate (FITC)-labelled Annexin V antibody was carried out. MDA-MB-231 cells treated with 84.08 µg/ml of the plant extract

and incubated for 24 hours were considered for the Annexin V & Dead Cell Reagent assay. Cells were gated against untreated control cells and analysed in a flow cytometer for apoptosis. Quadrant statistics were used to examine the fraction of the cell population in each quadrant. Cells in the lower right quadrant reflected early apoptosis, whereas cells in the upper right quadrant denoted late apoptosis **(Fig. 43).** From the apoptosis profile of the sample **(Fig. 43 D),** it is clear that the plant extract could induce 10% of the cells to the late apoptotic stage and 35.28% of cells into the final apoptotic stage. Thus, the exposure to the plant extract remarkably increased the apoptotic cell population in the treated cells when compared to the control **(Fig. 43 B),** which exhibited only 5 % of apoptotic cell population.

iv. Cell cycle analysis using flow cytometry

The activity of the most effective plant extract on cell cycle progression was evaluated using flow cytometry. MDA-MB 231 cells treated with 84.08 µg/ml of the extract of *O. trichocarpon* and incubated for 24 h were considered for the study. The flow cytometry histogram of the DNA content profile is given in **Fig. 44**. The diagram depicted the apoptotic effect of the extract on the cell count and the DNA content of the cell lines used.

From the DNA content profile, the stage of cell cycle at which the cell cycle arrest occurs was identified. As shown in the histogram **(Fig. 44),** the cell count in the G0/G1 population in treated cells is high (66.4%) when compared to the control (53.2%). The population in S phase declined from 24.3% in the control to 18.9% in treated cells. In the subsequent G2/M phase also; the cell population has remarkably reduced to 11.1%. High cell population in G0/G1 phase followed by highly reduced cell content in the S phase and the subsequent reduction in the G2/M phase in the treated cells indicated that the cell cycle arrest occurs at the G0/G1 phase. Thus, the cell cycle analysis by flow cytometry exhibited the potential of the extract of *O. trichocarpon* to

induce apoptosis by blocking the transition from G0/G1 phase to the S phase in the MDA-MB 231 cancer cells.

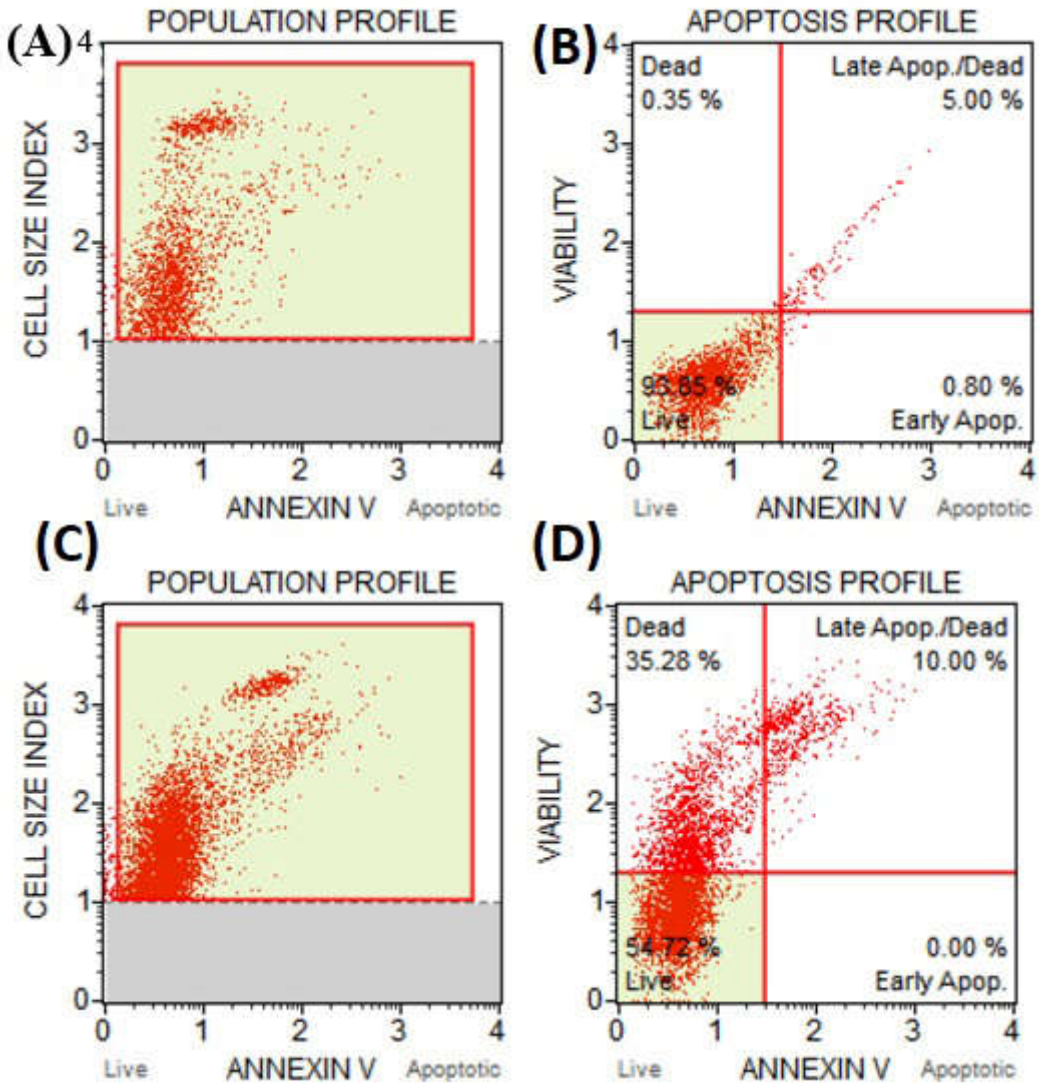
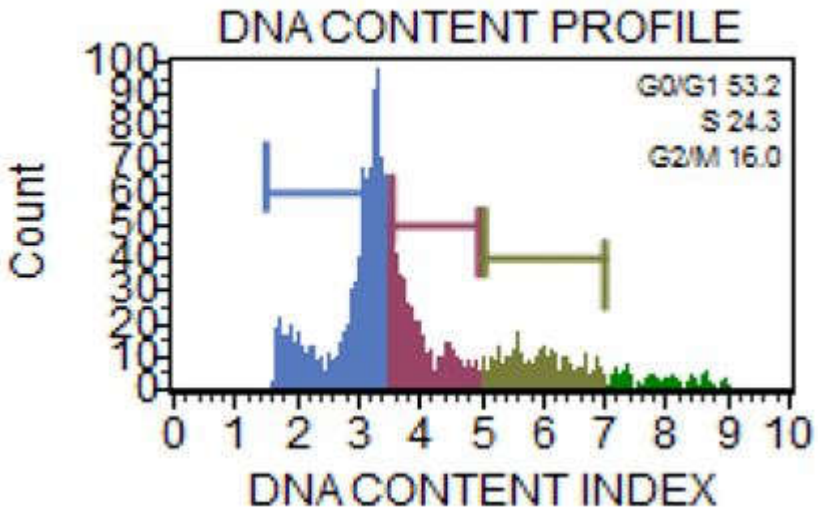


Fig. 43: Determination of apoptosis by FACS. (A) Population profile of untreated control cells; **(B)** Apoptotic profile of untreated control; **(C)** Population profile of *O. trichocarpon* treated cells **(D)** Apoptotic profile of *O. trichocarpon* treated cells

(A)



(B)

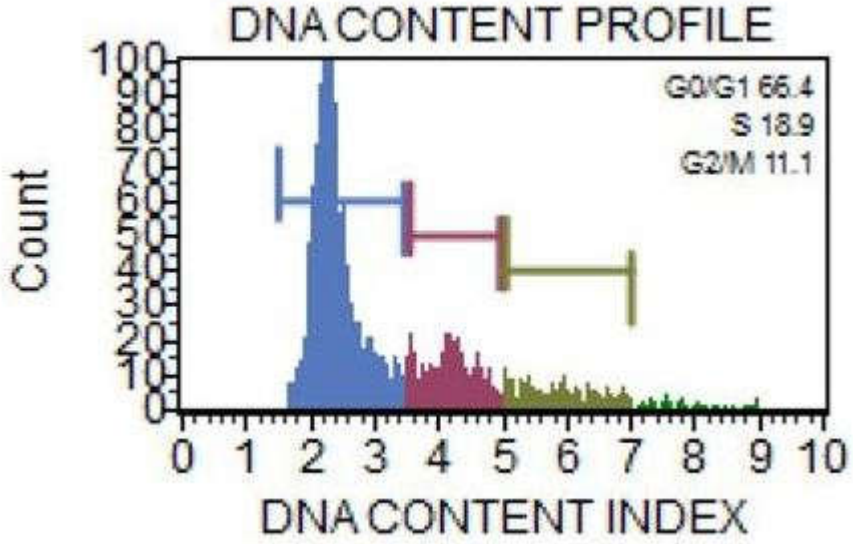


Fig. 44: DNA content profile of MDA-MB 231 cells obtained using flow cytometry. (A) Control; **(B)** MDA-MB 231 cells treated with the methanolic extract of *O. trichocarpon*

v. Gene expression studies

The effect of the methanolic extract of *O. trichocarpon* on the expression of two apoptotic related genes, AKT and ERK2, was determined using RT-qPCR followed by gel electrophoresis using GAPDH as the house keeping gene. MDA-MB 231 cells treated with 84.08 µg/ml of the extract and incubated for 24 h were considered for the study, by maintaining untreated cells as the control. The results are given in **Figs 45 & 46.** From the results, a significant upregulation in the expression of both the genes were observed, when compared to the control. The expression fold change value for the gene AKT was 2.01, while the other gene ERK2 expressed a fold change value of 1.85 **(Fig. 45).** The results indicated that the extract of *O. trichocarpon* could upregulate the genes almost the double times that of the control. The gel image analysis of the genes also confirmed the findings. It could be observed that the fluorescence emitted by the sample treated wells were relatively higher than the control, indicating the increased expression of the selected genes **(Fig. 46).** Thus, the study clearly shows that the methanolic extract of *O. trichocarpon* has the potential to upregulate the genes AKT and ERK2, which have significant role in the apoptotic activity of the cancer cells.

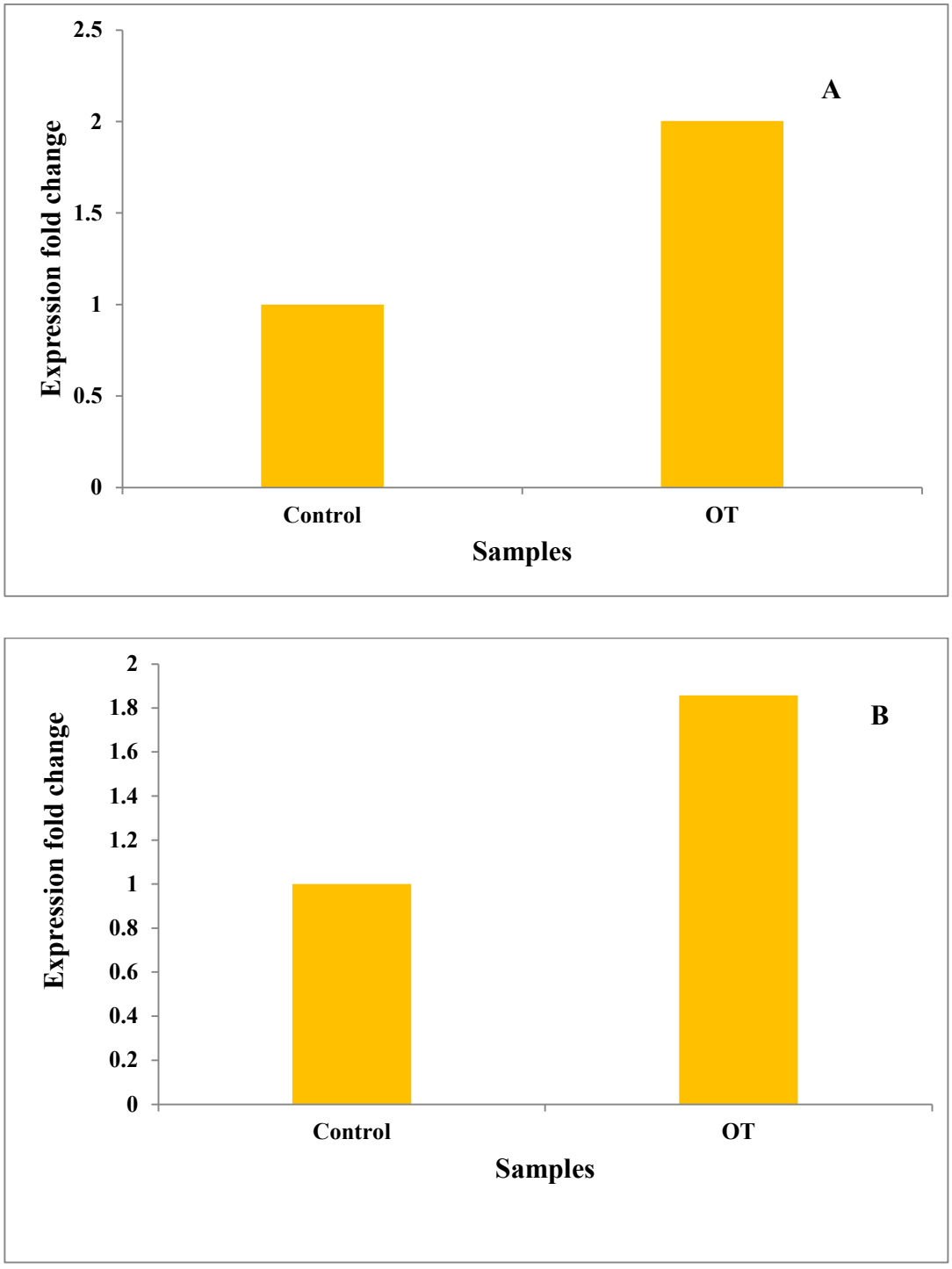


Fig. 45: Expression fold change of the genes in MDA-MB 231 cells treated with methanolic extract of *O. trichocarpon.* A: AKT; **B:** ERK2

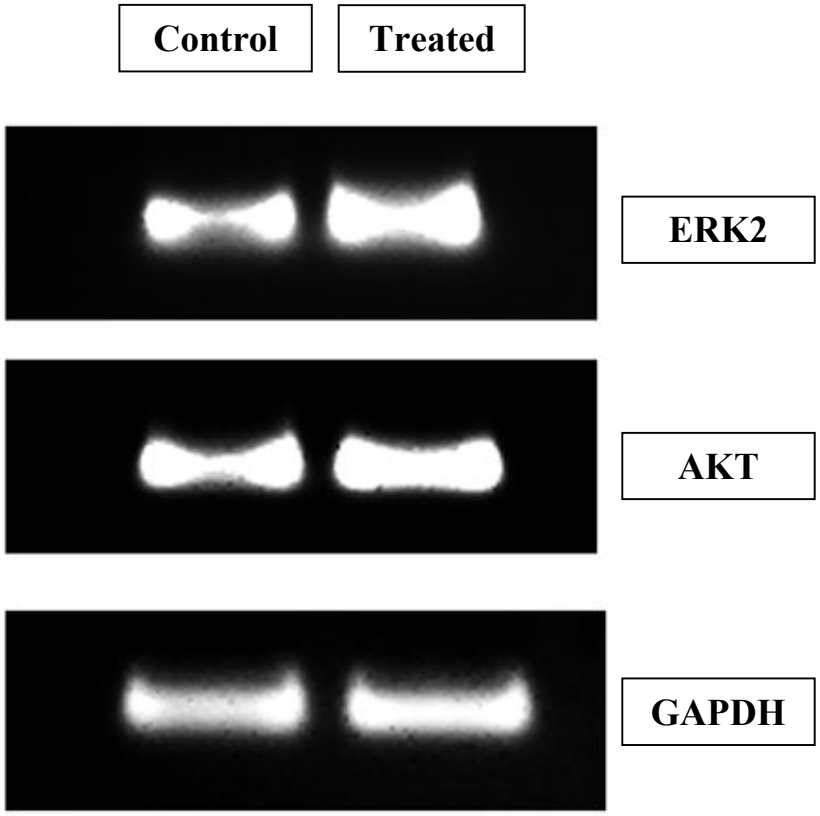


Fig. 46: Expression analysis of the genes using Real Time PCR

DISCUSSION

PART I – KARYOMORPHOLOGICAL STUDIES

Cytological studies help to explore systematic and evolutionary aspects of various taxa whose taxonomic identification is very difficult. Cytogenetical characterisation provides better knowledge on the evolutionary trends of different species (Prajitha & Thoppil, 2018). Karyological data can provide precise information on the morphology, size and number of chromosomes (Bhatnagar et al., 2019). Thus, karyotype and their component characteristics form the basic data to study karyoevolutionary trends in a given taxa (Lavania & Srivastava, 1992). Though, reports on the chromosome number of some species of *Ophiorrhiza* are available, detailed karyomorphometric studies are not accessible yet. The present study was thus designed to explore the karyomorphological details of the selected species of *Ophiorrhiza.*

Chromosome numbers are considered as the fundamental source of information of plant systematics and evolution of angiospermic plants (Kuo, 2013). They form the easiest karyotypic information to be obtained as they are usually well conserved in a population. However, wrongly identified chromosome numbers can lead to misidentification of the plant which can reduce the credibility of cytotaxonomy (Guerra, 2012). Cytotaxonomy mainly relies on the somatic chromosome number, base number and ploidy level (Lawand et al., 2019). In the present study, the somatic chromosome number of all the selected four species was determined using the mitotic squash preparation technique. Three species, *Ophiorrhiza grandiflora, O. pectinata* and *O. trichocarpon* exhibited the same number of somatic chromosomes (2n = 22) all with 11 pairs of chromosomes **(Plates 6, 7, 10, 11, 12, 13).** *O. jacobii* showed a chromosome number of 2n = 24 with 12 pairs of

chromosomes **(Plates 8, 9).** Furthermore, the ploidy level of chromosomes in all the species was found to be in the diploid condition.

Several other genera belonging to Rubiaceace also have shown similar chromosome number. Karyotype studies on five taxa of *Asperula,* revealed that all the studied taxa have a somatic chromosome number of 2n = 22 and the basic chromosome number was found as n = 11 (Minareci & Yildiz, 2011). Two species of *Coffea; Coffea canephora* and *C. dewevrei* also had 2n = 22 number of chromosomes (Pierozzi et al., 1999). The cytological study on *Kellogia chinensis* and *K. galioides* also revealed 2n = 22 number of chromosomes with x = 11 (Tu et al., 2006). In the present study, basic chromosome number of *Ophiorrhiza grandiflora, O. pectinata* and *O. trichocarpon* is x = 11 and that of *O. jacobii* is x = 12. The obtained results are in accordance with the findings of Kiehn (1995), who in his survey suggested that the tribe Ophiorrhizae in general is dibasic, with a basic chromosome number x = 11 or 12, and the predominant one is x =11. The basic ploidy level of the tribe was also considered as 2x or 4x.

Basic chromosome number is regarded as one of the stable and dependable markers at the generic and family level, which has important role in molding the existing concept of evolution (Mathew & Christopher, 2019). According to Guerra (2012), the basic chromosome number is the original chromosome number of a taxon which represents one of the haploid numbers seen within a taxon that clarifies chromosomal variations within the group and exhibits a direct relationship with the basic number of the related groups. Among the twelve haploid basic chromosome numbers observed in the family Rubiaceae; from x= 6 to x = 17, the basic chromosome number x = 11 shows notable preponderance (Raghavan & Rangaswamy, 1941). Due to the highest frequency of this basic chromosome number, they considered this to be the original basic number of Rubiaceae. Similarly, Lewis (1962) has also

assumed the number, x = 11 to be basic to majority of the taxa within the family and hence to the family.

Regarding the origin of the basic chromosome number x = 11, two theories were considered. (i) One postulated by Sharma and Chatterjee (1960), assumed that amphidiploidy arose from two related ancestral taxa with n = 6, and might have resulted in n = 12, from which n = 11 condition emerged by aneuploid reduction. (ii) The second theory was by Lewis (1962), who considered that amphidiploidy may arise from prototypes having n = 6 and n = 5, which directly resulted in n = 11 condition.

Raghavan and Rangaswamy (1941) studied the chromosomal characters of 20 species in the family Rubiaceae and found out that 11 is the basic number found in the maximum number of genera. In the report on the karyomorphological studies on the south-Indian Rubiaceae, Selvaraj (1987) suggests that the higher haploid numbers above the level of x =11 results due to aneuploidy and euploidy. Cytological studies on *Ophiorrhiza harrisiana* using pollen mother cells revealed the existence of secondary association of bivalents in the species, which offered clues that the theory of Lewis is more plausible. According to this system, the other basic numbers would have evolved from x = 11 in different lines either by ascending aneuploidy resulting in higher aneuploid derivatives such as x = 12 or by descending aneuploidy, resulting in lower numbers such as x = 10 (Philip & Mathew, 1988).

In the present work, the occurrence of x = 11, in three species corroborate with the findings of all other reports on the basic number in Rubiaceae family. The presence of x = 12 in *Ophiorrhiza jacobii* would be the result of ascending aneuploidy or euploidy. From the basic chromosome number, the corresponding somatic chromosome numbers might have arisen in the species selected for the study.

Chromosome size and structure are very stable characteristics within the family Rubiaceae (Kiehn, 1995). The chromosomal length ranges from 0.4 to 8.0 μm (Tu et al., 2006). The occurrence of small sized chromosomes forms the major cause of limited cytological investigations within the group. Furthermore, Selvaraj (1987), has found out that there is a close relation between the size and number of somatic chromosomes among the members of Rubiaceae. As the number of chromosomes increases, the size of the chromosomes reduces. In the present study, the range of chromosomal length was found to be 1.15 μm to 2.15 μm and also the range of ACL of all the four species was found as 1.40 μm to 1.56 μm, all of which indicates the tiny nature of the chromosomes **(Tables 4, 6, 8, 10).** This result is in accordance with the findings of Selvaraj (1987) regarding chromosome number and size.

Total chromosome length (TCL) for each species was found to be different with *Ophiorrhiza grandiflora* having 32.88 μm, *O. jacobii* with 37.62 μm, *O. pectinata* with 32.96 μm and *O. trichocarpon* with 30.86 μm. Differences in TCL and total chromosome volume come from the differential condensation of chromosomes and the association of varied quantities of histone and non-histone proteins during chromosome compaction, both of which are regulated by genes (Das et al., 1998). The detailed karyomorphometric data obtained from all the species showed striking differences in the total chromosomal length, short arm and long arm lengths, chromosome arm ratios as well as centromeric indices among the species **(Tables 5, 7, 9, 11).**

The position and nature of primary constriction is an important chromosomal parameter for karyomorphological analysis. Primary constriction is the region of the chromosome with significant chromosomal contraction and bright staining. On the basis of the position of primary constriction and the arm ratios, Abraham and Prasad (1983) proposed the

nomenclature system for chromosomes according to which the chromosomes can be classified into (i) median (M), (ii) nearly median (nm), (iii) nearly submedian (nsm- and nsm+), (iv) submedian (SM), (v) nearly subterminal (nst- and nst+), (vi) subterminal (ST), (vii) nearly terminal (nt) and (viii) terminal (T). In the study on Rubiaceae chromosomes, Selvaraj (1987) has mentioned the presence of different proportions of median and submedian chromosomes in higher frequencies. In the present study, the chromosomes of all the four selected species were nearly median (nm) or nearly submedian type nsm(-), which is in accordance with the former studies. Along with the primary constriction, features of the secondary constriction, its number, length and position on a chromosome also are important in cytogenetical studies. In the study, the number of chromosomes with secondary constrictions was found to be in equity. The first two pairs of chromosomes in all the species exhibited secondary constrictions. As attested by Stebbins (1971), chromosomes which are similar in size and have median or submedian centromeres constitute symmetric karyotype; whereas chromosomes which show marked differences in their sizes and have sub-telocentric or acrocentric centromeres constitute asymmetric karyotype. Thus, the findings of the present study, highlights the symmetrical nature of the karyotypes of the selected species.

Karyotypic evolution is an integral part of evolutionary processes. It is regarded as a mechanism that isolates species and has autonomous evolutionary trends from genetic evolution (Neethu, 2017) According to Imai et al. (2001), increasing chromosome number and the formation of terminal-centromeric chromosomes signifies karyotype evolution, whereas lesser chromosome number and formation of median centromeric chromosomes represents primitive nature. On the basis of these reports, the chromosome complements of the selected species of *Ophiorrhiza* can be considered as primitive karyotypes.

According to Stebbins (1971), asymmetrical karyotypes are more advanced than symmetrical karyotypes with regards to phylogeny and evolutionary processes. Rodrigues et al. (2012) suggested that karyotypic asymmetry leads to speciation, while symmetric karyotypes are ancestral in nature. The various parameters used in the study to assess the karyotypic symmetry or asymmetry include DI, VC, TF%, As K%, Syi and Rec indices, A1, A2 and A indices. Low values of TF%, Syi and Rec indices indicate increasing asymmetry, and higher values of As K%, A1, A2 and A indices also indicate asymmetry (Prajitha, 2018).

DI or Disparity index values are calculated for analysing the karyotypic asymmetry of the selected species. The values represent the variations in the chromosome size of the plants. Low DI value represents the omogeneity of chromosomes and high DI value indicates heterogenous assemblage of chromosomes; whereas an intermediate DI value indicates that the species is in an evolutionary pathway (Mohanty et al., 1991). DI obtained for *Ophiorrhiza grandiflora, O. jacobii, O. pectinata* and *O. trichocarpon* were 27.67, 30.30, 29.48 and 25.17 respectively. The values are quite lower indicating the primitive nature of the chromosomes.

VC or Variation coefficient determines minute variations among chromosome complements (Peruzzi & Eroğlu, 2013). The VC values obtained for *Ophiorrhiza grandiflora, O. jacobii, O. pectinata* and *O. trichocarpon* were 17.88, 18.02, 18.02 and 17.16 respectively. According to Stebbins (1959) low value of VC indicates homogeneity and primitiveness. Thus, the lower VC values obtained from the study ascertain the homogeneity of chromosome complements and depict the primitive nature of these plants.

TF% or total forma percentage or the mean centromeric index is a frequently used parameter to evaluate the karyotypic asymmetry or symmetry (Costa & Forni-Martins, 2003). TF% of 50 represents median centromeres

which depicts an absolute symmetrical karyotype. TF% of zero indicates terminal centromeres which imply complete asymmetric karyotype (Kapoor, 1977). In the present study, the TF% obtained for *Ophiorrhiza grandiflora, O. jacobii, O. pectinata* and *O. trichocarpon* were 43.24%, 44.71%, 43.75% and 42.51% respectively. The obtained values, which are all above 40%, clearly highlight the nearly median and nearly submedian nature of chromosomes with apparent symmetry. Thus, the symmetric nature of chromosomes regarding TF% denotes the primitive as well as slowly evolving character of the species studied.

Another important parameter considered to study karyotype asymmetry was As K% (Karyotype asymmetry index). The parameter can be used to determine the phylogenetic relations and chromosome evolution within and between the species of a genus (Arano, 1963). Value of As K% close to 50, indicates higher karyotype symmetry and primitiveness (Ye et al., 2014). The As K% for the studied species *Ophiorrhiza grandiflora* (56.56%), *O. jacobii* (54.27%), *O. pectinata* (56.01%) and *O. trichocarpon* (57.35%) indicated high karyotype symmetry and thus confirming the primitive nature of the species with respect to As K%.

Indices of symmetry are also important parameters to resolve the evolutionary pathway of the karyotype. Syi index (symmetry index) and Rec index (resemblance between chromosomes) are two such parameters. Syi index represents average centromere position; while Rec index represents the asymmetry in chromosome size within a chromosome complement (Greilhuber & Speta, 1976). The values of both these indices theoretically ranged from 0 to 100. Higher values of these two indices represent an evolutionarily primitive status (Venora et al., 2002). The Syi values obtained for *Ophiorrhiza grandiflora* (76.45), *O. jacobii* (82.37), *O. pectinata* (78.11) and *O. trichocarpon* (74.12) and the respective Rec values (73.62, 72.90,

74.16, 76.23) were higher values, which represented the less evolved nature of the species studied. The scatter plot representing the Rec and Syi indices show that all the selected species are less evolved in relation to chromosomal evolutionary processes, however, *O. trichocarpon* can be considered as more advanced than the other three species **(Fig. 5).**

Karyotype asymmetry indices are important parameters to analyse chromosomal evolution in plants (Paszko, 2006). Intrachromosomal asymmetry index (A1) and interchromosomal asymmetry index (A2) developed by Zarco (1986) can identify minute variations in the karyotypic asymmetry. The A1 index, which is independent of chromosome size or number, determines karyotypic asymmetry for the relationships between chromosomal arms. Without regard to chromosome number, the A2 index or Pearson's dispersion coefficient determines karyotypic asymmetry resulting from relationships between different chromosome sizes. The value of both these indices ranges from 0 to 1. The equations are constructed to provide lower values, when chromosomes are more likely to be metacentric. The A1 values observed for *Ophiorrhiza grandiflora*, *O. jacobii*, *O. pectinata* and *O. trichocarpon* were 0.21, 0.16, 0.21 and 0.24 respectively. The A2 values observed were 0.17, 0.18, 0.18 and 0.17. The values obtained for A1 and A2 in all the species were very low and indicates the more symmetric nature of chromosomes and thus the primitiveness of the species.

Watanabe et al. (1999) created a new indicator called the degree of asymmetry of karyotypes (A), which has the benefit of allowing a high level of sensitivity and precision when evaluating karyotype asymmetry. Greater values of the A index represent greater karyotypic heterogeneity. The A values obtained for the selected species; *Ophiorrhiza grandiflora* (0.12), *O. jacobii* (0.09), *O. pectinata* (0.12) and *O. trichocarpon* (0.14), clearly indicates minimal karyotypic heterogeneity. The scatter plot representing the

A1 and A2 indices show that all the selected species are less evolved in relation to chromosomal evolutionary processes, though; *Ophiorrhiza trichocarpon* can be considered more advanced than the other three species in having higher values of asymmetry indices **(Fig. 4).**

Cytogenetical investigation on *Ophiorrhiza grandiflora, O. jacobii* and *O. trichocarpon* were carried out for the first time. Thus, the present study would be the first report on the karyomorphological data on these species. The first report on the chromosome number of *Ophiorrhiza pectinata* was by Selvaraj (1987), in which the chromosome number was reported to be 2n = 44. In the present study, a deviant chromosome count of 2n = 22 was observed against this report, which may be a diploid cytotype. The cytotype reported earlier thus proves to be a tetraploid one. Kiehn (1995) suggested that polyploidisation of chromosomes can result in 4x chromosome numbers from lower numbers. The occurrence of chromosomal variation in *O. pectinata* might be the simultaneous existence of a diploid accession in *O. pectinata* with 2n = 22 as revealed by the present study and a polyploidy accession with 2n = 44 as reported earlier by Selvaraj (1987). Hence the diploid somatic chromosome number of *O. pectinata* was confirmed as 2n = 22 with 11 pairs of chromosomes **(Plate 10).** The range of chromosomal length was observed as 1.10 µm to 2.02 µm. The total chromosome length was 32.96 µm and the average chromosome length was 1.49 µm. In the previous report by Selvaraj (1987), the total length of the chromosomes was 28.8 µm and the range of chromosome length was 0.6 to 1.6 µm. On analysing both the findings, the higher chromosome number (2n = 44) in the tetraploid accession might be due to the polyploidisation from the lower chromosome number of 2n = 22 (Yano & Hoshino, 2007). Basic chromosome numbers also participate in the origin of higher ploidy levels in angiosperms (Mathew & Christopher, 2019).

Polyploidy is the most significant cytogenetic process that contributes to evolution and the emergence of new species in higher plants (Stebbins, 1971). Increased fertility and greater resistance to shifting environmental conditions are two benefits of polyploidy (Mathew & Christopher, 2019). Thus, polyploid individuals or populations may develop independently even within a single species (Tate et al., 2005) The previous report on *O. pectinata* might have obtained from the population samples consisting of tetraploid individuals. Chromosome number variations between individuals from various localities could be caused by epigenetic processes including DNA methylation and histone changes. These elements may have silencing effects on transposable elements, which can activate chromosomal evolution in different biogeographical regions (Li et al., 2017). Dysploidy, followed by interspecific hybridization and polyploidisation, may result in the creation of species complexes with different karyotypes (Haga & Noda, 1976; Choi et al., 2008). Even variations in diploid chromosomes within the species can cause intraspecific polyploidisation (Husband et al., 2013).

The present and previous reports on the chromosome number of *O. pectinata* as 2n = 22 and 44 respectively, provided evidence for the existence of polyploidisation in the genus. Transposable elements can assist gene silencing or proliferation processes, which can lead to differences in the karyotype of the genus. Thus, the result obtained from the present study, 2n = 22, can be confirmed as the original somatic chromosome number of *O. pectinata.*

In the present study, detailed karyomorphometrical analyses of the selected species of *Ophiorrhiza* were carried out. Individual parameters alone cannot make a compelling conclusion on karyotype symmetry/asymmetry studies and thus, a combination of various parameters was considered for the study. The karyograms and idiograms that were created can be a helpful tool

for the cytological identity of the species. Though the study reveals same karyotype formula and chromosomal number for some species, integrating diverse karyomorphological data enables species differentiation. In the present study, all the species exhibited nearly median and nearly submedian primary constriction revealing the nearly symmetrical karyotype. Furthermore, all the species expressed primitive karyomorphological data in respect to the different indices. Thus, the current investigation revealed the primitive status of the selected species of *Ophiorrhiza* in terms of their karyotype evolution. To understand the cytological stability of the species and the presence of variants within it, karyomorphological investigation on different populations of the same species is necessary. Additional in-depth research on molecular cytogenetics may resolve karyomorphological questions and clarify evolutionary relationships among the species.

PART II – PHYTOCHEMICAL PROFILING

In developing nations, almost 80% of the populace relies on conventional plant-based medications for their basic medical needs. The medicinal value of plants is the contribution of the bioactive phytocomponents within them. However, majority of plants have not yet undergone extensive chemical, pharmacological, and toxicological investigations (Riaz et al., 2012). Though 50% of medications used in medicine are of plant origin, phytochemical analysis of plants that are linked to ethnobotanical knowledge and are used in traditional medicines are few (Peteros & Uy, 2010). In the present study, efforts were made to analyse the phytochemicals present in the selected species of *Ophiorhiza*, which already have many reported uses in folklore medicines. Phytochemical profiling was carried out by qualitative phytochemical analyses, quantification of major phytocomponents, GC-MS and HR LC-MS analyses.

A. Qualitative phytochemical analyses

Basically, medicinal plants contain numerous phytochemical components, which act synergistically to produce pharmacological effects. So, phytochemical screening becomes an essential step to understand the various bioactive components present. In the qualitative phytochemical analyses, three different extracts of each plant selected were put into various tests according to standard protocols and the results are summarised in **Table 12.**

Phenols or the phenolic compounds are the most widely distributed secondary metabolites in the plant kingdom and they have attracted a lot of attention as potential natural antioxidants in terms of their ability to function as metal chelators and effective radical scavengers (Tayade et al., 2013). They are created as a defence mechanism in wounded plants against pathogens and are crucial for plant development and reproduction as well (Khatiwora et al., 2010). Flavonoids, phenolic acids, and polyphenols are the three main categories of dietary phenolics (Rubeena, 2021). Flavonoids exhibit a variety of biological effects including antibacterial, anti-inflammatory, anti-angiogenic, analgesic, anti-allergic, cytostatic, and antioxidant capabilities (Igbinosa et al., 2009). Alkaloid containing plants are prevalently used as spices, medications, colours, or poisons due to their various bioactivities. Alkaloids also have anti-malarial, anti-arrhythmic, antihypertensive and anti-cancer properties (Rubeena, 2021). In the present study, phenols, flavonoids, coumarins and alkaloids were present in the methanolic and aqueous extract of all the species, but absent in the hexane extracts.

Phytosterols are important compounds that have gained attention as antioxidants. They inhibit the production of ROS and prevent angiogenesis (Oyebodi et al., 2019). Sterol compounds also show antibacterial and antiviral activities along with cholesterol lowering and immune stimulating properties

(Igbinosa et al., 2009). As per the results obtained, phytosterols were observed in the methanolic and aqueous extract of *O. grandiflora, O. jacobii* and *O. pectinata.* In *O. trichocarpon* phytosterols were seen only in its methanolic extract.

Terpenoids have been discovered to be beneficial in the treatment and prevention of a number of illnesses, including cancer. The antibacterial, antifungal, anti-parasitic, antiviral, anti-allergenic, anti-spasmodic, anti-hyperglycemic, anti-inflammatory and immunomodulatory effects of terpenoids are also well recognised (Yadav et al., 2014). Anthraquinones are an important group of secondary metabolites with antiparasitic, bacteriostatic, antidepressant and antimicrobial properties (Peteros & Uy, 2010). In the present study, terpenoids and anthraquinones were seen in all the extracts of the four *Ophiorrhiza* plants.

Herbs that are primarily composed of tannins are astringent in nature and used to treat gastrointestinal ailments like dysentery and diarrhoea. They also possess anticancer properties (Igbinosa et al., 2009) and have the potential value as antimicrobial, antioxidant and antineoplastic agents (Peteros & Uy, 2010). Tannins were seen in the methanolic and aqueous extracts of *O. jacobii, O. pectinata* and *O. trichocarpon;* but in *O. grandiflora*, tannins were seen only in the methanolic extract. Saponins and phlobatannins were totally absent in the extracts of all the plants.

Plants rich in glycosides and coumarins are excellent dietary supplements since they are known to strengthen the body and the immune system (Yadav et al., 2014). Coumarins have also revealed their hepatoprotective, antioxidant, anti-inflammatory, antiviral and anti-carcinogenic properties (Peteros & Uy, 2010). Results showed that glycosides were present in the methanolic and aqueous extracts of *O. grandiflora, O. jacobii and O. trichocarpon;* but in *O. pectinata*, they were seen only in

the methanolic extract. Coumarins were observed in the methanolic and aqueous extract of all the selected species. Fixed oils and fats were observed in the methanolic extracts of *O. jacobii, O. pectinata* and *O. trichocarpon* only.

Hexane, methanol and water which are non-polar, semi-polar and polar respectively were used for the preparation of the extracts for the study. Among these, the presence of maximum number of compounds were observed in the methanolic extracts of the selected species and the intensity of coloured precipitates which indicated the quantity of secondary metabolites in respective tests were also higher in the methanolic extracts. Since methanol is a semi-polar solvent, numerous studies have shown that it has the highest extractive yield and a high concentration of different bioactive chemicals. Additionally, it was discovered that some non-polar chemical groups were also slightly soluble in methanol (Dhawan & Gupta, 2017; Truong et al., 2019). Though several solvents were used in the previous studies on *Ophiorrhiza*, methanolic extracts of the species have reported better results on different bioactivities analysed. Thus, in the present study, the methanolic extracts of the selected species were only considered for further analyses.

B. Quantification of major phytoconstituents

Bioactive phytoconstituents are the factors that provide medicinal properties to plants. The quantity of secondary metabolites within the plant determines the extent of healing capacity of the plant. Thus, their quantification is very important in analysing the pharmacological effects of therapeutically potential plants. Previous reports on the phytochemistry of the genus *Ophiorrhiza* indicated phenols, flavonoids, terpenoids and alkaloids as the predominant medicinal phytocompounds present within the members of the genus. So, after the qualitative screening of phytoconstituents, the major bioactive metabolites such as phenols, flavonoids, terpenoids and alkaloids

were considered for their quantitative analysis. The results of quantification of the major phytocomponents in the selected species of *Ophiorrhiza* are given in **Figs 6–13**.

Phenols are aromatic compounds with attached hydroxyl or carboxyl or methoxy groups, which have the power to neutralise radicals. The compounds release hydrogen atoms from these attached groups to the unstable free radicals and create stable phenoxyl radicals and play a crucial role in antioxidant activity. Thus, the assessment of the amount of phenolic compounds present in the extract is important to understand its antioxidant potential (Bajalan et al., 2017). The antioxidant capacity of phenol is due to its hydrogen donating, metal chelating, oxygen quenching and reducing properties (Narayanswamy & Balakrishnan, 2011). Other biological effects of phenols include inhibition of cell proliferation, anti-inflammatory, anti-aging, anti-atherosclerosis and cardiovascular protection (Ruwali et al., 2015). Phenols are usually present in all the plant parts in various forms, like tannins, phenolic acids, coumarins, stillbenes, lignans, and lignins (Dastoor et al., 2017).

In phenol quantification of the selected species, *O. grandiflora, O. jacobii and O. pectinata* revealed good and almost same quantity of the metabolite (446.83 ± 1.32, 454.12 ± 2.13 and 415.93 ± 0.51 mg GAE/g DW respectively), whereas; *O. trichocarpon* contained only half the phenol content of other plants (203.61 ± 1.01 mg GAE/g DW) **(Fig.7).** However, the obtained results suggested the abundance of phenol compounds in all the four species, giving a hint towards their antioxidant potential.

Flavonoids are significant phytochemicals that are found in abundance in fruits, flowers, seeds and bark. Isoflavonoids, chalcones, flavanones, flavones, dihydroflavonols, flavonols, anthocyanidins and catechins are some of its subclasses (Taher et al., 2020). Flavonoids can function in plants as

phytoalexins, pollinator attractants and ultraviolet filters (Iwashina, 2003). Flavonoids are subgroups of phenolic compounds and are well known for their antioxidant properties. Besides that, flavonoids possess anti-inflammatory, antimicrobial, antiallergic, anti-proliferative as well as anti-carcinogenic activities (Patil & Masand, 2018). Flavonoid compounds are generally found less in *Ophiorrhiza* species; but the isolated compounds from the genus have high pharmacological value. Luteolin-7-O-glucoside obtained from *O. mungos* and hyperin from *O. liukiuensis* have many reported medicinal properties. Thus, the estimation of flavonoids in the selected species of *Ophiorrhiza* was carried out.

As the review suggested, flavonoid content was comparatively lower in all the species selected. Among the four plants, *O. trichocarpon* exhibited higher flavonoid content (80.33 ± 0.40 mg QE/g DW) and *O. grandiflora, O. jacobii* and *O. pectinata* had a lower and almost equal flavonoid content (36.74 ± 1.16, 35.94 ± 0.26 and 42.02 ± 0.73 mg QE/g DW respectively) **(Fig. 9).**

Terpenoids are isoprenoid groups mostly found in the plant oils. They can be classified into monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, tetraterpenes, carotenoids and steroids (Shitan, 2016). Terpenoids are essential for plant growth and other physiological processes. Their bioactivities include anti-inflammatory, antibacterial, antiviral, antimalarial, anticancer, pro-transdermal absorptivity, cardiovascular disease prevention and treatment and hypoglycemic properties. Promising reports on the chemopreventive effect of terpenoid compounds on lung, liver, colon, cervical, breast, prostate, ovary and skin cancers are available. In addition, they have antioxidant, antiaging and neuroprotective properties (Yang et al., 2020). Ursolic acid, an important terpenoid compound observed in *Ophiorrhiza* species have been reported to have inhibitory effects

on prostate, breast and cervical cancers (Gai et al., 2016; Lewinska et al., 2017; Wang et al., 2017). Medicinal plants with high terpenoid content are considered as potent anticancer and anti-inflammatory agents. Thus, the extracts of all the four selected species were considered for terpenoid quantification.

In the case of terpenoid content, the plants showcased varying quantities of the metabolite. While *O. jacobii* possessed low quantity of terpenoids (89.22 ± 1.74 mg LE/g DW), all other species revealed double or triple or quadruple amounts of terpenoids in them. *O. pectinata* had the highest terpenoid content of 451.9 ± 0.96 mg LE/g DW. *O. grandiflora* and *O. trichocarpon* also revealed commendable terpenoid contents (248.85 ± 0.67 and 387.92 ± 3.90 mg LE/g DW) **(Fig. 11).** Thus, the selected plant species can be considered as terpenoid rich.

Alkaloids are low molecular weight compounds that contain at least one nitrogen atom in an amine-type structure (Taher et al., 2020). Usually seen in flowering plants, microorganisms and animals, alkaloids can exert both toxic and pharmacological activities and hence are used as pharmaceuticals, stimulants, and narcotics (John et al., 2014). Caffeine, nicotine and cocaine are a few examples of such plant-derived alkaloids. Alkaloid-rich plants grow more quickly than other plants because, in addition to their pharmacological effects, alkaloids are significant plant growth regulators (Waller and Nowacki, 1978). Alkaloid-containing plants typically have a bitter and pungent flavour (Ajanal et al., 2012). Isoquinoline, indole, pyrroloindole, piperidine, aporphine and pyridine alkaloids are among the several types of alkaloids, along with methylxanthine derivatives, indole beta-carboline and erythrine by-products. Alkaloids exhibit significant antibacterial, anti-inflammatory, anti-cancer, anti-human immunodeficiency virus (HIV), acetylcholinesterase (AChE)-inhibiting, and anti-depressant

pharmacological effects (Chikowe et al., 2020). Species of *Ophiorrhiza* are well known for being alkaloid-rich plants. The most significant alkaloids from the group are camptothecin and its derivatives, harman and ophiorrhine, which have strong anticancer activities. Thus, plants that are alkaloid-rich can be thought of as potent anticancer drugs.

The content of alkaloids in all the four selected species was found to be very low when compared to other secondary metabolites. Among them *O. trichocarpon* (14.5 ± 3.88 mg CE/g DW), followed by *O. jacobii* (13.87 ± 0.10 mg CE/g DW) exhibited the higher alkaloid contents. In *O. grandiflora*, the alkaloid content was 9.75 ± 1.00 mg CE/g DW and in *O. pectinata*, the least content of alkaloids (8.16 ± 1.79 mg CE/g DW) was observed **(Fig. 13).** Thus, the alkaloid concentration in the four species was very less when compared to the amount of other metabolites.

The methanolic extracts of the selected plant species had variable concentrations of secondary metabolites. Some compounds appeared in greater amounts, whereas others appeared in lesser amounts. The phytochemical review on the genus has shown that, though present in small quantity, certain classes of compounds exert potent pharmacological activity. This may be the probable reason that *Ophiorrhiza* plants are frequently used to cure a variety of diseases in traditional medicine. Therefore, rather than a single set of compounds, it must be the synergistic impact of the metabolites that provides their therapeutic characteristics.

C. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS is a very effective method for thorough analysis of volatile, low-molecular-weight metabolites as it combines high efficiency separation with mass detection that is adaptable, focused, and accurate (Koek et al., 2011). In the present study, the methanolic extracts of all the selected species,

were analysed by GC-MS technique to analyse the volatile compounds present within them.

The GC-MS analysis of the four species of *Ophiorrhiza*, revealed a total of 81 compounds. Among these, *O. trichocarpon* exhibited a higher number of compounds (34 compounds), followed by *O. pectinata* (33 compounds), *O. grandiflora* and *O. jacobii* (27 each). The list of compounds identified from the analysis of the selected species of *Ophiorrhiza* is given in **Table 13.** The gas chromatograms of each species and the mass spectra of compounds revealed are given in **Figs 14-18.**

The major classes of compounds identified from *O. grandiflora* include alkanes (24.84%), phytosterols (21.36%), fatty acids (16.12%), terpenoids (10.38%), terpene alcohols (7.32%), alkaloids (4.92%) and fatty acid esters (4.68%). In *O. jacobii,* the major classes of compounds observed were terpenoids (80.21%), phytosterols (9.90%), fatty acids (3.51%), alkanes (2.67%), terpene alcohols (1.89%) and fatty acid esters (1.15%). In *O. pectinata,* the major classes of compounds were fatty acids (28.05%), terpenoids (26.51%), phytosterols (15.28%), fatty acid esters (6.69%), anthraquinones (4.95%) and pyran compounds (4.79%). In *O. trichocarpon,* the major classes of compounds were terpenoids (65.59%), phenols (11.98%), phytosterols (8.79%), fatty acids (6.85%), fatty acid esters (2.04%), diterpene alcohols (1.46%) and coumarins (1.44%). Thus, the major classes of compounds observed in all the four species are terpenoids, fatty acids and their esters and phytosterols. Other classes like phenols, alkaloids and alkanes were found in minor percentages. Thus, the obtained data were in concurrence with the results of quantification of the phytoconstituents.

Maltol (0.64%) was the first eluted compound in the analysis at a retention time (RT) of 5.704 and was observed only in *O. pectinata*. This pyran compound, which is primarily used as flavour enhancer, has reported

antioxidant and neuroprotective activities (Limin et al., 1990; Hong et al., 2014). Pyranone (4.15%), another compound observed in *O. pectinata* has potent antioxidant properties (Čechovská et al., 2011). Ban et al. (2007), studied the anti-proliferative and pro-apoptotic effects of pyranone through the inactivation of NF-kB in human colon cancer cell lines.

2,3-Dihydrobenzofuran (0.56%) is the flavonoid fraction identified in *O. pectinata,* which has been reported to have antitumour, antioxidant and antimicrobial activities (Pieters et al., 1999; Chen et al., 2002; Ravi et al., 2012). 4-Vinyl-2-methoxy phenol (0.49%) is the phenol compound identified in the species which has already been reported to exhibit anti-inflammatory activities (Jeong et al., 2011). In the analysis, both of these phenolic compounds were detected in very few amounts.

Among the terpenoid compounds, neophytadiene is an important sesquiterpenoid observed in the methanolic extracts of all the selected plants, but in varying quantities. The percentage of neophytadiene were 7.15% in *O. grandiflora,* 0.24% in *O. jacobii,* 5.86% in *O. pectinata* and 0.36% in *O. trichocarpon.* Neophytadiene's mode of action in LPS-induced inflammation was examined by Bhardwaj et al. (2020) in both *in vitro* and *in vivo* settings, and it was discovered that the compound has anti-inflammatory potential. The compound has very high medicinal value with its antimicrobial, antioxidant, antipyretic and analgesic properties (Verma et al., 2015). The GC-MS analysis of ethanolic extract of *O. rugosa* var. *prostrata* leaves had revealed the presence of neophytadiene with a peak area of 0.86% (Adnan et al., 2019).

Loliolide (0.26%), found in the extract of *O. trichocarpon,* is actually a terpenoid compound usually seen in brown algae. The compound was reported to have antioxidant and cell protecting effects (Yang et al., 2011).

The GC-MS analysis of *O. rugosa* var. *prostrata* had also revealed the presence of loliolide with a peak area of 1.10% (Adnan et al., 2019).

The methanolic extract of *O. pectinata* alone exposed nerolidol–epoxyacetate (0.61%), a naturally occurring sesquiterpene, commonly known as peruviol. It is present in the essential oils of numerous species of plants in the whole plant body and in others in flowers. This compound is employed in detergents and cleansers as well as perfumes as an odorant. Nerolidol has a wide range of biological and pharmacological effects, including antioxidant, anti-microbial, anti-parasitic, insecticidal, anti-ulcer, skin penetration enhancer, anti-tumor, anti-nociceptive, and anti-inflammatory qualities (Chan et al., 2016).

In the analysis, seselin (0.44%) was exclusively found in the extract of *O. trichocarpon*. The compound is an angular pyranocoumarin that was initially isolated from Rutaceae plants. Seselin is a significant phytochemical with numerous biological functions. Seselin has been shown to have anti-inflammatory and antinociceptive effects *in vivo* by Lima et al. (2006). It has also been reported to have antiproliferative effects on a number of cancer cell lines, including leukaemia and lymphoma cells. Seselin inhibited mouse paw and ear edema as well as inflammation brought on by carrageenin. It has antimicrobial properties as well. Seselin obtained from *Plumbago zeylanica* has been reported to inhibit phytohemagglutinin (PHA)-stimulated cell proliferation in human peripheral blood mononuclear cells, which further expands its medicinal value (Tsai et al., 2008).

Phytol is an important diterpene. In the review on the biological effects of phytol, Islam et al. (2015) have reported the vast pharma-medico aspects of phytol and their derivatives which include antimicrobial, cytotoxic, antitumorous, antimutagenic, antibiotic, chemotherapeutic, antidiabetic, lipid lowering, antispasmodic, antinociceptive, antioxidant, anti-inflammatory,

antidepressant, immunoadjuvancy, hair fall defense and antidandruff activities. In addition, phytanic acid, which also has cytotoxic, anticancer, antidiabetic, and lipid-lowering properties, is a significant biometabolite of phytol. In the present study, the methanolic extracts of all the selected species revealed the presence of phytol. The peak area obtained were 7.32%, 0.80%, 1.66% and 1.46% for *O. grandiflora*, *O. jacobii, O. pectinata* and *O. trichocarpon* respectively. Phytol was reported previously from *O. rugosa* var. *prostrata* leaves with 15.50% of peak area (Adnan et al., 2019).

Braylin (0.99%), obtained from *O. trichocarpon,* is a member of natural coumarins, with a wide range of pharmacological properties. The pharmacological properties of braylin were analysed *in vitro, in silico* and *in vivo* in inflammatory and immune response models (Espirito-Santo et al., 2017).

1-Hydroxy-4-methylanthraquinone (0.42%), observed in *O. pectinata* is an anthraquinone derivative, first isolated from the methanolic extract of *Ceratotheca triloba* roots (Mohanlall et al., 2011) After its isolation, Mohanlall and Odhav (2013) reported the antibacterial effect of the compound on *Staphylococcus aureus* and *Micrococcus luteus*. The compound also exhibited antioxidant properties. The compound has been previously reported from the species, *O. liukiuensis* (Martins & Nunez, 2015).

Squalene is a very important bioactive compound, belonging to the group of triterpenes. In the present study, the methanolic extracts of all the species unveiled the presence of squalene with a peak area percentage of 2.09%, 1.31%, 3.37% and 0.88% for *O. grandiflora*, *O. jacobii, O. pectinata* and *O. trichocarpon* respectively. This compound which is predominantly seen in shark liver oil has various other plant sources such as amaranth seed, rice bran, wheat germ and olive. Squalene, an ingredient in olive oil, is thought to be the cause of olive oil's health benefits, which include a

decreased risk of cardiovascular disease and some cancers, such as colon and breast cancer (Rao et al., 1998). The non-cancer benefits of squalene include antioxidant, antiradioactive, immune stimulating, anti-histaminic and anti-allergic activities (Reddy & Couvreur, 2009). The compound was previously reported from *O. rugosa* var. *prostrata* as well (Adnan et al., 2019).

Chrysophanic acid (4.53%) is another anthraquinone derivative observed in *O. pectinata,* first isolated from the methanolic extract of *Ceratotheca triloba* roots (Mohanlall et al., 2011). After its isolation, Mohanlall and Odhav (2013) reported the antibacterial effect of the compound on *Staphylococcus typhimurium* and *E. coli*. The compound also exhibited antioxidant properties.

Solanesol is a non-cyclic terpene alcohol isolated from the tobacco plant. Solanaceous plants like tomato, potato, egg-plant, and pepper plants are the main sources of solanesol. Solanesol is notable as the biosynthetic precursor for the synthesis of ubiquinone drugs, such as coenzyme Q10 and vitamin K2. Its bioactive potentials include effects against bacteria, fungi, viruses, cancer, inflammation, and ulcers. Derivatives of solanesol are used to treat wounds, osteoporosis, acquired immune deficiency syndrome, and cardiovascular disease. They also have anti-oxidant and antitumor activities (Yan et al., 2017; Yan et al., 2015). In the present study, the compound was observed in *O. jacobii* (1.09%) and *O. trichocarpon* (0.19%).

Vitamin E (α-tocopherol) was identified from the extract of *O. jacobii* with the peak area of 0.20%. Previously reported from *O. rugosa* var. *prostrata* (Adnan et al., 2019), it has potent antioxidant and antiatherogenic properties (Patra et al., 2001; Carr et al., 2000) and is vital for the formation and natural functioning of red blood cells and muscles (Lukaski, 2004).

Campesterol is one of the most common plant sterols, which is widely distributed in seeds, nuts, cereals, beans, legumes, and vegetable oils (Ostlund et al., 2002; Phillips et al., 2005). Plant sterols, such as campesterol, have cholesterol-lowering properties due to their structural resemblance to cholesterol (Choi et al., 2007). There is evidence that campesterol has chemopreventive qualities against prostrate (McCann et al., 2005), lung (Schabath et al., 2005) and breast (Awad et al., 2000) cancers. In the present study, the compound was observed in *O. grandiflora* (3.96%), *O. jacobii* (1.76%) and *O. trichocarpon* (1.59%). Stigmasterol is another phytosterol that exhibits similar bioactivities. *O. rugosa* var. *prostrata* extract had the presence of both these compounds with a peak area of 5.00 and 7.92% respectively (Adnan et al., 2019). The hexane extract of *O. shendurunni* whole plant also has revealed the compound, stigmasterol (Rajan et al., 2016b).

γ -Sitosterol was investigated for its hypolipidemic activity against five target proteins and was proved to be a potent hypolipidemic agent (Balamurugan et al., 2015). The compound was found in all the plant extracts with a peak area of 11.49% (*O. grandiflora*), 4.12% (*O. jacobii*), 15.28% (*O. pectinata*) and 3.26% (*O. trichocarpon*). Previous reports reveal its occurrence in *O. rugosa* var. *prostrata* (Adnan et al., 2019).

Germanicen (0.83%) was seen only in *O. trichocarpon* in the present analysis. Osorio et al. (2012) reported that this triterpenoid, from Celastraceae species inhibited HIV replication targeting NF-kB and Sp1 dependent transcription. A triterpene of the lupane type, lupenone (4.43%), observed in the extract of *O. trichocarpon* has previous reports of having a number of pharmacological effects, including activities like anti-inflammatory, anti-viral, anti-diabetic, anti-cancer, and beneficial for Chagas disease (Xu et al., 2018).

Ursolic acid is a triterpene with several biological functions. It selectively inhibits cyclic AMP phosphotransferase and AMP-dependent protein kinase and also blocks the stimulation of lipoxygenase and cyclooxygenase, thereby providing anti-inflammatory effects. It selectively induces cell death with the aid of caspase-3, thereby limiting the cell overgrowth that may result in cancer. Ursolic acid is reported from the chloroform extract of *O. liukuensis* whole plant (Martins & Nunez, 2015). In *O. trichocarpon,* the compound was observed with a peak area of 0.76%. The alcoholic extract of *O. nicobarica* has revealed this compound earlier (Chattopadhyay et al., 2007).

Triterpenoid lupenyl acetate has been shown to have bioactivities against hyperglycemia, inflammation, bacteria, mutagenesis, and ulcers (Verma et al., 2015). In the analysis, the compound was seen in *O. jacobii* (2.08%), *O. pectinata* (2.49%) and *O. trichocarpon* (2.71%). Found widely in both plants and microorganisms, humulane-type sesquiterpenoids have reported anti-tumor, anti-inflammatory, antibacterial and antiviral properties (Jiao et al., 2018). Humulane-1,6-dien-3-ol is the most prominent compound identified in the extracts of *O. jacobii* (58.19%) and *O. trichocarpon* (54.89%). Cycloartenol (12.59%) identified in *O. pectinata*, is the fundamental triterpenoid found in plants that serves as the precursor of nearly all phytosterols. The substance from which cycloartenol is generated is squalene (Ohyama et al., 2009).

3-Epipapyriferic acid is the last eluted compound at a RT of 44.438 min. This triterpene, forms the major constituent of silver birch bark resin. In the present study, the compound was observed in the extract of *O. jacobii* with a peak area of 1.35%. Better known as a dammarane compound, it exhibits various biological activities. It can be useful in the treatment of

cancer, diabetes mellitus, ageing, neurodegenerative diseases, cardiovascular diseases, bone, liver, kidney and gastrointestinal diseases (Cao et al., 2015).

Fatty acids and their esters synthesised in plants exhibit anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic and vasodilatory characteristics, which in turn helps in the prevention of coronary heart disease, hypertension, type 2 diabetes, rheumatoid arthritis *etc*. (Simopoulos, 1999). Hence, cosmetic and pharmaceutical sectors frequently use fatty acids made from plant sources. Several fatty acids and their esters were identified in the GC-MS analysis of the plant extracts, which add to the bioactivity potential of the selected species.

Octadecanoic acid, sometimes referred to as stearic acid, is a saturated fatty acid that is typically found in a variety of animal and plant fats. Shea butter and cocoa butter both contain them. It is well-known that the fatty acid is a strong anti-inflammatory lipid. Additionally, it has antiviral properties and lowers plasma cholesterol (Khalil et al., 2000; Rasmussen et al., 2006). The compound was eluted in *O. jacobii* (0.35%), *O. pectinata* (6.73%) and *O. trichocarpon* (0.26%).

The most prevalent saturated fatty acid found in animals, plants and microorganisms is hexadecanoic acid, also known as palmitic acid. It is a significant part of the oil found in the fruit of oil palms, as its name suggests. The antioxidant and ant-inflammatory effects of palmitic acid are reported by Aparna et al. (2012). In the present analysis, the fatty acid was observed in all the plant species with a peak area percentage of 12.92%, 2.84%, 18.77% and 2.92% in *O. grandiflora*, *O. jacobii, O. pectinata* and *O. trichocarpon* respectively, indicating that all the species have good percentage of this compound. Similarly, good percentage of this compound (3.95%) was observed in *O. rugosa* var. *prostrata* (Adnan et al., 2019). The chloroform

extract of *O. shendurunni* whole plant also revealed this compound (Rajan et al., 2016b).

Tetradecanoic acid, also known as myristic acid, is a saturated fatty acid found in majority of animal and plant fats, including butterfat and the oils from coconut, palm, and nutmeg. They have antibacterial, antioxidant and anticancer properties, along with larvicidal and repellent activity (Verma et al., 2015; Sivakumar et al., 2011). The methanolic extract of *O. grandiflora* had a peak area of 0.97% of this compound.

Linoleic acid is an essential fatty acid abundant in nuts and fatty seeds. It is an unsaturated omega-6 fatty acid, which has wide applications due to its beneficial properties. They have anti-inflammatory, acne reducing, skin-lightening and moisture retaining capacities (Letawe et al., 1998; Ando et al., 1998). In the present analysis, all the plant species exhibited the presence of the compound with the peak area percentage as 1.14%, 0.31%, 2.55% and 3.66% for *O. grandiflora*, *O. jacobii, O. pectinata* and *O. trichocarpon* respectively. *O. rugosa* var. *prostrata* has been reported for the presence of this compound earlier (Adnan et al., 2019).

Esters of fatty acids like hexadecanoic acid, linoleic acid, octadecanoic acid, stearic acid, tetradecanoic acid, eicosanoic acid and palmitic acid were also observed in the analysis. Compounds like 2-2-methylenebis-phenol (0.26%), 2-4-bis (1-phenylethyl)-phenol (6.78%) and 2,4,6-tris-(1-phenylethyl)-phenol (4.93%) represented the phenolic fractions of *O. trichocarpon.* Very few alkaloid compounds like, 3-phenyl-1,2-dihydroquinoxalin-2-one, 1-anliinoisoquinoline, 1-decanoylpiperidine and aspidofractinine-3,8-dione were unveiled from the analysis. Many phytoconstituents like neophytadiene and squalene (terpenes), hexadecanoic acid, linoleic acid, linoleic acid methyl ester (fatty acids and ester), phytol

(diterpene alcohol) and γ-sitosterol (phytosterols) were found to be common in all the plant extracts.

GC-MS analysis of the extracts of selected *Ophiorrhiza* species revealed a large number of phytochemical compounds belonging to various classes and nearly all of them have distinct bioactivities. Even though, some are present in extremely small amounts, they may nevertheless play a role in the potential effects seen when screening the bioactivities of plant extracts, such as antioxidant, antiproliferative and anti-inflammatory activity. The presence of active metabolites provides strong support for the usage of the members of *Ophiorrhiza* in traditional medicine.

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

HR LC-MS is a very effective method for detailed analysis of non-volatile, low-molecular-weight compounds as it provides high efficiency separation with proper detection of compounds. In the present study, the methanolic extract of all the selected species, were analysed by HR LC-MS technique to detect the non-volatile compounds present within them.

The HR LC-MS analysis of the four species of *Ophiorrhiza*, revealed a total of 108 compounds and *O. trichocarpon* exhibited higher number of compounds (47 compounds), followed by *O. jacobii* (41 compounds), *O. pectinata* and *O. grandiflora* (30 and 24 compounds respectively). In the study, compounds are analysed using both positive and negative ionisation modes. The list of compounds identified from the analysis of the selected species of *Ophiorrhiza* is given in **Table 14.** The liquid chromatograms of each species and the mass spectra of compounds revealed are given in **Figs 19-23.**

The major classes of bioactive compounds observed in all the four species are fatty acids (19 compounds), flavonoids (15 compounds), terpenoids (13 compounds) and alkaloids (10 compounds). Other classes like phenols, glycosides, anthraquinones and sterols were also found. Out of the hundred compounds that were eluted, about 62 compounds were found to possess some kind of bioactivities.

The first eluted compound was Punaglandin 5 (RT- 0.991) from *O. grandiflora.* The first terpenoid fraction eluted from this plant was loganic acid. Loganic acid, first isolated from the seeds of *Strychnos potatorum*, is a strong antioxidant agent and has cytoprotective properties by preventing heavy metal toxicity by inhibiting ROS generation and lipid peroxidation (Abirami et al., 2019). *In vivo* studies on the rabbit model to analyse the intraocular pressure (IOP) reducing capacity of loganic acid obtained from Cornelian cherry fruit found out that the compound has strong IOP hypotensive effect which can be compared with the standard timolol. Thus, the compound has good glaucoma inhibiting properties. As per the results, IOP declined by about 25% within the initial three hours of treatment (Szumny et al., 2015). Loganic acid obtained from the ethanolic root extract of *Gentiana lutea* has antiadipogenic effects both *in vitro* and *in vivo* and can be studied for its utilisation in the treatment of obesity (Park et al., 2018). In *Ophiorrhiza liukiensis* also, this compound was observed (Krishnakumar et al., 2020).

Monotropein, seen in *Ophiorrhiza grandiflora*, is an iridoid monoterpenoid isolated from *Morinda officinalis.* It is a metabolite with strong anti-inflammatory (Choi et al., 2005) and antiosteoporosis activities (Zhang et al., 2016). Apiin is a natural flavonoid, observed in *O. grandiflora* and *O. pectinata.* The compound, which is a prominent constituent in winter–hardy plants has anti-inflammatory properties (Mencherini et al., 2007).

Pumiloside, is an important glycoside found in the extract of *O. trichocarpon*. The compound was first isolated from *O. pumila* and is present in many species of *Ophiorrhiza* **(Table 1).** Pumiloside is an important precursor for the biosynthesis of camptothecin (Kitajima, 2007). Stachyoside is a phenolic compound usually present in Lamiaceae and Rubiaceae members. In the study, the compound was observed only in *O. trichocarpon.* Being a monoterpene indole alkaloid, it may have cytotoxic, analgesic and anticancer activities (Pimenta et al., 2010).

Anatabine, identified in *O. jacobii* and *O. trichocarpon*, is a minor alkaloid usually seen in the family Solanaceae. Paris et al. (2013) investigated the potential of anatabine to lower Alzheimer's Aβ production *in vitro* as well as *in vivo* and found that the compound can regulate the brain Aβ production and thus can help in Alzheimer's treatment. The compound was also reported to express anti-inflammatory activity by inhibiting STAT3 phosphorylation (Paris et al., 2013).

Hesperidin is a natural flavonoid seen in the inner white peel of citrus fruits. The review on the biological activities of the compound has noted the efficacy of the compound to prevent various diseases like cardiovascular diseases, neurodegenerative diseases and cancer. The compound also exhibits antioxidant and anti-inflammatory properties (Aggarwal et al., 2020). In the present study, the compound was eluted from *O. grandiflora, O. pectinata* and *O. trichocarpon.*

6-Methoxyluteolin 7-[6" -(2-methylbutyl) glucoside] seen in the extract of *O. jacobii* is an important flavonoid compound. The compound earlier isolated from *O. mungos* as Luteolin-7-O-glucosides, is very important with antioxidant, anti-inflammatory and anti-cancer properties. Upon being modelled in rats, the compound is reported to protect the gastrointestinal tract

against ethanol and indomethacin-induced gastric ulcer (Baskar et al., 2011; Antonisamy et al., 2016).

Naringenin, identified in *O. pectinata* is a flavonoid compound predominant in fruits like citrus, bergamots and tomatoes and exhibit radioprotective, antiviral, antibacterial, antioxidant, anti-inflammatory, antitumor, antiadipogenic and cardioprotective effects (Kumar & Tiku, 2016; Salehi et al., 2019).

3-α(s)-Strictosidine, found in *O. jacobii* is an indole alkaloid, which acts as the precursor for various other indole alkaloids, including camptothecin and is also found in *O. pumila* (Rüffer et al., 1978). Lappaol B is a phenolic compound identified in the plant, which has reported mild anti-austeric activity on human pancreatic cancer PANC-1 cell lines (Tezuka et al., 2013). Amarogentin, a glycoside observed in *O. jacobii* is a compound usually seen in gentian roots. Due to its bitterness, the roots of the plant are used for treating digestive disorders in traditional medicines. The bitterness attributing compound is amarogentin. The compound has been reported to possess anticarcinogenic and antileishmanial activities (Ray et al., 1996; Medda et al., 1999).

Lespenefril, identified from the extract of *O. trichocarpon* is a flavonoid compound, commonly known as kaempferitrin. Wang and Zhao (2019) reported that the compound block NF-kB and AKT/mTOR pathways in human rheumatoid arthritis fibroblast-like synoviocytes to restrict cell growth, induce apoptosis, and reduce inflammation.

Manniflavanone, identified in the extract of *O. trichocarpon,* was first isolated from the bark of *Garcinia buchananii*. In Africa, bark of *Garcinia buchananii* trees is used for centuries to treat diabetes, dysentery, diarrhoea, and weight loss. Bibi et al. (2014) studied the anti-inflammatory and

antioxidant activities of the stem bark extracts and found that manniflavanone was the most effective antioxidant and anti-inflammatory phenolic component among the extracted bark phenolics. Their study showed that the underlying anti-inflammatory effects of manniflavanone are connected to NF-kB and NF-kB target gene suppression. Caribine, is an indole alkaloid, usually found in Amaryllidaceae and has potent antiviral activity (Perez, 2003). In the present analysis, *O. jacobii* and *O. trichocarpon* was found to possess this compound.

N-Caffeoyltryptophan, also known as Javamide II, is an aminoacid derived from coffee beans that functions as a strong SIRT 1/2 inhibitor. SIRT 1 and SIRT 2 are tumor-promoting deacetylase enzymes (Park, 2016; Choi et al., 2013). Thus, the compound can be a potent anticancer agent. In the study, the compound was observed only in *O. trichocarpon.*

Mosloflavone, a flavonoid identified in *O. trichocarpon,* was isolated from *Mosla soochowensis.* The reported bioactivities of this compound include antiviral activity on Enterovirus 71, antioxidant and anti-inflammatory activity (Choi et al., 2016; Rui et al., 2020).

1-Hydroxy-4-methylanthraquinone observed in *O. trichocarpon* is an anthraquinone derivative, first isolated from the methanolic extract of *Ceratotheca triloba* roots (Mohanlall et al., 2011). After its isolation, Mohanlall and Odhav (2013) reported the antibacterial effect of the compound on *Staphylococcus aureus* and *Micrococcus luteus*. The compound also exhibited antioxidant properties. The compound has been previously reported from the species, *O. liukiuensis* (Martins & Nunez, 2015) and also in *O. pectinata* by GC-MS analysis. Physalin O, observed in *O. trichocarpon*, is a phytosterol initially isolated from *Physalis* species and was reported to possess anti-inflammatory activities (Ji et al., 2012).

Limonin or limonic acid seen in the extract of *O. trichocarpon*, is a triterpenoid, usually seen in citrus plants. By sustaining the antioxidant defence system, this compound demonstrated considerable neuroprotective effect against glutamate toxicity in primary cultured rat cortical cells (Yoon et al., 2010). Chidambara Murthy et al. (2011) evaluated the anticancer potential of limonin, isolated from the seeds of *Citrus reticulata,* on human colon adenocarcinoma cell lines (SW480). The compound could inhibit cell proliferation and activate caspase-3 by the mitochondrial mediated intrinsic apoptotic pathway. Balestrieri et al. (2011) found out that the limonin has high antiviral activity against human retroviruses like HTLV-1 and HIV-1, by exhibiting antiviral action comparable to that of the standards used.

Chrysin is a flavonoid compound identified in the extracts of *O. jacobii* and *O. trichocarpon.* The compound which naturally occurs in many plants, honey and propolis has many reports on its therapeutic potential. It exhibits antioxidant, anti-inflammatory, anticancer and antiviral activities.

Rubiadin, the anthraquinone fraction exhibited by *O. jacobii,* was isolated from *Rubia cordifolia*. This compound had substantial hepatoprotective effect in rats when exposed to carbon tetrachloride (Rao et al., 2006) and was previously reported as a good antioxidant (Tripathi et al., 1997).

Swietenine, is a triterpene isolated from the seeds of *Swietenia macrophylla*. The *in vivo* hypoglycemic action of this compound was tested on neonatal streptozotocin induced type 2 diabetic rats. The oral administration of swietenine at doses of 25 and 50 mg/kg body weight per day was found to have considerable dose-dependent hypoglycemia and hypolipidemic action (Dewanjee et al., 2009). This compound, identified in the extract of *O. jacobii* was recently reported to be an anti-inflammatory agent that downregulate pro-inflammatory cytokines and mediators (Mak et al., 2021).

Euphornin, is a diterpene identified in the extracts of *O. jacobii* and *O. trichocarpon.* This compound, which was first isolated from *Euphorbia heliscopia* has been reported to exert antiproliferative activity on human cervical adenocarcinoma HeLa cells by inducing apoptosis and cell cycle arrest at the G2/M phase of cell division (Li et al., 2018).

In addition to the mentioned bioactive compounds, several fatty acids, phenols, flavonoids and alkaloids were also identified from the HR LC-MS analysis of the four *Ophiorrhiza* species. The major fatty acids were 3-iodo-hexadecanoic acid, 2-iodo-hexadecanoic acid, 9,12,13-trihydroxy-10-octadecenoic acid, pinellic acid, palmitic acid, hydroxyoctadecadienoic acid *etc*. The phenols like caffeic acid-3-glucoside, (-)-pinoresinol glucoside, *etc*. were also found. Flavonoids such as clematin, rutin and santin were also observed in the plant extracts. Alkaloids like tejedine, isoreserpine, thalidasine *etc*., were also eluted in the analysis. The last compound to get eluted was a biopeptide (RT-26.962). In the analysis, it was observed that there was no single compound common to all the species. However, compounds specific to each species was high. Each species had numerous compounds to its individual credit that would provide distinguishing medicinal properties to each species.

The GC-MS and HR LC-MS techniques yielded a plethora of phytochemical components that possess diverse bioactivity potentials. HR LC-MS study revealed some of the efficient bioactive components that were not found by GC-MS analysis. Thus, both of these analytical techniques are required for thorough investigation of plant metabolites. Many compounds identified from the analyses possessed significant therapeutic efficacies, preferably anticancer, anti-inflammatory, antioxidant and so on. So, the existence of these chemicals provided possible hints for the application of selected plants in screening various bioactive potentials.

PART III – BIOACTIVITY SCREENING

A. Free radical scavenging activity of selected species of *Ophiorrhiza*

The most common method for determining the antioxidant capacity of plant extracts is *in vitro* free radical scavenging assays (Pracheta et al., 2011). The ability of the extract or phytochemicals to donate hydrogen atoms or electrons and stabilise free radicals is used as an index for antioxidant capacity in all of these assays (Hajji et al., 2010). Since the antioxidant efficacy of the testing material is dependent on the physical and chemical factors available within the experimenting system, a single test model would not be sufficient to determine the antioxidant activity of a sample (Mathew & Abraham, 2006). In the present study, four different assays were performed to assess the ability of the selected species of plants to scavenge free radicals such as DPPH, hydroxyl, nitric oxide, and superoxide radicals.

i. DPPH free radical scavenging activity

The DPPH radical scavenging assay is a simple and easy *in vitro* method for determining the antioxidant capacity of a plant extract (Alam et al., 2013). DPPH is a stable purple/deep violet organic nitrogen radical that, unlike most other free radicals, does not dimerise. The delocalisation of the electron also produces a deep violet colour, which is marked by absorption in solution at 517-528 nm. In the technique, the purple-coloured DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical is reduced to yellow-coloured 2, 2-diphenyl-1-picrylhydrazine by antioxidants present in the sample (Nurul et al., 2013). The hydrogen donating ability of the sample is related to the enhanced discolouration, which leads to lower absorbance (Ndhlala et al., 2007). Due to the stability of DPPH, the radical scavenging process allows for a reaction with nearly any form of antioxidant. This means that even weak

antioxidants have enough time to react with the DPPH radical (Rubeena, 2021).

In the present study, the methanolic extracts of all the selected species of *Ophiorrhiza* exhibited a dose dependent increase in their potential to quench the radicals (**Fig. 24).** Though the highest percentage inhibition was showed by *O. jacobii,* the IC_{50} value was found to be better for *O. trichocarpon* (85.65 ± 2.48 µg/ml), followed by *O. jacobii* (92.64 ± 2.16 µg/ml), *O. pectinata* (123.43 ± 1.98 µg/ml) and *O. grandiflora* (139.4 ± 2.05 µg/ml). Thus, in this assay, *O. trichocarpon* was better. The standard, ascorbic acid showed an IC_{50} value of 38.42 ± 1.57 µg/ml (**Table 15)**. Eventhough, the activity of all the extracts seems to be less when compared to the standard, the IC_{50} values come within the range of selected concentrations, and hence all the plant extracts have considerable scavenging effects on the DPPH radical.

The DPPH radical scavenging potential of the ethanolic extract of *O. tirunelvelica* (whole plant), showing the moderate radical scavenging capacity of the plant was reported earlier (Prabha & Karuppusamy, 2017). The aqueous leaf extract of *O. pectinata* was previously reported to have moderate radical scavenging potential on the DPPH radicals (Baiju et al. (2017).

ii. Hydroxyl free radical scavenging activity

Hydroxyl radicals have the capacity to interact with all the biological molecules and cause lipid peroxidation, protein breakdown, and membrane disruption. Therefore are the most potent reactive oxygen species. These radicals are very dangerous because cells lack an enzyme mechanism that removes them. They attack DNA and produce protein crosslinks that are difficult to repair, resulting in cell death (Sharma et al., 2012). In hydroxyl radical scavenging assay, the free radicals are produced by Fenton reaction.

The potential of the plant extracts to quench these radicals is analysed by evaluating the inhibition of degradation of 2-deoxyribose by these radicals. The radicals breakdown 2-deoxyribose into fragments that become red when heated with TBA at a low pH (Vazhayil et al., 2016). So low red colour indicates high antioxidant potential.

The methanolic extracts of all the plants showed a dose dependent increase in the percentage of inhibition of free radicals with increasing concentration of the plant extracts from 125 to 2000 µg/ml **(Fig. 25).** Among the selected plants, *O. jacobii* was identified as the best scavenger with the highest inhibition percentage ranging from 15.48 ± 0.61 to 70.24 ± 0.9 and IC_{50} value of 767.11 ± 2.33 µg/ml. The standard, gallic acid revealed IC_{50} value of 477.49 ± 2.05 µg/ml. On analysing the IC_{50} values of other extracts for hydroxyl radical scavenging assay (**Table 15),** the activity shown by them were not so better than that of the standard and thus have the potential to scavenge hydroxyl radicals from a relatively higher level to moderate level.

iii. Nitric oxide free radical scavenging activity

Nitric oxide is a diffusible free radical produced from the amino acid L- arginine by the enzymes present in the vascular endothelial cells, neuronal cells and phagocytes. When nitric oxide radical reacts with superoxide radical, the extremely reactive peroxynitrite anion is formed (Boora et al., 2014). Under normal physiological conditions, low quantities of nitric oxide operate as a regulator of immune response, blood pressure, and neuronal communication. Overabundance, on the other hand, can cause tissue damage and is linked to a number of cardiovascular issues, including atherosclerosis and hypertension, as well as carcinomas and inflammatory disorders (Oyebode et al., 2019). The nitric oxide radical scavenging assay is based on the generation of nitric oxide in aqueous solution by sodium nitroprusside at physiological pH (7.2). The created nitric oxide combines with oxygen to

make nitrite ions, the amount of which can be calculated using the Griess reagent (Marcocci et al., 1994). Thus, the antioxidant potential of a sample is determined by its capacity to compete with oxygen to suppress nitrite ion production.

In the present study, the methanolic extracts of all the selected species of *Ophiorrhiza* exhibited a dose dependent increase in their potential to quench the nitric oxide radicals **(Fig. 26).** Though, all the extracts exhibited increasing inhibitory percentages, the IC_{50} values of the extracts **(Table 15)** were not that significant when compared to the standard. While the IC_{50} value of the standard, gallic acid was 108.80 ± 1.55 µg/ml, the least IC_{50} value among the plant extracts was for *O. jacobii* (783.35 ± 2.88 µg/ml) and hence the plant extracts could exhibit only moderate scavenging potential on nitric oxide radicals.

iv. Superoxide free radical scavenging activity

Superoxides are the initial ROS produced in the cell, which can generate secondary ROS and induce oxidative stress (Alam et al., 2013). They are nucleophilic reactants with both oxidising and reducing characteristics and have a brief lifetime. They can accept one electron and two protons to form hydrogen peroxide radical. Despite the fact that both superoxide and hydrogen peroxide radicals are weak, they can both contribute to the formation of the highly reactive hydroxyl radical (Sharma et al., 2012). As a result, their existence within the cells is extremely harmful since they might generate extra free radicals. In the experiment, superoxide anions are generated in riboflavin-nicotinamide adenine dinucleotide system by the oxidation of NADH. These radicals reduce nitroblue tetrazolium (NBT) resulting in the formation of blue formazan product. The assay measures the ability of the sample to suppress blue formazon production by scavenging

superoxide radicals produced in the riboflavin-NBT-system (Mulla et al., 2009).

The methanolic extracts of all the selected species exhibited a dose dependent increase in their potential to scavenge the superoxide radicals **(Fig. 27).** Same as in the previous assay, though, all the extracts exhibited increasing inhibitory percentages, the IC_{50} values of the extracts **(Table 15)** were higher when compared to the standard. While the IC_{50} value of the standard, ascorbic acid was 238.35 ± 2.17μg/ml, the least IC_{50} value among the plant extracts was for *O. pectinata* (737.48 ± 2.66 μg/ml) and hence the plant extracts could exhibit only moderate scavenging potential on nitric oxide radicals.

The evaluation of *in vitro* free radical scavenging potential of the selected species of *Ophiorrhiza* using different assays provided results which exposed that all the plant extracts have an increasing inhibitory potential on the selected free radicals. In DPPH radical scavenging assay, *O. trichocarpon* was the better inhibitor. *O. jacobii* was good at inhibiting hydroxyl and nitric oxide radical quenching. But, *O. pectinata* inhibited more of superoxide radicals. *O. grandiflora* performed poorly in all the assays. The results suggested that a single species was not found to be a predominant antioxidant candidate, but each species had specific free radical scavenging properties on specific ROS. The IC_{50} value of all extracts demonstrated that the extracts had modest antioxidant activity. This is understandable given that the standards utilised are pure and the plant extract is in its raw form. Since the findings of the multiple assays differed so widely, it is clear that a single assay is insufficient to assess antioxidant capacity.

Recent research has demonstrated that the antioxidant benefits of plant extracts are due to the synergistic action of their phytoconstituents, primarily phenols, flavonoids, terpenoids, sterols, tannins, and c oumarins (Oyebode et

al., 2019). All these secondary metabolites were present in the current study, and thus the free radical scavenging potential exhibited by the plant extracts could be attributed to these compounds. However, the fact that an antioxidant capability is related not just to the synergistic action of compounds or the overall phenolic content, but also to the existence of highly active individual antioxidant compounds, which should be taken into account (Augusto et al., 2014).

In the phytochemical analyses, the highest number of bioactive compounds was identified in *O. trichocarpon,* followed by *O. jacobii, O. pectinata* and *O. grandiflora*. This variation also might have contributed to the differential antioxidant activity. The most important compounds with antioxidant properties that were identified from the selected species were maltol, loliolide, n-hexadecanoic acid, 2,3-dihydrobenzofuran, 4-vinyl-2-methoxy phenol, neophytadiene, phytol, squalene, chrysophanic acid, solanesol, vitamin E, caffeic acid-3-glucoside, hesperidin, 6-methoxyluteolin 7-[6" -(2-methylbutyl) glucoside], naringenin, manniflavanone, mosloflavone, chrysin, *etc.*

B. Anti-inflammatory activity

The phytochemical screening of the selected species of *Ophiorrhiza,* revealed the presence of many secondary metabolites like flavonoids, phenols, alkaloids, sterols and terpenoids which have reported anti-inflammatory properties. The GC-MS and HR LC-MS analyses also identified many compounds with reported anti-inflammatory properties. On this background, the methanolic extracts of the selected species of *Ophiorrhiza* were considered for evaluating the anti-inflammatory activity. For the initial screening, *in vitro* biochemical assays were done and the most effective extract was then selected for further analyses.

i. Evaluation of anti-inflammatory activity using biochemical assays

a) Inhibition of protein denaturation

Protein denaturation occurs when there is a change in its physiological, chemical, and biological properties as a result of structural disruption, which eventually impacts their functional capacities (Eckersall, 2008). Inflammatory stimuli can promote protein denaturation, which can lead to serious health consequences (Kiranmayi et al., 2018). Almost all anti-inflammatory medications are intended to prevent protein denaturation (Gunathilake et al., 2018). Thus, the capacity to decrease protein denaturation appears to indicate anti-inflammatory efficacy.

In the present study, the methanolic extracts of all the selected species exhibited a dose dependent increase in the percentage of inhibition of protein denaturation. However, among the four species, *O. trichocarpon* exhibited the highest inhibition percentage ranging from 13.83 ± 1.80 to 64.25 ± 2.81 from the lowest concentration of 62.5 to the highest concentration of 500 µg/ml of the plant extracts (**Fig. 28)** and also exhibited better results with the lowest IC_{50} value (376.89 ± 3.92 µg/ml).

b) Proteinase inhibitory activity

Proteinases are hydrolases that breakdown the peptide links between the amino acids of proteins and peptides (Soualmia & El Amri, 2018). As part of the immune response, leukocytes release bactericidal enzymes and proteases that play an important role in tissue damage during inflammatory reactions (Omoregiel et al., 2014). Thus, proteinase inhibitors have significant role in cell protection as they regulate inflammation by inhibiting proteolytic activity of proteinases (Hiemstra, 2002).

Proteinase inhibitory potential of the methanolic extracts of all the selected species were found to be in a concentration-dependent manner. Though all the plant species exhibited considerable percentage inhibition (**Fig. 29),** *O. trichocarpon* exhibited the lowest IC_{50} value of 367.153 ± 4.57 µg/ml.

In vitro biochemical experiments for evaluating the anti-inflammatory potential of selected *Ophiorrhiza* species revealed that; *O. trichocarpon* has better anti-inflammatory activity and was further investigated for its effect on the RAW 264.7 macrophage cell line.

ii. Anti-inflammatory activity on RAW 264.7 macrophage cell line

Inflammatory responses are usually related with the activation and release of several inflammatory enzymes like cyclooxygenase, lipoxygenase, myeloperoxidase and inducible nitric oxide synthase. The effect of the methanolic extract of *O. trichocarpon* on the activity of these inflammatory enzymes and also on the cellular nitrite level was analysed on LPS induced RAW 264.7 macrophage cell line.

a) Cyclooxygenase (COX) activity

Prostaglandins are arachidonic acid metabolites that play an important role in inflammation. Their synthesis is dependent on the activity of prostaglandin synthases, also known as cyclooxygenases. COXs have two isoforms, namely; COX 1 and COX 2. Both of these isoforms generate prostanoids with distinct roles. COX 1 is constitutively expressed in most cells and produces prostanoids with housekeeping functions. COX-2 produce prostanoids in inflammation and in proliferative diseases (Ricciotti & FitzGerald, 2011). Thus, substances that suppress the generation of cyclooxygenase isoenzymes, notably COX-2, are regarded to have anti-inflammatory properties.

In the present study, the methanolic extract of *O. trichocarpon* displayed a concentration-dependent increase in the inhibition percentage of the inflammatory enzyme from the lowest to the highest concentrations of the extract. The three different concentrations of the sample were 25, 50 and 100 µg/ml and the range of inhibition was 26.84 ± 1.82 to 62.41 ± 1.72% (**Fig. 30)**. The IC_{50} value of *O. trichocarpon* was 56.09 ± 3.25 µg/ml and that of the standard was 33.15 ± 2.05 µg/ml, indicating that the plant extract has significant ability to suppress COX activity.

b) Lipoxygenase (LOX) activity

Lipoxygenases are oxidative enzymes that catalyse the conversion of fatty acids to metabolites that are harmful. They participate in the conversion of arachidonic acid into leukotrienes, a pro-inflammatory mediator that promotes inflammation. (Hu & Ma, 2018). Both acute and chronic inflammatory illnesses, such as asthma, rheumatoid arthritis, atherosclerosis and cancer, express LOX and their pro-inflammatory products. (Wisastra & Dekker, 2014). Thus, a strong LOX inhibitor can be an effective anti-inflammatory agent.

In this assay also, the extract showed a dose-dependent increase in the inhibition percentage of the inflammatory enzyme **(Fig. 31)**. While considering the IC_{50} values (*O. trichocarpon* - 80.15 ± 2.92 µg/ml), the plant extract showed moderate capacity to suppress LOX activity.

c) Myeloperoxidase (MPO) activity

Myeloperoxidase is a pro-inflammatory enzyme secreted by leukocytes, primarily neutrophils and monocytes. When neutrophils are activated, they produce superoxide radicals, which can form extremely reactive secondary oxidants such as hydrogen peroxide and hypochlorous acid. MPO produces hypochlorous acid from hydrogen peroxide and chloride

anions. Hypochlorous acid is a powerful neutrophil oxidant that interacts with most biological molecules, particularly proteins (Van et al., 2007). MPO is also a biomarker of inflammation associated with a number of cardiovascular disorders. (Olza et al., 2012). Thus, MPO inhibitors can be effective anti-inflammatory agents.

In the assay, at the highest concentration of 100 µg/ml, the methanolic extract of *O. trichocarpon* reduced the activity of the MPO enzyme to almost the same value obtained for the standard at the initial concentration **(Fig. 32),** showing that the plant extract has significant effect on MPO activity.

d) Inducible nitric oxide synthase (iNOS) activity

Nitric oxide (NO) is an important messenger involved in many biological processes. It has a pivotal role in regulating inflammatory responses as its overproduction can cause tissue damage. NO is synthesised from L-arginine by the enzyme nitric oxide synthase (NOS), which exists in three isoforms; neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). nNOS and eNOS produce NO at low levels. But, proinflammatory mediators upregulate the expression of iNOS and overproduction of NO takes place in the cells (Khallaf et al., 2016), causing cell damage. The extract which can down-regulate the action of iNOS can be considered to have anti-inflammatory potential.

In the present study, the sample showed a concentration-dependent increase in the inhibition percentage of the enzyme. The results from the assay (**Fig. 33),** annotated the activity of the plant extract on suppressing the iNOS activity as moderate.

e) Cellular nitrite level

From the previous assay, the ability of the plant extract to suppress the iNOS activity was found and the reduction in iNOS activity justifies the reduction in cellular nitrite levels. From the results obtained (**Fig. 34),** the

extract of *O. trichocarpon* has prominent ability to reduce nitrite level to about half.

Though the evaluation of anti-inflammatory potential of the methanolic extract of *O. trichocarpon* on LPS induced RAW 264.7 macrophage cell line showed varying results, the major findings indicated that the plant has the potential to be a strong candidate for production of an anti-inflammatory drug. The obtained results are very significant in the point that the standard used for the assay, diclofenac sodium, is a commonly used anti-inflammatory agent which produces several harmful effects on the body. Apart from the healing effects of diclofenac, many side effects of its usage like gastropathy, degenerative and inflammatory liver alterations as well as functional renal changes are also occurring (Schwaiger et al., 2004).

iii. Cytotoxic evaluation using MTT assay on RAW 264.7 cells

To confirm that the inhibitory effects of the plant extract observed during *in vitro* studies was due to the anti-inflammatory potential of the plant extract and not due to its cytotoxic effect, MTT assay was carried out (**Fig. 35, Plate 14).** The study authenticated the assumption and from the assay, the LC_{50} value of the extract was found to be 110.58 ± 1.92 µg/ml and was considered for further analysis.

iv. Estimation of inflammatory mediators using ELISA

Inflammatory mediators, particularly pro-inflammatory mediators, are primarily responsible for inducing inflammatory reactions. COX-2 is a key mediator involved in the manufacture of prostaglandins, which cause inflammation and pain (Cao et al., 2010). In this study, ELISA was utilised to quantify the inflammatory mediator COX-2, in LPS induced RAW 264.7 cells. An amount of 110.58 µg/ml of the extract was treated with the RAW cells and the level of COX-2 was evaluated. It was found that the extract

reduced the level of COX-2 from 0.46 ± 0.02 to 0.36 ±0.05 U/ml of cell lysate (**Fig. 36).** The acquired result was in concurrence with the extract's dose-dependent inhibition in the previous assay. Among the various inflammatory pathways, NF-κB pathway plays an important role in modulating COX-2 and iNOS production (Park et al., 2015). Thus, decreased COX-2 enzyme levels correlate with decreased iNOS activity and cellular nitrite levels, confirming the suppression of NF-κB signaling by the plant extract.

The anti-inflammatory potential of the methanolic extract of the plant *O. trichocarpon* would be due to the synergistic effect of several anti-inflammatory compounds present within the plant. The major compounds of the plant, identified from GC-MS and HR LC-MS analyses, with anti-inflammatory properties were neophytadiene, loliolide, seselin, phytol, braylin, solanesol, lupenone, ursolic acid, lupenyl acetate, humulane-1,6-dienol, hesperidin, lespenefril, manniflavanone, mosloflavone, physalin O and chrysin.

Eventhough, some reports are available on the anti-inflammatory property of different species of *Ophiorrhiza*, the study on the selected species are not found. Hence the present study would be the first report on these particular plants of *Ophiorrhiza*. The study, finally suggest a fairly good anti-inflammatory potential of these plants.

C. Screening for cytotoxicity with *Allium cepa* L.

Cytotoxicity assays are commonly used methods to determine the toxic nature of a sample. The easy and fast nature of these experiments helps in the analysis of the cytotoxic nature of substances intended to be used in pharmaceutical or cosmetic industries (Monteiro et al., 2017). Though many plants are used as model organisms for cytotoxicicity studies, *Allium cepa* is a better system to detect the genotoxic effects of plant extracts (Leme & Marin-Morales, 2009).

In the present study, the cytotoxic potential of the selected species of *Ophiorrhiza* on *Allium cepa* root tip cells were analysed using different concentrations of the plant extracts (12.5, 25, 50 and 100 µg/ml) at different time periods (1, 12, 24 and 48 h). Roots treated with distilled water were taken as the negative control and that with hydrogen peroxide (100 µl/ml) as the positive control. Mitotic index and abnormality percentage were evaluated to analyse the mitodepressive effect of the plant extracts on the meristematic cells. The inversely proportional nature of both these endpoints, are regarded as the indicators of cytotoxicity potential. Lower mitotic index and higher abnormality percentage indicates cytotoxic nature of the samples. In the assay, mitotic index was found to exhibit a dose dependent reduction from lower to higher concentrations, whereas, abnormality percentage showed a dose dependent increase from lower to higher concentrations **(Figs 37–40).** However, an increase in the time period was found to have no effect on these parameters.

In *O. grandiflora*, the lowest mitotic index was 27.34 ± 2.38% at the 12 h treatment and the highest abnormality percentage was 84.43 ± 2.09% at the 1 h treatment. In *O. jacobii*, the lowest mitotic index was 35.97 ± 3.76% at the 48 h treatment and the highest abnormality percentage was 85.68 ± 2.56% at the 1 h treatment. In *O. pectinata*, the lowest mitotic index was 25.34 ± 6.63% at the 12 h treatment and the highest abnormality percentage was 86.32 ± 1.79% at the 48 h treatment. In *O. trichocarpon*, the lowest mitotic index was 33.13 ± 2.58% at the 48 h treatment and the highest abnormality percentage was 87.21 ± 1.19% at the 1 h treatment. These results show that all the plant extracts have cytotoxic properties and induce aberrations at different stages of the cell cycle, thereby inhibiting cell division.

According to Prajitha and Thoppil (2016), if the mitotic index falls below 22%, it indicates lethality and a value below 50% indicates sub-lethal

toxic effects. The present study thus indicated the sub-lethal cytotoxicity of the plant extracts. The mitodepressive effect of the extracts revealed the antiproliferative potential of the plant extracts on actively diving cells. Reduced mitotic index, demonstrates the presence of cytotoxic components in the extract that hinder mitotic activities and produce aberrant cells via genotoxic effects on the treated root tip cells (Anjana & Thoppil, 2013). According to Anjana and Thoppil (2013), the lower mitotic activity may be due to a blockade in DNA synthesis or the G2 phase of the cell cycle, which prevents the cells from entering the mitotic phase.

Along with the lowering of mitotic index, the plant extracts could induce several chromosomal aberrations in almost all the stages of mitotic cell division. The dose dependent increase of abnormality percentage in root tip cells on treating with the plant extracts also supported the ability of the extracts to influence the structure of chromosomes. The abnormalities observed at various stages of cell cycle are documented in **Plates 16–20.** The normal mitotic stages of cell division are depicted in **Plate 15.** All the plant extracts have produced some kind of abnormalities and the same aberrations were produced by different plant extracts at different concentrations and time. Both single as well as multiple aberrations were observed in the study.

Chromosomal aberrations can be both clastogenic and non-clastogenic. Non-clastogenic aberrations or aneugenic aberrations occur when the mitotic spindle formation is disrupted, whereas clastogenic aberrations occur when the genetic material is damaged.

In the present study, majority of the aberrations observed were multiple aberrations comprising both clastogenic and non-clastogenic abnormalities. Nuclear lesion was one of the prominent clastogenic aberrations observed in the interphase and the prophase stages. It was also observed along with other aberrations in different stages **[Plates 16 b, c, d, e, f, g, i, j, k, l; 17 f, l; 20 h].**

It probably arises due to the inhibition of DNA biosynthesis by the phytoconstituents present in the plant extracts (Akaneme & Iyioke, 2008). Single as well as double nuclear lesions were seen in the study. Chromatin and nuclear erosions were seen in all the stages, either alone **[Plates 16 h, 17 a, 18 (I) d, 20 d, g]** or in combination with other abnormalities **[Plates 17 e; 19 (II) a; 20 e]**. This might have occurred due to the dissolution of the nucleoproteins (Karaismailoglu et al., 2013). Changes in protoplasmic viscosity should have caused chromatin disintegration **[Plates 17 b, c; 20 h].** Irregular cell expansion results in enlarged cells called giant cells, which were observed in interphase, prophase and metaphase stages **[Plates 16 f; 17 i, l; 18 (II) f].** Formation of giant cells reveal the potential of plant extracts to block normal cell division. The presence of large cells demonstrates plant extracts' potential to disrupt the regular cell cycle, particularly during the S phase. Incomplete cytoplasmic division results in the formation of giant cells. Before dying, these cells expand in size, undertake DNA replication, and nuclear division, reaching an unnatural size (Prajitha & Thoppil, 2016). Nuclear budding was another abnormality identified in the prophase **(Plate 17 h, j).** The abnormality was actually the expansion of nucleus to bud structure that helps in the evacuation of unwanted DNA material and act as precursors of micronuclei (Dutra et al., 2010).

Sticky nature of chromosomes was a notable aberration in the study. Stickiness typically occurs when specific non-histone proteins involved in chromosomal organisation, chromatid separation, and segregation fail to function properly. In the current investigation, sticky chromosomes were found in all mitotic stages. **[Plates 17 d; 18 (I) c; 18 (II) a; 18 (III) d; 19 (II) k; 20 b].** Because of the sticky nature of chromosomes, chromosomal clustering and coagulation can occur (Pagliarini et al., 2000). Such anomalies were also identified in the current investigation **[Plates 18 (II) h, m; 19 (I) i, j]**. Sticky chromosomes can also result from chromosomal DNA

depolymerisation (Kumari et al., 2009). As these abnormalities are permanent, they can cause cell death and hence lower the mitotic index. The cytotoxic potential of the compounds in the plant extract would have caused the sticky nature of chromosomes. Formation of chromosomal bridges is also linked to the sticky nature of chromosomes **[Plates 19 (I) e, h; 19 (II) a, g].** The uneven chromosomal translocation or inversion might be the cause for bridge appearance (Gömürgen, 2005). Pulverised chromatin was another abnormality observed in the study **[Plate 20 i],** that might have occurred due to the premature chromosomal condensation (Onwuamah et al., 2014). All the above mentioned aberrations reveal that the plant extracts have clastogenic cytotoxic properties.

In addition to these abnormalities, several aneugenic aberrations were also found in the study. Most of the aneugenic aberrations were found in the metaphase and anaphase stages of the cell cycle. C-metaphase was one such aberration found after the extract treatment **[Plates 18 (I) a; 18 (II) b, i; 18 (III) m, o].** That was an evidence of scattering of condensed chromosomes caused by spindle fibre inactivation (Dwivedi & Kumar, 2015). The tilt in equatorial plate was another abnormality that usually occur due to the distortion of the spindle apparatus **[Plate 18 (I) i]**. Misorientation of the chromosomes happens due to the abnormal functioning of mitotic apparatus. Several aberrations like disturbed metaphase and anaphase **[Plates 18 (II) e; 19 (I) h]**, diagonal metaphase and anaphase **[Plates 18 (I) k; 18 (II) b, i; 18 (III) m, o; 19 (I) d]**, pole to pole metaphase **[Plates 18 (III) b, h, p]** *etc.,* are examples for misoriented chromosomes. Similarly, chromosomal laggards and vagrants were observed in the study **[Plates 19 (I) e, h, k]**. These were the result of aberrant spindle development and the failure of spindle fibres to draw the corresponding chromosomes to the polar areas of the cells (Obute et al., 2016; Kalcheva et al., 2009).

Microtubule-organising center (MTOC) allows spindle fibres to be spatially positioned during cell division (Marc, 1997). The plant extracts could interfere with spindle formation and a shift in MTOC was demonstrated **[Plate 19 (II) j]**. When spindle fibres get destructed, chromosomes clump together and assume a ball shape resulting in ball metaphase and ball anaphase **[Plates 18 (II) n; 18 (III) c; 19 (I) c]**. Similarly, chromosomes clump together and assume a star shape to form stellate chromosomes **[Plates 18 (II) c; 18 (III) e].** All the above mentioned abnormalities indicate that the phytoconstituents of the plant extracts have the potential to induce non-clastogenic effects on the treated root cells.

In addition to the above ones, binucleate cells, hyperploid cells, hypoploid cells, polyploid cells, chromosome granulation, chromosome fragmentation, chromosome shrinkage, chained chromosomes, cytostasis, tropokinesis, *etc*., were identified in the study. In the present study, majority of the abnormalities identified were multiple aberrations, in which both the clastogenic and non-clastogenic effect of the plant extract could be visible. Some of them were; binucleate cell showing double nuclear lesions **[Plate 16 d],** bizarre nucleus with double nuclear budding **[Plate 17 j],** chained metaphase in a hyperploid cell **[Plate 18 (I) h],** tilt in equatorial plate at metaphase showing disturbed chromosomes in a hyperploid cell **[Plate 18 (I) i],** chromosome shrinkage in a hypoploid cell **[Plate 18 (I) j],** stellate sticky partial C-metaphase **[Plate 18 (II) c],** disturbed metaphase showing vagrants **[Plate 18 (II) d],** giant hyperploid cell **[Plate 18 (II) f],** diagonal metaphase in a polyploid giant cell **[Plate 18 (II) g],** pole to pole sticky metaphase **[Plate 18 (III) b],** diagonal anaphase showing chromosome bridges and vagrants **[Plate 19 (I) e],** vagrants at anaphase showing chromosome erosion **[Plate 19 (II) h],** coagulated chromatin at telophase showing oblique cell plate **[Plate 20 a],** and diagonal cell plate formation showing nuclear lesion **[Plate 20 j].**

The apoptosis inducing potential of the plant extracts was revealed from the occurrence of cell death causing aberrations like chromatin and nuclear disintegration, chromosome condensation, chromosome and cytoplasmic shrinkage, nuclear fragmentation, nuclear blebs, *etc*. Thus, the study points to the cytotoxic potential of the phytocompounds present in the plant extract, which could induce clastogenic, non-clastogenic as well as cytoplasmic abnormalities that eventually cause cell death. Hence, cytotoxicity study using *A. cepa* helped in the initial screening of the plant extracts for their antiproliferative effect on actively dividing cells. Cell death happen either due to necrosis or apoptosis. In this investigation, many manifestations of apoptotic cell death were observed and further anticancer study was carried out on human breast cancer cell line MDA-MB 231.

D. Antiproliferative activity

The studies on *A. cepa* root tip cells revealed that the plant extracts of the selected species of *Ophiorrhiza* have cytotoxic properties and hence the antiproliferative potential of these plants was evaluated on MDA-MB 231 cell line. Among the various cancers seen in females, breast cancer causes the highest deaths. According to the global cancer statistics, more than half percent of breast cancer cases occur in developing nations. The reasons can be mutated genes, radiation therapies, food habits, alcoholism, obesity, birth control pills, abortions, *etc*. (Bray et al., 2018). Thus, for the current study, a breast cancer cell line was selected. MTT assay was employed to analyse the cytotoxic potential of the selected plants on both the normal L929 cells and the cancerous MDA-MB 231 cells. The most effective extract was then evaluated for apoptosis using FACS. After the detection of apoptosis, cell cycle analysis by flow cytometry followed by gene expression studies were carried out to explicate the anticancer mechanism regulated by the most effective extract.

i. Cytotoxicity determination by MTT assay

The use of antineoplastic medicines is predicated on their specific toxicity to malignant cells and also minimal toxicity to normal cells. So, a normal cell line and a cancer cell line were selected for the study. The antiproliferative ability of the extracts on cancer cells was compared with that of the normal cells. The most effective extract would be one that has a better antiproliferative effect on the tested cancer cell line while causing minimal damage to normal cells (Baskar et al., 2011).

MTT test is a widely used method for determining the cytotoxic potential of a drug or substance on cell lines at various doses (Bahuguna et al., 2017). MTT assay measures mitochondrial activity in metabolically active cells. The assay works on the basis that mitochondrial reductases in living cells convert the water-soluble yellow tetrazolium salt MTT into purple formazan crystals that can be solubilised and optically read. As a result, the rise or reduction in formazan concentration reflected in optical density represents a measure of the number of viable cells (van Meerloo et al., 2011). It is suggested that the assay primarily measures the activity of intracellular NAD(P) dependent oxidoreductases, mainly dehydrogenases (Surin et al., 2017).

In the present study, the cytotoxicity of the selected species of *Ophiorrhiza* was first analysed on the normal L929 cells. Different concentrations of the extracts (6.25, 12.5, 25, 50 and 100 µg/ml) of all the species were treated on the cells for 24 h and the LC_{50} value for each extract was determined. Similarly, the cytotoxicity activity of the selected species of *Ophiorrhiza* on the MDA-MB 231 cancer cells was also evaluated. The results **(Figs 41 & 42)** indicated that all the extracts exhibited a dose dependent cytotoxicity effect on the cells. The LC_{50} value obtained for each species is given in **Table 16.** Furthermore, from the microscopic observation

of the treated L929 cells and MDA-MB 231 cells (**Plates 21-28),** several apoptotic features like nuclear fragmentation, condensed nucleus, cell shrinkage, membrane blebbing, apoptotic bodies, budding and echinoid spikes were clearly visible in the MDA-MB 231 cells. These findings indicated that the cytotoxic nature of the extracts was manifested through inducing apoptosis.

On analysing the cell viability percentages and the LC_{50} values of all the extracts in both the cell lines, *O. trichocarpon* significantly inhibited the proliferation of MDA-MB 231 cancer cells (LC_{50} = 84.08 ± 2.74 µg/ml) when compared to other extracts and the extent of toxicity was minimum against the normal L929 cells (LC_{50} = 107.26 ± 2.55 µg/ml), which indicated the cancer cell-specific toxic effect of the extract. Thus, the MTT assay indicated the efficient cytotoxic potential of *O. trichocarpon.*

Gopalakrishnan and Shankar (2014), had evaluated the cytotoxicity of the crude methanolic extracts and the isolated camptothecin (CPT) from the multiple shoot cultures of *O. decumbens* in five cell lines and compared their activity with the commercially available standard CPT. The crude extract of the plant was more effective than the isolated CPT in terms of the IC_{50} values, which indicated that the crude extract possesses better anticancer potential than the isolated compounds. In the present study also, the extracts used were crude, but that might not affect the cytotoxic potential of the plants on the cell line. Viet Cuong et al. (2019) analysed the cytotoxic effects of the isolated secondary metabolites from the aerial parts of *O. baviensis* on different cancer cell lines and 3 compounds showed cytotoxic activity against all tested cell lines with IC_{50} values ranging from 37.89 to 79.6 µg/ml. In the present study, the LC_{50} value obtained for the most effective extract was also in that range (84.08 ± 2.74 µg/ml). On analysing the above mentioned studies and the present study results, the conducted MTT assay can be considered valid and

give the lead for choosing the methanolic extract of *O. trichocarpon* for further analysis.

ii. Detection of apoptosis using FACS

The apoptotic features exhibited in the MTT assay revealed that the cytotoxic potential of the plant extract on the cancer cell line was because of its apoptosis inducing capacity. To analyse the extend of cell death triggered by apoptosis, FACS was carried out with FITC-Annexin V flow cytometry. Apoptosis is an important mode of programmed cell death that results in the genetically determined destruction of cells. It is a regulated energy dependent mechanism that affects individual or clusters of cells (Elmore, 2007).

Cells respond to particular apoptotic signals by initiating multiple intracellular processes such as cleavage and degradation of certain cellular proteins, compaction and fragmentation of nuclear chromatin and loss of membrane integrity in the later stages of apoptosis. The externalisation of phosphatidylserine (PS) to the cell surface is another key physiological alteration that occurs during apoptosis.

During apoptosis, changes in the lipid membrane asymmetry occur. As a result, PS, which is a membrane component normally localised to the internal face of the cell membrane are translocated to the outer surface of the cell membrane. This is the process, which is exploited to detect apoptosis using flow cytometry.

The Annexin V & Dead Cell Reagent assay detects cells in various stages of apoptosis in a quick and easy manner. The assay employs two stains to consistently identify and distinguish between live, dead, and apoptotic cells. The two stains are recombinant annexin V conjugated to fluorescein (FITC Annexin V) and 7-aminoactinomycin D (7-AAD).

Annexin V is a phospholipid-binding protein that is calcium-dependent and has a high affinity for PS. Annexin V is linked to a green fluorescent dye (FITC) to facilitate visual detection and is hence known as FITC-Annexin V. 7-AAD is a membrane impermeant red fluorescent dye that can infiltrate cells with compromised membrane integrity and stain DNA molecules. As a result, it will not stain live or healthy cells, and will only stain cells with damaged membranes, such as late-stage apoptotic and dead cells.

Different apoptotic stages can be examined after staining a cell population with FITC annexin V and 7-AAD in the provided binding buffer. In live cells, PS is present on the inner surface of the membrane and the membrane is intact in nature. So Annexin V cannot bind. Furthermore, 7-AAD cannot enter intact living cells. As a result, live cells are not stained by either of the dyes. In the early apoptotic stage, molecules of PS are translocated to the outer surface of the cell membrane where Annexin V can readily bind them. So, the cells undergoing early apoptosis will give a green fluorescence. During the late apoptotic stage or nearly dying stage of cells, the FITC green fluorescence is still obtained as the PS is still on the outer surface of the membrane. Also, at this stage, the cell membrane gets damaged which allows 7-AAD to penetrate into the cell. Hence, these cells will give red and green fluorescence. In dead cells, the entire membrane is damaged and thus 7-AAD, stain the cells, giving only red fluorescence. Thus, live cells show little or no fluorescence, early apoptotic cells show green fluorescence, late apoptotic and dying cells show red and green fluorescence and completely dead cells show red fluorescence. These four populations of cells can easily be distinguished using a flow cytometer.

The flow cytometry results represent the cell population divided into four quadrants. The lower left quadrant represents cell population that does not give signal for either of the two dyes and show no fluorescence and hence

indicating live cell population. The lower right quadrant represents the cell population that gives signal for only Annexin V and show green fluorescence and hence indicating early apoptotic cell population. The upper right quadrant represents the cell population that gives signal for both the dyes and show red and green fluorescence and hence indicating late apoptotic/dead cell population. The upper left quadrant represents cell population that give signal for 7-AAD and show red fluorescence and hence indicating completely dead cells due to apoptosis.

In the present study, MDA-MB-231 cells treated with 84.08 µg/ml of the plant extract and incubated for 24 hours were considered for the Annexin V & Dead Cell Reagent assay. **Fig. 43** represented the flow cytometry results, wherein each dot represented a single cell. The percentage of live cells decreased from 93.85% in the control **(Fig. 43 B),** to 54.72% in the treated cells **(Fig. 43 D).** It is clear that the plant extract could induce 10% of the cells to the late apoptotic stage and 35.28% of cells into the final apoptotic stage, which, in the control is 5% and 0.35% respectively. Thus, the assay confirmed that the methanolic extract of *O. trichocarpon* could trigger apoptotic pathways.

iii. Cell cycle analysis using flow cytometry

Anticancer compounds inhibit cell division by inducing cell cycle arrest (Fan et al., 2022). The cell cycle is a continuous pathway mediated by several regulators, primarily cyclins and cyclin-dependent kinases (CDKs). As a result, anticancer treatment focuses mostly on these compounds (Meeran & Katiyar, 2008). Cancer cells, unlike normal cells, have the ability to bypass cell cycle checkpoints; thus, chemotherapeutic medicines cause DNA damage, activating cell cycle checkpoints (Choumessi et al., 2012).

Cell cycle analysis aids in understanding the distribution of cells in various phases, which is really the measurement of the DNA content present at various stages of the cell cycle. The amount of DNA in the pre-replicative phase (G0/G1), replicative phase (S), and post-replicative phase (G2/M) indicates if the cell cycle is normal or has ceased. A powerful anticancer drug can cause cell cycle arrest at a specific stage of the cycle, lowering DNA content in the subsequent phase.

Flow cytometry was used in this study to determine the effect of the methanolic extract of *O. trichocarpon* on the cell cycle. The treated cells are first permeabilised in the experiment, followed by DNA staining with a fluorescent dye such as propidium iodide (PI). Although PI is a membrane impermeant dye, it can enter cells with compromised membrane integrity and stain DNA. Permeabilisation of cells is thus a key phase in cell cycle study. To boost the specificity of DNA labelling in each phase, PI discriminates cells at different stages of the cell cycle based on differential DNA content in the presence of RNAase. The DNA content at different phases is plotted as frequency histogram that provides information regarding the percentage of cells in different phases of the cell cycle.

From the result of the DNA content profile **(Fig. 44)**, a significant variation in the DNA content at different stages of cell cycle for the control versus the treated cells was observed. As shown in the histogram, the cell count in the G0/G1 population in treated cells is high (66.4%) when compared to the control (53.2%). The population in S phase declined from 24.3% in the control to 18.9% in treated cells. In the subsequent G2/M phase also, the cell population has remarkably reduced to 11.1%. This indicates that the treated cells have much lesser DNA content than the cells considered as control, as the cell cycle progress from the G0/G1 phase to the S phase. The relatively increased DNA concentration in the G0/G1 phase represents cell retention at

that stage (Reshmi, 2022). The analysis confirms that the extract has cell death inducing property via apoptosis.

On considering the DNA index of the MDA-MB 231 cells treated with the methanolic extract of *O. trichocarpon* **(Fig. 44 B),** the high cell population in G0/G1 phase is followed by highly reduced cell content in the S phase and the subsequent reduction in the G2/M phase. The data indicates that the extract has the ability to sustain its responsiveness all over the cell cycle (Wang et al., 2000). From the G0/G1 phase, there is a sharp decline in the DNA content during the S phase indicating that the cell cycle arrest occurs at the G0/G1 phase. Further reduction of DNA content in the G2/M phase shows the progressive responsiveness of the extract in inducing cell cycle arrest. DNA damage caused by anticancer compounds can activate cell cycle checkpoints, halting cell cycle progression and potentially triggering cell death (Zhou et al., 2018). Thus, the cell cycle analysis by flow cytometry exhibited the potential of the extract of *O. trichocarpon* to induce apoptosis by blocking the transition from G0/G1 phase to the S phase in the MDA-MB 231 cancer cells.

iv. Gene expression studies

Apoptosis is a highly regulated process which involves either the upregulation of apoptotic genes or the downregulation of the non-apoptotic genes. The methanolic extract of *O. trichocarpon* significantly inhibited the proliferation of MDA-MB 231 cancer cell lines and the extent of toxicity was minimum against the normal L929 cells, indicating the cancer cell-specific toxic effect of the extract. Furthermore, the cell death induced by the plant extract of *O. trichocarpon* on the cell lines was found to be due to apoptosis. The cell cycle analysis by flow cytometry exhibited the potential of the extract to induce apoptosis by blocking the transition from G0/G1 phase to the S phase in the MDA-MB 231 cancer cells. So, to analyse the potential of the

extract to regulate the apoptosis related genes, AKT and ERK2, was determined using RT-qPCR followed by gel electrophoresis using GAPDH as the house keeping gene. Using RT-qPCR, the change in the expression of the selected genes when compared to the control was analysed.

Housekeeping genes are those that are expressed consistently across tissues. They code for proteins that are constantly required by the cell; thus, they are essential to a cell and must be present at all times. They are conserved across species and perform cellular maintenance. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most commonly used housekeeping genes in gene expression data comparisons. It is a key regulatory enzyme that catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate during glycolysis. Since its expression is consistent across time and experimental manipulations, it is commonly used for quantitative RT-PCR analysis.

Protein kinase B, often known as AKT, is at the crossroads of cell death and survival, as are many other protein kinases. The gene is associated with a variety of interrelated cell signalling processes that are involved in cell metabolism, growth and division, apoptosis suppression and angiogenesis (Nitulescu et al., 2018). However, AKT expression in the nucleus has been shown to induce apoptosis by causing unscheduled activation of the cell cycle-regulatory kinase, Cdk2, either by direct phosphorylation or by inducing p27 degradation (Los et al., 2009).

ERK, which is extracellular signal-regulated kinase, is a member of mitogen-activated protein kinases (MAPKs) family. They include ERK1/2, c-Jun N-terminal kinases (JNK1/2/3) and the p38 MAP kinases (p38 α, β, γ, δ). It was previously been observed that ERK activation induce either intrinsic or extrinsic apoptotic pathways **(Fig. 2)** by causing mitochondrial cytochrome

C release or caspase-8 activation, long-term cell cycle arrest or autophagic vacuolisation (Cagnol et al., 2010).

In the present study, MDA-MB 231 cells treated with 84.08 µg/ml of *O. trichocarpon* extract and incubated for 24 h were considered, by maintaining untreated cells as the control**.** From the results (**Fig. 45),** a significant upregulation in the expression of both the genes were observed. The expression fold change value for the gene AKT was 2.01, while the other gene ERK2 expressed a fold change value of 1.85. The results indicated that the extract of *O. trichocarpon* upregulated the apoptotic genes almost two times than that of the control. The gel image analysis of the genes also confirmed these findings **(Fig. 46).** The fluorescence emitted from the sample treated wells was relatively higher than the control which indicated that the methanolic extract of *O. trichocarpon* has increased the expression of the mRNA of the selected genes. Thus, the study clearly shows that the methanolic extract of *O. trichocarpon* has the potential to upregulate the genes AKT and ERK2.

Antiproliferative efficacy of the selected species of *Ophiorrhiza* on the MDA-MB 231 cells have not been previously reported. The available report of cell cycle analysis and gene expression studies on different cell lines was on *O. pumila.* Liu et al. (2020) reported the anti-proliferative and anti-migratory activities of the ethanol extract of *O. pumila* on HepG2 and SMMC-7721 cells. The plant extract caused cell cycle arrest at the G2/M phase and boosted the expression of pro-apoptotic Bax and cleaved Caspase-3 while decreasing the expression of the anti-apoptotic protein Bcl-2. The extract was reported to reduce the phosphorylation of P65. Fan et al. (2022) reported anti-lymphoma effects of *O. pumila* extract on murine lymphoma cell line (A20). The extract caused cell cycle arrest at the S phase of cell division. The extract could lower cyclin A2 expression, a crucial mediator of

the S phase. The extract also increased the expression of apoptosis proteins, Bax and cleaved caspase-3.

Thus, the previous reports reveal that the cell cycle arrest and the expression of genes at specific apoptotic pathways are dependent on the plant extract and the cell line chosen. The extract of *O. trichocarpon* contained several compounds with anticancer activities; such as phytol, squalene, solanesol, campesterol, lupenone, ursolic acid, humulane-1,6-dienol, pumiloside, stachyoside, limonin, chrysin and euphornin. So, the activity of *O. trichocarpon* on MDA-MB cells by inducing cell cycle arrest at the G0/G1 phase followed by the upregulation of apoptotic related genes can be ascribed to the efficacy of these potential phytocompounds.

SUMMARY AND CONCLUSIONS

Plants and plant products form the most precious gift of nature to mankind. Humans have relied upon plants for food and medicines since time immemorial and the majority of the global population depend on these natural resources for their survival. Since plants are the reservoirs of pharmacologically active compounds, they are widely used in the traditional folklore medical systems. Furthermore, the plethora of bioactive compounds makes them an inevitable part of the development of modern drugs. Though there are records of the medicinal properties of many plants in ancient literature, scientific validation is lacking. The unique therapeutic action of plant-based compounds, which bypass the deleterious effects of synthetic drugs, has gained the interest of several researchers to explore the therapeutic properties of ethnobotanically useful plants. However, a great number of terrestrial flora still remains unexplored. The proper identification, validation of the therapeutic potential and understanding of the conservation status of the plants is essential for the protection, propagation and preservation of these biological resources. It is on this background, the present study was carried out.

Ophiorrhiza L., is a species-rich genus, belonging to the family Rubiaceae, whose members have been used in traditional and folk medicine as snake bite antitussive, analgesic and for the treatment of various other ailments. Though *Ophiorrhiza* is a species-rich genus, very few members are scientifically investigated and many others are still unexplored. The present study focuses on the cytogenetical, phytochemical and bioactivity analyses of four selected species of *Ophiorrhiza* (*O. grandiflora, O. jacobii, O. pectinata* and *O. trichocarpon*).

I. Karyomorphological studies

Cytogenetical studies on the selected species of *Ophiorrhiza* were carried out using mitotic squash preparation of the root tip cells. The study revealed the karyological identity and evolutionary position of the species. The karyomorphological analysis of the species disclosed the somatic chromosome number, ploidy level and many other karyomorphometric parameters which helped to establish their cytogenetical status. Three species; *Ophiorrhiza grandiflora, O. pectinata* and *O. trichocarpon* exhibited the same number of somatic chromosomes (2n = 22) all with 11 pairs of chromosomes. *O. jacobii* showed a chromosome number of 2n = 24 with 12 pairs of chromosomes. The karyomorphological analysis of all the selected species revealed that these plants possess small sized chromosomes within a range of 1.10 μm (*O. pectinata, O. trichocarpon*) to 2.15 μm (*O. jacobii*). The ploidy level of chromosomes in all the species was found to be in the diploid condition. The number of chromosomes with secondary constrictions was found to be in equity. The first two pairs of chromosomes in all the species exhibited secondary constrictions. The detailed karyomorphometric data of each species was made using the respective karyotypes. From the data, it was found that the majority of the chromosomes had nearly median nature of chromosomes. In *O. jacobii* and *O. pectinata*, all the chromosomes exhibited nearly median type of primary constriction. However, in both *O. grandiflora* and *O. trichocarpon,* 2 pairs of chromosomes evinced a nearly submedian nature of primary constriction and the rest had a nearly median type of primary constriction. Thus, the karyotype of all the studied species disclosed the predominance of nearly median type of primary constriction indicating the symmetric nature of chromosomes. The karyogram and idiogram of the chromosome complements of all the species were prepared according to the decreasing order of the chromosome size.

Various asymmetry indices were also calculated to understand the symmetry or asymmetry of chromosomes in each species. DI (disparity index), VC (variation coefficient), TF% (total forma percentage), As K% (karyotype asymmetry index), Syi (symmetry index) and Rec indices (resemblance between chromosomes), A1 (intrachromosomal asymmetry index), A2 (interchromosomal asymmetry index) and A (degree of asymmetry of karyotypes) indices revealed that the chromosomes are symmetric and hence disclose a primitive karyotype in all the selected species. The scatter plot representing the Rec versus Syi indices and A1 versus A2 indices showed that all the selected species are less evolved concerning chromosomal evolutionary processes. However, *Ophiorrhiza trichocarpon* can be considered more advanced than the other three species. The present investigation revealed the primitive status of the selected species of *Ophiorrhiza* in terms of their karyotype evolution. Thus, the complete karyomorphometric features of the four species add to the chromosomal database for future evolutionary investigations.

II. Phytochemical profiling

The phytoconstituents present in the selected species of *Ophiorrhiza* were determined by qualitative phytochemical analyses, quantitative estimation of major phytoconstituents, GC-MS and HR LC-MS analyses.

For the qualitative phytochemical analyses, three extracts of each plant; *viz*., hexane extract, methanolic extract and aqueous extract were considered for the tests. The tests revealed the presence of bioactive secondary metabolites like phenols, flavonoids, alkaloids, phytosterols, terpenoids, tannins, saponins, phlobatannins, anthraquinones, coumarins, glycosides and fixed oils and fats. As the maximum number of compounds was detected in the methanolic extract of each plant, further studies were carried out on the methanolic extract of the four selected species. Quantitative

estimation of the major class of compounds like phenols, flavonoids, terpenoids and alkaloids showed considerable amounts of these phytoconstituents in all the plants. The total phenolic content of the methanolic extracts of the plants was calculated from the calibration curve of gallic acid and the highest quantity of phenolic content was observed in *O. jacobii.* However, *O. grandiflora* and *O. pectinata* also revealed a good and almost the same quantity of phenols. Using quercetin, the total flavonoid content was determined. Flavonoid content was comparatively lower in all the species and *O. trichocarpon* exhibited the highest content of flavonoids. Amount of terpenoids present in the methanolic extracts of the selected plants was determined using the calibration curve of linalool and *O. pectinata* showed the highest terpenoid content. Alkaloids were present in comparatively smaller quantities in all the extracts of the plants and were determined using the calibration curve of caffeine. Out of the four species, the methanolic extract of *O. trichocarpon* showed the highest alkaloid content.

The volatile phytocompounds present in the methanolic extract of the selected species of *Ophiorrhiza* were identified by the technique of GC-MS analysis. The four species of *Ophiorrhiza*, revealed a total of 81 compounds. Among these, *O. trichocarpon* exhibited a higher number of compounds (34 compounds), followed by *O. pectinata* (33 compounds), *O. grandiflora* and *O. jacobii* (27 each). Many phytoconstituents like neophytadiene and squalene (terpenes), hexadecanoic acid, linoleic acid, linoleic acid methyl ester (fatty acids and ester), phytol (diterpene alcohol) and γ-sitosterol (phytosterols) were found to be common in all the plant extracts. The non-volatile components in the methanolic extract of the selected *Ophiorrhiza* species were recognised by HR LC-MS analysis. The four species of *Ophiorrhiza*, revealed a total of 108 compounds and *O. trichocarpon* exhibited a higher number of compounds (47 compounds), followed by *O. jacobii, O. pectinata* and *O. grandiflora* (41, 30 and 24 compounds

respectively). In the analysis, it was observed that there was no single compound common to all the species. However, compounds specific to each species were high. Among the compounds identified the important ones were acarbose, 9,12,13-trihydroxy-10-octadecenoic acid, isoreserpine, sedoheptulose, loganic acid, monotropein, hesperidin, apiin, anatabine, 3-α(S)-strictosidine, lappaol B, caribine, swietenine, euphornin, amarogentin, rutin, chrysin, rubiadin, retinoid, naringenin, lupanine, pumiloside, stachyoside A, N-caffeoyltryptophan, physalin O, limonin, quinic acids, lespenefril, manniflavanone, tejedine, piperolactam-C, mosloflavone and 1-hydroxy-4-methylanthraquinone. The presence of these active metabolites revealed the potential of the selected plants to be screened for further bioactivity studies.

III. Bioactivity screening

Free radical scavenging activity

The potential of the selected species of *Ophiorrhiza* to scavenge free radicals was analysed by DPPH, hydroxyl, nitric oxide and superoxide radical scavenging assays. The different assays provided results which exposed that all the plant extracts have an increasing inhibitory potential on the selected free radicals. In DPPH radical scavenging assay, *O. trichocarpon* was the better inhibitor. *O. jacobii* was good at inhibiting hydroxyl and nitric oxide radical quenching. But, *O. pectinata* inhibited more superoxide radicals. *O. grandiflora* performed poorly in all the antioxidant assays. The results suggested that a single species was not found to be a predominant antioxidant candidate, but each species had specific free radical scavenging properties on specific ROS. However, in all the assays, the standards used were having better IC_{50} values than the plant extracts. Thus, The IC_{50} values revealed that the extracts of *Ophiorrhiza* species had moderate antioxidant activity.

Anti-inflammatory activity

The anti-inflammatory potential of the selected species of *Ophiorrhiza* was first analysed by *in vitro* biochemical assays and the most effective plant extract was selected for further analysis of its activity on LPS-induced RAW 264.7 macrophage cell line. For the biochemical assays, *viz*., inhibition of protein denaturation and proteinase inhibitory activity, different concentrations (62.5, 125, 250 and 500 µg/ml) of the methanolic extracts of all the selected species were considered. From both the *in vitro* assays, *O. trichocarpon* was found to exhibit better anti-inflammatory potential with the lowest IC_{50} values among the selected species of *Ophiorrhiza*. So, for further analysis, *O. trichocarpon* was selected.

The effect of the methanolic extract of *O. trichocarpon* on the activity of inflammatory enzymes like cyclooxygenase, lipoxygenase, myeloperoxidase and inducible nitric oxide synthase and also on the cellular nitrite level was analysed on LPS induced RAW 264.7 macrophage cell line. The analyses were done using three different concentrations of the sample (25, 50 and 100 µg/ml). Diclofenac sodium was the standard used in all the assays. The plant extract showed noteworthy results in the cyclooxygenase, myeloperoxidase and cellular nitrite level assays. But the results were found to be moderate in lipoxygenase and inducible nitric oxide synthase assay.

MTT assay was carried out to evaluate the cytotoxic nature of the plant extract using various concentrations of the extract *viz.,* 6.25, 12.5, 25, 50 and 100 µg/ml. From the assay, the LC_{50} value of the extract was found to be 110.58 ± 1.92 µg/ml and this LC_{50} value was considered for the estimation of the inflammatory mediator, COX-2, using ELISA. It was found that the extract could significantly reduce the level of COX-2. From the results of all the anti-inflammatory assays done, the plant extract of *O. trichocarpon* can be considered a strong candidate for advanced studies.

Cytotoxicity studies using *Allium cepa*

The cytotoxic potential of the selected species of *Ophiorrhiza* on *Allium cepa* root tip cells was analysed using different concentrations of the plant extracts (12.5, 25, 50 and 100 µg/ml) at different time periods (1, 12, 24 and 48 h). Roots treated with distilled water were taken as the negative control and with hydrogen peroxide (100 µl/ml) as the positive control. All the plant extracts exhibited dose dependent cytotoxicity on the treated root tip cells. As concentration increased, a reduction in mitotic index and a rise in the abnormality percentage was observed, which indicated the mitodepressive potential of the extracts. However, time duration was found to have no significant role in determining the cytotoxic nature of the extracts. The microscopic observations of the treated root cells revealed several clastogenic and non-clastogenic aberrations at each mitotic stage. The apoptosis-inducing potential of the plant extracts was revealed from the occurrence of cell death causing aberrations like chromatin and nuclear disintegration, chromosome condensation, chromosome and cytoplasmic shrinkage, nuclear fragmentation, nuclear blebs, *etc*. Hence, a cytotoxicity study using *A. cepa* helped in the initial screening of the plant extracts, which revealed their cytotoxicity effect on actively dividing cells, which can be taken as a lead point for the further analysis of the extracts.

Antiproliferative activity

To evaluate the antiproliferative potential of the selected plants, MDA-MB 231 (human breast cancer) cell line was chosen. MTT assay was first carried out on the normal L929 cells to determine the cytotoxicity of the selected species of *Ophiorrhiza*. Different concentrations of the extracts (6.25, 12.5, 25, 50 and 100 µg/ml) of all the species were treated on the cells for 24 h and the LC_{50} value for each extract was determined. The same assay was followed on the MDA-MB 231cells. The microscopic observation of the

treated cancer cells showcased many apoptotic signals such as nuclear fragmentation, condensed nucleus, cell shrinkage, membrane blebbing, apoptotic bodies, budding and echinoid spikes. These findings indicated that the cytotoxic nature of the extracts was manifested through inducing apoptosis. On analysing the cell viability percentages and the LC_{50} values of all the extracts in both the cell lines, *O. trichocarpon* significantly inhibited the proliferation of MDA-MB 231 cancer cells (LC_{50} = 84.08 ± 2.74 µg/ml) when compared to other extracts and the extent of toxicity was minimum against the normal L929 cells (LC_{50} = 107.26 ± 2.55 µg/ml), which indicated cancer cell-specific toxic effect of the extract. Thus, the extract of *O. trichocarpon* was chosen for further procedures. Detection of apoptosis by FACS confirmed that the methanolic extract of *O. trichocarpon* could trigger apoptotic pathways. Further, flow cytometry was used to determine the effect of the methanolic extract of *O. trichocarpon* on the cell cycle. From the DNA content profile, a significant variation in the DNA content at different stages of the cell cycle was observed. From the G0/G1 phase, there was a sharp decline in the DNA content during the S phase indicating that the cell cycle arrest occurred at the G0/G1 phase. Following the cell cycle analysis, gene expression studies using two apoptosis-related genes, AKT and ERK2, were carried out with the GAPDH as the housekeeping gene. A significant upregulation in the expression of both genes was observed. The results indicated that the extract of *O. trichocarpon* upregulated the apoptotic genes almost two times that of the control. The gel image analysis of the genes also confirmed these findings.

In conclusion, the karyomorphological studies revealed the symmetric and primitive nature of the chromosomes of the selected species. The phytochemical profiling opened up the presence of numerous bioactive secondary metabolites, whose synergistic activity can produce significant

therapeutic effects. The identification of highly active phytocompounds was a lead to analyse different bioactivities of the plants. In the free radical scavenging assays, all the extracts exhibited moderate activity. In the anti-inflammatory assays, it was *O. trichocarpon* that exhibited commendable activity. In the antiproliferative studies also, *O. trichocarpon* was found to be the most effective plant among the selected species.

Deliverables:

- The cytogenetical studies on the selected plants of *Ophiorrhiza* revealed the actual chromosome number and other karyotypic details of the plants for the first time
- Karyomorphometric data exposed the primitive evolutionary status of all four species studied
- Profiling of the active phytocompounds of the plants by different techniques elucidated that these plants are reservoirs of numerous bioactive secondary metabolites
- Free radical scavenging potential was found to be worth mentioning in the selected species of *Ophiorrhiza*
- The anti-inflammatory potential of the plants was determined and *O. trichocarpon* was found to be a promising drug candidate
- Cytotoxicity screening with *Allium cepa* demonstrated the mitodepressive effect of the plant extracts
- The antiproliferative efficacy of *O. trichocarpon* on MDA-MB 231 breast cancer cells was analysed through MTT assay, FACS, cell cycle analysis and gene expression methods

RECOMMENDATIONS

Cytogenetical studies on *Ophiorrhiza* species are very few, especially in the south-Indian members. An extended study on the cytotaxonomical and cytogenetical features can be proposed as a future perspective. *In vivo* and clinical investigations on animal models can be undertaken to attest to the exhibited bioactivities by the selected plants. The identification of the specific compounds responsible for the exerted bioactivities, followed by their isolation and characterisation is recommended. In many reports of antiproliferative studies, the crude extract could induce better activity than the isolated compounds. Comparative research focusing on this aspect would also be considered.

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APPENDICES

Modified Carnoy's fluid **Appendix 1**

Acetic acid	:	10 ml
Ethanol	:	30 ml

Aceto-orcein stain **Appendix 2**

Orcein	:	2 g
45% acetic acid	:	100 ml

The stain is prepared using a reflex condenser and is filtered to remove the undissolved stain.

Wagner's reagent **Appendix 3**

Iodine	:	1.27 g
KI	:	2 g

Dissolve the above chemicals in 5 ml H_2SO_4 and make up to 100 ml using double distilled water.

Phosphate buffer (pH 4.7) **Appendix 4**

Sodium phosphate	:	2 M
Citric acid	:	0.2 M

pH of 2 M sodium phosphate is adjusted to 4.7 with 0.2 M citric acid.

BCG Solution **Appendix 5**

BCG	:	69.8 mg
NaOH	:	3 ml (2 N)
Distilled water	:	5 ml

Heat the BCG with NaOH and distilled water until completely dissolved and then dilute the solution to 1000 ml.

Griess reagent **Appendix 6**

Naphthylethylenediamine HCl	:	0.1% in distilled water
Sulfanilamide	:	1% in 5% H_3PO_4

Both the solutions are mixed in 1:1 ratio

Phosphate buffer saline **Appendix 7**

NaCl	:	8 g
KCl	:	0.2 g
Na_2HPO_4	:	1.44 g
KH_2PO_4		0.24 g
Distilled water		1 litre

The above ingredients are mixed in 800 ml of distilled water and pH was adjusted to 7.4 with HCl. The total volume was then made up to 1 litre with distilled water. The solution was sterilized by autoclaving (20 min, 121ºC) and stored at room temperature.

Tris-HCl buffer **Appendix 8**

Tris base	:	121.1 g
HCl	:	70 ml

The tris base is dissolved in 800 ml distilled water and 70 ml concentrated HCl is added. Then the volume is adjusted to 1 litre with distilled water.

DMEM (Dulbecco's modified Eagle's medium) **Appendix 9**

Sodium bicarbonate	:	1.85 g
HEPES	:	2.95 g
DMEM powder	:	0.67 g
Distilled water	:	1 litre

Vaccum sterilized and stored at 4ºC.

Potassium phosphate buffer **Appendix 10**

A) KH_2PO_4	:	916 mg
B) Na_2HPO_4		950 mg

Dissolve both A) and B) in 100 ml double distilled water separately. Take 8.5 ml of A) and add 91.5 ml of B) and adjust the pH to 6.

HEPES buffer **Appendix 11**

NaCl	:	6.72 g
$CaCl_2$	:	133 mg
$MgCl_2$	:	114 mg

K_2HPO_4	:	418 mg
HEPES	:	4.77 g
H_2O	:	1 litre

Adjust the pH to 7.4 with HCl or NaOH. HEPES buffer can be stored refrigerated for several weeks.

Blocking buffer **Appendix 12**

Gelatin	:	0.2%
TWEEN 20	:	0.005%

The ingredients are freshly prepared in PBS

Acetocarmine stain **Appendix 13**

Carmine	:	2 g
45% Acetic acid	:	100 ml

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

TE buffer **Appendix 14**

Tris HCl	:	10 mM, pH 8
EDTA	:	0.1 mM, pH 8

RESEARCH PUBLICATIONS AND PRESENTATIONS

Research publications from the Ph. D. work

1. **Preethamol, S. N.,** & Thoppil, J. E. (2022). Antioxidant potential and chemical composition of *Ophiorrhiza jacobii*: A promising medicinal plant endemic to Western Ghats. *Indian Journal of Pharmaceutical Sciences*, *84*(2), 407-414. OMICS International, Medknow publications **(IF – 0.97).**

2. **Preethamol, S. N.,** & Thoppil, J. E. (2020). Phenolic content, flavonoid content and antioxidant potential of whole plant extract of *Ophiorrhiza pectinata* Arn. *Indian Journal of Pharmaceutical Sciences*, *82*(4), 712-718. OMICS International, Medknow publications **(IF - 0.97).**

3. **Preethamol, S. N.,** & Thoppil, J. E. (2019). Evaluation of cytotoxicity and apoptotic potential of *Ophiorrhiza pectinata* Arn. - A potent anticancer agent. *Asian Journal of Pharmaceutical and Clinical Research*, *12*(8), 97-102. Innovare Academic Science Publishers.

Research publications co-authored

1. Hareesh, V. S., **Preethamol, S. N.,** Sabu, M., & Thoppil, J. E. (2020). Revisiting the taxonomy of *Ophiorrhiza codyensis* and lectotypification of *O. pykarensis* (Rubiaceae), two endemic taxa of the Western Ghats. *Phytotaxa*, *452*(2), 173-179. Magnolia press **(IF- 1.2).**

Paper presentations

1. **Preetha Mol S. N.,** & Thoppil, J. E. (2018). 'Evaluation of phytochemical constituents in *Ophiorrhiza pectinata* Arn.', in the International Conference on Phytomedicine held at the Department of Botany, Bharathiyar University, Coimbatore (Oral presentation).

2. **Preetha Mol S. N.,** & Thoppil, J. E. (2019). 'Phytochemical profiling of *Ophiorrhiza brunonis* Wight & Arn. (Rubiaceae)', in the MESMAC International Conference held at MES College, Mampad, Malappuram (Oral presentation).

3. **Preetha Mol S. N.,** & Thoppil, J. E. (2019). 'Cytotoxic and apoptotic effect of *Ophiorrhiza pectinata* Arn. (Rubiaceae)', in the International Conference on Recent Innovations in Biosustainability and Environmental

Research (RIBER) held at the Department of Zoology, Annamalai University, Tamil Nadu (Oral presentation).

4. **Preetha Mol S. N.,** & Thoppil, J. E. (2020). 'Evaluation of antioxidant potential of *Ophiorrhiza pectinata* Arn.', in the National Conference on Current Trends and Advances in Biological Sciences (CTAB) held at the Post Graduate Department of Botany and Biotechnology, Bishop Moore College, Mavelikkara, Kerala (Oral presentation).