

**Bioactivity screening and phytochemical
analysis of some selected species of
Gomphostemma Wall. ex Benth.
(Lamiaceae)**

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

DOCTOR OF PHILOSOPHY IN BOTANY

By

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Sajitha Menon K.

ABBREVIATIONS

µg/mL	:	Microgram/milliliter
µM	:	Micromolar
AFM	:	Atomic Force Microscopy
AgNPs	:	Silver nanoparticles
BSA	:	Bovine serum albumin
CA	:	Chromosome aberrations
COX-2	:	Cyclooxygenase-2
DMEM	:	Dulbecco's Modified Eagle's Medium
DMRT	:	Duncan's Multiple Range Test
DMSO	:	Dimethyl sulphoxide
DPPH	:	1, 1-diphenyl-2-picrylhydrazyl
DW	:	Dry weight
EDTA	:	Ethylene diamine tetra acetic acid
EDX	:	Energy dispersive X-ray analysis
ELISA	:	Enzyme linked immunosorbent assay
ESI	:	Electrospray ionization
FeCl ₃	:	Ferric chloride
FTIR	:	Fourier-transform infrared spectroscopy
GAE	:	Gallic acid equivalent
GC/MS	:	Gas Chromatography/Mass Spectrometry
GSH	:	Glutathione
h	:	Hour
H ₂ O ₂	:	Hydrogen peroxide
H ₂ SO ₄	:	Sulphuric acid
HCl	:	Hydrochloric acid
HEPES	:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	:	High Performance Liquid Chromatography
HR-LC/MS	:	High Resolution-Liquid Chromatography/ Mass Spectrometry
HRP	:	Horseradish peroxidase

IC ₅₀	:	Inhibition concentration 50%
IL	:	Interleukins
iNOS	:	Inducible nitric oxide synthase
LOX	:	Lipoxygenase
LPS	:	Lipopolysaccharide
M	:	Molar
<i>m/z</i>	:	mass-to-charge ratio
MCF-7	:	Michigan Cancer Foundation-7
mg	:	Milligram
MI	:	Mitotic index
min	:	Minute
mM	:	Millimolar
MPO	:	Myeloperoxidase
MTT	:	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
N	:	Normal
Na ₂ CO ₃	:	Sodium carbonate
NaOH	:	Sodium hydroxide
NBT	:	Nitro blue tetrazolium
NCCS	:	National Centre for Cell Science
NF-κB	:	Nuclear factor kappa light chain enhancer of activated B cells
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
PCR	:	Polymerase chain reaction
PG	:	Prostaglandins
QE	:	Quercetin equivalent
Q-TOF	:	Quadrupole time-of-flight

RAW 264.7	:	Mouse leukaemic monocyte macrophage cell line
RNS	:	Reactive nitrogen species
ROS	:	Reactive oxygen species
RT	:	Retention time
SDS	:	Sodium dodecyl sulphate
SE	:	Standard error
SEM	:	Scanning electron microscopy
SPR	:	Surface plasmon resonance
TAE	:	Tris acetate EDTA
TBA	:	Thio barbituric acid
TCA	:	Trichloroacetic acid
TEM	:	Transmission electron microscopy
TNF	:	Tumour necrosis factor
v/v	:	volume per volume
w/v	:	weight per volume
WHO	:	World Health Organization

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INTRODUCTION

Plants are nature's treasure chest and a gift to mankind with a plethora of pharmacologically active compounds. The use of plants and plant derived compounds in managing infectious diseases and disorders is a time-honoured tradition. The current spate of increasing life threatening diseases affecting human population across the world has compelled the researchers to develop novel and effective therapeutic strategies. Many existing therapies using synthetic drugs are flawed by their profound and persistent side effects. Thus, natural compounds are preferred as an alternative to conventional synthetic drugs. However, there exist a paradox in the use of plants and plant derived compounds as medication. Complexity of phytocomponents, lack of documentation, incomplete knowledge and insufficient verification are some of the reasons for this contradiction. Hence, the past few decades have witnessed profound research focussed on the validation of efficacy of plant extracts and phytochemicals on various human health conditions. Most of the biomolecules developed as drugs are from traditional medicinal plants, but there are many more in nature still unexplored and unexploited.

Plant products, either pure compound or extracts provide unlimited scope for new drug leads because of their unmatched chemical diversity. The therapeutic effect of plants may be attributed to the synergic effect of the diverse constituents in them. In general, plant metabolites can be classified as primary metabolites and secondary metabolites. Primary metabolites include carbohydrates, proteins, nucleic acids, fats and lipids which are directly involved in growth and metabolism. Secondary metabolites are usually the end products of primary metabolism or biosynthesised from one or more primary metabolites by wider variety of pathways. They are not necessary for the plant to live, but play a vital role in plant protection against biotic and

abiotic stress. Studies have shown that secondary metabolites are responsible for the therapeutic potential of the plants. Alkaloids, terpenoids, flavonoids, cardiac glycosides, *etc.* are some of the major secondary metabolite groups assigned with bioactivity (Pagare et al., 2015). Alkaloids include a broad group of compounds with a prominent place in modern pharmacotherapy. Quinine, ephedrine, nicotine, morphine, *etc.* are examples of plant alkaloids with notable biological activity (Schläger & Dräger, 2016). Terpenoids are a vast group of amazingly diverse compounds with a wide range of biological activity against a variety of infectious and non-infectious diseases. Flavonoids are polyphenol class of compounds broadly distributed in plants and are noted for their extensive bioactivities including anti-ageing, anticancer, anti-inflammatory, anti-viral and cardioprotective efficacy (Wang et al., 2018). Cardiac glycosides have been a part of folk medicines as heart tonics, diuretics and have proved their potential as anticancer agents (Patel, 2016). Thus, plants with assorted secondary metabolites are valuable source for compounds with therapeutic potential and constitute a pool for new therapeutic leads. However, scientific validation of plant derived drugs need high-throughput screening and clinical trials (Altaf et al., 2019).

Phytochemical techniques play a significant role in the isolation and characterization of phytoconstituents. Preliminary phytochemical screening is a quick and inexpensive procedure to reveal various types of chemical compounds in a mixture, whereas quantitative estimation conveys an approximate amount of a particular compound in a mixture (Alamgir, 2018). Metabolomics is regarded as the large scale qualitative and quantitative study of all metabolites in a biological system. Hyphenated analytical techniques like GC/MS and LC/MS, combining chromatographic and spectral methods helps in the analysis and identification of biomaterials especially natural products. Chromatography coupled with MS offers an excellent solution for the analysis of complex mixtures. GC/MS is ideally suited for the analysis of

volatile, thermally and energetically stable compounds. Volatiles are a specialized class of metabolites with prominent roles in providing aromas to fruits and vegetables. They are equally known for their contributions to plant defence responses. Current technology uses GC/MS coupled with headspace solid-phase microextraction (HS-SPME) and single quadrupole MS detectors for high sensitivity. However, GC/MS is less amenable to large polar metabolites due to their poor volatility. To analyse non-volatile compounds, usually LC/MS is employed. Development of faster and efficient platforms like HPLC and HR-LC/MS has increased the chromatographic resolution which enables accurate screening and identification of metabolites in a complex mixture (Patel et al., 2010; Lei et al., 2011). Since, study of plant metabolome includes analysis of a massive range of chemical species with diverse physicochemical properties; MS based analytical tools form powerful platforms for the separation, characterization and quantification of vast compound diversity present in plant matrices (Jorge et al., 2016).

Metabolite profiling in the context of drug development has helped not merely in identifying and quantifying the metabolites in a mixture, but has also provided an insight into the anticipatory safety concerns when applied to biological systems (Ufer et al., 2017). Plant metabolome is complex and diverse, especially secondary metabolites which are coupled with characteristic species and agronomic differences making it extremely difficult to identify a single active compound. The spectrum of pharmacological efficacy exhibited by the plants usually arises from the synergistic action of multiple ingredients in them. However, the application of biological technologies and approaches like genomics and metabolomics to phytomedicine research can assist in evidence based phytotherapeutics and in developing a biological finger print of medicinal plant extracts. Metabolomics approaches with GC/MS and LC/MS are effective to provide a proof for the toxicology and safety of phytopreparations in animals. In addition, plant

metabolomics strategies also provide unique and important insights to medicinal herb research, linking putative bioactivity with the constituent phytochemicals of plants (Shyur & Yang, 2008).

Globally, there has been a tremendous increase in initiatives for the screening of natural products as active agents against cancer, malaria and innumerable contagious diseases for which only limited remedies are available. But, drug discovery from plants is a time and cost dependent scientific cumbersome task. It begins with appropriate selection of plants for biological screening followed by techniques to identify the compounds and their biological activity. An obvious challenge in this process is the occurrence of secondary metabolites as complex mixtures, making it difficult to identify the lead compound responsible for the bioactivity shown. However, multidisciplinary approaches involving bioassays in conjunction with fractionation of plant extracts and newly evolved techniques for structural elucidation of pure compounds have made the task more feasible (Lahlou, 2007).

Antioxidants are substances or compounds that can inhibit oxidation caused by free radicals. Free radicals are unstable atoms with a single unpaired electron in outer shell. Free radicals are produced in cells as a result of normal cellular metabolism. The unpaired electron of free radicals makes them highly reactive, transient and unstable. Because of their high reactivity, they attract electrons from other atoms to attain stability. As a result, attacked atom loses its electron and becomes a free radical itself. Thus, a series of reactions are launched which finally damage the living cells. Free radicals in biological systems are subsets of reactive oxygen species (ROS) or reactive nitrogen species (RNS). A major part of ROS originate as by-products of aerobic metabolism in mitochondria. The RNS on the other hand, is produced mainly in biological tissues like vascular endothelial cells by nitric oxide

synthase. Both ROS and RNS play a two-fold job in living cells. At low to moderate levels they have beneficial effects with involvement in various physiological functions and innumerable cellular signalling pathways. But at higher concentrations, accumulation of ROS and RNS induce oxidative stress and nitrosative stress respectively in cells which can cause potential damage to biomolecules including lipids, proteins and DNA. The excess of ROS is of more concern to human beings because of the potential damage they can bring about to the cells which eventually leads to many human diseases like arthritis, cancer, cardiovascular diseases, diabetes mellitus, respiratory diseases, *etc.* It is also supposed to be a catalyst in speeding the ageing process (Jensen, 2003; Phaniendra et al., 2015).

Reactive oxygen species (ROS) are also produced in the cells due to environmental conditions like air pollution, cigarette smoking, exposure to heavy metals and ionizing radiations. ROS are extremely reactive and can cause damage to biomolecules altering their structure. As far as cells are concerned, regulation of redox state is critical for its viability, proliferation and function. For this, cells have innate antioxidant mechanisms to tackle the free radicals and other ROS and RNS. But, excess accumulation of oxidants can happen which may lead to oxidative stress with adverse effects on the normal functioning of the cells. The three important ROS that are of physiological significance are superoxide anion, hydroxyl radical and hydrogen peroxide. The prime site of superoxide anion production in the cell is mitochondria. Superoxide is converted to hydrogen peroxide by the action of superoxide dismutases. Hydrogen peroxide is also synthesised in various other metabolic reactions that involve enzymes like xanthine oxidase, amino acid oxidase and NAD(P)H oxidase. Hydroxyl radicals are the most reactive among ROS with the potential to damage biomolecules like proteins, lipids, carbohydrates and DNA. They are either produced in a succession of reactions called Haber-Weiss and Fenton reactions in which hydrogen

peroxide (H_2O_2) is broken down to OH^- in presence of transition metals like Fe^{2+} , Cu^{2+} , *etc.* or by O_2^- itself which reacts with H_2O_2 and generate hydroxyl radicals. These reactions occur naturally in the body of all organisms and every organism including humans are equipped with an endogenous antioxidant mechanism to counterbalance the effect of oxidants (Birben et al., 2012). However, oxidative stress can develop from an inefficient endogenous antioxidant mechanism or due to exogenous factors. The oxidative stress, especially the one resulting from life style reasons, may at times become detrimental to human health and can lead to chronic diseases like cancer, inflammation, diabetes as well as other cardiovascular and respiratory complications.

Antioxidants significantly delay or prevent oxidation by scavenging the free radicals. Oxidative stress resulting from insufficient endogenous antioxidant mechanisms or which are induced by exposure to toxic environmental conditions can be ameliorated to a greater extent by the intake of exogenous antioxidants. Plants are an excellent source of antioxidants. Natural exogenous antioxidants are derived chiefly from fruits, vegetables, cereals, mushrooms, spices, beverages, medicinal plants, *etc.* These natural antioxidants are mainly secondary metabolites like polyphenols, carotenoids and vitamins. These metabolites also exhibit a broad range of biological activities like anti-ageing, anticancer, anti-inflammatory and antimicrobial properties (Xu et al., 2017).

Plants have efficient enzymatic and non-enzymatic antioxidant defence systems to avoid toxic effects of free radicals. Non-enzymatic antioxidants are mainly secondary metabolites that are synthesised and accumulated in plants. These metabolites apart from their role in ROS metabolism also have a vital role in adapting plants to the fluctuating environmental conditions (Kasote et al., 2015). However, there are increasing evidences to suggest that the

advantageous effects of plant food consumption are not attributable to a single specific compound, but to the synergetic effect of complex mixtures of phytochemicals and nutrients. Hence, intake of fruits, vegetables, grains, *etc.* in daily diet is more advisable than consuming isolated compounds or high dose of compounds like food supplements (Bouayed & Bohn, 2010).

The term “inflammation” is derived from a Latin word *inflammar* which means “to set on fire.” Inflammation is regarded as an adaptive response of the body provoked by stimuli like infection or tissue injury. It is a part of body’s defence mechanism to restore homeostasis by eliminating foreign particles and damaged tissue debris to initiate healing processes and to reinstate normality. A controlled inflammatory response is beneficial, but its dysregulation can bring more harm to the organism than the initiating stimuli itself (Lin & Li., 2018). Usually acute inflammatory responses contribute to mitigate injury or infection to restore tissue homeostasis. But under certain conditions, chronic inflammation may occur when acute inflammatory mechanisms fail to eliminate tissue injury (Chen et al., 2018a). Inflammation which is initiated as a protective response is characterized by many events like enhanced permeability of capillaries, increased blood flow, vascular dilation and leukocyte recruitment to the effected site. Polymorphonuclear neutrophils are among the first leukocyte responders to accumulate in the inflamed site. This is followed by mononuclear cells, monocytes and macrophages which enter the inflammatory site and clear cellular debris and apoptotic polymorphonuclear neutrophils by phagocytosis without prolonging inflammation. However, failure to remove noxious materials produced by neutrophils via phagocytosis or failure to clear apoptotic inflammatory cells and a delay of apoptosis may lead to chronic and pathological conditions. This may ultimately lead to human inflammatory pathologies including arthritis, asthma, cancers, cardiovascular diseases and many other periodontal diseases (Freire & Van Dyke, 2013). To overcome the complications of

inflammation, anti-inflammatory agents are given. Anti-inflammatory agents function either by reducing or inhibiting inflammation mediators like COX-1, COX-2, TNF- α , interleukins, *etc.*

Macrophages are mononuclear cells capable of phagocytosis. They are distributed throughout mammalian organs and their morphology varies depending on their state of activity (Elhelu, 1983). Macrophages are highly versatile, large white blood cells that portray a central role in all stages of inflammatory response. They are derived from blood monocytes and reside in all tissues where they act as sentinels responding to damage. Once activated, macrophages are efficient phagocytes and eliminate unwanted materials including apoptotic cells. They also secrete a diverse array of cytokines and chemokines that direct the inflammatory response and aid in tissue repair. Macrophages have the ability to acquire distinct functional phenotypes as directed by tissue type and environmental cues. This enables the macrophages to play multiple roles in inflammatory responses and in the repair of tissues, both encouraging and discouraging these processes (Dunster, 2016). Macrophages release a variety of agents that can influence inflammatory responses, microbial killing, antigen presentation, wound healing and many more. Enzymes secreted by macrophages include proteases, lipases, deoxyribonucleases, phosphatases, collagenases, elastases, *etc.* Cytokine secretion by macrophages plays important roles in many stages of immune response. Small molecular weight molecules such as O_2^- and NO are also released by macrophages in response to various inflammatory stimuli. These reactive molecules are the products of macrophage NADPH and iNOS enzyme activity. Macrophages also produce many arachidonic acid intermediates which have pivotal roles in the process of inflammation (Fox & Rossi, 2010).

Prostaglandins are lipid autacoids with vital role in the generation of inflammatory response. They are arachidonic acid metabolites and their production depends on the activity of prostaglandin G/H synthases, colloquially known as COXs. COXs are bifunctional enzymes that contain both cyclooxygenase and peroxidase activities. They exist as distinct isoforms referred to as COX-1 and COX-2. COX-1 is expressed constitutively in most cells and is the dominant source of prostanoids that have housekeeping functions. On the other hand, COX-2 is induced by inflammatory stimuli, hormones and other growth factors. COX-2 is the important source of prostanoid formation in inflammation and in proliferative diseases like cancer. Nonsteroidal anti-inflammatory drugs (NSAIDs) usually inhibit the biosynthesis of cyclooxygenase isoenzymes particularly COX-2 to produce the desirable effect (Ricciotti & FitzGerald, 2011).

Lipoxygenases (LOX) are a group of oxidative enzymes with a non-heme iron atom in their active site. They are involved in the regulation of inflammatory responses by the generation of pro-inflammatory mediators called leukotrienes or anti-inflammatory mediators known as lipoxins. In general, lipoxygenases are identified as 5-, 8-, 12- and 15-lipoxygenases according to their selectivity to oxygenate fatty acids in a specific position. Biosynthesis of leukotrienes is regulated by the activity of 5-lipoxygenase (Wisastra & Dekker, 2014). Thus, the inhibition of LOX can reduce leukotriene production there by producing an anti-inflammatory effect (Hu & Ma, 2018).

Cytokines are small soluble proteins secreted mainly by helper T cells and macrophages. These small proteins have specific effect on cell interaction and communication. They carry out a predominant role in inflammation. Pro-inflammatory cytokines are produced primarily by activated macrophages and are involved in the up-regulation of inflammatory reactions. Studies have

shown that TNF- α , IL-1 β , IL-6, IL-8 and IL-12 are the major pro-inflammatory cytokines produced by macrophages when exposed to inflammatory stimuli. TNF- α also known as cachectin, is indispensable in several signalling pathways including apoptotic pathways (Zhang & An, 2007). It has a crucial role in acute and chronic inflammatory conditions. Current studies suggest that it can be a potential therapeutic target in treating inflammation (Popa et al., 2007).

Nitric oxide (NO) is one of the prominent mediators involved in inflammation. It is synthesised by three different isoforms of NO synthase: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) synthases (Cassini-Vieira et al., 2015). Inducible nitric oxide synthase (iNOS) catalyzes a reaction that converts O₂ and l-arginine to nitric oxide (NO). Macrophages in response to inflammatory stimuli express iNOS (Robinson et al., 2011). Many anti-inflammatory agents like glucocorticoids are reported to inhibit NO production and iNOS expression. Studies have shown that glucocorticoids exert their inhibitory effect on NO production and iNOS expression through the inhibition of a transcription factor called nuclear factor-kappa B (NF- κ B) (Matsumura et al., 2001; Hämäläinen et al., 2008).

Nuclear factor- κ B (NF- κ B) represents a family of inducible transcription factors, which regulates a large array of genes involved in immune function and inflammatory responses. This family is composed of five structurally related members including NF- κ B1 (also named p50), NF- κ B2 (also named p52), RelA (also named p65), RelB and c-Rel which mediate transcription of target genes by binding to a specific DNA element, κ B enhancer as various hetero or homodimers. The NF- κ B proteins are normally suppressed in the cytoplasm by a family of inhibitory proteins including I κ B family members and other related proteins. The activation of NF- κ B involves two major signalling pathways, the canonical and non-

canonical or alternative pathways. Both pathways are important for regulating immune and inflammatory responses even though they differ in their signalling mechanism. Chronic inflammatory diseases are often associated with deregulated NF- κ B activation. Therapeutic strategies targeting NF- κ B regulation can be of great significance in treating inflammatory diseases (Liu et al., 2017).

Neutrophils are implicated in tissue damages that occur in a broad spectrum of inflammatory diseases. Myeloperoxidase (MPO) is an enzyme mostly expressed in neutrophils and to a lesser extent in monocytes. This enzyme is known to play a major role in the initiation and progression of acute and chronic inflammatory diseases, fundamentally cardiovascular diseases (CVD). It is also reported to be an early biomarker of inflammation associated with cardiovascular risk in obese children at the prepubertal age (Olza et al., 2012). When stimulated, neutrophils discharge superoxide which is converted into more reactive secondary oxidants including hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). The heme enzyme myeloperoxidase (MPO) uses H_2O_2 and Cl^- to produce hypochlorous acid (HOCl) which is a potent neutrophil oxidant. It is extremely cytotoxic and reacts readily with most biological molecules especially proteins. It can readily inactivate the major plasma protease inhibitor α_1 -antitrypsin and can activate neutrophil collagenase. Thus, HOCl may promote tissue damage indirectly by facilitating proteolysis at inflammatory sites. Therefore, specific inhibitors of MPO can act as good anti-inflammatory drugs (Kettle & Winterbourn, 1991; Van et al., 2007).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most popularly prescribed medications for treating inflammatory diseases and many of them are excellent analgesic and anti-inflammatory agents. But long-term consumption of NSAIDs may cause gastrointestinal and cardiovascular

complications. Hence, there has always been a search for non-NSAIDs with efficient anti-inflammatory property (Sostres et al., 2010). There are many plants used traditionally in treating inflammatory conditions. The search for anti-inflammatory agents with lesser side effects has lead to the identification and characterization of a hand full of natural plant derived compounds with effective anti-inflammatory activity and the search is nevertheless going on for more naturally active compounds with promising activity.

Cytotoxicity studies are universally regarded as a preliminary but obligatory step in the evaluation of toxicity of test substances. This is especially valid in the screening of biological activity of plant extracts or active compounds isolated from plants. If the screening is for cosmetic or pharmaceutical use, minimum or no toxicity is preferred. Alternatively, if the extract or compound is intended to act as anticancer drugs selective cytotoxicity to cancerous cells is recommended. This process helps to identify and prioritize test substances for further studies (McGaw et al., 2014).

Plants have invariably been used as an indicator organism to study toxicity and mutagenesis. Among the plants, *Allium cepa* L. is widely used as a model for the assessment of cytotoxicity. They are excellent genetic models especially for evaluating chromosomal aberrations. It is considered as one of the best test system to evaluate genotoxicity potential of environmental chemicals because of its sensitivity and correlation with mammalian test system (Pandey et al., 2014). In the *A. cepa* bioassay, meristem cells of roots are evaluated for chromosomal aberrations and mitotic index to determine the influences of the extract or compound being tested. This is a quick and rapid test to assess cytogenetic effects (Bianchi et al., 2016). Cytotoxicity screening in general is considered as an initial screening process, directed towards the identification and isolation of specific compound or mixture with anticancer activity.

Cancer is the most discussed and fearful disease of the era. It is not just a single disease but comprises more than 270 different diseases characterized by uncontrolled growth and spread of abnormal cells. It is regarded as the second largest cause of mortality next to heart diseases worldwide. In men, the highest percentages of cancer types are reported in the prostate, lung, colon, rectum and urinary bladder. In women, cancer prevalence is highest in breast, colon, lungs, rectum, thyroid and uterine corpus. Among children, blood cancer and cancers related to the brain and lymph nodes are found in more significant percentage. Cancer occurs by a series of successive mutations in genes which ultimately effects cell function, disrupts cellular relations and finally affects the cell cycle leading to abnormal proliferation. Abnormal diet, chemical substances with toxic properties, polluted environment, smoking and several infectious agents are responsible for cancer (Hassanpour & Dehghani, 2017).

Cancer cells differ from normal cells in many aspects. Cancer cells often assume an abnormal and variable appearance. They lack adherence quality as normal cells and hence show a tendency to spread which culminates in metastasis. Normal cells have a systematic path of growth, division and death. Cells usually follow a normal physiological path of cell death called programmed cell death or apoptosis. Cancer cells neither have a systematic path of growth, nor they experience apoptosis and as a result they continue to grow and divide, becoming more and more abnormal. Lack of communication between the cells and absence of cellular repairing mechanism add to the abnormality of cancer cells.

Apoptosis is not the only mode of death followed by the cells. But there are other types like necrosis, pyroptosis, entosis and mitotic catastrophe. Many of their mechanisms are currently unclear. Out of these, apoptosis and necrosis are the main models involved in cell death. Apoptosis literally means

“falling off” and is a normal process inevitable in development and ageing. It is a form of programmed cell death, a homeostatic mechanism to maintain cell population. This type of cell death is an energy dependent pathophysiological phenomenon, which leads to cellular shrinkage and cell death. But, it is devoid of cell membrane rupture and inflammatory responses. Cell shrinkage, pyknosis and extensive plasma membrane blebbing are visible signs of apoptosis. It is the fastest mode of cell death while necrosis and autophagy are seen after the culmination of apoptosis. Extrinsic pathway, intrinsic pathway and perforin/granzyme pathway are the three main apoptotic pathways. All the three pathways converge to a common execution point. Caspases play a significant role in extrinsic and intrinsic pathways. Caspases are the highly conserved cysteine dependent aspartate specific proteases that act as biochemical regulators and executioners of apoptotic cells. They are present in almost all body cells in their inactive form and need activation by protease cascade which ultimately ignites the apoptotic process (Kumar et al., 2015).

Necrosis is another possible pathway of cell death. It is considered as a toxic process where the cell is a passive victim and follows an energy independent mode of death. Necrosis is a Greek word meaning “death or dead body.” Since necrosis refers to a degradative process that occurs after cell death, it is also regarded as an unsuitable term to describe the mechanism of cell death. The term “oncosis” is suggested alternatively, to describe the process that leads to necrosis by karyolysis and cell swelling. This is in contradiction to apoptosis which is accompanied by cell shrinkage, pyknosis and karyorrhexis. Some of the major morphological changes associated with necrosis include swelling of the cells, formation of cytoplasmic vacuoles, clumping of chromatin, swelling of organelles, mitochondrial damage, loss of calcium homeostasis, accumulation of oxygen-derived free radicals, defects in membrane permeability and finally disruption of the cell membrane. All these

changes finally provoke inflammatory responses in contrast to apoptosis (Elmore, 2007; Chen et al., 2018b).

A number of methods have been developed to identify the DNA damage and apoptosis by evaluating morphological, biochemical and molecular alterations. Changes in plasma membrane composition and function can be detected by its altered permeability to certain dyes. Appearance of phosphatidylserine on the external plasma membrane surface which can be labelled with annexin V conjugated to fluorochromes (Darzynkiewicz & Bedner, 2000). Apoptotic cells are recognized either on the basis of their reduced DNA-associated fluorescence as well as with diminished DNA content (sub-G1) or morphological changes. Activation of caspases can be examined through colorimetric, immunoblot or immunohistochemical methods. Activated caspases cleave many cellular proteins and the resulting fragments may also serve as useful biomarkers (Plesca et al., 2008).

There are many sophisticated techniques available to detect and examine apoptotic cells. Light microscopy, TEM analysis and SEM studies can provide qualitative and quantitative data. MTT assay is a colorimetric method used to determine the number of viable cells in proliferation assays. Double staining procedures often correlate to the phenotype of apoptotic cells. Conventional, pulse field and field inversion gel electrophoresis can be used to separate DNA molecules to get the characteristic ladder patterns of discontinuous DNA fragments which are hall marks of apoptosis. Single cell gel electrophoresis otherwise called comet assay helps to visualize DNA damage at the level of individual cells. Apoptotic cells appear as comet-like structures with large tails and small heads. Flow cytometry is a reliable technique for the accurate quantification of apoptosis. This technique distinguishes apoptotic cells from non-apoptotic cells by means of DNA

staining. It enables counting, examining and sorting of microscopic particles suspended in a stream of fluid. It allows simultaneous multiple parametric analyses of the physical and chemical characteristics of single cells flowing through an optical or electronic detection apparatus. Other immunohistochemical techniques like ELISA help to detect apoptosis using antibodies against a wide range of substrates especially caspases (Archana et al., 2013). Finally, gene expression studies can be made with powerful techniques like RT-qPCR. Gene expression is a process by which information from a gene is used for the synthesis of a functional product like proteins or RNAs. Gene expression studies with real-time polymerase chain reaction usually investigate the changes in the expression of a particular gene or set of genes by measuring the abundance of the gene-specific transcript. RT-PCR products can then be analysed with gel electrophoresis to examine the expression change.

Cancer is a multifactorial disease with extreme complexity in its genesis and progression (Kumar et al., 2017). Conventional anticancer drug therapy is primarily based on the cytotoxic and/or cytostatic activity of the drug. In most instances these drugs affect not only the cancer cells, but also disturb the normal cell metabolism causing harmful side effects. Major challenge in anticancer drug development is to design a drug with high selectivity and specificity. However, recent developments in molecular biology and an understanding of pharmacology of cancer at molecular level have provoked the researchers to come up with targeted anticancer drugs. Such anticancer drugs usually aim at specific molecular targets that play a part in the development, progression and spread of a given neoplasm. Selection of appropriate molecular target for inhibition or modification, such that the target is tumour specific, non-redundant and able to influence the outcome of tumour progression is the significant challenge in this approach. Compounds of natural origin have often provided new leads in novelty of

compounds with anticancer activity which can well be explored for targeted anticancer therapies (Narang & Desai, 2009; Zajac et al., 2016).

Nano is a prefix used to describe “one billionth” of something. Nanotechnology involves creation and manipulation of materials at nanoscale. Nanotechnology is considered as a potential branch that can contribute much to drug development, communication technologies and in the production of stronger and lighter materials. Nanoparticles have a special place in nanoscience and nanotechnology. Ordinary materials when reduced to the nanoscale often acquire and exhibit novel and unpredictable characteristics like extraordinary strength, chemical reactivity, electrical conductivity, *etc.* that the same material does not possess at the micro or macro scale (Benelmekki, 2015).

Among the different metallic nanoparticles, silver nanoparticles (AgNPs) have a vital role in biomedical applications and nanomedicine. Silver nanoparticles have unique physical and chemical properties that make them useful in the pharmaceutical field as drug delivering agents. Synthesis of nanoparticles is generally carried out using three approaches – physical, chemical and biological. In physical method nanoparticles are synthesised by evaporation-condensation. Even though the method is faster and involves no hazardous chemicals, it has some disadvantages like low productivity and lack of uniform size distribution. Chemical methods usually employ metal precursors, reducing agents and capping agents for the synthesis of nanoparticles. The main advantage is a higher yield compared to physical method. But these methods are usually expensive. Involvement of hazardous chemicals and low purity make chemical methods inappropriate for pharmaceutical purposes. To overcome the shortcomings of physical and chemical methods, a green chemistry approach has emerged that uses biological systems such as bacteria, fungi, plant extracts and even small

molecules like vitamins and amino acids for the synthesis of silver nanoparticles. This green approach is more simple, cost effective, eco-friendly and above all free from hazardous chemicals. It also facilitates to control particle size and shape, which is a crucial factor for biomedical applications (Zhang et al., 2016).

Characterization of nanoparticles is critical to evaluate the functional aspects of synthesised particles. There are many techniques available for the determination of various parameters. It includes UV-Vis spectroscopy, X-ray diffractometry (XRD), Fourier-transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) (Natsuki et al., 2015).

India is one of the seventeen megadiverse countries in the world, bestowed with three biodiversity hot spots. It has a rich repository of medicinal plants, many of them well explored and many more unexplored for their medicinal properties. Lamiaceae, formerly called Labiatae and popularly known as “mint family” is one of the most diverse and widespread gamopetalous families in terms of medicinal value. As of now, it consists of seven subfamilies with ten genera not placed in any of the subfamilies. The family as a whole consist of about 236 genera and over 7,200 species with cosmopolitan distribution. Plants belonging to this family are mostly herbs or under shrubs with quadrangular stem. Leaves are usually opposite or whorled, simple, often with serrate margin and oil secreting glands. Flowers are bisexual, zygomorphic in racemes or cymes, in whorls or leaf axils. Generally flowers are bilabiate with 5 sepals. Petals 5, united and two lipped. Androecium is didynamous with 2-4 anthers. Ovary is 4 lobed with gynobasic style. Fruits are seen as nutlets. Many species belonging to this family are aromatic because of the presence of external glandular structures that produce

volatile oils. It includes *Mentha*, *Salvia*, *Lavandula*, *Orthosiphon*, *Ocimum*, *Leucas*, *Anisomeles*, *Colebrookea*, *Coleus*, *Hyptis*, *Origanum*, *Scutellaria*, *Thymus*, *Lamium*, *Pogostemon*, etc. (Trivellini et al., 2016). They are also known for their medicinal properties and have a prime position in traditional herbal preparations. Members like *Ocimum*, *Mentha*, *Coleus*, *Thymus*, *Leucas*, *Pogostemon*, etc. are well known for their antimicrobial, anti-inflammatory, anti-diabetic, anti-depressant and anticancer properties. Many of them are used in herbal preparations for treating infertility, digestive disorders, heart diseases, etc. (Debbarma et al., 2017).

However, it is intriguing to note that the family Lamiaceae also have oil poor species. Lamioideae is a subfamily within Lamiaceae, and most of the genera in it are essentially oil poor (Bianco et al., 2016). *Gomphostemma* Wall. ex Benth. is a genus of subfamily Lamioideae with about 30 species. Most of them are distributed in South East Asia, China and Indian sub-continent as rainforest herbs or sub-shrubs. Even though Lamioideae is the second largest subfamily in Lamiaceae, it is a poorly investigated group. The restricted distribution of its members seems to be the reason for their least exploration (Roy & Lindqvist, 2015; Walsingham & Bramley, 2010).

Since, no ample records have been available till date on the phytochemical and pharmacological aspects of *Gomphostemma*, present study focuses on a detailed phytochemical profiling and bioactivity screening of selected species of *Gomphostemma*.

The work aims to fulfil the following objectives:

- To screen for major phytocomponents by qualitative analysis and quantitative estimation.
- To elucidate the volatile and non-volatile metabolites by GC/MS and HR-LC/MS analysis.

- To determine the antioxidant potential by *in vitro* assays.
- To evaluate the anti-inflammatory potential by biochemical assays.
- To screen the *in vitro* anti-inflammatory activity on RAW 264.7 cells.
- To estimate the role of inflammatory mediators using ELISA.
- To determine the cytotoxic potential by *Allium cepa* bioassay.
- To screen the anti-proliferative efficacy on breast cancer (MCF-7) cell line.
- To evaluate the apoptotic potential by comet assay, double staining, cell cycle analysis and ELISA.
- To elucidate the antiproliferative mechanism by gene expression analysis.
- To demonstrate the green synthesis of silver nanoparticles by selected species of *Gomphostemma*.
- To characterize the silver nanoparticles by UV-Visible spectroscopy and SEM analysis.

REVIEW OF LITERATURE

A. Background and rationale

Man has depended on plants not just for food and shelter but also for health and wellness. With the advent of civilization, humans started to categorize plants suited for diverse needs among which, plants with healing properties hold a remarkable position. Use of plants and their extracts for alleviating various illnesses led to the development of plant based medicinal systems. Even now, over 80% of population in developing countries depend on herbal medicines for curing and preventing sickness. Advances in science and technology contributed to the use of a single or mixture of chemical entities for treating diseases in place of crude herbal preparations. The sheer diversity of plant derived compounds in terms of structural complexity and activity has prompted the scientific world to exploit them as therapeutic agents. As a result, a number of compounds of plant origin are approved as drugs and many are in clinical trials.

Lamiaceae is a prominent family of flowering plants with high species diversity and cosmopolitan distribution. Many species of this family retain a rich tradition as flavouring agents and as ingredients in herbal preparations for treating many ailments. Studies have proved that complex mixture of bioactive compounds in these plants contributes to their curative and preventive properties (Carović-Stanko et al., 2016). It is commonly called mint family and includes many culinary and aromatic plants like *Ocimum*, *Salvia*, *Mentha*, *Thymus*, *Pogostemon*, *Lavandula*, etc. Most of the members of this family are attributed with medicinal properties and are regularly included in the preparation of home remedies as infusions or decoctions to cure flu, cough, cold, skin irritations, symptoms of indigestion, etc. (Raja, 2012). Plants belonging to Lamiaceae have been well acknowledged for their pleasantly scented and commercially valuable essential oils. However, the

family also contains plant species with poor oil content. Lamioideae, a subfamily within Lamiaceae is basically oil poor with genera like *Leucas*, *Lamium*, *Phlomis*, *Stachys*, *Leonotis*, *Galeopsis*, etc. Nevertheless, a few exceptions such as *Pogostemon* and *Sideritis* with complex chemical constitution are also members of this subfamily. As might be expected, only little information is available on these oil poor genera, but even then many of them like *Leucas*, *Stachys*, *Lamium*, *Sideritis*, etc. are valued for their medicinal properties especially in folk medicines. Apparently their medicinal properties are not confined to the perfumery and culinary flavouring compounds (El-Gazzar & Watson, 1970; Matkowski et al., 2008). Members of Lamioideae seen in Western Ghats include species of *Anisomeles*, *Colebrookea*, *Gomphostemma*, *Leucas*, *Leonotis*, *Leonurus* and *Pogostemon*,

Gomphostemma represents a genus of wild flowering plants belonging to the subfamily Lamioideae. It is seen as undershrubs or herbs in tropical rain forests. When compared to other taxa of the family, they are comparatively less explored for their phytochemical and biological aspects probably due to their restricted and limited distribution. However, many species are reported to be used in traditional medicines especially in North-Eastern states of India, Bangladesh, China and Malaysia. Mainly leaves are credited with medicinal properties and are typically administered as decoctions or infusions. The leaf paste of *G. heyneanum* var. *heyneanum* is used by the tribes of Wayanad district to cure dysentery and diarrhoea (Prasad et al., 2013). Other species like *G. niveum* and *G. parviflorum* are used by the ethnic tribes of Assam for treating malaria. Leaf decoction and paste of *G. niveum* are also used as a cure for tuberculosis and insect stings (Dutta, 2014; Shankar et al., 2012). In Bangladesh, the leaves of *G. parviflorum* var. *parviflorum* are traditionally used for treating inflammations (Shams-Ud-Doha et al., 2013). The leaves of *G. javanicum* crushed with turmeric are used as external application for treating skin diseases by the rural folks of North Andaman (Prasad et al., 2008). *G. microdon* is used as a drug for treating menstrual problems as well as for the treatment of coughs and for eliminating phlegm (Zhang et al.,

2009). Like so, many species of *Gomphostemma* are used in different folklore medicines according to their local availability.

This review is a compilation of available literature reports relevant to the present study, related to plants belonging to Lamiaceae with emphasis on subfamily Lamioideae.

B. Phytochemical constituents

Phytochemicals are chemicals or metabolic compounds of plant origin and are attributed with various bioactivities. But, since they occur as multi-component mixtures, their separation, identification and characterization are arduous. It requires a combination of various techniques ranging from simple phytochemical screening assays to complex analytical techniques for the identification, purification and isolation of biologically active components from crude plant extracts (Sasidharan et al., 2011). Major phytocomponents identified in various members of Lamiaceae and subfamily Lamioideae are consolidated and presented in Table 1 and Table 2 respectively.

Table 1: Some selected reports on major phytochemical components identified in various members of Lamiaceae

Sl No.	Plant	Compounds	Reference
1.	<i>Ocimum tenuiflorum</i>	α -pinene, camphene, sabinene, β -pinene, cymene, limonene, linalool, borneol, α -terpineol, α -cubebene, eugenol	(Joshi & Hoti, 2014)
2.	<i>Hyptis suaveolens</i>	α -thujene, α -pinene, camphene, sabinene, myrcene, α -phellandrene, δ -3-carene, p-cymene, limonene, camphor, thymol, δ -elemene, eugenol, β -caryophyllene, α -humulene, spathulenol	(Benelli et al., 2012)

3.	<i>Salvia officinalis</i>	(Z)-salvene, α -pinene, camphene, myrcene, p-cymene, limonene, linalool, α -thujone, camphor, α -humulene, β -caryophyllene, caryophyllene oxide	(Baj et al., 2013)
4.	<i>Plectranthus amboinicus</i>	Terpinolene, p-cymene, γ -terpinene, terpinen-4-ol, methyl thymol, thymol, β -caryophyllene, α -humulene	(da Costa et al., 2010)
5.	<i>Mentha piperita</i>	Menthone, menthol, menthyl acetate, menthofuran, limonene, α -pinene, germacrene D, myrcene, β -caryophyllene, α -terpinene, piperitone, linalool	(Derwich et al., 2010)
6.	<i>Thymus vulgaris</i>	α -pinene, α -thujene, camphene, myrcene, limonene, β -phellandrene, γ -terpinene, p-cymene	(Ahmad et al., 2014)
7.	<i>Scutellaria barbata</i>	Linalool, camphor, menthone, menthol, α -terpineol, thymol, eugenol, α -copaene, β -bourbonene, β -guaiene, β -himachalene, globulol	(Yu et al., 2004)
8.	<i>Lavandula angustifolia</i>	α -pinene, camphene, β -myrcene, delta-3-carene, p-cymene, limonene, linalool, lavandulol, borneol, nerol, terpineol, citral, geranyl acetate, α -humulene, germacrene D, caryophyllene oxide	(Singh et al., 2015)
9.	<i>Agastache rugosa</i>	Sabinene, β -myrcene, limonene, menthan-3-one, caryophyllene, germacrene D, elemene, spathulenol, caryophyllene oxide, α -cadinol	(Haiyan et al., 2016)

10.	<i>Isodon rugosus</i>	α -phellandrene, α -pinene, sabinene, o-cymene, β -linalool, terpinen-4-ol, caryophyllene, trans-p-mentha-2, 8-dienol, germacrene D, caryophyllene oxide	(Hussain et al., 2017)
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The ethanolic extract of leaves and roots of *Gomphostemma parviflorum* were analysed for their phytochemical constituents by Shams-Ud-Doha et al. (2013). Root extract was found to be rich in tannins, alkaloids, flavonoids, carbohydrates, terpenoids and steroids, whereas tannins were found to be absent in leaf extract.

Table 2: Some selected reports on major phytochemical components identified in various members of subfamily Lamioideae of Lamiaceae

Sl NO.	Plant	Compounds	Reference
1.	<i>Leucas aspera</i>	α -thujene, α -pinene, α -phellandrene, o-cymene, limonene, β -ocimene, β -elemene, α -bisabolol, β -caryophyllene, β -gurjunene, α -humulene, caryophyllene oxide	(Joshi, 2015)
2.	<i>Leucas virgata</i>	Camphene, β -pinene, α -phellandrene, p-cymene, limonene, linalool, pinocarvone, carvone, β -caryophyllene, α -humulene, caryophyllene oxide, β -bisabolol	(Mothana et al., 2013)
3.	<i>Lamium amplexicaule</i>	α -thujene, α -pinene, myrcene, p-cymene, limonene, germacrene-D, caryophyllene oxide, phytol	(Sajjadi & Ghannadi, 2012)

4.	<i>Lamium album</i>	Carvone, 4-vinyl-2-methoxy phenol, loliolide, palmitic acid, phytol, γ -sitosterol	(Iordache et al., 2009)
5.	<i>Stachys cretica</i>	α -pinene, 1-octen-3-ol, limonene, linalool, cis-verbenol, β -cubebene, β -elemene, β -caryophyllene, α -humulene, caryophyllene oxide, phytol	(Oztürk et al., 2009)
6.	<i>Stachys inflata</i>	α -pinene, p-cymene, limonene, linalool, α -terpineol, carvone, trans-caryophyllene, cubebol, spathulenol, caryophyllene oxide, β -bourbonene	(Sajjadi & Somae, 2004)
7.	<i>Phlomis bracteosa</i>	Sabinene, linalool, δ -elemene, eugenol, β -bourbonene, β -elemene, β -caryophyllene, β -gurjunene, α -humulene, germacrene D, cubenol	(Joshi & Pande, 2010)
8.	<i>Phlomis cashmeriana</i>	α -pinene, sabinene, o-cymene, limonene, 3-thujanone, 1-terpene-4-ol, α -citral	(Ullah et al., 2013)
9.	<i>Leonotis nepetifolia</i>	α -thujene, α -pinene, sabinene, myrcene, β -ocimene, linalool, eugenol, α -copaene, β -bourbonene, β -elemene, β -caryophyllene, α -humulene, caryophyllene oxide	(Oyedeji et al., 1999)
10.	<i>Pogostemon cablin</i>	Patchoulol, δ -guaiene, α -guaiene, α -patchoulene, β -caryophyllene, α -gurjunene, valencene	(Kusuma & Mahfud, 2017)

11.	<i>Anisomeles malabarica</i>	α -pinene, camphene, linalool, α -thujone, thymol, eugenol, δ -cadinene, β -caryophyllene, caryophyllene oxide, α -bisabolol, phytol	(Yogesh & Krishnakant, 2011)
12.	<i>Leonurus sibiricus</i>	α -pinene, myrcene, p-cymene, β -phellandrene, limonene, linalool, thymol, β -caryophyllene, β -elemene, α -copaene	(Sitarek et al., 2017)

Phytochemical screening of methanolic extract of *Gomphostemma niveum* by Dutta (2014), revealed the presence of alkaloids, terpenoids, phenols, flavonoids, tannins and saponins. The total phenol, flavonoid and alkaloid contents were found to be 75.04 mg/g, 28.23 mg/g and 0.083 mg/g in terms of gallic acid, quercetin and colchicine equivalents respectively. The study thus provided evidence for the presence of bioactive compounds in *G. niveum* and validated its ethnomedicinal use.

A chemosystematic study of selected species of *Gomphostemma* and related genera *Chelonopsis* and *Bostrychantha* belonging to the tribe Gomphostemmatae of Lamioideae with reference to their flavonoid and phenolic acid compounds was conducted by Bongcheewin et al. (2014). The study revealed thirty five flavonoid glycosides, eight phenolic acids and its derivatives as identified by LC-UV-MS/MS analysis of aqueous 80% methanol extracts based on their UV and mass spectra, retention times and comparison with in-house library. The study thus clearly displayed the compound diversity in plants analysed.

Phytochemical investigation of chloroform extract of *Gomphostemma niveum* leaves led to the isolation of two new diterpenes. Their structures were elucidated by spectroscopic and single crystal XRD techniques. First

compound was named as gomphostenin 1 and its structure was elucidated as 8-ethyl (5H-furan-2-one, 14-hydroxy, 2-oxo 3, 20 Z (17) diene clerodane, while second compound was found to be an acetyl derivative of first compound and was named as gomphostenin A. The *in vitro* antimalarial activity evaluated against *Plasmodium falciparum* showed that gomphostenin A was more active than gomphostenin 1 and chloroform extract itself (Sathe & Kaushik, 2010).

Wang et al. (2007) isolated two new nortriterpenoids, gomphoparvin A and gomphoparvin B from methanol extract of *Gomphostemma parviflorum*. Five known compounds ergosta-7, 22-diene-3, 5, 6, 9-tetrol, ergosta-7, 22-dien-3-ol, asperphenamate, β -sitosterol and daucosterol were also identified. Their structures and relative configurations were elucidated on the basis of 1D- and 2D-NMR as well as with MS analysis.

Zhang et al. (2009) isolated two new clerodane type diterpenoids from aerial parts of methanol extract of *Gomphostemma microdon*. They were named as microdon A and microdon B. Seven known compounds *viz.*, phytol, betulinic acid, 3-taraxeranol, 4, 5-dihydroxy-7-methoxyflavone, 5-hydroxy-2 methoxybenzoic acid, 3-hydroxystigmast-7-en-11-one and apigenin were also identified.

A study on the phytochemical evaluation in selected species of *Galeopsis*, *Melittis*, *Sideritis* and *Stachys* belonging to the subfamily Lamioideae by Frezza et al. (2019), led to the isolation and identification of nineteen iridoidic compounds: 8-epiloganic acid, 8-epiloganin, ajugoside, reptoside, harpagide, 8-O-acetyl-harpagide, monomelittoside, melittoside, allobetonoside, virginioside, geniposidic acid, 6-deoxy-harpagide, bartsioside, 6-O-acetyl-ajugol, 5-O-allosyloxy-aucubin, asperulosidic acid, deacetyl-asperulosidic acid, 5-O- β -D-glucopyranosyl-harpagide and 4'-O-galactopyranosyl-teuhircoside.

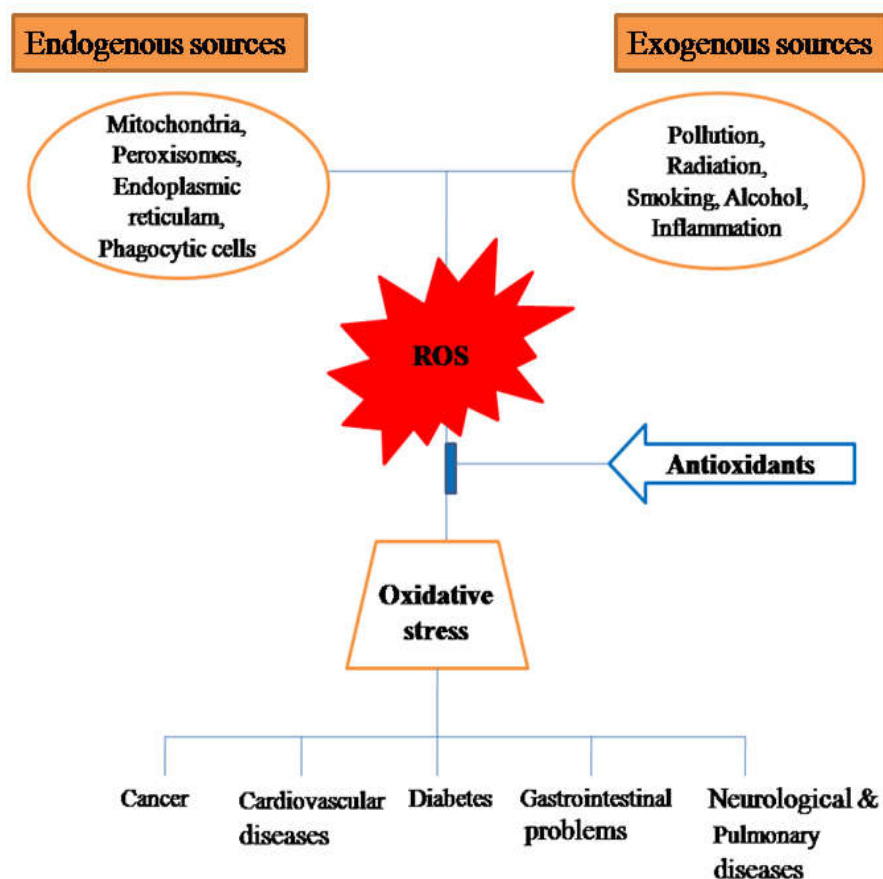
C. Antioxidant studies

Free radicals are highly reactive and unstable molecules that are generated in the body both by exogenous sources like pollution, alcohol, heavy metals, *etc.*, and from endogenous sources like mitochondria, peroxisomes, endoplasmic reticulum, *etc.* as a result of normal cell metabolism. Free radicals collectively include reactive oxygen species (ROS) and reactive nitrogen species (RNS). They play a duplex role in the body as toxic and beneficial molecules. Body has its own antioxidant mechanisms to tackle the excess free radicals. But, at times it may become ineffective and an overload of free radicals leads to a condition called oxidative stress. This is a deleterious condition which can bring about damages to biomolecules especially to proteins and DNA, culminating in cytotoxicity and mutations. Cancers and a variety of cardiovascular, neurological and pulmonary diseases are known to be induced by excess of free radicals. Free radicals can be counteracted by antioxidants. Antioxidants are produced either endogenously or supplied exogenously to equip the body to prevent and repair damages caused by oxidative stress (Pham-Huy et al., 2008). A brief outline on the deleterious effect of free radicals and their scavenging by antioxidants are represented in Fig. 1.

Lamiaceae has been a subject of extensive studies in terms of its antioxidant potential. Studies have shown that members of this family are potential source of natural antioxidants with elaborate free radical scavenging activity owing to their high polyphenol content (Tzima et al., 2018). Lamioideae, the second largest subfamily within Lamiaceae are reported to be rich in iridoid glycosides, phenylpropanoids and phenylethanoid glycosides. Caffeic and chlorogenic acids are the main free phenolic acids in this group (Zgórk & Głowniak, 2001). Presence of phenolic compounds may be taken as an indication for the antioxidant potential of its members. Since, not many

reports are available on the antioxidant property of *Gomphostemma*; this review will focus on notable antioxidant works in taxa closely related to *Gomphostemma* in Lamiioideae.

Fig. 1: Mechanism of free radical scavenging by antioxidants



Five medicinal plants from the subfamily Lamiioideae were tested for antioxidant activity and screened for their polyphenol content. Methanolic extracts of aerial parts of *Ballota nigra*, *Lamium maculatum*, *Leonurus cardiaca*, *Marrubium vulgare* and *Galeopsis tetrahit* were subsequently partitioned by liquid-liquid extraction between petroleum ether, dichloromethane, ethyl acetate and n-butanol. All 25 extracts and sub fractions were assayed for DPPH scavenging, hydroxyl dependent 2-deoxy-D-ribose degradation and phosphomolybdenum reduction. Total polyphenols

and free hydroxycinnamic acids were determined. All species possessed remarkable antioxidant capacity and the relative differences between species and fractions were attributed to the method of testing. The butanol fraction of *L. cardiaca* had a lowest EC₅₀ (4.45 µg/mL) for DPPH scavenging. The maximum inhibition of deoxyribose degradation was demonstrated for *B. nigra* ethyl acetate and butanol fractions with inhibition percentage of 79.32 ± 1.62 and 82.04 ± 2.28 respectively. Ethyl acetate fraction of *B. nigra* and butanol fraction of *L. cardiaca* also exhibited good reducing capacity of 318.6 ± 14.7 mg/g and 271.4 ± 2.4 mg/g ascorbic acid equivalents respectively (Matkowski et al., 2008).

Chew et al. (2012) evaluated methanolic extracts of root, flower, leaf and stem of *Leucas aspera* for their antioxidant, antimicrobial and cytotoxic activities. Antioxidant potential was determined by DPPH assay with BHT and vitamin E as positive controls. The root extract demonstrated strong antioxidant activity with IC₅₀ value of 6.552 µg/mL, whereas other extracts showed moderate activity.

Methanolic extracts of aerial flowering parts of *Stachys anisochila*, *Stachys beckeana*, *Stachys plumosa* and *Stachys alpina* ssp. *dinarica* were investigated for their total phenolic content and antioxidant activity. The extracts were studied for total antioxidant activity along with DPPH and hydroxyl radical scavenging activity as well as for lipid peroxidation. A high correlation was observed between total phenolic content and total antioxidant activity. All *Stachys* extracts, with the exception of *S. plumosa*, exhibited high activity. IC₅₀ values were 17.90 mg/mL, 20.90 mg/mL, 26.14 mg/mL and 101.61 mg/mL for *S. anisochila*, *S. beckeana*, *S. alpina* ssp. *dinarica* and *S. plumosa* extracts respectively. In the concentration range from 6.25 to 50 mg/mL, all extracts scavenged hydroxyl radical above 40% with maximal inhibitions for *S. anisochila*, *S. alpina* ssp. *dinarica* and *S. beckeana* extracts

with 50.22%, 50.94% and 64.97% respectively. As for lipid peroxidation, IC₅₀ values for *S. beckeana* and *S. alpina* ssp. *dinarica* extracts were 25.07 and 49.00 mg/mL respectively, while *S. anisochila* and *S. plumosa* extracts failed to reach 50% of lipid peroxidation inhibition (Kukić et al., 2006).

Many species of *Phlomis* are used in traditional Chinese and Turkish medicines. Keser et al. (2012) determined antioxidant capacities of *Phlomis pungens*. Aqueous and ethanol extracts of leaves and flowers were evaluated for ABTS radical scavenging, DPPH scavenging, superoxide anion radical scavenging, reducing activity and ferrous metal chelating activity. Flower water extract showed highest ABTS (90.6%), superoxide (98.3%) and metal chelating activity (23.43%) at 100 µg/mL. The leaf water extract was efficient in DPPH scavenging activity with a scavenging effect of 77.19% at 100 µg/mL.

Vijayalakshmi and Ranganathan (2011) evaluated the antioxidant potential of ethyl acetate extract of *Anisomeles malabarica* leaves using various assays. It was observed that the extract effectively scavenged hydroxyl and superoxide anion radicals with IC₅₀ of 93.61 µg/mL and 82.92 µg/mL respectively. It also scavenged DPPH radicals with an IC₅₀ of 294.15 µg/mL and ABTS radicals with an IC₅₀ of 91.06 µg/mL. The result thus indicated that *A. malabarica* extract could be considered as a potential source of natural antioxidants.

Oyedemi and Afolayan (2011) examined the antioxidant activity of aqueous extract of leaves of *Leonotis leonurus* by evaluating the reducing power, DPPH, ABTS and nitric oxide scavenging activity. The reducing power was observed to be concentration dependent. The plant extracts (0.8 mg/mL) efficiently scavenged DPPH, ABTS and nitric oxide with inhibition percentages of 72.48, 78.02 and 70.43% respectively. The result thus substantiated the ethno-therapeutic usage of this plant.

D. Anti-inflammatory studies

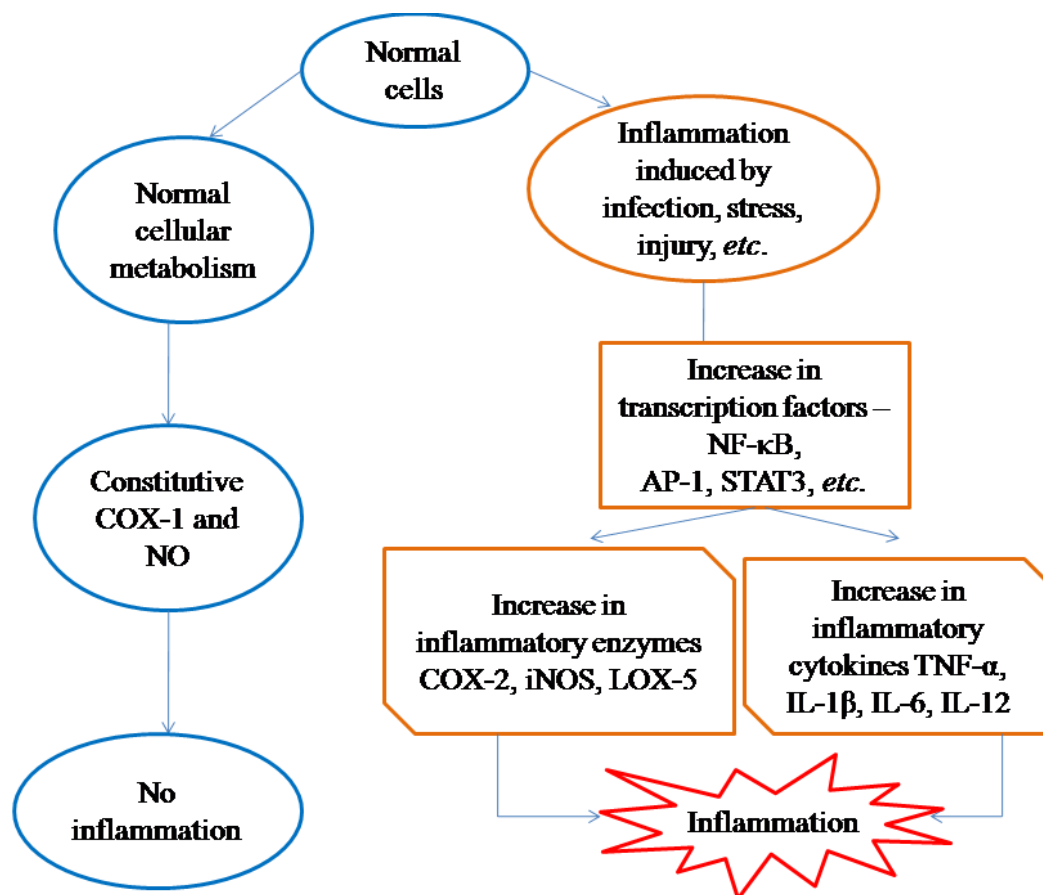
Inflammation is regarded as a normal response of living cells to stimuli like injury or infections (Rankin, 2004). Inflammatory responses are classified as acute and chronic depending on the duration. Acute inflammatory responses are usually short lived and are triggered by stimuli like microbial infections. It is resolved by macrophages and mast cells along with inflammatory mediators including chemokines, cytokines, vasoactive amines, eicosanoids and products of proteolytic cascades. The immediate effect of this cascade is that plasma proteins and leukocytes mainly neutrophils, that are normally restricted to the blood vessels now gain access to the extravascular tissues at the site of infection or injury. The neutrophils attempt to kill the invading agents by releasing the toxic contents of their granules, which include reactive oxygen species and reactive nitrogen species, proteinase, cathepsin G and elastase. These are highly potent effectors and do not discriminate between microbial and host targets. A successful acute inflammatory response eliminates infectious agents followed by a resolution and repair phase, which is mediated mainly by tissue-resident and recruited macrophages. If the acute inflammatory response fails to eliminate the damage, the neutrophil infiltrate is replaced with macrophages finally leading to chronic inflammation (Medzhitov, 2008).

Tissue macrophages are cells bestowed with the ability to initiate acute phase response and have a crucial role in chronic inflammation. Acute phase response is a complex but early and unspecific reaction of living organisms to a variety of injuries and infections. The local reaction is manifested as acute inflammation which is usually beneficial to restore homeostasis. But, excess of inflammatory mediators can bring about lethal side effects. As a part of acute phase response, activated macrophages release a wide spectrum of mediators like free radicals, prostaglandins and cytokines. Free radicals

include peroxide and nitric oxide (NO). The major cytokines released are TNF- α , IL-1, IFN γ , IL-6 and IL-8 (Koj, 1996).

A variety of chemical mediators contribute to inflammatory responses. It includes vasoactive amines, eicosanoids, leukotrienes, prostaglandins and cytokines. Arachidonic acid, which is a main component of membrane phospholipids in all cells, is one of the most important substrates in the synthesis of eicosanoids (Abdulkhaleq et al., 2018). Apart from these mediators, the nitric oxide (NO) induced by iNOS, enzymes like MPO and transcription factors especially NF- κ B are known to play a crucial role in inflammatory response (Laroux et al., 2001). A brief representation of the inflammatory cascade is depicted in Fig. 2.

Fig. 2: Mechanism of inflammatory cascade

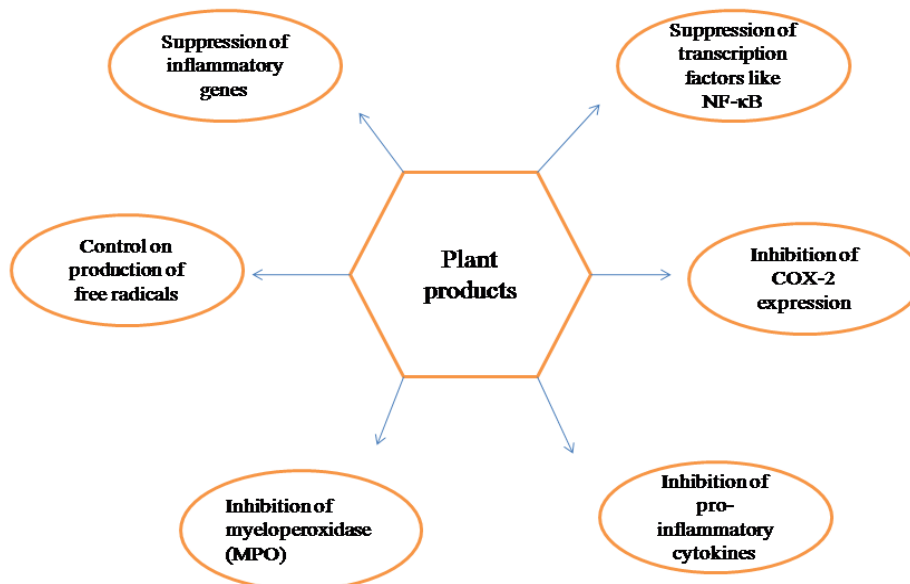


Transcription factors are specific proteins which are involved in enhancing gene expression by interacting with matching sequences in the promoter region of DNA molecule. Transcription factors such as nuclear factor kappa-B (NF- κ B), activator protein-1 (AP-1), signal transducer and activator of transcription (STAT), *etc.* are involved in the expression of a variety of proteins controlling inflammatory response. NF- κ B is present in the cytosol of many cell types. It exists as a heterodimer composed of p50 and p65 subunits, held in inactive state by I κ B inhibitory subunit (Grimm & Baeuerle, 1993). NF- κ B activation occurs via two major pathways: the canonical and the non-canonical signalling pathways. Activation of NF- κ B results in the induction of a variety of inflammatory, developmental and survival genes. The rapid and reversible inflammatory and immune response typically occurs through the activation of canonical pathway, while the slower and irreversible developmental response typically occurs through the non-canonical pathway (Shih et al., 2011). The canonical pathway mediates the activation of NF- κ B1 p50, RELA (also known as p65) and c-REL, whereas the non-canonical NF- κ B pathway selectively activates p100-sequestered NF- κ B members, predominantly NF- κ B2 p52 and RELB (Sun, 2017). In canonical pathway, upon stress-induced phosphorylation and subsequent ubiquitination, I κ B is degraded and the heterodimer p50/p65 is translocated to nucleus where it binds to specific DNA sequences and stimulates the transcription of corresponding genes (Karin & Delhase, 2000). AP-1 refers to a family of protein dimers, which after phosphorylation enter the nucleus and upon binding to consensus sequences enhance the expression of appropriate genes (Karin et al., 1997).

Herbal remedies which include plant extracts and plant derived compounds have a rich tradition of being used for treating many inflammation related disorders. Studies have shown that many natural chemicals such as phenols, alkaloids, glycosides and carbohydrates are responsible for this

bioactivity (Bodîrlău et al., 2009). Some of the possible anti-inflammatory targets of plant natural products are shown in Fig. 3.

Fig. 3: Anti-inflammatory targets of plant derived products



A study was conducted by Stalinska et al. (2005) using transcription factors as targets of anti-inflammatory treatment in cell cultures treated with extracts from some Mediterranean diet plants. Human hepatoma (HepG2) and human umbilical vein endothelial cells (HUVEC) were used as model systems. NF-κB and AP-1 were activated by cytokine IL-1 in the absence and presence of twenty one selected plant extracts and the effect was evaluated by electrophoretic mobility shift assay (EMSA). The extracts from *Scandix australis* and *Artemisia alba* clearly inhibited the activation of NF-κB in two cell types studied, whereas extracts from *Amaranthus* sp., *Eryngium campestre*, *Thymus pulegioides* and *Reichardia picroides* elicited cell-type dependent response. The IL-1 induced activation of AP-1 in HUVEC was more potently diminished by the extracts from *Scandix australis*, *Amaranthus* sp. and *Artemisia alba*, while extracts from *Urospermum picroides* and *Scandix pecten-veneris* affected HepG2 cells. Inhibitory activities of plant

extracts towards cytokine activated NF- κ B and AP-1 seemed to be depending on the order of addition of IL-1 and plant extract to the cell culture.

Gomphostemma parviflorum var. *parviflorum* was analysed for its anti-inflammatory activity (Shams-Ud-Doha et al., 2013). Leaves and roots were extracted with ethanol and anti-inflammatory activity was investigated using carrageenan-induced paw edema in rats. Ethanolic extract of leaves significantly and dose-dependently reduced edema in rats with inhibition percentage of 19.85 and 35.54% at a dose of 200 and 400 mg/kg body weight respectively. The rate of anti-inflammatory activity of leaf extract (400 mg/kg) increased with time and reached peak level at fourth hour of the study which was comparable to that of standard drug diclofenac sodium with 40.26% of inhibition. The ethanolic extract of roots also significantly protected the rats against carrageenan-induced edema with 23.06 and 28.17% inhibition of edema after fourth hour of carrageenan injection at a dose of 200 and 400 mg/kg body weight respectively.

Different extracts of *Leucas zeylanica* were investigated to determine the anti-inflammatory and anti-gout potential by measuring the inhibition of 5-lipoxygenase (5-LO), microsomal prostaglandin E2 synthase (mPGES)-1 and xanthine oxidase activity. Potent suppression of 5-LO activity in neutrophils was observed for dichloromethane extract and n-hexane extract, whereas extracts prepared with methanol were found to be less effective. More detailed concentration-response studies using this cell-based assay revealed an IC₅₀ value of 5.5 μ g/mL for dichloromethane extract. The effect of extracts was also evaluated on a cell-free assay using isolated human recombinant 5-LO as enzyme source. Again, the dichloromethane and n-hexane extracts efficiently blocked 5-LO activity while methanol extract was much less efficient. The dichloromethane extract showed concentration-dependent inhibition of 5-LO activity with IC₅₀ of 2.2 μ g/mL. The

dichloroethane extract also inhibited the microsomal prostaglandin E synthase-1 derived from human A549 cells with an IC₅₀ value of 0.4 µg/mL. Among the extracts tested, only the dichloromethane extract of *L. zeylanica* exhibited pronounced suppression of human xanthine oxidase activity in a cell-free assay with more than 50% enzyme inhibition (Napagoda et al., 2018).

Rao et al. (2009) isolated chemical constituents from the whole plant of *Anisomeles indica* and investigated their anti-inflammatory activity. HPLC purification procedures led to the isolation of one cembrane-type diterpenoid, two benzenoids, five flavonoids and six phenyl propanoids. The isolated compounds were examined for their inhibitory effects on LPS stimulated macrophages. Among these, diterpenoid compound exhibited potent inhibition on NO, TNF and IL-12 production at a concentration of 5 µM, followed by other compounds. The study thus concluded that these potent compounds from *A. indica* have the potential to be used in anti-inflammatory therapeutics.

The anti-inflammatory bioactivity of compounds isolated from aqueous-methanolic extract of *Lamium album* was screened in human neutrophils by Czerwińska et al. (2018). The effect of compounds on inhibition of selected inflammatory markers released by neutrophils such as cytokines (IL-8, TNF-α) was studied. The level of cytokine production after incubation with the compounds was measured by ELISA. Two new derivatives of quercetin were isolated in this study. Among the compounds analysed, phenylpropanoids (verbascoside and phlinoside D), iridoids and flavonoids were found to be significant inhibitors of IL-8 secretion as compared to their activity on TNF-α. The compounds at a concentration of 25 µM inhibited secretion of IL-8 in the range from 29.1 to 50.0%. The compound verbacoside is also known to inhibit iNOS activity by reducing

NF- κ B activation and nuclear translocation leading to a decreased concentration of pro-inflammatory mediators (Speranza et al., 2010).

Polyphenolic rich extract of *Stachys officinalis* was investigated to determine the effect on lipoxygenases, cyclooxygenase-1 and cyclooxygenase-2 activity. Significant inhibitory activity was obtained for lipoxygenases (IC_{50} - 1.22 μ g/mL), cyclooxygenase-1 (IC_{50} - 10.1 μ g/mL) and cyclooxygenase-2 (IC_{50} - 1.2 mg/mL). Further, *in vivo* studies also proved the anti-inflammatory effect of *S. officinalis* (Paun et al., 2018).

Chemical profiling and *in vitro* testing of biological activities with *Phlomis thapsoides* resulted in the isolation of one new compound called 6, 10, 17-trimethyl-2-octadecanone and three known compounds (sitosterol-3-O- β -glucoside, ipolamiide and lamiide). In anti-inflammatory studies using isolated compounds, lamiide was found to inhibit 5-lipoxygenase with an IC_{50} value of 72.92 μ g/mL. The anti-inflammatory activity was also evaluated with *in silico* molecular modelling method. Sitosterol-3-O- β -glucoside and the iridoid glucoside lamiide demonstrated a good inhibition of 5-lipoxygenase. The new compound 6, 10, 17-trimethyl-2-octadecanone was shown to inhibit 5-lipoxygenase activating protein as evidenced from its higher fitting scores (Sobeh et al., 2016).

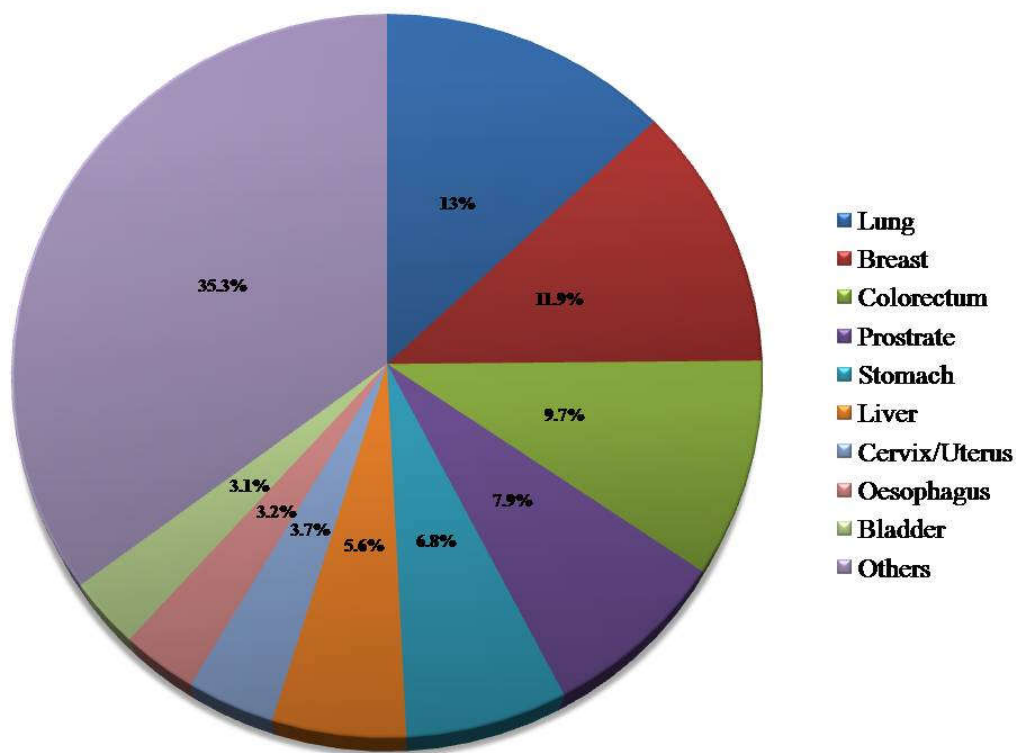
A study was designed by Li et al. (2015) to investigate the anti-inflammatory effect of phlomisioside F, a diterpene glycoside isolated from *Phlomis younghusbandii*. Anti-inflammatory effects were evaluated by using carrageenan-induced rat paw edema test, dimethylbenzen-induced ear edema test, acetic acid-induced vascular permeability and cotton pellet granuloma test. Furthermore, ELISA was used to determine the release of pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β). To explore the mechanisms, quantitative real-time PCR and western blotting assays were employed to measure the expressions of iNOS and COX-2. The expressions

of nuclear p65, cytosolic p65, I κ B, p38, p-p38, p-ERK1/2, ERK1/2, JNK and p-JNK were also determined by western blotting assay. The results indicated that phlomiside F administered orally could not only decrease rat paw edema in rats and ear edema in mice, but also reduced the vascular permeability and granuloma weights in rats. Further, *in vitro* studies with LPS-induced RAW 264.7 cells, upon treatment with phlomiside F, a significant reduction in pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β) and enzymes (iNOS and COX-2) was noted. In addition, the treatment also reduced the production of nuclear p65, p38, p-p38, p-ERK1/2, JNK and p-JNK proteins in LPS-induced RAW 264.7 cells. It was thus suggested that the compound is a promising potential anti-inflammatory drug capable of down-regulating the expressions of pro-inflammatory cytokines and mediators by inhibiting the NF- κ B/MAPK pathways.

E. Anticancer studies

Cancer is not a single disease but encompasses a cluster of diseases characterized by an autonomous proliferation of neoplastic cells that have a variety of alterations as well as mutations and genetic instability (Meza-Junco et al., 2006). It is the uncontrolled growth of abnormal cells in the body with potential to spread to other parts of the body. Cancer is caused by exposing human cells to undesirable chemicals, drugs, foods, and agents including ultraviolet light and radiation. Cancer is the result of a chemically damaged cell's fight for survival. Over hundred different cancers affect humans and some are deadly (Cole & Kramer, 2015). Fig. 4 represents estimated world cancer incidence proportions in percentage by major sites.

Fig. 4: An estimated percentage of world cancer incidence proportions by major sites



Source: (Stewart & Wild, 2014)

There are several methods for the treatment of cancer like surgery, radiotherapy, immunotherapy, chemotherapy, photodynamic therapy, stem cell transformation, *etc.* They are often accompanied by severe side effects. Chemotherapeutic agents are usually cytostatic and cytotoxic drugs and side effects include neutropenia, sensory neuropathy, diarrhoea, cardiotoxicity, *etc.* One of the harmful side effects of chemotherapeutic drugs like melphalan, carboplatin, cisplatin and cyclophosphamide is that, apart from destroying the cancer cells they also affect the normal cells (Iqbal et al., 2017). Development of an effective anticancer therapy without side effects is the major trend in pharmaceutical research. Bioactive phytochemicals are preferential, as they are pleiotropic in their functions which enable them to target the multiple signalling events in cancer cells in a more effective manner

with fewer side effects. Hence, phytochemicals are the most suitable candidates for anticancer drug development. Many phytochemicals have manifested their anticancer potential both *in vitro* and *in vivo*. Some of them are andrographolide from *Andrographis paniculata*, ashwagandhanolide from *Withania somnifera*, betulinic acid from *Betula utilis*, curcumin from *Curcuma longa*, eugenol from *Ocimum sanctum*, vinblastine and vincristine from *Catharantus roseus*, gingerol from *Zingiber officinale*, and many more (Singh et al., 2016).

• Cytotoxicity

The ability of a new compound or plant extract to induce cytotoxicity is a part of screening programme to identify the anticancer potential of the agent being tested. Plant based assays are important tools for screening toxicity in environmental, pharmaceutical and cosmetic risk assessment. The most frequently used plant models are *Allium cepa*, *Vicia faba*, *Zea mays*, *Tradescantia*, *Nicotiana tabacum*, *Crepis capillaris* and *Hordeum vulgare* (Khanna & Sharma, 2013). Higher plants are preferred over animal based bioassays due to their sensitivity to detect toxic agents and the procedure is inexpensive. They also offer evaluation on the basis of a number of genetic endpoints, ranging from point mutations to DNA damage. The chromosome assays on plant models are rapid and inexpensive. (Patlolla , 2013).

Among the different plant models, *Allium cepa* is considered as an efficient test organism to identify the presence of mutagenic chemicals. Large and countable chromosomes with high proportion of cells in mitotic stage make *A. cepa* root tips a suitable model for screening cytotoxicity. The growing root tips of *A. cepa* is useful for evaluating DNA damages that are expressed as chromosomal aberrations, disturbances in mitotic cycle, nuclear alterations and micro nuclei in meristem cells. The decrease in the mitotic index (MI) of *A. cepa* meristem cells can be considered as a reliable method

to determine the presence of cytotoxic agents. Changes in chromosomal structure or in total number come under the category of chromosomal aberrations. Chromosomal aberrations such as chromosome bridges and breaks are indicators of a clastogenic action. The numerical aberrations like aneuploidy and polyploidy are consequences of abnormal segregation of chromosomes, which can occur either spontaneously or by the action of aneugenic agents. Chromosome without telomeres become sticky and may fuse with other broken chromosome ends resulting in chromosomal rearrangements like acentric fragments, dicentric bridges or micronuclei (Nefic et al., 2013). A short list of cytotoxic studies of different lamiaceous plant extracts using *A. cepa* assay are shown in Table 3.

Table 3: A summary of cytotoxic studies of different lamiaceous plant extract using *Allium cepa* assay

Sl No.	Plant	Assay	References
1.	<i>Ocimum gratissimum</i>	Genotoxicity	(Borooah, 2011)
2.	<i>Origanum vulgare</i>	Cytotoxicity	(Dragoeva et al., 2014)
3.	<i>Rosmarinus officinalis</i>	Cytotoxicity	(Cardoso et al., 2014)
4.	<i>Pogostemon auricularius</i>	Cytotoxicity	(Anjana & Thoppil, 2013)
5.	<i>Hyptis suaveolens</i>	Cytotoxicity	(Sharma et al., 2019)
6.	<i>Ocimum basilicum</i> , <i>Leucas zeylanica</i>	Genotoxicity	(Sinha & Kumar, 2014)
7.	<i>Hyssopus officinalis</i>	Cytotoxicity, genotoxicity	(Dragoeva et al., 2010)
8.	<i>Lavandula stoechas</i>	Cytotoxicity, genotoxicity	(Çelik & Aslantürk, 2007)
9.	<i>Isodon coetsa</i>	Cytotoxicity	(Neelamkavil & Thoppil, 2014)
10.	<i>Hyptis suaveolens</i> , <i>Leucas indica</i>	Genotoxicity	(Sumitha & Thoppil, 2016)

● Antiproliferative activity

Lamiaceae is a plant family with myriad number of biologically active compounds and hence is a plausible source for effective anticancer drugs. Mechanisms involved in antiproliferative activity include cell cycle arrest, apoptosis and DNA repair mechanisms. Table 4 represents some relevant works with selected members of Lamiaceae on different cell lines.

Since studies on anticancer activity with *Gomphostemma* are scarce, notable anticancer studies with members of subfamily Lamioideae in Lamiaceae are reviewed here.

Table 4: Antiproliferative activity shown by some members of Lamiaceae on cell lines

Sl NO.	Plant	Cell lines	References
1.	<i>Ocimum tenuiflorum</i>	MCF-7, MDA-MB-231 and HS-27	(Lam et al., 2018)
2.	<i>Salvia chinensis</i>	MCF-7, A549, HCT-116, COLO 205 and MiapaCa-2.	(Zhao et al., 2015)
3.	<i>Mentha</i> spp.	Vero, Hep2 and HeLa	(Rahimifard et al., 2010)
4.	<i>Thymus kotschyanus</i>	A549 and HeLa	(Doosti et al., 2018)
5.	<i>Ocimum canum</i>	MCF-7	(Selvi et al., 2015)
6.	<i>Lavandula angustifolia</i>	A375 and MDA-MB-231	(Alexa et al., 2018)
7.	<i>Isodon rosthornii</i>	A-549, HL-60, MCF-7, SMMC-7721 and SW-480	(Zhan et al., 2013)

Stachys pilifera was examined for its cytotoxic effect and cell death mechanisms with methanolic extract as well as with its alkaloid and terpenoid

fractions on the HT-29 colorectal cell line. The HT-29 cells were cultivated and then incubated for 24 h with various concentrations in methanolic extract of *S. pilifera* and its fractions. Cell viability was measured by MTT assay. Morphology of the cells was evaluated with contrast microscopy. Further, the extract and fractions were tested on some major regulators of apoptosis like caspase-8, caspase-9, nuclear factor- κ B (NF- κ B) and nitric oxide (NO). Cisplatin was used as positive control. The estimated IC₅₀ values of the methanolic extract, alkaloid and terpenoid fractions and cisplatin against HT-29 cells after 24 h were found to be 612, 48.12, 46.44 and 4.02 μ g/mL respectively. Cells treated with the extract and fractions showed morphological changes such as plasma membrane blebbing, cell size reduction and apoptotic bodies. It was concluded that *S. pilifera* extract and its fractions inhibited NF- κ B and NO together with activation of caspase-8 and caspase-9 there by inducing apoptosis. These findings displayed the therapeutic potential of *S. pilifera* in the treatment of colon cancer (Kokhdan et al., 2018).

Khanavi et al. (2012) evaluated four species of *Stachys* viz., *S. laxa*, *S. trinervis*, *S. subaphylla* and *S. turcomanica* for their cytotoxic activity. Total extracts were obtained with methanol and then partitioned between chloroform, ethyl acetate and methanol. These fractions and total extracts were examined for their *in vitro* cytotoxic activity against colon carcinoma (HT-29), colorectal adenocarcinoma (Caco-2), breast ductal carcinoma (T47D) and Swiss mouse embryo fibroblast (NIH 3T3) cell lines using MTT assay. Doses of 3.125, 6.25, 12.5, 25, 100, 200, 400 and 800 μ g/mL in 1% (v/v) DMSO were tested. Highest cytotoxic activity (IC₅₀ < 50 μ g/mL) was displayed by the ethyl acetate and chloroform fractions of *S. laxa* against proliferation of T47D and HT-29 cell lines, whereas chloroform and ethyl acetate fractions were more effective towards T47D cell line alone. All other extracts except *S. trinervis* represented moderate cytotoxic activity (IC₅₀ < 70

µg/mL) on both cell lines. Fractions of *S. trinervis* demonstrated no effective cytotoxic activity. IC₅₀ values established that the growth and proliferation of HT-29 and T47D cells were most affected by chloroform and ethyl acetate fractions of *S. laxa* and *S. turcomanica* which may be attributed to the cytotoxic activity of nonpolar components in them.

A biological investigation of *Phlomis lanceolata* was carried out by Soltani-Nasab et al. (2014) to find out the *in vitro* cytotoxic activity of total extract and different fractions on four cell lines. Cytotoxic activity of total methanol extract and partition fractions of chloroform, ethyl acetate and petroleum ether of flowering aerial parts were examined with MTT assay on colon carcinoma (HT-29), breast ductal carcinoma (T47D), colonorectal carcinoma (Caco-2) and Swiss mouse embryo fibroblast (NIH 3T3) cell lines. Petroleum ether fraction showed highest cytotoxic activity against proliferation with IC₅₀ of 830.64 ± 37.13 µg/mL against HT-29, 263.16 ± 30.97 µg/mL against Caco-2, 238.40 ± 27.61 µg/mL against T47D and 175.52 ± 7.90 µg/mL against NIH 3T3. Presence of heavy triterpenes and lipophil compounds as recognized by TLC test in petroleum ether fraction were assumed responsible for high cytotoxic activity exhibited.

Anisomelic acid isolated from *Anisomeles malabarica* was tested for its cytotoxicity and apoptosis inducing potential in breast (MCF-7 and MDA-WIB-231) and cervical cancer (SiHA and ME-180) cell lines. The MTT assay indicated that the compound is cytotoxic to all of the four cell lines tested. Acridine orange, Ethidium Bromide and Hoechst 33258 staining of cells revealed typical apoptotic morphology such as condensed chromatin and formation of apoptotic bodies. The comet assay revealed DNA strand breaks indicating that the treatment with compound induced DNA damage which culminated in apoptosis. Thus, the study revealed the anti-proliferative and

apoptosis-inducing properties of anisomelic acid and its potential in cancer therapy (Preethy et al., 2013).

Vinayagam and Sudha (2014) evaluated *in vitro* cytotoxicity activity of acteoside isolated from the crude methanolic extract of *Leucas indica* flowers on MCF-7 cell lines. It revealed significant cytotoxicity in a concentration range between 1.0 µg/mL to 250 µg/mL as measured with MTT assay with IC₅₀ value of 7.7 µg/mL.

Mohan et al. (2017) assessed the anticancer activity of *Leucas aspera* nanoparticles and compared it with methanolic crude extract in PC3 prostate cancer cells. The *in vitro* cytotoxicity of *L. aspera* nanoparticles and crude extract measured by MTT assay for PC3 cells was concentration and time-dependent. Silver nanoparticles showed superior activity than crude extract. Furthermore, the anticancerous activity of *L. aspera* in prostate cancer was established with *in vitro* hemolysis assay, cellular uptake studies, cell aggregation studies and cell migration assays.

A comparative study on cytotoxicity between wild and *in vitro* cultured *Lamium album* was made by Veleva et al. (2014). Methanol and chloroform extracts were used for the study. Cytotoxicity was assessed on human lung cell line A549 and cervical cancer cell line HeLa with MTT assay. Genotoxic effect was measured with comet assay. For A549 cell line after 48 h treatment, IC₅₀ values for wild plants were 0.5 and 0.8 mg/mL for chloroform and methanol extracts respectively. The *in vitro* propagated plants showed less activity and IC₅₀ value was calculated as 0.8 and 0.9 mg/mL for chloroform and methanol extracts respectively. Cytotoxic effect on HeLa carcinoma cells after 48 h treatment showed IC₅₀ values as 1.65 and 1.00 mg/mL of methanol and chloroform extracts respectively. Genotoxic effect as measured by comet assay showed a mean 70% DNA damage in A549 cells and 50% in HeLa.

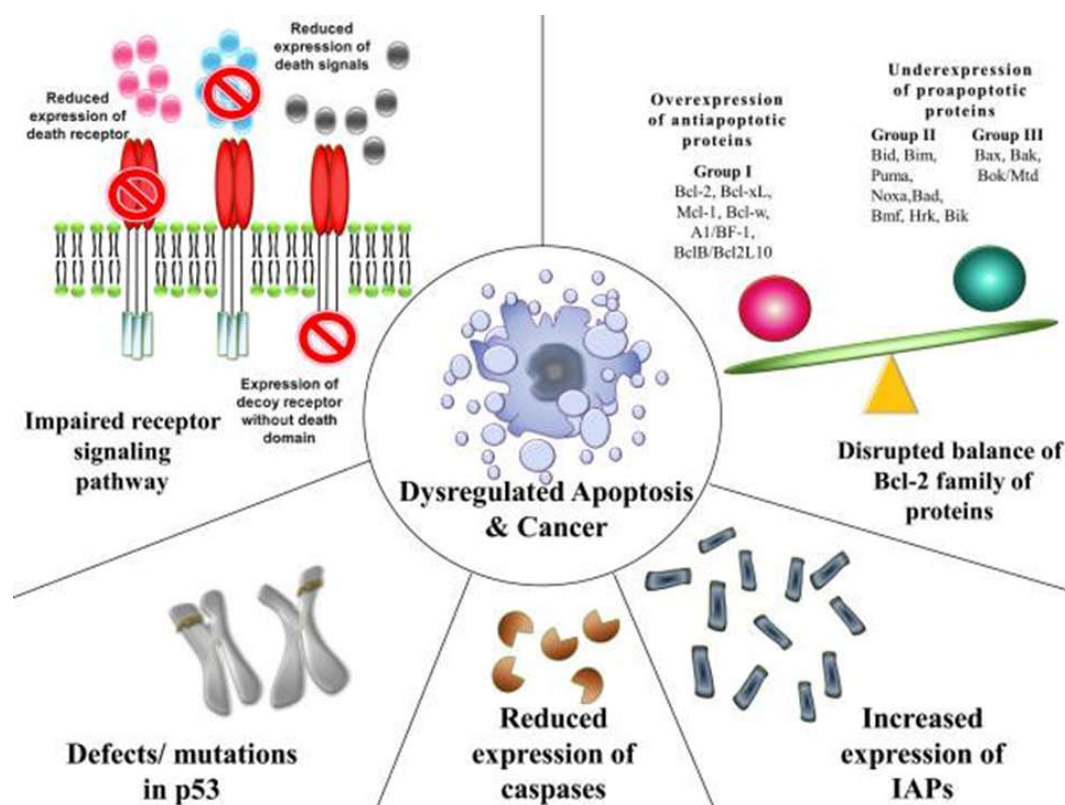
Veerabadran et al. (2013) investigated the antioxidant and antiproliferative potential of *Leonotis nepetifolia* leaves. The screening of antioxidant activity with chloroform, ethyl acetate and methanol extracts showed that methanol extract has a higher potential so that, antiproliferative activity was done with methanolic extract alone. MTT assay was performed on MCF-7 (Breast cancer cell line) and Hep-2 (Liver cancer cell line) cells. The MCF-7 cells showed a cell viability of 45.2% at 1.25 mg/mL and for Hep-2 cells it was 56.25%. Further, DNA fragmentation studies also revealed significant genotoxic effect induced by the plant extract on both cell lines.

Rai et al. (2016b) investigated the cytotoxicity of *Pogostemon heyneanus* leaves with methanol, ethanol and chloroform extracts in MCF-7 and MDA-MB-231 cell lines by MTT assay. The results showed that chloroform extract of *P. heyneanus* was effective against both the cell lines. The IC_{50} values against MCF-7 and MDA-MB-231 cells were found to be 56.66 ± 0.88 μ g/mL and 152.0 ± 0.75 μ g/mL respectively.

● Apoptosis

Apoptosis is defined as a form of programmed cell death. It is one of the most researched topics in cell biology. Cancer disease results from a condition where there is a loss of balance between cell division and cell death. It is a condition when cells that should have died did not receive the signals to do so. Defect that occurs at any point in the mechanism of apoptosis can lead to cancer. The possible cellular mechanisms leading to evasion of apoptosis and carcinogenesis are shown in Fig. 5. There are two initiation pathways for apoptosis: extrinsic pathway and intrinsic pathway. Both these pathways ultimately lead to a common execution pathway. A third lesser known initiation pathway is intrinsic endoplasmic reticulum pathway (Wong, 2011).

Fig. 5: Possible cellular mechanisms leading to evasion of apoptosis and carcinogenesis



Source: (Wong, 2011)

● Extrinsic pathway

Extrinsic pathway is initiated by death receptors which are members of the tumour necrosis factor (TNF) receptor gene super family that consists of more than 20 proteins. All of them are endowed with a broad range of biological functions including regulation of cell death and survival. The TNFRs are type I transmembrane proteins possessing 2 - 4 similar cysteine-rich domains in the extracellular portion. In addition, death receptors are defined by a cytoplasmic domain of about 80 amino acids called 'death domain', which play a crucial role in transmitting the death signal from the cell's surface to intracellular signaling pathways. The best-characterized death

receptors that have been identified to regulate apoptosis either directly or indirectly are TNFR1 (TNFRSF1A), Fas/Apo1/CD95 (TNFRSF6), DR3 (TNFRSF25), DR4 (TNFRSF10A), DR5 (TNFRSF10B) and DR6 (TNFRSF21). Of these, Fas, DR4 and DR5 primarily mediate apoptosis, although they can trigger alternative signaling pathways. The corresponding ligands of the TNF superfamily comprise death receptor ligands such as TNF- α that bind to TNFR1, CD95 ligand (CD95L), Apo3L/TWEAK for DR3, TRAIL/Apo2L for DR4 and DR5 and TRADD for DR6 (Fulda & Debatin, 2006; Nair et al., 2014). Death receptors that directly signal apoptosis activation namely Fas, DR4 and DR5 trigger the cell-extrinsic apoptotic cascade by forming a death-inducing signaling complex (DISC), which most often contains the adaptor protein FADD and the apoptosis-initiating protease caspase-8 and -10. Further recruitment of these multiple zymogen molecules results in cross-activation and autocatalysis, leading to the generation and release of active caspase molecules into the cytoplasm (van Raam & Salvesen, 2012).

● **Intrinsic pathway**

The intrinsic apoptotic pathway is a mitochondria-dependent pathway. It is mediated by intracellular signals that converge at the mitochondrial level in response to different stress conditions. External stimuli like irradiation, treatment with chemotherapeutic agents or internal stimuli such as irreparable genetic damage, hypoxia, extremely high concentrations of cytosolic Ca^{2+} ions and severe oxidative stress are some of the triggers involved in the initiation of the intrinsic mitochondrial pathway. Subsequent activation of pro-apoptotic BH3-only members of the Bcl-2 family (Bax, Bak) neutralizes the antiapoptotic proteins Bcl-2, Bcl-xL and Mcl-1 which leads to the disruption of outer membrane permeability of mitochondria (MOMP). The result is proteins which include the apopto-genetic factors like cytochrome-*c*

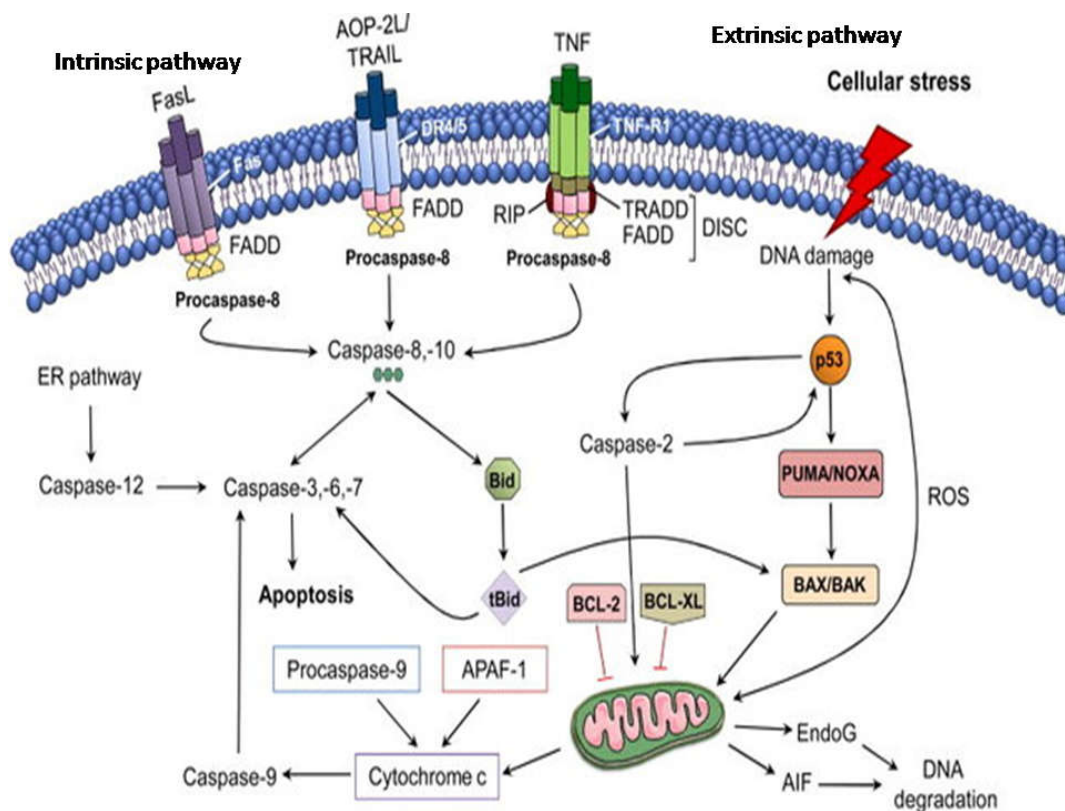
which is normally confined in the intermembrane space spread into the cytosol. Cytochrome-*c* binds to the cytosolic Apaf-1 (apoptosis protease activating factor-1) and triggers the formation of a complex named apoptosome, which in turn recruits initiator pro-caspase-9 to its caspase recruitment domain (CARD), allowing auto-activation and then proteolysis. The process consecutively activates downstream executor caspases-3, -6 and -7 for cleavage of cellular substrates leading to apoptotic cell death (Pistritto et al., 2016).

The extrinsic and intrinsic pathways of apoptosis converge on a common point of execution where caspases drive the terminal events of the cell cycle. Caspases are a family of proteins and one of the main effectors of apoptosis. About 14 mammalian caspases have been identified so far. They are synthesised as inactive zymogens with a prodomain followed by p20 (large) and p10 (small) subunits which are later cleaved to form active enzymes following the induction of apoptosis. Based on their function, the caspases can be classified into inflammatory caspases, apoptotic initiator caspases and apoptotic effector caspases. Inflammatory caspases include caspase-1, -4, -5, -11, -12, -13 and -14, which are involved in inflammation. Apoptotic initiator caspases are caspase-2, -8, -9 and -10 which mediate the interaction with upstream adaptor molecules. Apoptotic effector caspases are executioners and include class caspase-3, -6 and -7. Effector caspases are typically processed and activated by upstream caspases and perform the downstream execution steps of apoptosis by cleaving multiple cellular substrates (Jin & El-Deiry, 2005). Fig. 6 is a schematic representation of different pathways of apoptosis.

Various gene families such as caspases, B cell lymphoma (Bcl) -2 family of genes, tumour necrosis factor (TNF) receptor gene super family and p53 gene are involved and collaborated in the process of apoptosis.

Among which, p53 controls the expression of a large set of genes involved in significant cellular processes including DNA damage detection, cell cycle arrest, apoptosis, DNA repair and senescence. It has involvement in both intrinsic and extrinsic pathways of apoptosis. It has the ability to activate the intrinsic pathway of apoptosis by inducing the transcription of especially apoptotic Bcl-2 family genes such as PUMA. Mutations in p53 gene are considered as one of the most common genetic changes in cancer. Mutant p53 proteins not only lose their native tumour suppressor activity but also facilitate active tumour development (Kiraz et al., 2016).

Fig. 6: Different pathways of apoptosis



Source: (Ghavami et al., 2014)

Methods to detect apoptosis

The detection of apoptosis includes methods related to membrane alterations, DNA fragmentation, cytotoxicity, cell proliferation, mitochondrial damage, immunological detection and mechanism based assays (Banfalvi, 2017).

● MTT assay

Cell-based assays are used for screening compounds or extracts to determine if they have any impact on cell proliferation or to demonstrate direct cytotoxic effects that eventually lead to cell death. The tetrazolium reduction, resazurin reduction and protease activity assays measure certain features of general metabolism. An array of tetrazolium compounds have been used to distinguish viable cells. The principle of MTT assay is the conversion of water-soluble yellow dye MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to insoluble purple formazan by the action of mitochondrial reductase. The quantity of formazan formed is recorded by changes in absorbance at around 540 – 570 nm using a plate reading spectrophotometer. Viable cells with active metabolism alone can convert MTT into a purple coloured formazan product. When cells die, they lose the ability to convert MTT into formazan. Thus, colour formation serves as a useful and convenient marker of only viable cells (Kumar et al., 2018).

● Dual AO/EB staining to detect apoptosis

Dual acridine orange/ethidium bromide (AO/EB) fluorescent staining is used to identify apoptosis-associated changes of cell membranes occurring during the process of apoptosis which can be visualized under a fluorescent microscope. This method can accurately distinguish cells in different stages of apoptosis. Acridine orange can penetrate normal and early apoptotic cells with intact membranes and impart fluorescing green colour when bound to

DNA. Ethidium bromide can enter only to cells with damaged membranes, such as late apoptotic and dead cells, emitting orange-red fluorescence when bound to concentrated DNA fragments or apoptotic bodies (Liu et al., 2015).

- **Comet assay**

Comet assay is a reliable method to measure genotoxic effect and to monitor DNA damage in individual cells. Since apoptotic cells are characterized by fragmentation of DNA, comet assay can easily detect the extent of DNA damage produced after treatment (Gopalakrishna & Khar, 1995). The method is otherwise known as single cell electrophoresis assay. It is popularly called as comet assay as the image of the electrophoresed DNA appears like a comet, with undamaged DNA in the head and fragmented DNA migrating to form a tail. Comet images are captured with a fluorescent microscope as staining is done with fluorescent stains like propidium iodide (Husseini et al., 2005).

Lee and Steinert (2003), reviewed studies that used comet assay to determine DNA strand breaks using parameters like DNA tail moment, percentage of DNA in the tail, *etc.* and concluded it as a valid method to assess DNA damage and apoptosis.

- **Caspase activity**

Caspases are a family of cysteine proteases and are major regulators of apoptotic cell death. These enzymes are involved in initiation and execution of apoptosis. Thus, it can be used as a biochemical marker for identifying apoptosis induced by diverse stimuli. Determination of caspase activity and detection of caspase cleavage products can be performed using different methodological approaches. These include detection of caspase processing (electrophoresis, immunoblotting), analysis of enzymatic activity using labeled synthetic caspase substrates or inhibitors (e.g., spectrophotometry,

fluorimetry, chemiluminescence techniques, ELISA) or detection of the target caspase substrate cleavage (e.g., immunoblotting). The choice of the method depends on several factors including the experimental system used, cell type, *etc.* (Vaculova & Zhivotovsky, 2008).

Saunders et al. (2000) has described an enzyme-linked immunosorbent assay (ELISA) for quantifying relative amounts of active caspase-3 in apoptotic cells. Covalent modification of caspase-3 active sites with a biotinylated inhibitor differentiates active from latent caspases. An ELISA plate with an antibody specific for caspase-3 helps for the capture and makes the assay specific for caspase-3. Detection is with horseradish peroxidase (HRP)-conjugated streptavidin that binds to the biotinylated inhibitor covalently bound to caspase-3. The assay detected 6.6 ng active caspase-3 per 10^6 apoptotic staurosporine-treated jurkat cells. Specificity of the assay for caspase-3 was established by lack of signals with purified caspases-2, -7, -8, and -10 that were modified by a biotinylated inhibitor. Specificity was also demonstrated by lack of signals with apoptotic MCF-7 cells which do not express caspase-3.

● Flow cytometry

Flow cytometry is a preferred platform for rapid assessment of multiple apoptotic attributes at a single cell level. The major advantages of flow cytometry include the possibility of multiparameter measurements, single cell analysis and rapid analysis. Flow cytometry thus over comes the problems of traditional bulk techniques such as fluorimetry, spectrophotometry or gel techniques. There are a handful of commonly used cytometric assays based on the assessment of mitochondrial transmembrane potential, caspase activation, plasma membrane remodelling and DNA fragmentation. Fragmented and low molecular weight DNA can be extracted during the process of cell staining in aqueous solutions. Such extractions are

made when cells are treated with precipitating fixatives such as ethanol or methanol. As a result of DNA extraction, apoptotic cells exhibit a deficit in DNA content and following staining with a DNA-specific fluorochrome they can be recognized by flow cytometry as cells having fractional DNA content. On frequency distribution histograms they are characterized by a distinctive “sub-G1” peak that represents oligonucleosomal DNA fragments. The sub-G1 peak represents apoptotic cells and are seen separated with little or no overlapping from the G1 peak of the non-apoptotic cell population (Wlodkowic et al., 2009).

Lu et al. (2016) determined the effect of phlomisioside F, a diterpene glycoside from *Phlomis younghusbandii* on the proliferation, migration and invasion of human non-small cell lung cancer cell line A549. The compound exhibited significant anti-proliferative effect on A549 cells in concentration-dependent and time-dependent manners, with half maximal inhibitory concentration (IC₅₀) of 54.51 μ M. Treatment with different concentrations (10, 20 and 40 μ M) for 48 h resulted in significantly decreased migration and invasion in A549 cells. In addition, concentrations of 25, 50 and 75 μ M induced cell cycle arrest in G0/G1phase and enhanced cell apoptosis. Furthermore, caspase-3, caspase-9 and Bax protein expressions were up-regulated while Bcl-2 and COX-2 protein expressions were found significantly down-regulated.

● Gene expression studies

Gene expression refers to a process by which functional products like proteins are produced from the information encrypted in a gene. The expression studies are aimed to detect and quantify messenger RNA (mRNA) levels of a specific gene. Many novel techniques are available for the quantification of RNA like qPCR, expression microarrays and RNAseq for the transcriptome analysis. Conventional polymerase chain reaction (PCR) is

a technique that amplifies a specific segment of DNA to obtain hundreds of millions of copies in few hours. Nowadays, real-time PCR or quantitative Polymerase Chain Reaction (qPCR) is employed in gene expression studies, as both amplification and detection is possible simultaneously. There are two methods to quantify the expression of a gene under study. They are absolute quantification and relative quantification. In the first case, the absolute expression amount is expressed as number of copies obtained. In the second, the quantification is based on a calibrator, whose value is benchmark for all others, assigning it a value of one. The microarrays technique is based on complementarity between nucleic acid strands allowing detection of specific sequences by hybridization. In the field of gene expression, it could study between 500 and 18,000 cDNAs. The transcriptome and RNA sequencing is yet another method for gene expression studies. The transcriptome is a set of ribonucleic acids in the cell, including messenger ribonucleic acid, transfer ribonucleic acid, ribosomal ribonucleic acid, small nuclear ribonucleic acid and others. The interpretation of transcriptome is however complex, as it needs extensive building of cDNA libraries with massive sequencing system and efficient analysis of data with bioinformatics tools (San Segundo-Val & Sanz-Lozano, 2016).

A polysaccharide fraction extracted from the rhizomes of *Stachys floridana* by hot water extraction followed by its sequential purification was found to inhibit the proliferation and growth of human colon cancer HT-29 cells in a duration and dose-dependent manner. It was found to induce apoptosis and flow cytometric studies showed increased accumulation in G₂/M phase of HT-29 cells. Furthermore, gene expression studies with RT-qPCR showed that it could decrease Bcl-2 mRNA level and increase the mRNA expressions of Bax and p53. Spectrophotometric analysis showed a significant increase in the activity of caspase-3. These results indicated that the polysaccharide fraction induced apoptosis in HT-29 cells through

regulation of apoptosis-associated gene expressions such as Bcl-2, Bax and p53 with the activation of caspase-3 (Ma et al., 2013).

F. Synthesis of silver nanoparticles

Nanotechnology is that branch of technology that deals with nano-meter size objects. Nanoparticles are the engineered materials with atomic dimensions in nano range (1-100 nm). They are classified into different classes based on their properties, shapes or sizes. The different groups include carbon based, metal, ceramic, semiconductor, lipid based and polymeric nanoparticles. The high surface area and nanoscale size impart unique physical and chemical properties to nanoparticles. Their optical properties are dependent on the size, which imparts different colours due to absorption in the visible region. Their reactivity, toughness and other properties are also dependent on their size, shape and structure (Gross et al., 2016). Due to these characteristics, they are suitable for various pharmaceutical, commercial and domestic applications. Two main approaches for the synthesis of nanoparticles are bottom-up method and top-down synthesis. The top-down approach involves decreasing the size of a large uniform piece of material to create the required nanostructures from it. This method is much used in the field of electronics. However, it poses some difficulties to generate uniform nanoparticles and there are chances for internal stress, structural defects and contaminations. The bottom-up approach arranges the basic building blocks such as atoms or molecules into large nanostructures using chemical or physical forces. The growth and self-assembly of the building blocks allow designing of nanoparticles with more precise structures, shape, size and chemical composition as compared to the other approach (Su & Chung, 2018).

Among various nanoparticles, metal nanoparticles are the most promising ones and this is due to their unique properties contributed by the high surface to volume ratio. In recent years, silver nanoparticles have attracted a lot of attention due to their good conductivity, chemical stability

and their applications in various industries including medical sciences, food industries, electronics and mechanical industry and in environment management. Silver nanoparticles (AgNPs) are one of the most investigated and explored nanostructures mainly because of their promising characteristics suitable for various biomedical applications. Many silver nanoparticles have shown genuine characters and remarkable potential for the development of antimicrobial agents, drug-delivery formulations, diagnosis platforms, biomaterial coatings, tissue restoration and other therapeutic alternatives (Burdusel et al., 2018).

A wide range of physical and chemical approaches are available to produce nanoparticles with varying size, shape and composition. Conventionally, nanoparticles have been synthesised and stabilised by appropriate physical and chemical methods. The physical approach includes techniques like laser ablation, lithography and high-energy irradiation, whereas the chemical methods use techniques like chemical reduction and electrochemistry. Studies have shown that the size, shape, stability and physicochemical properties of nanoparticles are affected by various factors such as pH, temperature, concentrations, metal ion precursors, reducing agent, *etc.* Hence, one of the challenging aspects in synthesis of nanoparticles is the development of a protocol that can control the size, shape, stability and physicochemical properties of nanoparticles. Conventional chemical and physical processes are expensive and often use hazardous toxic materials. Some nanoparticles produced by these processes have been found to be toxic due to factors like composition, size, shape and surface chemistry. As a consequence, the presence of these toxic formation agents on the synthesised nanoparticles and potentially the nanoparticles themselves have prevented and limited their clinical and biomedical applications. All these factors can be potentially controlled to certain extend through biological mediated production. Hence, extensive efforts have been made to develop reliable, biologically compatible and environment-friendly green processes for the synthesis of nanoparticles (Shah et al., 2015).

Biosynthesis of nanoparticles using unicellular and multicellular biological entities such as actinomycetes, bacteria, fungi, plants, viruses and yeast is an environment-friendly green chemistry based approach. Biological entities have become excellent medium for nanoparticle synthesis with a wide range of sizes, shapes, compositions and physicochemical properties (Mohanpuria et al., 2008). As compared to microorganisms, the plant approach is more preferred, as it does not demand for complex multi-step procedures such as isolation, culture preparation and culture maintenance. Furthermore, synthesis with plants tends to be faster and is more cost-effective than with microorganisms (Iravani, 2011). Table 5 represents a brief list of biogenic synthesis of silver nanoparticles by various lamiaceous plants.

Table 5: A brief list of biogenic synthesis of silver nanoparticles by various lamiaceous plants

Sl NO.	Plant	Part used	Shape and size	References
1.	<i>Leucas aspera</i>	Leaves	Spherical, 22–43 nm	(Elumalai et al., 2017)
2.	<i>Anisomeles indica</i>	Leaves	Spherical, 18–35 nm	(Govindarajan et al., 2016)
3.	<i>Stachys lavandulifolia</i>	Sepals	Spherical, 7.08 nm	(Azizi et al., 2016)
4.	<i>Phlomis</i> sp.	Leaves	Spherical, 19–30 nm	(Allafchian et al., 2016)
5.	<i>Salvia leucantha</i>	Whole plant	Spherical, 15–20 nm	(Rajendran & Prabha, 2016)
6.	<i>Salvia spinosa</i>	Whole plant	Spherical to oval, 19–125 nm	(Pirtarighat et al., 2018)
7.	<i>Coleus forskohlii</i>	Root	Needle, 82.46 nm	(Baskaran & Ratha Bai, 2013)
8.	<i>Ocimum sanctum</i>	Leaves	Spherical, 18–25 nm	(Ramteke et al., 2012)

9.	<i>Origanum vulgare</i>	Aerial parts	Spherical, 2–25 nm	(Shaik et al., 2018)
10.	<i>Mentha piperita</i>	Leaves	Spherical, 20–50 nm	(Gabriela et al., 2017)

Plant extracts are rich in bioactive components like alkaloids, phenolic acids, polyphenols, proteins, sugars and terpenoids. These biomolecules are believed to play an important role in reducing the metallic ions and then stabilizing them. The synthesis of nanoparticles from reducing metal salts via plants is relatively a simple process that can occur in room temperature. The process involves mixing of plant extract with a metal salt solution which is followed by biochemical reduction of salts. The formation of nanoparticles is indicated by a change in the colour of reaction mixture. Synthesis process involves an initial activation period when metal ions are converted from their mono or divalent oxidation states to zero-valent states and nucleation of the reduced metal atoms take place (Malik et al., 2014). This is followed by a period of growth when smaller neighbouring particles amalgamate to form larger nanoparticles that are more stable while further biological reduction of metal ions takes place. As growth progresses, nanoparticles aggregate to form a variety of morphologies such as cubes, spheres, triangles, hexagons, pentagons, rods, *etc.* The plant extract's ability to stabilize the nanoparticle ultimately determines the most energetically favourable and stable morphology (Akhtar et al., 2013). Fig. 7 is a schematic representation showing the mechanism of synthesis of nanoparticles with plant extracts.

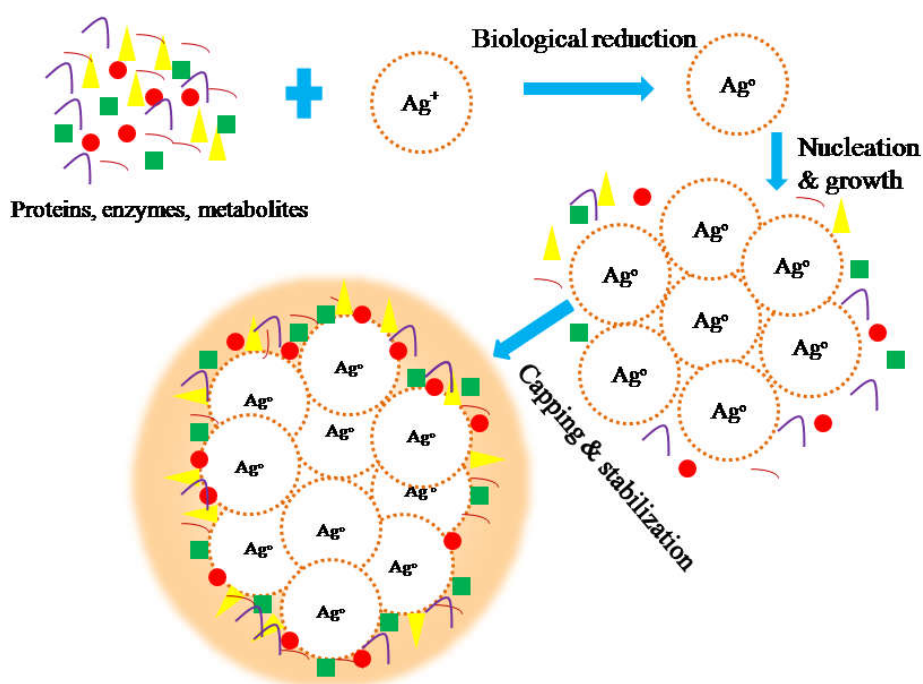
CHARACTERIZATION OF SILVER NANOPARTICLES

● UV-Visible spectroscopy

UV-Visible spectroscopy is a reliable technique to measure the surface plasmon resonance. Noble metal nanoparticles show surface plasmon

resonance (SPR) in the visible range as well as in the infrared range. The surface plasmon resonance phenomenon is due to the collective oscillation of free electrons of the metal nanoparticles in resonance with the frequency of light waves interacting with the metal nanoparticles. Basically, SPR absorption peak occurs in metal nanoparticle only. Hence, the existence of SPR peak is the primary signature of metal nanoparticle formation (Das et al., 2010).

Fig. 7: Mechanism of synthesis of silver nanoparticles with plant extracts



- **Fourier-transform infrared spectroscopy (FTIR)**

Fourier-transform infrared spectroscopy (FTIR) is a technique used to analyze the chemical composition of many organic chemicals, polymers, paints, coatings, adhesives, lubricants, semiconductor materials, coolants,

gases, biological samples and minerals. The IR spectra provide information about the local molecular environment of the organic molecules on the surface of nanoparticles. The FTIR spectral measurements help to identify the potential biomolecules responsible for reducing and capping the bio-reduced silver nanoparticles (Devaraj et al., 2013).

- **SEM and TEM analysis**

Scanning electron microscope (SEM) functions by imaging the sample surface by scanning it with a high-energy beam of electrons. It shows detailed images with high magnifications. But, since images are made without light waves they are black and white. The surface structure of nanocomposites, nanofibers, nanoparticles and nanocoatings can be imaged through SEM with high clarity. In contrast to SEM, Transmission electron microscopy (TEM) transmits a beam of electrons through an ultra thin specimen. The electrons transmitted through the specimen produce magnified image that is focussed by an objective on an imaging screen. It has application in both material science and biological science. TEM images can reveal the distribution and dispersion of nanoparticles and nanocoatings. The extent of intercalation and orientation of nanoparticles can be visualized using TEM micrographs (Joshi et al., 2008).

- **Energy Dispersive X-ray (EDX) analysis**

The Energy Dispersive X-ray (EDX) microanalysis is an elemental analysis technique combined to electron microscopy and is based on the generation of characteristic X-rays that can divulge the presence of elements present in the specimens. EDX analysis of silver nanoparticles is performed to confirm the presence of elemental silver, as well as its percentage compared to other elements in the nanoparticles (Scimeca et al., 2018).

- **Atomic Force Microscopy (AFM)**

Atomic force microscopy (AFM) is a scanning probe technique that can disclose a range of information about nanoparticles, the biomolecules and the nanoparticle-biomolecule interaction on a single particle basis. Unlike TEM and SEM, which are performed with conducting or semiconducting samples under vacuum conditions, AFM can be applied to nonconductive, wet and soft samples, allowing different types of materials to be analyzed in physiological environments. Samples can be analyzed using various scanning modes including static and dynamic mode, which provide information about various sample parameters including morphological information about the size, shape, *etc.* of the nanoparticles (Sapsford et al., 2011).

The literature survey confirms the importance of natural products in drug development. Even though much studies are available on many popular members of Lamiaceae, phytochemical and bioactivity studies with respect to the selected species of *Gomphostemma* appear to be meagre. Hence, the review suggests more exploration on *Gomphostemma* with emphasis on species endemic to Western Ghats to reveal their therapeutic potential.

MATERIALS AND METHODS

The present study has been designed to carry out phytochemical characterization and bioactivity screening of selected species of *Gomphostemma*. The protocols and experimental procedures adopted for the entire study are presented in this section. Phytochemical profiling was carried out by qualitative and quantitative estimation, as well as with GC/MS and HR-LC/MS techniques. Bioactivity screening mainly emphasised on the antioxidant, anti-inflammatory, cytotoxic and antiproliferative potential of selected species of *Gomphostemma*. Green synthesis of silver nanoparticles was also attempted.

1. PLANT MATERIALS

The plants selected for the present study were *Gomphostemma heyneanum* var. *heyneanum*, *Gomphostemma heyneanum* var. *rottleri* and *Gomphostemma eriocarpum* (Plate 1). Among these, *G. heyneanum* var. *heyneanum* and *G. heyneanum* var. *rottleri* were collected from Nelliampathy, Palakkad district, Kerala and *G. eriocarpum* was collected from Ranni forest area of Pathanamthitta district, Kerala. Plants were authenticated by Dr A. K. Pradeep, Assistant Professor, Department of Botany, University of Calicut and Dr K. M. Prabhukumar, Senior Scientist, Plant Systematic and Genetic Resources Division, Centre for Medicinal Plants Research, Arya Vaidya Sala, Kottakkal. Voucher specimens were deposited at the Herbarium (CALI) of Department of Botany, University of Calicut, Kerala, India. Taxonomic description of plant specimens selected for the study is given below:

- ***Gomphostemma heyneanum* Wall. ex Benth. var. *heyneanum* (CALI No. 123772)**

Habit: Subshrubs

Habitat: Ever green and moist deciduous forest

Flowering and fruiting: October - December

Distribution: Endemic to Southern Western Ghats

Description: Stem obtusely 4-angular, stellate tomentose. Leaves 19-25 x 1-15 cm, broadly elliptic, acute at both ends, stellate tomentose below, sparsely hairy above, crenate, nerves 5-7 pairs; petiole 4-6 cm long. Flowers in racemes 16-18 x 2-2.5 cm; flowers 10-20 together; bracts elliptic, acute; calyx lobes lanceolate; corolla 1.3-1.5 cm long, tube 7-8 cm broad, cylindric, midlobe of flower lip obtuse, emarginated; filaments unequal, glabrous. Nutlets glabrous (Narayanan, 2009).

- ***Gomphostemma heyneanum* var. *rottleri* Prain (CALI NO. 123773)**

Habit: Subshrubs

Habitat: Moist deciduous forest

Flowering and fruiting: August - December

Distribution: Endemic to Southern Western Ghats

Description: Stem sub-tetragonous and furrowed with ligneous base. Leaves 17 x 8 cm, elliptic-ovate, obtuse, base acute, crenate, subscabrous above and stellately pubescent below; lateral nerves 6-8 pairs; petiole 5-5.5 cm long. Flowers in racemes 5-8 cm long, floral leaves elliptic-ovate, 1.5 x 0.8 cm; bracts and bracteoles elliptic-lanceolate. Calyx tube 5 mm long, lobes ovate-

acute, 6 mm long. Corolla pale yellow, 2 cm long. Nutlets ovoid, orange-yellow (Sasidharan, 1993).

- ***Gomphostemma eriocarpum* Benth. (CALI NO. 123774)**

Habit: Subshrubs

Habitat: Evergreen forest

Flowering and fruiting: July - December

Distribution: Endemic to Southern Western Ghats

Description: Stem terete, puberulus. Leaves 15 x 5 cm, serrate, glabrescent above, densely covered with stellate pubescent below, nerves 4 pairs; petiole 1.5 cm long. Flowers 5-10 together, sessile; bracts oblong, obtuse; calyx 10 mm long, densely hairy, lobed to the middle, lobes acuminate; corolla 25 mm long; tube slender, 15 mm long, midlobe of lower lip broad, entire, yellow, upper lip oblong; filaments unequal, puberulus. Nutlets hairy, globose (Augustine, 2000).

2. METHODOLOGY

A. PHYTOCHEMICAL CHARACTERIZATION

Standard qualitative tests were carried out to identify the presence and absence of metabolites in methanolic extracts of selected species of *Gomphostemma*. Quantitative estimation of major components like phenols, terpenoids and flavonoids were also done. Analytical techniques like GC/MS and HR-LC/MS were employed to ascertain the volatile and non-volatile compounds in the extract.

- **Preparation of methanolic extract**

Aerial parts of the plant specimens were collected and thoroughly washed. Shade dried samples were powdered in a blender. Dried powder (50 g) was extracted with 500 mL of 100% methanol for 6-8 h in soxhlet apparatus. The extract thus obtained was cooled, filtered and concentrated to remove solvent in a rotary evaporator. The final concentrated dried extracts were stored in amber coloured glass bottles at 4 °C for further experiments.

- a. Qualitative phytochemical screening**

Methanolic extracts of selected plants were subjected to qualitative phytochemical analysis to detect the presence/absence of various phytocomponents using standard procedures (Trease & Evans, 1989; Harborne, 1998; Edeoga et al., 2005).

- i. Test for phenols**

- **Phosphomolybdic acid test**

The extract was spotted on a filter paper. Few drops of phosphomolybdic acid was added to the spot and then exposed to ammonium vapours. Formation of blue colouration in the spotted area was taken as indication for the presence of phenols.

- **Ferric chloride test**

To 2 mL of extract 0.5 mL of 5% aqueous ferric chloride solution was added and observed for the colour change. Formation of deep blue or black colour was taken as positive for the presence of phenols.

ii. Test for flavonoids

● Alkaline reagent test

To about 3 mL of extract 2 mL of 20% sodium hydroxide was added and observed for the formation of intense yellow colour, which became colourless on further addition of dilute hydrochloric acid indicating the presence of flavonoids.

● Shinoda's test

A fraction of extract was mixed with 1 mL of concentrated hydrochloric acid and to this mixture a piece of magnesium ribbon was added. Formation of pink colouration was taken as evidence for the presence of flavonoids.

iii. Test for alkaloids

● Hager's test

To 2 mL of extract, 3 mL of Hager's reagent (saturated aqueous picric acid solution) was added. Formation of yellow coloured precipitate was observed as indication for the presence of alkaloids.

● Wagner's test

Few drops of Wagner's reagent were added to about 2 mL of the extract and observed for the formation of reddish brown precipitate which indicated the presence of alkaloids.

● Mayer's test

Few drops of Mayer's reagent were added to a fraction of extract. Formation of white or pale yellow precipitate showed the presence of alkaloids.

iv. Test for terpenoids

- **Salkowski test**

To 5 mL of extract, 3 mL of chloroform was added and mixed well. To this mixture concentrated sulphuric acid was added along the sides of the test tube to form a layer. Formation of a reddish brown colour in the interface was taken as evidence for the presence of terpenoids.

v. Test for steroids

- **Liebermann-Burchard test**

A fraction of extract was mixed with 1 mL of chloroform and 3 mL of acetic anhydride. To this mixture a few drops of concentrated sulphuric acid was added along the sides of the test tube to form a layer underneath. Formation of bluish to green colouration was observed as positive for the presence of steroids.

vi. Test for tannins

- **Braymer's test**

A portion of extract was mixed with 2 mL of water followed by the addition of few drops of 10% ferric chloride solution. Formation of bluish black or greenish black colouration was taken as positive for the presence of tannins.

- **Lead acetate test**

To about 3 mL of extract, 3 mL of lead acetate was added. Formation of white precipitate indicated the presence of tannins.

vii. Test for saponins

• Foam test

About 10 g of extract was shaken vigorously with 20 mL of water and observed for the formation of persistent foam, which indicated the presence of saponins.

viii. Test for carotenoids

• Chloroform and H₂SO₄ test

A fraction of extract was mixed with 10 mL of chloroform and filtered. To the filtrate few drops of concentrated sulphuric acid was added. Formation of bluish colouration in the interface was inferred for the presence of carotenoids.

ix. Test for cardiac glycosides

• Keller Kiliani's test

To 2 mL of extract, 1 mL of glacial acetic acid and few drops of 5% ferric chloride were added. Concentrated sulphuric acid was added and observed. Formation of a brown ring at the interface indicated the presence of deoxysugar, which is a characteristic feature of cardenolides. Formation of violet colouration below the ring and a greenish ring in the acetic acid layer was inferred for the presence of cardiac glycosides.

x. Test for quinones

• HCl test

A small amount of extract was treated with 2 mL of concentrated hydrochloric acid and observed for the formation of yellow precipitate which indicated the presence of quinones.

xi. Test for Coumarins

• Sodium hydroxide test

To 1 mL of extract, 1 mL of 10% sodium hydroxide was added and observed for the formation of yellow colour which indicated the presence of coumarins.

xi. Test for iridoid glycosides

A fraction of extract was mixed with 5 mL of 1% aqueous hydrochloric acid and kept for 3-6 h. About 0.1 mL of the mixture was decanted to another tube containing Trim-Hill reagent. The tube was heated for a short time in a flame and development of colouration was noted. Iridoids like asperulose, aucubin and monotropein give blue colour where as others like herpagide form a red-violet colouration.

b. Quantitative estimation of major phytochemicals

Estimation of major phytochemicals like phenols, flavonoids and terpenoids were done following standard methods as detailed below:

i. Estimation of total phenolic content

The total phenolic content of plant extract was estimated with Folin-Ciocalteu reagent as described by Oueslati et al. (2012) using gallic acid as standard. An aliquot of sample/standard was mixed with 0.5 mL of distilled water and 0.125 mL of 1 N Folin-Ciocalteu reagent. The mixture was incubated for 5 min and 1.25 mL of 7% Na₂CO₃ was added. The volume of whole reaction mixture was made to 3 mL with distilled water and mixed well. It was then incubated for 90 min. The absorbance of the standard and samples was measured at 760 nm in a spectrophotometer (Elico SL 218, India). Calibration curve of gallic acid was plotted and phenolic content was

calculated using regression equation. Total phenolic content was expressed in milligram of gallic acid equivalent per gram of dry weight (mg GAE/g DW). All the samples were analysed in triplicates.

ii. Estimation of total flavonoid content

The total flavonoid content was determined by the aluminium chloride method (Dewanto et al., 2002; Akanitapichat et al., 2010) with slight modifications. Quercetin was used as the standard. An aliquot of standard/sample was mixed with 75 μ L of 7% NaNO₂ solution and incubated for 6 min. To this, 0.15 mL of AlCl₃ (10%) was added and kept for 5 min. To this mixture 0.5 mL of NaOH (1 M) was added and the whole solution was adjusted to 2.5 mL with distilled water. The absorbance was measured at 510 nm against blank *i.e.*, methanol. Calibration curve of quercetin was plotted and the total flavonoid content was expressed as milligram of quercetin equivalent per gram of dry weight (mg QE/g DW) as determined using regression equation. Samples were analysed in triplicates.

iii. Estimation of total terpenoid content

The total terpenoid content of selected species of *Gomphostemma* was determined by the method described by Ghorai et al. (2012) with slight modifications. Linalool was used as the standard. An aliquot of reaction mixture obtained after Salkowski test was transferred to a colorimetric cuvette and absorbance was measured at 538 nm against blank (95% methanol). Linalool calibration curve was prepared at varying concentrations. The total terpenoid content expressed as milligram of linalool equivalent per gram of dry weight (mg linalool/g DW) was determined from regression equation. Samples were analysed in triplicates.

c. Gas Chromatography/Mass Spectrometry (GC/MS) analysis

The identification and quantification of volatile compounds present in selected species of *Gomphostemma* was done by GC/MS analysis of methanolic extracts. GC/MS analysis was done with Shimadzu QP-2010 Plus with thermal desorption system TD 20, fitted with a 60 m $\times$ 0.25 mm $\times$ 0.25 m WCOT column coated with diethylene glycol (AB-Innowax 7031428, Japan). Helium was the carrier gas with a flow rate of 1.21 mL/min at a column pressure of 77.6 kPa. The injector and detector temperatures were set at 260 °C. Samples (8 μ L) were injected into the column with a split ratio of 10:0. Component separation was achieved with a linear temperature program of 70 - 260 °C with a total run time of 65.32 min. The MS parameters were: electron ionization (EI) voltage 70 eV, peak width 2 s, mass range 40 - 700 m/z and detector voltage 1.5 V. The constituents were identified by comparing their linear retention indices and MS fragmentation pattern with those of other compounds of known composition, as well as by matching fragmentation patterns with National Institute of Standards and Technology (NIST) and Wiley mass spectra libraries. Finally, their quantification was performed on the basis of their GC peak areas and expressed in terms of percentage.

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

The identification of non-volatile components in selected species of *Gomphostemma* was done with High Resolution - Liquid Chromatography/Mass Spectrometry (HR-LC/MS) analysis of methanol extracts of selected plants. Chromatographic separations were performed in an HR-LC/MS Q-TOF (Agilent G6550A, USA) equipped with an electrospray ionization source (Dual AJS ESI) maintained at 35 °C. The column employed for separation was CAPCELL C18, MG-II type (4.6 mm $\times$ 250 mm) with a particle size of 5 μ m. Elution was achieved using a mobile phase consisting of

95% A (water) and 5% B (acetonitrile) at a flow rate of 0.2 mL/min. The injection volume was 2 µL and the total run time was 30 min. Eluted compounds were detected with MS Q-TOF equipped with an electrospray ion source in positive ion modes with nebulizer gas nitrogen (13 L/min), gas temperature at 250 °C and nozzle voltage as 1000 V.

B. ANTIOXIDANT ACTIVITY

Free radical scavenging activity of three selected taxa of *Gomphostemma* was done with five different *in vitro* assays using methanol extracts at various concentrations. All the experiments were carried out in triplicates.

a. DPPH radical scavenging activity

DPPH (1, 1-diphenyl-2-picryl hydrazyl) is a stable free radical with pink colour which turns yellow when scavenged by antioxidants. Antioxidants react with DPPH and reduce it to DPPH-H which can be measured by the variation in absorbance as read spectrophotometrically. The degree of discolouration indicates the scavenging potential of the antioxidant compounds. The DPPH radical scavenging activity of plant extracts was done according to the method described by Chang et al. (2001) with modifications. Extracts of various concentrations (12.5, 25, 50, 100 and 200 µg/mL) made from a stock concentration of 10 mg/mL were made up to a final volume of 20 µL with DMSO. Ascorbic acid was used as standard. To the sample/standard 1mL DPPH (0.1 mM) solution was added followed by 450 µL of 50 mM Tris-HCl buffer (pH 7.4). Distilled water was the control. The reaction mixture was incubated for 20 min in dark at room temperature. After which, the absorbance of the mixture was read at 517 nm with 3 mL of DPPH as blank. The percentage of inhibition was calculated using the equation:

$$\text{Percentage inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c is the absorbance of the control and A_s is the absorbance of the sample.

b. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of selected plant extracts was measured as per the protocol of Kunchandy and Rao (1990). In this assay, the ability of various concentrations of plant extracts to scavenge the hydroxyl radicals generated by the Fe^{3+} - ascorbate - EDTA - H_2O_2 system (The Fenton reaction) is measured. The reaction mixture contained 2 deoxy 2 ribose (2.8 mM), FeCl_3 (100 μM), EDTA (100 μM), H_2O_2 (1.0 mM) and ascorbic acid (100 μM) in phosphate buffer (20 mM, pH 7.4) with various concentrations of plant extract (125, 250, 500, 1000 and 2000 $\mu\text{g/mL}$) finally made up to a volume of 1 mL. Distilled water without test compound served as the control and gallic acid was used as the standard. The whole reaction mixture was incubated for 1 h at room temperature. Then, 1 mL of 2.8% TCA and 1 mL of 1% aqueous TBA was added. The mixture was then incubated at 90 °C for 15 min. After cooling, the absorbance was measured at 532 nm against a blank. The percentage inhibition was calculated as follows:

$$\text{Percentage inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c is the absorbance of the control and A_s is the absorbance of the sample.

c. Nitric oxide free radical scavenging activity

Nitric oxide free radical scavenging activity was measured using Griess reagent following standard method (Kumaran, 2006). Sodium nitro prusside in aqueous solution at physiological pH generates nitric oxide which

on interaction with oxygen produces nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen resulting in reduced production of nitrite ions which can be measured spectrophotometrically. Sodium nitro prusside (10 mM) in phosphate buffered saline (PBS) at pH 7.4 was mixed with different concentration of samples (125, 250, 500, 1000 and 2000 µg/mL) and incubated at 25 °C for 30 min. Distilled water was taken as the control. Then, 1.5 mL of the incubated solution was removed and mixed with 1.5 mL of Griess reagent. Gallic acid was used as the standard. Absorbance was measured at 546 nm. The percentage of scavenging activity was measured as follows:

$$\text{Percentage inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c is the absorbance of the control and A_s is the absorbance of the sample.

d. Superoxide scavenging activity

Superoxide is a radical with important biological activity. But, over production of this radical can cause redox imbalance with harmful physiological consequences. Superoxide scavenging activity was assayed as described by Valentão et al. (2002). The assay is based on the reduction of nitroblue tetrazolium (NBT) by the superoxide generated from riboflavin. The reaction mixture contained different concentration of samples (125, 250, 500, 1000 and 2000 µg/mL), 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) made up to 2.64 mL with phosphate buffer (67 mM, pH 7.8). A control with all reagents except extract but with an equivalent amount of distilled water was also taken. All the tubes were uniformly illuminated with an incandescent light for 10-15 min and the absorbance was measured at 560 nm before and after

illumination. Ascorbic acid served as standard and the percentage of scavenging activity was evaluated as follows:

$$\text{Percentage inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c is the absorbance of the control and A_s is the absorbance of the sample.

e. Reducing power activity

Reducing power activity was measured by the method of Yen and Duh (1993). Various concentrations of extracts (125, 250, 500, 1000 and 2000 $\mu\text{g/mL}$) were mixed with 2.5 mL of 200 mM phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The reaction mixture was boiled for 20 min at 50 °C. Control was prepared with all reagents excluding the extracts but with equal amount of distilled water. After cooling, 2.5 mL of 10% TCA was added followed by centrifugation at 3000 rpm for 10 min. The upper layer of solution (5 mL) was mixed with 5 mL of distilled water and 0.1% ferric chloride (1 mL) was added and the absorbance was read at 700 nm. Quercetin was used as the standard. Increase in absorbance was taken as indication for increase in reducing power.

C. ANTI-INFLAMMATORY ACTIVITY

Anti-inflammatory potential of selected species of *Gomphostemma* was initially screened with biochemical assays. The most effective extract was further analysed for its activity on LPS induced RAW 264.7 macrophage cell line. ELISA was also performed to estimate and identify the major inflammatory mediators involved in the activity.

a. Evaluation of anti-inflammatory activity with biochemical assays

Preliminary screening of anti-inflammatory potential of selected species of *Gomphostemma* was performed with biochemical assays. Four different concentrations (62.5, 125, 250 and 500 µg/mL) were analysed. Diclofenac sodium was taken as the standard. The percentage of inhibition was calculated and the effective plant extract eligible for further analysis was identified.

i. Inhibition of protein denaturation

The ability of plant extracts to inhibit albumin denaturation was measured with standard protocol of Mizushima and Kobayashi (1968). The test solution consisted of 0.45 mL of bovine serum albumin and 0.05 mL of various concentrations (62.5, 125, 250 and 500 µg/mL) of plant extract. Diclofenac sodium served as the standard. The pH of all the above solutions was adjusted to 6.3 using 1 N HCl. The reaction mixture was initially incubated at 37 °C for 20 min and further the temperature was increased to 57 °C for 3 min. After cooling, 2.5 mL of phosphate buffer was added to the solutions. The absorbance was measured spectrophotometrically at 660 nm. The percentage inhibition was calculated as follows:

$$\text{Percentage inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c is the absorbance of the control and A_s is the absorbance of the sample.

ii. Proteinase inhibitory activity

Proteinase inhibitory activity was performed as described by Oyedepo and Femurewa (1995) with slight modification. The reaction mixture (2 mL) contained trypsin (0.06 mg), 1 mL of Tris-HCl buffer (20 mM, pH 7.4) and 1

mL of different concentrations (62.5, 125, 250 and 500 µg/mL) of plant extract prepared from a stock concentration of 10 mg/mL. The mixture was incubated for 5 min at 37 °C. Then 1 mL of 0.8% (w/v) casein was added. The mixture was again incubated for another 20 min. Further, 2 mL of perchloric acid (70%) was added to terminate the reaction. Cloudy suspension formed was centrifuged for 10 min at 3000 rpm. The absorbance of the supernatant was read at 210 nm. Buffer was used as the blank. The experiment was performed in triplicates. The percentage of proteinase inhibitory activity was calculated as follows:

$$\text{Percentage inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c is the absorbance of the control and A_s is the absorbance of the sample.

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

Anti-inflammatory activity of the effective extract was evaluated for its activity on RAW 264.7 macrophage cell line. The extract was analysed for its effect on cyclooxygenase activity (COX-2), lipoxygenase activity (LOX), myeloperoxidase activity (MPO) as well as for cellular nitrite level (NO) and inducible nitric oxide synthase (iNOS) activity.

● Cell culture

Raw 264.7 cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India (Plate 2 A) and maintained in Dulbecco's Modified Eagles Medium (DMEM). The cell line was supplemented with 10% fetal bovine serum (FBS), L-glutamine, sodium bicarbonate and antibiotic solution containing penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (2.5 µg/mL). Cell line was maintained at 37 °C in a humidified 5% CO₂ incubator.

• Preparation of plant extract and treatment

Cultured cells were grown to 60% confluency followed by activation with 1 μ L lipopolysaccharide (LPS) (1 μ g/mL). A stock of extract at 1 mg was weighed and dissolved in 1 mL DMEM using a cyclomixer. The solution was filtered and used for further assays. LPS stimulated RAW cells were exposed with different concentrations (25, 50 and 100 μ g/mL) of extract. Diclofenac sodium, a standard anti-inflammatory drug in varying concentration corresponding to the test solution was used as the positive control. All the treated cells were incubated for 24 h. After incubation, anti-inflammatory assays were performed using the cell lysate.

i. Cyclooxygenase (COX) activity

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

$$\text{Percentage inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c is the absorbance of the control and A_s is the absorbance of the sample.

ii. Lipoxygenase (LOX) activity

The effect of plant extract on LOX activity was determined as per Axelrod et al. (1981). The reaction mixture (2 mL) contained Tris-HCl buffer (pH 7.4), 50 μ L of cell lysate and sodium linoleate (200 μ L). The absorbance was read at 234 nm which reflected the formation of 5-hydroxyeicosatetraenoic acid as a measure of LOX activity. Percentage inhibition of the enzyme was calculated with the formula mentioned previously.

iii. Myeloperoxidase (MPO) activity

Cell lysate was homogenized in a solution containing potassium phosphate buffer (50 mM) and 0.57% of hexadecyltrimethyl ammonium bromide (HTAB). The reaction mixture was frozen in liquid nitrogen and thawed. The samples were then centrifuged at 2000 rpm for 30 min at 4 °C and supernatant was assayed for MPO activity. The MPO in the sample was activated by the addition of 50 mM of phosphate buffer (pH 6) containing guaiacol (1.67 mg/mL) and hydrogen peroxide (0.0005%). The change in absorbance was measured at 460 nm (Bradley et al., 1982). MPO activity was represented as units per mL of cell lysate. One unit of MPO activity is defined as that quantity degrading 1 μ M of peroxide per minute at 25 °C.

iv. Cellular nitrite level

The level of cellular nitrite (NO) was determined by the method of Lepoivre et al. (1990). To 0.5 mL of cell lysate, sulphosalicylic acid (0.1 mL) was added and vortexed for 30 min. The samples were then centrifuged at 5,000 rpm for 15 min. The supernatant was used for the estimation of nitrite levels. To 200 μ L of the supernatant, 30 μ L of NaOH (10%) was added. Further, 300 μ L of Tris-HCl buffer was added and mixed well. To this, 530 μ L of Griess reagent was added and incubated in dark for about 10-15 min.

The absorbances of solutions were measured at 540 nm against Griess reagent as blank.

v. Inducible nitric oxide synthase (iNOS) activity

Nitric oxide synthase was measured by the method described by Salter et al. (1996). Cell lysate was homogenized in 2 mL of HEPES buffer. The reaction mixture consisted of 0.1 mL of L-Arginine, 0.1 mL of manganese chloride, 0.1 mL of 30 µg dithiothreitol (DTT), 0.1 mL of NADPH, 0.1 mL of tetrahydropterin, 0.1 mL of oxygenated haemoglobin and 0.1 mL of cell lysate. Increase in absorbance was noted at 401 nm.

c. MTT assay of RAW 264.7 cells

MTT assay was performed to determine the appropriate concentration of the plant extract to be used for further analysis so as to overrule the probable cytotoxicity of the extract and its influence on anti-inflammatory activity. RAW 264.7 cell line maintained in DMEM medium with supplements were grown to 60% confluency in culture flask. Cells were trypsinized and suspended in 10% growth medium. 100 µL of cell suspension was seeded in 96 well tissue culture plate and incubated at 37 °C in a humidified 5% CO₂ incubator. After 24 h, the growth medium was removed. 100 µL of freshly prepared extract in various concentrations (62.5, 125, 250 and 500 µg/mL in 500 µL of 5% DMEM) were added in triplicates to the respective wells and incubated at 37 °C in a humidified 5% CO₂ incubator. Cells without any treatment were also maintained as control. After 2 h of incubation, the sample content in wells were removed and 30 µL of reconstituted MTT solution was added to all test and cell control wells. The plate was gently shaken and additionally incubated for another 4 h at 37 °C in a humidified 5% CO₂ incubator. After the incubation period, the supernatant was removed and 100 µL of DMSO was added as MTT solubilization

solution. The wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance was measured by using microplate reader at a wavelength of 540 nm (Chow et al., 2004). Percentage of cell viability was calculated and further half maximal inhibitory concentration (IC₅₀) was determined.

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

d. Estimation of inflammatory mediators using ELISA

Estimation of inflammatory mediators like COX-2, NF-κB (p65) and TNF-α in LPS stimulated RAW 264.7 cells was done with ELISA. The LPS treated cells were exposed to 75 µg/mL of the extract. Cell lysate (100 µL) was added to the 96 well plate and kept in incubation for 37 °C overnight. After incubation, the wells were drained and washed with PBS for 3 to 5 times. Blocking buffer (200 µL) was added and kept for 1 h at room temperature. It was then washed twice with PBS. To this 100 µL of primary antibodies (COX-2, NF-κB (p65) and TNF-α) were added and left for 2 h at room temperature. It was then washed two times with PBS. Following this, 100 µL of secondary antibody (anti-human HRP conjugated secondary antibody) was added and incubated for 1 h at room temperature. It was then washed twice with PBS and 200 µL of O-dianizidine hydrochloride was added and left as such for 30 min at room temperature in dark. The reaction was stopped by adding 5 N HCl (50 µL) and absorbance was read at 415 nm using an ELISA reader.

D. SCREENING FOR CYTOTOXICITY WITH *ALLIUM CEPA* L.

All the three selected taxa of *Gomphostemma* were scrutinized for their antiproliferative potential using various assays. Preliminary screening for cytotoxicity and cell death was done on *A. cepa* root tips. Cytotoxicity of

extracts was also analysed on human breast cancer cell line MCF-7 (Michigan Cancer Foundation-7). The effective extract was subjected for more screening with comet assay, estimation of caspases, apoptotic detection, cell cycle analysis and gene expression studies.

a. Cytotoxicity studies using *Allium cepa* bioassay

Cytotoxicity of plant extracts was evaluated with *A. cepa* L. root meristems. Prior to use, healthy onion bulbs were selected and outer scales of the bulbs were carefully removed without destroying the root primordia. They were kept for rooting in distilled water for 1-2 days. Germinated bulbs with healthy roots were collected at the time of peak mitotic activity (between 9.00 am and 10.00 am on normal sunny days). They were washed with distilled water and subjected to treatment. Various concentrations (5, 25, 50, 75 and 100 µg/mL) were prepared from a stock of 1 mg/mL of methanolic extract using distilled water. The selected bulbs were placed at the rim of the vials containing various concentrations of the extract and ensured that the roots were immersed in it. The experimental set up was kept undisturbed for different time intervals (1, 3, 12 and 24 h). The sprouted bulbs were also treated with distilled water and H₂O₂, which served as negative and positive controls respectively. The treated root tips were collected and fixed in modified Carnoy's fluid for 1 h. Mitotic squash preparations were made with improved techniques of Sharma and Sharma (1990) and stained with acetocarmine. The slides were scanned under 40x of microscope (Leica DM2000) and photomicrographs were taken using the top mounted camera (Leica DMC2900). Total number of cells, dividing cells, non-dividing cells and damaged cells in 5 different fields of view were recorded. Mitotic index (MI) and chromosome aberrations (CA) were calculated as follows:

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

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

b. *In situ* visualization of cell death

Assessment of cell death was done with *A. cepa* root tips using Evans blue staining technique. Five different concentrations (5, 25, 50, 75 and 100 µg/mL) and 24 h treatment period were selected for the study. Distilled water and H₂O₂ served as negative and positive controls respectively. Control and treated bulbs with intact roots were placed in Evans blue staining solution for 15 min followed by washing in distilled water for 30 min (Baker & Mock, 1994). Roots with dead cells that stained blue were photographed and ten root tips of equal length were collected from control and treatment. These root tips were soaked separately in 3 mL of N, N-dimethylformamide for 1 h at room temperature and absorbance of Evans blue released was measured at 600 nm.

E. ANTIPROLIFERATIVE STUDIES WITH MCF-7 CELL LINE

Antiproliferative activity of selected species of *Gomphostemma* was initially screened using MTT assay on MCF-7 cell line. The effective extract was also tested for its activity on L929 cell line. Further, the effective extract was analysed for its apoptotic activity using various assays followed by gene expression studies.

• Preparation of plant extract

A stock solution of 1mg/mL of plant extract was prepared in PBS. The sample solution was filtered through 0.22 µm millipore syringe filter to ensure the sterility. Further, various concentrations (6.25, 12.5, 25, 50, and 100 µg/mL) were prepared from stock using appropriate volume of cell culture medium DMEM and dissolved completely using a cyclomixer.

● Culture of cell lines

MCF-7 (Human breast adenocarcinoma) cells and L929 (Fibroblast) cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India (Plate 2, B & C) and maintained in Dulbecco's Modified Eagles Medium (DMEM). The cell lines were cultured in tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing, streptomycin (100 µg/mL), penicillin (100 µg/mL) and amphotericin B (2.5 µg/mL). Cultured cell lines were kept in a humidified 5% CO₂ incubator at 37 °C.

a. Cytotoxic evaluation using MTT assay on MCF-7 cell line

The yellow coloured tetrazolium dye MTT (3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide) is reduced by mitochondrial succinate dehydrogenase within mitochondria of metabolically active live cells to its insoluble formazan, which has a purple colour. This insoluble formazan can be dissolved by a solubilization solution to a coloured solution whose absorbance can be quantified spectrophotometrically. This assay thus helps to assess the cell metabolic activity and indirectly reflects the number of viable cells present in the medium. In the present study, two days old confluent monolayer of cells was trypsinized and the cells were suspended in 10% growth medium. 100 µL of cell suspension was seeded in 96 well tissue culture plate and incubated in a humidified 5% CO₂ incubator at 37 °C. After 24 h, the growth medium was removed and freshly prepared plant extract in various concentrations (6.25, 12.5, 25, 50, and 100 µg/mL) were added and incubated for another 24 h at 37 °C in a humidified 5% CO₂ incubator. Non-treated control cells were also maintained. All experiments were maintained in triplicates. After desired incubation period, entire plate was observed in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observations were recorded as

images. Any detectable morphological changes in the cells like rounding or shrinking of cells, granulation, vacuolization in the cytoplasm, *etc.* were considered as indicators of cytotoxicity.

MTT (15 mg) was reconstituted in 3 mL of PBS until completely dissolved and sterilized by filter sterilization. After 24 h of incubation period, the sample content in wells were removed and 30 μ L of reconstituted MTT solution was added to all test and cell control wells. The plate was gently shaken and then incubated for 4 h at 37 °C in a humidified 5% CO₂ incubator. After which, the supernatant was removed and 100 μ L of DMSO was added and the wells were mixed gently to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (Chow et al., 2004). Percentage of cell viability was calculated and further half maximal inhibitory concentration (IC₅₀) was determined.

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

b. Cytotoxic evaluation using MTT assay on L929 cell line

The effective extract was analysed for its cytotoxicity on normal L929 cells. Various concentrations (6.25, 12.5, 25, 50, and 100 μ g/mL) were tested as described previously and IC₅₀ value was determined.

c. Comet assay

The effective extract was further analysed for its genotoxicity by comet assay (Singh, et al., 1988; Dhawan, et al., 2009). The comet assay is used to detect the DNA damage caused by double strand breaks, single strand breaks, alkali labile sites, oxidative base damage and DNA cross-linking with DNA or protein. It requires only small number of cells per sample and is a sensitive test for detecting DNA damage. MCF-7 cells were cultured in 6 well plates and after attaining 80% growth confluency, the growth medium was removed.

The cells were treated with IC₅₀ concentration of plant extract and subjected to overnight incubation. Untreated control cells were also maintained. After incubation, the cells were trypsinized, washed with fresh media and used further for comet assay.

Fully frosted microscope slides were pre-coated with 1 mL of normal melting agarose (1%) and stored at 4 °C. This layer was removed before use and 120 µL of 1% normal melting agarose (NMA) was pipetted on to the slides, which were then covered with cover slips. Cell suspensions (1×10^4 /5-30 µL) were mixed with 50 µL of low melting point agarose (LMPA) and pipetted over the first layer of agarose. Normal melting agarose (80 µL) was used as a final protective layer. After each step, the slides were incubated at 4 °C for 10 min to allow agarose to set. Slides were then placed in cold lysing solution for 1 h. After which, slides were placed in electrophoresis buffer for 20 min to allow unwinding of DNA. Electrophoresis was conducted in the same buffer by applying an electric current of 0.8 V/cm (300 mA) for 20 min using an electrophoresis supply. Finally slides were washed in neutralization buffer three times for 5 min each, dried and stained with 50 µL ethidium bromide (EtBr) for 15 min followed by washing with PBS to remove excess of stain. The slides were then photographed using inverted epifluorescence microscope (Olympus CKX41 with Opioka Pro5 CCD camera). Comets were scored using Triton comet scoring software (version 1.5) and various parameters were determined.

d. Detection of apoptosis

DNA-binding dyes acridine orange and ethidium bromide (AO/EB) were used for the morphological detection of apoptotic and necrotic cells (Zhang et al, 1998). Acridine orange is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into DNA whereas; ethidium bromide is taken up only by non-viable cells and emits red

fluorescence by intercalation into DNA. After treatment with plant extract at IC_{50} concentration for 24 h, the cells were washed with cold PBS and then stained with a mixture of acridine orange (100 $\mu\text{g/mL}$) and ethidium bromide (100 $\mu\text{g/mL}$) at room temperature for 10 min. The stained cells were washed twice with 1X PBS; they were then observed and photographed under fluorescence microscope (Olympus CKX41 with Optika Pro5 camera).

e. Cell cycle analysis

MCF-7 cells were cultured as described earlier and treated with IC_{50} concentration of effective extract for 24 h. The cells were then centrifuged at 3000 rpm for 5 min. The supernatant was removed and repeatedly washed with PBS. 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 $-20\text{ }^{\circ}\text{C}$. After overnight incubation, the samples were centrifuged at 3000 rpm for 5 min at room temperature. The supernatant was removed, washed twice with 250 μL PBS and finally 250 μL of cell cycle reagent was added which includes the nuclear DNA intercalating stain propidium iodide (PI) which discriminates cells at different stages of the cell cycle based on the differential DNA content. This was incubated in dark for 30 min (which is light sensitive). After this, it was analysed using MUSETM Cell Analyser (Merck-Millipore, Germany). Gating was performed with reference to untreated control cells.

f. Determination of caspase activity with ELISA

The MCF-7 cells were cultured and after attaining 80% growth confluency, the growth medium was removed and cells were exposed to IC_{50} concentration of effective extract. It was incubated for 24 h at $37\text{ }^{\circ}\text{C}$ in a humidified 5% CO_2 incubator. Untreated cells were maintained as the control.

Cell lysate (100 μ L) was added to the 96 well plate and kept in incubation for 37 °C overnight. After incubation, the wells were drained and washed twice with PBS. Then, 200 μ L of freshly prepared blocking buffer in PBS was added and incubated for 1 h at room temperature. Further, it was washed twice with PBS. Further, 100 μ L each of primary antibodies (caspase-9 and caspase-7) were added and left for 2 h at room temperature. After incubation, it was washed twice with PBS followed by the addition of 100 μ L of secondary antibody (HRP Conjugate) and incubated for 1 h at room temperature. It was then washed with PBS and 200 μ L of O-dianizidine hydrochloride was added and left for 30 min at room temperature. The reaction was stopped by adding 5 N HCL (50 μ L) and absorbance was measured at 415 nm.

g. Gene expression studies

Regulation of genes p21 and p53 in MCF-7 cell lines by effective extract was analysed as follows:

i. Isolation of total RNA (TRIZol method)

Total RNA was isolated using the total RNA isolation kit according to the manufacture instruction (Invitrogen). Addition of TRIZol solution causes the disruption of cells and subsequent release of RNA. MCF-7 cells were cultured as described previously and after attaining 70% confluency in 6 well plate, the cells were treated with IC₅₀ concentration of effective plant extract. It was incubated for 24 h at 37 °C in a CO₂ incubator. A set of untreated cells was maintained as the control. After incubation, DMEM media was removed aseptically and 200 μ L of TRIZol reagent was added to culture well plate and again incubated for 5 min. The contents were then transferred to a fresh sterile eppendorf tube followed by the addition of 200 μ L of chloroform. It was shaken vigorously for 15 seconds and incubated for 2-3 min at room

temperature. After which, 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. It was incubated for 10 min at room temperature and then centrifuged at 14000 rpm for 15 min at 4 °C. The supernatant was discarded and pellet thus obtained was washed with 200 µL of 75% ethanol. It was then centrifuged at 14000 rpm for 5 min at 4 °C in a cooling centrifuge. The RNA pellet was dried and suspended in Tris-EDTA buffer.

ii. Synthesis of cDNA

The cDNA synthesis was performed using cDNA preparation kit (Thermo scientific, Verso cDNA synthesis kit). About 4 µL of 5X cDNA synthesis buffer, 2 µL of dNTP mix, 1 µL of anchored oligo dT, 1 µL of RT enhancer, 1 µL of verso enzyme mix and 5 µL of RNA template (1ng of total RNA) were added to an RNase free tube. Then, the total volume was made to 20 µL with the addition of sterile distilled water and the solution was mixed gently. 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	30	1
Inactivation	95	2	1

iii. Gene expression analysis by RT-qPCR

Real-Time qRT-PCR analysis was carried out using SYBR Green Master Mix (Applied Biosystem, Life technologies). For each 50 µL reaction mixture, 25 µL of master mix, 2 µL of forward primer (0.1-1.0 µM), 2 µL of reverse primer (0.1-1.0 µM) and 10 µL of template DNA (10 pg-1 µg) were used. The house keeping gene used was β-actin. All reactions were performed

in triplicates and data were analysed according to $\Delta\Delta C_t$ method (Light Cycler 96 SW 1.1 Software).

The various steps involved, the time taken and temperature of each step are given below:

Steps	Time required	Temperature
Initial activation step	2 minutes	95 °C
3 step cycling:		
Denaturation	10 seconds	94 °C
Annealing	1 minutes	55 °C
Extension	1 min/kb	72 °C
Number of cycles	40 cycles	68 °C
End of PCR cycling	indefinite	4 °C

The primer sequences used:

Human β -actin Forward	5'-TCACCCACACTGTGCCCATCTACGA-3'
Human β -actin Reverse	5'-CAGCGGAACCGCTCATTGCCAATGG-3'
Human p21 Forward	5'-GAGGCCGGATGAGTTGGGAGGAG-3'
Human p21 Reverse	5'-CAGCCGGCGTTTGGAGTGGTAGAA-3'
Human p53 Forward	5'-CCCCTCCTGGCCCCTGTCATCTTC-3'
Human p53 Reverse	5'-GCAGCGCCTCACAACCTCCGTCAT-3'

iv. Agarose gel electrophoresis

The PCR product was subjected to agarose gel electrophoresis. Agarose gel (1.5%) was prepared in 1x TAE buffer and melted in hot water bath at 90 °C. The melted agarose was cooled down to 45 °C. Ethidium

bromide (6 μ L, 10 mg/mL) was added and poured into a gel casting apparatus with gel comb. After setting, the comb was removed from the gel. The electrophoresis buffer was poured to gel tank and the platform with the gel was placed in it. The gel was loaded with samples and run at 50 V for 30 minutes. The stained gel was visualized using a gel documentation system (E gel imager, Invitrogen).

F. GREEN SYNTHESIS OF SILVER NANOPARTICLES AND THEIR CHARACTERIZATION

Present study attempted for an eco-friendly technique for the synthesis of silver nanoparticles using selected species of *Gomphostemma*. Characterization of silver nanoparticles with various analytical techniques was also undertaken.

a. Synthesis of silver nanoparticles

i. Preparation of plant extract

About 10 mg of the methanolic extract of plant was dissolved in 20 mL of deionized water. It was blended uniformly and filtered using Whatman No. 1 filter paper.

ii. Synthesis of silver nanoparticles

About 10 mL of the collected filtrate was treated with 90 mL of silver nitrate (AgNO_3) (2 mM) and boiled at 80 $^{\circ}\text{C}$ for 10 minutes. The above solution was incubated for 30 min and observed for the formation of brownish yellow coloured solution, which indicated the synthesis of silver nanoparticles (Gnanadesigan et al., 2012).

iii. Collection of silver nanoparticle pellets

The solution was centrifuged at 12,000 rpm for 10 min and redispersed in sterile distilled water. The centrifugation and redispersion were repeated five times to ensure the complete separation of silver nanoparticles. The pellets thus obtained were stored at room temperature for further characterization studies.

b. Characterization of silver nanoparticles

i. UV-Vis spectral analysis

About 1 mL of the synthesised solution was monitored to detect the reduction of Ag^+ ions and formation of peaks at a wavelength range of 300-800 nm were monitored in UV-Vis spectrophotometer.

ii. FE-SEM/EDX analysis

The dried pellets of silver nanoparticles were mounted on an aluminium stub using carbon tape and dried by keeping it in a desiccator overnight and examined using a field emission scanning electron microscope (Zeiss GeminiSEM 300) equipped with energy dispersive spectrometer (Gemini 300/EDS, Octane Plus).

● Statistical analysis

All the data obtained were subjected to statistical analysis using SPSS version 20. One-way ANOVA was performed followed by DMRT (Duncan's multiple range test) to confirm the variability of the data and validity of the results. All results were expressed as mean $\pm$ SE. Results with $P < 0.05$ was considered as statistically significant.

RESULTS

In the present study, selected species of *Gomphostemma* endemic to South Western Ghats were screened for their phytochemical constituents and bioactivity potential. *Gomphostemma heyneanum* var. *heyneanum*, *G. heyneanum* var. *rottleri* and *G. eriocarpum* were the three taxa selected for the study. The selected species were screened for their phytochemical constituents, antioxidant potential, anti-inflammatory and anticancer efficacy by appropriate assays. The most effective and potential plant among the selected taxa was subjected to in-depth studies. Green synthesis of silver nanoparticles with the three selected taxa of *Gomphostemma* and their characterization was also attempted.

A. PHYTOCHEMICAL CHARACTERIZATION

Phytochemical constituents in the selected species of *Gomphostemma* were analysed by preliminary qualitative tests and quantitative estimation. Volatile components were revealed by GC/MS and non-volatile components by HR-LC/MS technique.

a. Qualitative phytochemical screening

Qualitative phytochemical screening of selected species of *Gomphostemma* revealed the presence of various classes of phytoconstituents like phenols, flavonoids, alkaloids, terpenoids, steroids, carotenoids, cardiac glycosides, quinones, coumarins and iridoid glycosides, whereas tannins and saponins were found to be absent in all the three taxa. The results of preliminary phytochemical tests are presented in Table 6.

Table 6: Qualitative phytochemical screening of selected species of *Gomphostemma*

Sl NO.	Phytochemical components	Tests	GHH	GHR	GE
1.	Phenols	Phosphomolybdic acid test	+	+	+
		Ferric chloride test	+	+	+
2.	Flavonoids	Alkaline reagent test	+	+	+
		Shinoda's test	+	+	+
3.	Alkaloids	Hager's test	+	+	+
		Wagner's test	+	+	+
		Mayer's test	+	+	+
4.	Terpenoids	Salkowski test	+	+	+
5.	Steroids	Liebermann-Burchard test	+	+	+
6.	Tannins	Braymer's test	—	—	—
		Lead acetate test	—	—	—
7.	Saponins	Foam test	—	—	—
8.	Carotenoids	Chloroform & H ₂ SO ₄ test	+	+	+
9.	Cardiac glycosides	Keller Kiliani's test	+	+	+
10.	Quinones	HCl test	+	+	+
11.	Coumarins	NaOH test	+	+	+
12.	Iridoid glycosides	Trim-Hill reaction	+	+	+

(+): presence, (-): absence. GHH - *Gomphostemma heyneanum* var. *heyneanum*; GHR - *Gomphostemma heyneanum* var. *rottleri*; GE - *Gomphostemma eriocarpum*

b. Quantitative estimation of major phytocomponents

Estimation of total phenolic, terpenoid and flavonoid contents were conducted in methanolic extract of selected species of *Gomphostemma* using standard protocols. Quantitative estimation revealed an approximate amount of major classes of compounds in the plants selected for the study.

The total phenolic content in the extracts was estimated with Folin-Ciocalteu reagent and expressed in terms of gallic acid equivalent (mg GAE/g DW) using standard equation from the linear calibration curve ($y = 0.005x$, $R^2 = 0.991$; Fig. 8). The highest phenolic content was detected in *G. heyneanum* var. *heyneanum* (260.31 ± 11.62) followed by *G. heyneanum* var. *rottleri* (178.78 ± 9.32) and *G. eriocarpum* (32.56 ± 3.18) (Fig. 9).

Total flavonoid content was quantified using aluminium chloride and expressed in terms of quercetin equivalent (mg QE/g DW). The content was calculated from the standard curve equation ($y = 0.009x$, $R^2 = 0.984$; Fig. 10). The total flavonoid content (Fig. 11) was found to be highest in *G. heyneanum* var. *heyneanum* (129.22 ± 10.65) followed by *G. heyneanum* var. *rottleri* (88.96 ± 0.55) and *G. eriocarpum* (33.69 ± 0.53).

Total terpenoid content was measured and expressed as linalool equivalent (mg linalool/g DW) from the standard curve equation ($y = 0.004x$, $R^2 = 0.975$; Fig. 12). The content in the extracts was estimated to be 279.56 ± 8.53 , 143.75 ± 7.66 and 118.21 ± 7.79 for *G. heyneanum* var. *heyneanum*, *G. heyneanum* var. *rottleri* and *G. eriocarpum* respectively. Total terpenoid content was noted to be highest in *G. heyneanum* var. *heyneanum*. Nevertheless, considerable amount of terpenoids was detected in other two taxa also (Fig. 13).

Quantitative estimation revealed significant amounts of phenols, terpenoids and flavonoids in selected species of *Gomphostemma*. Presence of

considerable amounts of these phytoconstituents can contribute to the bioactivity potential of these plants.

c. Gas Chromatography/Mass Spectrometry (GC/MS) analysis

The volatile phytoconstituents in the methanolic extract of selected species of *Gomphostemma* was determined by GC/MS analysis. A total of 72 compounds were identified which belonged to various classes like terpenoids, flavonoids, phenolics, fatty acids, aldehydes, *etc.* Compounds identified are enlisted in Table 7 and gas chromatograms are given in Figs 14, 15 & 16. Mass spectra of compounds detected are shown in Figs 17 (i)–17(xv).

The methanolic extract of *G. heyneanum* var. *heyneanum* revealed the presence of 38 compounds in GC/MS analysis. The major compounds detected were 2,6,8-trimethylbicyclo[4.2.0]oct-2-ene-1,8-diol (32.49%), α -thujene (14.63%), neophytadiene (6.22%), β -caryophyllene (5.48%), limonene (3.96%), stearic acid (3.29%), stigmasterol (3.15%), quinic acid (3.08%), phytol (2.60%) and eucalyptol (1.96%). Terpenes were the predominant class of compounds identified and a variety of them like α -thujene, α -pinene, β -phellandrene, β -myrcene, p-cymene, geranyl isovalerate, α -humulene, β -elemene, *etc.* were present in the extract. Phenolic compounds were represented by quinic acid (3.08%) and α -tocopherol (1.24%). Sterols detected include campesterol and stigmasterol. Apart from this, fatty acids (stearic acid) and fatty acid esters, together with significant numbers of organic compounds, ketones, alcohols and aldehydes were noticed.

GC/MS analysis of methanolic extract of *G. heyneanum* var. *rottleri* showed a total of 39 compounds. Dianhydromannitol (19.64%), phytol (18.75%), stigmasterol (10.68%), neophytadiene (6.71%) and β -sitosterol (5.61%) were the major compounds detected. In this extract also terpene class of compounds dominated with significant percentage and number, followed

by phytosterols and organic compounds. Terpenes like β -caryophyllene, valencene, caryophyllene oxide, loliolide, neophytadiene and squalene were present in notable amounts. Phenolic compounds were represented by 2-methoxy-4-vinylphenol, α -tocopherol and cinnamaldehyde. Other class of compounds were alcohols, fatty acids, fatty acid esters, aldehydes, ketones and lactones.

The extract of *G. eriocarpum* contained 26 compounds with terpenes as major class of compounds. Phytol (47.65%), stigmasterol (15.73%) and neophytadiene (11.21%) were the major compounds identified. Terpenes like α -humulene, γ -elemene, squalene and loliolide were present in the extract. Phenolic compounds were represented by eugenol and α -tocopherol. Sterols like stigmasterol, campesterol and β -sitosterol were noted in good percentage. Other class of compounds like fatty acids, fatty acid esters, organic compounds, ketones, alcohols, aldehydes and lactones were also detected in the extract.

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

The non-volatile phytoconstituents in selected species of *Gomphostemma* were revealed by HR-LC/MS analysis. The identified compounds, their retention time, mass to charge ratio and observed molecular mass are listed in Table 8. The liquid chromatograms are represented in Figs 18–20 and mass spectra of the identified compounds are shown in Figs 21 (i)–21 (vii).

The analysis revealed a total of 34 components belonging to various classes like terpenoids, phenols, alkaloids, flavonoids, fatty acids, *etc.* The extract of *G. heyneanum* var. *heyneanum* showed 11 components which included alkaloid like cephaeline, flavonoids like theaflavin and genistein,

and a polyphenol ester chlorogenic acid. Seventeen components including a variety of biopeptides were identified from *G. heyneanum* var. *rottleri*. An alkaloid cuscohygrine, a flavanone diosmetin, triterpenes dihydrocelastrol and swietenine were the interesting compounds identified. *G. eriocarpum* showed 10 constituents with major compounds belonging to triterpene classes. Among the compounds noted, theaflavin and 3-(4-hydroxyphenyl) pyruvic acid were found to be present in both *G. heyneanum* var. *heyneanum* and *G. heyneanum* var. *rottleri*. Dihydrocelastrol and swietenine were identified to be common in *G. heyneanum* var. *rottleri* and *G. eriocarpum*. The present analysis also revealed a variety of biologically active biopeptides. Thus, HR-LC/MS provided a vivid picture of phytoconstituents which were not revealed by GC/MS analysis.

B. ANTIOXIDANT ACTIVITY

The methanolic extracts of selected species of *Gomphostemma* were subjected to antioxidant screening against DPPH radical, hydroxyl radical, nitric oxide radical, superoxide radical and also for their reducing power potential using different *in vitro* assays. The activities were compared with known antioxidants.

a. DPPH radical scavenging activity

DPPH assay is widely used as an index for measuring the antioxidant potential of crude extracts of plants. In the present study, DPPH radical quenching activity of methanolic extracts of selected species of *Gomphostemma* was investigated with five different concentrations (12.5, 25, 50, 100 and 200 µg/mL). Ascorbic acid was taken as the standard. The results are illustrated in Fig. 22 and IC₅₀ values are shown in Table 9. All the extracts exhibited significant dose-dependent response. Among the plants tested, *G. heyneanum* var. *heyneanum* showed an effective increase in

percentage of inhibition from 25.36 ± 2.46 to 85.40 ± 1.05 for concentrations 12.5 $\mu\text{g/mL}$ to 200 $\mu\text{g/mL}$ with an IC_{50} value of $46.63 \pm 2.26 \mu\text{g/mL}$ which is comparable to the IC_{50} of ascorbic acid ($48.93 \pm 1.75 \mu\text{g/mL}$). The percentage inhibition for *G. heyneanum* var. *rottleri* ranged from 18.58 ± 2.98 to 72.83 ± 0.96 and for *G. eriocarpum* it was 16.01 ± 1.49 to 69.41 ± 1.83 for five different concentrations tested. The IC_{50} values for *G. heyneanum* var. *rottleri* and *G. eriocarpum* were $80.41 \pm 1.87 \mu\text{g/mL}$ and $101.61 \pm 1.22 \mu\text{g/mL}$ respectively.

b. Hydroxyl radical scavenging activity

The hydroxyl radicals are regarded as the most reactive among the free radicals. The hydroxyl radical scavenging activity of methanolic extracts of selected species of *Gomphostemma* is presented in Fig. 23 and IC_{50} values are shown in Table 9. Gallic acid served as the standard. All the extracts displayed a dose-dependent hydroxyl radical scavenging activity for tested concentrations (125, 250, 500, 1000 and 2000 $\mu\text{g/mL}$). Among the extracts, *G. eriocarpum* showed highest percentage of inhibition with a range of 7.88 ± 1.78 to 78.11 ± 3.68 . It also had a lowest IC_{50} value of $587.17 \pm 2.97 \mu\text{g/mL}$. The scavenging ability of *G. heyneanum* var. *heyneanum* was also of the same range with percentage inhibition of 13.44 ± 2.41 to 76.87 ± 3.23 for tested concentrations and the IC_{50} value noted was $745.17 \pm 2.03 \mu\text{g/mL}$. The methanolic extract of *G. heyneanum* var. *rottleri* showed least activity with percentage of inhibition ranging from 5.90 ± 1.26 to 68.44 ± 2.65 and the IC_{50} value was found to be $1108.56 \pm 2.12 \mu\text{g/mL}$. All the extracts showed a better performance than gallic acid.

c. Nitric oxide free radical scavenging activity

In nitric oxide scavenging assay 125, 250, 500, 1000 and 2000 $\mu\text{g/mL}$ extract concentrations were tested. Gallic acid was taken as the standard. The

results are depicted in Fig. 24 and IC₅₀ values are shown in Table 9. All the extracts displayed a concentration-dependent scavenging activity. The highest activity was displayed by *G. heyneanum* var. *heyneanum* and the percentage of inhibition ranged from 25.50 ± 2.04 to 74.58 ± 1.27 with an IC₅₀ value of 803.86 ± 1.05 µg/mL. Among the other two extracts, *G. heyneanum* var. *rottleri* showed a moderate activity with inhibition percentage in the range of 16.67 ± 1.08 to 57.18 ± 5.35 and the IC₅₀ value was 1415.04 ± 2.25 µg/mL. The methanolic extract of *G. eriocarpum* showed least scavenging activity with inhibition percentage ranging from 9.65 ± 4.17 to 40.51 ± 0.98 for tested concentrations and the IC₅₀ value (2471.08 ± 1.97 µg/mL) was higher compared to other extracts of selected species of *Gomphostemma*. Since nitric oxide free radicals along with other free radicals play a pivotal role in many pathological conditions like cancer and inflammation, the scavenging potential can be explored further for ameliorating such conditions.

d. Superoxide scavenging activity

Superoxides are formed enormously in biological system as a result of innumerable metabolic reactions. Excess of superoxide free radicals are toxic to cells which can contribute to tissue disintegration and other disorders. In the present study, superoxide scavenging potential of methanolic extracts of selected species of *Gomphostemma* was assayed with five different concentrations (125, 250, 500, 1000 and 2000 µg/mL). Ascorbic acid was the standard. From the results displayed in Fig. 25 and IC₅₀ values shown in Table 9, it can be inferred that all the three plant extracts have optimal superoxide scavenging potential. A dose-response relationship was noted. Among the plant extracts tested, *G. heyneanum* var. *heyneanum* exhibited highest activity with inhibition percentage in the range of 27.54 ± 2.12 to 77.36 ± 2.4 and IC₅₀ value (699.471 ± 2.31 µg/mL) noted was found to be least, compared to other two plant extracts. The inhibition percentages

observed for *G. heyneanum* var. *rottleri* and *G. eriocarpum* were in the range of 17.63 ± 4.71 to 60.32 ± 3.13 and 8.45 ± 2.18 to 53.57 ± 1.94 respectively. The IC_{50} value obtained for *G. heyneanum* var. *rottleri* was 1280.32 ± 3.81 $\mu\text{g/mL}$ and it was 1627.18 ± 2.32 $\mu\text{g/mL}$ for *G. eriocarpum*. However, the activities of the extracts analysed were less as compared to the standard ascorbic acid.

e. Reducing power activity

Measurement of reducing power is an ideal assay for understanding the antioxidant potential of plant extracts. In the present study, reducing power of methanolic extracts of selected species of *Gomphostemma* was measured in terms of absorbance for five different concentrations (125, 250, 500, 1000 and 2000 $\mu\text{g/mL}$). Quercetin served as the standard. All the extracts showed a dose-dependent increase in reductive potential as inferred from the absorbance noted (Fig. 26). The methanolic extract of *G. heyneanum* var. *heyneanum* showed an increase in absorbance from 0.44 ± 0.06 to 1.71 ± 0.08 . For *G. heyneanum* var. *rottleri* the absorbance was found to increase from 0.108 ± 0.04 to 1.16 ± 0.07 and for *G. eriocarpum* it increased from 0.15 ± 0.01 to 0.84 ± 0.03 indicating the dose-dependent increase in the reducing power of extracts.

Present study revealed the antioxidant potential of selected species of *Gomphostemma* from South Western Ghats. The plant extracts responded to various assays in a moderate to higher level. In all the assays performed, *G. heyneanum* var. *heyneanum* showed efficient scavenging potential. *G. eriocarpum* was effective in scavenging hydroxyl radicals, but it was found to be less efficient in other assays. *G. heyneanum* var. *rottleri* showed moderate activity in all the assays. Thus, it can well be said that the selected species of *Gomphostemma* under scrutiny have the potential to scavenge different free radicals and hence are promising candidates for further screening.

C. ANTI-INFLAMMATORY ACTIVITY

Inflammation is a physiological response to a variety of conditions like injuries, allergies, infections, certain cancers and other autoimmune diseases. In the present study, selected species of *Gomphostemma* were evaluated for their anti-inflammatory potential by *in vitro* biochemical assays and the most effective extract was further analysed for its activity on RAW 264.7 macrophage cell line.

a. Evaluation of anti-inflammatory activity with biochemical assays

i. Inhibition of protein denaturation

Denaturation of protein is well documented to be associated with many inflammatory conditions and agents which can inhibit protein denaturation are considered significant in anti-inflammatory drug development. Inhibition of protein denaturation was analysed using bovine serum albumin. Diclofenac sodium, a known nonsteroidal anti-inflammatory drug was used as the standard. The protein denaturation inhibition potential was determined with four different concentrations (62.5, 125, 250 and 500 µg/mL) and the percentage of inhibitions caused by the methanolic extracts of selected species of *Gomphostemma* are summarized and presented in Fig. 27. All extracts exhibited a concentration-dependent inhibition of protein denaturation. Among the extracts tested, *G. heyneanum* var. *heyneanum* showed potential activity with inhibition percentage ranging from 39.67 ± 1.55 to 80.99 ± 1.71 . *G. heyneanum* var. *rottleri* showed considerable effect with a range of activity from 36.16 ± 2.34 to $75.11 \pm 1.46\%$. The extract of *G. eriocarpum* showed least activity with inhibition range of 21.90 ± 1.04 to $49.79 \pm 0.66\%$.

ii. Proteinase inhibitory activity

Proteinases or proteases are proteolytic enzymes which have a vital role in many physiological functions of the cell. However, a variety of inflammatory diseases are associated with excessive activity of proteinases. It is because of this that they form excellent targets for treatment of many diseases with proteinase inhibitors as potential therapeutic agents. Present analysis was conducted with four different concentrations (62.5, 125, 250 and 500 µg/mL) of methanolic extracts of selected species of *Gomphostemma* and diclofenac sodium served as the standard. The data on the effect of extracts on proteinase inhibition are given in Fig. 28. In the present evaluation also, the most effective extract was found to be *G. heyneanum* var. *heyneanum* with an inhibition percentage in the range of 49.54 ± 1.28 to 81.15 ± 1.14 . The proteinase inhibition rate of *G. heyneanum* var. *rottleri* was in the range of 24.03 ± 1.18 to $68.95 \pm 1.13\%$. In this assay also *G. eriocarpum* showed least activity among the extracts with an inhibition of 15.89 ± 1.14 to $56.56 \pm 1.09\%$ for the concentrations tested.

In the *in vitro* biochemical assays conducted for evaluating the anti-inflammatory activity among selected species of *Gomphostemma*, a significant activity was portrayed by *G. heyneanum* var. *heyneanum* and hence it was further analysed for its activity on RAW 264.7 macrophage cell line.

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

The methanolic extract of *G. heyneanum* var. *heyneanum* was tested for its activity on RAW 264.7 macrophage cell line. Inflammation involves the activation and release of many key enzymes like cyclooxygenase, lipoxygenase, myeloperoxidase and nitric oxide synthase. Mitigating inflammatory responses by targeting these key inflammatory enzymes

provides an attractive approach for developing anti-inflammatory agents. The extract was analysed for its effect on cyclooxygenase activity, lipoxygenase activity, myeloperoxidase activity as well as for cellular nitrite level and inducible nitric oxide synthase (iNOS) activity.

i. Cyclooxygenase (COX) activity

The evaluation of cyclooxygenase enzyme activity is used as a method for the assessment of anti-inflammatory potential. In the present study, the effect of methanolic extract of *G. heyneanum* var. *heyneanum* was analysed for its effect on COX-2 activity in LPS induced RAW 264.7 macrophage cell line. The activity was measured with three different concentrations (25, 50 and 100 µg/mL) and expressed in terms of percentage of inhibition. Diclofenac sodium was used as the standard. The dose-dependent inhibition of COX-2 by the plant extract and standard is depicted in Fig. 29. The plant extract showed 29.04 ± 0.84 percentage of inhibition at 25 µg/mL of concentration. For concentrations 50 µg/mL and 100 µg/mL, percentage of inhibitions noticed were 49.58 ± 1.50 and 53.56 ± 1.21 respectively. For the same concentrations, the standard diclofenac sodium showed percentage of inhibitions in the range of 72.02 ± 0.59 to 84.91 ± 1.14 . The IC_{50} value calculated for the plant extract was 78.81 ± 4.30 µg/mL and for diclofenac it was found to 21.70 ± 3.21 µg/mL.

ii. Lipoxygenase (LOX) activity

Lipoxygenases are a versatile group of enzymes associated with arachidonic acid metabolism and are well known for their role in mediating inflammatory responses. In the present screening, three different concentrations (25, 50 and 100 µg/mL) were tested with diclofenac sodium as standard. The results are presented in Fig. 30. Plant extract showed only a moderate inhibitory effect with percentage of inhibition in the range of 11.04

± 0.66 to 40.74 ± 1.65 as compared to the standard diclofenac which exhibited a good inhibitory activity in the range of 71.94 ± 1.15 to 98.02 ± 0.34 for the concentrations tested. The IC_{50} value for plant extract was 126.11 ± 5.65 $\mu\text{g/mL}$ and for diclofenac it was 23.62 ± 2.24 $\mu\text{g/mL}$.

iii. Myeloperoxidase (MPO) activity

Myeloperoxidase is an enzyme that is released abundantly during the onset of inflammation and hence is identified as an early biomarker of inflammation. In the present analysis 25, 50 and 100 $\mu\text{g/mL}$ of plant extract and standard diclofenac sodium was tested for their effect on MPO activity in LPS treated RAW 264.7 cells. The plant extract significantly reduced the enzyme activity in a dose-dependent manner. A marked decrease in activity was noted as depicted in Fig. 31 and at highest concentration tested (100 $\mu\text{g/mL}$) a much reduced MPO activity of 0.0015 U/mL of cell lysate was recorded which is almost comparable to that of diclofenac at the same concentration.

iv. Cellular nitrite level

Many inflammatory conditions are often seen associated with high levels of cellular nitrites (NO). Agents which are potent inhibitors of NO production are considered as good candidates for developing anti-inflammatory drugs. In the present study, three concentrations (25, 50 and 100 $\mu\text{g/mL}$) of plant extract and standard diclofenac sodium were evaluated for their potential to inhibit NO production in LPS stimulated RAW 264.7 cells. The plant extract significantly inhibited nitrite accumulation in a concentration-dependent manner (Fig. 32). There has been a marked decrease in cellular nitrite level from 587.38 ± 1.41 $\mu\text{g/mL}$ to 359.95 ± 0.93 $\mu\text{g/mL}$ of cell lysate. This effect is commendable when compared to that of diclofenac

which reduced the nitrite level from 729.60 ± 0.84 to 343.53 ± 1.48 $\mu\text{g/mL}$ of cell lysate.

v. Inducible nitric oxide synthase (iNOS) activity

Inducible nitric oxide synthase (iNOS) is a chief regulator of inflammatory processes, responsible for high output nitric oxide (NO) synthesis. Since the plant extract exhibited good performance in reducing cellular nitrite levels in LPS induced RAW 264.7 cells, the same concentrations (25, 50 and 100 $\mu\text{g/mL}$) of plant extract were evaluated for their effect on iNOS levels with diclofenac sodium as standard. The activity of iNOS was significantly inhibited by the plant extract in a dose-dependent manner (Fig. 33). At concentrations of 25 to 100 $\mu\text{g/mL}$, the extract inhibited iNOS activity in a range of 32.46 ± 0.73 to $69.74 \pm 1.10\%$ with IC_{50} value of 62.19 ± 0.34 $\mu\text{g/mL}$. For diclofenac, the percentage of inhibition was in the range of 55.38 ± 0.67 to 90.77 ± 1.19 for the same concentrations and IC_{50} value was calculated as 22.47 ± 0.93 $\mu\text{g/mL}$. Thus it can be inferred that the reduction in cellular nitrite levels is due to the reduced activity of iNOS.

Present screening with LPS induced RAW 264.7 cells using five parameters of inflammation thus revealed that the methanolic extract of *G. heyneanum* var. *heyneanum* has anti-inflammatory activity at a moderate level. It has a greater impact on COX-2, MPO activity, cellular nitrite levels and in inhibiting iNOS activity as compared to its effect on lipooxygenase.

c. MTT assay of RAW 264.7 cells

MTT assay was used to analyse the cytotoxic effect of *G. heyneanum* var. *heyneanum* on RAW 264.7 cells (Plate 3). Various concentrations in the range of 10 $\mu\text{g/mL}$ to 50 $\mu\text{g/mL}$ were used (Fig. 34). The IC_{50} value was found to be 86.83 ± 1.54 $\mu\text{g/mL}$. A concentration of 75 $\mu\text{g/mL}$, a

concentration lower than IC_{50} value was considered appropriate for further analysis.

d. Estimation of inflammatory mediators using ELISA

The *in vitro* anti-inflammatory activity of *G. heyneanum* var. *heyneanum* was monitored by evaluating the levels of major inflammatory mediators with ELISA. The levels of inflammation related enzyme COX-2 and proinflammatory cytokine TNF- α were determined. The level of NF- κ B (p65) transcription factor was also measured. The RAW 264.7 cells ignited with LPS and treated with extract were used for the assay. Cells treated with LPS alone served as the control. A concentration of 75 μ g/mL was taken to exclude the effects of cytotoxicity. As shown in Fig. 35, treatment with extract considerably decreased COX-2 level from 1.98 ± 0.07 to 1.36 ± 0.11 U/mL and that of TNF- α was reduced from 2.03 ± 0.14 to 1.55 ± 0.09 U/mL of cell lysate. The level of NF- κ B (p65) transcription factor was significantly attenuated from 1.89 ± 0.11 to 1.18 ± 0.07 U/mL of cell lysate.

Hence, it can be presumed that the anti-inflammatory potential of methanolic extract of *G. heyneanum* var. *heyneanum* is due to its ability to effect inflammatory enzymes, cellular nitrite levels and inflammatory mediators like TNF- α . This is further supported by the suppressed iNOS activity and reduced levels of NF- κ B (p65) transcription factor.

D. SCREENING FOR CYTOTOXICITY WITH *ALLIUM CEPA* L.

a. Cytotoxicity studies using *Allium cepa* bioassay

The root meristems of *Allium cepa* L. are most frequently used as experimental materials for the study of induced cytotoxicity and chromosomal aberrations. In the present study, root tip meristems of *A. cepa* were treated with different concentrations (5, 25, 50, 75 and 100 μ g/mL) of methanolic

extracts of selected species of *Gomphostemma* for different time intervals (1, 3, 12 and 24 h). Mitotic index and chromosome aberrations were the two important parameters noted to study the cytotoxic effect of plant extracts on *A. cepa* root meristems. The aberrations induced were apparently different from normal mitotic stages (Plate 4). The effect exhibited was dose and duration-dependent. The results are depicted in Figs 36–39 and Plates 5–18.

Mitotic index (MI) is an accepted parameter to measure the cytotoxic and mitodepressive potential of plant extracts. There was a decrease in mitotic index in a concentration and time-dependent manner. In lowest concentration (5 µg/mL) after 1h incubation, mitotic index was found to be $73.63 \pm 1.52\%$ for *G. heyneanum* var. *heyneanum* (Fig. 36 A). In the same concentration and duration, it was 74.80 ± 2.13 for *G. heyneanum* var. *rottleri* and $75.83 \pm 4.77\%$ for *G. eriocarpum*. At the highest concentration (100 µg/mL), mitotic index declined and was found to be 61.74 ± 2.13 and $69.41 \pm 2.79\%$ respectively for *G. heyneanum* var. *heyneanum* and *G. heyneanum* var. *rottleri*. However, *G. eriocarpum* at the same concentration showed a mitotic index of $70.52 \pm 1.01\%$.

At 3 h incubation, mitotic index showed further reduction with respect to concentration and time of treatment. For *G. heyneanum* var. *heyneanum* at highest concentration of 100 µg/ mL it was $58.36 \pm 1.31\%$. In the case of *G. heyneanum* var. *rottleri* and *G. eriocarpum*, at the same concentration it was 64.60 ± 4.45 and $65.43 \pm 3.28\%$ respectively (Fig. 37 A). At the time period of 12 h incubation, mitotic index showed a significant reduction such that, at highest concentration (100 µg/mL) it was found to be 48.74 ± 5.03 and $56.28 \pm 5.49\%$ respectively for *G. heyneanum* var. *heyneanum* and *G. heyneanum* var. *rottleri*. At the same concentration *G. eriocarpum* showed a mitotic index of $60.31 \pm 1.71\%$ (Fig. 38 A)

At the final time period of 24 h incubation, there was a sharp and notable decline in mitotic index (Fig. 39 A). In the case of *G. heyneanum* var. *heyneanum* at the highest concentration there was a sharp decline in mitotic index to $31.47 \pm 2.28\%$ and for *G. heyneanum* var. *rottleri* it was $48.52 \pm 5.25\%$. For *G. eriocarpum*, mitotic index at the same concentration was noted as $55.86 \pm 3.59\%$. Thus in all time intervals and concentrations tested, *G. heyneanum* var. *heyneanum* showed comparatively high mitodepressive activity when compared to other two extracts as indicated by the lowest mitotic indices in all treatments.

Chromosome aberration percentage is yet another important parameter for the direct measure of cytotoxicity. In the present study, an array of chromosomal and cytological aberrations were noticed with different concentrations at different time intervals. The frequency of aberrations increased with increase in concentration and duration of treatment. During 1 h treatment with lowest concentration, *G. heyneanum* var. *heyneanum* showed chromosome aberration percentage of 19.80 ± 1.83 which increased to $27.63 \pm 1.02\%$ at $100 \mu\text{g/mL}$. Similarly, chromosome aberration percentage ranged from 16.2 ± 1.81 to $26.63 \pm 2.36\%$ and 15.43 ± 1.15 to $25.27 \pm 1.32\%$ respectively for *G. heyneanum* var. *rottleri* and *G. eriocarpum* (Fig. 36 B). As the duration of treatment was increased to 3 h, a slight increase in chromosome aberration percentage was noted and at $100 \mu\text{g/mL}$ it was found to be $37.43 \pm 1.59\%$ for *G. heyneanum* var. *heyneanum*, whereas for *G. heyneanum* var. *rottleri* and *G. eriocarpum* it was 35.87 ± 4.58 and $33.80 \pm 2.24\%$ respectively (Fig. 37 B).

The effect of plant extract to induce chromosome and cytological aberrations became more evident at 12 h treatment as there was a drastic increase in chromosome aberration percentage with respect to highest concentration (Fig. 38 B). *G. heyneanum* var. *heyneanum* showed

chromosome aberration percentage of 54.03 ± 2.84 at $100 \mu\text{g/mL}$. At the same concentration, *G. heyneanum* var. *rottleri* and *G. eriocarpum* showed 50.26 ± 0.89 and 44.27 ± 1.88 percentage of chromosome aberration respectively. However, greater chromosome aberration percentage was noted at 24 h of treatment (Fig. 39 B). At $100 \mu\text{g/mL}$ for *G. heyneanum* var. *heyneanum* the percentage was observed to be $73.87 \pm 1.47\%$. It was $64.13 \pm 1.80\%$ for *G. heyneanum* var. *rottleri* and, while that for *G. eriocarpum* it was noted as $57.20 \pm 1.44\%$ for the same concentration. Nevertheless, positive control showed greater aberration percentage, irrespective of concentration and duration in all treatments.

Present study to reveal the cytotoxic potential of selected species of *Gomphostemma* using *A. cepa* assay disclosed a diverse range of clastogenic, turbagenic and cytological aberrations. Major clastogenic aberrations observed include nuclear lesions, nuclear budding, nuclear peak, giant cells, nuclear erosion, pulverized chromatin, chromosome gaps, ring chromosomes, hyperchromatia, anaphase and telophase bridges, *etc.* Turbagenic abnormalities resulting from mitotic spindle disturbances were also observed. Some of the aberrations observed which resulted from spindle failure include hypoploidy, lagging chromosomes, vagrance, shift in microtubular organizing centers (MTOC), ball metaphase, misorientation, binucleate cell, *etc.* Many of the abnormalities observed were multiple aberrations like nuclear lesion together with cytoplasmic vacuolation, nuclear and cytoplasmic shrinkage, nuclear lesion and blebs with cytoplasmic vacuolation, *etc.* (Plates 5–18).

It can thus be suggested that methanolic extracts of selected species of *Gomphostemma* have the ability to induce nuclear, chromosomal and cytological aberrations, together with the inhibition of mitotic index. It can be inferred that these plants have cytotoxic and mitodepressive activity and have the potential to interfere with the cell cycle.

b. *In situ* visualization of cell death

Evans blue staining technique was used to visualize the cell death induced on *A. cepa* roots by different concentrations of plant extracts (5, 25, 50, 75 and 100 µg/mL) after 24 h treatment. The results are shown in Fig. 40 and Plate 19. N, N-dimethylformamide was used to release the Evans blue from the roots and the estimation of cell death was carried out spectrophotometrically. The intensity of dye taken up by the root cells is found proportional to the cell death induced, as the dye is absorbed only by the dead cells.

At 24 h incubation, negative control root tips showed negligible absorbance (0.027 ± 0.001), whereas positive control exhibited maximum absorbance (0.966 ± 0.005). The cell death and absorbance pattern was found to be dose-dependent. The extract of *G. heyneanum* var. *heyneanum* showed maximum cell death with absorbance in the range of 0.307 ± 0.007 to 0.687 ± 0.011 . The absorbance range for *G. heyneanum* var. *rottleri* was 0.175 ± 0.002 to 0.574 ± 0.015 . *G. eriocarpum* showed minimum absorbance range from 0.104 ± 0.021 to 0.457 ± 0.034 . Thus, it is clear from the result that *G. heyneanum* var. *heyneanum* is more effective in inducing cell death in *A. cepa* roots as compared to other two plant extracts.

E. ANTIPROLIFERATIVE STUDIES WITH MCF-7 CELL LINE

a. Cytotoxic evaluation using MTT assay on MCF-7 cell line

In vitro cytotoxic tests on mammalian cell lines are primarily adopted for safety evaluation before proceeding to animal models. In the present study, cytotoxic evaluation was conducted with different concentrations of plant extracts against MCF-7 cell line using MTT assay. MCF-7 cells were incubated in various concentrations (6.25, 12.5, 25, 50 and 100 µg/mL) of plant extract for 24 h. IC₅₀ value for each extract was determined. The results

are summarized and represented in Fig. 41 and Plates 20–22. As shown in Fig. 41, percentage of viability decreased with increase in concentration, displaying the cytotoxic potential of plant extracts. MTT assay of *G. heyneanum* var. *heyneanum* showed significant effect on MCF-7 cells with cell viability percentage in the range of 74.38 ± 2.40 to 35.57 ± 0.44 for concentrations from 6.25 to 100 $\mu\text{g/mL}$. The IC_{50} value was calculated as 45.97 ± 1.48 $\mu\text{g/mL}$. In the case of *G. heyneanum* var. *rottleri*, viability percentage showed a gradation in the range of 86.33 ± 1.92 to 58.53 ± 1.06 and IC_{50} value obtained was 116.18 ± 1.14 $\mu\text{g/mL}$. Among the extracts tested *G. eriocarpum* displayed lowest cytotoxicity with IC_{50} value of 127.26 ± 1.77 $\mu\text{g/mL}$ and the viability percentage ranged from 88.50 ± 1.78 to 60.11 ± 1.31 . The results showed that *G. heyneanum* var. *heyneanum* has the lowest IC_{50} value with significant cytotoxic potential. Morphological investigation of treated cells (Plate 22) clearly displayed many apoptotic features like cell shrinkage, cell rounding, membrane blebbing and formation of apoptotic bodies. Thus, methanolic extract of *G. heyneanum* var. *heyneanum* at 45 $\mu\text{g/mL}$ (based on IC_{50} value) was selected for further screening and analysis.

b. Cytotoxic evaluation using MTT assay on L929 cell line

The effective extract of *G. heyneanum* var. *heyneanum* was analysed for its cytotoxicity on normal L929 cells. Various concentrations in the range of 6.25 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$ were tested and IC_{50} value was determined (Fig. 42). It was found that the extract has only insignificant cytotoxicity against L929 cells (Plate 23). The cell viability percentage observed at 100 $\mu\text{g/mL}$ was 77.64 ± 1.37 and IC_{50} value was found to be 220.23 ± 1.16 $\mu\text{g/mL}$, a value much higher as compared to the activity of extract on MCF-7 cell line. This makes it clear that the extract has selective cytotoxic activity towards the selected cancer cell line.

c. Comet assay

Comet assay, also called single cell gel electrophoresis was conducted to analyse the DNA damage induced by the extract on individual cells. It is called so because, the resulting image resembles a comet with distinct head and tail. It is a rapid and sensitive technique to evaluate genotoxicity as the amount of DNA liberated from the head of the comet is proportional to the amount of DNA damage. Five parameters *viz.*, comet length, tail length, tail DNA percentage, tail moment and olive tail moment were determined for the assessment of DNA damage induced by 45 µg/mL of extract on MCF-7 cells. The results are shown in Fig. 43 and Plate 24. As evident from the results, a considerable increase in comet length and tail length was noticed as compared to control (Fig. 43 A & B). The tail DNA percentage was found to be 42.91 ± 2.24 for treated cells, whereas for control it was found to be 11.09 ± 1.20 (Fig. 43 C). Tail moment and olive tail moment were also found significantly elevated when compared to control (Fig. 43 D & E), clearly revealing the efficiency of *G. heyneanum* var. *heyneanum* to induce DNA damage and its apparent genotoxic potential on MCF-7 cell line.

d. Detection of apoptosis

Induction of apoptosis was assessed with AO/EB dual staining technique. MCF-7 cells were treated with 45 µg/mL of *G. heyneanum* var. *heyneanum* extract and incubated for 24 h followed by staining. Morphological changes of cells treated with extract were examined under fluorescent microscope. This staining technique enabled to visualize and segregate normal cells from apoptotic cells. Early apoptotic cells, late apoptotic cells and necrotic cells were distinguished from normal cells distinctly by their typical morphological features and variable staining pattern (Plate 25). Control (untreated cells) showed uniform green, whereas treated cells showed morphological changes, which are characteristic symptoms of

apoptosis like chromatin condensation, nuclear fragmentation, apoptotic bodies, *etc.* Most of the treated cells were in early and late apoptotic stages. Early apoptotic cells were marked by granular greenish yellow colour. Late apoptotic cells with orange red staining and fragmented nuclei were also detected. The study thus revealed the potential of *G. heyneanum* var. *heyneanum* extract to induce apoptosis on MCF-7 breast cancer cell line.

e. Cell cycle analysis

The effect of *G. heyneanum* var. *heyneanum* extract on cell cycle progression was analysed with flow cytometry. The results are summarized and represented in Fig. 44, 45 and 46. The MCF-7 cells were treated with 45 µg/mL of plant extract and incubated for 24 h. Cell cycle progression of treated and untreated cells was determined by examining the DNA content and population profile. As shown in flow cytometry histograms of cell cycle analysis (Fig. 44 B), there is a prominent increase in G0/G1 population in treated cells with a corresponding decrease in S and G2/M population when compared to the control (Fig. 44 A) indicating the cell cycle arrest at this phase and, a sub-G1 population seen to the left of G0/G1 peak is possibly representing the accumulation of apoptotic cells. The population profile (Fig. 45 B) also shows the accumulation of treated cells in G0/G1 phase when compared to the control (Fig. 45 A). The DNA content index revealed a higher percentage of G0/G1 population (74.86 ± 1.82) in treatment as compared to control (65.81 ± 1.26). The percentage of cells in S phase declined from 16.3 ± 0.78 in control to 4.7 ± 0.69 in treated cells. The treated cells also showed a remarkable reduction in G2/M population (Fig. 46). Increased G0/G1 population associated with concurrent decrease in cell population in S phase and G2/M phase in treated cells clearly marks the induction of apoptosis with cells cycle arrest in G1 phase. Thus, it can be concluded that extract of *G. heyneanum* var. *heyneanum* has the potential to

induce apoptosis by arresting cell cycle progression at G0/G1 phase in MCF-7 cells.

f. Determination of caspase activity with ELISA

The effect of extract of *G. heyneanum* var. *heyneanum* on caspase-9 and caspase-7 were determined by ELISA. The MCF-7 cells were treated with 45 µg/mL of plant extract and incubated for 24 h. Untreated cells served as control. The activity of caspase-9 and caspase-7 was estimated and compared with the control. The activities of caspases in response to plant treatment are shown in Fig. 47. The activity of caspase-9 was found to be 0.329 U/ mg of cell lysate and for control it was obtained as 0.225 U/mg of cell lysate. Similarly, the activity of caspase-7 was also found to be elevated (0.342 U/mg of cell lysate) as compared to control (0.185 U/mg of cell lysate). Thus, caspases are found significantly activated by treatment with the plant extract indicating their possible role in inducing apoptosis.

g. Gene expression studies

The potential of methanolic extract of *G. heyneanum* var. *heyneanum* to induce apoptosis was studied by analysing its effect on the expression of two apoptosis related genes. Expression of tumour suppressor gene p53 and its downstream effector p21 was examined by RT-qPCR followed by gel electrophoresis for the separation and visualization of DNA fragments using β-actin as loading control. The MCF-7 cells treated with 45 µg/mL of *G. heyneanum* var. *heyneanum* extract for 24 h were used for the study and untreated cells served as the control. The results are presented in Figs 48 and 49. The results showed that the treatment significantly induced an up-regulation in the expression of both p53 and p21. The expression fold change for p21 was found to be 1.21 ± 0.06 and that of p53 was noted as 1.82 ± 0.09 . The gene p53 is known for its role in cell cycle arrest and in inducing

apoptosis, whereas p21gene is a p53 dependent mediator involved in cell cycle arrest in response to DNA damage. Thus, it can be accomplished that methanolic extract of *G. heyneanum* var. *heyneanum* has the potential to induce cell cycle arrest followed by apoptosis.

F. GREEN SYNTHESIS OF SILVER NANOPARTICLES AND THEIR CHARACTERIZATION

a. Synthesis of silver nanoparticles

The synthesis of silver nanoparticles by biological method is considered as an efficient and non-toxic approach to obtain nanoparticles with better sizes and morphologies. In the present study, green synthesis of silver nanoparticles with selected species of *Gomphostemma* was attempted. The silver nanoparticles obtained were characterized by its colour formation, surface plasmon resonance, SEM and EDX analysis. The optimal ambient conditions for the synthesis of silver nanoparticles were determined and fixed to be (Temperature - 80 °C, pH of reaction mixture - 7, metal ion (silver nitrate) concentration - 2 mM AgNO₃, stoichiometric proportion of 2 mM AgNO₃ and methanolic extract - 9:1 and the incubation time - 30 min).

The reduction of silver nitrate into silver nanoparticles by the plant extracts was evidenced by the visual colour change of the reaction mixture from pale green to yellowish brown or dark brown colour. All the three plant extracts evaluated showed the colour change with slight variations in intensity (Plate 26). It was brownish for *G. heyneanum* var. *heyneanum* and the rate of synthesis was moderate. But, *G. heyneanum* var. *rottleri* produced dark brown coloration and the rate of synthesis was rapid as compared to other two extracts. *G. eriocarpum* produced a light brown solution and a slow rate of synthesis was noticed. The blank solution remained colourless throughout, even after the addition of silver nitrate solution.

b. Characterization of silver nanoparticles

i. UV-Vis spectral analysis

The synthesised silver nanoparticles by the plant extracts were subjected to optical measurements with UV-Vis spectrophotometry to observe absorption peaks resulting from surface plasmon excitation. The UV spectra of silver colloids were observed in the range of 300–800 nm (Fig. 50). The synthesised silver nanoparticles exhibited a UV-Vis absorption maximum in the range of 400–500 nm. In the case of *G. heyneanum* var. *heyneanum* maximum absorbance was centered on 437 nm. Whereas, *G. heyneanum* var. *rottleri* showed an absorption peak around 435 nm and for *G. eriocarpum*, it was found to be 448.5 nm.

ii. FE-SEM/EDX analysis

SEM technique was employed to validate the morphology and size of nanoparticles (Plate 27, 28 & 29). The silver nanoparticles were nearly spherical in shape. The average size of the silver nanoparticles synthesised by *G. heyneanum* var. *heyneanum* was in the range of 24.46–38.67 nm (Plate 27). The size of *G. heyneanum* var. *rottleri* mediated nanoparticles was in the range of 23.93–34.18 nm, slightly smaller as compared to that of the first variety (Plate 28). The silver nanoparticles synthesised with *G. eriocarpum* were the largest among the three extracts tested, with the size in the range of 59.97–65.23 nm (Plate 29).

The EDX analysis of silver nanoparticles synthesised by extracts of *Gomphostemma* confirmed strong signals at 3 keV from silver atoms in the nanoparticles (Plate 30). It showed higher percentage of silver which confirmed the formation of nanosilver and its elemental nature. Elemental analysis of nanoparticles synthesised by *G. heyneanum* var. *heyneanum*

showed silver (73%) followed by trace amounts of chlorine (9.5%) carbon (9.16%), oxygen (6.85%), silicon (1.16%) and phosphorus (0.33%). Elemental profile of *G. heyneanum* var. *rottleri* showed silver (77.83%), chlorine (12.94%), carbon (4.58%), oxygen (3.43%), silicon (0.59%) and phosphorus (0.63%). Silver nanoparticles by *G. eriocarpum* also showed strong signal of silver (74.44%) followed by weak signals of chlorine (9.83%), carbon (6.66%), oxygen (7.25%), silicon (1.31%) and phosphorus (0.51%). Present study thus manifested an eco-friendly approach for the green synthesis of silver nanoparticles with selected taxa of *Gomphostemma*.

DISCUSSION

The practice of using plant derived products with therapeutic potential for treating various ailments is as old as human civilization. But with the advent of industrial revolution and development of organic chemistry, synthetic compounds acquired preferential importance and drugs from natural sources began to be blindly considered as an option for the poorly educated with mere superstitious values. Alternative therapy has however stood the test of time and recent years have witnessed profound interest in plant derived products with therapeutic values. Abusive and long term usage of synthetic drugs have rendered them with harmful side effects and this might be the possible reason for the recent interest in drugs of plant origin. Still, the potential use of plants as source of drugs is poorly explored and only a small percentage has been properly studied and screened phytochemically in terms of their pharmacological properties. Further, drug development from plant sources is a multidisciplinary approach and it takes years of effort to derive and isolate an active compound with desirable biological activity (Rates, 2001). Even with all the prevailing challenges in drug discovery, plants continue to be important in providing leads against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria and inflammation (Balunas & Kinghorn, 2005).

A. PHYTOCHEMICAL CHARACTERIZATION

Phytochemicals are responsible for the medicinal and therapeutic potential displayed by the plants. Detailed phytochemical analysis helps in systematic characterization and identification of chemical constituents imparting pharmacological properties to plants. Successful determination of bioactive components from plants requires a multifaceted approach and is influenced by a number of factors like sample preparation, extraction solvent,

method of extraction, *etc.* Screening with crude extracts prefers those solvents which can extract maximum components from sample (Liu, 2008). Among the various conventional methods, soxhlet extraction is still considered as one of the effective and reliable methods for extracting components from plant materials. Developments of modern chromatographic techniques like GC/MS and HR-LC/MS as well as other spectrometric techniques have made bioactive compound analysis and identification easier than before (Azmir et al., 2013).

In the present study, qualitative and quantitative estimation of major phytochemicals together with GC/MS and HR-LC/MS studies were used for the phytochemical characterization of methanolic extracts of *Gomphostemma* species.

a. Qualitative phytochemical screening

Preliminary phytochemical screening of all the three selected taxa of *Gomphostemma* revealed the presence of various classes of phytochemicals (Table 6). Major phytoconstituents like phenols, flavonoids, alkaloids, terpenoids, steroids, carotenoids, cardiac glycosides, quinones, coumarins and iridoid glycosides were identified to be present, but tannins and steroids were found to be absent in selected plants. The detection of major phytochemicals from selected taxa of *Gomphostemma* has been published (Sajitha & Thoppil, 2018) and is in agreement with the previous reports on phytochemical studies with other species of *Gomphostemma* (Shams-Ud-Doha et al., 2013; Dutta, 2014). It is also in concurrence with the major class of compounds reported from various members of Lamiaceae and Lamioideae (Table 1 & 2).

Studies have shown that plant derived phenols and phenolic acids have good antimicrobial activity and many of them have even displayed good synergism with synthetic antibiotics against antibiotic resistant

microorganisms (Aldulaimi, 2017). Plant phenolic compounds are also potent antioxidant (Sakihama et al., 2002) and anti-inflammatory (Brezani et al., 2018) agents. Flavonoids are low molecular weight polyphenolic compounds (Samanta et al., 2011). Flavonoids from plant sources are known for their versatile benefits. They are proved to have antioxidant and free radical scavenging capacity. Many are shown to have role in coronary heart disease prevention, hepatoprotective, anti-inflammatory and anticancer activities, while some flavonoids have also shown potential antiviral activities (Kumar & Pandey, 2013). Plant alkaloids are one of the largest and highly diverse groups of chemical entities with potent pharmacological effects. Many plant alkaloids are known for their narcotic, analgesic, muscle relaxant, antidepressant, antimicrobial and anticancer properties (Bribi, 2018). Terpenoids include a diverse array of compounds with applications in cosmetic and perfumery sectors. They also constitute one of the largest natural bioactive compounds and many of them like limonene, cineole, betulinic acid, sitosterol, *etc.* are under clinical trials for the treatment of various types of diseases and disorders (Singh & Sharma, 2015). Plant steroids are unique class of compounds with therapeutic potential especially in treating inflammatory disorders and some are also reported to have anticancer efficacy (Patel & Savjani, 2015). Cardiac glycosides are unique group of steroids and are known for their effect in the treatment of heart failure. Some cardiac glycosides have also displayed antitumour and antiviral activities (Morsy, 2017). Carotenoids are natural isoprenoid pigments with antioxidant and immunomodulating activities, which may prevent degenerative diseases like cardiovascular diseases, diabetes and several types of cancers, especially prostate and digestive tract tumours (Strati et al., 2012). Quinones are ubiquitous class of compounds seen as secondary metabolites and are principally isolated from plants. Quinones have shown to exhibit numerous biological activities like neurological, antibacterial, antiplasmodial,

antioxidant, antitumour and anti-HIV. These activities displayed by quinones are attributed to the redox properties of their carbonyl functions (Eyong et al., 2013). Coumarins are benzopyrone class of organic compounds found in many plants (Poumale et al., 2013). Many coumarins and their analogues of plant origin are known for anticoagulant property. They have also been shown to be useful in inflammation-modulating, edema-reducing and anticancer therapies. However, coumarin and coumarin rich herbs have a small risk of inducing hepatotoxicity (Yarnell & Abascal, 2009). Lamiaceae, especially members of Lamioideae is reported to have a rich variety of iridoid glycosides (Kooiman, 1972). Iridoid glycosides are monoterpenoids and most of them like harpagoside, catalposide, geniposide, *etc.* have anti-inflammatory, analgesic, antimicrobial and antitumour properties (Villasenor, 2007). Preliminary screening thus revealed the diversity of phytochemicals in selected plants and gave an insight to their bioactive potential.

b. Quantitative estimation of major phytocomponents

Estimation of major secondary phytocomponents like phenols, flavonoids and terpenoids was done on extracts of selected plants using standard protocols. Total phenolic content estimated with Folin-Ciocalteu reagent revealed highest phenolic content (Fig. 9) in *G. heyneanum* var. *heyneanum* (260.31 ± 11.62 mg GAE/g DW) and lowest in *G. eriocarpum* (32.56 ± 3.18 mg GAE/g DW). Phenolic compounds are the most widespread class of metabolites in nature. They are natural antioxidants with the potential to neutralize free radicals (Lopez et al., 2003). Phenolics have the ability to act as antioxidants in a number of ways. Hydroxyl groups of phenolics are good hydrogen donors, which can react with reactive oxygen and reactive nitrogen species leading to termination reaction and thus preventing the generation of new radicals. Their antioxidant capacity is also due to their ability to chelate metal ions involved in the production of free radicals.

Phenolic structures have the potential to strongly interact with proteins largely due to their hydrophobic benzenoid rings and hydrogen bonding potential of their hydroxyl groups. The ability of phenolics to act as antioxidants is also by virtue of their capacity to inhibit some enzymes involved in radical generation. Synergistic effects of phenolic compounds with other antioxidants have also been reported (Brewer, 2011).

Flavonoid content was estimated and expressed in terms of quercetin equivalent (mg QE/g DW). It was found to be highest in *G. heyneanum* var. *heyneanum* (129.22 ± 10.65) and lowest in *G. eriocarpum* (33.69 ± 0.53) (Fig. 11). Flavonoids are polyphenolic compounds with benzo- γ -pyrone structure with a variety of pharmacological activities reported. They are well known for their capacity as antioxidant agents. Functional hydroxyl groups in them mediate their antioxidant effects. Flavonoids also have the ability to induce protective enzyme systems as a part of their antioxidant efficacy. To add on to this, several flavonoids such as catechin, apigenin, quercetin, rutin, venoruton, *etc.* are reported to have hepatoprotective activities. Flavonoid rich plant extracts from different species have also been reported to possess antibacterial, anti-inflammatory, anticancer and antiviral activity (Panche et al., 2016).

Total terpenoid content was measured and expressed as linalool equivalent (mg linalool/g DW). Highest content was estimated in *G. heyneanum* var. *heyneanum* (279.56 ± 8.53) and lowest in *G. eriocarpum* (118.21 ± 7.79) (Fig. 13). Chemical profile of Lamiaceae is rich in terpenoids especially diterpenes (Hussein, 2018) and results of the present study is in agreement with the same. Terpenoids include a wide array of natural compounds and all are synthesised from two five-carbon building blocks. Based on the number of building blocks they are classified as monoterpenes, sesquiterpenes, diterpenes, *etc.* and almost all of them display a wide range of

biological activities. Monoterpenes are mainly found as constituents in essential oils, floral scents and resins of aromatic plants. Studies have shown that they are good antitumour agents and that they not only have the ability to prevent the formation or progression of cancer but also have the potential to regress the existing malignant tumours. Sesquiterpenes are less volatile as compared to monoterpenes and are known for their anti-inflammatory property. Many of them are also widely used in treating headaches, fever, asthma and numerous skin diseases. Diterpenes constitute a large group with ubiquitous distribution. A variety of them like phytol, paclitaxel, retinol, *etc.* have shown anticancer, antidiabetic and antimicrobial properties (Wang et al., 2005b).

Quantitative analysis gave an estimate of major phytocomponents in selected species of *Gomphostemma* which can be synchronised to the bioactivities exhibited by them.

c. Gas Chromatography/Mass Spectrometry (GC/MS) analysis

GC/MS technology is a widely used technique for metabolite profiling. It facilitates the identification and robust quantification of relatively broad classes of compounds in plant extracts (Lisec et al., 2006). GC/MS analysis is particularly useful in the identification and quantification of volatile and semi-volatile components in a complex mixture like plant extracts (Cappelaro & Yariwake, 2015). Present study analysed selected species of *Gomphostemma* with GC/MS technique for the identification of various volatile constituents in them. An array of 72 compounds belonging to various classes of compounds like terpenoids, phenolic compounds, flavonoids, fatty acids, *etc.* were identified (Table 7). Terpenes were the predominant class of compounds identified and this is in concurrence with the results of quantitative estimation studies which showed highest content of terpenoids in all the three taxa analysed. The bioactivities shown by selected species of *Gomphostemma* in

the present study may be justified on the basis of these active chemical entities present in them.

The GC/MS analysis of methanolic extract of *G. heyneanum* var. *heyneanum* revealed the presence of 38 compounds (Table 7). The major components identified with prominent biological activities include α -thujene, neophytadiene, β -caryophyllene, limonene, eucalyptol, phytol, stearic acid, stigmasterol, quinic acid and tocopherol. These compounds have already been detected in other members of Lamiaceae (Table 1) and also in the subfamily Lamioideae (Table 2). The monoterpene α -thujene (14.63%), which was found in fairly good percentage, is a major component in many plant derived essential oils with insecticidal, anti-inflammatory and antimicrobial activities (Singh & Atal, 1986; Terblanché & Kornelius, 1996; Sadhasivam et al., 2016). Limonene (3.96%) is a cyclic monoterpene naturally occurring in the peels of citrus and other plants. It is reported to have antioxidant activity together with apoptotic and immune modulation effects. It is noted to have strong potential for development as a chemopreventive agent, particularly for cancers with multifactorial etiologies (Miller et al., 2011). Eucalyptol (1.96%), a monoterpene and an aromatic constituent of eucalyptus oil has also been identified in many members of Lamiaceae like *Salvia*, *Mentha*, etc. (Mander & Liu, 2010; Mesquita et al., 2019). It is a well known anti-inflammatory agent (Juergens et al., 2003). Studies have shown that it has prooxidant, antioxidant and anticancer properties (Özkan & Erdogan, 2013; De Lima et al., 2014).

Other notable monoterpenes identified were α -pinene, β -phellandrene, β -myrcene and p-cymene. The monoterpene, α -pinene (1.29%) has antioxidative, anticancer and genotoxic properties (Aydin et al., 2013). Another monoterpene, β -phellandrene (1.33%) which is a major constituent in many essential oils derived from *Lavandula*, *Mentha*, *Stachys*, etc. is reported

to have minty and citrusy odour (Lis-Balchin, 2003; Telci et al., 2010; Javidnia et al., 2010; Singh et al., 2015). The acyclic monoterpene β -myrcene (1.44%) has antioxidant property (Ciftci et al., 2011). Studies have shown that β -myrcene in the essential oil of *Pinus koraiensis* cones has anti-invasive effect on metastatic MDA-MB-231 human breast cancer cells (Lee et al., 2015). Another monoterpene p-cymene (1.23%) is a natural antioxidant usually with a woody and spicy odour. It is regarded as the main antimicrobial compound in thyme with analgesic and anti-inflammatory properties (Marchese et al., 2017a).

The sesquiterpene identified as β -caryophyllene (5.48%) has various biological activities. It is a major plant volatile found in large amounts in the essential oils of many plants like *Origanum vulgare*, *Cinnamomum* spp., *Piper nigrum*, *Cannabis sativa*, etc. (Gertsch et al., 2008). It has displayed pronounced antimicrobial and strong antioxidant activity (Dahham et al., 2015). This sesquiterpene is also reported to have antineoplastic, analgesic and anti-inflammatory properties (Fidyt et al., 2016; Sain et al., 2014). Another noted sesquiterpene, β -elemene (1.17%) is reported to have anticancer activity. It has displayed anticancer effect on laryngeal (Tao et al., 2006) and prostate cancer cells (Li et al., 2010). Its antitumour effect on non-small-cell lung cancer (NSCLC) has been shown to be mediated by cell cycle arrest followed by apoptosis (Wang et al., 2005a).

Phytol (2.60%) is a diterpene alcohol and a major constituent of chlorophyll. Many works have documented its biological activities. It has shown promising antiradical activity towards hydroxyl radical, superoxide anion radical, methoxy radical, carbon-dioxide anion radical, nitric oxide radical and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. It is also known for its antimicrobial activity against many bacterial and fungal strains (Pejin et al., 2014b). Studies have suggested that phytol has the ability to attenuate

inflammatory response by reducing cytokine production and oxidative stress (Silva et al., 2014). Phytol is found to be cytotoxic to many cancer cell lines like A549 (human lung carcinoma), MCF-7 (breast cancer), HeLa (cervical cancer), HT-29 (colon cancer), *etc.* Detailed study with A549 cell line have shown that phytol can induce ROS mediated apoptosis by induction of caspases and is also found to inhibit the known tumour proliferation factor glucose-6- phosphate dehydrogenase, displaying its innate potential to become a good anticancer drug candidate (Pejin et al., 2014a; Thakor et al., 2017). Neophytadiene (6.22%) is a diterpene which is present in plants as well as in green microalgae and is reported to have the potential to be used as biofuel blended with other fossil fuels (López-Rosales et al., 2019).

Quinic acid (3.08%) is a phenolic acid found in coffee beans, cinchona and other plant materials. It is the hydrolysis product of chlorogenic acid. Phenolic acids in general are potential cancer preventing agents (Newmark, 1996). It has a wide spread occurrence in woody dicotyledons and has been extracted extensively from many plant tissues (Yoshida et al., 1975; Barco et al., 1997). It has shown good antioxidant property (Pero et al., 2009), radioprotective (Cinkilic et al., 2013) and anti-inflammatory activity (Zeng et al., 2009). Studies have shown that quinic acid has antiviral activity especially anti-herpes activity (Özçelik et al., 2011).

Tocopherols are a group of fat soluble phenolic compounds and a class of vitamin E, naturally found in many fruits, vegetables and seeds. Present study identified α -tocopherol (1.24%) in considerable percentage. They have strong antioxidant properties and hence are suggested to reduce the risk of cancer (Engin, 2009; Das & Suh, 2016). Vitamin E together with vitamin C has shown to be effective in protecting cisplatin induced nephrotoxicity and the protection is mediated partially by preventing the decline of renal antioxidant status (Ajith et al., 2007).

Stigmasterol (3.15%) is a phytosterol with therapeutic potential. Dietary plant sterols especially stigmasterol has been shown to reduce plasma cholesterol levels in the body (Batta et al., 2006). Apart from this, it is also ascribed with anti-inflammatory property (Gabay et al., 2010). They are also known to induce apoptosis in cancer cell lines thus displaying their anticancer potential (O'Callaghan et al., 2010; Kim et al., 2014). Campesterol (1.42%) is a natural plant derived sterol with cholesterol lowering capacity and anticancer property. Campesterol isolated from *Chrysanthemum coronarium* is shown to have potential antiangiogenic action, which could possibly be responsible for the antiproliferative activity of this compound (Choi et al., 2007).

GC/MS analysis of methanolic extract of *G. heyneanum* var. *rottleri* showed a total of 39 compounds (Table 7). Here also terpenes constituted major class of compounds but, in contrast to *G. heyneanum* var. *heyneanum* monoterpenes were scarce. Two major diterpenes identified were phytol (18.75%) and neophytadiene (6.71%). Sesquiterpenes identified include β -caryophyllene (1.97%), caryophyllene oxide (1.65%) and valencene (0.77%) along with minor percentages of spathulenol, α -cubebene, cubenol, etc. Caryophyllene oxide is an oxygenated terpenoid with antifungal activity against dermatophytes (Yang et al., 2000). Caryophyllene oxide isolated from essential oils of medicinal plants such as *Psidium guajava*, *Origanum vulgare*, *Cinnamomum* spp., *Eugenia caryophyllata* and *Piper nigrum* have induced apoptosis in human prostate and breast cancer cells through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation (Park et al., 2011). Valencene is an aroma component in citrus fruits used widely as a flavouring agent in beverage industry. Valencene derived from *Cyperus rotundus* is reported to have biological effects like anti-allergic, antimelanogenesis and anti-inflammatory activities (Yang et al., 2016). Spathulenol (0.63%) is a sesquiterpene with earthy aromatic odour and this

component isolated from *Salvia mirzayanii* has shown immunomodulatory effect on peripheral blood lymphocytes (Ziaei et al., 2011).

Squalene (1.03%) is a widely occurring natural triterpene. It has several beneficial properties as a natural antioxidant, as emollient in adjuvants for vaccines, has a preventive effect on breast cancer, possesses tumour protective, cardioprotective properties and has the ability to decrease the serum cholesterol level (Spanova & Daum, 2011).

Loliolide (0.97%) is a monoterpenoid lactone, ubiquitous in plants and animals including marine organisms. It is usually present in small quantity. Plants containing this component are used in the treatment of diabetes and depression. Apart from this, a wide range of bioactivities including anti-inflammatory and antioxidant properties are assigned to this compound (Grabarczyk et al., 2015). Another monoterpene noticed was dihydroactinidiolide (0.46%).

The phenolic compounds identified were 2-methoxy-4-vinylphenol (1.43%), α -tocopherol (1.69%) and cinnamaldehyde (0.38%). The compound 2-methoxy-4-vinylphenol is responsible for the natural aroma of buckwheat and is used as a flavouring agent. This component isolated from pine needles has shown anti-inflammatory effect mediated by the suppression of NF- κ B and MAPK activation in LPS-stimulated RAW 264.7 cells. It induced cell cycle arrest in BaP treated NIH 3T3 cells by regulating the expression of cell cycle related proteins (Jeong & Jeong, 2010). Cinnamaldehyde, the phenolic compound responsible for the aroma of *Cinnamomum* spp. is known to inhibit a wide spectrum of food pathogenic and spoilage microorganisms (Sanla-Ead et al., 2012). It has shown to induce apoptosis by ROS-mediated mitochondrial permeability transition in human promyelocytic leukemia HL-60 cells. Cinnamaldehyde and its derivatives have been found to be effective in treating various types of cancer, including breast, prostate and

colon cancers. Its ability to inhibit metastasis and its anti-invasion effects would be beneficial for cancer treatment (Hong et al., 2016).

Phytosterols identified were stigmasterol (10.68%) and β -sitosterol (5.61%). Among the two, β -sitosterol is one of the main dietary sterols found in plants. It is reported to have anti-inflammatory activity (Saeidnia et al., 2014). Treatment with β -sitosterol inhibited the growth of HT-29 cell line (Awad et al., 1998) and stimulated apoptosis in MDA-MB-231 human breast cancer cells (Awad et al., 2000).

The extract of *G. eriocarpum* showed 26 compounds with terpenes as major class of compounds (Table 7). Phytol (47.65%) and neophytadiene (11.21%) were the two main diterpenes noted. Monoterpenes were represented by loliolide (1.18%) and dihydroactinidiolide (0.45%). Dihydroactinidiolide is volatile with tea-like odour. It is a natural analogue of loliolide and a degradation product of carotenoid. It has displayed good antioxidant potential and *in silico* studies have supported its effect in treating Alzheimer's disease (Das et al., 2018).

A variety of sesquiterpenes like β -caryophyllene (0.96%), α -humulene (3.39%), germacrene D (0.23%), γ -elemene (0.79%) and spathulenol (0.90%) were identified. One triterpene, squalene (1.14%) was also identified. Among the sesquiterpenes, α -humulene has shown to exhibit marked anti-inflammatory properties in murine model of airways allergic inflammation, which seemed to be mediated *via* reduction of inflammatory mediators, adhesion molecule expression and transcription factors activation (Rogerio et al., 2009). The α -humulene from *Salvia officinalis* has exhibited cytotoxicity in colon cancer (HCT-116) and breast cancer (MCF-7) cell lines (El Hadri et al., 2010). Volatile oils from Verbenaceae members like *Lippia*, *Lantana*, *Aloysia*, etc. have shown to be rich source of sesquiterpenes like caryophyllene and germacrene D. Essential oils from these plants have also

shown significant antimicrobial properties which were attributed to these sesquiterpenes present abundantly in them (Montanari et al., 2011). The component γ -elemene is a structural isomer of β -elemene. Elemene is a natural sesquiterpene found in many plants and β -elemene is found as a common component, whereas γ -elemene is seen in minor quantities. Elemene is considered as an active pharmaceutical ingredient with its wide spectrum of antineoplastic activity. Its ability to regulate cell cycle and to induce apoptosis makes it a good candidate that can be exploited as an anticancer agent (Wang et al., 2012).

Phenolic compounds were represented by eugenol (0.57%) and α -tocopherol (0.45%). Eugenol is a hydroxyphenyl propene found in the essential oils of several plants belonging to Lamiaceae, Lauraceae, Myrtaceae and Myristicaceae. It is largely used in both foods and cosmetics as a flavouring agent. Recent scientific evidences have claimed that it has excellent antimicrobial, antioxidant and anti-inflammatory activities (Marchese et al., 2017b).

Sterols like stigmasterol (15.73%), campesterol (1.62%) and β -sitosterol (1.41%) were noted in good percentage in GC/MS result of *G. eriocarpum*. As discussed earlier, all of them are reported to have significant biological activities.

A variety of fatty acids and their esters are synthesised in plants. Fatty acids derived from plant sources are popular ingredients in pharmaceutical and cosmetic industries (Freije et al., 2013). Stearic acid is a fatty acid which constituted 3.29% of peak area in *G. heyneanum* var. *heyneanum* and 0.66% in *G. heyneanum* var. *rottleri*. Studies have shown that it has antibacterial and antifungal activities (Agoramoorthy et al., 2007; da Silva et al., 2002). Methyl esters of palmitic acid and linolenic acid were noticed in all the three selected taxa of *Gomphostemma*.

GC/MS analysis thus unveiled the presence of a variety of compounds with potent biological activity in selected species of *Gomphostemma* which can be taken as a possible lead to the therapeutic efficacy of the selected plants. *G. heyneanum* var. *heyneanum* was found to be rich especially in monoterpenes. The compounds β -caryophyllene, neophytadiene and phytol were the terpenes found to be common in all the three selected taxa. Stigmasterol was the phytosterol prominent in all the selected plants. The phenolic compound α -tocopherol was also found to be common in the three selected plants of *Gomphostemma*.

d. High Resolution-Liquid Chromatography/Mass spectrometry (HR-LC/MS) analysis

Liquid chromatography/mass spectrometry (LC/MS) is a technique to identify the non-volatile components in a mixture. Coupling of MS to chromatographic techniques makes it highly sensitive and specific compared to other chromatographic detectors. Mass spectrometers operate by converting the analyte molecules to a charged state followed by the analysis of the ions and any fragment ions that are produced during the ionisation process, on the basis of their mass to charge ratio (m/z). LC/MS has a wider application in the identification of biological molecules. For unknown analyses, LC/MS require comparison of full-scan spectra against pre-established libraries (Pitt, 2009). HR-LC/MS offers mass assignments with better accuracy and allows for better resolution (Wu et al., 2012).

The HR-LC/MS analysis revealed the presence of a total of 34 components (Table 8). *G. heyneanum* var. *heyneanum* showed 11 components, where as *G. heyneanum* var. *rottleri* had 17 and from *G. eriocarpum* 10 constituents were identified. The extracts showed great variation in their constituents and no compounds were found common in all the three extracts. Two compounds theaflavin and 3-(4-hydroxyphenyl)

pyruvic acid were found to be common in *G. heyneanum* var. *heyneanum* and *G. heyneanum* var. *rottleri*. Dihydrocelastrol and swietenine were the compounds found to be common in *G. heyneanum* var. *rottleri* and *G. eriocarpum*. No compounds were noted to be common between *G. heyneanum* var. *heyneanum* and *G. eriocarpum*.

Cephaeline identified from *G. heyneanum* var. *heyneanum* is an alkaloid. It has originally been isolated from *Cephaelis ipecacuanha* and is known to be present in other plant species like *Psychotria acuminata*. It has the ability to stimulate stomach lining thereby inducing vomiting and hence recommended as an emergency treatment for accidental poisoning (Asano et al., 2001). Cephaeline has shown the potential to inhibit Zika and Ebola virus infections by inhibiting viral replication and decreasing viral entry. Thus, it has been suggested as a pharmaceutical therapy against these viruses (Yang et al., 2018).

Chlorogenic acid which is a polyphenol ester of caffeic acid and quinic acid is an intermediate in lignin synthesis. It was originally isolated from green coffee beans but has also been found in the seeds and leaves of many dicotyledonous plants. It has radical scavenging ability and hence has antioxidant activity (Izawa et al., 2010). It is a biologically active polyphenol with therapeutic roles such as hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, antiviral and anti-microbial activity (Naveed et al., 2018).

Theaflavin identified from the extracts of *G. heyneanum* var. *heyneanum* and *G. heyneanum* var. *rottleri* is a flavonoid, previously reported to be present in the leaves of *Camellia sinensis* and related plants. Theaflavin and its derivatives have antioxidant properties (Leung et al., 2001). It has shown potent antiproliferative and genotoxic properties against human colon carcinoma, breast cancer and stomach cancer cell lines (Koňariková et al.,

2015; Hibasami et al., 1998). Studies have indicated that theaflavin derivatives are potential compounds with anti-influenza and anti-inflammatory properties (Zu et al., 2012). Genistein is an isoflavonoid primarily reported from soybeans and legumes as well as from many medicinal plants. Genistein has antiproliferative property against different types of cancers and has the potential to alter apoptosis, cell cycle and is an inhibitor of angiogenesis, thereby inhibiting metastasis. Studies have shown that the intake of soy products can lower the incidence of breast and prostate cancers which is attributed to the presence of genistein. It has a structural similarity to 17- β -estradiol and hence possesses weak estrogenic activity thereby playing a favourable role in the treatment of hormone-related cancers (Spagnuolo et al., 2015).

Dihydrocelastrol and swietenine were identified in the extracts of *G. heyneanum* var. *rottleri* and *G. eriocarpum*. Dihydrocelastrol is a triterpene and is a dihydro-analog of celastrol, originally isolated from the traditional Chinese medicinal plant *Tripterygium wilfordii*. It has shown to inhibit cellular proliferation and induce cell apoptosis in myeloma cells mediated through caspase dependent way, possibly by inhibiting the IL-6/STAT3 and ERK1/2 pathways (Hu et al., 2017). Swietenine is a tetranortriterpenoid originally isolated from the seeds of *Swietenia macrophylla*. It has shown significant dose dependant hypoglycemic and hypolipidemic activity in type 2 diabetic rats (Dewanjee et al., 2009).

Cuscohygrine is an alkaloid found as a natural constituent in *Atropa belladonna* (Van Haga, 1954) and *Erythroxylon coca* (Leete, 1983). It is also reported from plants like *Withania somnifera*, *Convolvulus arvensis*, etc. and has shown cytotoxic and genotoxic activities (Rai et al., 2016a; Todd et al., 1995).

A variety of biopeptides were identified in the methanol extracts of *Gomphostemma* through HR-LC/MS analysis. Bioactive peptides are defined as specific protein fragments that can induce a positive impact on body functions or conditions. Thus, bioactive peptides have the potential to affect major body systems like cardiovascular, digestive, immune and nervous systems depending on their amino acid sequence (Korhonen & Pihlanto, 2006). An array of biological activities including antioxidative, antihypertensive, antidiabetic and immunomodulating has been ascribed to different types of biopeptides derived from various sources. Hence, biopeptides are getting much attention among researchers with regard to their mode of action, biological and medicinal health benefits as well as on their therapeutic applications. However, many aspects need to be extensively addressed with regard to their production through advance strategies, mechanisms of action and multiple biological functionalities (Saadi et al., 2015).

Peptides represent a unique class of pharmaceutical compounds, molecularly positioned between small molecules and proteins, but with distinct biochemical and molecular properties. Since peptides are signalling for many physiological functions, they present an opportunity for therapeutic interventions that closely mimic natural pathways. Hence, the areas of highest concentration for peptide development in pharmaceutical industry are mostly concerned with metabolic diseases, oncology and cardiovascular diseases (Lau & Dunn, 2018). Present HR-LC/MS analysis of selected species of *Gomphostemma* showed the presence of various tripeptides. Tripeptides are reported to have many biological activities. Studies have shown that tripeptides with cysteine and tryptophan amino acid residues have antioxidant properties (Tian et al., 2015). A tripeptide alanine-cysteine-glutamine was found to have significant

antioxidant activity and which was clearly demonstrated by *in vitro* and *in vivo* animal models (Choi et al., 2015).

Collagen tripeptides with highly repetitive sequence of glycine-x-y where x and y are arbitrarily occupied by proline/ hydroxyproline and alanine have displayed anti-inflammatory effect in atopic dermatitis (Hakuta et al., 2017). Peptides are a novel class of anticancer agents that could specifically target cancer cells with lower toxicity to normal tissues, which offers new opportunities for cancer prevention and treatment. Anticancer peptide therapy is an emerging field that uses biopeptides to kill cancer cells. Bioactive peptides can be designed using bioinformatics tools to target proteins of interest, including amino acid sequences and interaction partners of many oncogenic proteins. Dipeptides and tripeptides are easily absorbed by the cells as compared to high molecular bioactive peptides. Legume derived peptides have displayed appreciable physiological effects against cancer cells by inducing apoptosis and affecting the tubulin-microtubule equilibrium (González-Montoya et al., 2017). Even though many studies are available on the bioactive potential of peptides, rigorous studies are required to validate their role as therapeutic agents.

Phytochemical analysis with GC/MS and HR-LC/MS techniques revealed a multitude of compounds in the extracts of selected taxa of *Gomphostemma*. GC/MS analysis unfolded the volatile components particularly terpenes which constitute the main fraction of compounds in the members of Lamiaceae (Sonmezdag et al., 2017). HR-LC/MS analysis detected many bioactive components which were not revealed in GC/MS analysis. This shows that a combination of these modern techniques is more effective in providing a comprehensive result in untargeted metabolomics profiling. Most of the compounds identified were found to have efficient biological activities. The present phytochemical studies and the above

discussed facts, thus provided a strong lead for the screening of bioactivities of selected species of *Gomphostemma* that were deciphered in this study.

B. ANTIOXIDANT ACTIVITY

A free radical is any chemical species, an atom or molecule capable of independent existence with one or more unpaired electrons in valence shell which makes it highly unstable, short lived and reactive. Because of its high reactivity, it attacks the nearest stable molecules and as a result the attacked molecule becomes a free radical itself beginning a chain reaction. The most important free radicals in biological system are radical derivatives of oxygen and nitrogen (Tandon et al., 2005). Some of the most important reactive oxygen species (ROS) in biological systems are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, *etc.* Reactive nitrogen species (RNS) include nitric oxide, peroxyxynitrite, nitroxyl anion, *etc.* (Griending et al., 2016). Antioxidants are molecules stable enough to donate electrons to the free radicals thus neutralizing and reducing their rampaging capacity. Antioxidants therefore delay or inhibit cellular damage mainly by their free radical scavenging activity. Free radicals are produced in cells as a result of metabolic processes as well as under the influence of external sources such as pollutants and chemicals. Cells have an innate antioxidant mechanism to counteract the free radicals normally produced in the cells. Cells are equipped against antioxidant stress with a network of antioxidant enzymes like superoxide dismutase, catalase, *etc.* and compounds like ascorbic acid, tocopherols, *etc.* But, loss of balance between free radical generation and antioxidant defence system of the cells leads to an unfavourable oxidative damage state called as oxidative stress. Oxidative stress leads to the damage of a wide range of molecules like nucleic acids, proteins and lipids. It is held responsible for many inflammatory conditions, certain cancers and mechanisms associated with ageing (Lobo et al., 2010).

Studies have shown that dietary consumption of foods rich in antioxidants can mitigate the negative effects of ROS and can effectively enhance the immune function of the body (Bendich, 1993).

Plants have always been a rich source of exogenous antioxidants. Since research on natural antioxidants has become increasingly active in various fields, many assays have been developed to evaluate the antioxidant activity of plants and food constituents (Moon & Shibamoto, 2009). In the present study, five different assays were used to analyse the antioxidant potential of selected species of *Gomphostemma*.

a. DPPH radical scavenging activity

DPPH free radical scavenging method is one of the primary methods for evaluating the antioxidant potential of a compound or other biological extracts. It is relatively a simple and inexpensive method to measure the free radical scavenging activity. In this method, the prospective compound or extract is mixed with DPPH solution and absorbance is recorded after a defined period. DPPH has an odd electron which makes it a stable free radical and gives a deep violet colour with absorption around 517 nm. On mixing DPPH solution with an antioxidant that can donate a hydrogen atom, it gets reduced to its non-radical form. Once the DPPH free radical is scavenged, the colour of the reaction mixture changes from deep violet to yellow with corresponding reduction in absorbance at 517 nm (Kedare & Singh, 2011). In the present study, methanol extracts of selected species of *Gomphostemma* were analysed for their DPPH scavenging potential and all of them showed significant activity in a dose-dependent manner (Fig. 22). *G. heyneanum* var. *heyneanum* was identified as the best scavenger with an IC₅₀ value of 46.63 ± 2.26 µg/mL which is commensurable to that of the standard ascorbic acid used (Table 9). It was followed by *G. heyneanum* var. *rottleri*

with an IC_{50} of $80.41 \pm 1.87 \mu\text{g/mL}$ and the least activity was shown by *G. eriocarpum* with an IC_{50} of $101.61 \pm 1.22 \mu\text{g/mL}$.

b. Hydroxyl radical scavenging activity

Hydroxyl radical is the most virulent free radical with high reactivity among reactive oxygen species. It has the ability to react and induce damage to biological molecules especially to proteins and DNA by a variety of mechanisms (Dizdaroglu & Jaruga, 2012). Present assay utilized the hydroxyl radicals formed by the Fenton reaction and its ability to degrade 2-deoxy-d-ribose in the reaction mixture. The amount of degradation product was determined photometrically after a reaction with 2-thiobarbituric acid yielding a pink pigment. Active free radical scavengers can inhibit the 2-deoxy-d-ribose degradation by hydroxyl radicals revealing their antioxidant potential (Chobot, 2010). Among the selected plants, *G. eriocarpum* showed highest percentage of inhibition with lowest IC_{50} value of $587.17 \pm 2.97 \mu\text{g/mL}$. The IC_{50} value noted for *G. heyneanum* var. *heyneanum* was $745.17 \pm 2.03 \mu\text{g/mL}$ and for *G. heyneanum* var. *rottleri* it was found to be $1108.56 \pm 2.12 \mu\text{g/mL}$ (Table 9). All the three selected taxa of *Gomphostemma* showed a better antioxidant activity than the standard gallic acid (Fig. 23). The study thus disclosed the scavenging ability of selected plants against the most reactive oxygen species.

c. Nitric oxide free radical scavenging activity

Nitric oxide is a diffusible free radical with diverse roles in biological systems. It is generated from amino acid L-arginine by the enzymes in the vascular endothelial cells as well as by certain neuronal cells and phagocytes. Even low concentrations of nitric oxide are sufficient for physiological functions. Presence of higher amounts of nitric oxide radical is found to be associated with various carcinomas and inflammatory conditions. The toxicity

of nitric oxide radical is found to increase greatly by its reaction with superoxide radical forming the highly reactive peroxynitrite anion. Present assay is based on the principle that, nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH on interacting with oxygen produces nitrite ions. These nitrite ions estimated with Griess reagent gives a quantitative measure of nitric oxide formed. Scavengers of nitric oxide will compete with oxygen resulting in reduced production of nitrite ions (Boora et al., 2014). The nitric oxide scavenging activity of selected species of *Gomphostemma* was measured with varying concentrations of extracts using gallic acid as standard. All the extracts displayed scavenging activity in a dose-dependent manner (Fig. 24). Extract of *G. heyneanum* var. *heyneanum* showed highest activity with an IC_{50} value of $803.86 \pm 1.05 \mu\text{g/mL}$. Extract of *G. heyneanum* var. *rottleri* displayed a moderate activity with an IC_{50} of $1415.04 \pm 2.25 \mu\text{g/mL}$, whereas *G. eriocarpum* showed least scavenging and the IC_{50} value was found to be $2471.08 \pm 1.97 \mu\text{g/mL}$ (Table 9). Even though the scavenging activity displayed by all the three extracts was less as compared to that of standard gallic acid, the activity showed by *G. heyneanum* var. *heyneanum* is appreciable.

d. Superoxide scavenging activity

Superoxide radical is a reactive oxygen species predominantly produced by mitochondrial complexes and endoplasmic reticulum with a role in intracellular signalling cascade. It is relatively weak compared to hydroxyl radical but, its accumulation is capable of causing damage to biomolecules posing threat to cell viability (Weidinger & Kozlov, 2015). Studies have shown that it is a potent nucleophilic base capable of lipid peroxidation. Superoxide by itself has only a weak reactivity with DNA. However, hydroxyl radical which can be generated by the reaction of superoxide radical with hydrogen peroxide is a DNA oxidising reactive oxygen species.

Superoxide is also capable of reacting with nitric oxide whose product can mediate lipid peroxidation and DNA damage (Hileman et al., 2001). The superoxide scavenging activity revealed an optimal activity (Fig. 25) with *G. heyneanum* var. *heyneanum* exhibiting an IC_{50} value of 699.471 ± 2.31 $\mu\text{g/mL}$. Among the other two extracts, *G. heyneanum* var. *rottleri* showed an IC_{50} value of 1280.32 ± 3.81 $\mu\text{g/mL}$ and for *G. eriocarpum* it was 1627.18 ± 2.32 $\mu\text{g/mL}$ (Table 9). In view of the crucial role exhibited by superoxide radicals in inflammation and age-related diseases, the optimal activity shown by *G. heyneanum* var. *heyneanum* could be a promising lead for solving diseases caused by superoxide accumulation.

e. Reducing power activity

Measure of reducing power is an indicator of antioxidant potential (Ngonda, 2013). Compounds with reducing power are regarded as potential electron donors with the ability to reduce oxidized intermediates of lipid peroxidation processes so that, they can act as primary and secondary antioxidants (Chanda & Dave, 2009). The reducing power assay evaluate the ability of extracts to reduce Fe^{3+} to Fe^{2+} and concurrent increase in the absorbance depending on the reducing power of the compounds which can be read at 700 nm (Irshad et al., 2012). The dose response curves for reducing powers of methanolic extracts of selected species of *Gomphostemma* are depicted in Fig. 26. The reducing power of the extracts was found to increase with increase in the concentration. The extract of *G. heyneanum* var. *heyneanum* showed highest reducing power which ranged from 0.44 ± 0.06 to 1.71 ± 0.08 and for *G. heyneanum* var. *rottleri* it was from 0.108 ± 0.04 to 1.16 ± 0.07 . Among the extracts, *G. eriocarpum* showed the least reducing power with the absorbance in the range of 0.15 ± 0.01 to 0.84 ± 0.03 . The reducing power of the standard was found to be higher than all the test extracts. Since reducing power is a measure of hydrogen donating ability, it

can be assumed that methanolic extract of *G. heyneanum* var. *heyneanum* has higher amount of reducing agents than other two plant extracts, which enable it to convert free radicals to more stable products thereby terminating the free radical chain reaction.

Present study measured the antioxidant potential of selected species of *Gomphostemma* with various assays. The plant extracts showed moderate to high free radical scavenging activity. In all the assays, *G. heyneanum* var. *heyneanum* showed predominating antioxidant potential. The methanolic extract of *G. eriocarpum* was efficient in scavenging hydroxyl radicals, but its performance was not impressive in other assays. Among the three taxa, *G. heyneanum* var. *rottleri* showed moderate activity in all the assays. However, since the results of various assays varied considerably it is obvious that a single assay is not sufficient to measure the antioxidant potential. Further, different antioxidant assays differ from one another in terms of reaction conditions, assay principles, oxidant species used, *etc.* So, a summarized result obtained from various assays alone can be considered as a reliable and accurate measure of antioxidant property (Dontha, 2016; Rahman et al., 2015).

In the present study, phytochemical analysis of selected species of *Gomphostemma* revealed the presence of many potential phytochemicals like phenols, flavonoids, terpenoids, alkaloids, steroids, carotenoids, quinones, coumarins and iridoid glycosides. Presence of such bioactive components in these selected plants may be the reason for the antioxidant potential exhibited by them. Polyphenols which include phenolic acids and flavonoids are the largest group of phytochemicals attributed with antioxidant properties. Other main natural antioxidants from plants are carotenoids and vitamins (Xu et al., 2017). Polyphenols are the most ubiquitous class of metabolites in nature. Structurally phenolic compounds consist of one or more

aromatic rings with hydroxyl groups attached to them. These hydroxyl groups are good hydrogen donors imparting antioxidant property to the phenolics. Apart from this, antioxidant activity of phenolic compounds is also due to their ability to chelate metal ions involved in the production of free radicals. Many studies have also shown that phenolic compounds by virtue of their ability to interact with proteins have the potential to inhibit many enzymes involved in the generation of free radicals (Pereira et al., 2009). Studies have shown that there is a good correlation between phenolic concentration determined by Folin-Ciocalteu method and antioxidant capacity revealed by DPPH assay. But it is worth considering that the antioxidant activity is not merely related to the total phenolic content but is also related to the presence of individual active phenolic compounds (Piluzza & Bullitta, 2011; Augusto et al., 2014).

Flavonoids are the largest group of naturally occurring phenolic compounds. They are known for a variety of pharmacological activities including antioxidant property. Their activities depend upon their structural class, degree of hydroxylation and other substitutions. Antioxidant property is one of the best described properties of almost every group of flavonoids. They are one among the most effective scavengers of peroxy, superoxide and peroxynitrite radicals (Aron & Kennedy, 2008). However, many reports have also shown that the antioxidant activity is not fully contributed by phenolic compounds alone and that other constituents like ascorbates, reducing carbohydrates, tocopherols, carotenoids, terpenes and pigments as well as the synergistic effect among them could possibly contribute to the total antioxidant activity (Babbar et al., 2011).

Qualitative and quantitative phytochemical analysis in the present study has revealed considerable amounts of phenol and flavonoids in selected species of *Gomphostemma*. Highest phenol and flavonoid contents were

identified in *G. heyneanum* var. *heyneanum* which justifies the high performance of this species in all the antioxidant assays. Among the extracts, *G. eriocarpum* in general showed least activity in all assays except hydroxyl scavenging activity. The high hydroxyl scavenging activity of *G. eriocarpum* may be attributed to a specific compound or to the synergistic effect of compounds present in them. GC/MS and HR-LC/MS analysis of *G. heyneanum* var. *heyneanum* has identified many phenolic and flavonoid compounds like quinic acid, α -tocopherol, theaflavin, chlorogenic acid, *etc.* with antioxidant potential. Apart from phenols and flavonoids a variety of other components like α -pinene, β -caryophyllene, phytol, β -myrcene, *etc.* are also known to have antioxidant property. Presence of these components thus substantiates the antioxidant activity exhibited by the plant extract.

In general, the antioxidant potential of selected species of *Gomphostemma* as unveiled through different assays may be attributed to the variety of phytochemicals present in them. Evidences based on researches suggest that antioxidants play a vital role in the prevention of free radical formation thereby preventing and reducing the incidence of various disorders such as cancer, ageing, cardiovascular disease, cataracts, immune system decline, *etc.* (Alok et al., 2014, Willcox et al., 2004). Many antioxidants have also been recommended as adjuvants in cancer therapy (Borek, 2004; Thyagarajan & Sahu, 2018). In the light of above discussed facts about antioxidants and their importance, present study revealing the antioxidant property of selected species of *Gomphostemma* is noteworthy and is a manifestation of the bioactive potential of these plants.

C. ANTI-INFLAMMATORY ACTIVITY

Inflammation is body's natural response to stimulations like invading pathogens or to endogenous signals such as damaged cells. It is a mode of protective immune response to ensure tissue repair and to restore homeostasis

(Kulkarni et al., 2016). Majority of the drugs used for treating inflammations are accompanied by serious side effects. Because of the significant side effects of steroidal and nonsteroidal anti-inflammatory medications, there has been a greater interest in natural compounds and herbal remedies to reduce pain and inflammation (Maroon et al., 2010). The present study is an attempt to elucidate the anti-inflammatory potential of selected species of *Gomphostemma*.

a. Evaluation of anti-inflammatory activity with biochemical assays

The anti-inflammatory potential of selected species of *Gomphostemma* was initially evaluated with biochemical assays and the most effective extract was further subjected to detailed studies with RAW 264.7 cells.

i. Inhibition of protein denaturation

Protein denaturation is the net effect of alterations in the biological, chemical and physical aspects of the protein by mild disruption of its structure which badly affects its functional properties (Eckersall, 2008). Protein denaturation is well correlated with the occurrence of inflammatory response and the ability of a substance to inhibit the denaturation of protein apparently signifies its anti-inflammatory activity (Saso et al., 2001; Osman et al., 2016).

As a part of the study, selected species of *Gomphostemma* were analysed for their ability to inhibit protein denaturation (Fig. 27). Among the extracts tested, *G. heyneanum* var. *heyneanum* showed maximum activity with inhibition percentage ranging from 39.67 ± 1.55 to 80.99 ± 1.71 , whereas *G. heyneanum* var. *rottleri* showed moderate effect with a range of activity from 36.16 ± 2.34 to 75.11 ± 1.46 and *G. eriocarpum* showed least activity with inhibition range of 21.90 ± 1.04 to 49.79 ± 0.66 . As discussed earlier, the ability of a compound to inhibit protein denaturation is an indication of its anti-inflammatory potential.

ii. Proteinase inhibitory activity

Proteinases are proteolytic enzymes that catalyze the hydrolysis of the peptide bonds between the amino acids of proteins and peptides and, proteinase inhibitors that can treat inflammatory diseases are gaining academic attention (Soualmia & El Amri, 2018). Proteinase inhibitors can regulate inflammation by inhibiting proteolytic activity of proteinases (Hiemstra, 2002). Neutrophils which play a major role in inflammation are rich source of proteinases especially serine proteinases. Since, proteinases are key regulators involved in inflammatory responses they are considered as potential targets for therapeutic interventions for controlling inflammation (Pham, 2008).

In the present study, methanolic extract of three selected taxa of *Gomphostemma* were tested for their proteinase inhibitory activity. The most effective extract was found to be *G. heyneanum* var. *heyneanum* with an inhibition percentage in the range of 49.54 ± 1.28 to 81.15 ± 1.14 followed by *G. heyneanum* var. *rottleri*. In this assay also *G. eriocarpum* showed least activity.

Thus, the *in vitro* biochemical assays conducted for evaluating the anti-inflammatory property of selected species of *Gomphostemma* revealed that among the plants tested, *G. heyneanum* var. *heyneanum* has a promising activity and hence it was further analysed for its effect on RAW 264.7 macrophage cell line.

b. Anti-inflammatory activity of *G. heyneanum* var. *heyneanum* on RAW 264.7 macrophage cell line

Inflammation is the response of body's immune system to infection and injury. It has been implicated in the pathogenesises of arthritis, cancer, neurodegenerative and cardiovascular diseases. Acute phase of inflammation

is intrinsically beneficial as it leads to the removal of offending factors followed by the restoration of tissue structure and function. However, failure of acute inflammatory programme to resolve the situation may lead to persistence of inflammation leading to chronic inflammation and excessive tissue damage. Unresolved chronic inflammation can lead to the development of more complex diseases like cancer, inflammatory bowel diseases, pulmonary and cardiovascular diseases, *etc.* depending on the organ affected (Sansbury & Spite, 2016; Tsoupras et al., 2018).

i. Cyclooxygenase (COX) activity

Prostaglandins which are formed from arachidonic acid play a vital role in the generation of inflammatory response. They are produced by enzymes called cyclooxygenase (COX) which exist in two distinct isoforms, COX-1 and COX-2. Among the two, COX-1 is produced constitutively in all cells. On the other hand, COX-2 is produced in response to inflammatory stimuli and plays an important role in the production of prostaglandins (Rouzer & Marnett, 2009). In the present study, the extract of *G. heyneanum* var. *heyneanum* which was found to be effective in inhibiting protein denaturation and proteinase inhibitory activity was subjected to analyse its effect on COX-2 activity in LPS induced RAW 264.7 macrophage cell line. Three different concentrations (25, 50 and 100 µg/mL) were used and the activity was expressed in terms of percentage of inhibition (Fig. 29). A dose-dependent inhibition of COX-2 by the plant extract was noted and IC₅₀ value was calculated as 78.81 ± 4.30 µg/mL. Study thus displayed the ability of *G. heyneanum* var. *heyneanum* to inhibit the activity of COX-2, which is a manifestation of its anti-inflammatory potential.

ii. Lipoxygenase (LOX) activity

Lipoxygenases (LOX) are dioxygenase enzymes which are expressed in immune, epithelial and tumour cells with a variety of physiological functions like inflammation, allergies, skin disorder, tumourigenesis, *etc.* (Mashima & Okuyama, 2015). They play a crucial role in the conversion of arachidonic acid into leukotrienes, which mediate inflammatory responses. Hence, potential LOX inhibitors can effectively inhibit inflammatory responses (Boudreau et al., 2017). In the present investigation, three different concentrations (25, 50 and 100 µg/mL) of *G. heyneanum* var. *heyneanum* methanol extract were tested. As shown in Fig. 30, the extract showed only a moderate activity with an IC₅₀ value of 126.11 ± 5.65 µg/mL.

iii. Myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) is a heme-containing peroxidase expressed mainly in neutrophils and to a lesser degree in monocytes. In the presence of hydrogen peroxide and halides, it catalyzes the formation of reactive oxygen intermediates, including hypochlorous acid. It has also been demonstrated to be a local mediator of tissue damage and the resulting inflammation may cause various inflammatory diseases. These findings have implicated MPO as an important therapeutic target in the treatment of inflammatory conditions (Aratani, 2018). Studies have shown that estimation of myeloperoxidase activity is a reliable indicator of inflammation and is as good as the measurement of interleukin-6 concentration as marker of inflammation (Faith et al., 2008). Inhibition of myeloperoxidase activity has shown to attenuate inflammatory responses and compounds which can reduce the activity of MPO are counted to be with anti-inflammatory potential (Kim et al., 2018). In this study, different concentrations (25, 50 and 100µg/mL) of methanol extract of *G. heyneanum* var. *heyneanum* were evaluated for their effect on MPO activity in LPS treated RAW 264.7 cells. The extract induced a

significant reduction in MPO activity comparable to that of the standard drug diclofenac sodium (Fig. 31). At highest concentration tested (100 µg/mL) MPO activity was found to be 0.0015 U/mL of cell lysate. A notable reduction in MPO activity upon treatment with plant extract is an indication of its potential anti-inflammatory property.

iv. Cellular nitrite level

Nitric oxide (NO) is a biological messenger with diverse role in many physiological and pathological processes. Numerous studies have shown that it act as a mediator in inflammatory response and that its over production can induce tissue damage. It is synthesised from L-arginine by the enzyme nitric oxide synthase (NOS). This enzyme occurs in three isoforms, neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) of which nNOS and eNOS generally produce low levels of nitric oxide required for normal physiological functions. But, the expression of iNOS is up-regulated by many proinflammatory mediators which result in overproduction of nitric oxide in the cells (Khallaf et al., 2016). In this study, three different concentrations (25, 50 and 100 µg/mL) of methanol extract of *G. heyneanum* var. *heyneanum* were used to measure the ability of the plant under study to inhibit NO production in LPS stimulated RAW 264.7 cells. It was noted that the plant extract could significantly inhibit the accumulation of nitrite in a dose-dependent manner (Fig. 32). Cellular nitrite level was reduced from 587.38 ± 1.41 µg/mL to 359.95 ± 0.93 µg/mL of cell lysate and the effect is commensurable to the standard diclofenac sodium.

v. Inducible nitric oxide synthase (iNOS) activity

As discussed earlier, among the three isoforms of nitric oxide synthase, inducible nitric oxide synthase (iNOS) plays a pivotal role in nitric oxide (NO) production suggesting it as a potential regulator of inflammation

(Nakazawa et al., 2017). The extract of *G. heyneanum* var. *heyneanum* (25, 50 and 100 µg/mL) was evaluated for its effect on iNOS levels in LPS induced RAW 264.7 cells. A dose-dependent inhibition in iNOS activity was noticed (Fig. 33) with an IC₅₀ value of 62.19 ± 0.34 µg/mL. This reduction in iNOS activity thus justifies the reduction in cellular nitrite levels observed in the previous assay. A decrease in cellular nitrite level through the inhibition of iNOS activity is a useful step in assessing the anti-inflammatory efficacy of compounds. Compounds and extracts which can down-regulate iNOS activity with subsequent decrease of its product are regarded to have anti-inflammatory potential (El-Ashmawy et al., 2018).

c. MTT assay of RAW 264.7 cells

MTT assay of RAW 264.7 cells was carried out to check the cytotoxicity so as to exclude the possibility that the inhibitory effect observed during *in vitro* studies was due to the cytotoxic effect of plant extract (Chauhan et al., 2011). Cytotoxic studies ruled out the possibility of cytotoxicity on RAW 264.7 cells and confirmed that the inhibitory effects were indeed due to the anti-inflammatory activity of plant extract (Fig. 34; Plate 3). As IC₅₀ value obtained from MTT assay of RAW 264.7 cells was found to be 86.83 ± 1.54 µg/mL, a lower concentration of 75 µg/mL of plant extract was considered for further analysis. Since the IC₅₀ values obtained for COX (78.81 ± 4.30 µg/mL) and iNOS (62.19 ± 0.34 µg/mL) assays were almost similar to this value, it seems to be appropriate for further analysis.

d. Estimation of inflammatory mediators using ELISA

ELISA test is a suitable method for the quantitative measurement of inflammatory mediators (Amsen et al., 2009). The effect of *G. heyneanum* var. *heyneanum* on the levels of COX-2, TNF-α and NF-κB (p65) were determined in LPS induced RAW 264.7 cells (Fig. 35). The COX-2 enzyme is

involved in the synthesis of prostaglandins that have a major role in causing inflammation and hence COX-2 inhibitors are potential anti-inflammatory agents (Cao et al., 2010). The plant extract (75 µg/mL) considerably reduced COX-2 level from 1.98 ± 0.07 to 1.36 ± 0.11 U/mL which is in concurrence to the dose-dependent inhibition displayed by the extract in the previous assay (Fig. 29).

NF-κB family of transcription factors have an essential role in inflammation as they are involved in the transcriptional induction of many pro-inflammatory cytokines (IL-1, IL-2, IL-6, TNF-α) and inducible enzymes (COX-2, iNOS). In unstimulated cells, NF-κB occurs in cytoplasm as an inactive heterodimer, complexed with an inhibitory protein IκB-α preventing it from entering the cell nucleus. Upon activation by inflammatory signals, this complex breaks and follows the canonical pathway to release the two sub-units p50 and p65. They are then translocated to nucleus, where they recognise specific promoter regions to bring about gene expression (Calixto et al., 2003; Shih et al., 2011). Quantification of NF-κB (p65) component of complex is considered as a parameter for measuring NF-κB activation (Maguire et al., 2011). Present assay showed that the level of NF-κB (p65) transcription factor was significantly reduced from 1.89 ± 0.11 to 1.18 ± 0.07 U/mL of cell lysate. NF-κB has a major role in controlling the expression of COX-2 and iNOS (Park et al., 2015). The reduction in cellular nitrite levels and iNOS activity as well as reduced COX-2 levels can thus be justified by the suppression of NF-κB by the plant extract. The level of TNF-α, a cytokine involved in inflammation was also found to be reduced from 2.03 ± 0.14 to 1.55 ± 0.09 U/mL in cell lysate.

In general, it can be said that the extract of *G. heyneanum* var. *heyneanum* has moderate anti-inflammatory property. It seems that its anti-inflammatory effect is mainly associated with the suppression of NF-κB

activation with subsequent effect on COX-2 and iNOS activity. Phytoconstituents may be held responsible for the anti-inflammatory potential exhibited by the plant extract (Beg et al., 2011; Sajitha & Thoppil, 2019). Active inflammatory cells apart from producing inflammatory mediators also release ROS causing an oxidative stress environment. Numerous studies have shown that the flavonoid and phenolic content in plants are excellent antioxidants which can scavenge free radicals. This suggests that good antioxidants can also function as good anti-inflammatory agents (Arulselvan et al., 2016). Phenolic compounds execute anti-inflammatory property by a variety of inhibitory activities of pro-inflammatory mediators and have shown to regulate transcriptional factors like NF- κ B (Ambriz-Pérez et al., 2016). Many of the components in *G. heyneanum* var. *heyneanum* as revealed by GC/MS and HR-LC/MS analysis are reported to have anti-inflammatory properties (Table 7 & 8). The component α -pinene is reported to have exhibited anti-inflammatory activity through the suppression of mitogen-activated protein kinases (MAPKs) and the nuclear factor-kappa B (NF- κ B) pathway in mouse peritoneal macrophages (Kim et al., 2015). Eucalyptol treatment has shown to diminish leukocyte infiltration, myeloperoxidase activity and cytokine production in LPS induced pulmonary inflammation in mouse model (Caceres et al., 2017). Studies have provided information that the anti-hyperalgesic and anti-inflammatory properties of p-cymene is by its potential to modulate pro-inflammatory cytokines and NO production (de Santana et al., 2015). Other components like limonene, β -caryophyllene, quinic acid, *etc.* are also known for their anti-inflammatory potential.

Even though many species with in Lamiaceae and Lamioideae are reported to have anti-inflammatory property, only a few studies are available on the anti-inflammatory activity of *Gomphostemma*. Present study showed that species of *Gomphostemma* endemic to South Western Ghats and *G.*

heyneanum var. *heyneanum* in specific, has fairly good anti-inflammatory potential.

D. SCREENING FOR CYTOTOXICITY WITH *ALLIUM CEPA* L.

Cytotoxicity studies are useful as an initial step in determining the potential toxicity of a test substance, including plant extracts or biologically active compounds isolated from plants. Cytotoxicity assays are widely performed to identify the impact of toxicity. This is especially important in the case of plant extracts and purified compounds intended to be used in pharmaceuticals or cosmetics in which case, minimal to no toxicity is important. In the case of compounds designed to act as anticancer drugs, selective cytotoxicity to cancerous cells is preferred (Monteiro et al., 2017).

Several animal and plant based models are available for the screening of cytotoxicity. Among the various plant species used for cytotoxic studies *Allium cepa* L. is regarded as the most favourable genetic model to assess chromosomal damages and disturbances in mitotic cycle. Larger and limited numbers of chromosomes are the main characteristic features for making *A. cepa* an excellent test system for evaluating cytotoxicity. The *A. cepa* assay enables for the assessment of different genetic endpoints like chromosome aberrations (CA), mitotic index (MI), nuclear abnormalities and micronucleus formation. The decrease in the mitotic index of *A. cepa* meristematic cells is considered as a reliable method to determine cytotoxicity (Leme & Marin-Morales, 2009).

Present study assessed the cytotoxicity of selected species of *Gomphostemma* using *A. cepa* assay (Figs 36–39). Five different concentrations (5, 25, 50, 75 and 100 µg/mL) and four different time intervals (1, 3, 12 and 24 h) were selected for the study. Hydrogen peroxide (H₂O₂) served as the positive control and distilled water was used as the negative

control. Mitotic index (MI) and chromosomal aberrations (CA) were the two end points considered for the study. Mitotic index which is characterized by the total number of dividing cells in cell cycle is a cell proliferation biomarker. Since it is a measure of proportion of cells in the mitotic phase of the cell cycle, decrease in the mitotic index of *A. cepa* meristematic cells may be interpreted as due to the mitodepressive and cytotoxic effect of the compound being tested (Yuet Ping et al., 2012). All the extracts selected for the study induced a dose and duration dependent drop in mitotic index in treated roots. Among the plants selected for the screening, highest reduction in mitotic index was caused by *G. heyneanum* var. *heyneanum* and it was found to be $31.47 \pm 2.28\%$ after 24 h treatment for the highest concentration (100 $\mu\text{g/mL}$). This reduction in mitotic index may be attributed to the ability of the extracts to cause cell cycle arrest. It is suggested that the mitodepressive effect may be due to the inhibition of DNA synthesis provoking cell cycle retardation at G1/S phase preventing the cells from entering the cell cycle (Soltys et al., 2011). It is reported that the mitodepressive effects which inhibits the biosynthesis of DNA and nucleoproteins apart from reducing the number of dividing cells can also influence the structure of chromosomes (Akinboro & Bakare, 2007).

A variety of chromosomal aberrations (Plats 5–18) were observed as a result of treatment with various concentrations of plant extracts. Cytological and chromosomal aberrations increased with the corresponding increase in the concentration of plant extracts. At highest concentration (100 $\mu\text{g/mL}$) after 24 h treatment, CA induced by *G. heyneanum* var. *heyneanum* was found to be $73.87 \pm 1.47\%$, for *G. heyneanum* var. *rottleri* it was $64.13 \pm 1.80\%$ and for *G. eriocarpum* it was noted as $57.20 \pm 1.44\%$ (Fig. 39 B). Cytotoxic effect of plant extracts was visible in all stages of cell cycle. Present study clearly revealed the ability of plant extract to interfere with different stages of cell cycle inducing clastogenic and spindle disturbances causing alterations in the

cells. The manifestation of plant extract to induce apoptosis was observed in the form of chromatin condensation, nuclear disintegration, formation of micronucleus, nuclear blebs, cytoplasmic shrinkage, *etc.*

Nuclear lesions and erosions which were commonly observed in interphase and prophase stage is an indication of nuclear disintegration. It is suggested to be a cytological evidence for nuclear poison and the inhibitory action of phytochemicals on DNA biosynthesis (Akaneme & Iyioke, 2008; Ngozi, 2011). Nuclear erosion which possibly resulted from the disintegration of nuclear proteins represents irreversible and toxic effect of plant extract (Karaismailoglu et al., 2013). Nuclear buds which are regarded as expansion of the nucleus into bud shaped structures are said to be responsible for the expulsion of undesirable DNA content, such as amplified DNA. Some reports indicate that they are the precursors of micronuclei (Dutra et al., 2010). Nuclear blebs which were observed as excrescences on the main nucleus consisting of nuclear material reflect the genotoxic effect of the extracts. Evidences have shown that these blebs may later get detached from the main nucleus to form micronucleus. However, the mechanisms responsible for the formation of micro nucleus have not yet been fully understood. It is proposed that micronuclei are formed either from the acentric chromosome fragments or from the whole chromosomes that fail to be included in the daughter nuclei at the completion of telophase. These displaced chromosomes or chromosome fragments which eventually get enclosed by a nuclear membrane may get eliminated after a few cycles of cell divisions (Fenech et al., 2011; Wang et al., 2016).

Nuclear fragmentation together with chromatin condensation observed in the study is common in apoptotic cells (Yamada et al., 2006). Hyperchromasia characterized by intense staining and nuclear shrinkage caused by chromatin condensation are also characteristic features of apoptosis

(Liu et al., 2018). Cytoplasmic shrinkage and vacuolation which were prominently noted are regarded as hallmarks of apoptosis (Tang et al., 2009). Studies have shown that cell shrinkage beyond a particular magnitude itself is a sufficient mediator of cell apoptosis. The volume decrease alone has been shown to be sufficient to cause apoptosis even in the absence of a death ligand. Shrinkage of cells beyond a point is probably inducing the activation of enzymes/receptors there by increasing the rate of apoptosis (Ernest et al., 2008). The occurrence of binucleated cells may be attributed to the inhibition of cytokinesis by the phytoconstituents in the extracts (Aşkin Çelik & Aslantürk, 2010). Pulverized chromatin observed may be due to the abnormal and premature condensation of chromosomes (Onwuamah et al., 2014).

Chromosome stickiness was a prominent abnormality recorded in the present study. Chromosome stickiness is characterized by intense chromosome clustering. Coagulation of chromosome usually results from the stickiness. It is postulated that, the stickiness may result from defective functioning of specific non-histone proteins involved in chromosome organization, chromatid separation and segregation. The altered functioning of these proteins leading to stickiness may be due to the action of phytocomponents in the extracts (Pagliarini et al., 2000). It is suggested that stickiness might also be due to the degradation or depolymerization of chromosomal DNA (Kumari et al., 2009). Stickiness is an indication of high toxicity of the chemical substance on the chromosomes and is probably an irreversible effect (Fiskesjo, 1997). The stickiness of metaphase chromosomes reinforces the toxic effect of plant extracts. Sticky chromosomes in metaphase lose their normal appearance and are seen with a sticky surface resulting in chromosome agglomeration. Stickiness has also been attributed to the effect of chemical compounds on the physical and chemical properties of DNA, protein or both, on the formation of complexes with phosphate groups in DNA, on DNA condensation or on formation of inter-and-intra-chromatid

cross links. Since this type of aberration is generally irreversible, it may finally lead to cell death and is likely to have contributed to the decline in mitotic index observed (Andrade et al., 2008).

Clastogenic aberrations were observed in the form of chromosome rings, breaks, fragments and gaps. Compounds that can induce chromosome breakage are known as clastogens and their action on chromosomes is generally regarded to involve an action on DNA (Türkoğlu, 2007). Ring chromosomes are formed by the breakage and subsequent fusion of broken ends forming cyclic chromosomes (Firbas & Amon, 2014). These types of aberrations highlight the clastogenic effect of the components in plant extract.

Most of the aberrations noticed in metaphase and anaphase were due to the disturbances caused to the spindle fibers. Displaced, scattered and misoriented arrangement of chromosomes hints to the turbagenic effect of the extracts. C-mitosis observed in the root tip meristems of *A. cepa* on treatment with the extracts is an indication of the random scattering of the condensed chromosomes caused by the inactivation of spindle fibres. Induction of C-mitosis is commonly associated with spindle poisons, indicating the turbagenic effect (Dwivedi & Kumar, 2015). In this study, the orientations as well as the functioning of the spindles were found to be greatly disturbed. Disturbed metaphases and diagonal anaphases observed indicate abnormal or distorted orientation of mitotic apparatus which reflects the hostile effect of plant extract on the microtubules of the spindle fibres. This adverse effect of extracts on spindle formation and interference with cell plate formation may be held responsible for the micro and macro cell formation (Tripathy et al., 2013). Similarly, lagging chromosomes or laggards are formed due to abnormal spindle formation and failure of spindle fibre to pull the respective chromosomes to the polar regions of the cells (Obute et al., 2016). Presence of vagrant chromosomes also points to the spindle poisoning induced by the

extracts (Kalcheva et al., 2009). Chromosome bridges noticed in anaphase and telophase can be attributed to the chromosomal stickiness and subsequent failure of free anaphase separation. Such bridges are also reported to be due to the unequal translocation or inversion of chromosome segments (Gömürgen, 2005). Microtubule-organizing centres (MTOC) are involved in the arrangement of microtubules facilitating the spatial positioning of spindle fibers during cell division. Shift in MTOC displays the ability of plant extracts to interfere with the spindle formation (Marc, 1997). Ball metaphase is attributed to the disturbances caused to the spindle fibres followed by abnormal clumping of chromosomes into a ball shaped structure (Yih et al., 1997). Effect of plant extracts on spindle fibers is again evident from tropokinesis and other disturbances in different stages of cell cycle.

Presence of giant cells reflects the ability of plant extracts to interfere with normal cell cycle, especially the S phase. Giant cells are formed due to incomplete cytoplasmic division. Such cells grow in size and undergo DNA replication and nuclear division attaining abnormal size before they die (Prajitha & Thoppil, 2016). Presence of ghost cells which have no nucleus but have cytoplasm and cellular outline is yet another manifestation of cytotoxic potential of plant extracts analysed (Farhan & Tawfiq, 2016). In addition to the above discussed conditions, several other anomalies like strap cells, hypercondensation of chromosomes, hypoploidy, nuclear peaks, *etc.* were also noticed which probably reflects the intense cytotoxicity of plant extracts. High frequency of multiple aberrations in treated cells is worth notable because, the presence of many abnormalities in the same cell is an indirect measure of the clastogenic, aneugenic and turbagenic property of the plant species used for the study. The results obtained strongly emphasize on the ability of phytocomponents in the selected extracts to interfere with the cell cycle process and to induce apoptosis.

The cytotoxic effect of selected species of *Gomphostemma* was further confirmed by evaluating the cell death of *A. cepa* root cells using Evans blue staining method. Evans blue is a reliable vital stain used for the assessment of cell viability in both plants and animals. It is an acidic, non-permitting exclusion dye which stains dead or damaged cells. The dye does not enter live cells with stable membranes but can easily penetrate dead cells. Further it can be extracted and analysed spectrophotometrically (Vijayaraghavareddy et al., 2017). Intensity of dye absorbed by the cells is directly proportional to the cell death. In the present study, Evans blue staining method provided indirect evidence to the ability of the plant extracts to induce cell death. The cell death and absorbance pattern was found to be positively related to concentration of treatment. Among the plants selected, extract of *G. heyneanum* var. *heyneanum* showed maximum absorbance in the range of 0.307 ± 0.007 to 0.687 ± 0.011 (Fig. 40). Intensity of staining (Plate 19) also suggests that the extract of *G. heyneanum* var. *heyneanum* has induced severe cell death in *A. cepa* root cells than the other two plant extracts.

The cytotoxic and cell death studies with *A. cepa* can be considered as initial screening procedures to determine the antiproliferative properties of compounds or extracts. Monitoring chromosomal aberrations with *A. cepa* assay is considered as one of the classical cytogenetical method to assess genotoxic effects. Besides its sensitivity, Allium test has shown high correlation to other test systems. Present study showed the clastogenic, aneugenic and other cytological effects of the extracts analysed. Clastogenic effect is characterized by the induction of chromosomal breakage during cell division, while aneugenic action comprises the inactivation of a cell structure, such as the mitotic spindle, leading to chromosomal losses (Leme et al., 2008). Apart from these, cytoplasm of the cells were also seen adversely affected by the treatment. All these effects possibly culminated in cell death as revealed by Evans blue staining. These observations points to the cytotoxic

and the possible apoptotic potential of plant species selected for the study. The results obtained points to the potential of phytochemicals in the extracts to interfere with the cell cycle, to induce cytotoxicity finally leading to cell death. Phytochemical studies of plants selected for the study have showed the presence of many components like α -pinene, β -humulene, elemene, phytol, cuscohygrine, squalene, *etc.* which are reported to have cytotoxic and anticancer activities. However, cytotoxicity primarily measures the potential of a compound to induce cell death, either by necrosis or apoptosis. Among the two mechanisms of cell death, apoptosis is more specialized and require different methods for its evaluation. Detailed studies on dose and time dependence of toxicity together with the effects on cell cycle is required to provide valuable information about mechanisms and type of toxicity, including necrosis, apoptosis and other events (Yang & Xiong, 2012).

E. ANTIPROLIFERATIVE STUDIES WITH MCF-7 CELL LINE

a. Cytotoxic evaluation using MTT assay on MCF-7 cell line

Antiproliferative mechanism involving apoptosis has always been a target of cancer therapy. Since many anticancer drugs developed so far are associated with severe side effects, there is an increasing demand for effective anticancer drugs. Plants with extremely diverse range of chemical compounds often with structural complexity are superior chemists which make them preferable for developing anticancer drugs (Howes, 2018). Since present study with selected species of *Gomphostemma* displayed promising cytotoxic effects on *A. cepa* root tips, these plants were evaluated further for their antiproliferative potential on MCF-7 cell line. The MCF-7 cells represent a suitable *in vitro* model for the breast cancer cell experiment. The study is particularly relevant because, breast cancer is the most frequent cause of cancer death among females. All the three selected taxa of *Gomphostemma* were screened with MTT assay to check the cytotoxic potential and the most

effective extract was analysed further for its genotoxic and apoptotic activity. It was also checked for its cytotoxicity on normal L929 cells. Genotoxic potential was evaluated with comet assay and the effect of extract on caspases activity was also analysed. Dual AO/EB staining was employed to detect apoptosis and arrest of phase in cell cycle was determined with flow cytometry. Gene expression studies with two apoptosis related genes p53 and p21 was also attempted to elucidate the pathway of anticancer mechanism moderated by the most effective extract among selected species of *Gomphostemma*.

All the three selected taxa of *Gomphostemma* were analysed for its cytotoxicity on MCF-7 cells with MTT assay. The MTT assay can measure viable cell in relatively high throughput without the need for elaborate cell counting. The most common use of the assay is to measure the *in vitro* cytotoxic effect of drugs at different concentrations on cell lines. Basic principle of MTT assay is related to the mitochondrial activity of cells. For viable cells, the mitochondrial activity will be constant and any increase or decrease in the number of viable cells thus becomes linearly related to the mitochondrial activity. Mitochondrial activity in this assay is reflected by the conversion of tetrazolium salt MTT into formazan crystals which can be solubilised for optical reading. Thus, any increase or decrease in formazan concentration reflected in optical density is a measure of increase or decrease in viable cell numbers (van Meerloo et al., 2011). It mainly determines the activities of intracellular NAD(P) dependent oxidoreductases, predominately dehydrogenases (Surin et al., 2017). In the present experiment, different concentrations (6.25, 12.5, 25, 50 and 100 µg/mL) and a time period of 24 h treatment was applied. There was a considerable decrease in cell viability with increase in concentration (Fig. 41; Plates 20–22). Among the extracts, the effect exerted by *G. heyneanum* var. *heyneanum* was noteworthy, since at the highest concentration (100 µg/mL) the cell viability percentage was found

to be as low as 35.57 ± 0.44 and the IC_{50} value was found to be 45.97 ± 1.48 $\mu\text{g/mL}$. In the case of *G. heyneanum* var. *rottleri*, IC_{50} value was calculated as 116.18 ± 1.14 $\mu\text{g/mL}$ and for *G. eriocarpum* it was 127.26 ± 1.77 $\mu\text{g/mL}$. In addition, distinct morphological changes like cell shrinkage, membrane blebbing, cell rounding and formation of apoptotic bodies were observed in treated cells. The morphological features noticed in treated cells are standard apoptotic markers which indirectly points to the induction of apoptosis by the plant extract (Akter et al., 2012). The characteristic morphological features portrayed by apoptotic cells may be attributed to the profound cytoskeleton reorganizations induced in them (Ndozangue-Touriguine et al., 2008). Based on MTT assay as well as on the performance displayed in *A. cepa* assays, extract of *G. heyneanum* var. *heyneanum* at a concentration of 45 $\mu\text{g/mL}$ (based on IC_{50} value) alone was considered for further scrutiny.

b. Cytotoxic evaluation using MTT assay on L929 cell line

The methanol extract of *G. heyneanum* var. *heyneanum* was assessed for its toxic effect on normal L929 cells. The L929 cell line is derived from an immortalized mouse fibroblast cell line and is internationally recognized as a representative of normal cells in *in vitro* cytotoxicity assessments (Liu et al., 2008). In this study, five different concentrations of *G. heyneanum* var. *heyneanum* were tested (6.25 - 100 $\mu\text{g/mL}$). It was found that even at the highest concentration (100 $\mu\text{g/mL}$), $77.64 \pm 1.37\%$ cells were viable (Fig. 42) which is quite high compared to the viability percentage of MCF-7 cells ($35.57 \pm 0.44\%$) at the same concentration. The IC_{50} value obtained was also high (220.23 ± 1.16 $\mu\text{g/mL}$). The typical morphological features noticed after treatment with plant extract in MCF-7 cells (Plate 20) as manifestation of apoptosis were not observed in L929 cells (Plate 23). It is generally accepted that a successful anticancer drug should kill cancer cells without causing excessive damage to normal cells such as L929 (Graidist et al., 2015). The

result of this study showed that the plant extract tested has selective cytotoxicity to cancer cells with only mild toxicity on normal cells. This can be taken as an indication of the efficient anticancer property of *G. heyneanum* var. *heyneanum*.

c. Comet assay

The genotoxic potential of *G. heyneanum* var. *heyneanum* was assessed with comet assay. It is a simple, sensitive and fast method for measuring DNA damages in cells. It quantifies the amount of denatured DNA fragments migrating out of the nuclei during electrophoresis. During separation across an applied electric field, DNA remaining in the place of the flooded cell, partly anchored to residual nucleus structures gets migrated towards the anode with a speed proportionate to its fragmentation. The degree of DNA migration in agarose is directly proportional to the degree of DNA damage. The image obtained by this procedure appear like a “comet” with a distinct head and tail of which, tail constitute of relaxed loops and damaged DNA fragments (Widziewicz et al., 2012). Five parameters namely comet length, tail length, tail DNA percentage, tail moment and olive tail moment were determined for the evaluation of DNA damage induced by 45 µg/mL of extract on MCF-7 cells (Plate 24). Increase in comet length and tail length (Fig. 43 A & B) is an indication of DNA damage induced by the plant extract (Malyapa et al., 1998). The relative increase in tail length, which is a measure of distance of DNA migration from the body of the nuclear core represents the extent of DNA damage (Kumaravel et al., 2009). A considerably elevated tail DNA percentage (42.91 ± 2.24) was noticed for treated cells as compared to the control (Fig. 43 C). The percentage of DNA in the tail is considered as directly proportional to the percentage of DNA damage and DNA breaks (Horváthová et al., 2004; Beedanagari et al., 2014).

Tail moment and olive tail moment were the other two tail parameters considered. The tail moment has been suggested as an index of induced DNA damage as it considers both the migrating DNA as well as the relative amount of DNA in the tail (Hellman et al., 1995; Mozaffarieh et al., 2008). The olive tail moment which is defined as the fraction of tail DNA multiplied by the distance between the profile centres of gravity for DNA in the head and tail is one of the best parameters capable of reflecting the DNA damage (Moneef et al., 2003). Tail moment and olive tail moment were also found to be elevated as compared to the control (Fig. 43 D & E). The result clearly demonstrated the genotoxic effect induced by the extract of *G. heyneanum* var. *heyneanum* on MCF-7 cell line. The DNA fragmentation is one of the morphological changes commonly noticed in cells undergoing apoptosis and is regarded as a marker of apoptosis. Among the different methods developed for detecting DNA fragmentation, comet assay is a rapid and increasingly used method in genotoxicity testing especially for detecting DNA strand breaks (Majtnerová & Roušar, 2018). The DNA damage in MCF-7 cells exerted by the extract of *G. heyneanum* var. *heyneanum* may be considered as a lead regarding the ability of the extract to induce apoptosis in cancer cells.

d. Detection of apoptosis

Double staining method using acridine orange and ethidium bromide (AO/EB) for detecting apoptosis is a reliable method to identify and distinguish apoptotic cells (Behzada et al., 2016). Acridine orange is taken up by both viable and non-viable cells giving out green fluorescence. Ethidium bromide is taken up only by non-viable cells and emits red fluorescence upon intercalation into DNA. Viable cells have uniform bright green nuclei with organized structure. Early apoptotic cells which still have intact membranes but have started to undergo DNA cleavage usually have granular greenish yellow colour and late apoptotic cells are seen to have orange to red nuclei

with condensed or fragmented chromatin. Necrotic cells have a uniformly orange to red nuclei. Thus, AO/ EB staining distinguishes several subpopulations of apoptotic cells from viable cells to necrotic cells. In addition, variable staining patterns and other morphological features like cell rounding, surface blebbing, shrinkage, chromatin condensation, nuclear fragmentation, *etc.* provide a complete morphological profile of an apoptotic cell (Baskić et al., 2006; Afsar et al., 2016).

In this study, MCF-7 cells were treated with 45 µg/mL of *G. heyneanum* var. *heyneanum* extract. After incubation and staining, morphological changes of cells were examined under fluorescent microscope (Plate 25). Green uniformly stained cells were noted only in control. The MCF-7 cells treated with the extract were found to be in different stages of apoptosis. Early apoptotic cells were identified by granular greenish yellow colour and late apoptotic cells were marked by orange red staining with fragmented appearance. Negligible number of necrotic cells with uniform red staining was also seen. In addition to variable staining pattern, morphological features like chromatin condensation, nuclear fragmentation, apoptotic bodies, *etc.* which are manifestations of apoptosis were also noticed. The variable staining pattern and morphological changes observed by AO/EB staining in treated cells confirm the apoptotic potential of the plant extract analysed.

The three main hall marks of cancer cells are uncontrolled growth, angiogenesis and apoptosis evasion. Apoptosis is a type of programmed cell death that is characterized by many morphological features like cell membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, DNA fragmentation, *etc.* (Su et al., 2015). Programmed cell death is a natural barrier that restricts the survival of malignant cells. Resistance to apoptosis allows cancer cells to survive longer and gives more time for the accumulation of mutations which can increase its

invasiveness. Hence, one of the effective methods to treat cancer is to gain control or terminate the uncontrolled growth of cancer cells. There are many anticancer drugs that target various stages in both intrinsic and extrinsic pathways of apoptosis. In the light of increasing resistance and toxicity of traditional therapies, a new non-toxic anticancer treatment that is able to differentiate between tumour cells and normal cells is highly recommended. Many plant based compounds that can induce apoptosis in cancer cells and are typically non-toxic to normal cells have been identified. The ability to activate an apoptotic pathway would be an intriguing approach in anticancer therapies (Pfeffer & Singh, 2018).

e. Cell cycle analysis

The effect of methanolic extract of *G. heyneanum* var. *heyneanum* on cell cycle was evaluated using flow cytometric analysis. Most of the chemotherapeutic drugs used for cancer treatment usually exert their cytotoxicity by inducing DNA damage which eventually activates cell cycle checkpoints and coordinate cell cycle progression with DNA repair (Choumessi et al., 2012). Since, the extract of *G. heyneanum* var. *heyneanum* has demonstrated good cytotoxic, genotoxic and apoptotic activity, it was analysed for its effect on cell cycle for a better understanding of mechanism of apoptosis. DNA content is the most frequently measured cellular constituent in cell cycle analysis. Its quantification serves to assess DNA ploidy level, cell position in the cell cycle and helps to reveal the presence of apoptotic cells that are characterized by fractional DNA content. Distribution of cells within the major phases of the cell cycle is based on differences in DNA content between the pre-replicative phase cells (G₀/G₁) versus the cells that actually replicate DNA (S phase) versus the post-replicative plus mitotic (G₂ + M) cells and the cells in G₂/M phase have double the DNA content as compared to G₀/G₁ phase. DNA content measured by cytometry is generally

expressed as DNA index and the results of cellular DNA content measurement are most frequently presented in the form of frequency histograms. As a result of extensive DNA fragmentation and loss of nuclear DNA content during apoptosis, the cells undergoing apoptosis acquire hypodiploid (or sub-G1) DNA profile during flow cytometry and are defined as “sub-diploid” or “sub-G1” cell population (Darzynkiewicz, 2010; Pereira et al., 2016).

The flow cytometry analysis was done with MCF-7 cells treated with 45 µg/mL of plant extract for 24 h. A significant increase in percentage of cells in G0/G1 phase of cell cycle was observed in the case of treated cells (Figs 44, 45 & 46). This was accompanied by a decrease in the percentage of cells in the S and G2/M phase of the cell cycle as compared to the control (Fig. 46). The increase in G0/G1 population and a sub-G1 population as shown in flow cytometry histogram of treated cells (Fig. 44 B) is a clear indication of cell cycle arrest and inhibition of cell proliferation at G0/G1 phase accompanied by apoptosis. The induction of cell cycle arrest at a specific checkpoint followed by apoptosis is a common mechanism of the cytotoxic effects of anticancer drugs (Song et al., 2017). Studies have shown that DNA damage induced by anticancer agents can activate DNA damage check points leading to the arrest of cell cycle progression which may trigger DNA damage repair, or lead to cell death (Zhou et al., 2018). The results of the present study thus provided evidence to the ability of *G. heyneanum* var. *heyneanum* to inhibit proliferation of MCF-7 cells by blocking the progression of cell cycle at G0/G1 phase followed by induction of apoptosis. The anti-proliferative activity exhibited may be attributed to the phytoconstituents present in the extract of *G. heyneanum* var. *heyneanum*.

f. Determination of caspase activity with ELISA

Caspases are a family of endoproteases that play critical roles in linking various cell regulatory networks controlling the initiation and execution of apoptotic cell death. Caspases involved in apoptosis have been sub-classified by their mechanism of action as initiator caspases (caspase-8 and -9) and executioner caspases (caspase-3, -6, and -7). Initiator caspases activate executioner caspases that subsequently coordinate their activities to demolish key structural proteins and activate other enzymes. These result in the morphological hallmarks of apoptosis including DNA fragmentation and membrane blebbing. Caspase-8 is involved in extrinsic apoptosis which is triggered by extracellular cues delivered in the form of ligands binding to death receptors. Caspase-9 is the executor caspase of intrinsic apoptosis which is also known as mitochondrial apoptosis as it depends on factors released from the mitochondria and is activated by a vast array of cellular stresses, including cytoskeletal disruption, DNA damage and accumulation of unfolded proteins, hypoxia and many others (McIlwain et al., 2013). Among the executor caspases, caspase-3 and caspase-7 are popularly known to be involved in the execution of apoptosis (Li & Yuan, 2008).

As indications of apoptosis were revealed by staining techniques and cell cycle analysis, the extract of *G. heyneanum* var. *heyneanum* was analysed for its influence on caspases activity. The MCF-7 cells treated with 45 µg/mL of plant extract for 24 h were evaluated for the activity of caspase-9 and caspase-7. The activity of caspase-9 and caspase-7 were found to be increased in treated cells as compared to the control (Fig. 47). Caspase-9 is an initiator in the apoptotic cascade which initiates the proteolytic cleavage of other downstream proteins like caspase-7 (Cagnol et al., 2011). The increased caspases activity observed in the present study suggests that caspases mediated intrinsic apoptotic pathway is responsible for the antiproliferative effect of *G. heyneanum* var. *heyneanum* on MCF-7 cells.

g. Gene expression studies

In order to substantiate the underlying mechanism of antiproliferative activity exhibited by the extract of *G. heyneanum* var. *heyneanum* on MCF-7 cells, the expression changes of genes which are known to be involved in cell cycle arrest and induction of apoptosis were examined. Changes in the expression of p53 and p21 were studied by RT-qPCR followed by gel electrophoresis for the separation and visualization of DNA fragments using β -actin as loading control (Fig. 48). The results showed that the treatment of MCF-7 cells with 45 μ g/mL of extract significantly induced an up-regulation in the expression of p53 with an expression fold change of 1.82 ± 0.09 over the control. The expression fold change for p21 was found to be 1.21 ± 0.06 (Fig. 49). Studies have shown that up-regulation of p53 induces the expression of p21 which can lead to a p21 mediated inhibition of cyclin-dependent kinase and cell cycle arrest in G0/G1 phase (Whyte et al., 2007). Since the up-regulation of p53 and p21 is observed together with cell cycle arrest in G0/G1 phase, it is possible that the plant extract might have exerted its effect on MCF-7 cells through these genes.

The p53, also known as TP53 is a tumour suppressor gene that is found to be frequently inactivated in many cancer types including breast, colon and lung cancers. The p53 gene product is a nuclear transcription factor which regulates many downstream genes involved in the induction of cell cycle arrest, DNA repair and apoptosis (Cooper & Hausman, 2000). Functionally active p53 transactivates an appropriate set of its target genes to induce cell cycle arrest at a checkpoint until the DNA damage is repaired. When DNA repair is complete, cells re-enter the normal cell cycle, but if the cells have serious DNA damage which is irreversible, p53 triggers its pro-apoptotic function to eliminate cells with serious DNA damage (Ozaki & Nakagawara, 2011; Kadam & Abhang, 2016).

Genetic instability is one of the most prominent features of malignant tumours and p53 responds to DNA damage either by inducing cell cycle arrest

or apoptosis depending upon the extent of DNA damage. The p53 induce cell cycle arrest through the up-regulation of p21. The p21 gene was the first to be identified as induced by wildtype p53 protein. The p53 is the main transcriptional regulator of p21 and the p21 gene has two conserved p53 responsive elements in its promoter. The arrest of cell cycle in G1/S transition has been identified as a p53-dependent process. The induction of cell cycle arrest at G1 by p53 is believed to be through the activation of transcription of the cyclin-dependent kinase (CDK) inhibitor p21. The CDK inhibitor p21, also known as p21waf1 is a small protein with 165 amino acids and belongs to the CIP/Kip family of CDKs inhibitors. The p21 is a well-known inhibitor of cell cycle which can arrest the cell cycle progression in G1/S and G2/M transitions. Progression of the mammalian cell cycle is regulated by CDKs and regulatory subunit cyclins. Cell cycle progression is triggered by partial phosphorylation of pRb (Retinoblastoma protein) by CDK-Cyclin and p21 disrupts these interactions and inhibit cell cycle progression. Transitions of cell cycle from G1 to S and G2 to mitosis is regulated by the sequential activation and inactivation of CDK family of proteins which is a family of serine/threonine protein kinases. Cyclin is the regulatory sub-unit of CDK and is degraded or synthesised during cell cycle. Of more than 21 CDKs identified so far, only CDK1, CDK2, CDK4 and CDK6 have been found to be required for cell cycle regulation. CDKs activity (CDK2, CDK4 and CDK6 for G1 phase, CDK2 for S phase and CDK1 for G2 and M phases) remain stable during cell cycle. However, the fluctuations in the cyclin levels during cell cycle can lead to activation of CDKs. Cyclin Ds including D1, D2 and D3 are the first among cyclins sensing the mitogenic signals and they activate CDK4 and CDK6 in G1 phase. Similarly, the activation of CDK2 by cyclin E1 and E2 can lead to G1/S transition. During S phase, the degradation and replacement of cyclin E proteins by cyclin A leads to transition from S phase to mitosis and finally, at the end of G2 phase, CDK2 interacts with cyclin B. But, hyper-activation of CDKs can cause unregulated cell division and can cause tumour development. It has been

shown that CDK activity is controlled by CDK inhibitors (CKIs) such as CIP/KIP and INK. The CIP/KIP family includes p21, p27 and p57 and the INK family comprises p15, p16, p18 and p19 proteins. INK family members bind to CDK4 and CDK6 and block their interaction with cyclin D. The CIP/KIP family members bind to cyclin A, E, D/CDK complex and suppress their catalytic activity causing cell cycle arrest. The p21 was the first member of CKI proteins to be identified which binds to cyclin A/CDK2, E/CDK2, D1/CDK4 and D2/CDK4 complex and as such, inhibits the phosphorylation of pRB protein. Induction of p21 by p53 upon DNA damage inhibits cyclin E/CDK2 and thereby inhibits G1/S transition. The p53-mediated induction of p21 has also been found to be causing the down regulation of ICBP90 (inverted CCAAT box binding protein, 90 kD) whose expression is observed during G1/S and G2/M phases and is found to be involved in DNA replication and cell cycle regulation. Hence, its down-regulation by p21 is an important mechanism for arresting G/S transition (Karimian et al., 2016; Abbas & Dutta, 2009; Giono & Manfredi, 2006).

The p53 is also known to mediate apoptosis. It participates directly in the intrinsic apoptosis pathway by interacting with the multidomain members of the Bcl-2 family to induce mitochondrial outer membrane permeabilization. The direct action of p53 results in robust mitochondrial outer membrane permeabilization (MOMP), unleashing the enzymatic apoptotic machinery of caspases and chromatin degradation. The MOMP is governed by pro- and anti-apoptotic Bcl-2 family members. Characteristic of these proteins is the presence of Bcl-2 homology (BH) motifs. About 20 members of this family have been identified and classified on the basis of their role in apoptosis. They are classified into 3 functional groups: (i) anti-apoptotic proteins such as Bcl-2 itself, Bcl-xL, Bcl-w, Mcl-1 and A1, which inhibit cell death through direct interactions with the pro-apoptotic members (ii) pro-apoptotic activators with only a single BH3 motif are important mediators in the cellular response to stresses such as DNA damage and (iii)

pro-apoptotic executors like Bax and Bak, which are believed to participate directly in MOM permeabilization (Garcia-Saez, 2012; Happonen et al, 2012).

Most of the BH3-only proteins are direct transcriptional targets of p53. The p53 in response to stress interacts with and neutralizes the anti-apoptotic members and activate pro-apoptotic members. The BH3-only proteins exist at low levels under normal conditions in the cell. In the presence of apoptotic stimuli they are activated to induce apoptosis. As a result of BH3-only stimulation, Bax and Bak become activated. Upon activation, Bax and Bak insert into the outer mitochondrial membrane and oligomerize to form dynamic lipid pores that release lethal proteins and cytochrome c from the mitochondrial intermembranous space to cytosol (Vaseva & Moll, 2009). Once in cytosol, cytochrome c binds to its cytosolic partner Apaf-1, the human homologue of *Caenorhabditis elegans* apoptotic protein CED-4 and induces the oligomerization of Apaf-1 cytochrome c complex in a dATP/ATP-dependent manner. This complex namely apoptosome is sufficient to recruit procaspase-9 and induce its autoactivation. The activated caspase-9 then cleaves and activates other downstream caspases such as caspase-3 and caspase-7 which begins the process of apoptosis (Jiang & Wang, 2000; Wu et al., 2016; Li et al., 2017).

Present study demonstrated the cytotoxicity and genotoxicity potential of *G. heyneanum* var. *heyneanum* as revealed by MTT and comet assay respectively. The apoptotic activity confirmed by AO/EB staining was further assured by the flow cytometry analysis which showed cell cycle arrest in the G0/G1 phase with a notable sub-G1 peak in the histogram. Elevated activity of caspase-9 which is often found involved in intrinsic pathway of apoptosis and caspase-7 as compared to the control, pointed to the induction of intrinsic pathway of apoptotic cell death. Finally, the up-regulation in the expressions of p53 and p21 asserted the apoptotic potential of *G. heyneanum* var. *heyneanum*. Cells often respond to DNA damage by activating the p53-p21 pathway halting their cell cycle progression in the G0/G1 phase. The

genotoxicity caused by the plant extract might have activated the expression of p53 gene which induced the activation of p21 to bring about cell cycle arrest in G0/G1 phase. As discussed earlier irreversible DNA damage might have triggered the apoptotic function of p53 leading to the recruitment of initiator caspase-9 followed by the activation of the downstream caspase-7 which finally culminated in apoptotic cell death.

The antiproliferative activity of *G. heyneanum* var. *heyneanum* can be attributed to the combined activity of various phytoconstituents in it. Phytochemical profiling has shown that *G. heyneanum* var. *heyneanum* is rich in terpenes and a variety of them like limonene, α -pinene, β -phellandrene, β -myrcene, p-cymene, *etc.* were identified in it by GC/MS analysis. Terpenes especially monoterpenes have antiproliferative activity (Sobral et al., 2014). The diversity of components in *G. heyneanum* var. *heyneanum* need to be emphasised, as a plethora of them like β -caryophyllene, phytol, β -elemene, quinic acid, stigmasterol, theaflavin, genistein, *etc.* were identified by GC/MS and HR-LC/MS analysis, many of which are appreciated for their anticancer property. The synergistic interaction of these compounds might have contributed to the antiproliferative activity of *G. heyneanum* var. *heyneanum* on MCF-7 cells. Synergy literally means that the effect of combination is greater than the sum of the individual effects and this is true in the case of phytomedicines. In pharmacodynamic synergy, different constituents in the extract act at different receptor targets involved in the disease to enhance the overall therapeutic effect. Even the minor constituents with little or no activity on the causative agent may assist the main active principle to reach the target by divergent ways either by improving bioavailability, or by decreasing metabolism or by the modulation of adverse effects (Rasoanaivo et al., 2011). Plants have been an important source of therapeutic resources and frequently it is observed that herbal extracts have pronounced biological activities rather than isolated constituents. This may be due to the interactions between the bioactive compounds with varying chemical structures that may act at different drug targets through multiple

mechanisms or signalling pathways for the same disease. They may synergistically function even as multi-target drugs at low doses and has been particularly promising in the treatment of complex diseases. However, there remain significant challenges in developing methods to analyse, understand and standardise the synergistic interactions of multiple components in herbal preparations (Casanova & Costa, 2017; Ni et al., 2018).

Along with these observations, the anti-inflammatory activity exhibited by the extract of *G. heyneanum* var. *heyneanum* deserves a special mention as inflammation especially chronic inflammation create a microenvironment suitable for the induction of cancer. Several studies have suggested that the inflammation-associated DNA damage in cancer stem-like cells can lead to cancer development with aggressive clinical features (Murata, 2018). Many anti-inflammatory agents which are effective in inhibiting inflammatory mediators like, COX-2, iNOS, LOX, NF- κ B and TNF- α are effective adjuvants for combination therapy with conventional cancer therapies to improve the effect of treatment (Rayburn et al., 2009). In this study, the extract of *G. heyneanum* var. *heyneanum* extract has been effective in reducing the levels of inflammatory mediators like COX-2, cellular nitrite levels, iNOS, MPO and NF- κ B activity. These findings suggest that the extract of *G. heyneanum* var. *heyneanum* can be effective in treating and preventing cancer types associated with inflammation.

F. GREEN SYNTHESIS OF SILVER NANOPARTICLES AND THEIR CHARACTERIZATION

Nanoparticles include particles in the size range of 1–100 nm and this size reduction renders them with unique abilities to interact with biological systems at the molecular level. Among the different types of nanoparticles, gold and silver nanoparticles have superior characteristics which make them useful in detection, diagnosis and treatment of various complex diseases including cancer. Silver is the most profit oriented precious metal with a long

history of usage in pharmaceutical industry for various purposes. Also, small silver nanoparticles have many advantages as drug carriers which include their adjustable size and shape, enhanced stability, high-density surface ligand attachment, transmembrane delivery without harsh transfection agents, protection of the attached therapeutics from degradation and potential for improved timed/controlled intracellular drug-delivery (Ivanova et al., 2018). Green synthesis of nanoparticles is a key branch of nanotechnology where the nanoparticles are produced with the help of biological entities like microorganisms and plant extracts. The green synthesis of nanoparticles is preferred over physical and chemical methods as it is environment friendly, cost effective without any need for high temperature, energy and toxic chemicals. The use of plant extracts is potentially beneficial over microorganisms because plant extracts reduce metal ions at a faster rate and does not involve the strenuous process of maintaining the microbial cell cultures (Mathur et al., 2018). Present study is an attempt to synthesize silver nanoparticles with selected species of *Gomphostemma*.

a. Synthesis of silver nanoparticles

Many factors like nature and concentration of plant extract and salt, temperature, pH and time influence the reduction of metal salts from positive oxidation state to zero oxidation state. Size of the metallic nanoparticles is strongly determined by the biomolecules in the extract. In general, presence of strong reducing agents in the extract promotes a fast reaction rate and favours the formation of smaller nanoparticles (Roy et al., 2019). In this study, fastest synthesis of silver nanoparticles was observed at a concentration of 2 mM of silver nitrate with an alkaline pH and the whole mixture required boiling at 80 °C followed by incubation of 30 min. The optimal conditions for the synthesis of silver nanoparticles may vary from one plant to another depending upon the phytocomponents and their reducing abilities. In general, an alkaline pH is preferred suitable for the synthesis of stable silver nanoparticles. Formation of silver nanoparticles is preliminarily noticed by

the colour change exhibited by the solution upon the synthesis of silver nanoparticles. Here, the colour of reaction mixtures changed from pale green to yellowish brown or dark brown colour for all the three plant extracts evaluated with slight variations in intensity (Plate 26). Metal nanoparticles exhibits different colours in solution due to their optical properties depending upon the excitation of free electrons in the nanoparticles. In the green synthesis of silver nanoparticles, change in colour of solution to shades of brown which can be visually observed is an indication of formation of silver nanoparticles (Vanaja et al., 2013).

b. Characterization of silver nanoparticles

i. UV-Vis spectral analysis

Ultraviolet visible spectroscopy is a reliable and useful technique for the characterization of silver nanoparticles. A strong, broad absorption band was observed in the range of 400–500 nm (Fig. 50). For *G. heyneanum* var. *heyneanum* maximum absorbance was centered at 437 nm; for *G. heyneanum* var. *rottleri* it was at 435 nm and for *G. eriocarpum*, it was located at 448.5 nm. The appearances of peaks show the characteristics of surface plasmon resonance of silver nanoparticles. Free electrons of silver nanoparticles yield the surface plasmon resonance (SPR) absorption band due to the mutual vibration of electrons of metal nanoparticles in resonance with light wave (Anandalakshmi et al., 2016). The appearance of peaks assigned to surface plasmons are well-documented for various metal nanoparticles with sizes ranging from 2 to 100 nm and in general silver nanoparticles (AgNPs) show the absorption spectrum under SPR in the range of 400–490 nm (Safaepour, et al., 2009; Ibrahim, 2015). Since the same range was observed in this study, formation of silver nanoparticles is validated.

ii. FE-SEM/EDX analysis

The Field-Emission Scanning Electron Microscopy (FE-SEM) technique was employed to visualize the size and shape of silver

nanoparticles. The SEM images of the AgNPs synthesised by selected species of *Gomphostemma* are shown in Plates 27, 28 and 29. Their morphological dimensions as seen in SEM images demonstrated that the AgNPs formed are nearly spherical in shape. The average size of the silver nanoparticles synthesised by *G. heyneanum* var. *heyneanum* was 24.46–38.67 nm (Plate 27). The size of silver nanoparticles by *G. heyneanum* var. *rottleri* was in the range of 23.93–34.18 nm which is slightly smaller as compared to the silver nanoparticles synthesised by the other variety (Plate 28). The silver nanoparticles synthesised by *G. eriocarpum* were the largest among the three taxa of *Gomphostemma* analysed with the size in the range of 59.97–65.23 nm (Plate 29). Studies have shown that the size and shape of silver nanoparticles can be varied and is immensely affected by many factors like temperature, concentration of silver nitrate, concentration of extract, pH, *etc.* (Skandalis et al., 2017; Rauwel et al., 2015). The size and shape of silver nanoparticles synthesised by selected taxa of *Gomphostemma* are commendable and comparable with the previous reports of other lamiacean members (Table 5). The biological effect of silver nanoparticles is mostly determined by the stability of particles and nanoparticles of smaller size usually demonstrate a higher biological activity (El-Nour et al., 2010; Rónavári et al., 2017).

Additionally, EDX analysis conducted confirmed the presence of silver nanoparticles as indicated by the strong signals displayed in the silver region of EDX spectra (Plate 30). Silver nanocrystallites usually display an optical absorption band peak at approximately 3 keV, which is typical about the absorption of metallic silver nanocrystals due to surface plasmon resonance and the same was observed in this study also. The biogenic silver nanoparticles were found to be with less impurity. Elements C, O, Si and P which were also found in the EDX spectrogram possibly indicate the presence of organic components in the biogenic nano-materials (Yu et al., 2019). The variable number of phytochemicals such as organic acids, terpenoids, flavonoids, phenols, proteins, *etc.* are said to be responsible for the immediate

reduction of silver ions into silver nanoparticles as well as for stabilizing AgNPs to prevent agglomeration by acting as capping agents (Jain & Mehata, 2017).

Development of biogenic metallic nanoparticles, particularly silver nanoparticles has received increasing attention because of their applications in many fields including drug delivery and nanomedicine. Although biogenic metallic nanoparticles are considered to be safer than chemically synthesised nanoparticles; several studies have shown similar toxicities of both types of nanoparticles including nephrotoxicity and neurotoxicity (Katas et al., 2018). Hence, further studies should be performed to investigate and confirm the potential toxicity of biogenic nanoparticles before implementing them for medicinal applications.

In conclusion, present study divulged the phytochemical and pharmacological properties of selected species of *Gomphostemma*. This is also a scientific validation of the therapeutic potential of *Gomphostemma*, an underutilized genus within Lamiaceae with the potential to be explored for its bioprospective values.

SUMMARY AND CONCLUSIONS

Nature has nurtured plants with an inexhaustible array of molecular entities that forms an infinite resource for drug development towards a multitude of diseases. From time immemorial, man has relied on plants for treating various ailments and plant-based system still forms the backbone of the health care system throughout the world. Plants have always been an important source of novel pharmacologically active compounds and have played a critical role in modern drug development. The molecular diversity and specific biological activity of plant derived compounds make them valuable for drug development. Despite intensive investigation a vast majority of terrestrial flora virtually remains unexplored both phytochemically and pharmacologically.

Lamiaceae is one among the most diverse families in the plant kingdom reputed for its fragrance and medicinal properties. Even though many species in Lamiaceae has been extensively studied for their pharmacological properties some members like *Gomphostemma*, a genus of subfamily Lamioideae within Lamiaceae are underutilized and poorly investigated in terms of their phytochemistry and bioactivity. Therefore, the principal aim of the present study was to probe selected species of *Gomphostemma* Wall. ex Benth., for their phytochemical and pharmacological aspects. The three taxa selected for the study were *G. heyneanum* var. *heyneanum*, *G. heyneanum* var. *rottleri* and *G. eriocarpum*. The aerial parts of these plants were subjected to detailed phytochemical profiling. Screening for bioactivities with emphasis to antioxidant, anti-inflammatory and antiproliferative potential was performed. Finally, green synthesis of silver nanoparticles with these plants was also attempted.

The major findings of the study are summarized as follows:

Phytochemical characterization

The methanolic extract obtained by soxhlet extraction of dried aerial parts of selected taxa of *Gomphostemma* was concentrated and used for the entire study. The qualitative phytochemical tests performed to detect various secondary metabolites showed the presence of phenols, flavonoids, alkaloids, terpenoids, steroids, carotenoids, cardiac glycosides, quinones, coumarins and iridoid glycosides. Whereas, tannins and steroids were found to be absent in the extracts of selected plants. Quantitative estimation of major phytoconstituents done by standard assays showed highest phenolic, flavonoid and terpenoids content in *G. heyneanum* var. *heyneanum* followed by *G. heyneanum* var. *rottleri*. Detailed phytochemical profiling with GC/MS and HR-LC/MS revealed an array of volatile and non-volatile components in the methanolic extract of *Gomphostemma* species. GC/MS analysis showed a total of 72 compounds belonging to various classes and included some notable components like α -thujene, α -pinene, β -phellandrene, β -myrcene, p-cymene, limonene, eucalyptol, neophytadiene, β -caryophyllene, β -elemene, phytol, stearic acid, quinic acid, tocopherol, squalene, stigmasterol, β -sitosterol, *etc.* with prominent biological activities. A total of 34 components were detected by HR-LC/MS in the extracts of plants analysed. The extracts were shown to contain a variety of bioactive components like theaflavin, cephaeline, chlorogenic acid, dihydrocelastrol, swietenine, cuscohygrine, *etc.* along with a number of biopeptides. The phytochemical studies thus provided a strong lead to the bioactivity potential of selected species of *Gomphostemma*.

Antioxidant activity

The antioxidant property of selected species of *Gomphostemma* was assessed by DPPH radical assay, hydroxyl radical scavenging, nitric oxide

radical scavenging, superoxide radical scavenging activity and reducing power assay. Among the plants analysed, *G. heyneanum* var. *heyneanum* displayed reasonable antioxidant activity in all the assays. The extract of *G. eriocarpum* was effective in scavenging hydroxyl radicals but its activity was feeble in other assays, whereas the performance of *G. heyneanum* var. *rottleri* was moderate in all the assays.

Anti-inflammatory activity

The anti-inflammatory potential of selected plants was initially evaluated with *in vitro* biochemical assays. Among the three taxa of *Gomphostemma* analysed, *G. heyneanum* var. *heyneanum* was effective in inhibiting protein denaturation and proteinase activity and hence it was further subjected to detailed studies with LPS induced RAW 264.7 cells. The effect of *G. heyneanum* var. *heyneanum* on inflammatory mediators like COX-2, LOX, MPO, cellular nitrite and iNOS activity was examined. It was found that the extract was effective in inhibiting COX-2, MPO, cellular nitrite and iNOS activity. Further evaluation showed that the anti-inflammatory property exhibited by the plant is possibly associated with the suppression of NF- κ B activation with subsequent effect on COX-2 and iNOS activity. In general, *G. heyneanum* var. *heyneanum* displayed a moderate anti-inflammatory activity.

Cytotoxicity and antiproliferative activity

The anticancer activity of selected species of *Gomphostemma* was determined by evaluating their cytotoxic and antiproliferative potential. The preliminary screening of cytotoxicity was done with *Allium cepa* assay. Five different concentration and four different time intervals were selected. The two end points considered for the study were mitotic index and chromosomal aberrations. Dose and duration dependent drop in mitotic index indicated the mitodepressive effect of extracts. A wide spectrum of chromosomal

aberrations and spindle disturbances were observed. Some of the common aberrations noticed were nuclear lesions, buds, blebs, fragmentation, stickiness, chromosome gaps, rings, micronucleus, disturbed metaphase and anaphase, *etc.* The ability of the plant extracts to induce apoptosis was observed in the form of chromatin condensation, nuclear disintegration, formation of micronucleus, nuclear blebs, cytoplasmic shrinkage, *etc.* Presence of multiple aberrations specified the acute cytotoxic potential of the extracts. The cell death of *A. cepa* induced by the plant extracts were visualized using Evans blue staining and was quantified spectrophotometrically. All these provided a strong base for further antiproliferative studies. Among the plants analysed, the extract *G. heyneanum* var. *heyneanum* was found to be effective in inducing cytotoxicity.

The antiproliferative potential of selected species of *Gomphostemma* was tested on MCF-7 cell line. The cytotoxicity was assessed with MTT assay. Five different concentrations and 24 h treatment period was considered for the study. Among the selected species of *Gomphostemma* evaluated, *G. heyneanum* var. *heyneanum* showed remarkable activity and the IC₅₀ was found to be 45.97 ± 1.48 µg/mL. The extract of *G. heyneanum* var. *heyneanum* was also tested on L929 cells to assess its toxicity towards normal cells. It was found to be less toxic towards L929 cells suggesting that the plant extract is selectively toxic to cancer cells. Since *G. heyneanum* var. *heyneanum* was found to have good antiproliferative potential it was selected and subjected to further studies to elucidate the mechanism by which it suppressed the cell line. The genotoxicity induced by the extract of *G. heyneanum* var. *heyneanum* as evaluated by comet assay showed its ability to induce DNA damage. The variable staining pattern and morphological changes observed by AO/EB displayed the induction of apoptosis in MCF-7 cells by the extract. Further, cell cycle analysis with flow cytometry

demonstrated the ability of the extract to induce cell cycle arrest at G0/G1 phase which confirmed that the antiproliferative effect of the extract was by apoptosis. The mechanism by which the extract induced apoptosis was further elucidated by measuring the activity of caspases. The increased activity of caspase-9 and caspase-7 suggested that the intrinsic apoptotic pathway is responsible for the antiproliferative effect of *G. heyneanum* var. *heyneanum* on MCF-7 cells. This was further substantiated by monitoring the expression of apoptosis related genes p53 and p21 by RT-qPCR which displayed an up-regulation in the expressions of both genes. Cells often respond to DNA damage by activating the p53-p21 pathway and halting their cell cycle progression in the G0/G1 phase. The genotoxic stress caused by the plant extract might have activated the expression of p53 gene which caused the activation of p21 to bring about cell cycle arrest in G0/G1 phase. The irreparable DNA damage might have triggered the apoptotic function of p53 leading to the recruitment of initiator caspase-9 followed by the activation of the downstream caspase-7 which finally culminated in apoptotic cell death.

Green synthesis of silver nanoparticles and their characterization

Green synthesis of silver nanoparticles with selected species of *Gomphostemma* was attempted and characterized by UV-Vis spectral analysis and FE-SEM/EDX analysis. All the three plants selected for the study effectively synthesised silver nanoparticles as visualized by the change in the colour of reaction mixtures from pale green to yellowish brown or dark brown with slight variations in intensity. The UV-Vis spectral analysis showed a strong, broad absorption band in the range of 400–500 nm, which is characteristic for silver nanoparticles. For *G. heyneanum* var. *heyneanum* maximum absorbance was centered at 437 nm, for *G. heyneanum* var. *rottleri* it was at 435 nm and for *G. eriocarpum*, it was located at 448.5 nm. SEM images showed that the AgNPs formed were nearly spherical in shape. The

average size of the silver nanoparticles synthesised by *G. heyneanum* var. *heyneanum* was 24.46–38.67 nm. The silver nanoparticles by *G. heyneanum* var. *rottleri* were slightly smaller with size in the range of 23.93–34.18 nm and that synthesized by *G. eriocarpum* were the largest among the three taxa of *Gomphostemma* analysed with the size in the range of 59.97–65.23 nm. Further, the EDX analysis confirmed the presence of silver at approximately 3 keV which is typical of metallic silver nanocrystals.

Conclusion and recommendations

In conclusion, the phytochemical and bioactivity screening of selected species of *Gomphostemma* from South Western Ghats revealed the medicinal attributes of these underutilised taxa in Lamiaceae. These plants were found to be rich in a variety of phytochemicals and showed modest antioxidant activity. Among the taxa evaluated, *G. heyneanum* var. *heyneanum* showed moderate anti-inflammatory potential and effective antiproliferative property. The plants demonstrated efficient synthesis of silver nanoparticles, which is admirable in the panorama of green technology. The collaborative interaction of diverse phytochemicals may be credited for the bioactive potential exhibited by these plant species. As a future lead, *in vivo* and clinical studies are suggested on animal models to attest the anti-inflammatory and antiproliferative activity. Identification, isolation and structural elucidation of compounds responsible for the bioactivities observed are recommended. An extended study on the green synthesis of silver nanoparticles and their bioactivity screening is also proposed.

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Appendix 1

Acetocarmine

Carmine – 2g

45% Acetic acid – 100 mL

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

Appendix 2

Blocking buffer

Gelatin – 0.2%

Tween 20 – 0.005%

The ingredients are freshly prepared in PBS.

Appendix 3

DMEM Medium

Sodium bicarbonate – 1.85 g

HEPES – 2.95 g

DMEM powder – 1 packet

Distilled water – 1 L

Vacuum sterilized and stored at 4 °C.

Appendix 4

Electrophoresis Buffer (300 mM NaOH / 1 mM EDTA)

Stock solutions:

10 N NaOH – 200 g/500 mL distilled water

200 mM EDTA– 14.89 g/200 mL distilled water

pH – 10

Store the stock solutions at room temperature.

For 1X Buffer (made fresh before each electrophoresis run) add 30 mL NaOH and 5 mL EDTA, per 1L and mix well. Ensure pH as >13 prior to use.

Appendix 5

Ethidium bromide stain

Ethidium bromide – 20 µg/mL

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

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

Appendix 6

Evans Blue (0.25%)

Evans Blue – 0.25 g

Distilled water – 100 mL

Appendix 7

Griess Reagent

Naphthylethylenediamine HCl – 0.1% in distilled water

Sulfanilamide – 1% in 5% H₃PO₄

Mix both in 1:1 ratio.

Appendix 8

HEPES Buffer

NaCl – 6.72 g

CaCl₂ – 133 mg

MgCl₂ – 114 mg

K₂HPO₄ – 418 mg

HEPES – 4.77 g

All ingredients dissolved in distilled water and made up to 1L.

Appendix 9

Lysing Solution (1000 mL)

2.5 M NaCl – 146.1 gm

100 mM EDTA – 37.2 gm

10 mM Trizma base – 1.2 gm

1%SDS – 10gm

Add ingredients to about 700 mL of distilled water and stir the mixture. Add 8 gm NaOH and allow the mixture to dissolve for about 20 min. Then, adjust the pH to 10.0 using concentrated HCl or NaOH and store at room temperature. To this mixture, 10% DMSO and 1% Triton X 100 is added prior to use.

Appendix 10

Mayer's reagent

Mercuric chloride – 1.36 g

Potassium iodide – 5 g

Distilled water – 100 mL

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

Appendix 11

Modified carnoy's fluid

Acetic acid – 10 ml

Ethanol – 30 mL

Appendix 12

Neutralization Buffer

0.4 M Tris – 48.5 gm

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

Appendix 13

Phosphate buffer saline (PBS)

NaCl – 8 g

KCl – 0.2 g

Na₂HPO₄ – 1.44 g

KH_2PO_4 – 0.24 g

Distilled water – 1 L

pH – 7.4

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

Appendix 14

TAE buffer (50x)

Tris base – 242 g

EDTA – 18.61 g

Glacial Acetic Acid – 57.1 mL

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

Appendix 15

Trim-Hill reagent

Acetic acid – 10 mL

Copper sulphate – 0.2%

Con. HCl – 0.5 mL

To prepare the reagent 10 mL of acetic acid is mixed with 1 mL of 0.2% copper sulphate followed by the addition of 0.5 mL of concentrated hydrochloric acid.

Appendix 16

Tris-EDTA buffer

Tris-HCl – 10 mM

EDTA – 1 mM

Appendix 17

Tris-HCl buffer

Tris base – 242.2 g

HCl – 80 - 85 mL

Appendix 18

Wagner's reagent

Iodine – 2 g

Potassium iodide – 6 g

Distilled water – 100 mL

2 g of iodine and 6 g of potassium iodide was dissolved in 100 mL of distilled water to prepare Wagner's reagent.

Research Publications

1. Sajitha, M. K., & Thoppil, J. E. (2018). Phytochemical evaluation and *in vitro* antioxidant studies of selected species of *Gomphostemma* Wall. ex Benth. from Western Ghats. *Journal of Drug Delivery and Therapeutics*, 8(6), 32-37.
2. Sajitha, M. K., & Thoppil, J. E. (2019). Screening selected species of *Gomphostemma* Wall. ex Benth. from Western Ghats for anti-inflammatory activity. *International Journal of Pharmaceutical Sciences and Research*, 10(4), 2012-2017.

Paper Presentations

1. Sajitha M. K., & Thoppil, J. E. (2017). Identification of bioactive compounds in *Gomphostemma heyneanum* Wall. ex Benth. var. *heyneanum* by GC-MS analysis. Poster presented in the third international conference on Frontiers of Mass Spectrometry organised by Inter University Instrumentation Centre and School of Environmental Sciences, M. G. University, Kottayam, Kerala from 11th to 14th December 2017.
2. Sajitha M. K., & Thoppil, J. E. (2018). Phytochemical profiling and antioxidative potential of *Gomphostemma heyneanum* Wall. ex Benth. var. *rottleri* Prain. Paper presented in ICPM – 2018 at Bharathiar University, Coimbatore, Tamil Nadu from 29th to 31st August 2018.
3. Sajitha M. K., & Thoppil, J. E. (2019). Phytochemical screening and cytotoxicity potential of *Gomphostemma eriocarpum* Benth. Paper presented in the MESMAC international conference organised by MES Mampad College, Mampad, Malappuram, Kerala from 15th to 17th January 2019.

Fig. 8: Standard calibration curve of gallic acid for the determination of total phenolic content

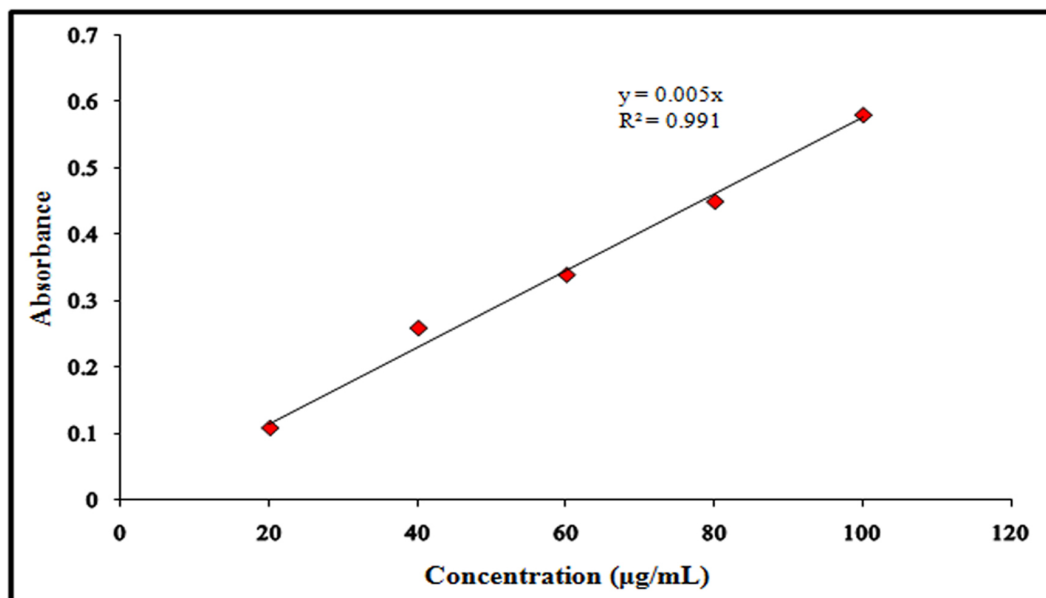
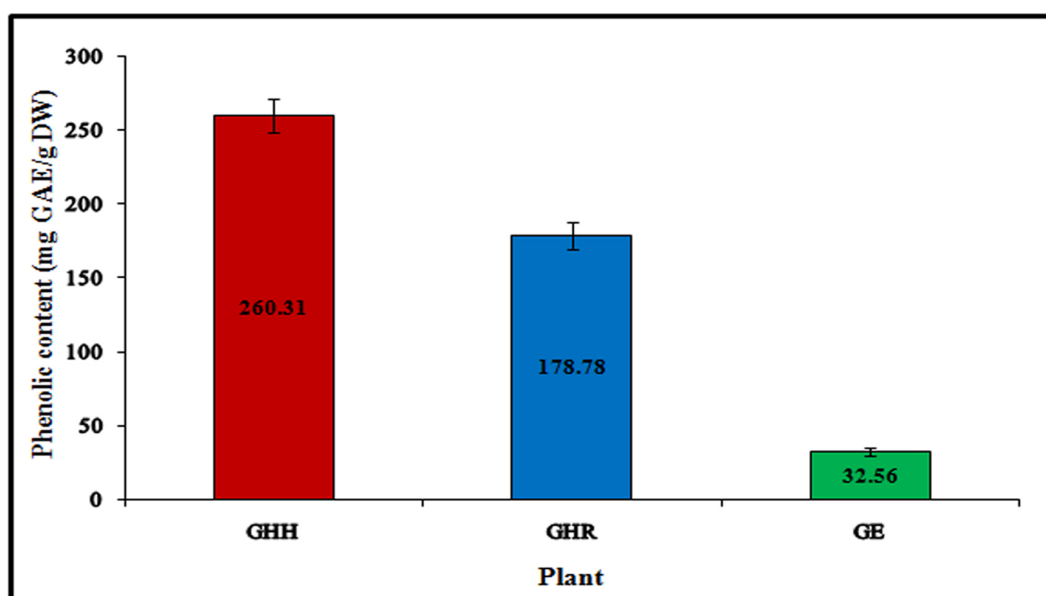


Fig. 9: Quantitative estimation of total phenolic content in selected species of *Gomphostemma*



GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*

Fig. 10: Standard calibration curve of quercetin for the determination of total flavonoid content

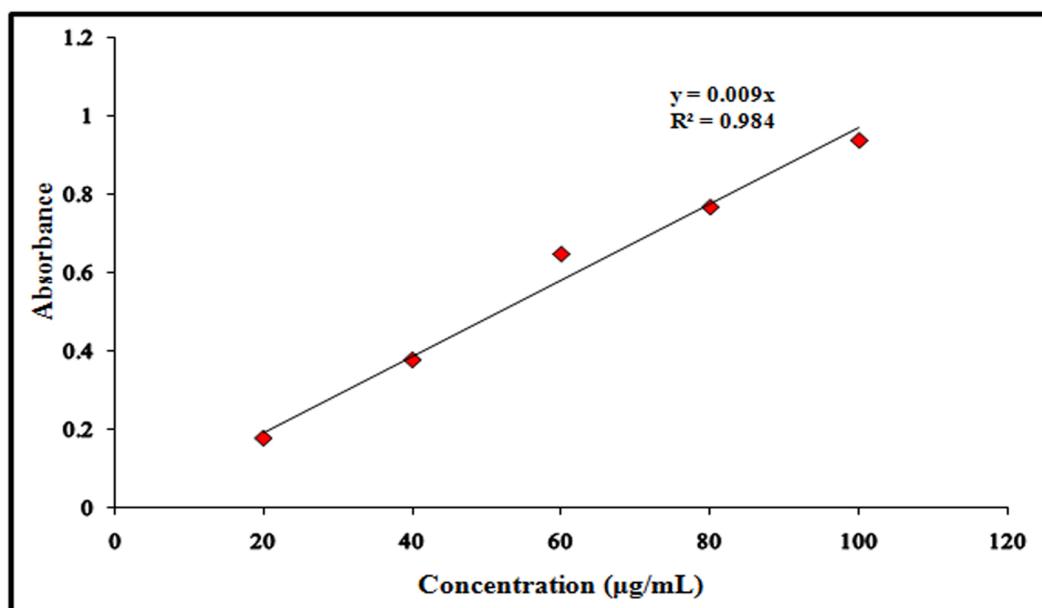
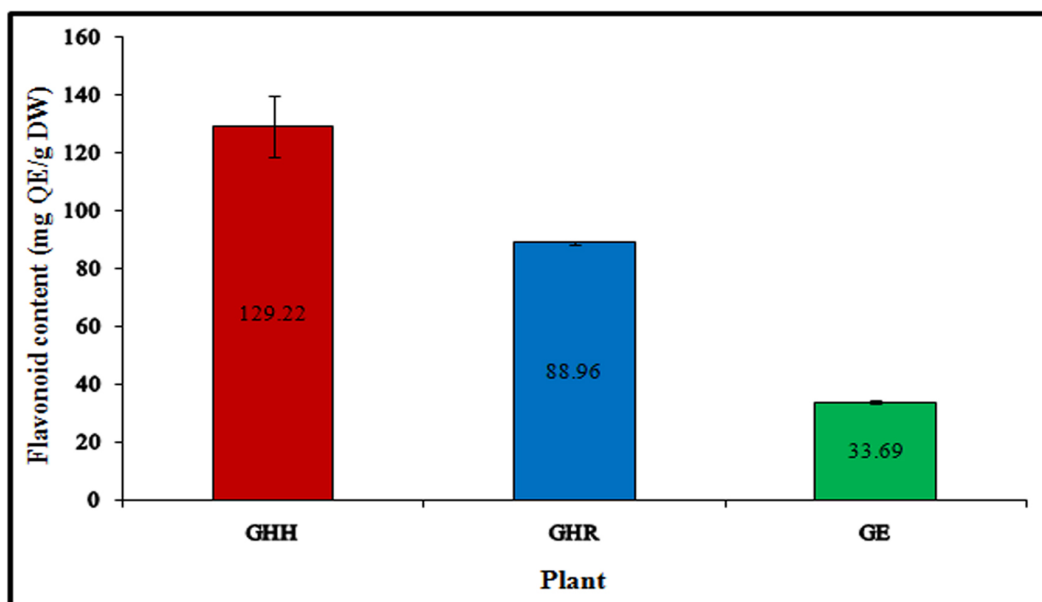


Fig. 11: Quantitative estimation of total flavonoid content in selected species of *Gomphostemma*



GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*

Fig. 12: Standard calibration curve of linalool for the determination of total terpenoid content

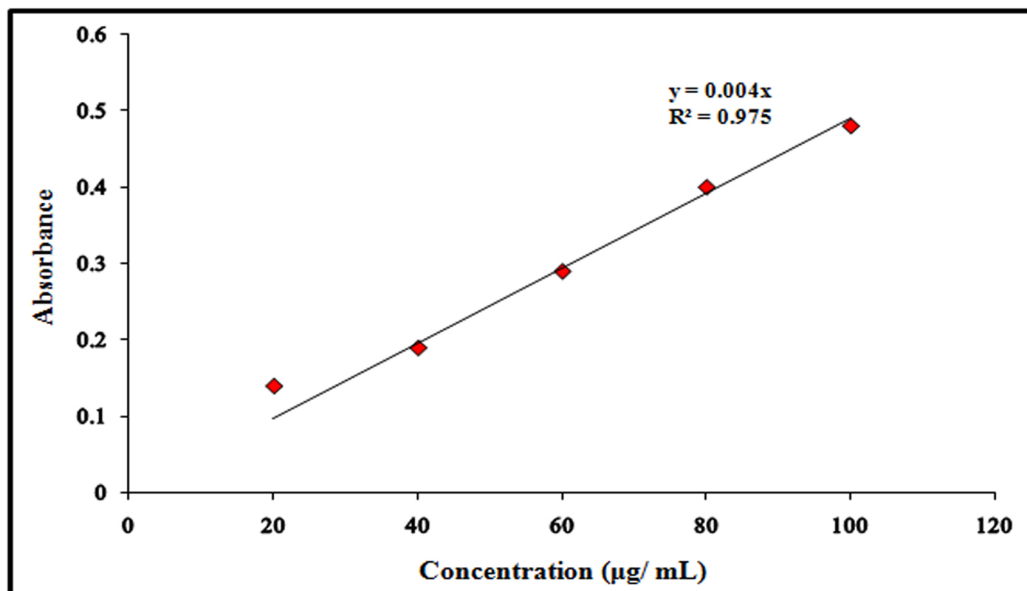
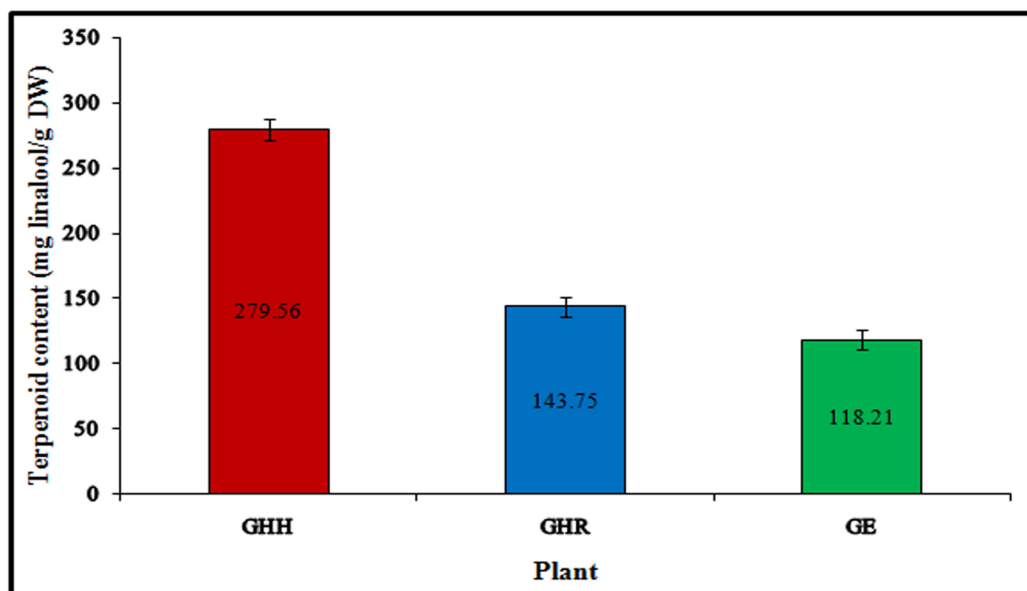


Fig. 13: Quantitative estimation of total terpenoid content in selected species of *Gomphostemma*



GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*

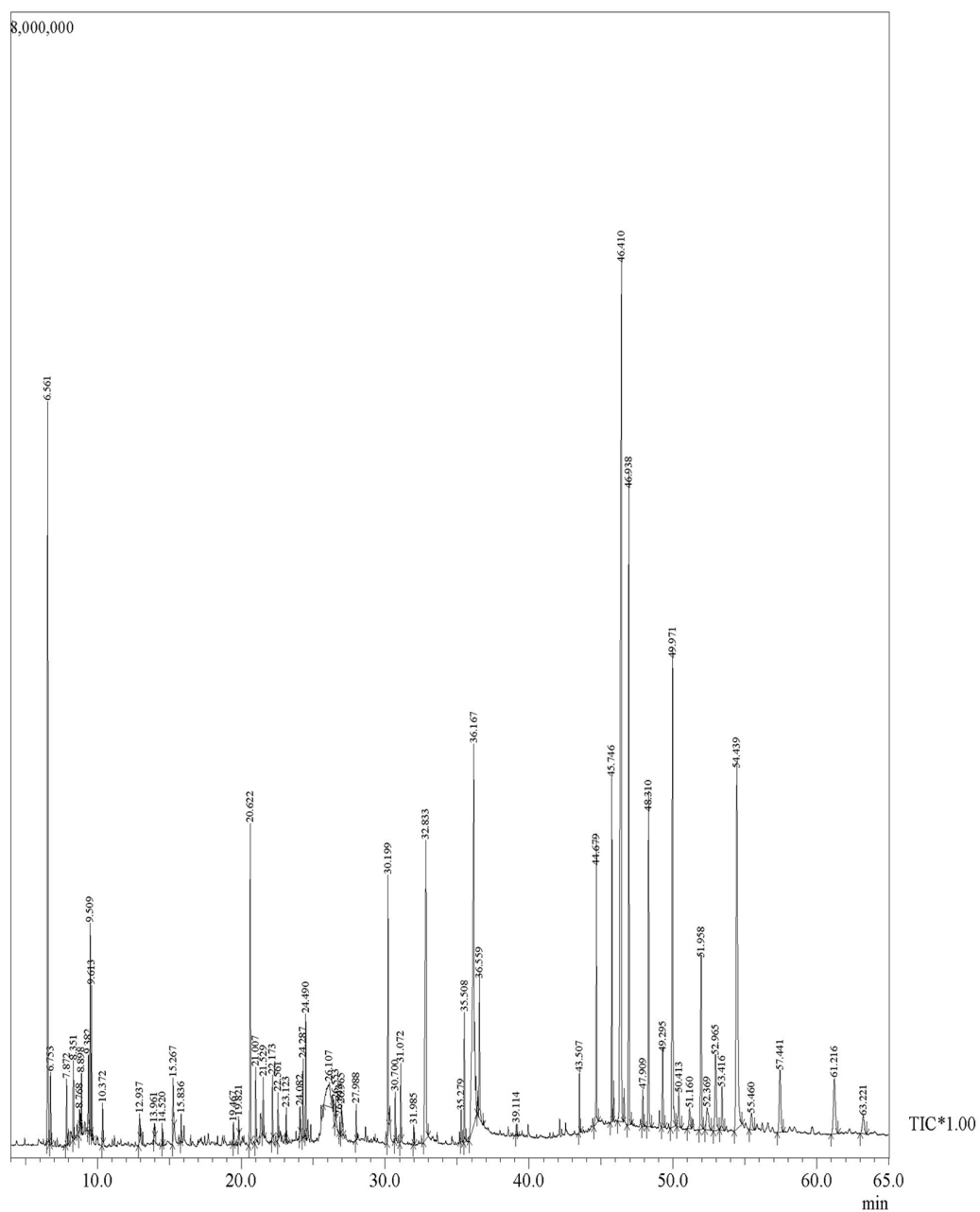


Fig. 14: Gas chromatogram of methanolic extract of *G. heyneanum* var. *heyneanum*

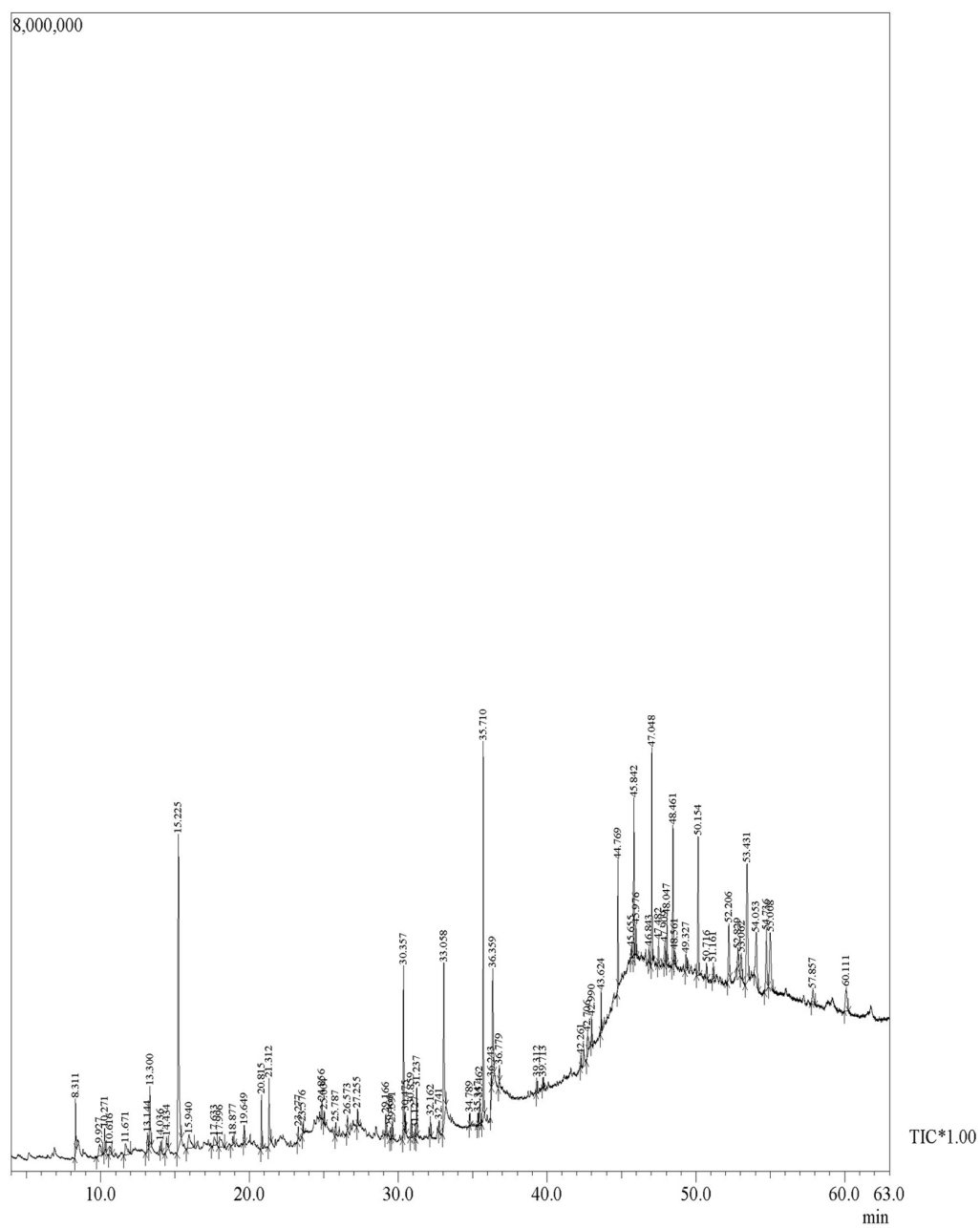


Fig. 15: Gas chromatogram of methanolic extract of *G. heyneanum* var. *rottileri*

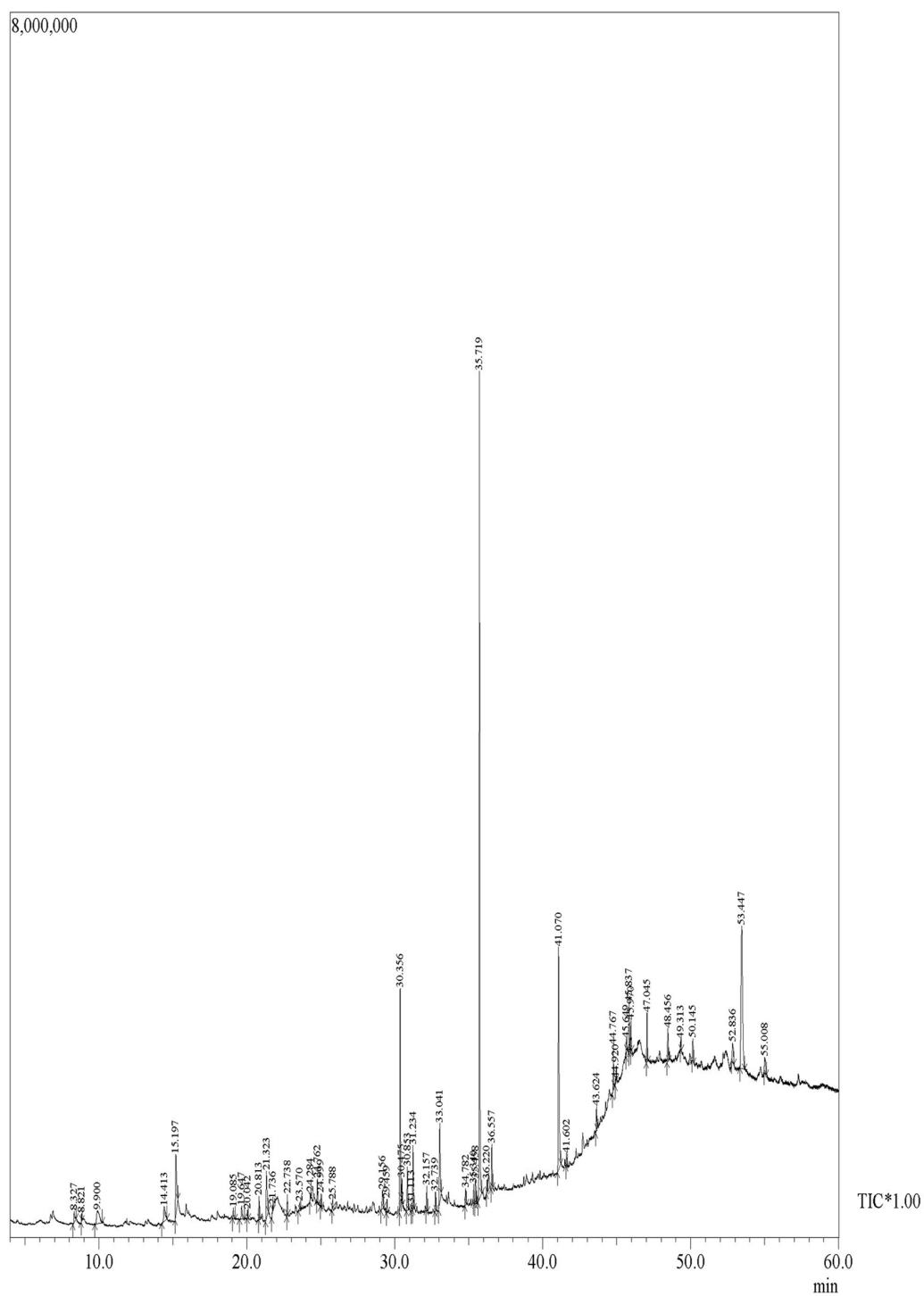


Fig. 16: Gas chromatogram of methanolic extract of *G. eriocarpum*

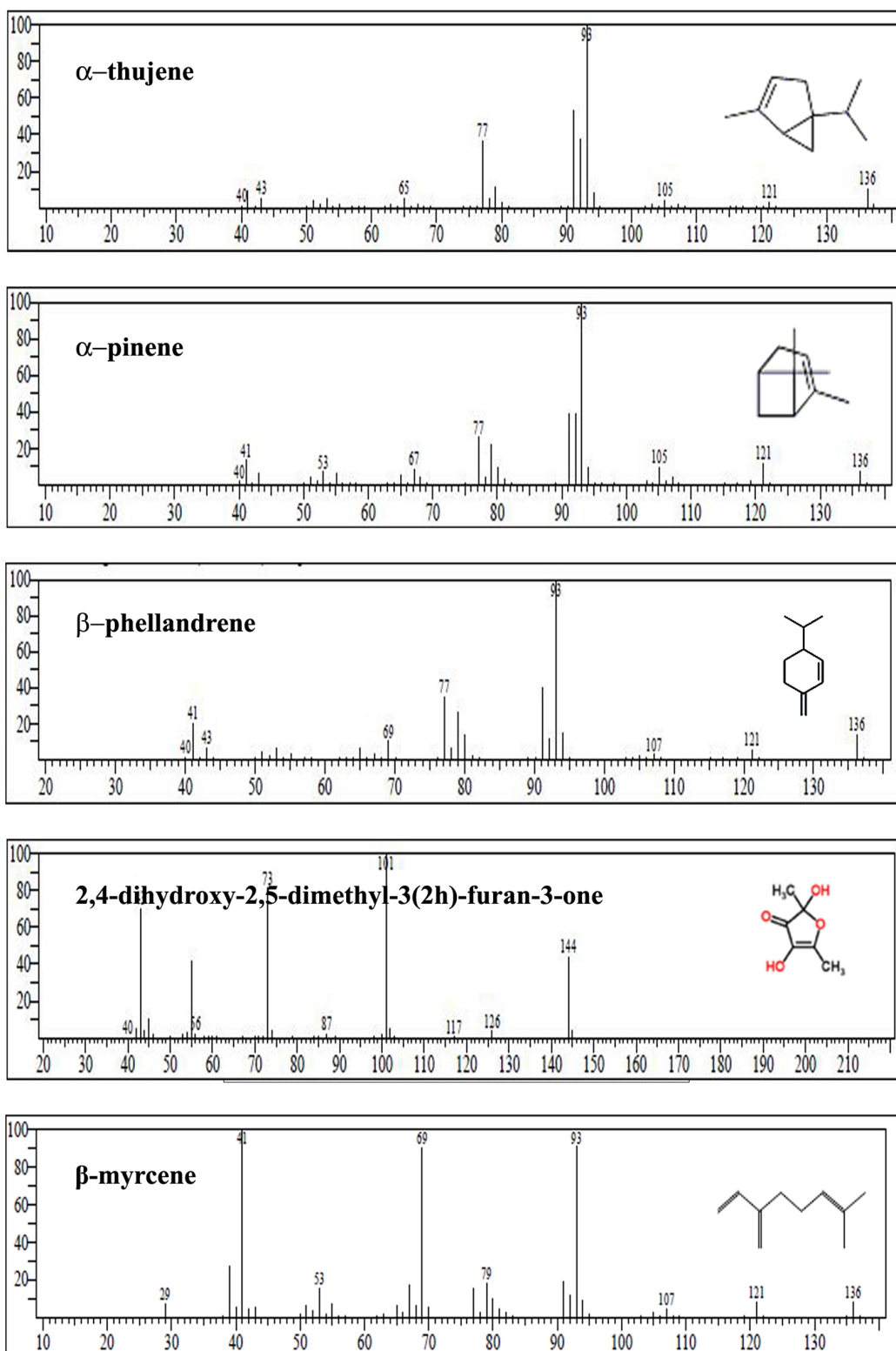


Fig. 17 (i): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

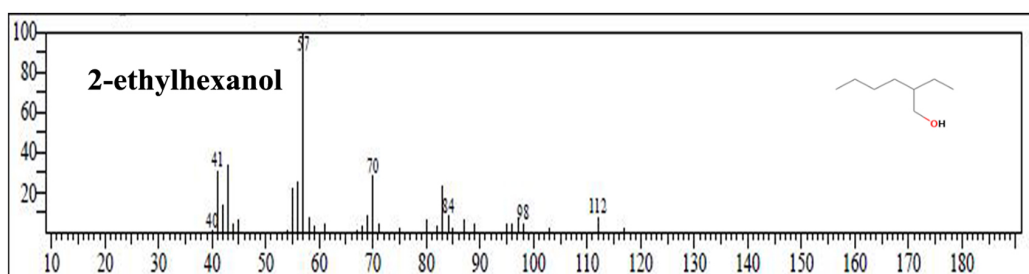
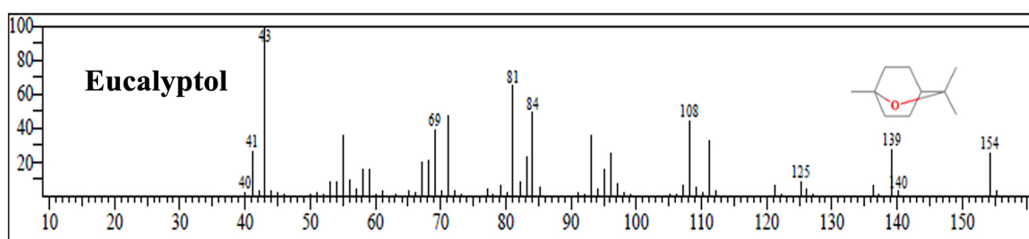
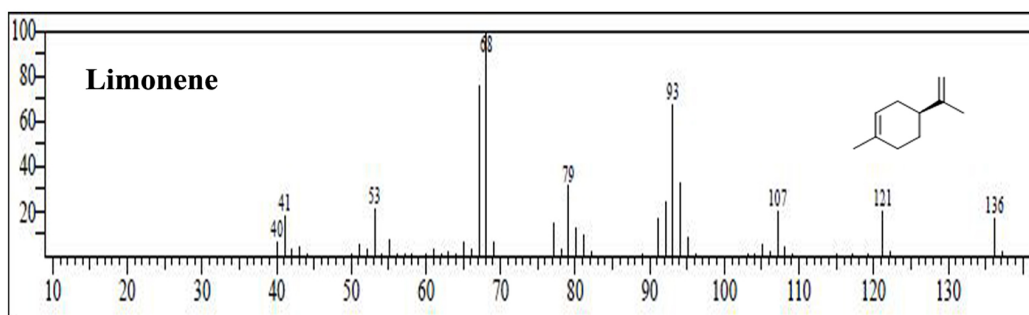
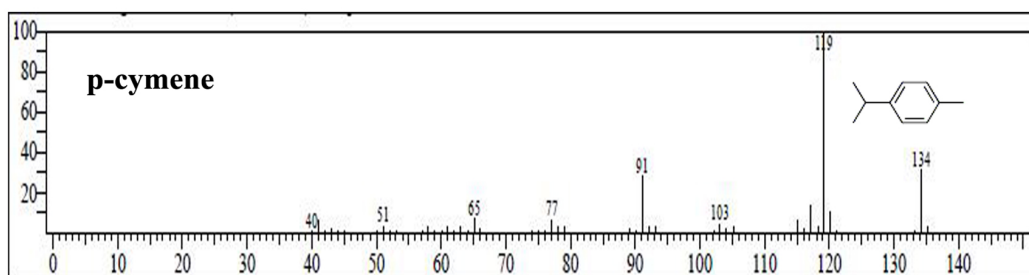
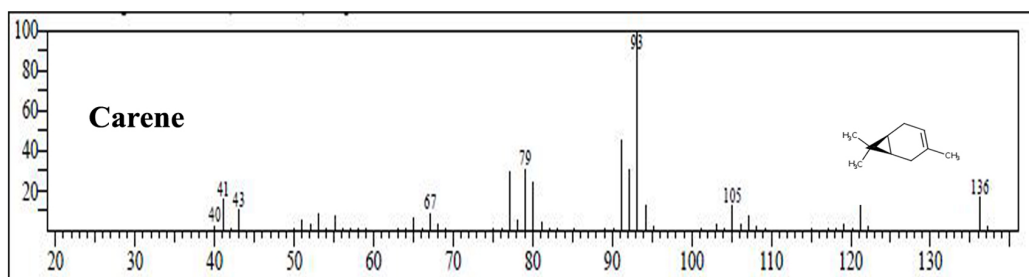


Fig. 17 (ii): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

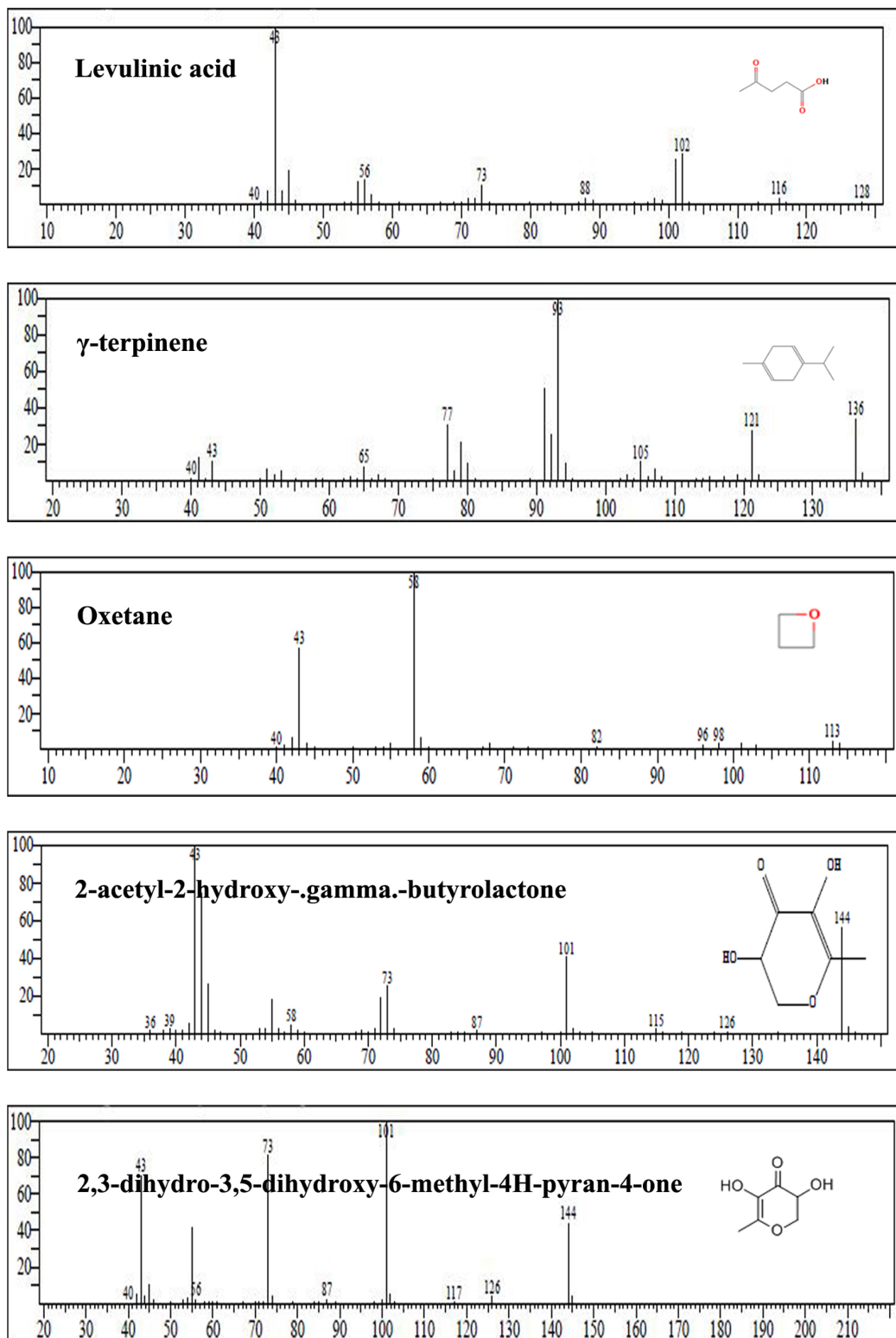


Fig. 17 (iii): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

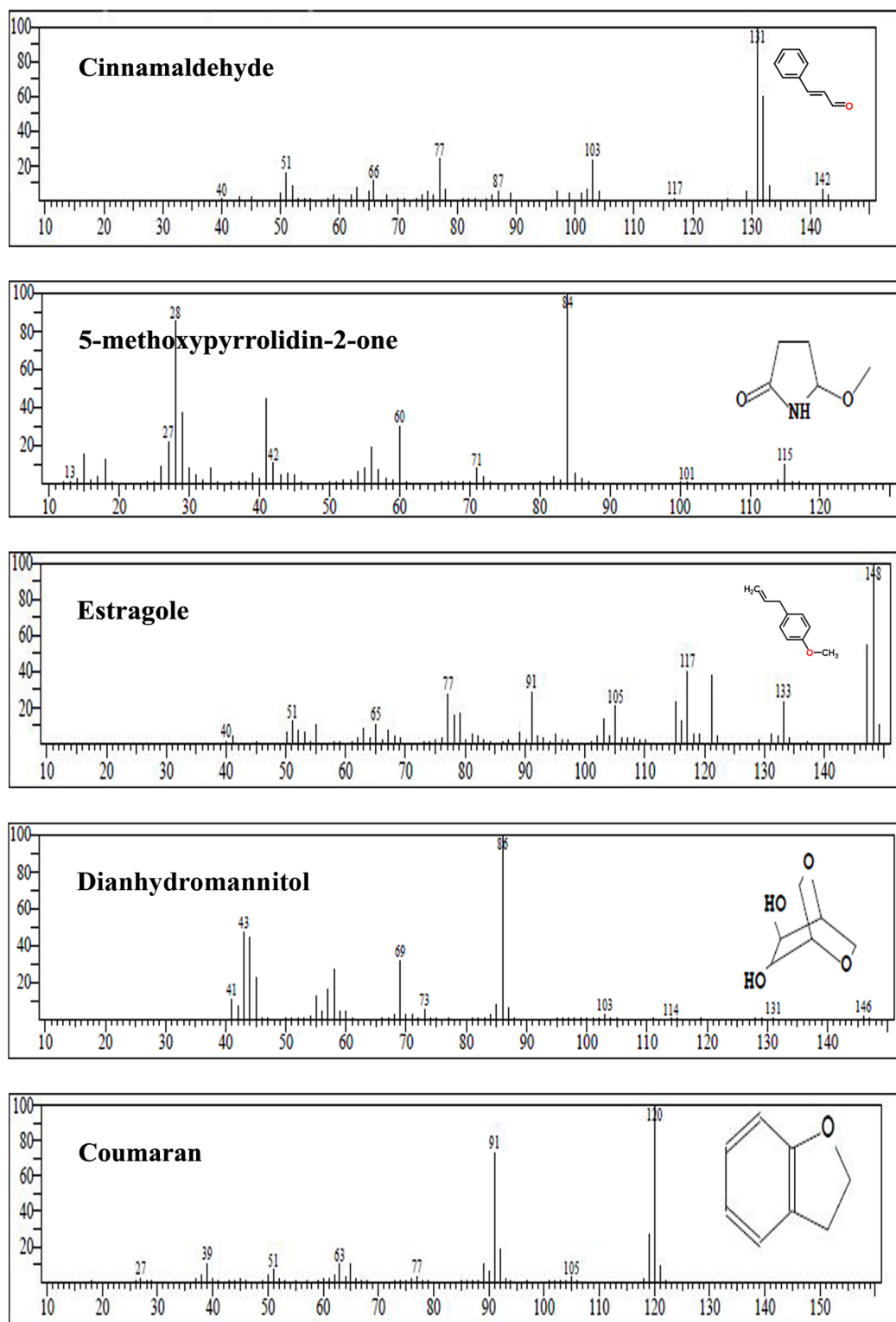


Fig. 17 (iv): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

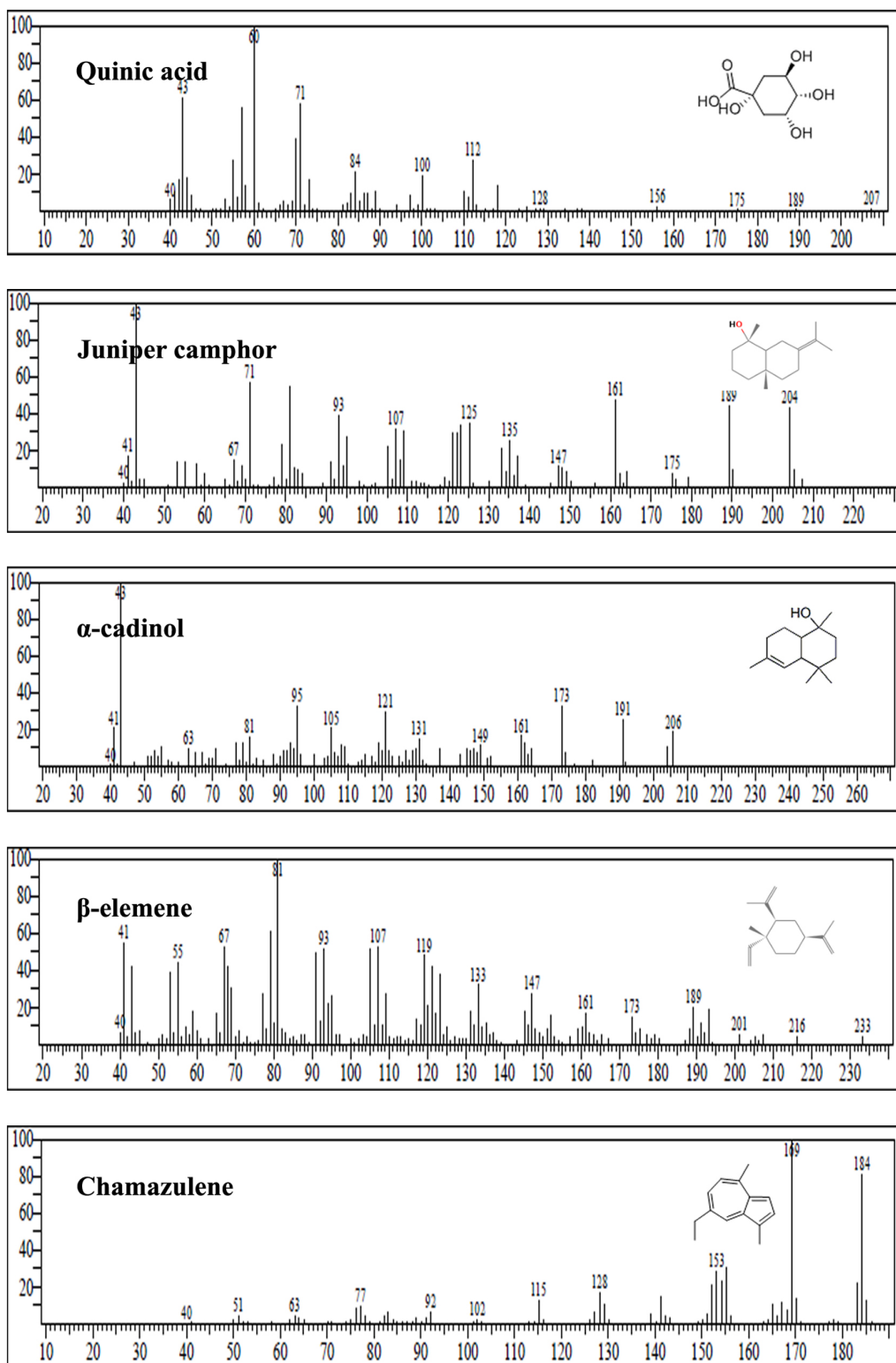


Fig. 17 (ix): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

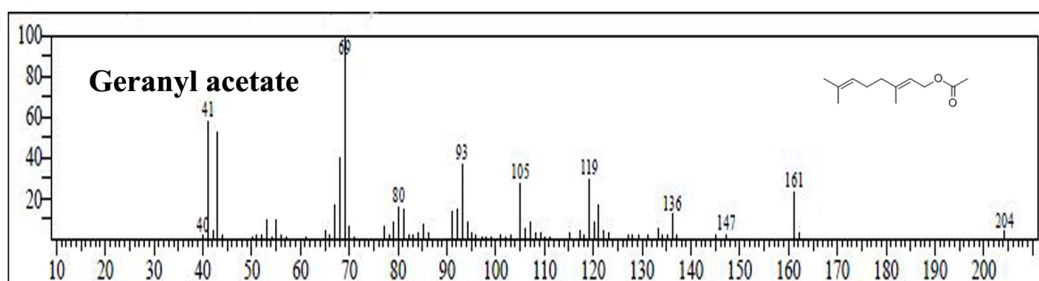
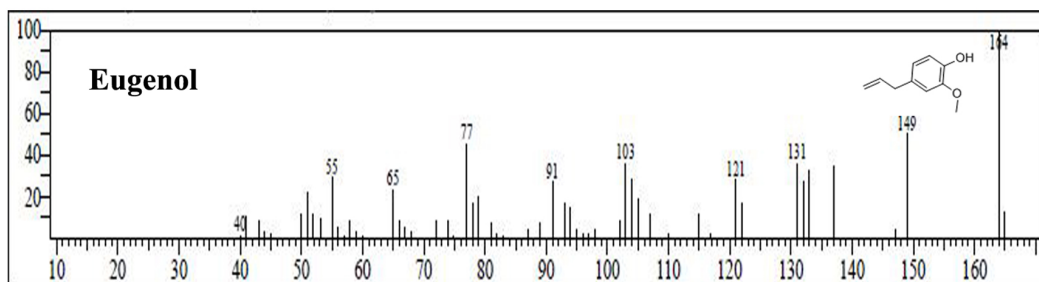
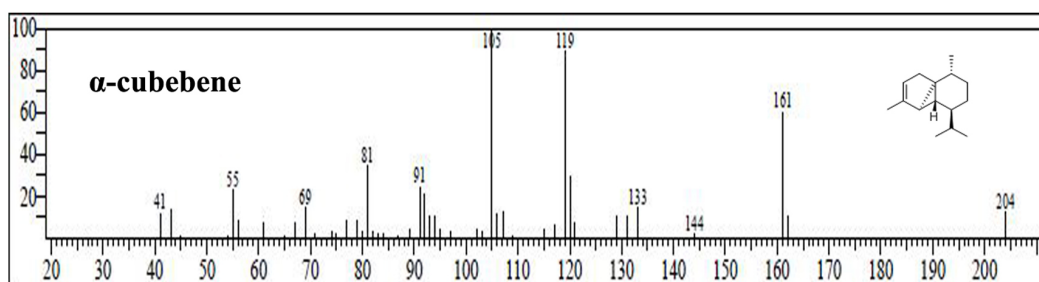
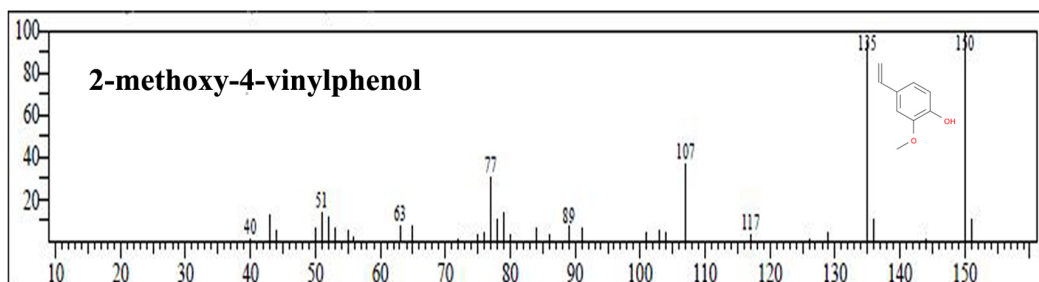
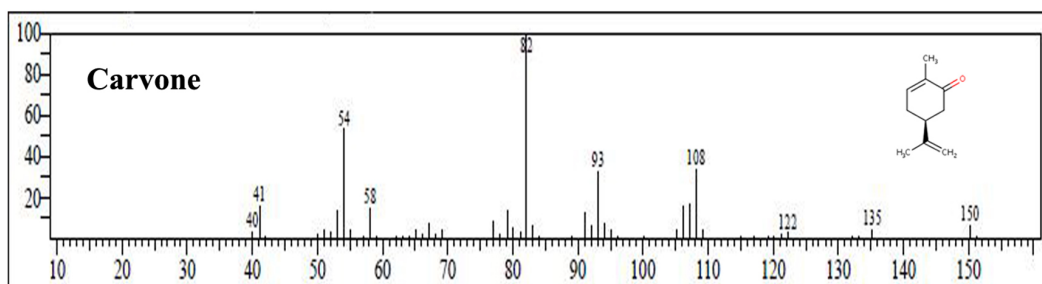


Fig. 17 (v): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

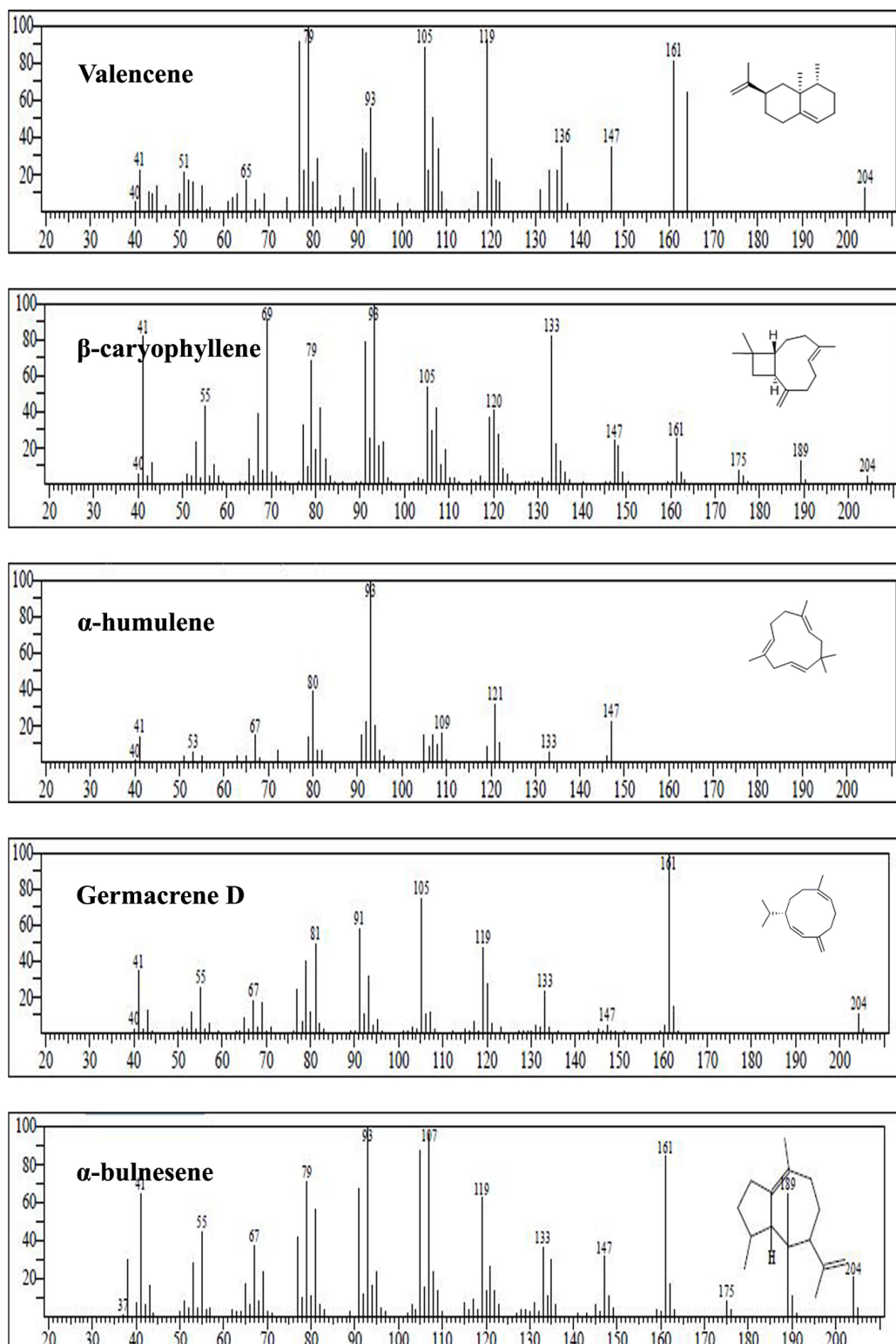


Fig. 17 (vi): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

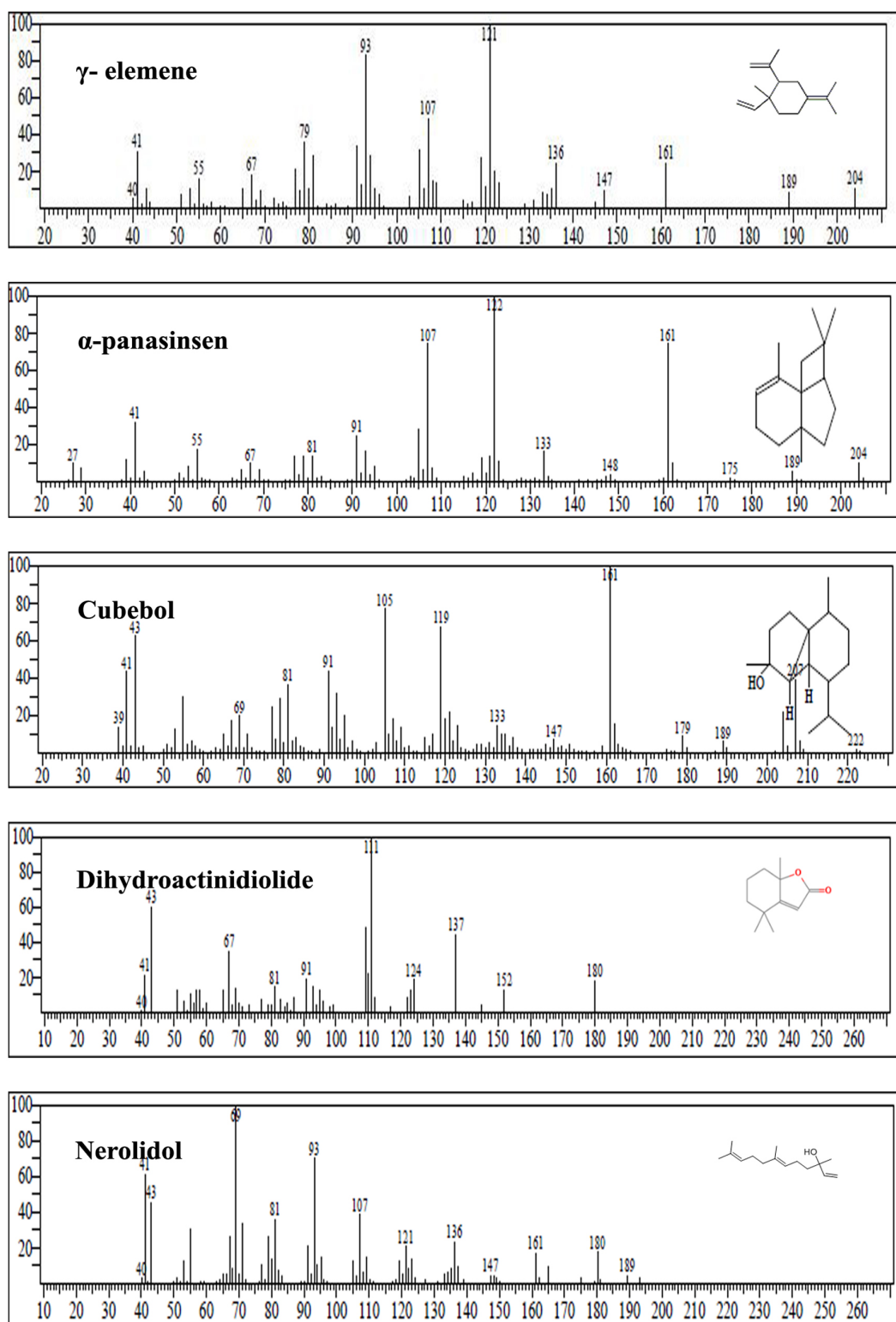


Fig. 17 (vii): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

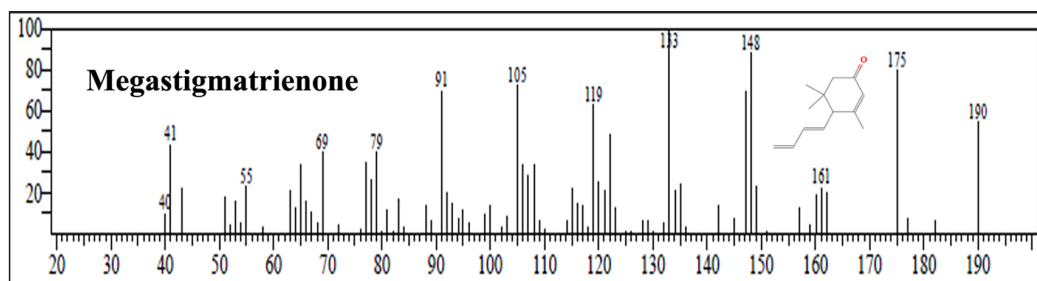
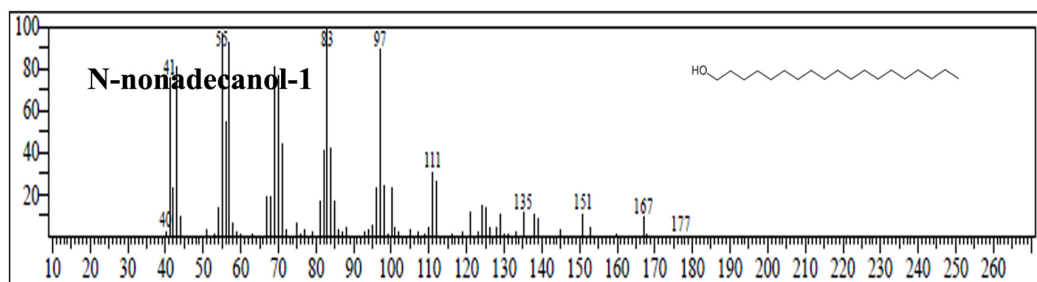
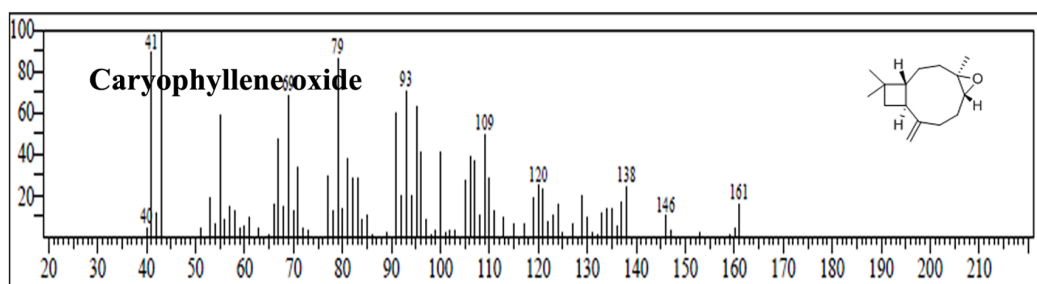
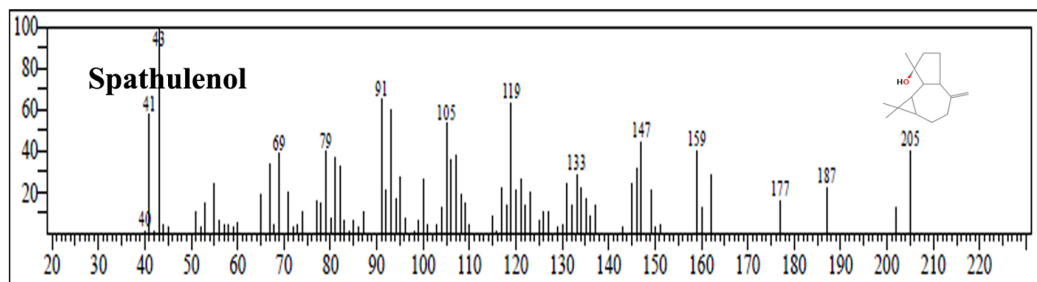
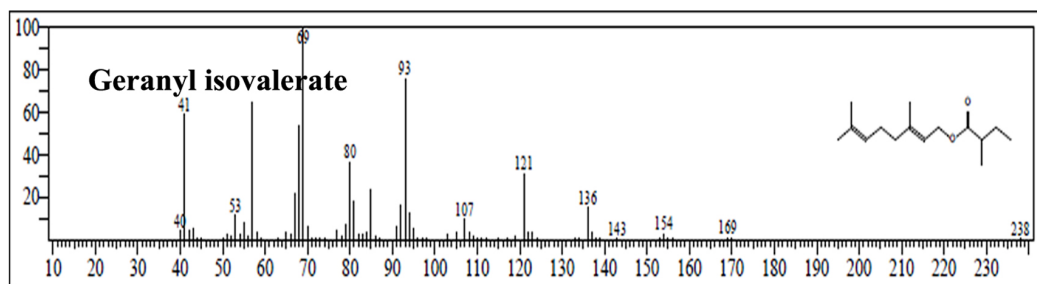


Fig. 17 (viii): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

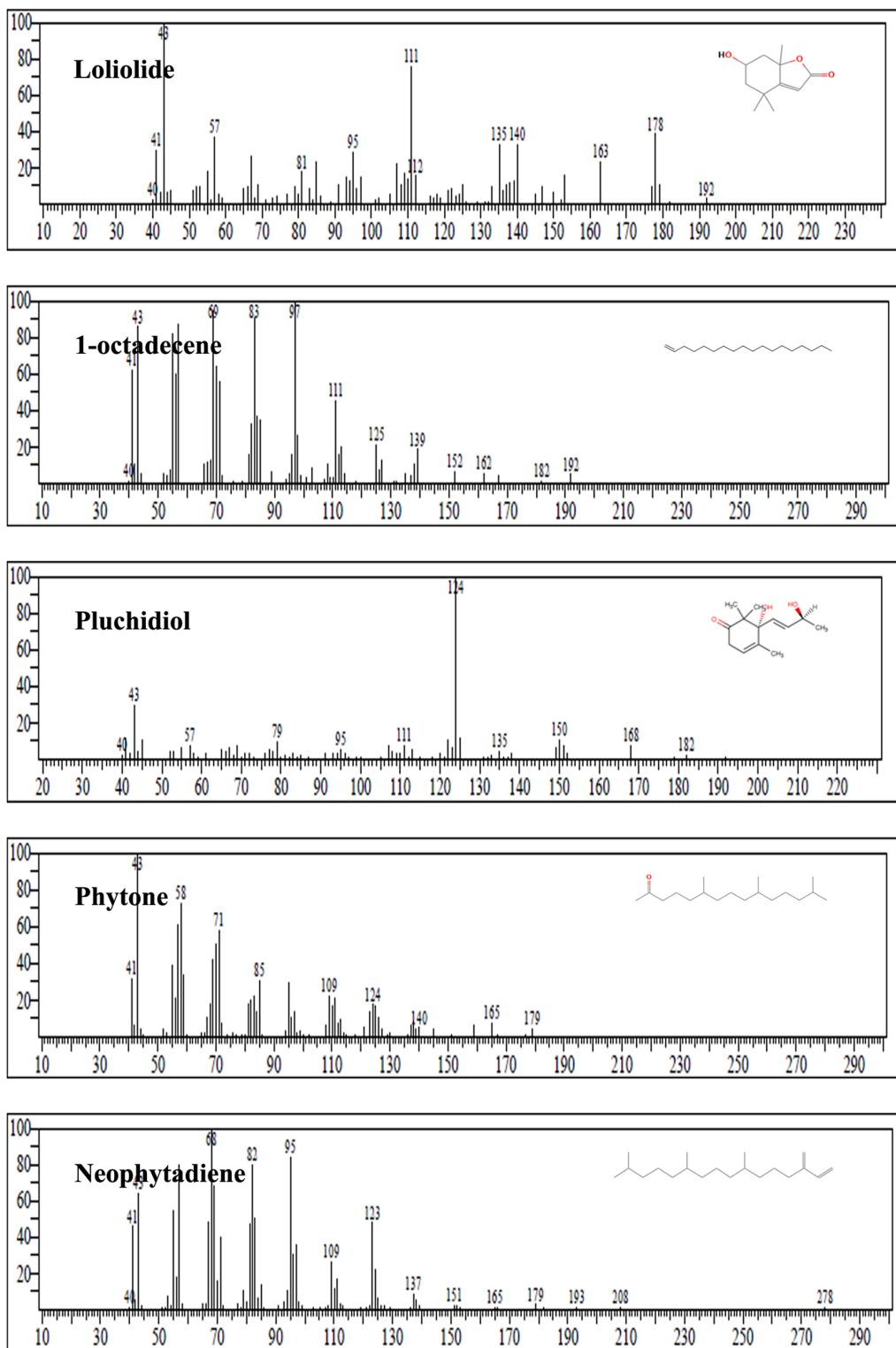


Fig. 17 (x): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

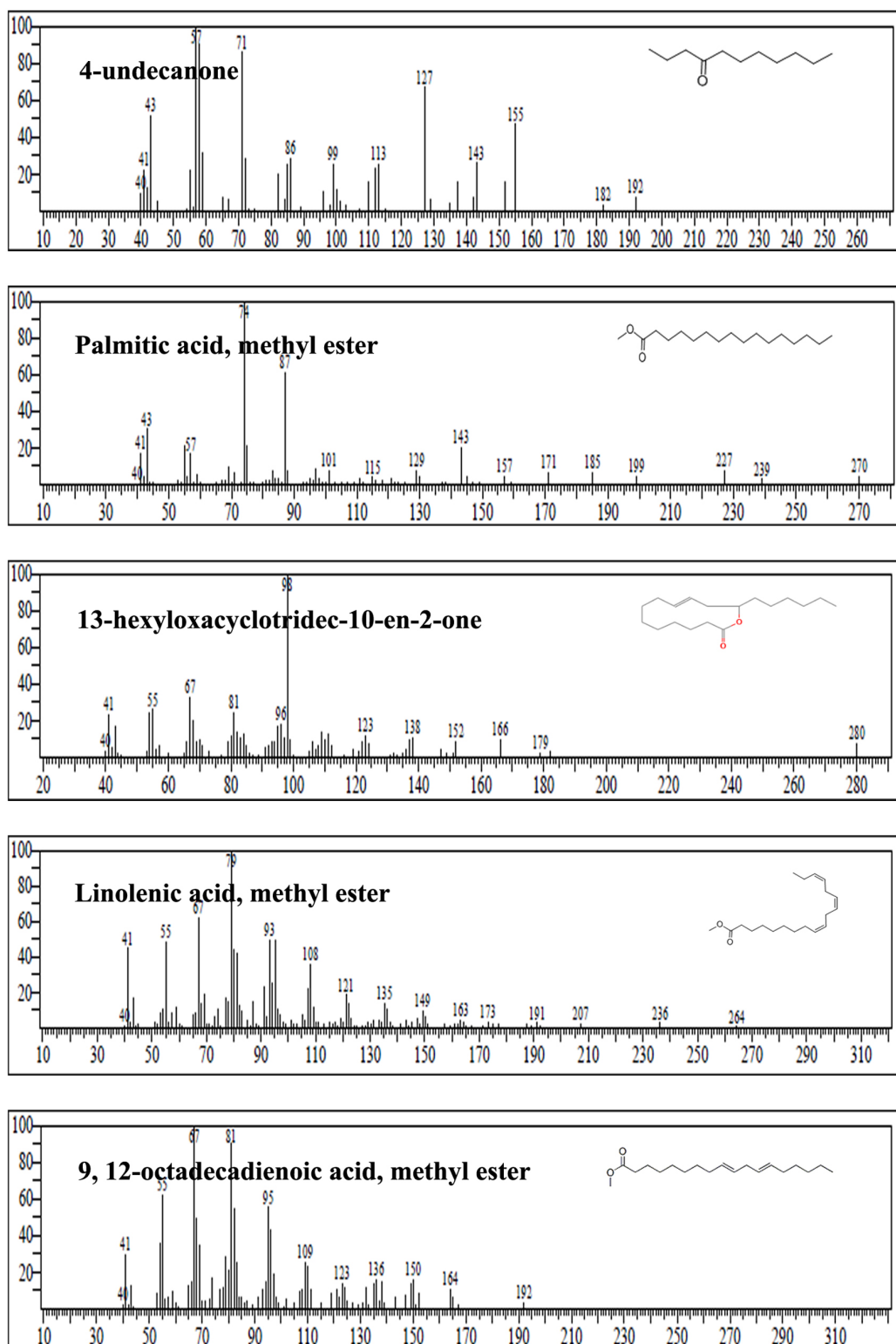


Fig. 17 (xi): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

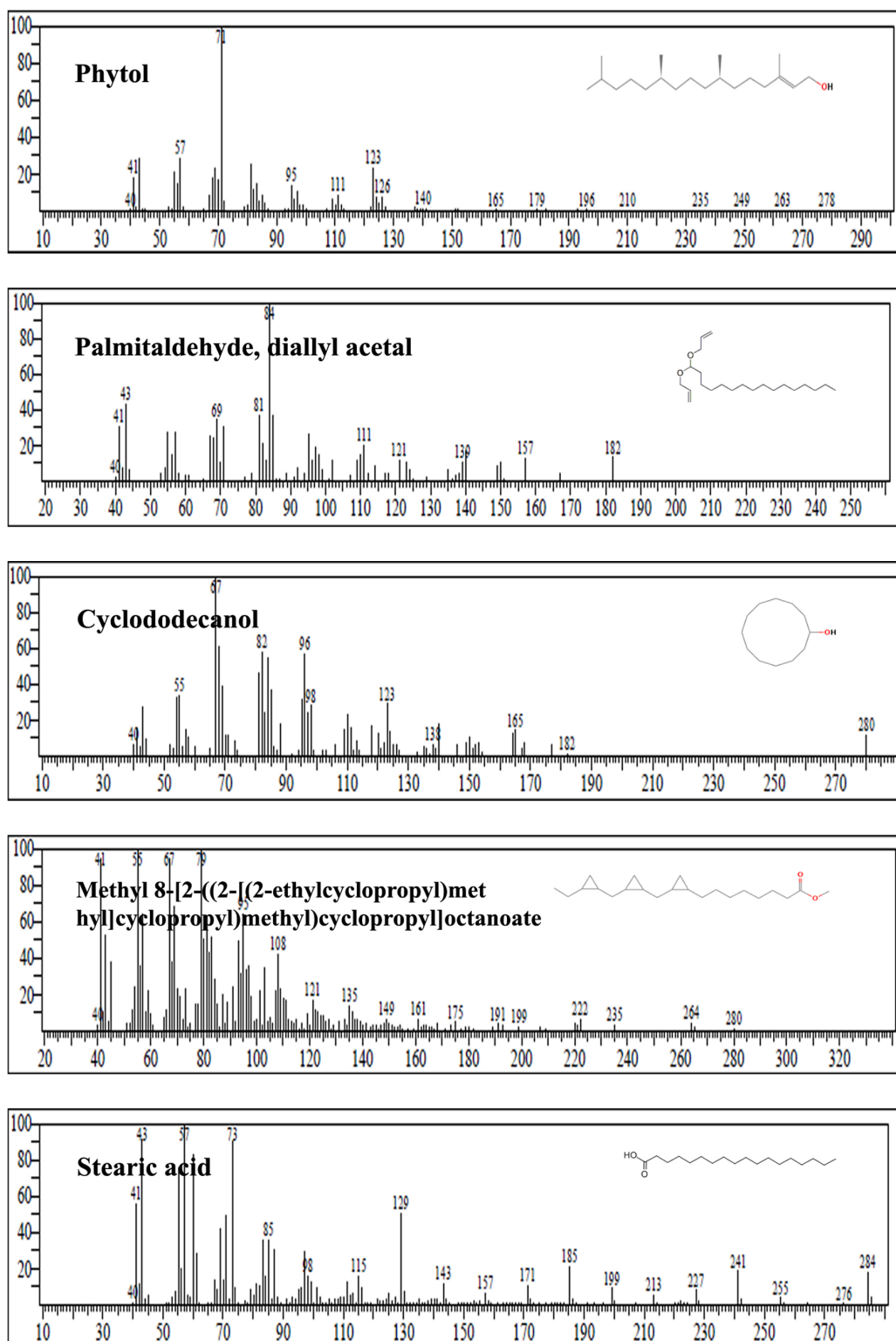


Fig. 17 (xii): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

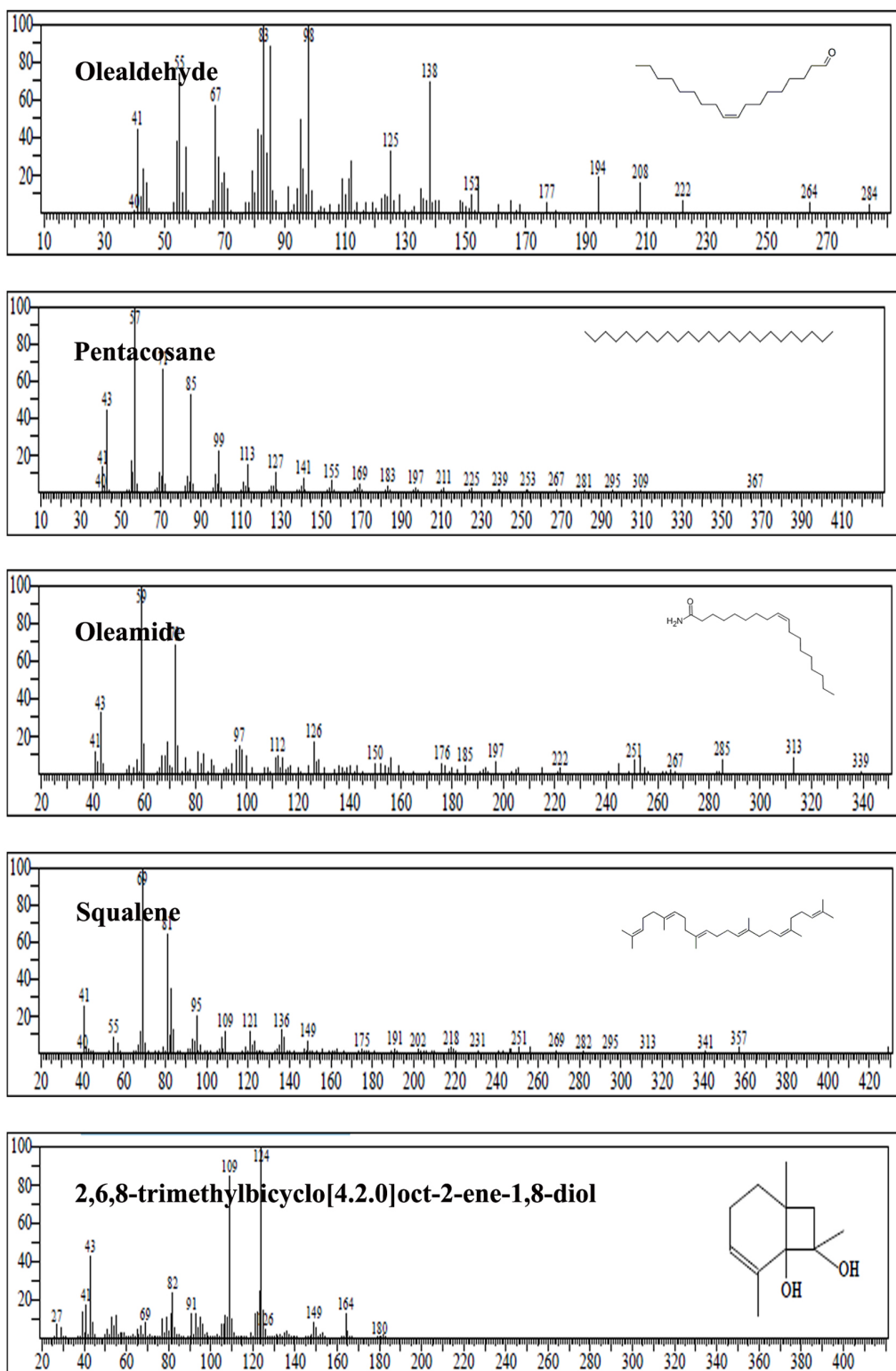


Fig. 17 (xiii): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

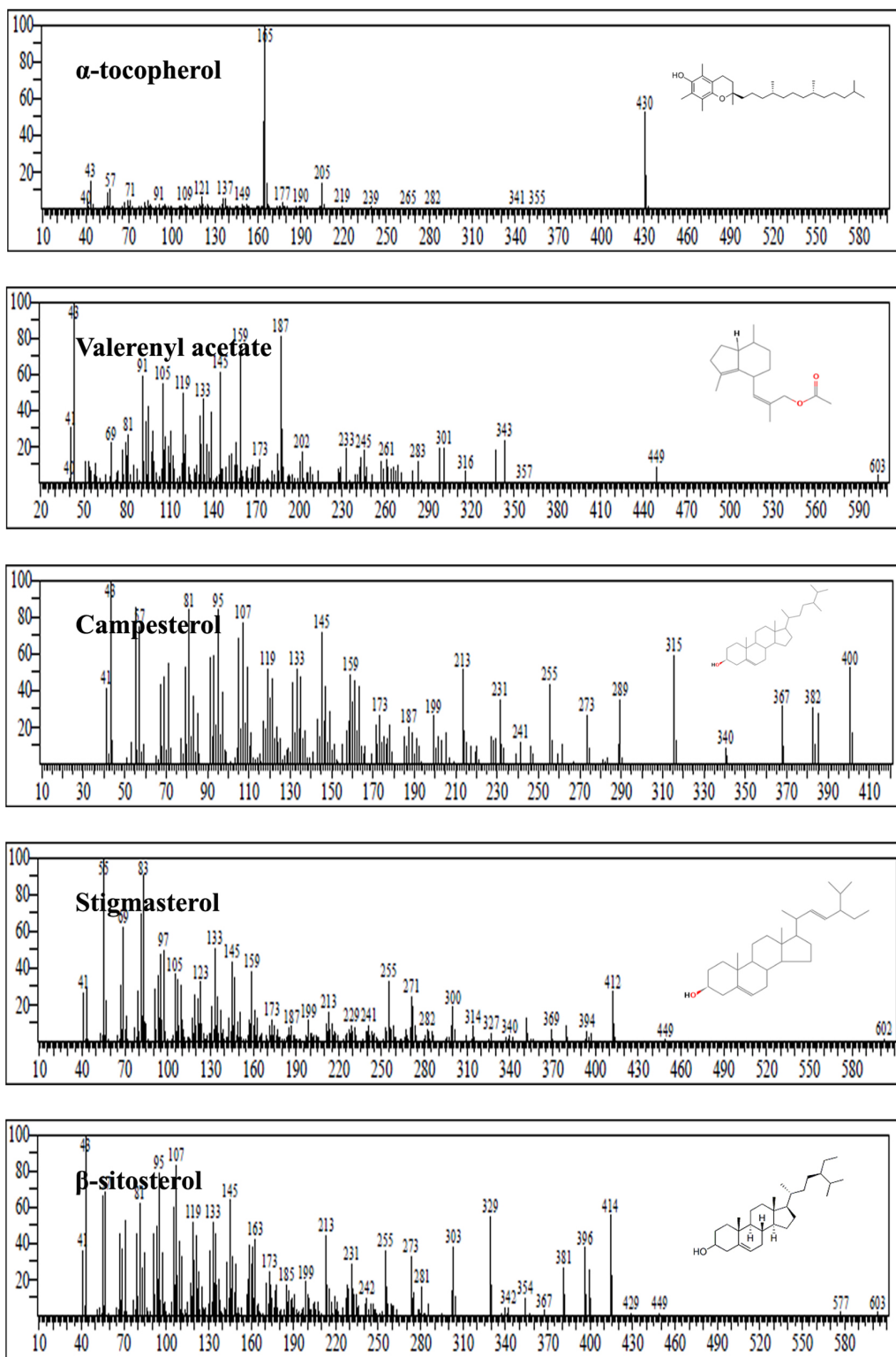


Fig. 17 (xiv): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

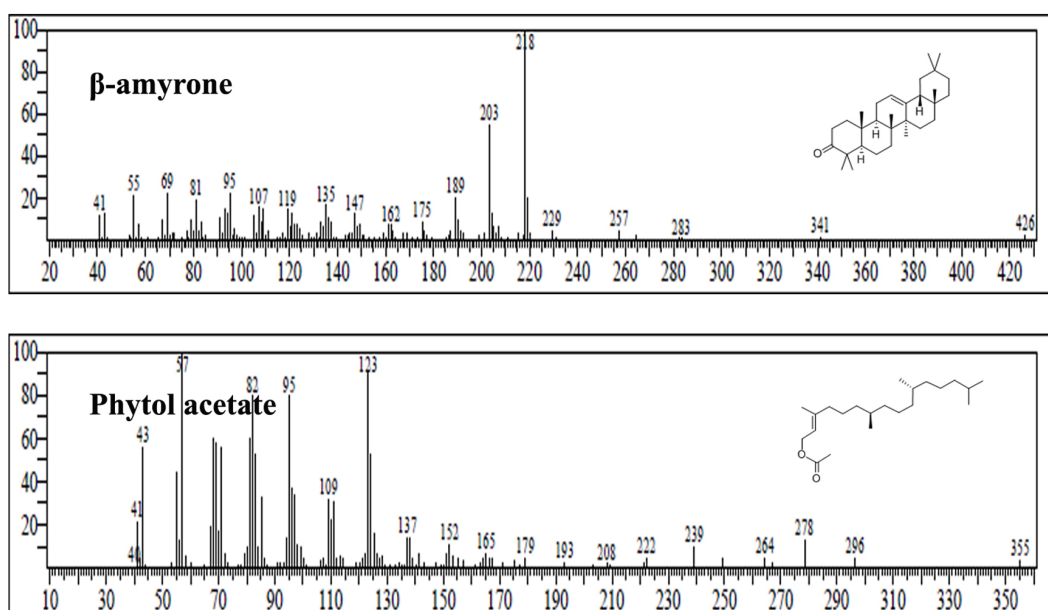


Fig. 17 (xv): Mass spectra of compounds detected by GC/MS in the methanolic extract of selected species of *Gomphostemma*

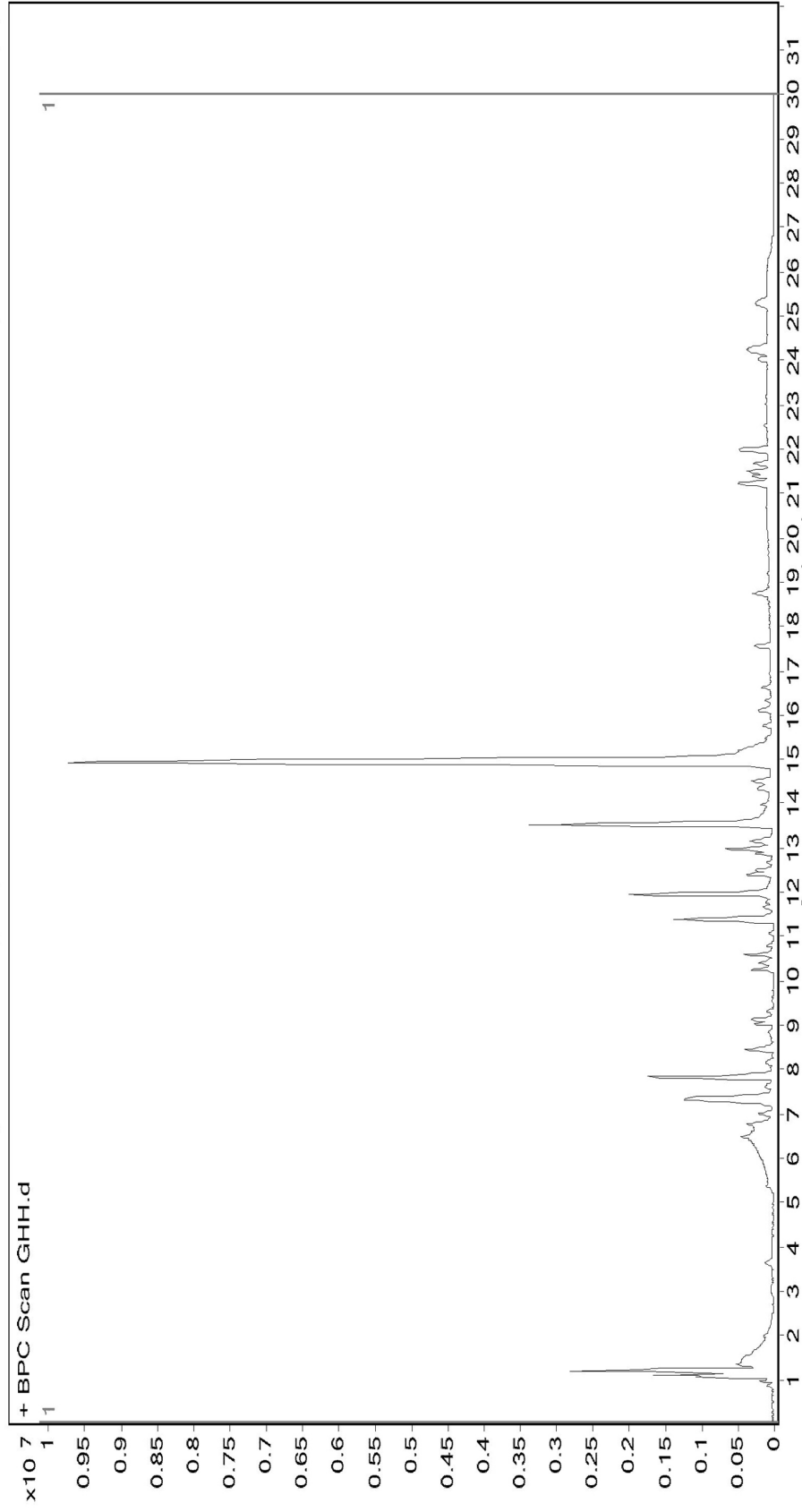


Fig. 18: Liquid chromatogram of methanolic extract of *G. heyneanum* var. *heyneanum*

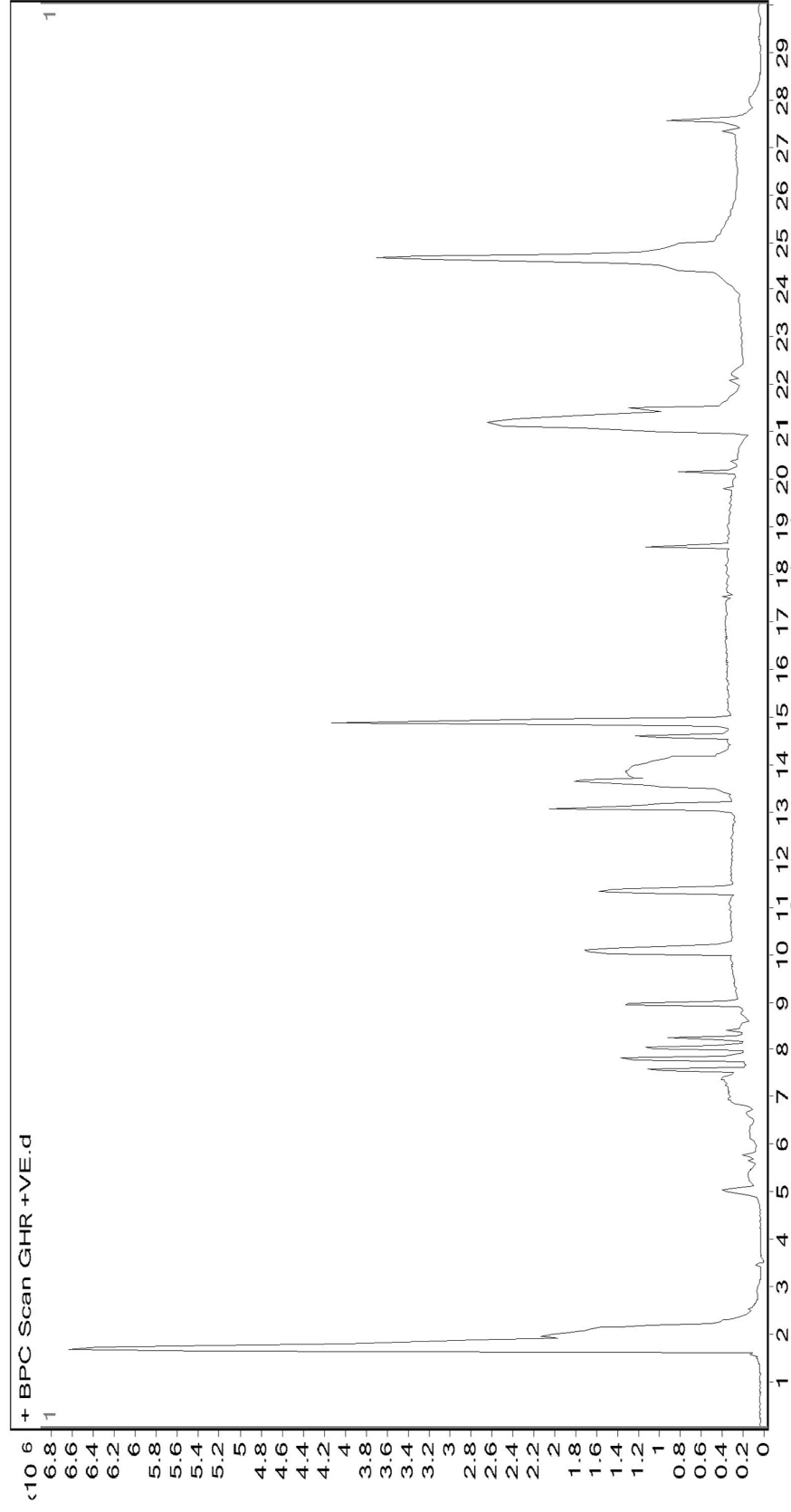


Fig. 19: Liquid chromatogram of methanolic extract of *G. heyneanum* var. *rotleri*

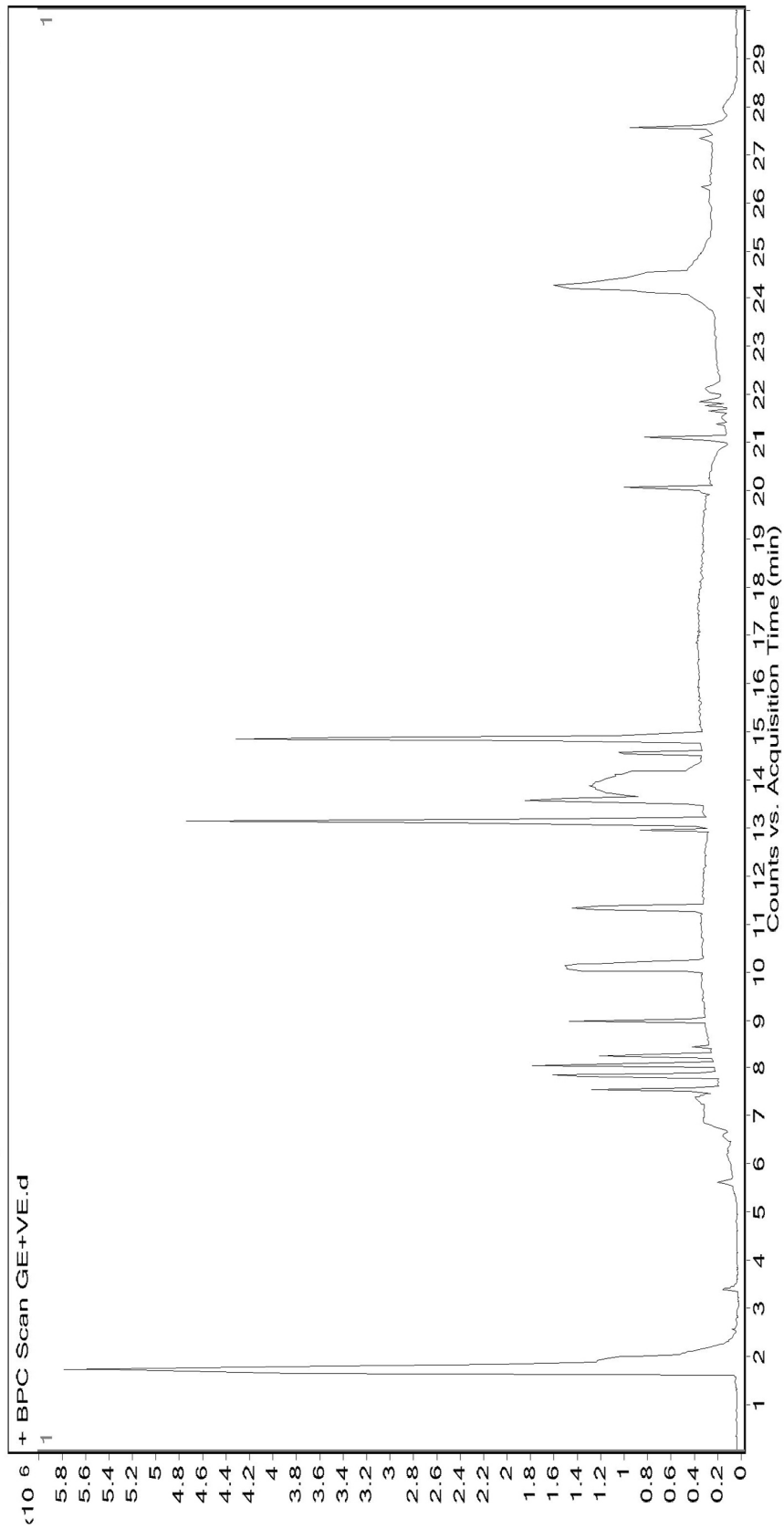


Fig. 20: Liquid chromatogram of methanolic extract of *G. eriocarpum*

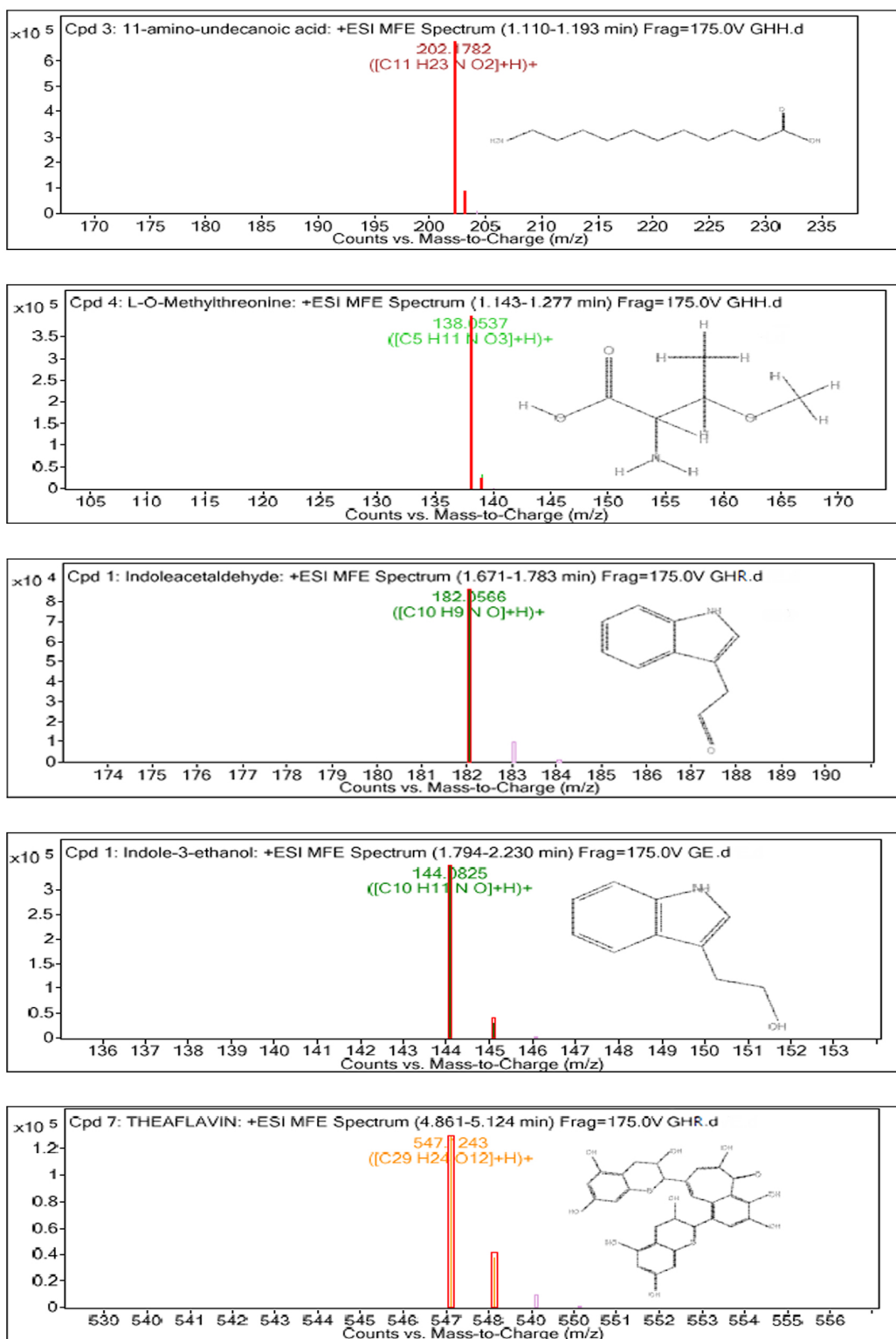


Fig. 21 (i): Mass spectra of compounds detected by HR-LC/MS in the methanolic extract of selected species of *Gomphostemma*

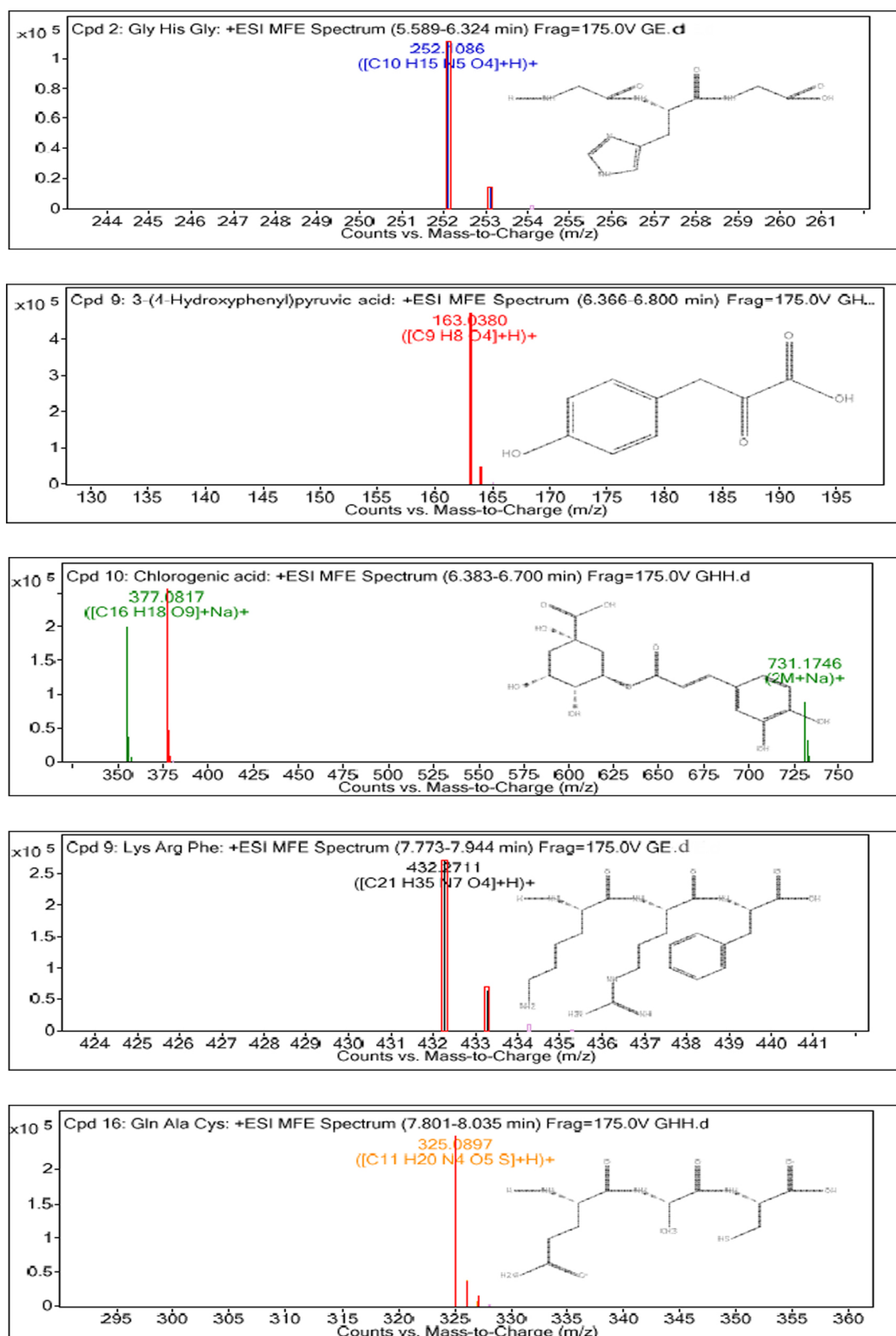


Fig. 21 (ii): Mass spectra of compounds detected by HR-LC/MS in the methanolic extract of selected species of *Gomphostemma*

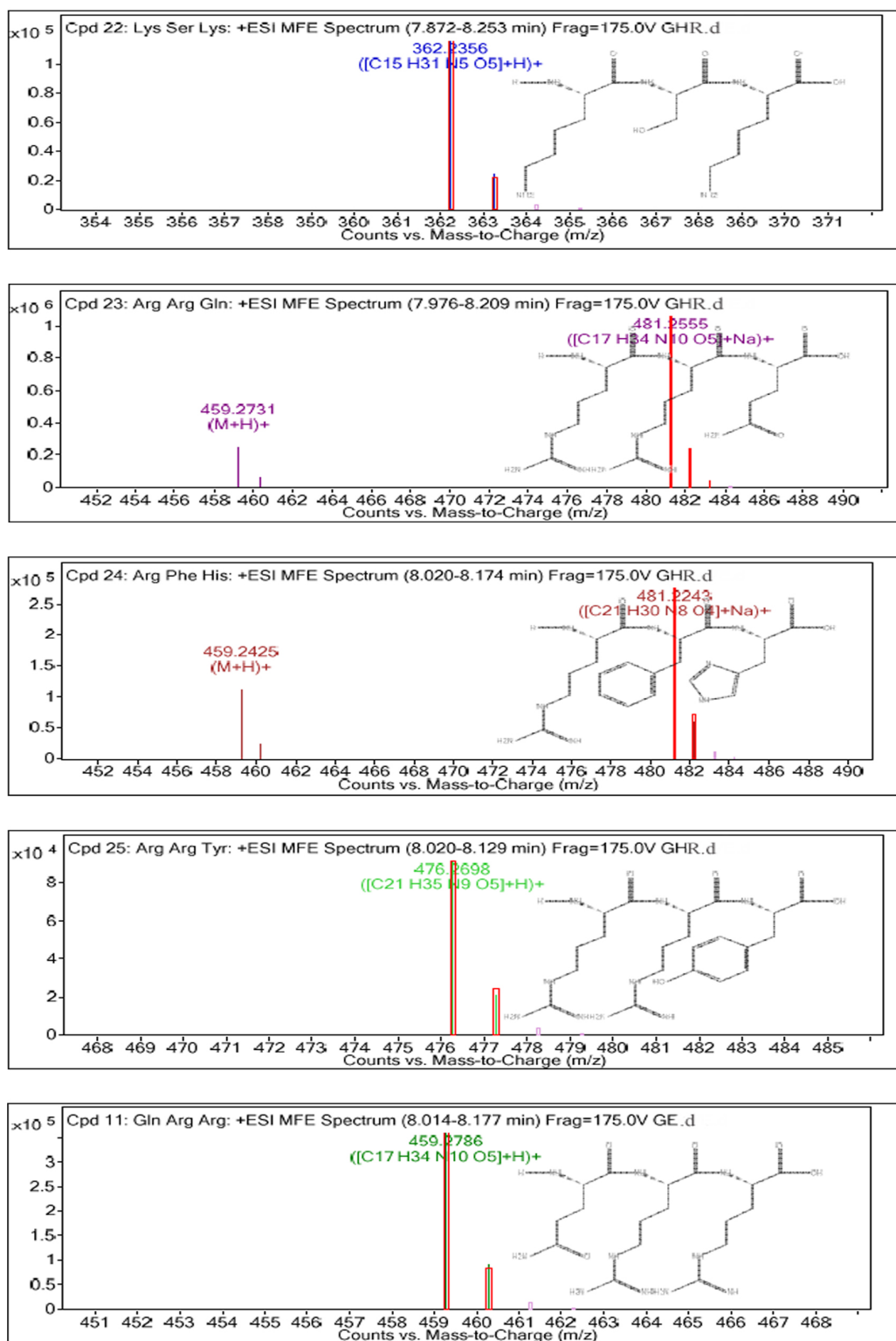


Fig. 21 (iii): Mass spectra of compounds detected by HR-LC/MS in the methanolic extract of selected species of *Gomphostemma*

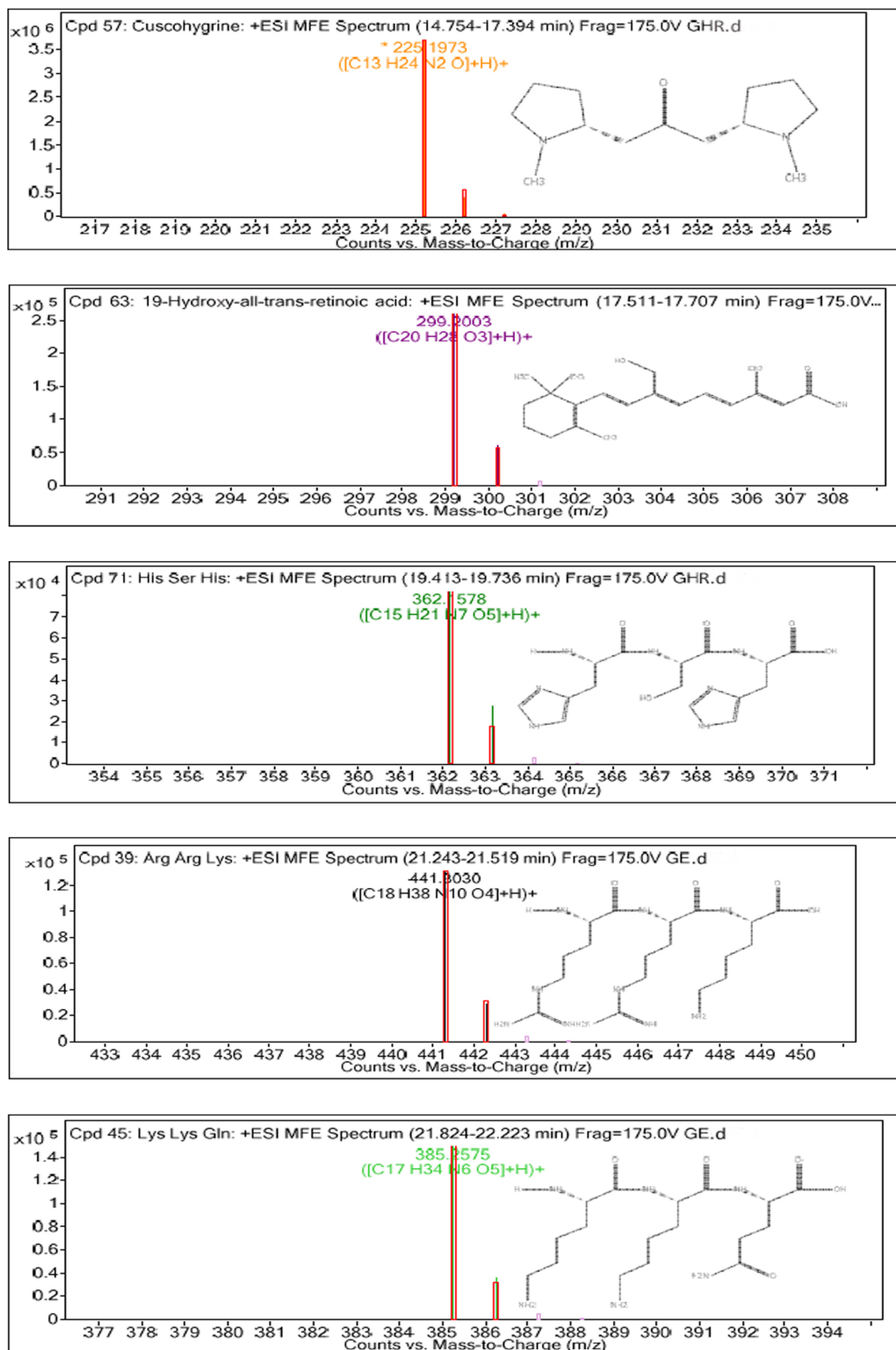


Fig. 21 (vi): Mass spectra of compounds detected by HR-LC/MS in the methanolic extract of selected species of *Gomphostemma*

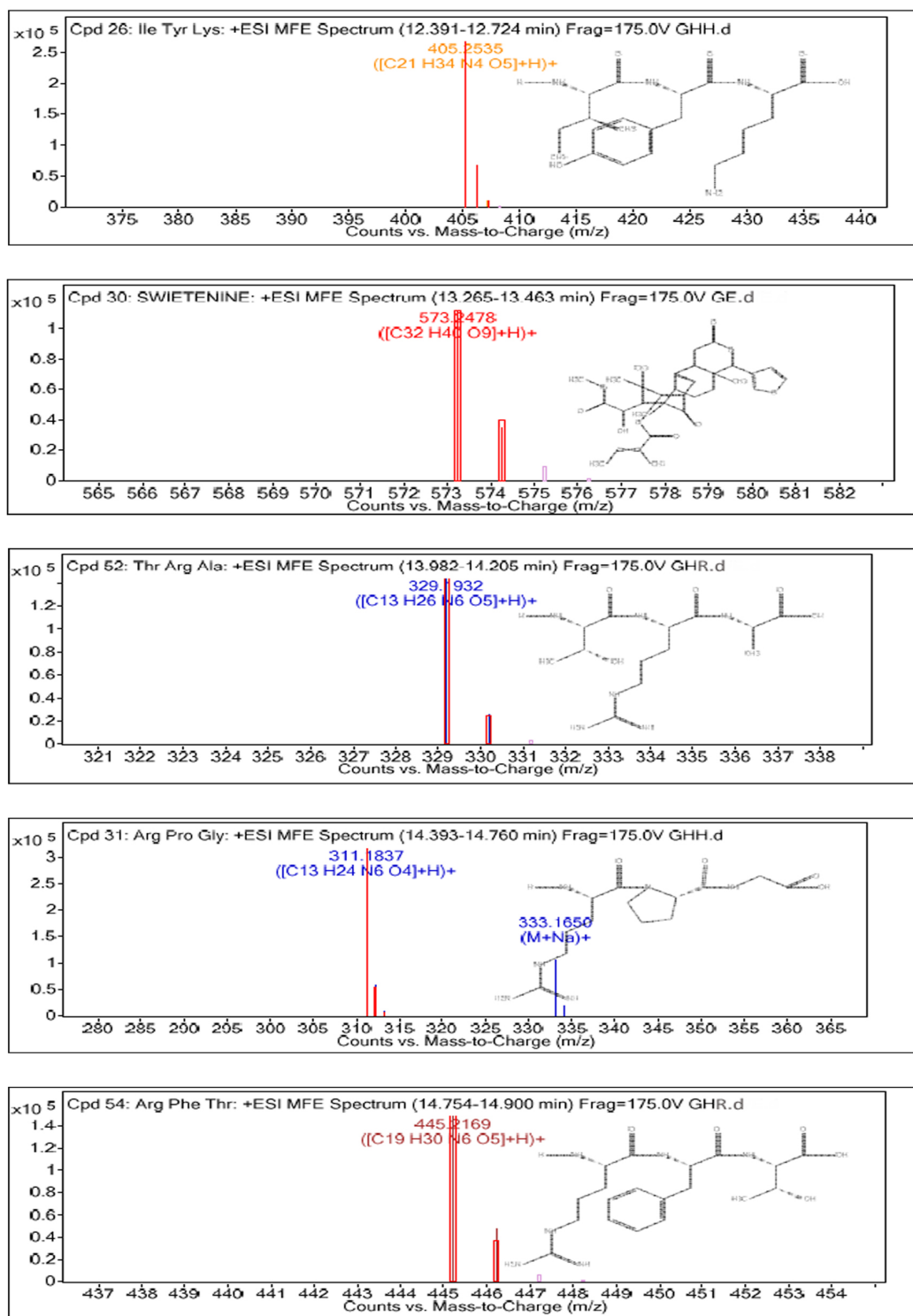


Fig. 21 (v): Mass spectra of compounds detected by HR-LC/MS in the methanolic extract of selected species of *Gomphostemma*

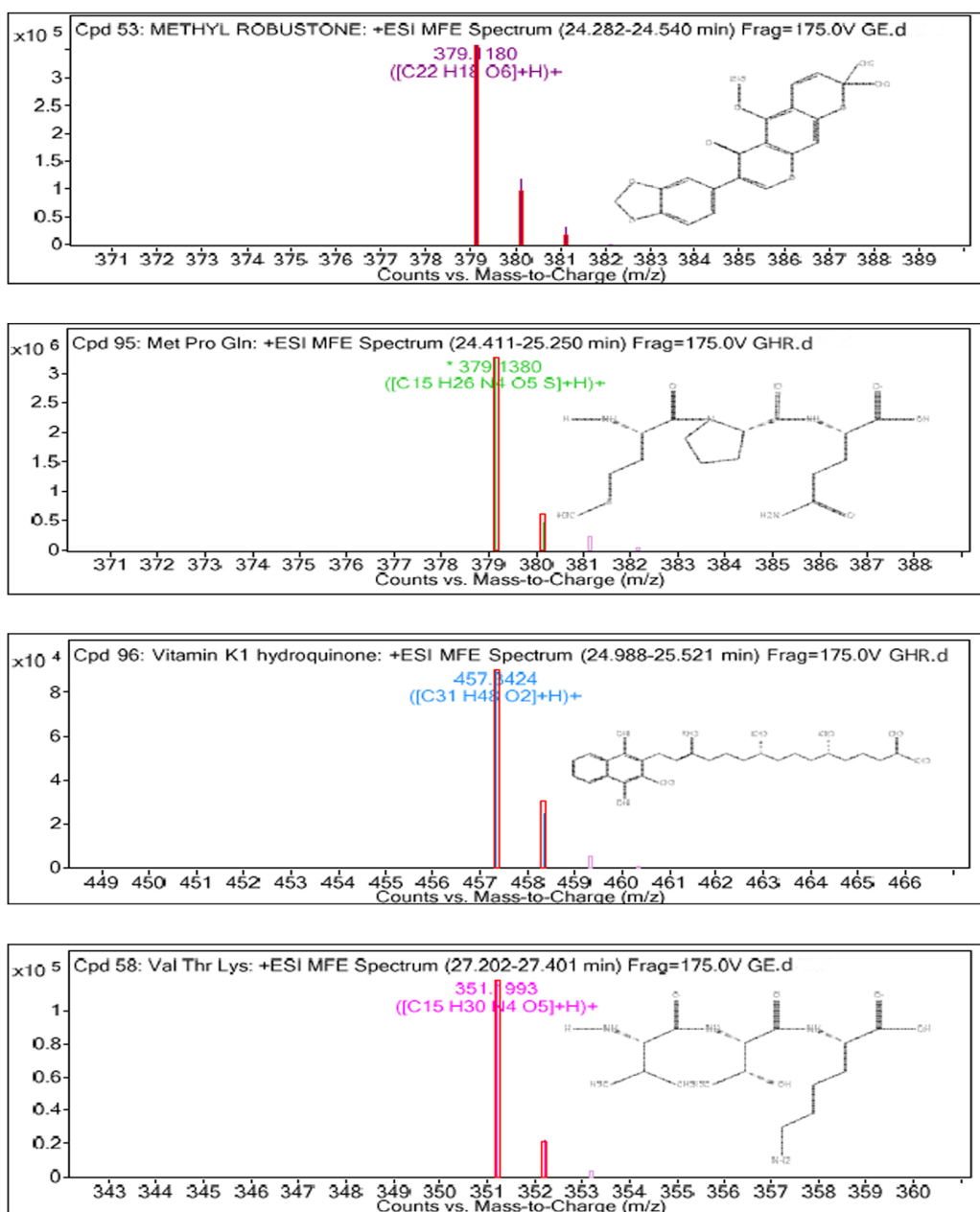


Fig. 21 (vii): Mass spectra of compounds detected by HR-LC/MS in the methanolic extract of selected species of *Gomphostemma*

Fig. 22: *In vitro* DPPH radical scavenging activity of selected species of *Gomphostemma*

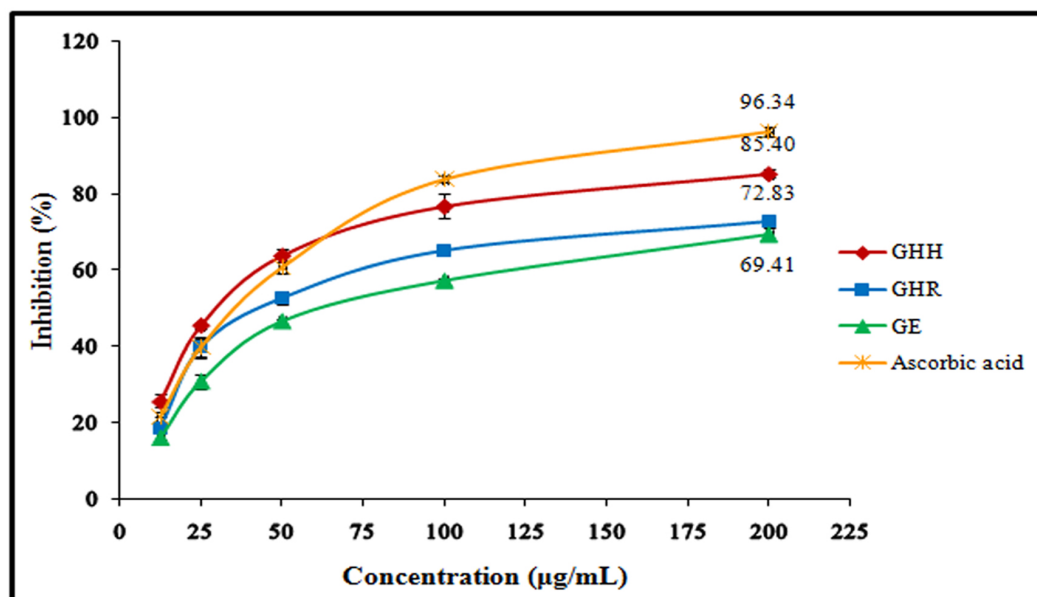
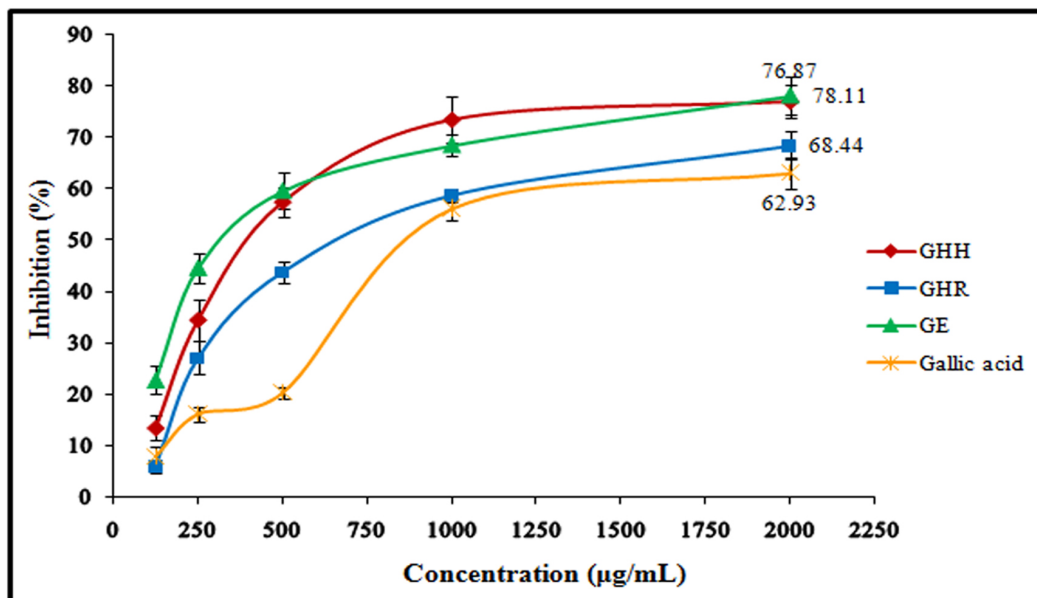


Fig. 23: *In vitro* hydroxyl radical scavenging activity of selected species of *Gomphostemma*



GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*

Fig. 24: *In vitro* nitric oxide free radical scavenging activity of selected species of *Gomphostemma*

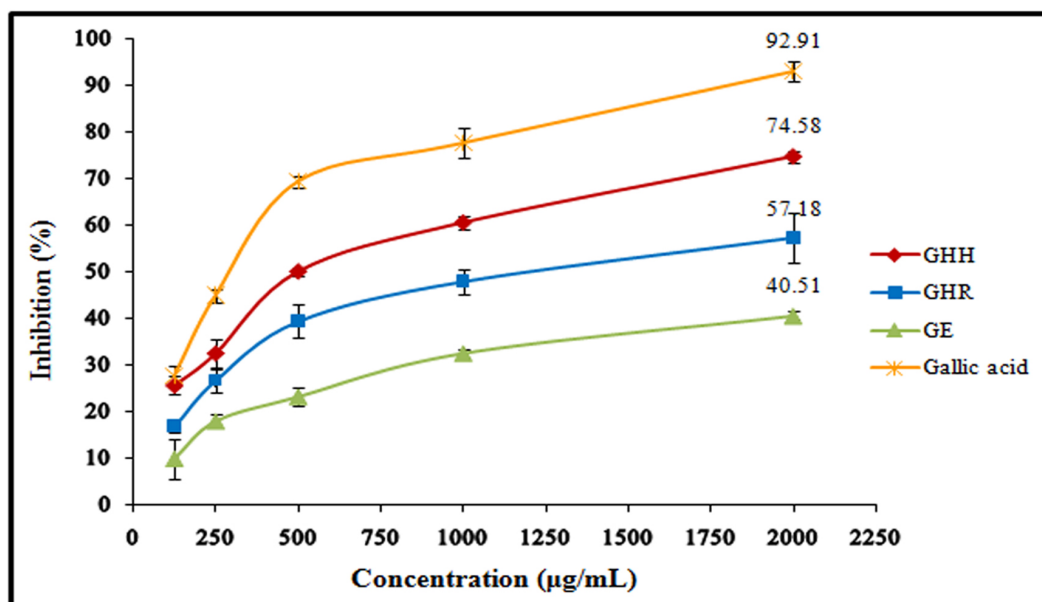
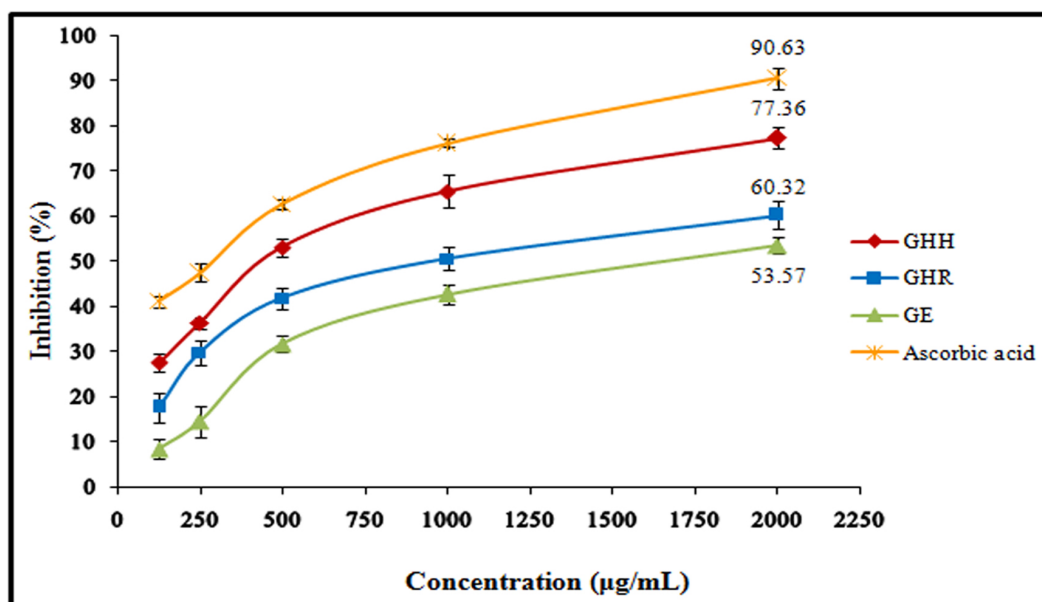
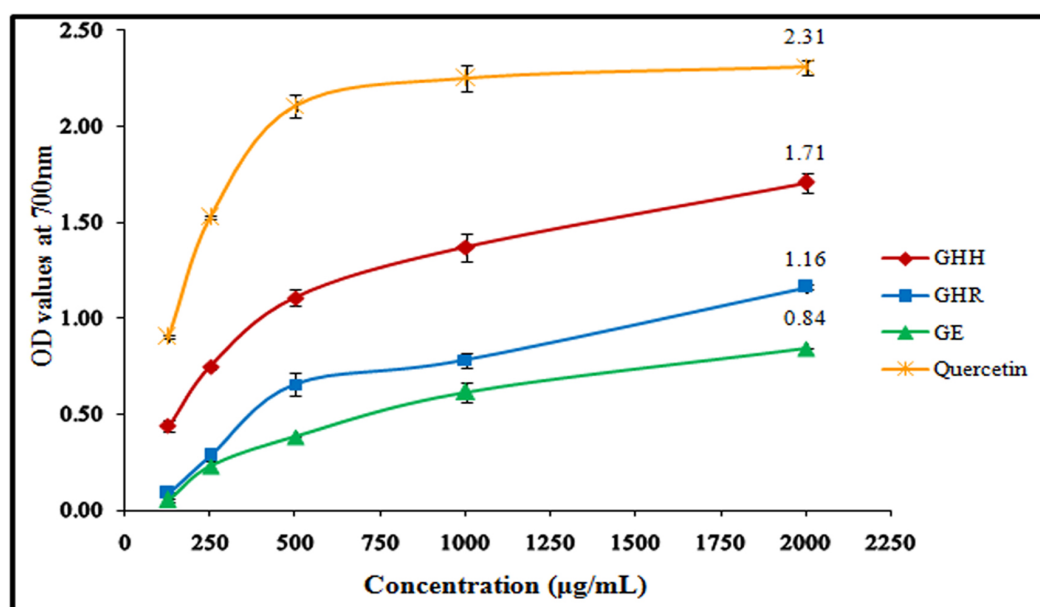


Fig. 25: *In vitro* superoxide radical scavenging activity of selected species of *Gomphostemma*



GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*

Fig. 26: *In vitro* reducing power activity of selected species of *Gomphostemma*



GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*

Fig. 27: Effect of methanolic extracts of selected species of *Gomphostemma* on protein denaturation

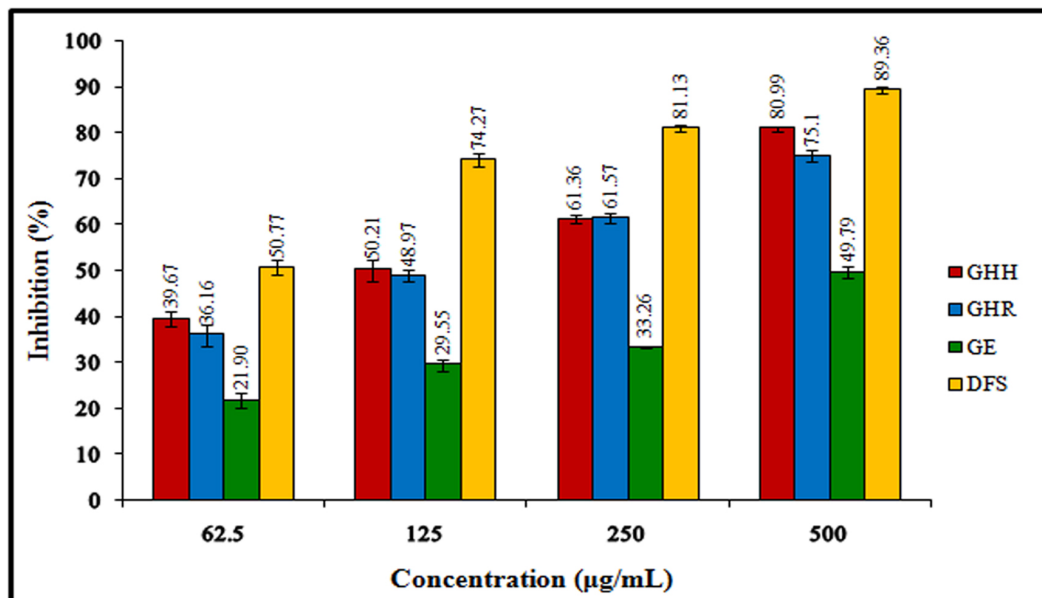
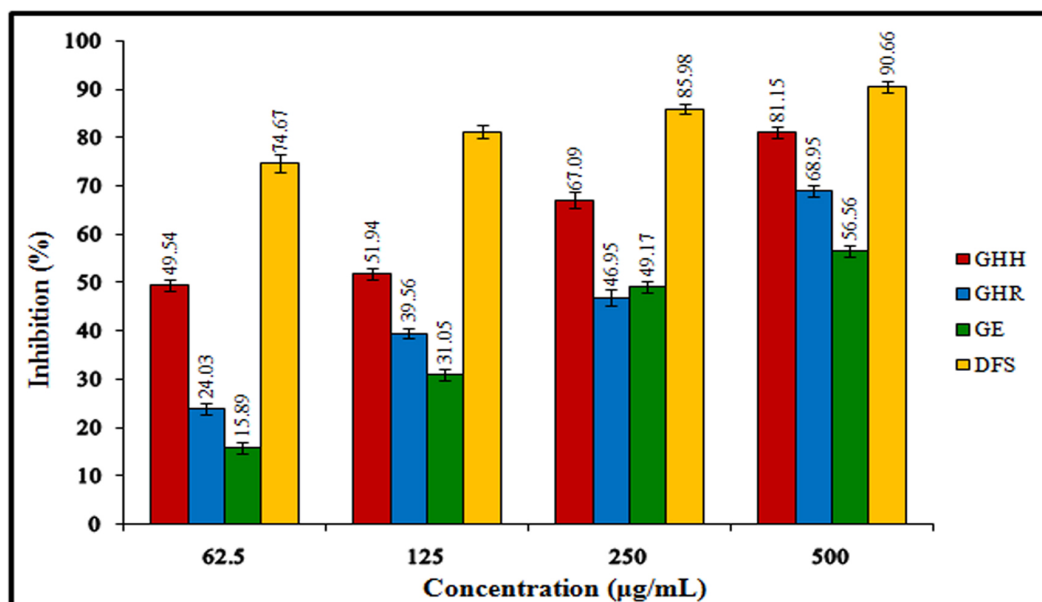


Fig. 28: Effect of methanolic extracts of selected species of *Gomphostemma* on proteinase inhibition



GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*; DFS - Diclofenac sodium

Fig. 29: Effect of methanolic extract of *G. heyneanum* var. *heyneanum* on cyclooxygenase enzyme activity

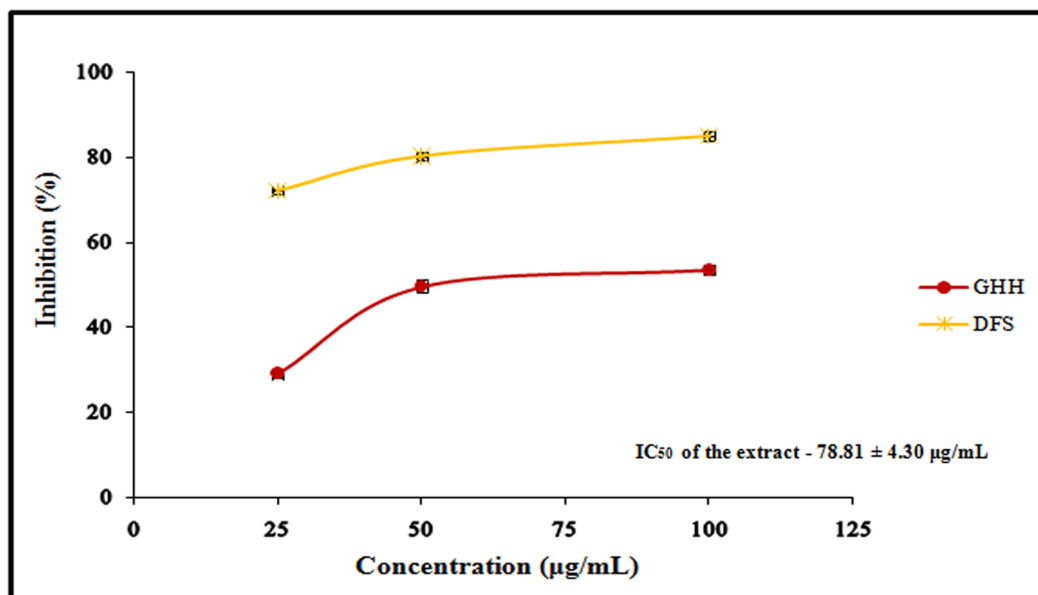
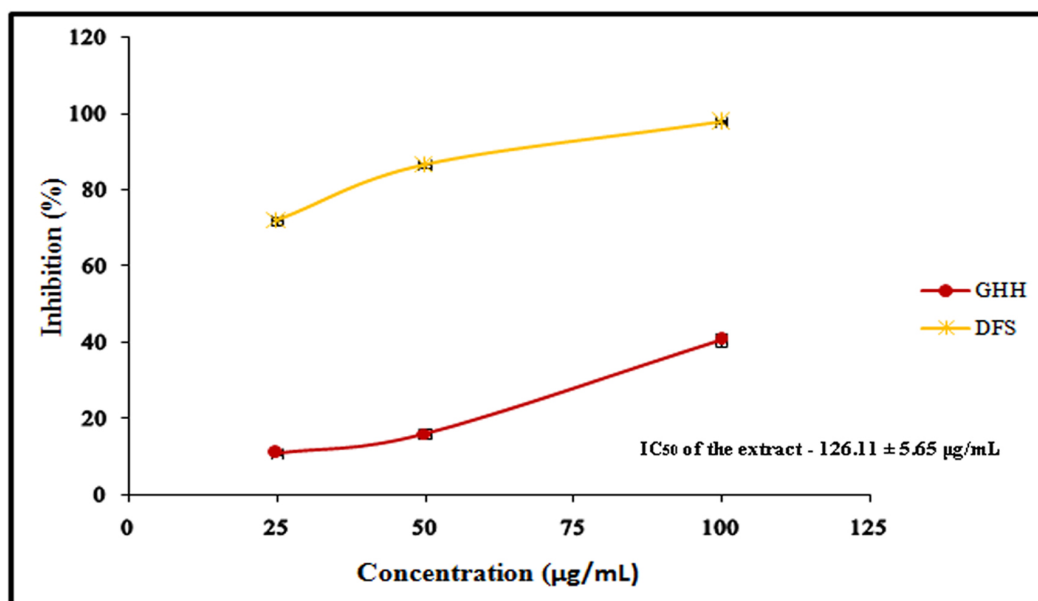


Fig. 30: Effect of methanolic extract of *G. heyneanum* var. *heyneanum* on lipoxigenase enzyme activity



GHH - *G. heyneanum* var. *heyneanum*; DFS - Diclofenac sodium

IC₅₀ value – Concentration of sample causing 50% inhibition

Fig. 40: Cell death assessed in *Allium cepa* root tips by extracts of *Gomphostemma* as determined by Evans blue staining

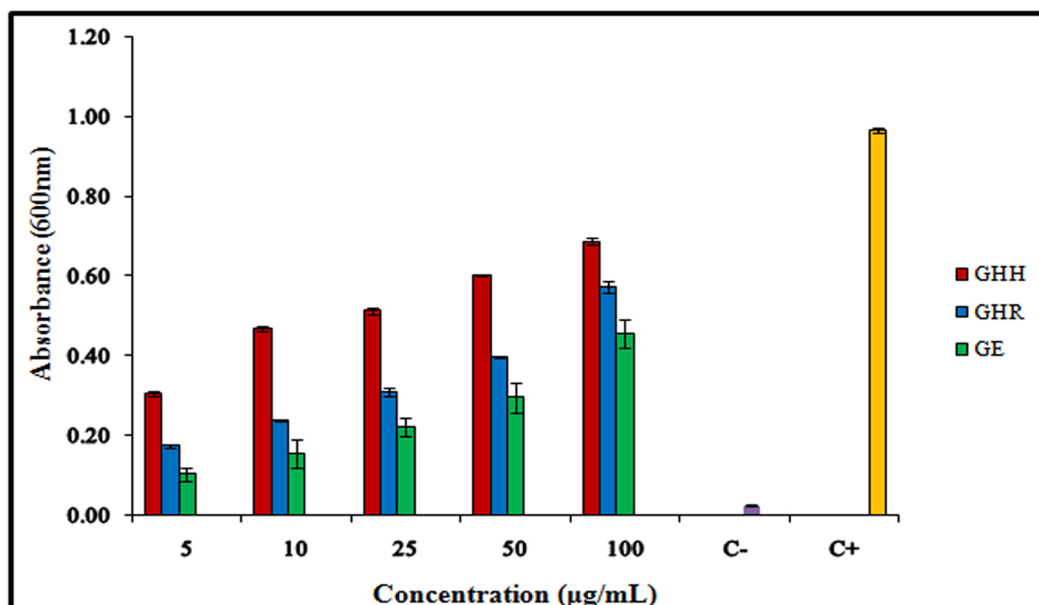
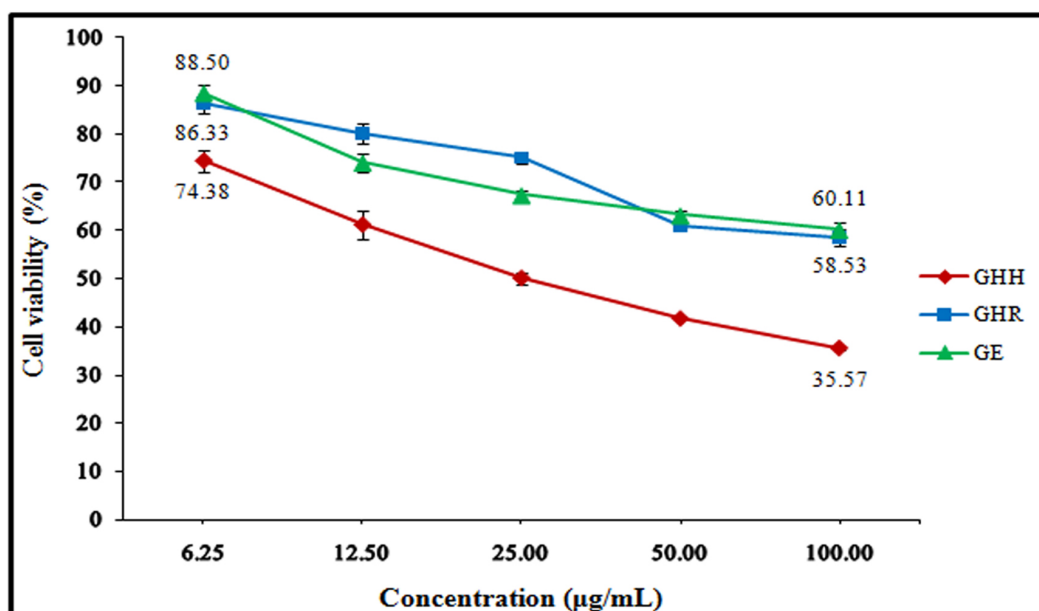


Fig. 41: Evaluation of cytotoxic potential of methanolic extracts of selected species of *Gomphostemma* on MCF-7 cells using *MTT* assay



GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*

Fig. 31: Effect of methanolic extract of *G. heyneanum* var. *heyneanum* on myeloperoxidase enzyme activity

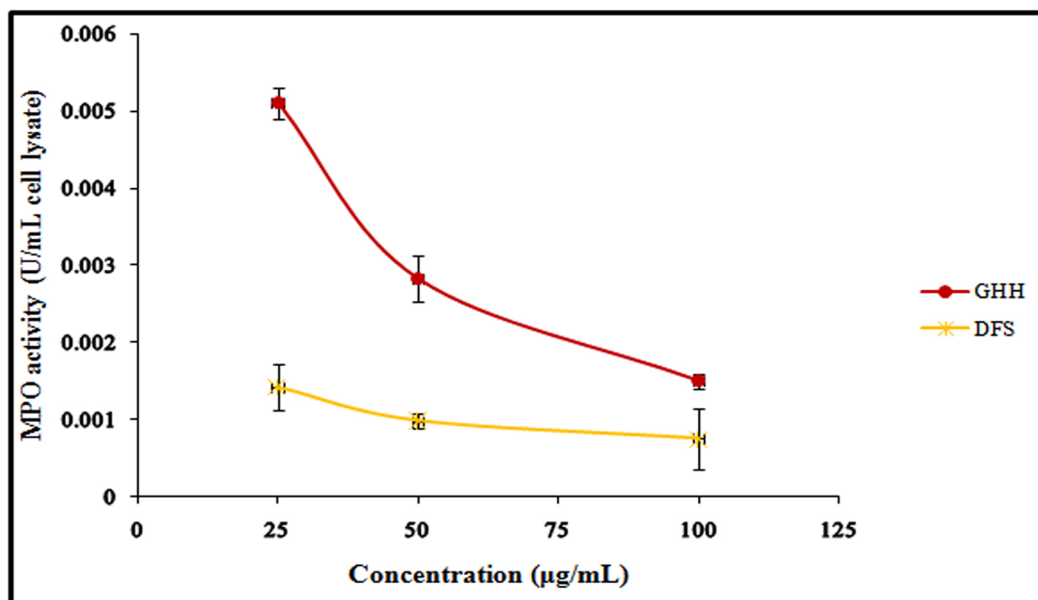
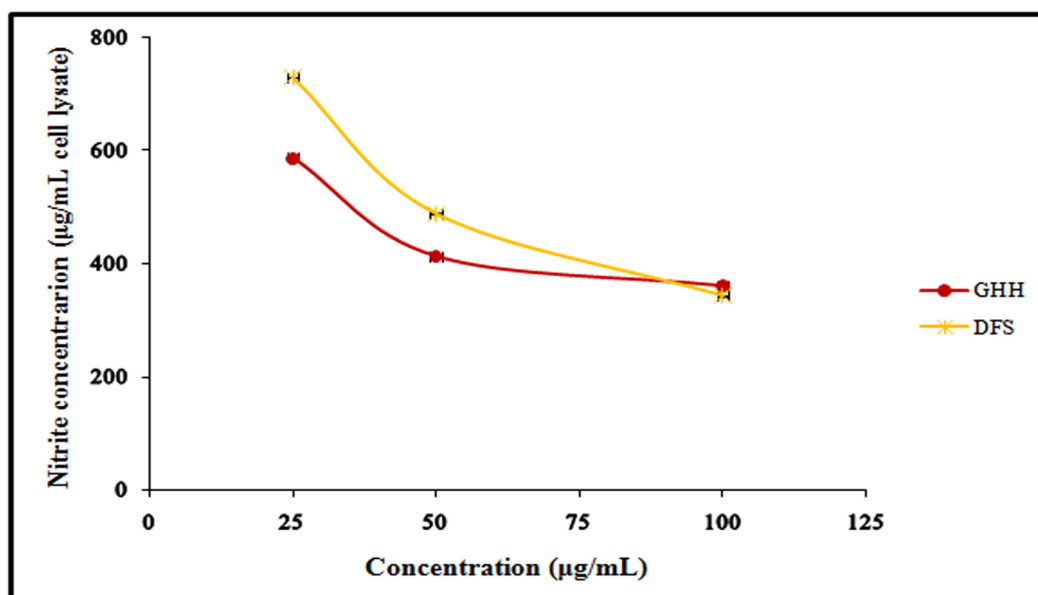


Fig. 32: Effect of methanolic extract of *G. heyneanum* var. *heyneanum* on cellular nitrite level



GHH - *G. heyneanum* var. *heyneanum*; DFS - Diclofenac sodium

Fig. 33: Effect of methanolic extract of *G. heyneanum* var. *heyneanum* on iNOS activity

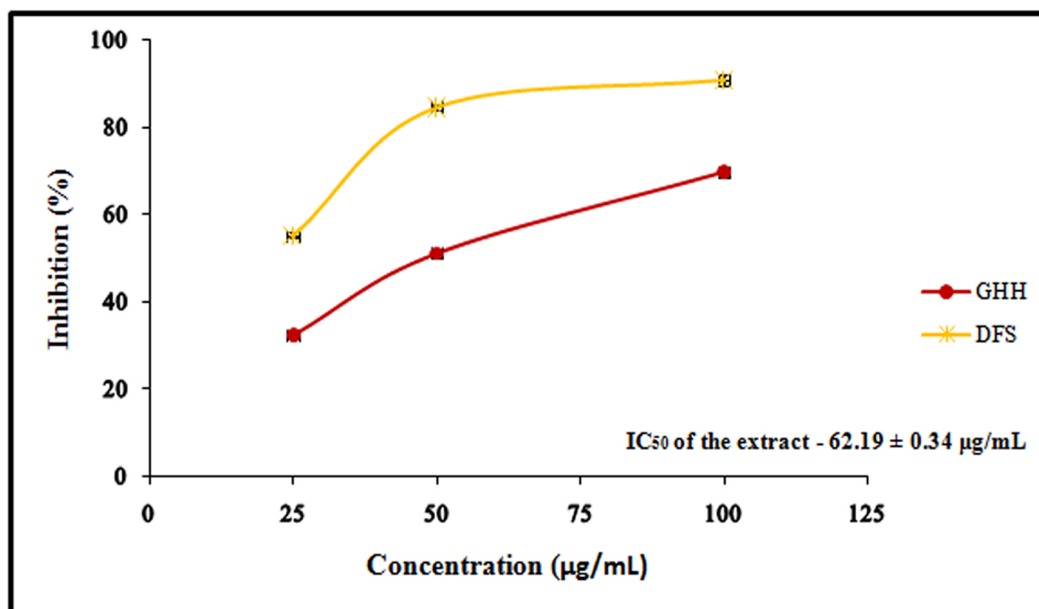
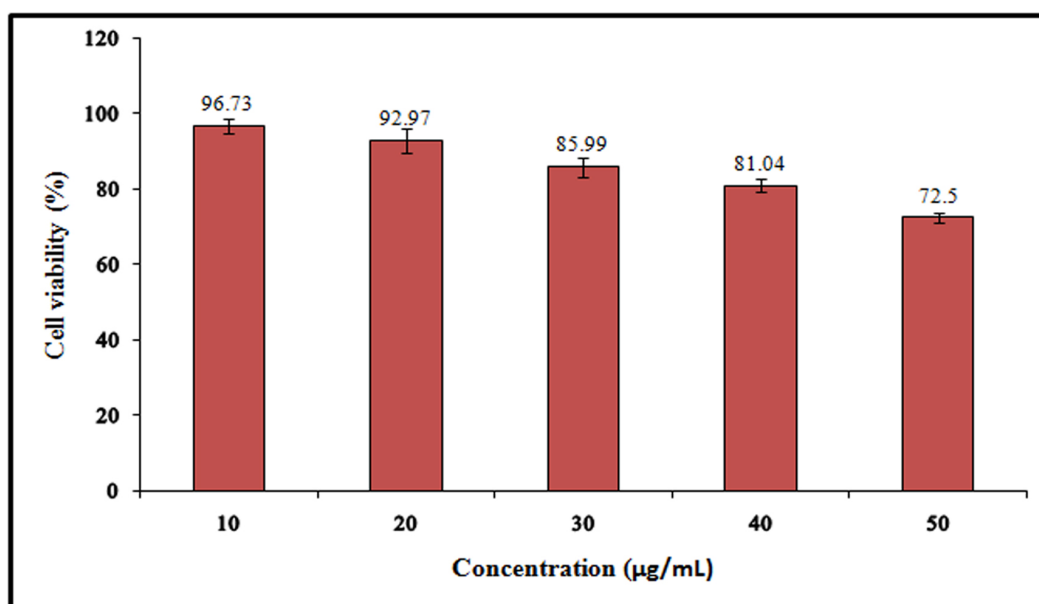
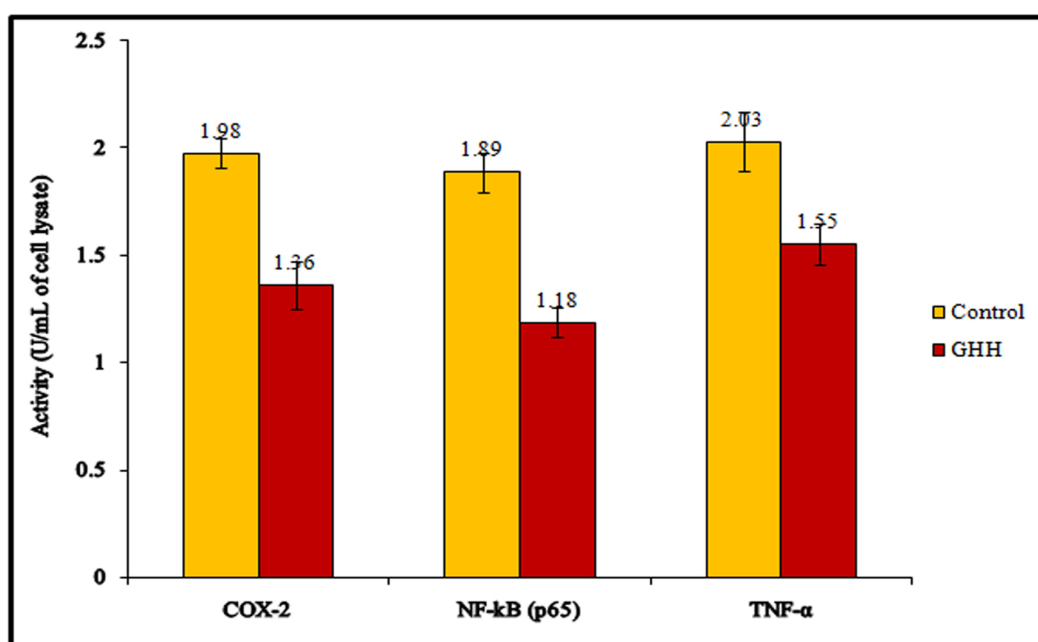


Fig. 34: Cytotoxic effect of methanolic extract of *G. heyneanum* var. *heyneanum* on RAW 264.7 cells



GHH - *G. heyneanum* var. *heyneanum*; DFS - Diclofenac sodium
 IC₅₀ value – Concentration of sample causing 50% inhibition

Fig. 35: Effect of methanolic extract of *G. heyneanum* var. *heyneanum* on production of inflammatory mediators



GHH - *G. heyneanum* var. *heyneanum*

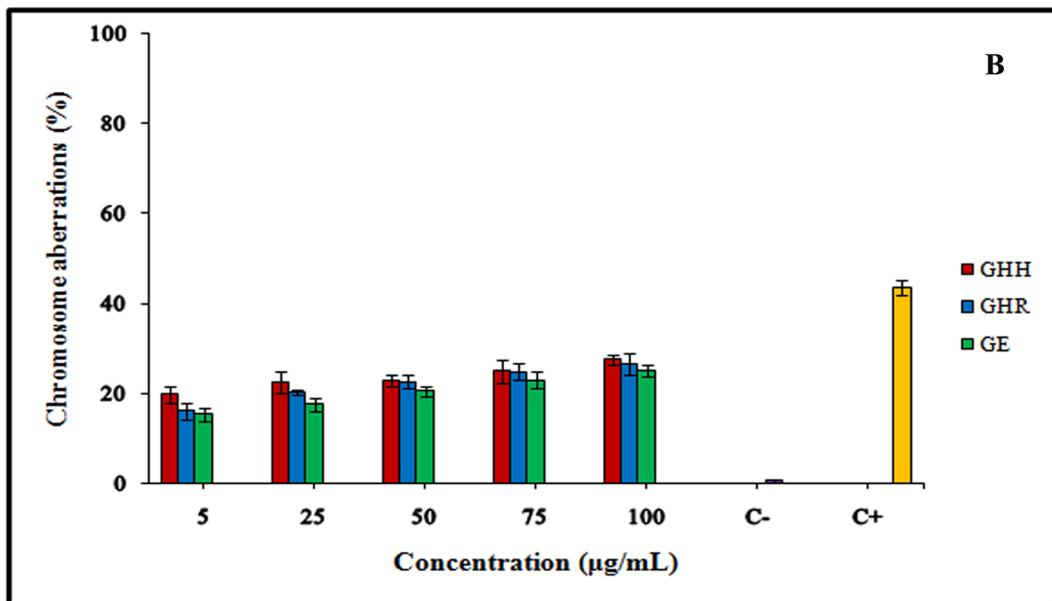
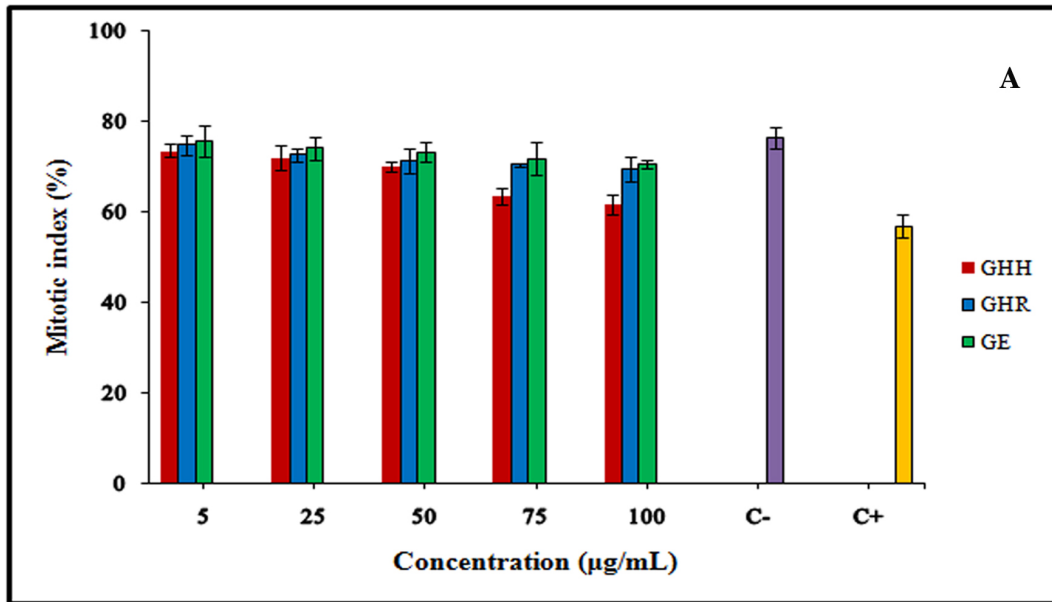


Fig. 36: The effect of varying concentrations of methanolic extracts of selected species of *Gomphostemma* on *Allium cepa* root meristems after 1 h of treatment period. A: Mitotic index B: Chromosome aberrations
 GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*
 Negative control - C-; Positive control - C+

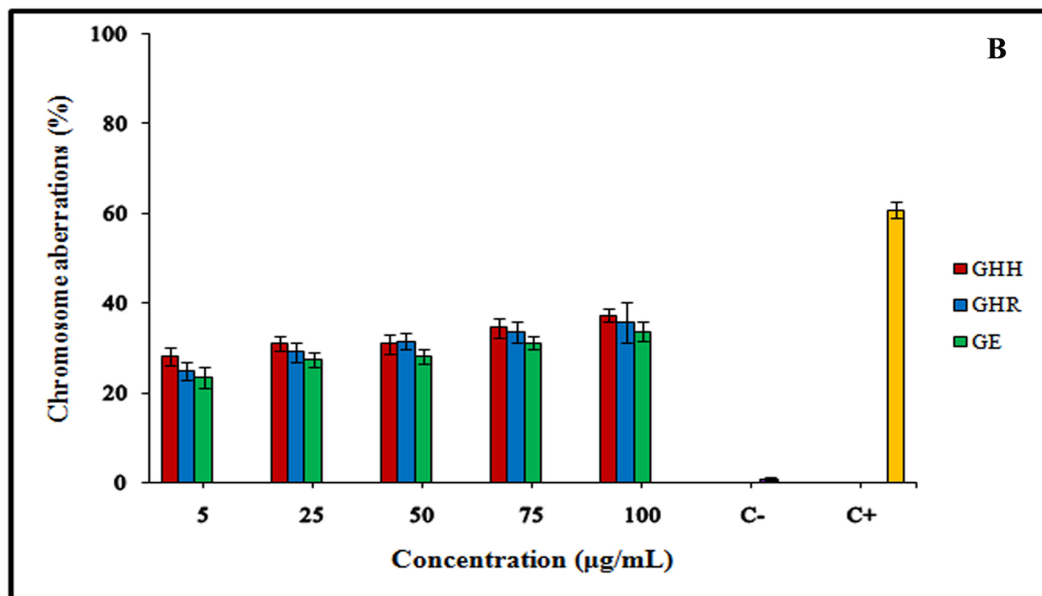
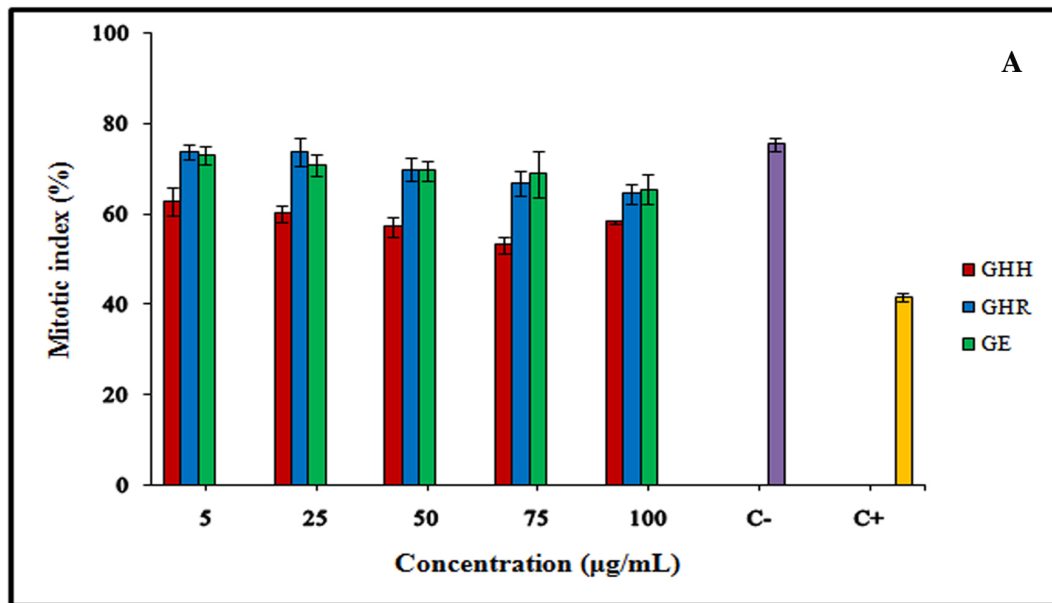


Fig. 37: The effect of varying concentrations of methanolic extracts of selected species of *Gomphostemma* on *Allium cepa* root meristems after 3 h of treatment period. A: Mitotic index B: Chromosome aberrations
 GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*
 Negative control - C-; Positive control - C+

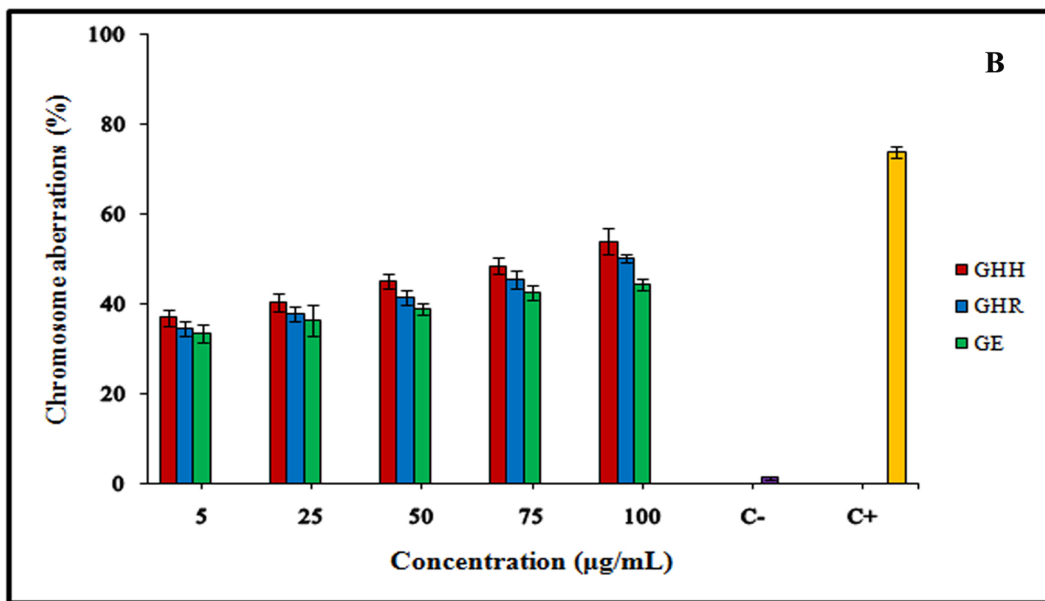
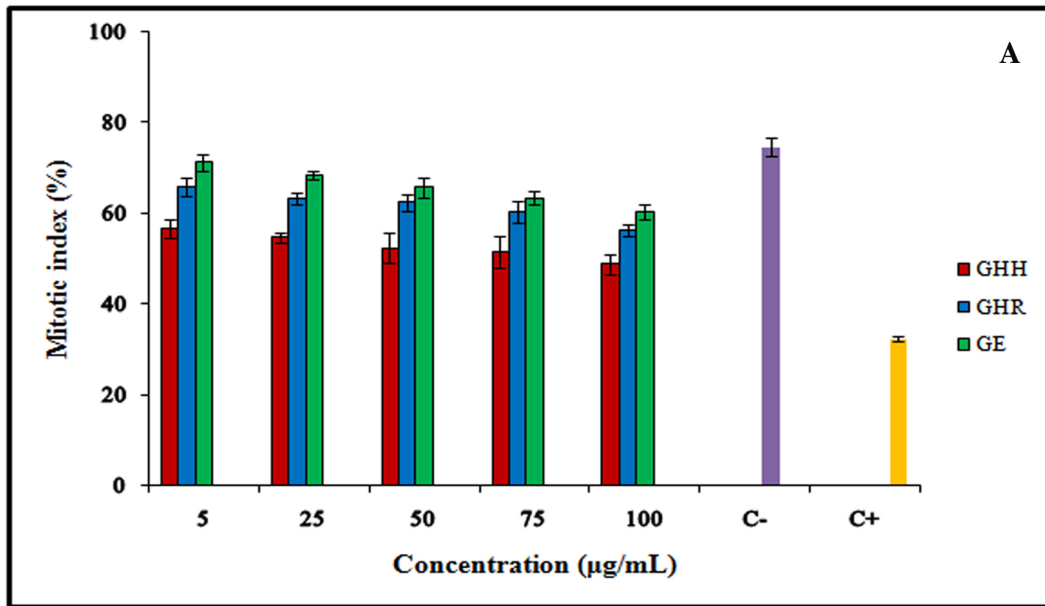


Fig. 38: The effect of varying concentrations of methanolic extracts of selected species of *Gomphostemma* on *Allium cepa* root meristems after 12 h of treatment period. **A:** Mitotic index **B:** Chromosome aberrations
 GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*
 Negative control - C-; Positive control - C+

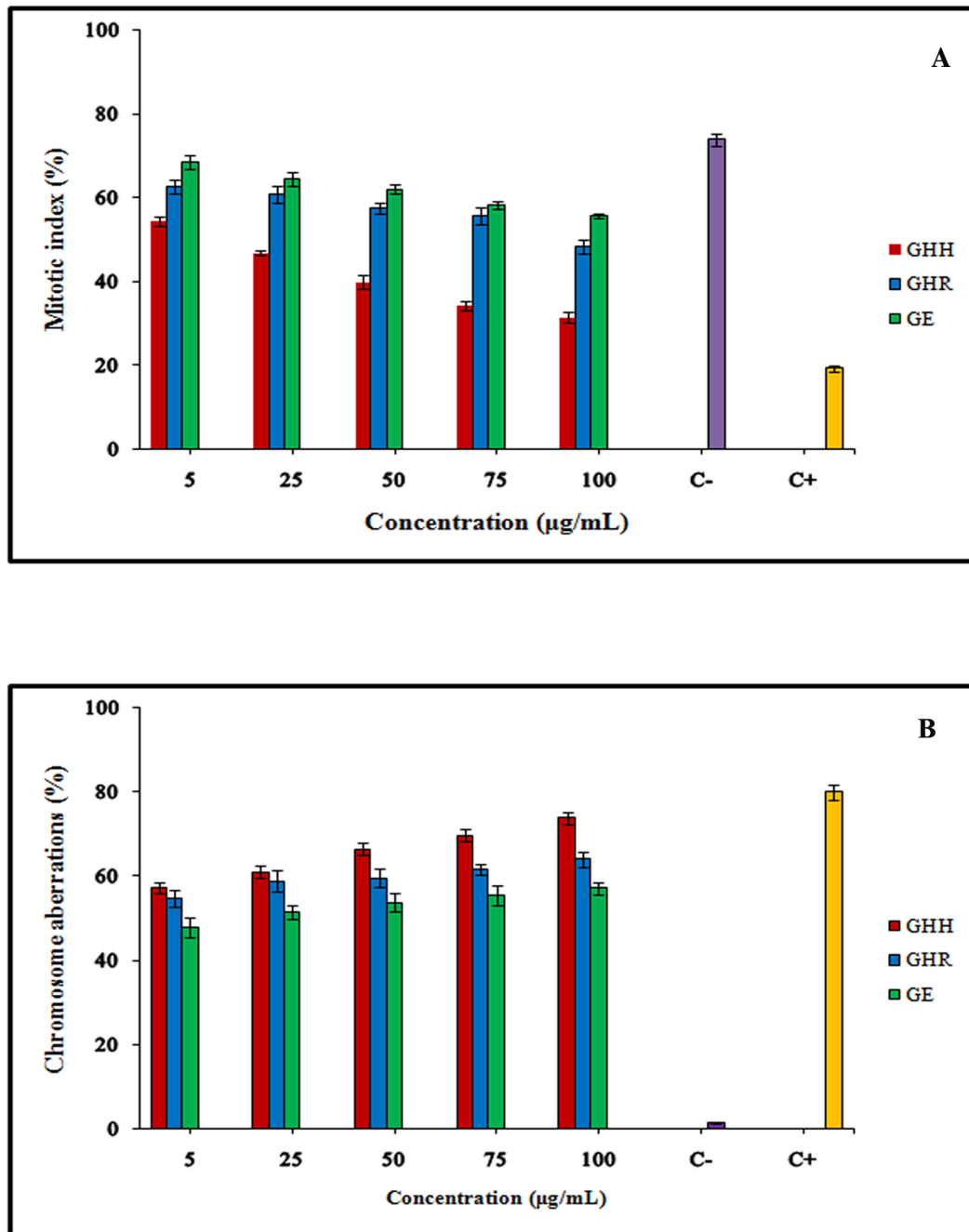


Fig. 39: The effect of varying concentrations of methanolic extracts of selected species of *Gomphostemma* on *Allium cepa* root meristems after 24 h of treatment period. A: Mitotic index B: Chromosome aberrations
 GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*
 Negative control - C-; Positive control - C+

Fig. 40: Cell death assessed in *Allium cepa* root tips by extracts of *Gomphostemma* as determined by Evans blue staining

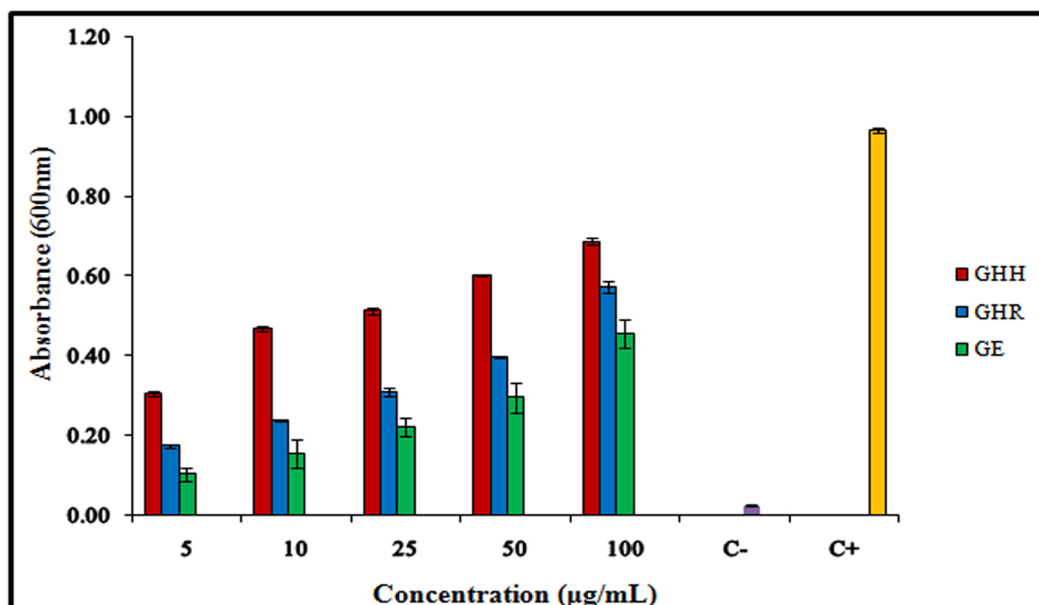
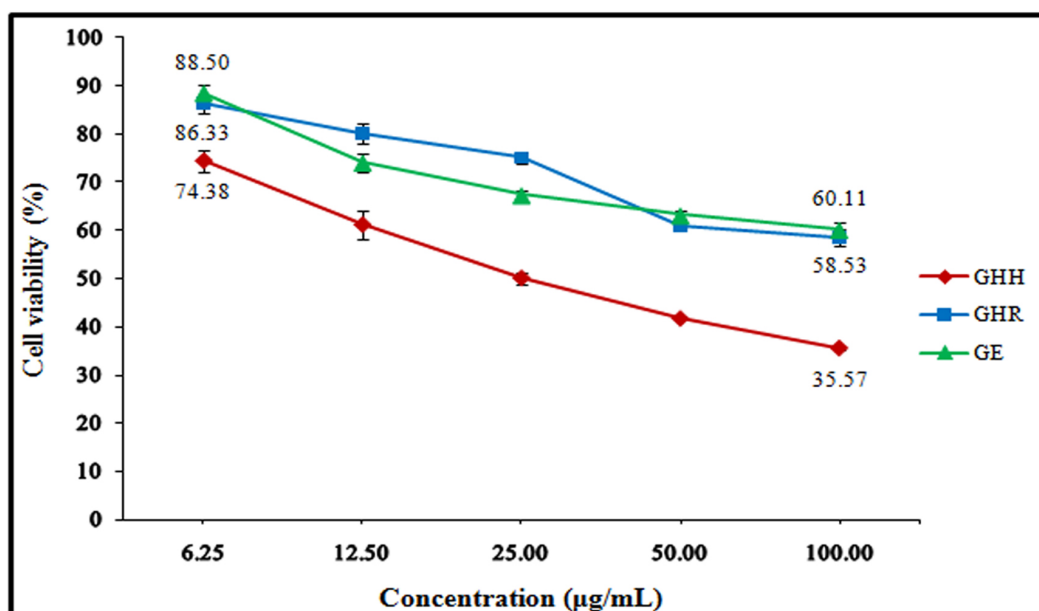


Fig. 41: Evaluation of cytotoxic potential of methanolic extracts of selected species of *Gomphostemma* on MCF-7 cells using MTT assay



GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*; Negative control - C-; Positive control - C+

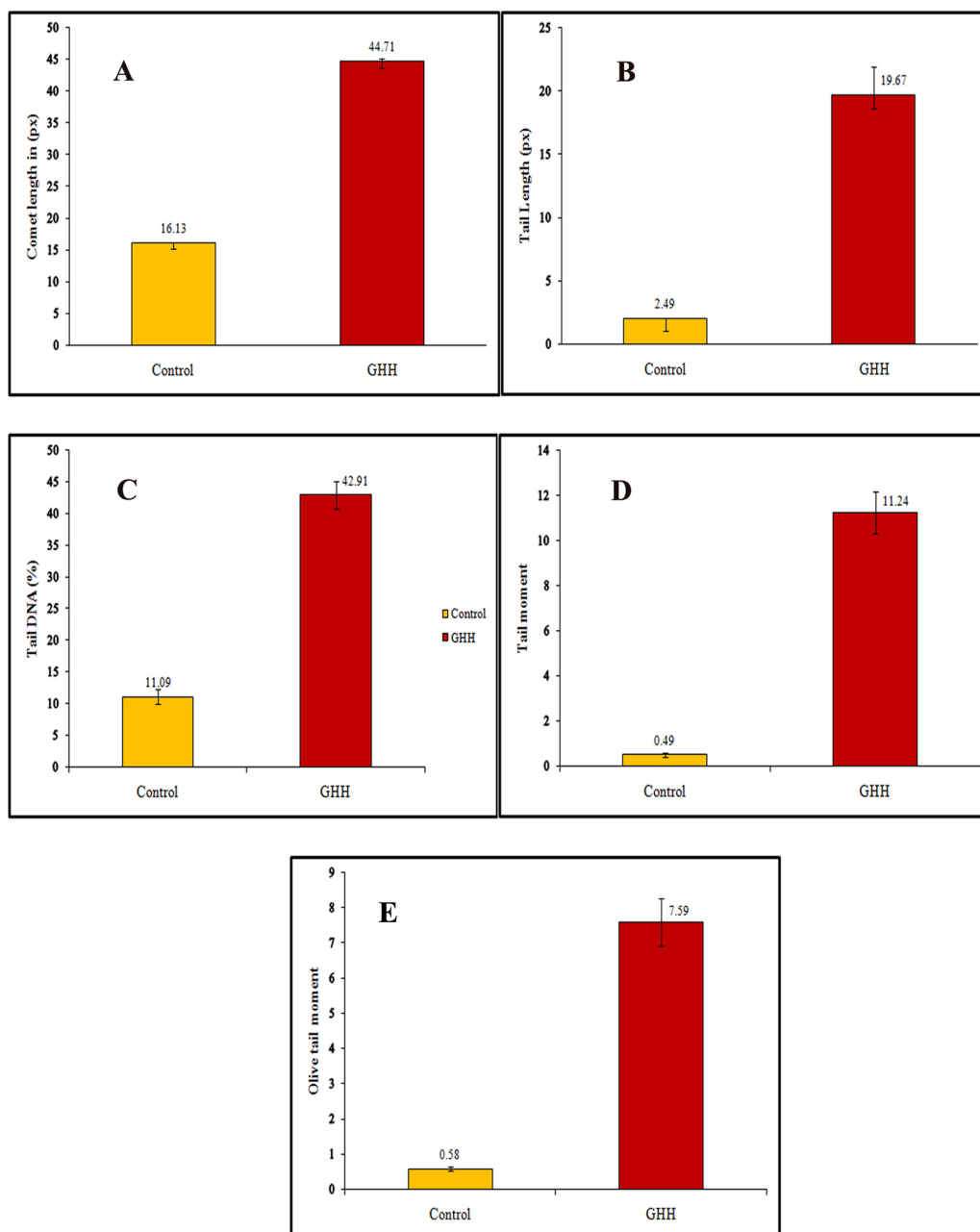
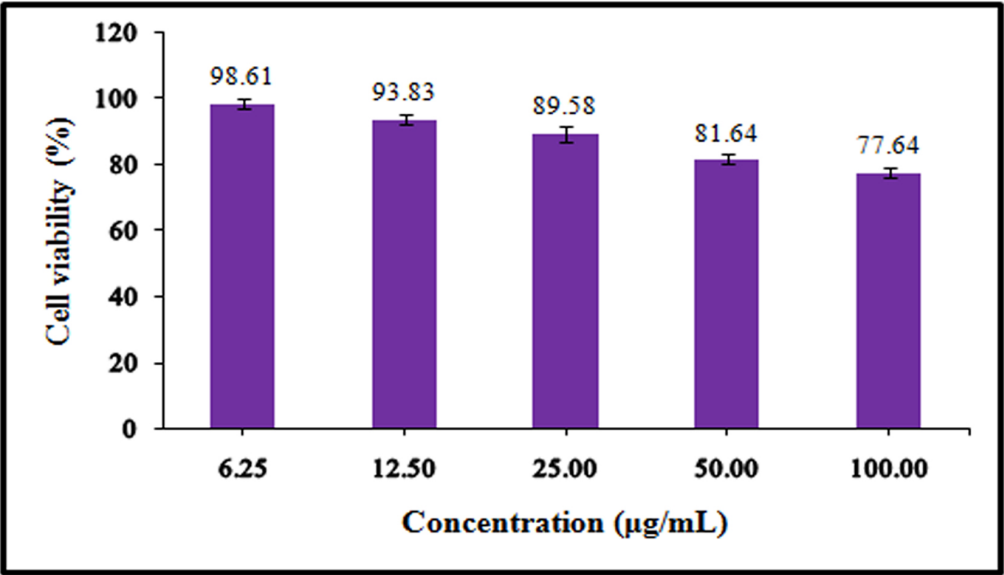


Fig. 43: Assessment of DNA damage induced by methanolic extract of *G. heyneanum* var. *heyneanum* on MCF-7 cells with comet assay. A: Comet length B: Tail length C: Tail DNA percentage D: Tail moment E: Olive tail moment

Fig. 42: Effect of methanolic extract of *G. heyneanum* var. *heyneanum* on L929 cells



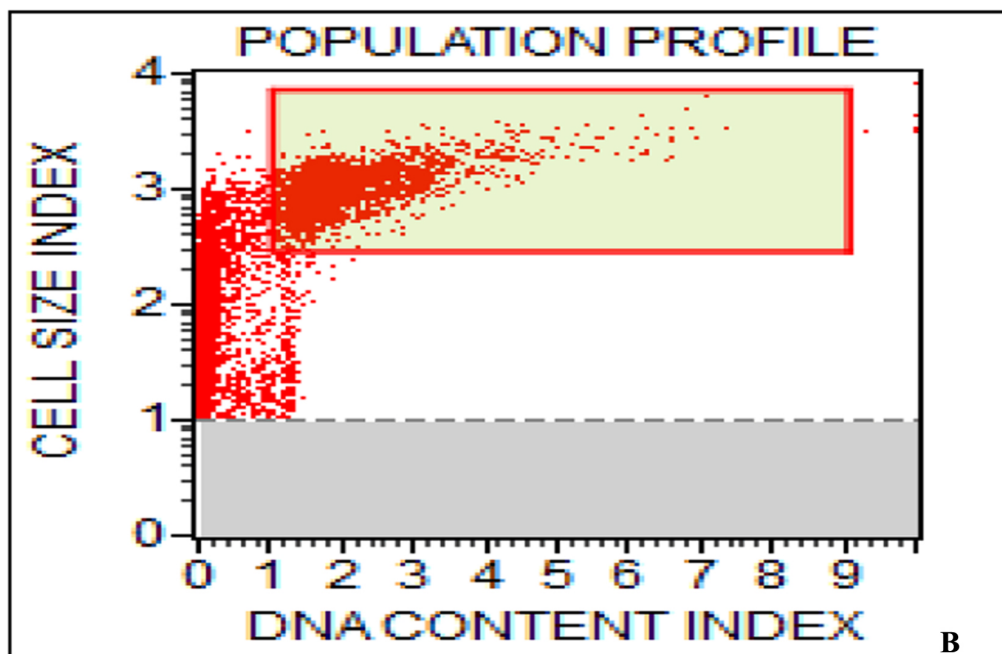
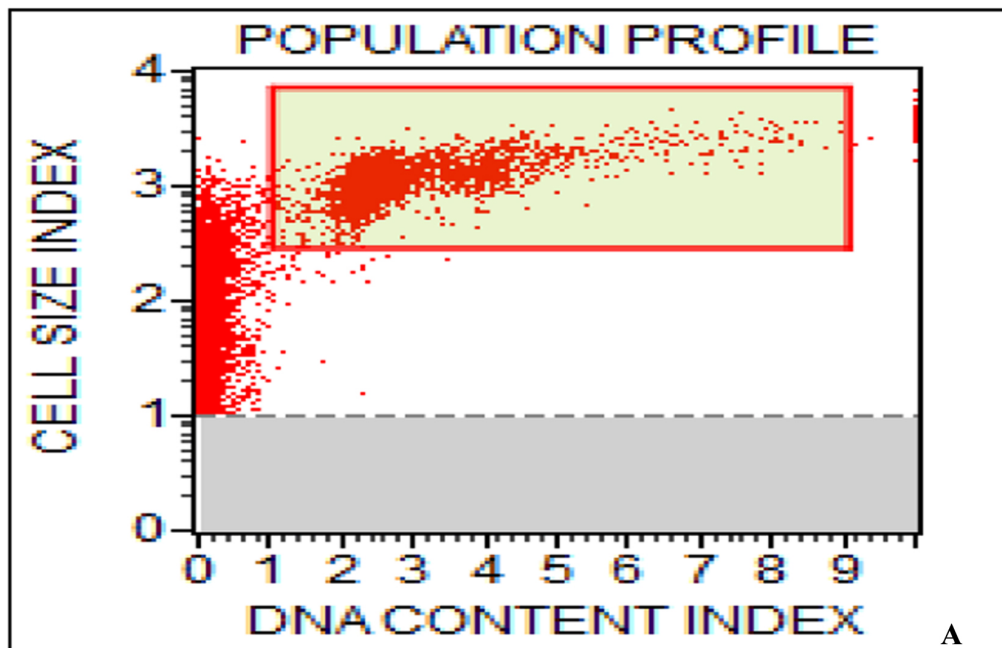


Fig. 45: Flow cytometric analysis showing population profile of MCF-7 cells.
A: Control **B:** MCF-7 cells treated (24 h) with methanolic extract (45 $\mu\text{g/mL}$) of *G. heyneanum* var. *heyneanum*.
 The rectangle represents the cells of interest excluding the cellular debris.

Fig. 46: Percentage of cell count of MCF-7 cells treated with extract of *G. heyneanum* var. *heyneanum*

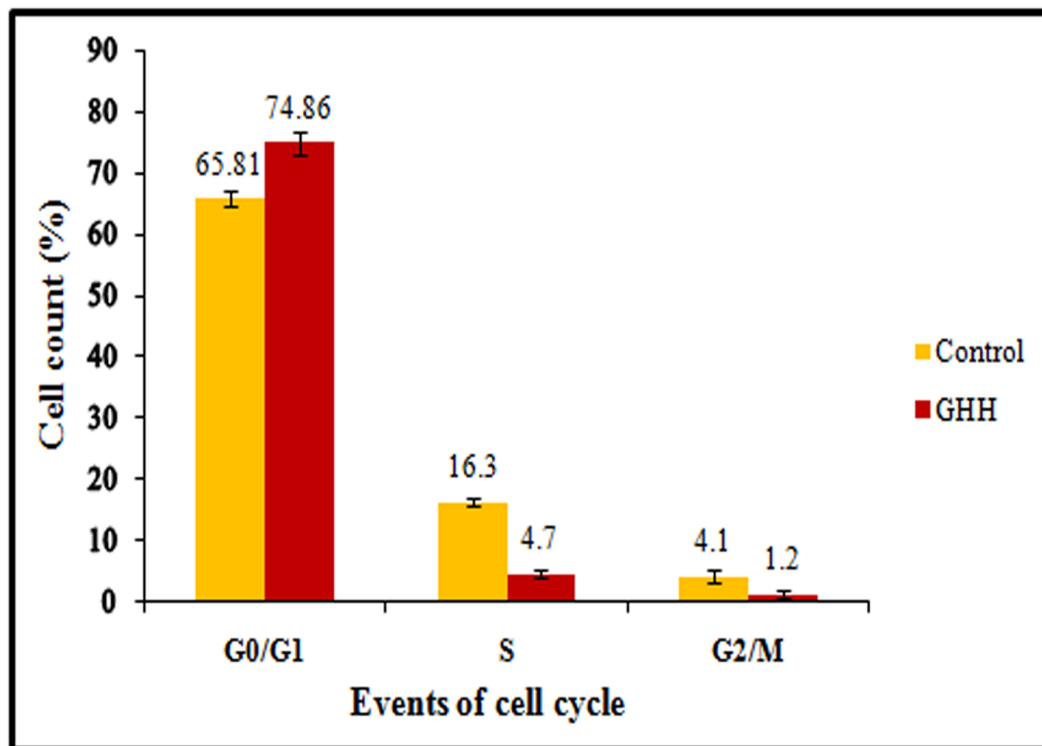
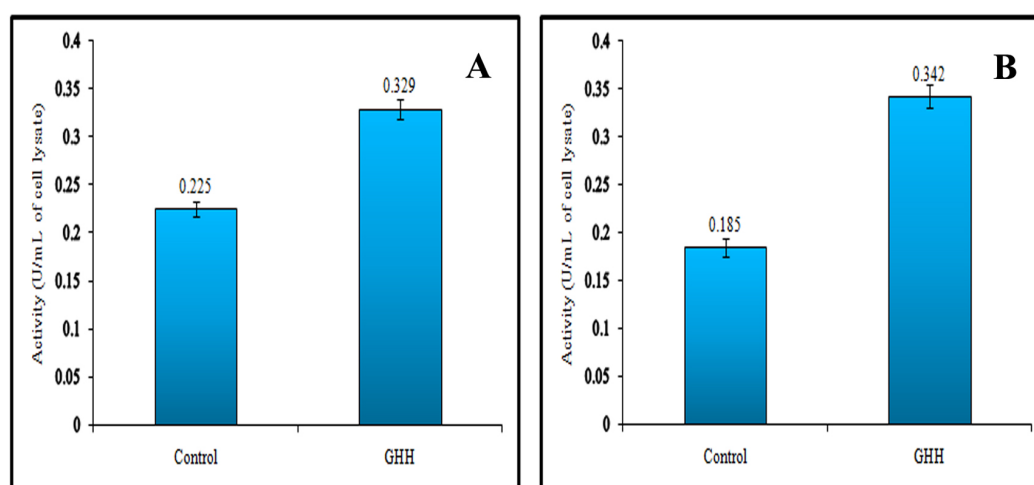


Fig. 47: Effect of methanolic extract of *G. heyneanum* var. *heyneanum* on caspase activity. A: Caspase-9 B: Caspase-7



GHH - *G. heyneanum* var. *heyneanum*

Fig. 46: Percentage of cell count of MCF-7 cells treated with extract of *G. heyneanum* var. *heyneanum*

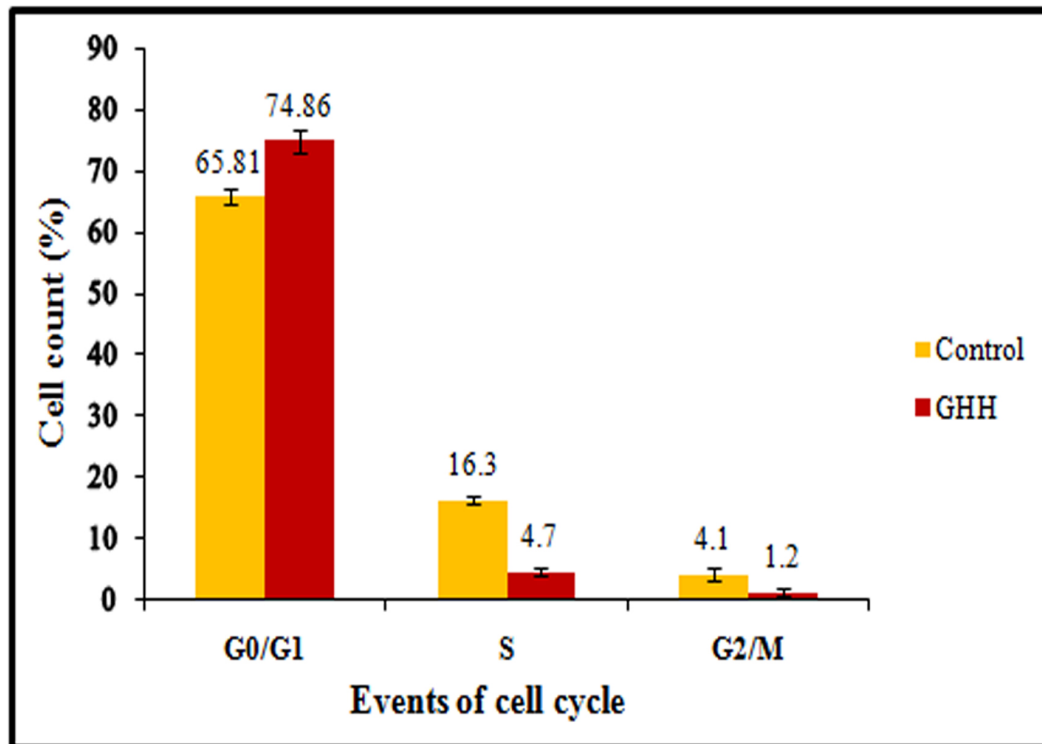
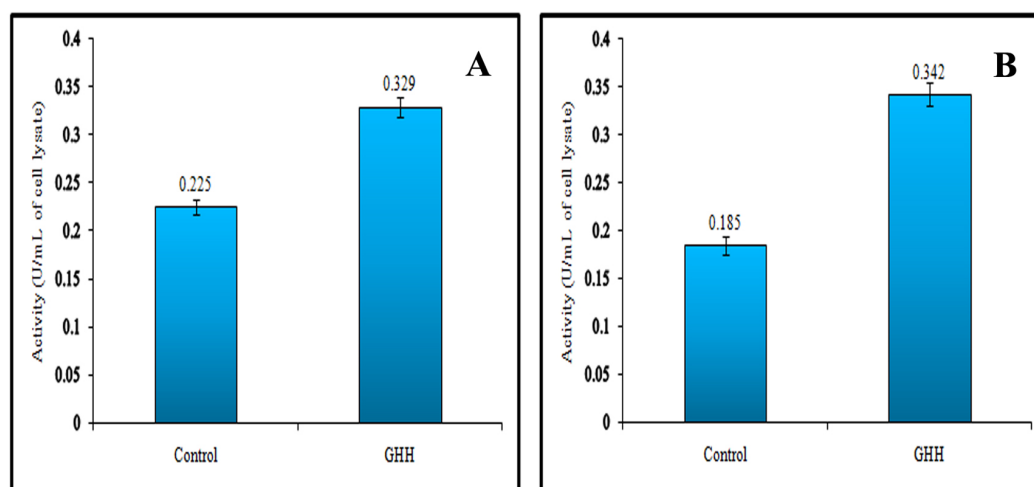


Fig. 47: Effect of methanolic extract of *G. heyneanum* var. *heyneanum* on caspase activity. A: Caspase-9 B: Caspase-7



GHH - *G. heyneanum* var. *heyneanum*

Fig. 48: Expression analysis of gene p53, p21 and β -actin using Real Time PCR

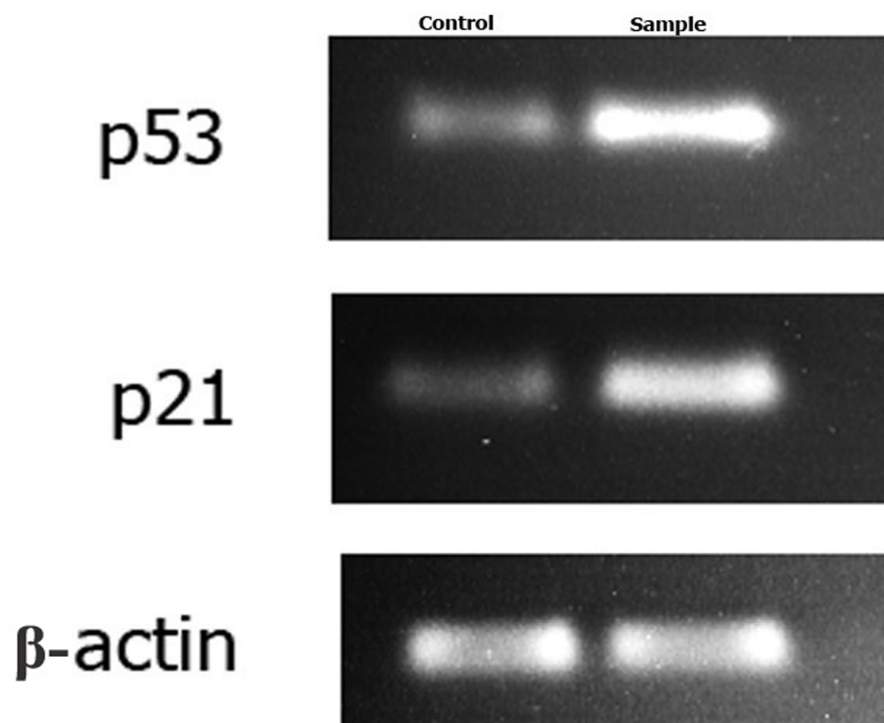
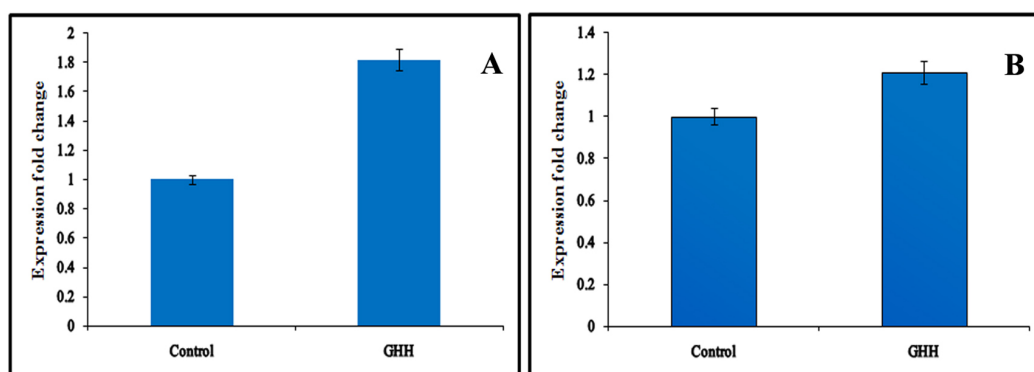


Fig. 49: Expression fold change of apoptosis related genes in MCF-7 cells treated (24 h) with methanolic extract (45 μ g/mL) of *G. heyneanum* var. *heyneanum*. A: p53 B: p21



GHH - *G. heyneanum* var. *heyneanum*

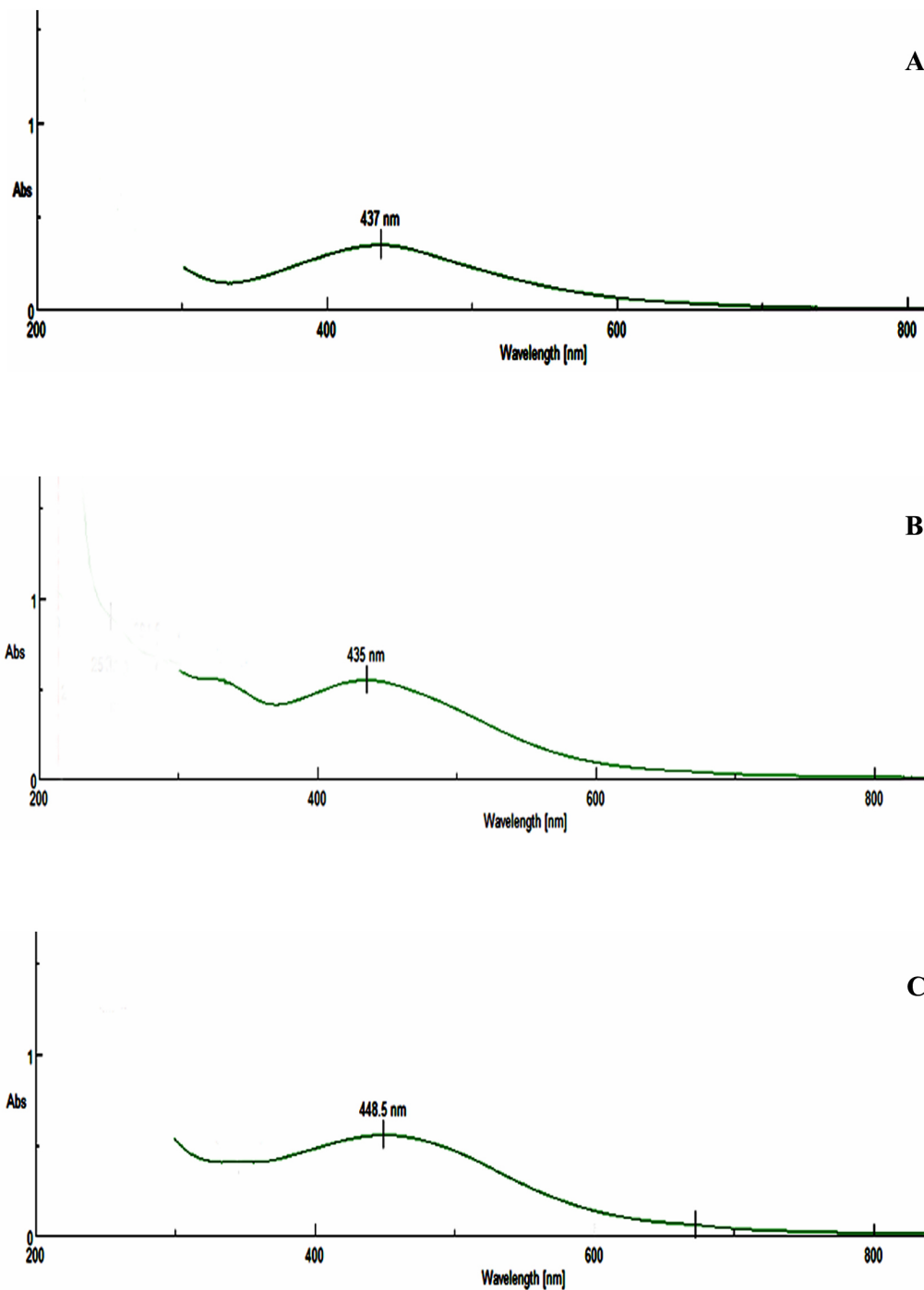


Fig. 50: UV- Vis spectra of silver nanoparticles synthesised by selected species of *Gomphostemma*. A: *G. heyneanum* var. *heyneanum* B: *G. heyneanum* var. *rottleri* C: *G. eriocarpum*

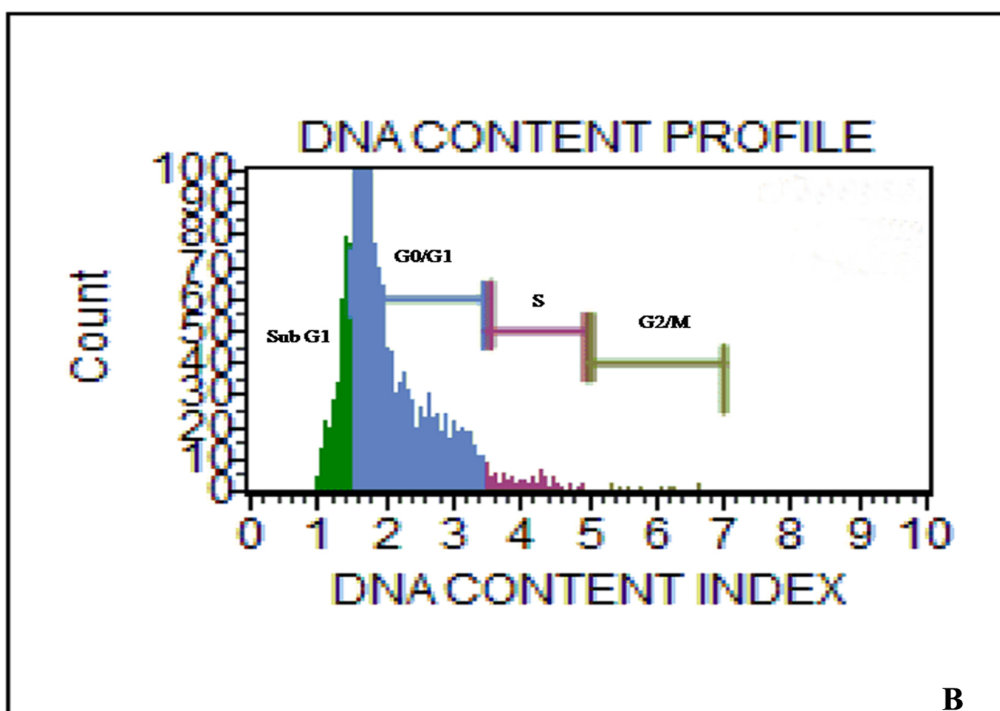
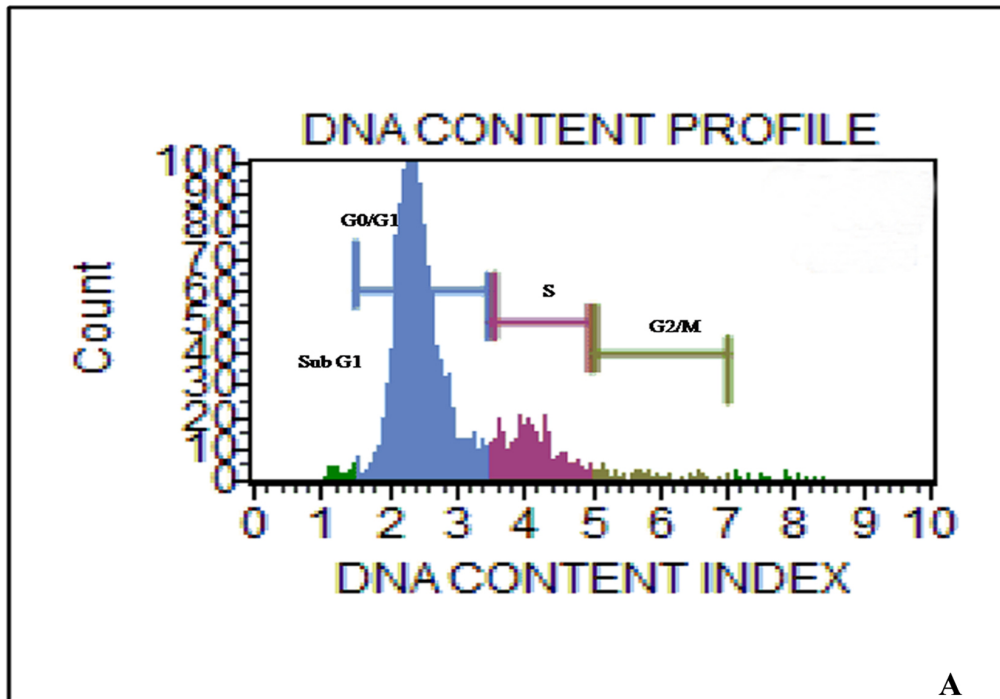


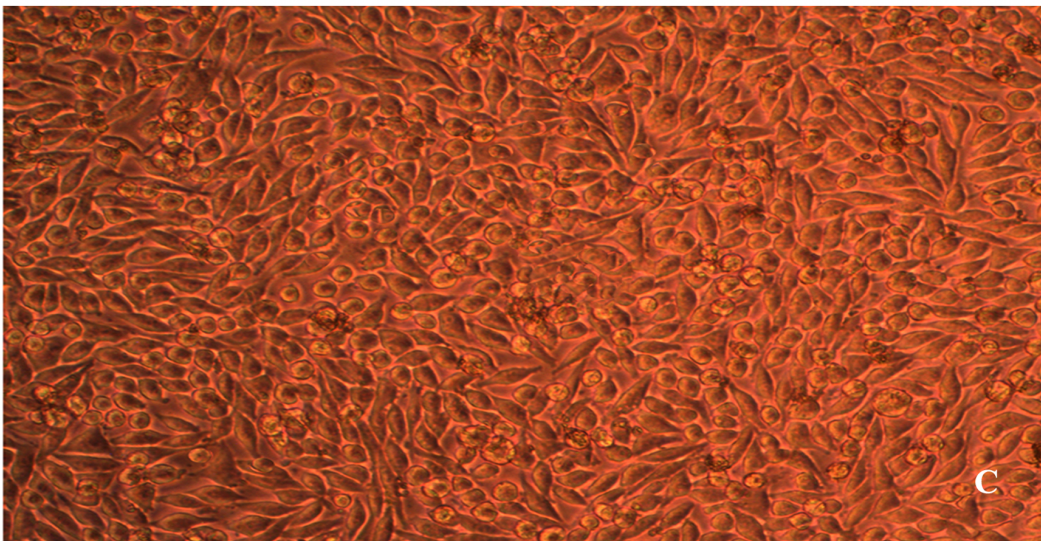
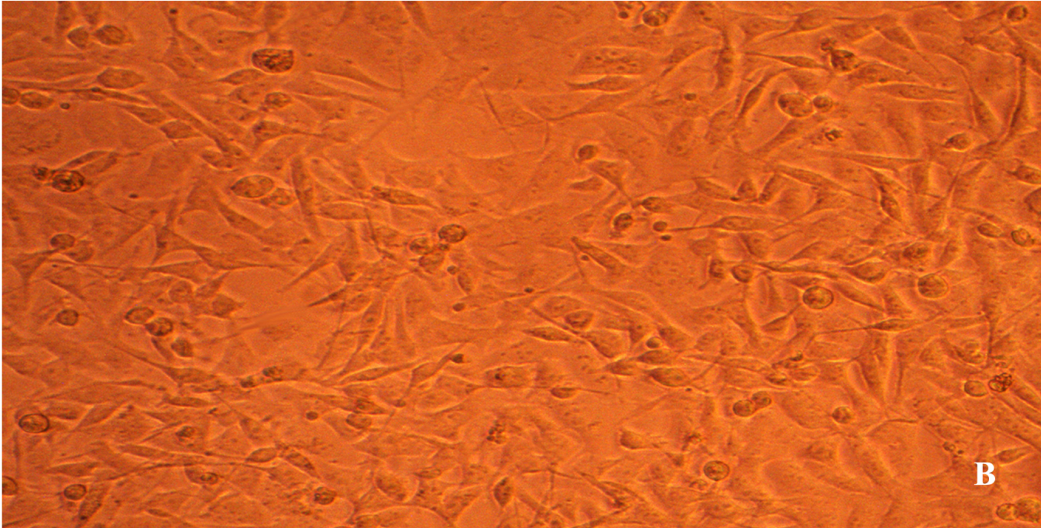
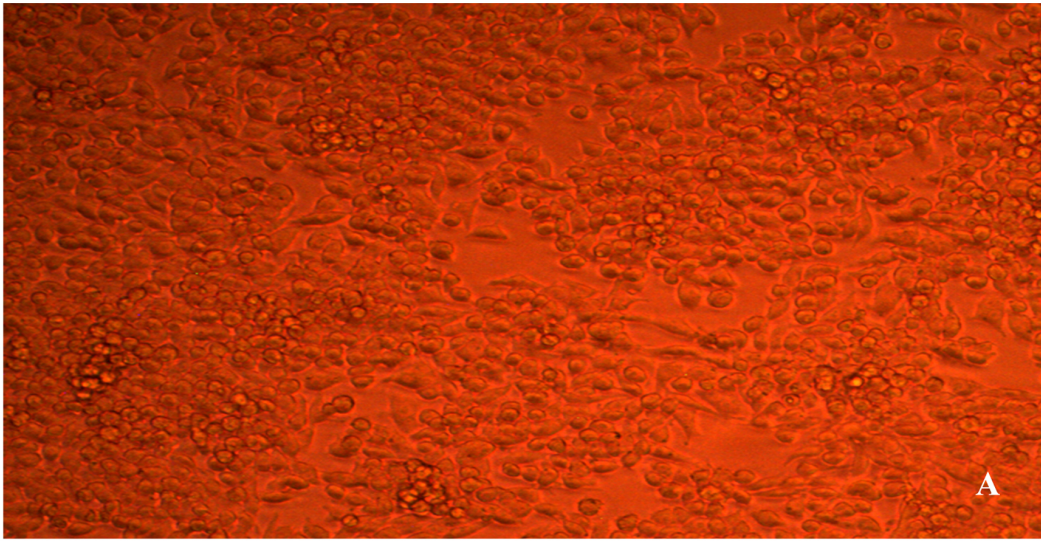
Fig. 44: Flow cytometry histograms of cell cycle analysis of MCF-7 cells.
A: Control **B:** MCF-7 cells treated (24 h) with methanolic extract (45 $\mu\text{g/mL}$) of *G. heyneanum* var. *heyneanum*

PLATE 1



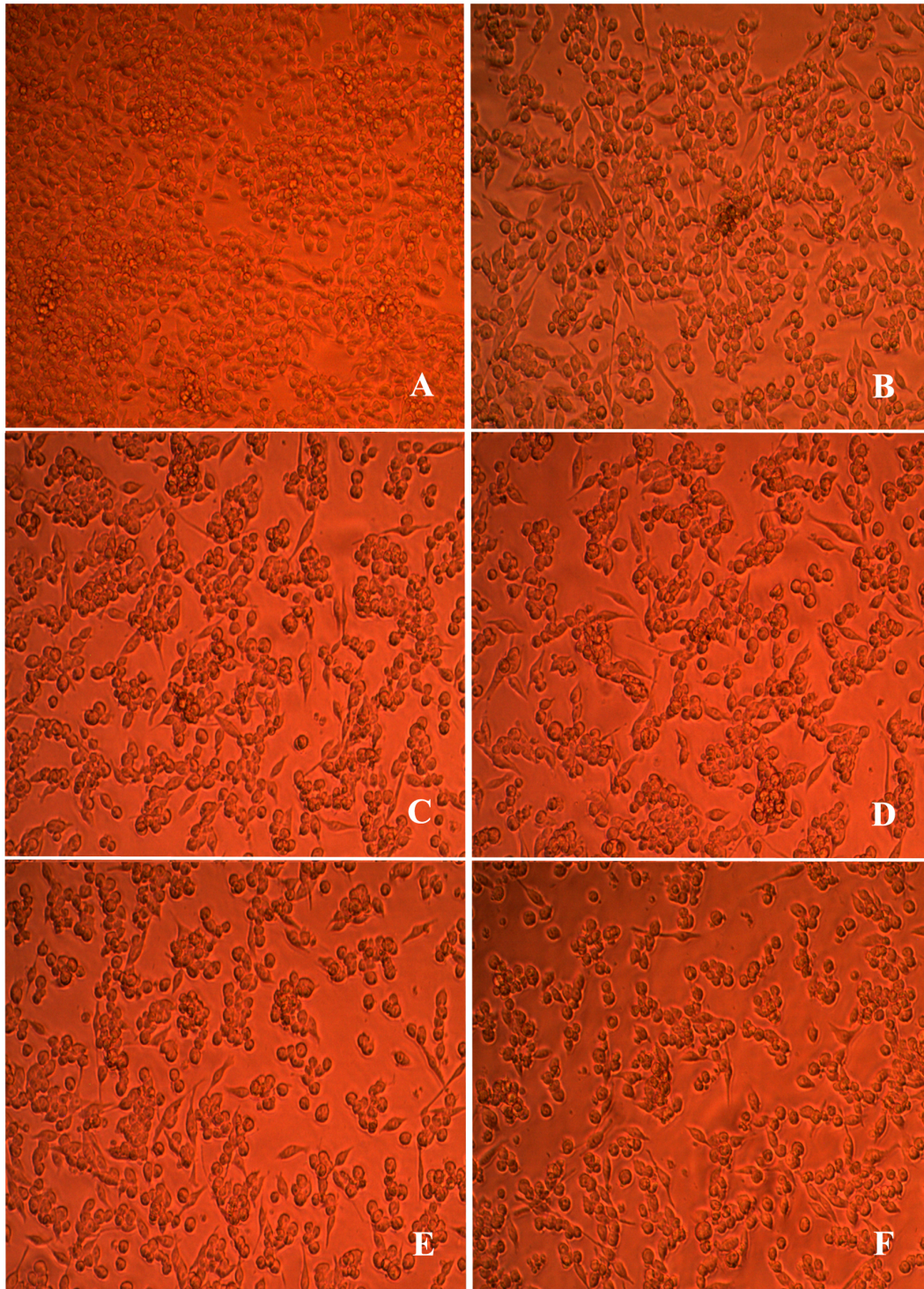
Habit - A: *Gomphostemma heyneanum* Wall. ex Benth. var. *heyneanum* **B:** *Gomphostemma heyneanum* var. *rotteri* Prain **C:** *Gomphostemma eriocarpum* Benth.
Inset: View of inflorescence of respective plants

PLATE 2



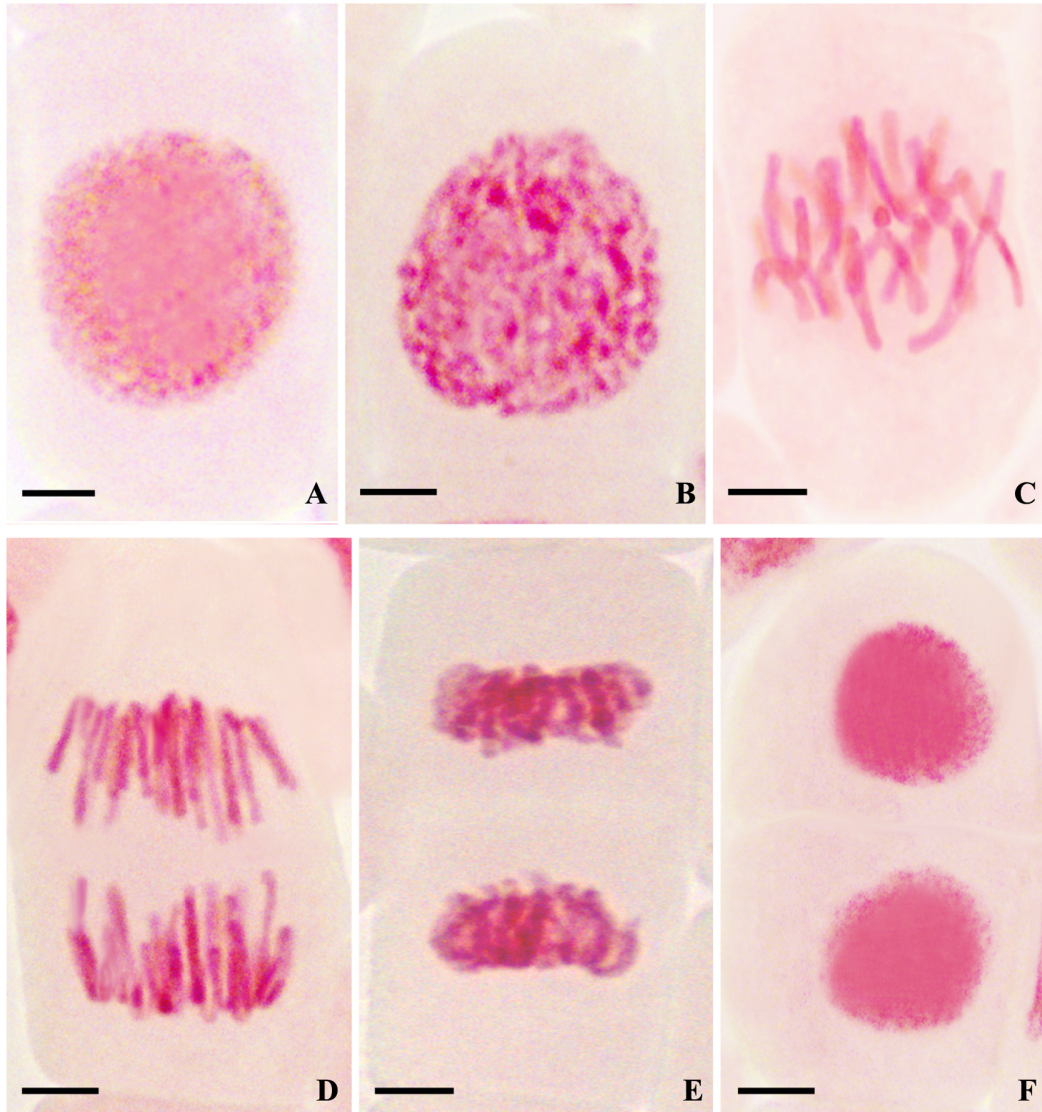
Cell lines used in the study A: RAW 264.7 B: MCF-7 C: L929

PLATE 3



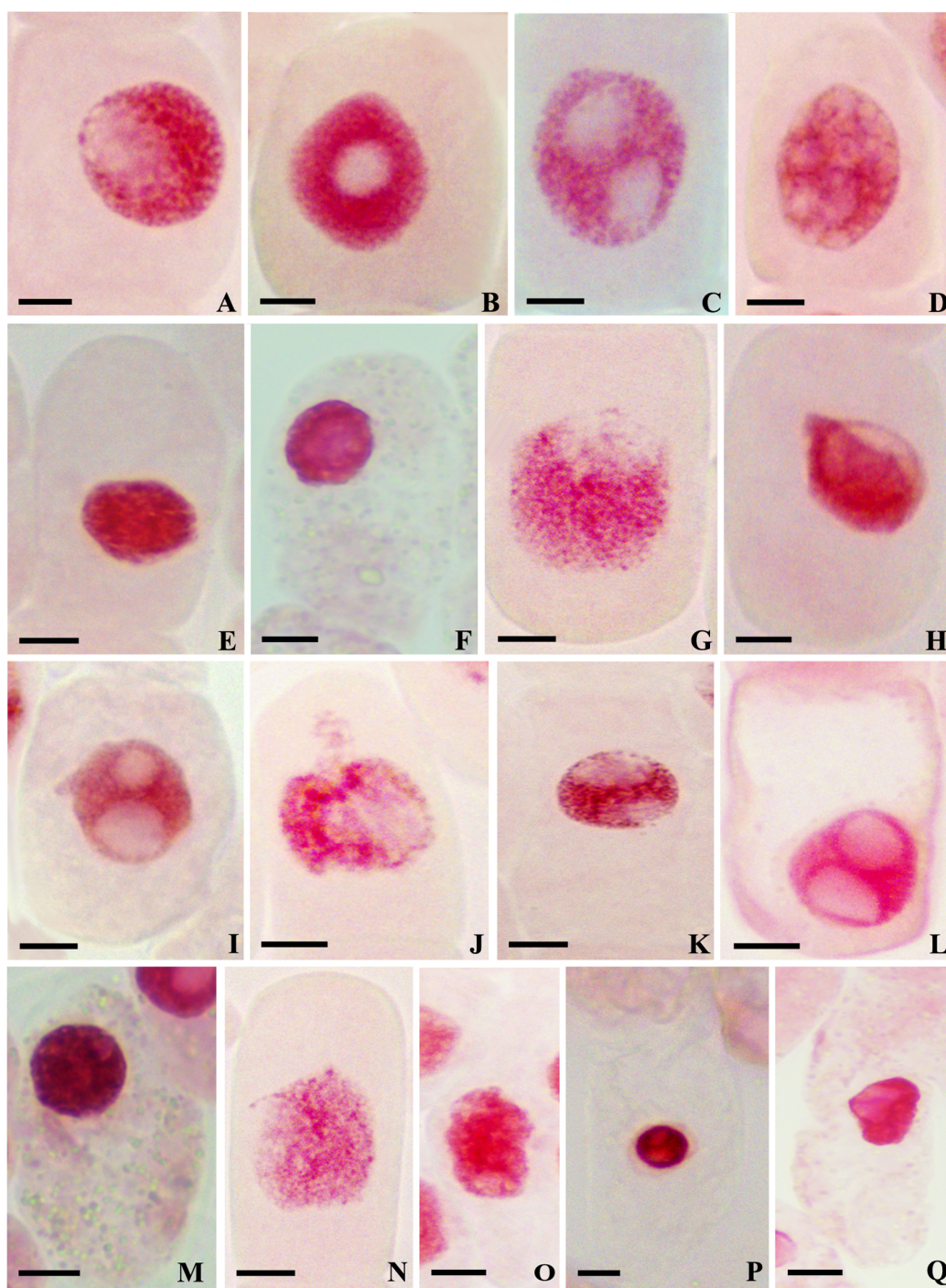
Cytotoxic effect of *G. heyneanum* var. *heyneanum* on RAW 264.7 cells. A: Control B: 10 µg/mL C: 20 µg/mL D: 30 µg/mL E: 40 µg/mL F: 50 µg/mL

PLATE 4



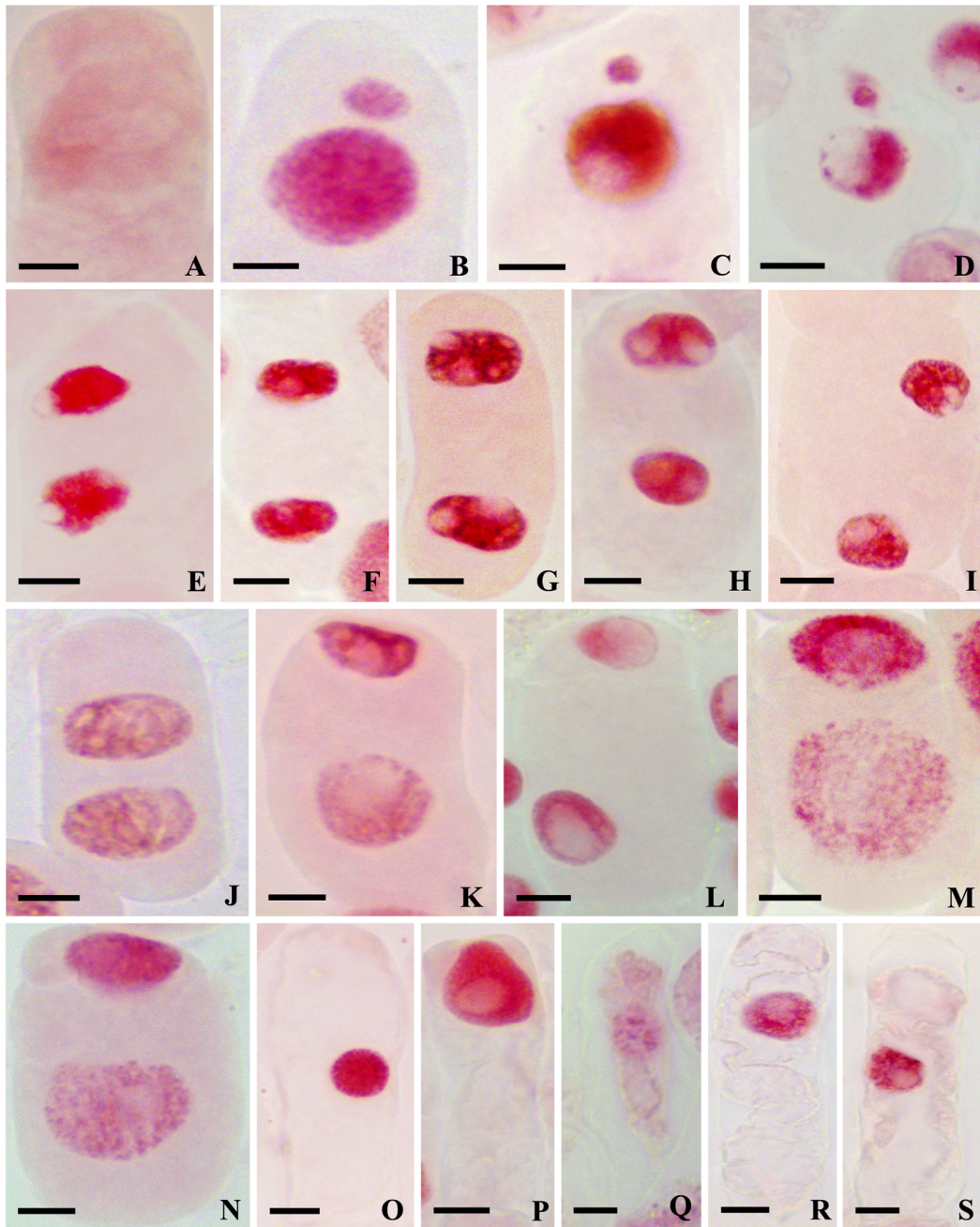
Normal mitotic stages in *Allium cepa* root . A: Interphase B: Prophase C: Metaphase D: Anaphase E: Telophase F: Cytokinesis.
Bar – 10 µm

PLATE 5



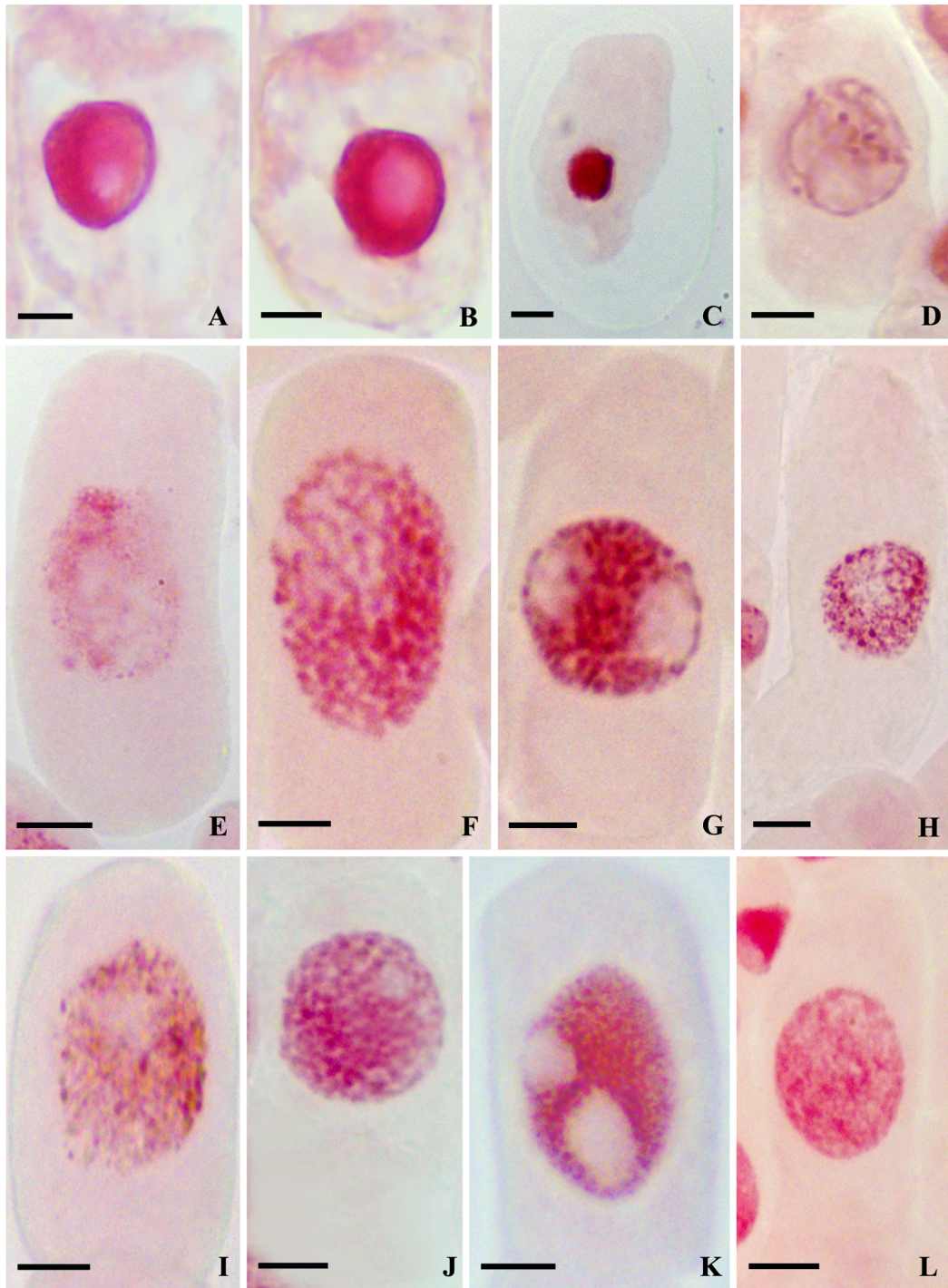
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root: Interphase - **A:** Single nuclear lesion formation **B:** Single nuclear lesion **C:** Double nuclear lesion **D:** Multiple nuclear lesion **E:** Nuclear breakage and abnormal condensation **F:** Nuclear displacement **G:** Nuclear erosion **H:** Nuclear lesion and bleb formation **I:** Nuclear lesion and nuclear blebbing **J:** Nuclear budding and lesion **K:** Double nuclear lesion and erosion **L:** Double nuclear lesion and cytoplasmic vacuolation **M:** Hyperchromasia with cytoplasmic shrinkage **N:** Nuclear peak and erosion **O:** Chromatin condensation, nuclear disintegration and blebbing **P:** Nuclear diminution **Q:** Bizarre nucleus showing lesion and cytoplasmic fragmentation. **Bar – 10 μm**

PLATE 6



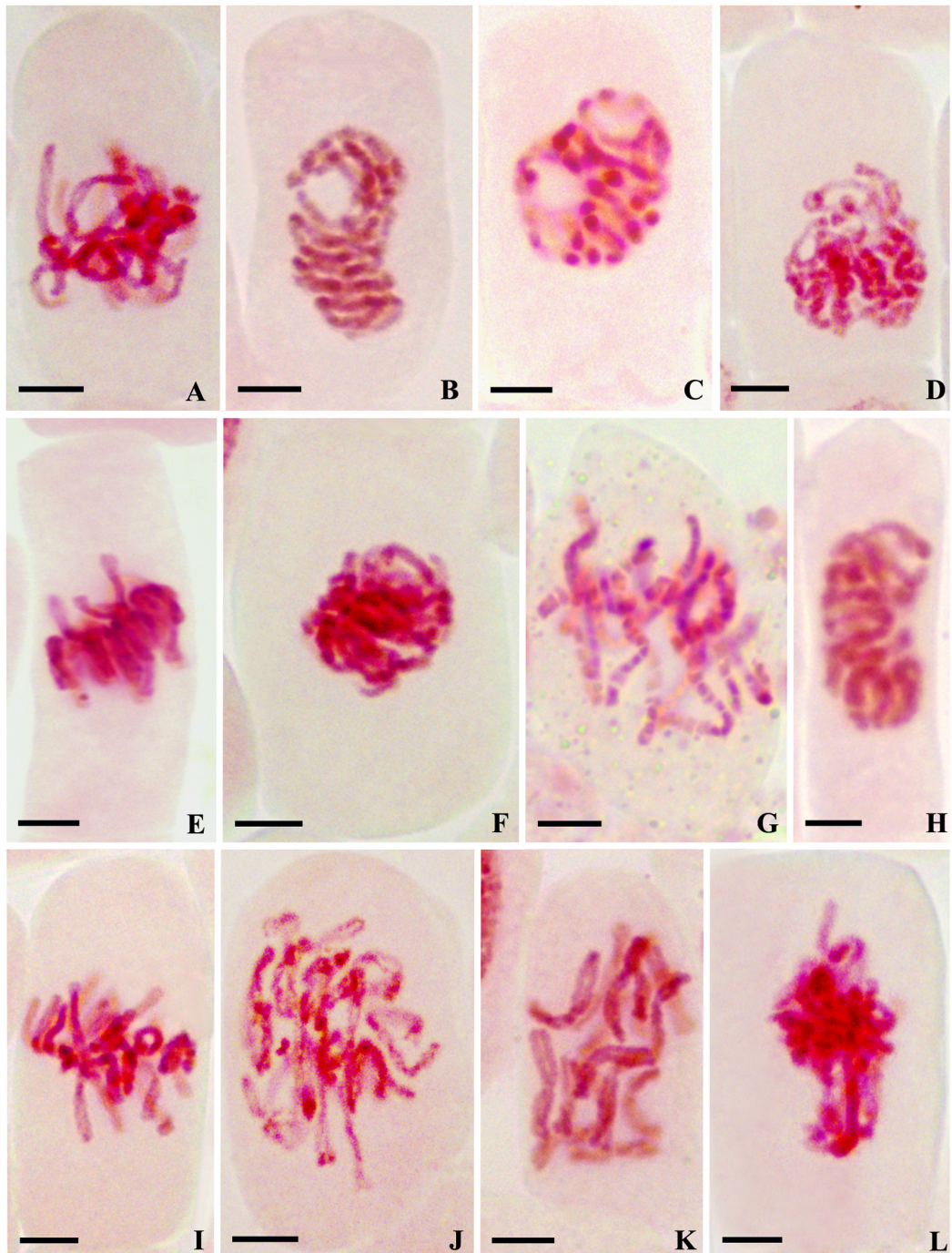
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Interphase - A: Ghost cell **B:** Micronucleus **C:** Nuclear lesion and micronucleus **D:** Nuclear erosion and pulverized micronucleus **E:** Bizarre binucleate cell showing lesion **F:** Nuclear lesion in binucleate cell **G:** Multiple nuclear lesion in binucleate cell **H:** Double nuclear lesion in binucleate cell **I:** Binucleate cell showing nuclear displacement and multiple nuclear lesion **J:** Nuclear erosion in binucleate cell **K:** Non-synchronized macro and micro nucleus at early and late interphase **L:** Macro and microcell formation showing nuclear lesion **M:** Macro and micro cell showing nuclear erosion **N:** Micro and macro cell **O:** Displaced nucleus showing hyperchromasia **P:** Nuclear peak, lesion and cytoplasmic shrinkage **Q:** Receding and breakage of cytoplasm showing nuclear fragmentation **R:** Cytoplasmic shrinkage and vacuolation **S:** Cytoplasmic and nuclear shrinkage. **Bar – 10 μ m**

PLATE 7



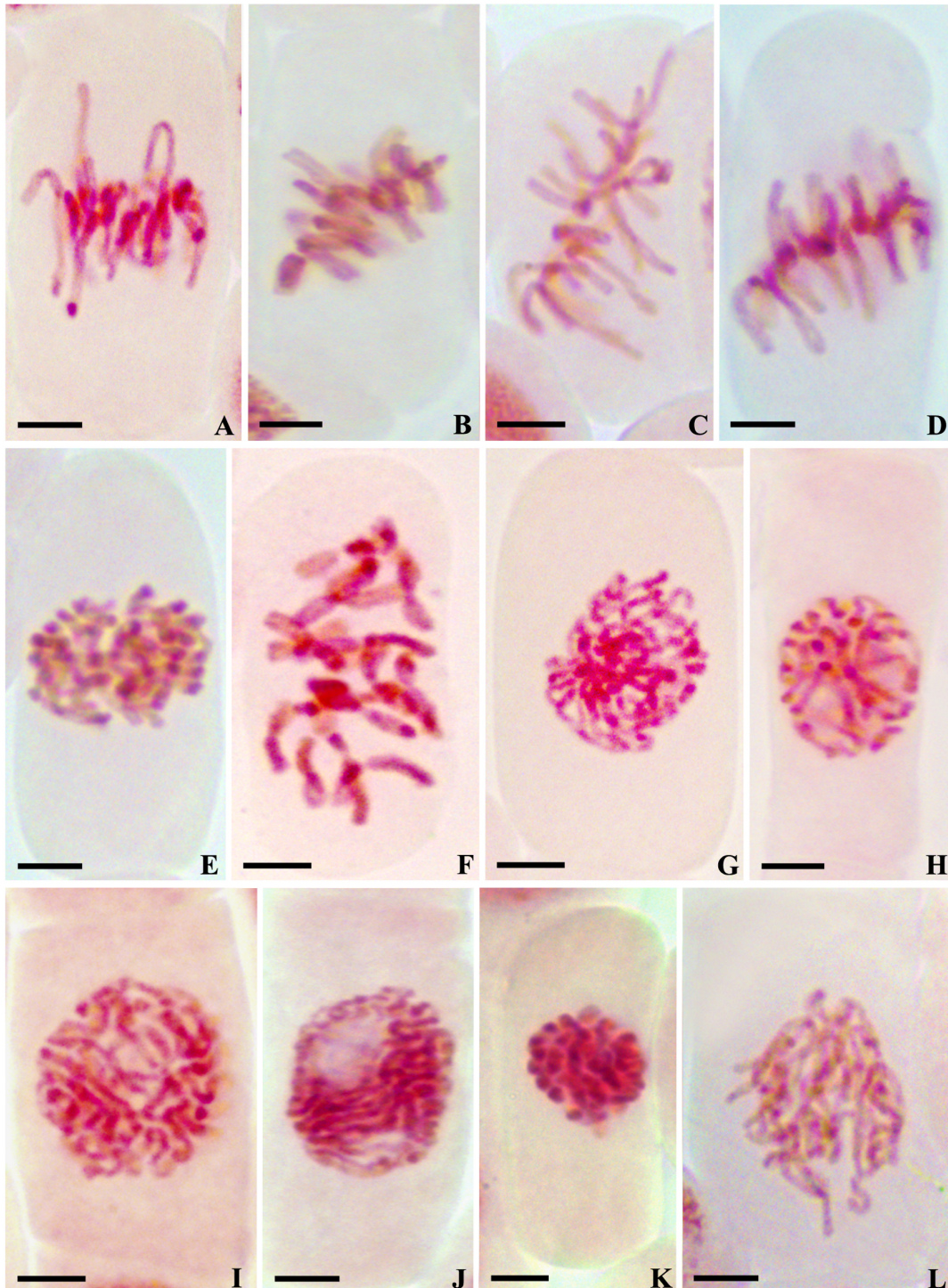
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Interphase - A: Nuclear lesion and cytoplasmic vacuolation B: Nuclear lesion and blebs with cytoplasmic vacuolation C: Cytoplasmic shrinkage and hyperchromasia D: Nuclear fragmentation Prophase - E: Fragmented and disintegrated nucleus F: Chromatin erosion G: Double nuclear lesion H: Pulverized nucleus showing formation of lesion I: Disintegrated nucleus J: Single nuclear lesion K: Double nuclear lesion L: Pulverized chromatin at early prophase. Bar – 10 μm

PLATE 8



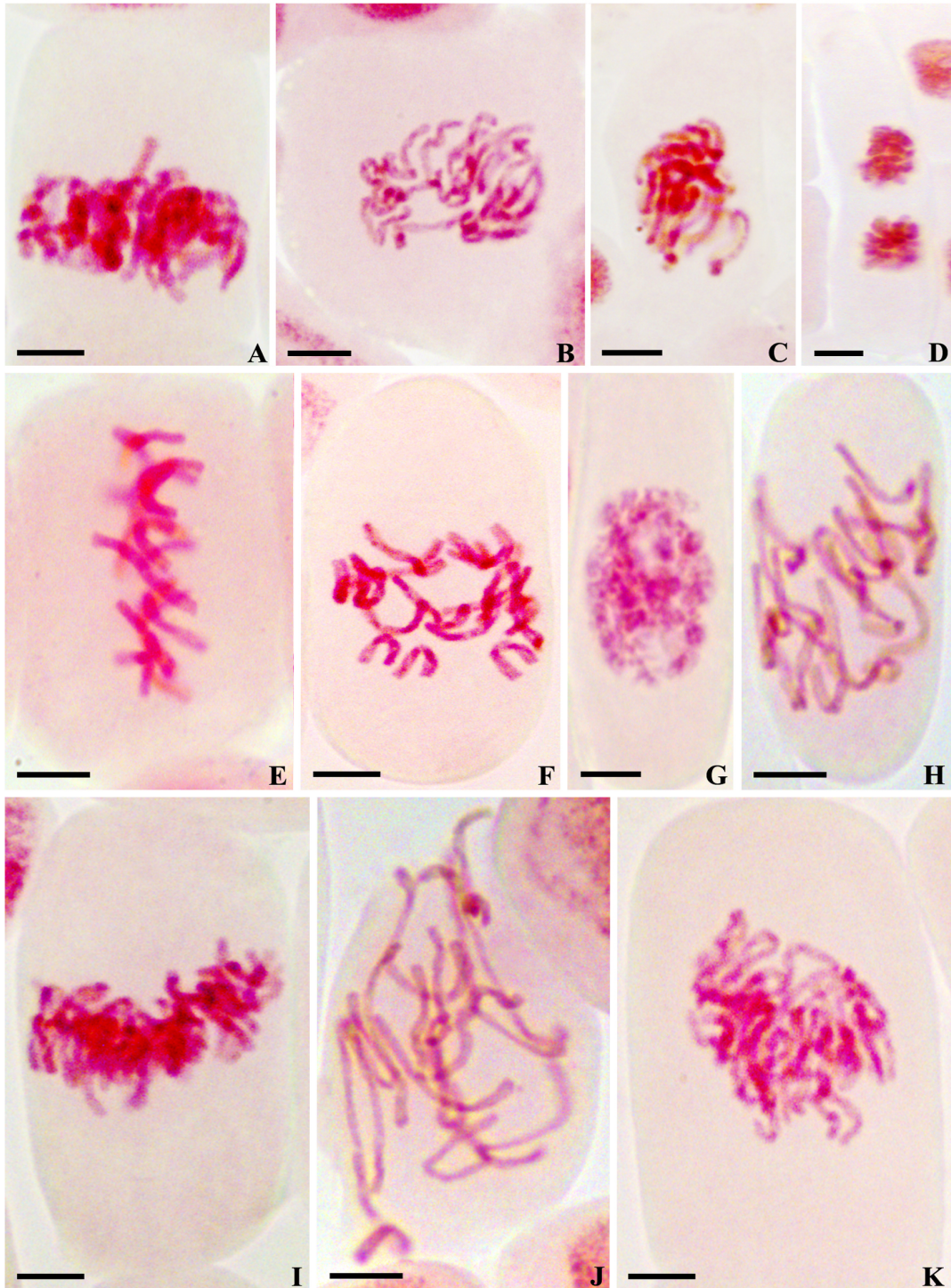
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Metaphase - A: Abnormal condensation **B:** Chained early metaphase **C:** Chained early metaphase showing chromosome erosion **D:** Chained metaphase **E:** Chromosome clumping showing displacement **F:** Chromosome erosion in hypoploid cell **G:** Chromosome gaps at disturbed metaphase **H:** Chromosome fragments in pole to pole metaphase **I:** Chromosome ring at metaphase **J:** C-metaphase **K:** C-metaphase showing misorientation **L:** Coagulated metaphase. **Bar – 10 μ m**

PLATE 9



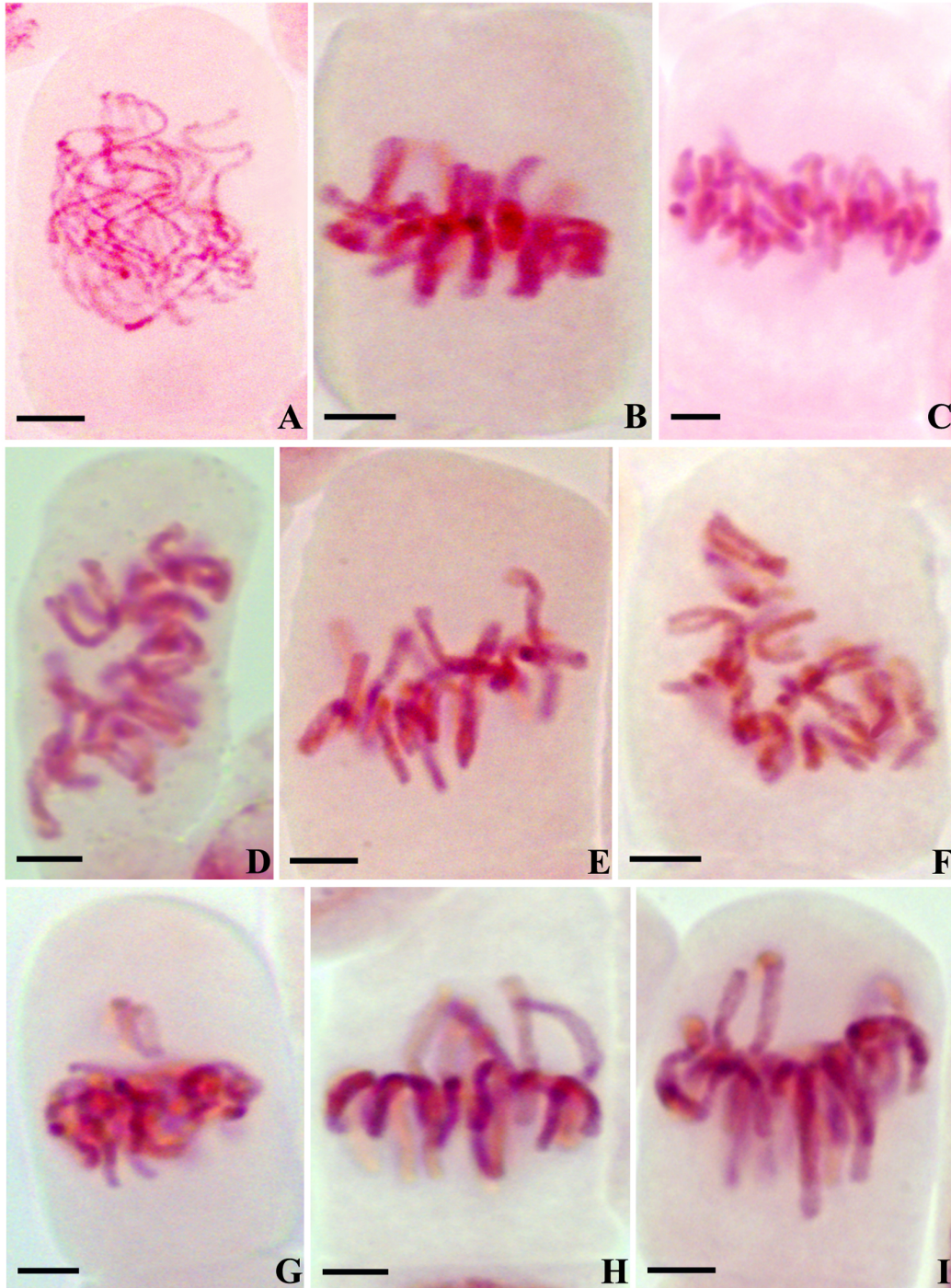
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Metaphase – A: Disturbed metaphase **B:** Diagonal partial C-metaphase in hypoploid cell **C:** Diagonal partial C-metaphase **D:** Diagonal sticky metaphase **E:** Disturbed ball metaphase **F:** Cytostasis at metaphase **G:** Early ball metaphase **H:** Early ball metaphase showing abnormal condensation **I:** Early ball metaphase showing chromosome gaps **J:** Formation of lesion zone in ball metaphase **K:** Sticky ball metaphase **L:** Disturbed early metaphase in hyperploid cell. **Bar – 10 μ m**

PLATE 10



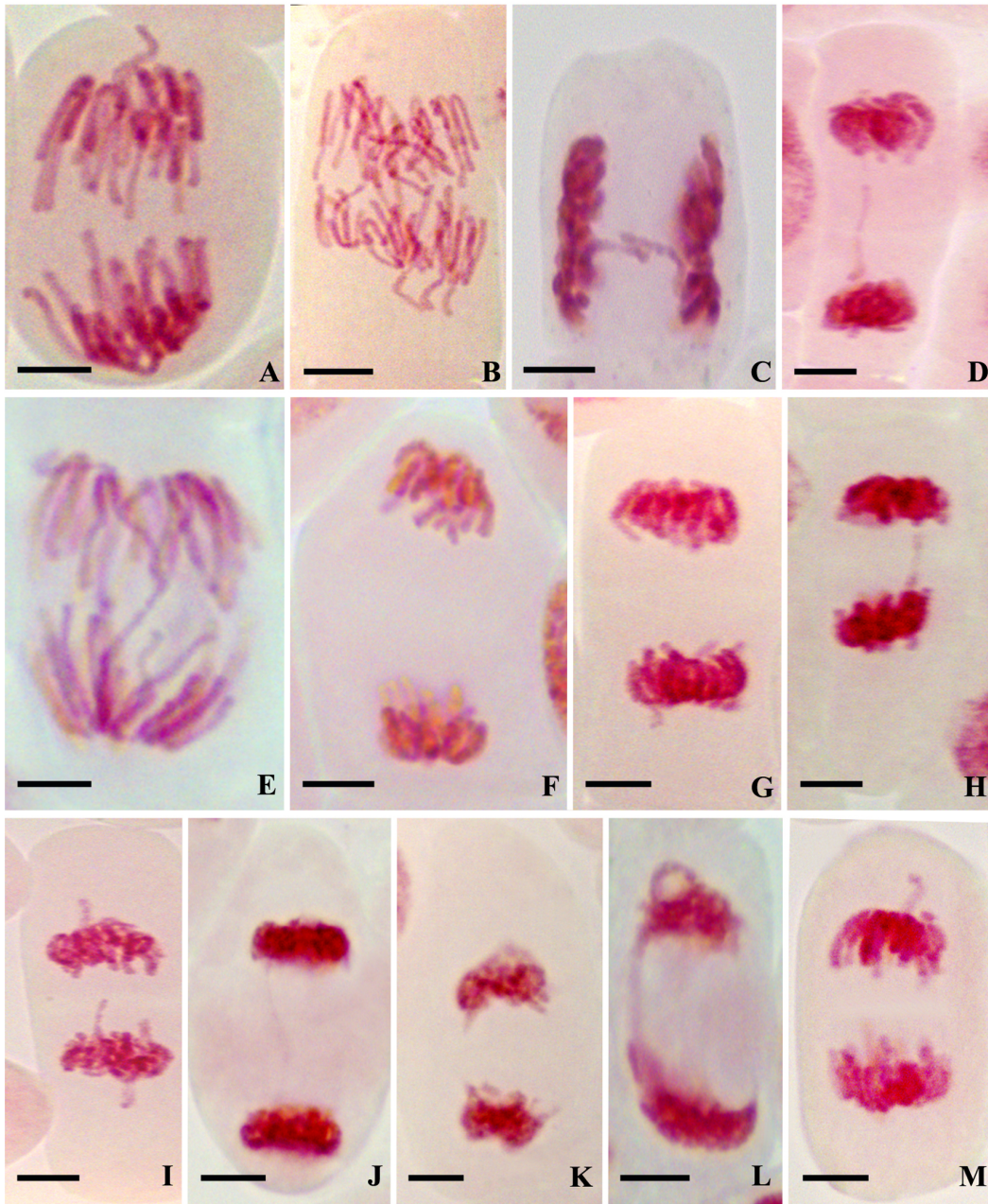
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Metaphase – A: Misorientation and chromosome gaps **B:** Early metaphase in hypoploid cell **C:** Misorientation at metaphase **D:** Hypercondensed double metaphase in binucleate cell **E:** Pole to pole metaphase **F:** Partial C-metaphase **G:** Pulverized ball metaphase **H:** Scattered metaphase **I:** Polyploid cell showing chromosome clumping **J:** Scattered early metaphase showing unequal condensation **K:** Slightly disturbed early metaphase in hyperploid cell.
Bar – 10 μ m

PLATE 11



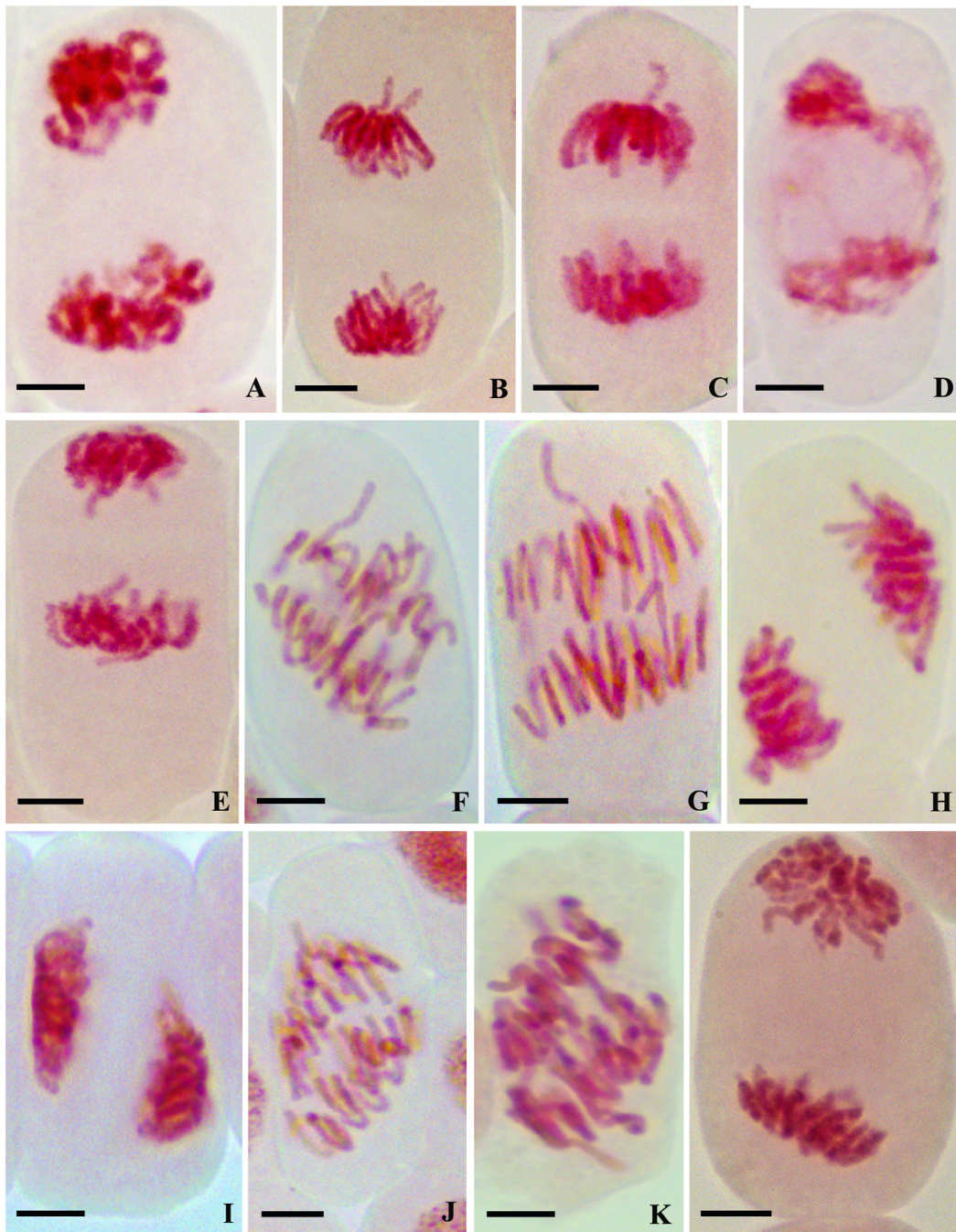
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Metaphase – A: Somatic association at early metaphase B: Sticky metaphase C: Sticky metaphase in hyperploid cell D: Sticky diagonal C-metaphase E: Sticky partial C-metaphase F: Sticky C- metaphase showing misorientation G: Sticky metaphase showing chromosome clumping and vagrants H: Vagrants at partial C- metaphase I: Tropokinesis. Bar – 10 μm

PLATE 12



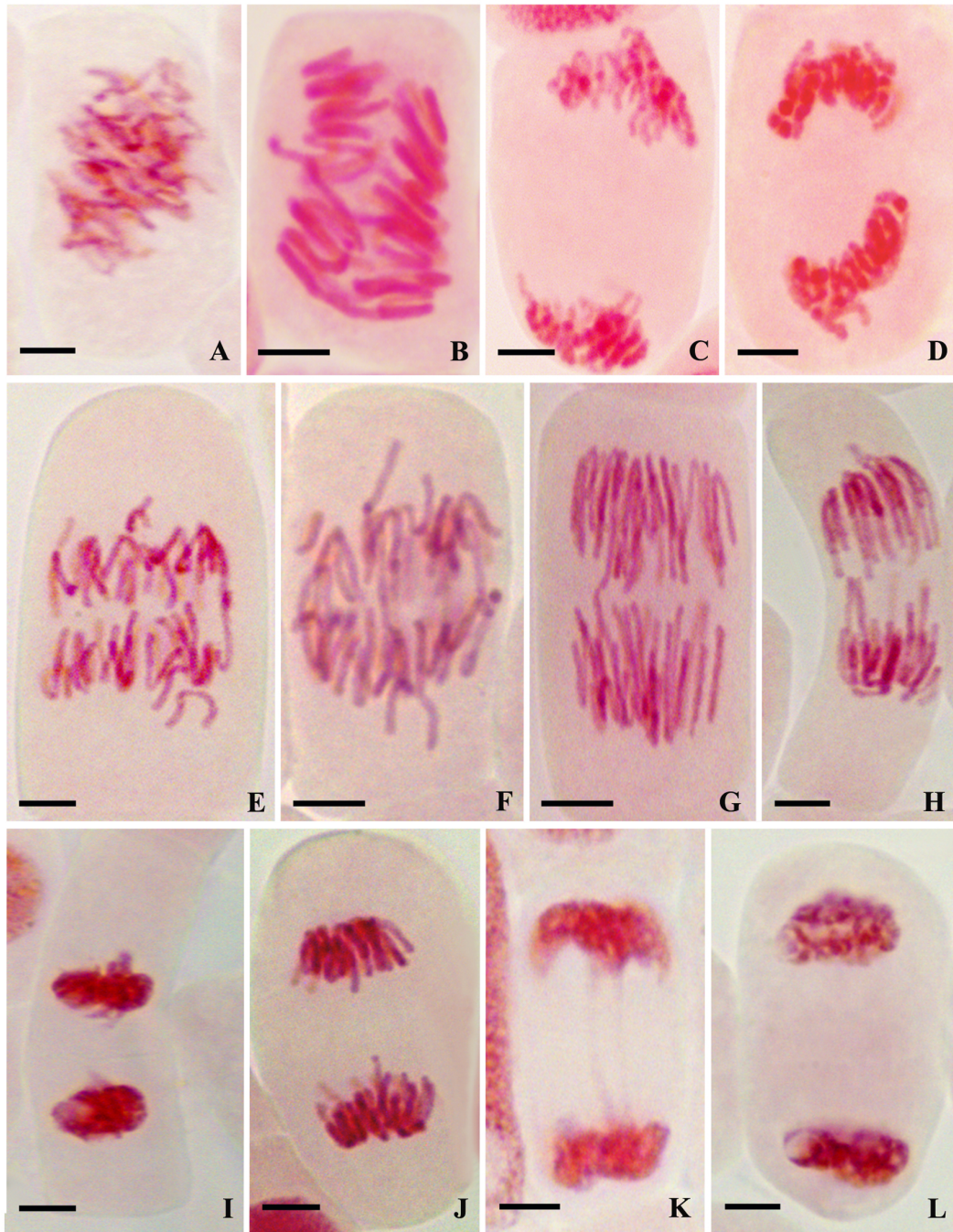
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Anaphase – A: Chromosome vagrants B: Bridges and vagrants C: Broken chromosome bridge after equatorial separation D: Broken chromosome bridge E: Chromosome bridges F: Chromosome erosion G: Chromosome fragments H: Coagulated anaphase showing bridge I: Chromosome clumping and vagrants J: Coagulated anaphase showing chromosome bridge K: Coagulated anaphase showing chromosome fragments L: Coagulated anaphase showing compound bridges M: Coagulated anaphase showing early cell plate formation. Bar – 10 μm

PLATE 13



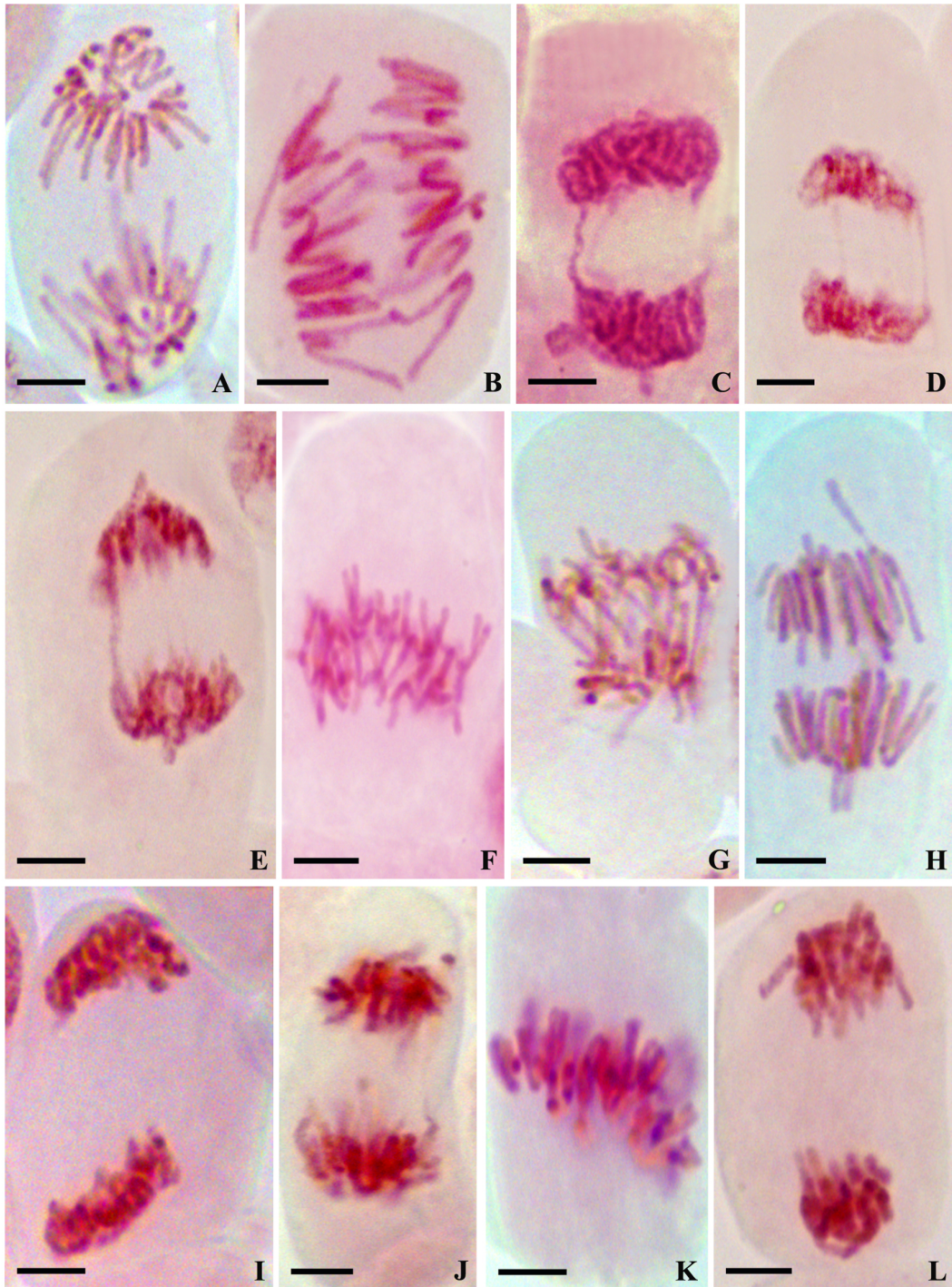
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Anaphase – A: Coagulated anaphase showing misorientation **B:** Coagulated late anaphase **C:** Coagulated anaphase showing vagrants and early cell plate formation **D:** Compound coagulated chromosome bridges showing chromosome erosion **E:** Misorientation at sticky anaphase **F:** Diagonal anaphase showing early movement **G:** Diagonal anaphase showing vagrants **H:** Diagonal anaphase showing laggards and vagrants **I:** Equatorial separation and chromosome erosion **J:** Diagonal scattered anaphase **K:** Diagonal anaphase showing chromosome bridges **L:** Hemistellate anaphase. **Bar – 10 μm**

PLATE 14



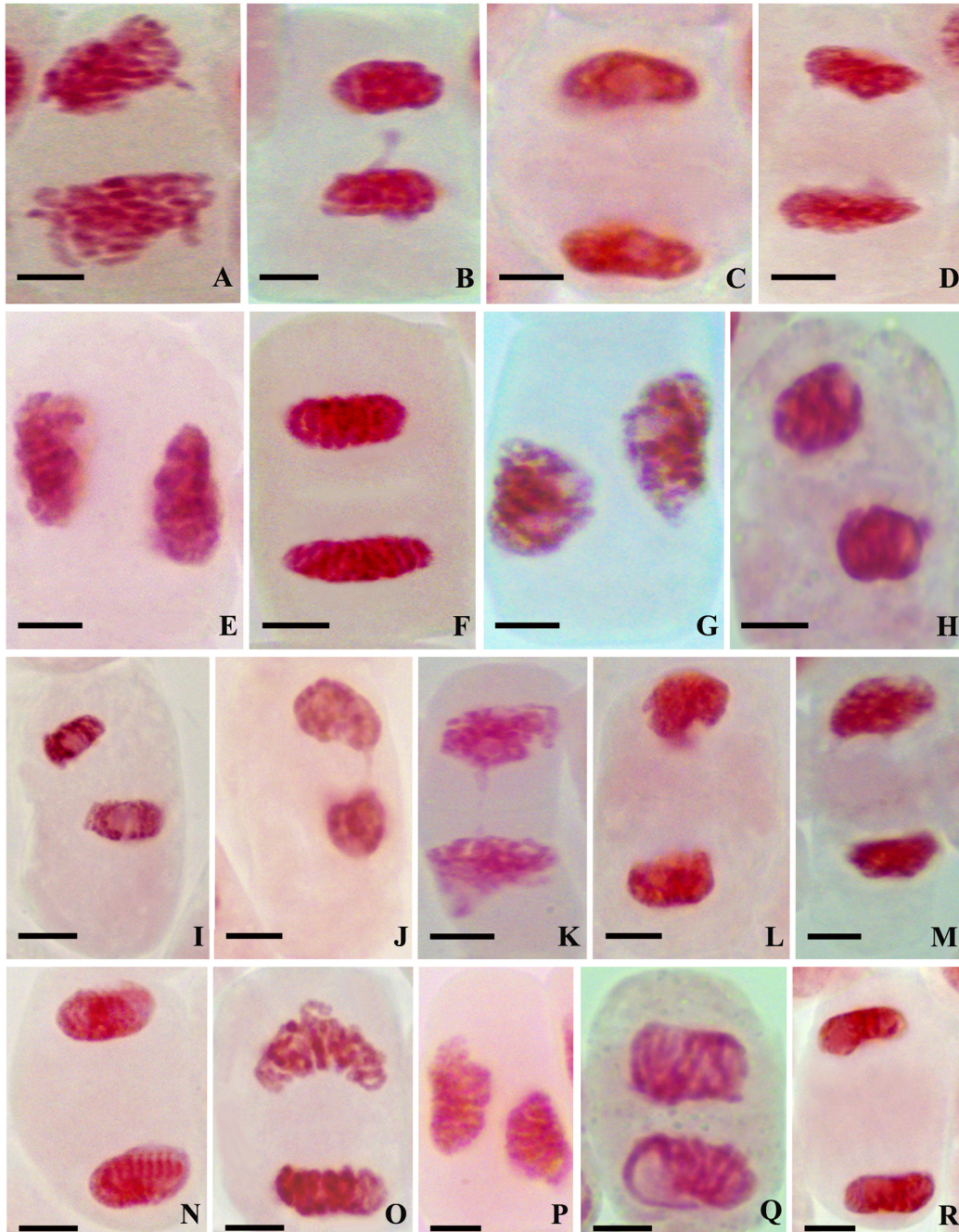
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Anaphase – A: Diagonal sticky stathmo-anaphase **B:** Equatorial separation, chromosome bridges and vagrants **C:** Diagonal partial chained late anaphase **D:** Shift in MTOC **E:** Early movement and vagrants **F:** Multiple bridges and vagrants **G:** Scattered anaphase **H:** Laggards at anaphase **I:** Misoriented coagulated anaphase showing early cell plate formation **J:** Sticky anaphase **K:** Sticky bridges at coagulated anaphase **L:** Pulverized chromosomes at late anaphase. **Bar – 10 μ m**

PLATE 15



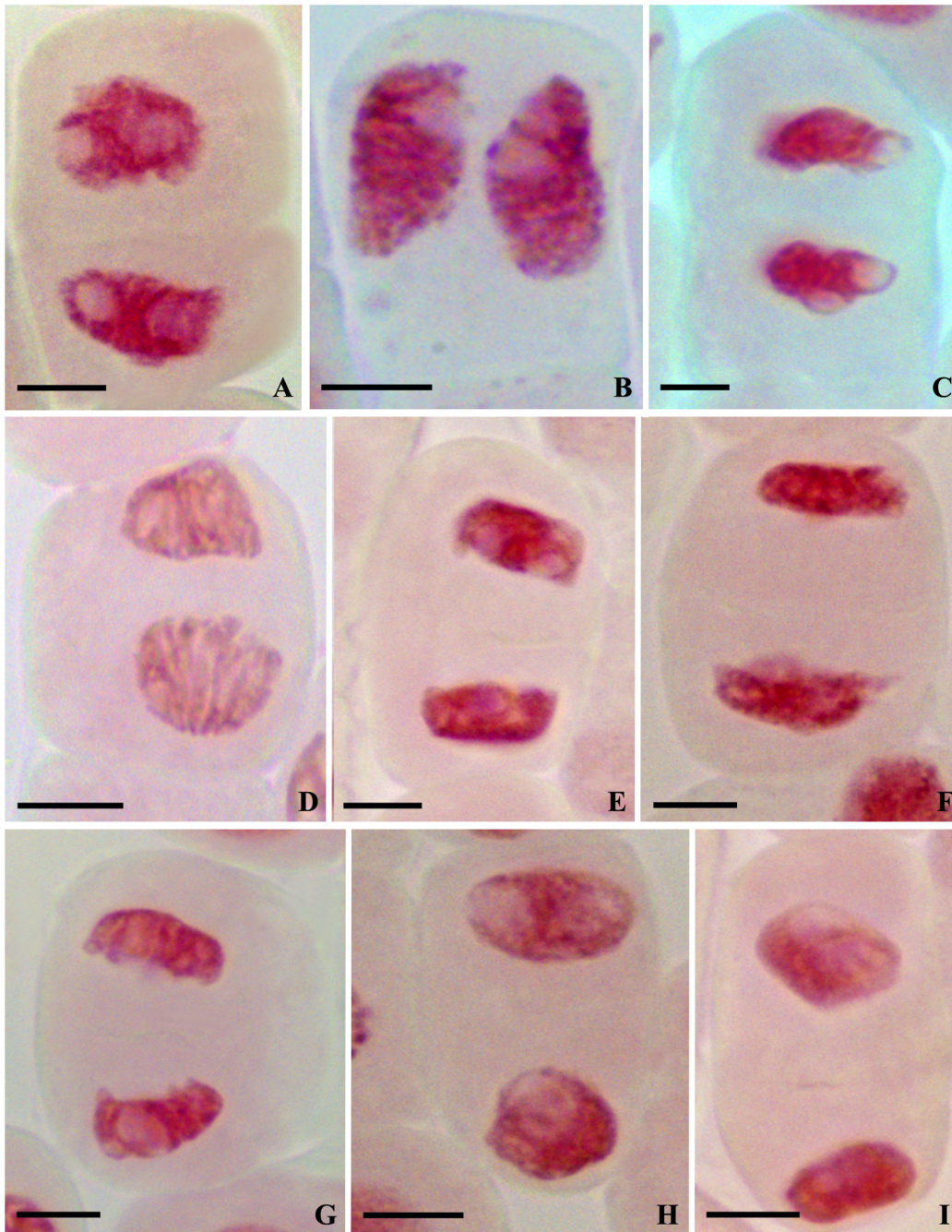
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Anaphase – A: Stellate anaphase **B:** Sticky bridges at anaphase after equatorial separation **C:** Sticky bridges at late anaphase **D:** Sticky bridges at anaphase **E:** Sticky complex bridges showing chromosome erosion at anaphase **F:** Sticky stathmo-anaphase **G:** Misoriented stathmo-anaphase **H:** Vagrants at anaphase **I:** Shift in MTOC at late anaphase **J:** Sticky stellate anaphase showing chromosome fragment **K:** Sticky unipolar anaphase **L:** Unequal separation at late anaphase. **Bar – 10 μ m**

PLATE 16



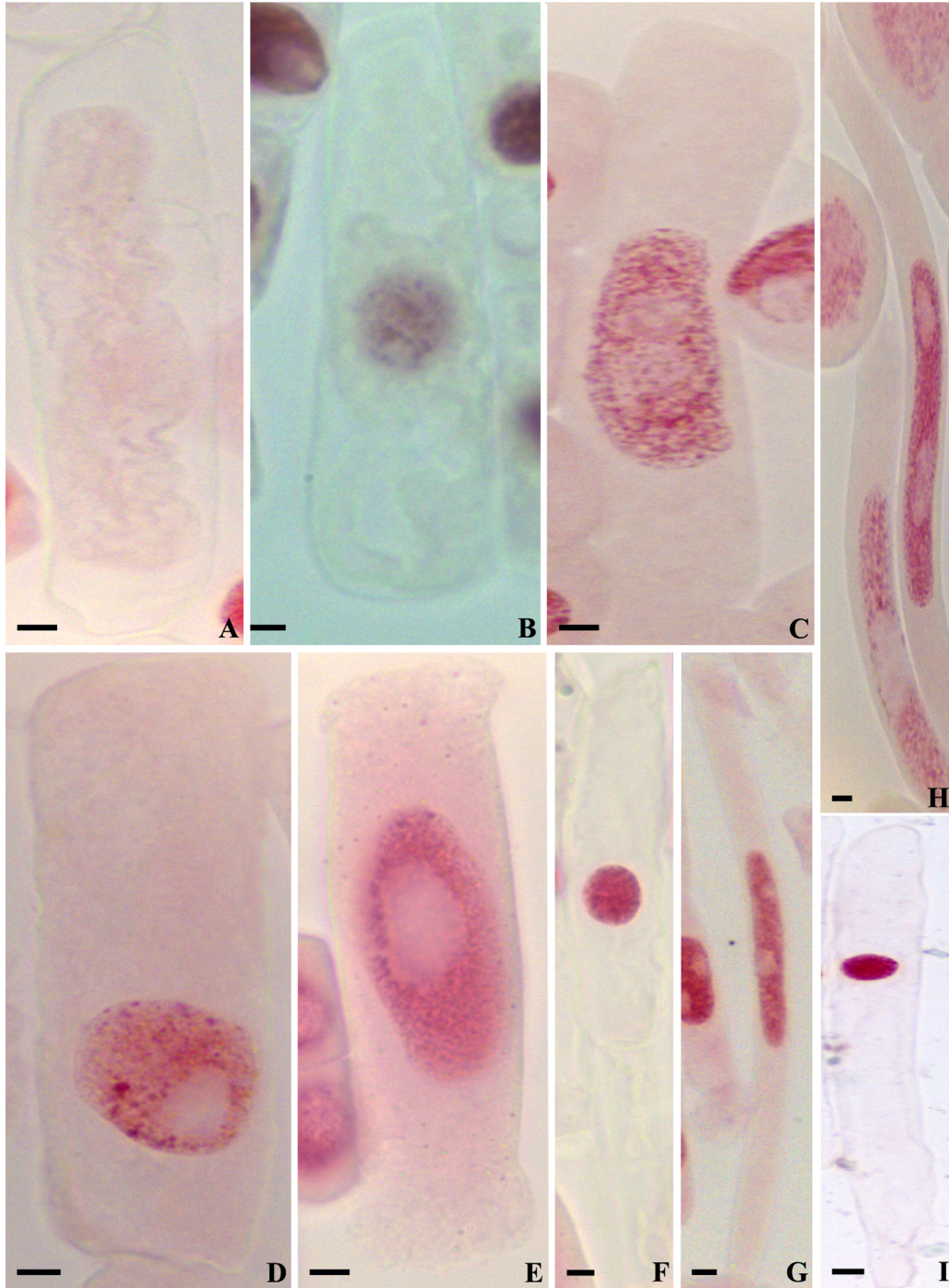
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Telophase – **A:** Bizarre late telophase showing vagrants **B:** Broken bridge **C:** Chromosome erosion **D:** Chromosome erosion at coagulated telophase **E:** Coagulated chromosome groups after equatorial separation **F:** Coagulated telophase with early cell plate formation **G:** Misoriented chromosome groups after equatorial separation **H:** Diagonal coagulated telophase showing chromosome fragment **I:** Shift in MTOC, formation of lesion and abnormal decondensation **J:** Persistent chromosome bridge at telophase showing chromosome erosion **K:** Persistent uneven bridge **L:** Coagulated telophase **M:** Coagulated unequal chromosome groups **N:** Pulverized chromatin **O:** Non-synchronized condensation **P:** Sticky telophase after equatorial separation **Q:** Sticky telophase showing chromosome erosion **R:** Sticky late telophase. **Bar – 10 μ m**

PLATE 17



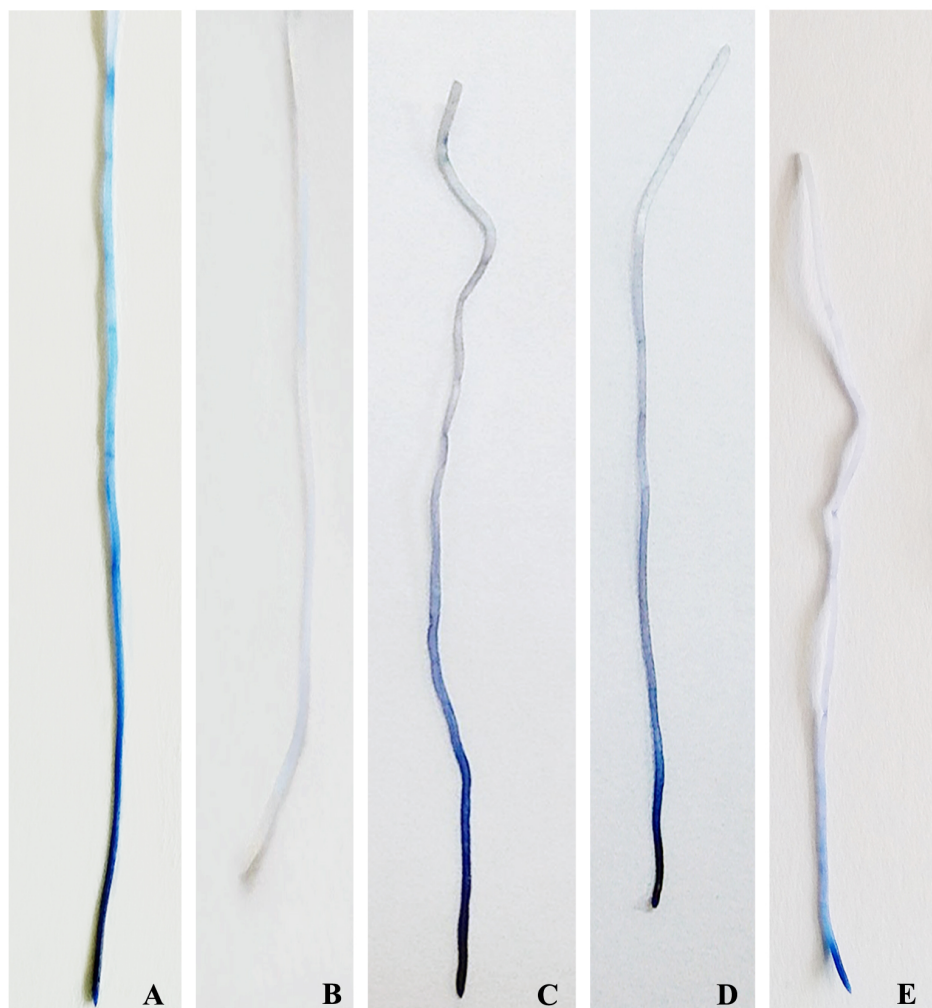
Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Cytokinesis – A: Chromatin erosion at cytokinesis B: Chromosome groups showing chromatin lesions after equatorial separation C: Coagulated chromosome with lesion D: Unequal chromosome groups E: Multiple nuclear lesion F: Pulverized chromatin G: Lesion and erosion at early cytokinesis H: Nuclear lesion at coagulated cytokinesis I: Nuclear lesion at sticky cytokinesis. Bar – 10 µm

PLATE 18



Chromosomal aberrations induced by selected species of *Gomphostemma* on *Allium cepa* root. Strap & Giant cells – A: Giant ghost cell showing cytoplasmic shrinkage **B:** Giant cell showing pulverized chromatin at interphase **C:** Giant cell showing formation of nuclear lesion at interphase **D:** Giant cell showing nuclear lesion and abnormal condensation **E:** Giant cell showing nuclear lesion **F:** Giant cell showing cytoplasmic shrinkage **G:** Strap cell with double nuclear lesion **H:** Strap cells showing lesion **I:** Strap cell showing nuclear diminution. **Bar – 10 μ m**

PLATE 19



Evans blue staining showing cell death in *Allium cepa* roots treated with 100 $\mu\text{g/mL}$ of extracts of selected species of *Gomphostemma* after 24 h of incubation.

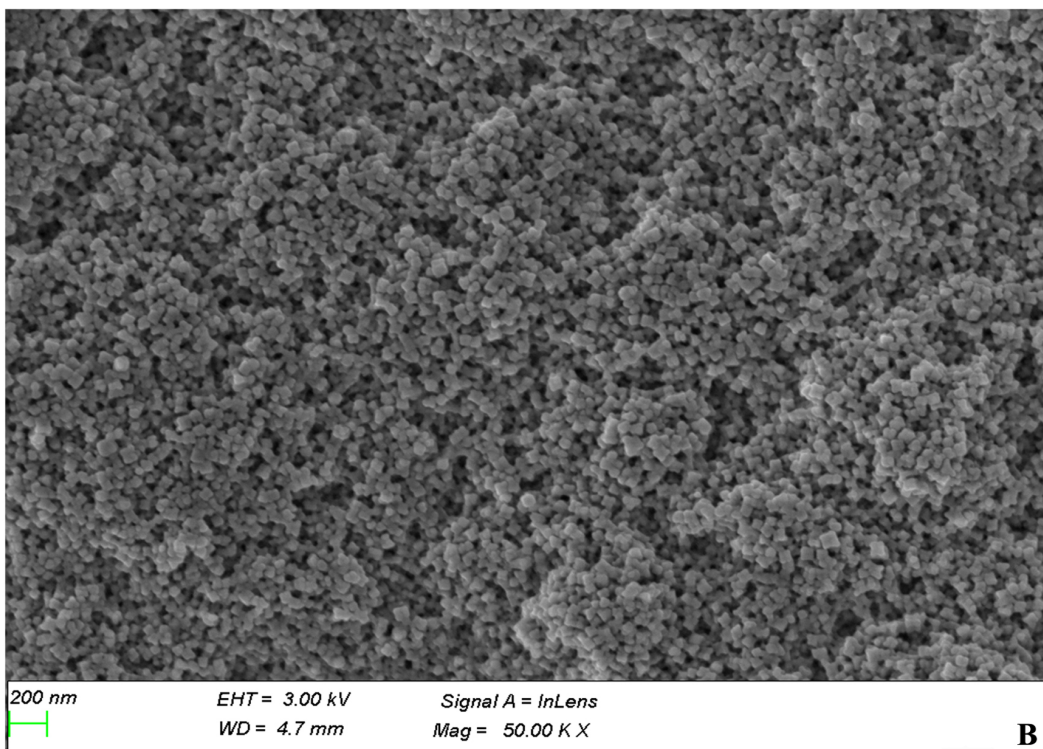
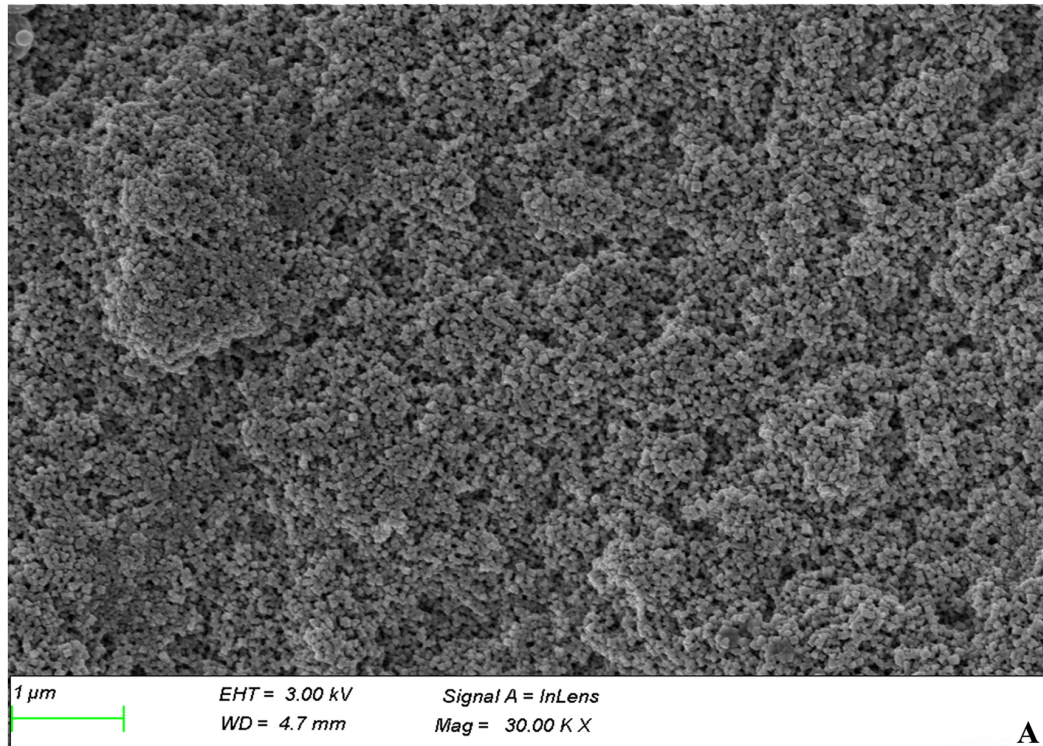
A. Positive control **B.** Negative control

C. *G. heyneanum* var. *heyneanum*

D. *G. heyneanum* var. *rottleri*

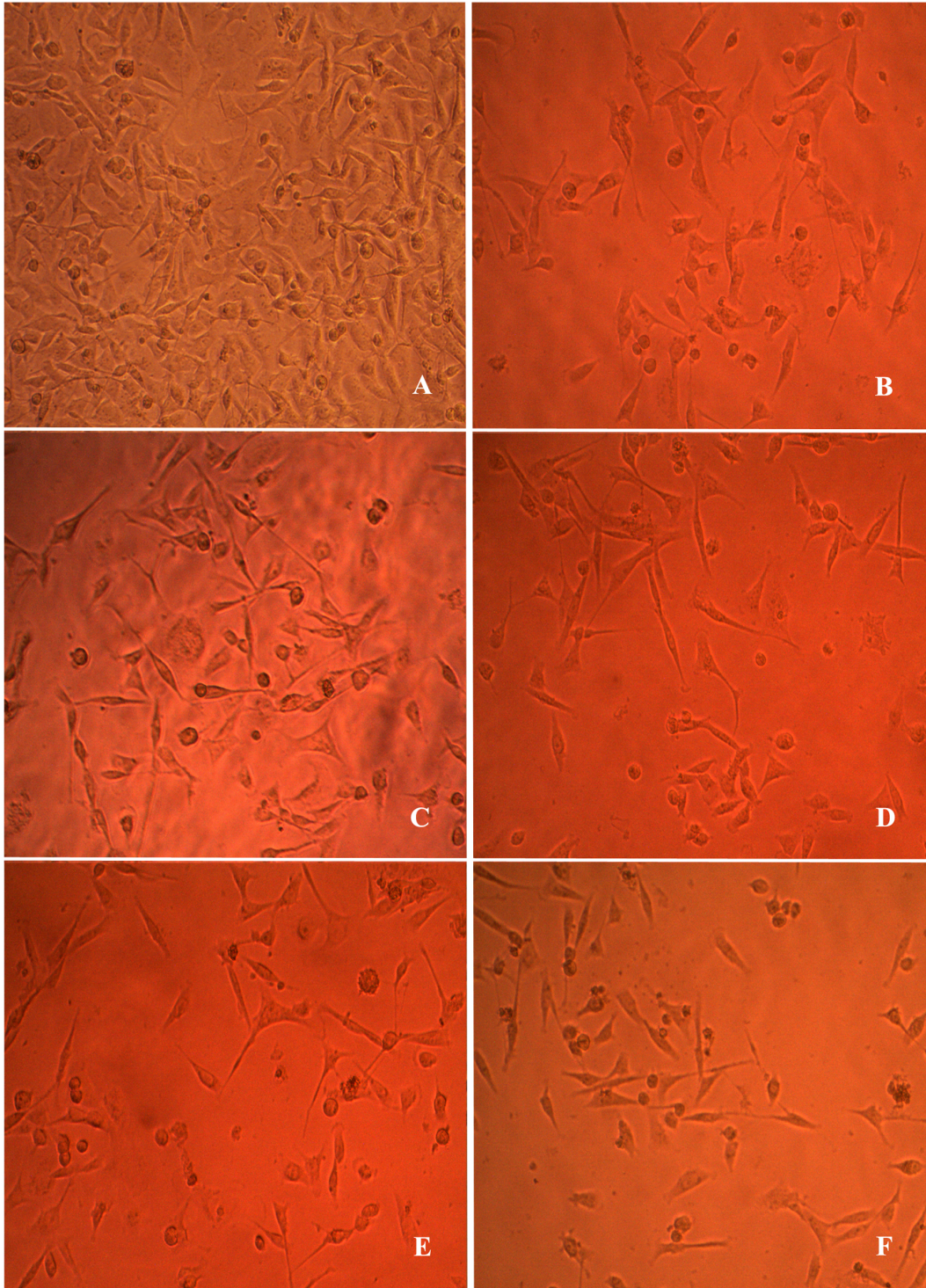
E. *G. eriocarpum*

PLATE 29



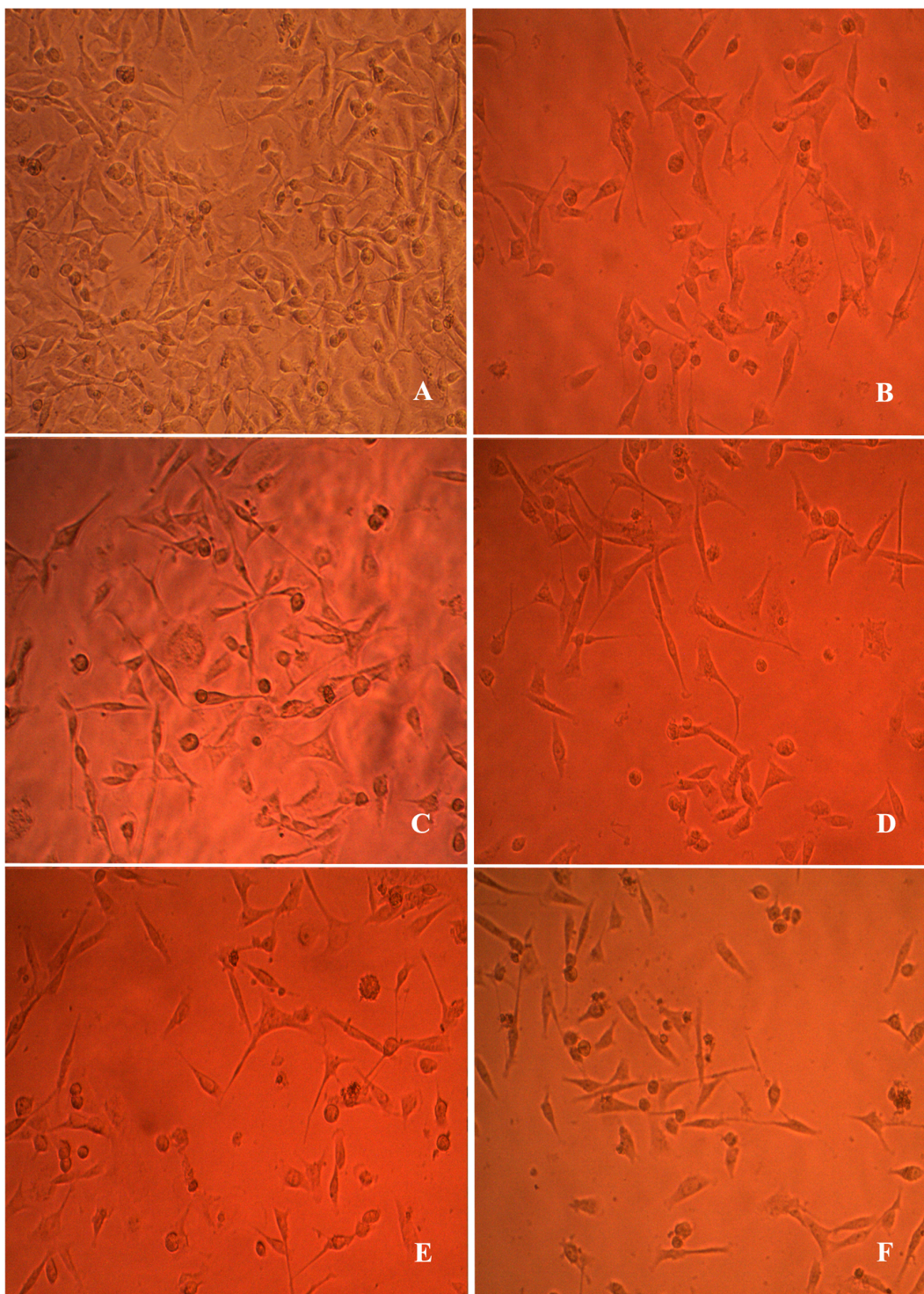
Scanning electron micrographs of silver nanoparticles synthesised by *G. eriocarpum* extract. **A:** Low magnification view **B:** High magnification view

PLATE 21



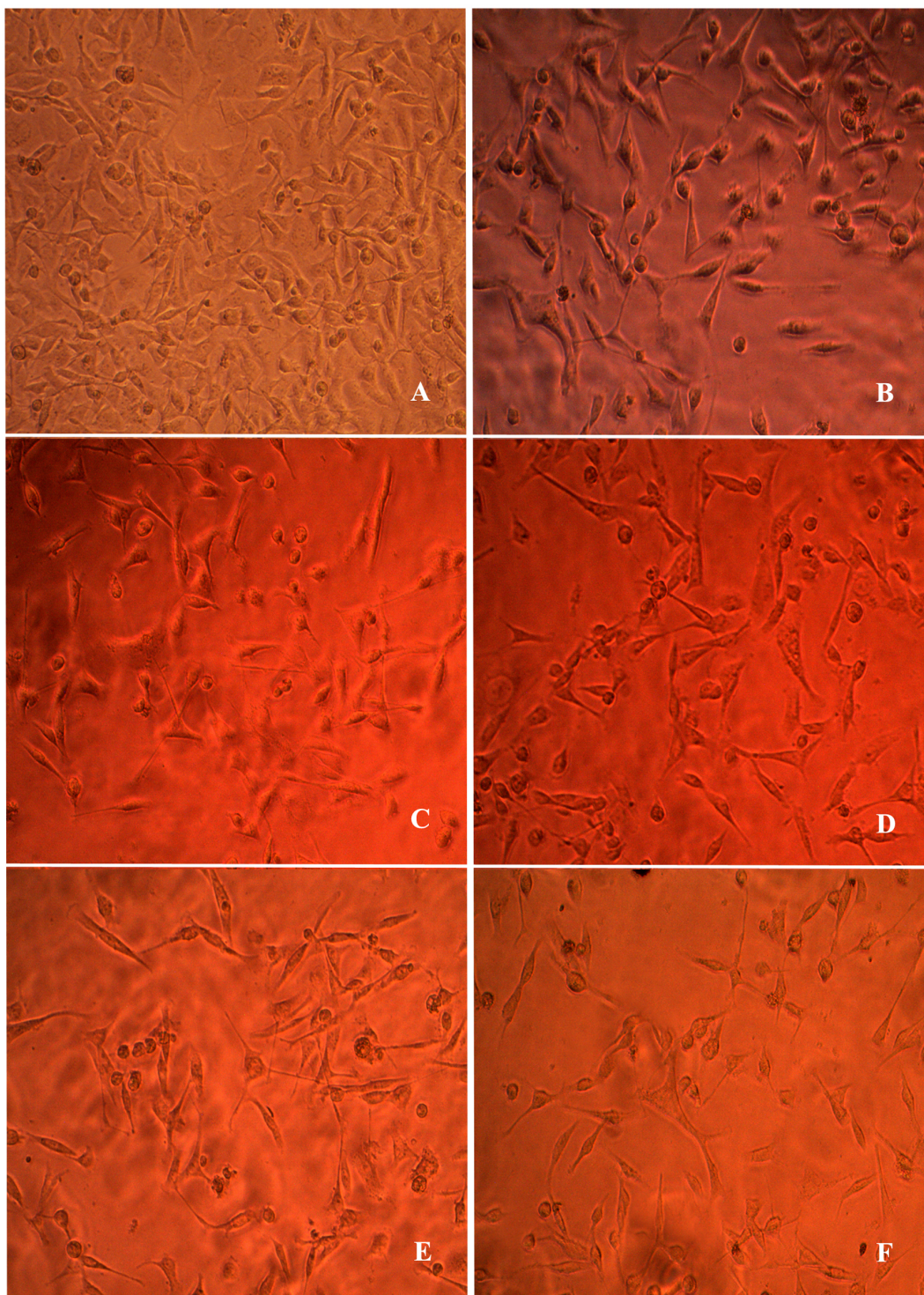
Cytotoxic effect of methanolic extract of *G. heyneanum* var. *rottleri* on MCF-7 cells. A: Control B: 6.25 $\mu\text{g/mL}$ C: 12.5 $\mu\text{g/mL}$ D: 25 $\mu\text{g/mL}$ E: 50 $\mu\text{g/mL}$ F: 100 $\mu\text{g/mL}$

PLATE 21



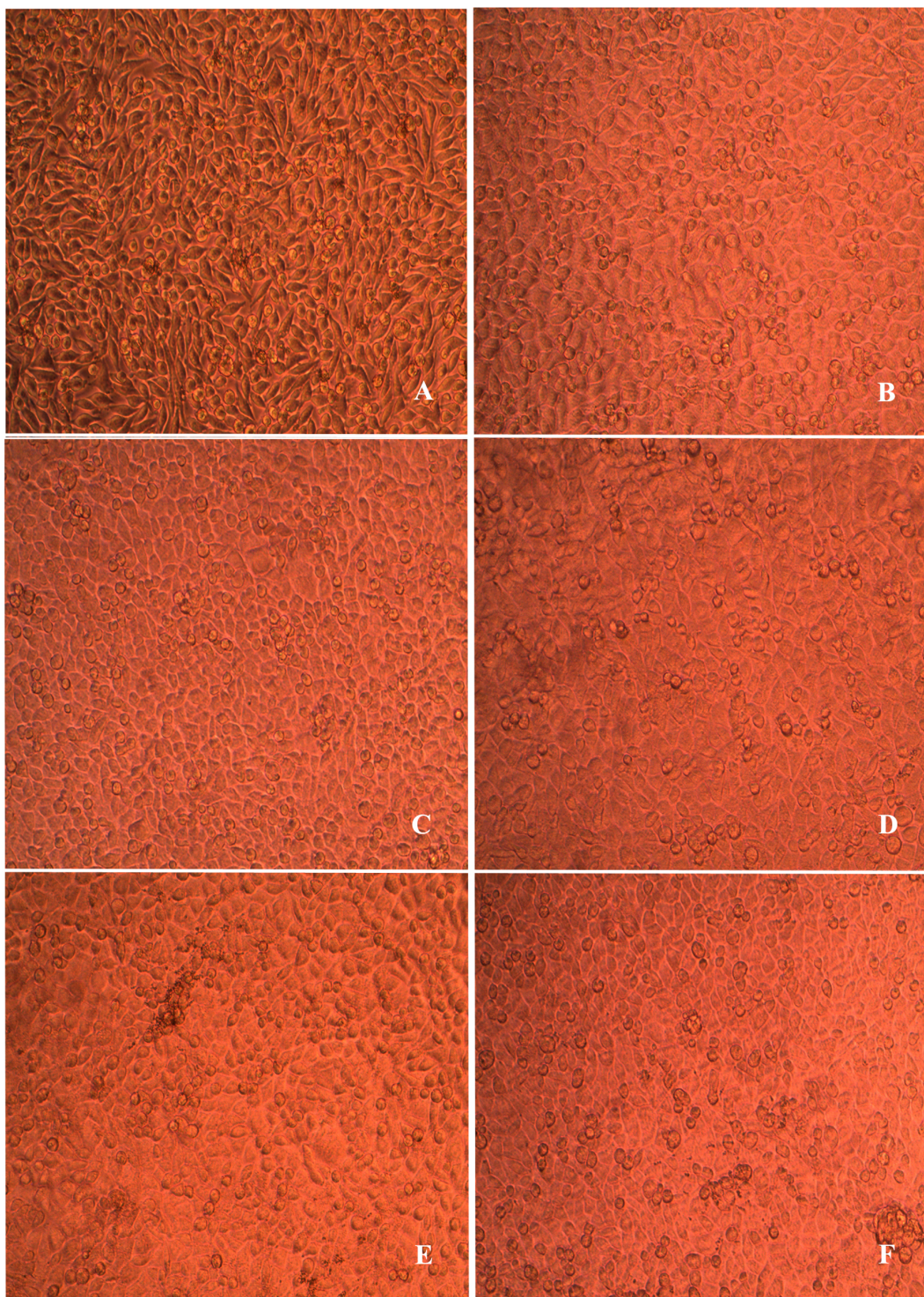
Cytotoxic effect of methanolic extract of *G. heyneanum* var. *rottleri* on MCF-7 cells. A: Control B: 6.25 µg/mL C: 12.5 µg/mL D: 25 µg/mL E: 50 µg/mL F: 100 µg/mL

PLATE 22



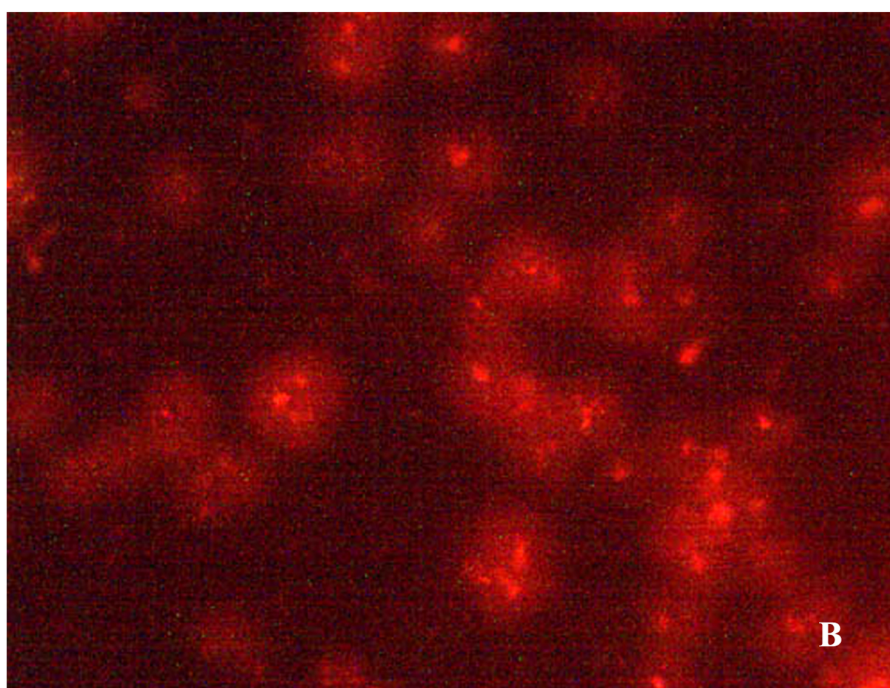
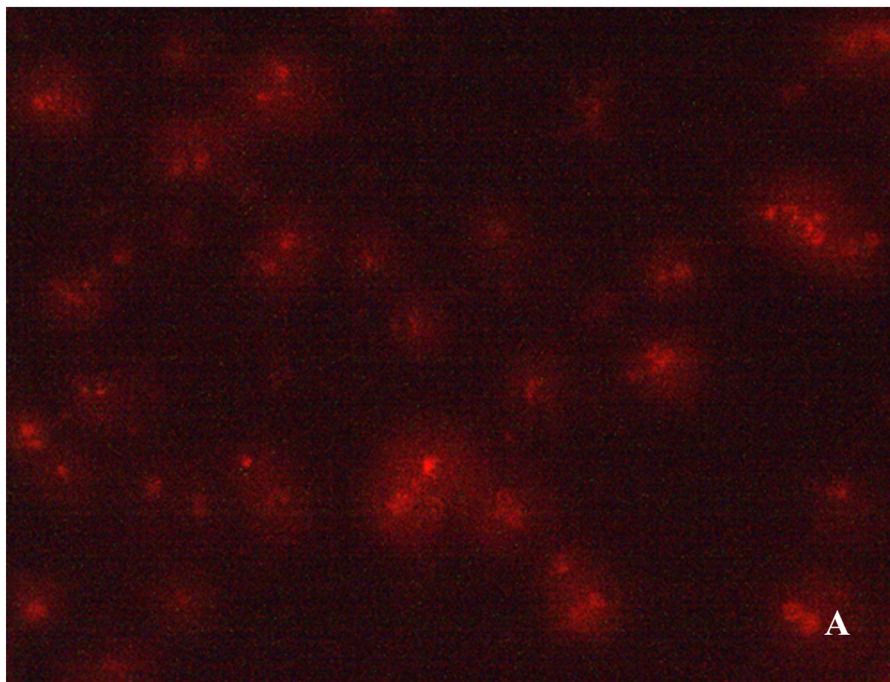
Cytotoxic effect of methanolic extract of *G. eriocarpum* on MCF-7 cells. A: Control B: 6.25 µg/mL C: 12.5 µg/mL D: 25 µg/mL E: 50 µg/mL F: 100 µg/mL

PLATE 23



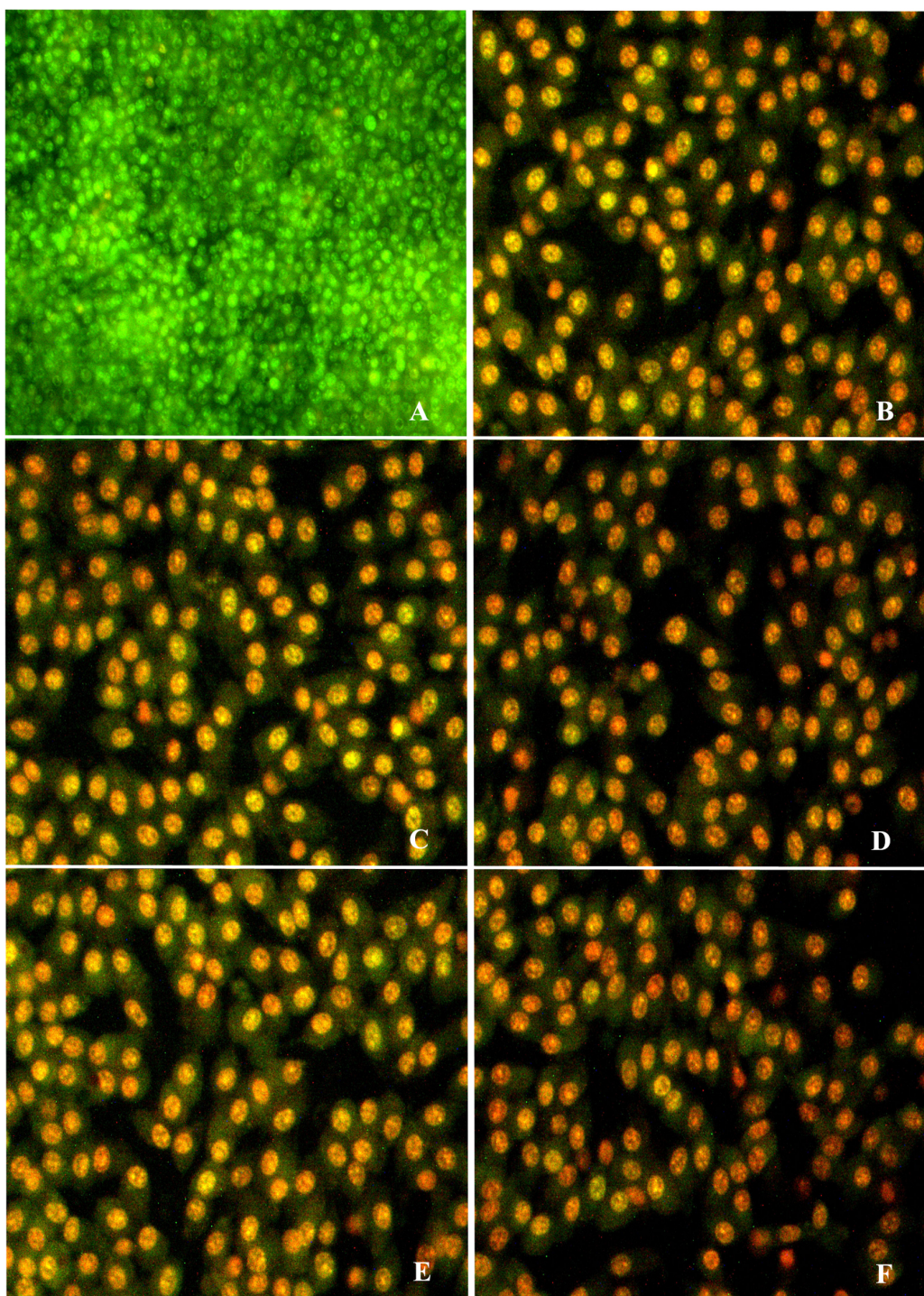
Effect of methanolic extract of *G. heyneanum* var. *heyneanum* on L929 cells. A: Control **B:** 6.25 µg/mL **C:** 12.5 µg/mL **D:** 25 µg/mL **E:** 50 µg/mL **F:** 100 µg/mL

PLATE 24



Comet assay showing the DNA damaging potential of *G. heyneanum* var. *heyneanum* on MCF-7 cells. **A:** Control **B:** Cells treated with *G. heyneanum* var. *heyneanum* extract

PLATE 25



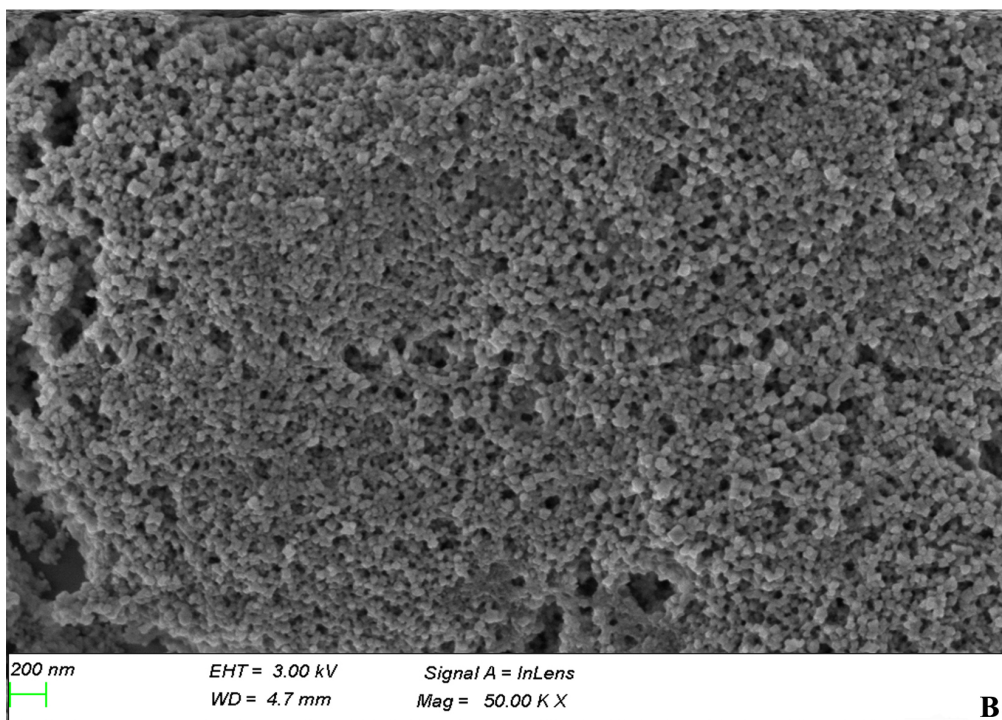
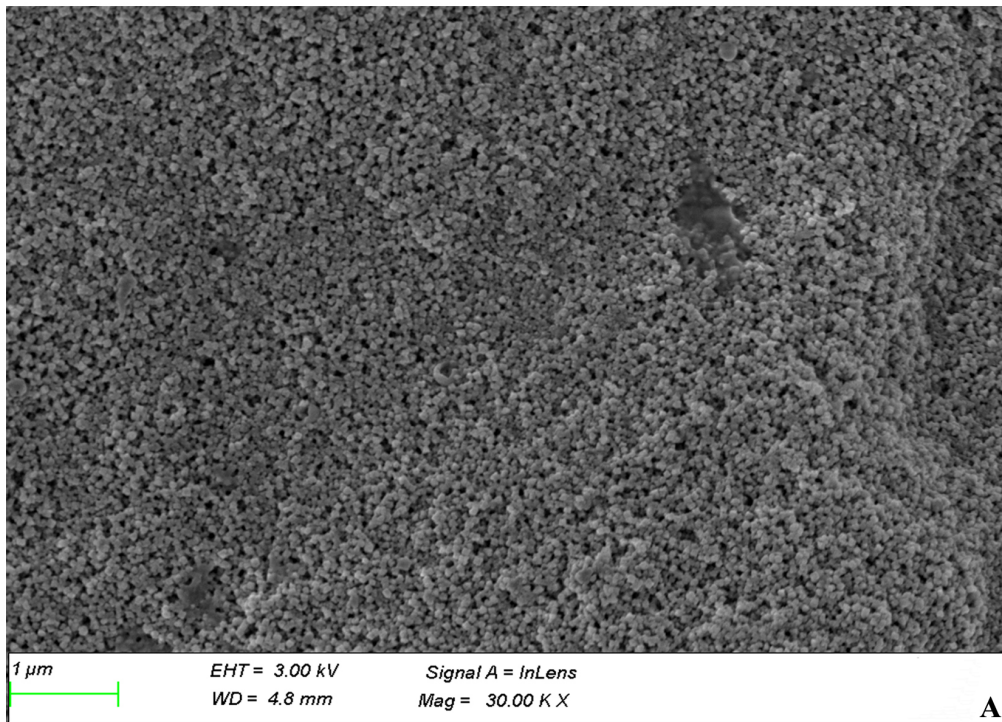
Detection of apoptosis by AO/EB staining on MCF-7 cells. A: Control **B - F:** Cells treated with methanolic extract of *G. heyneanum* var. *heyneanum*

PLATE 26



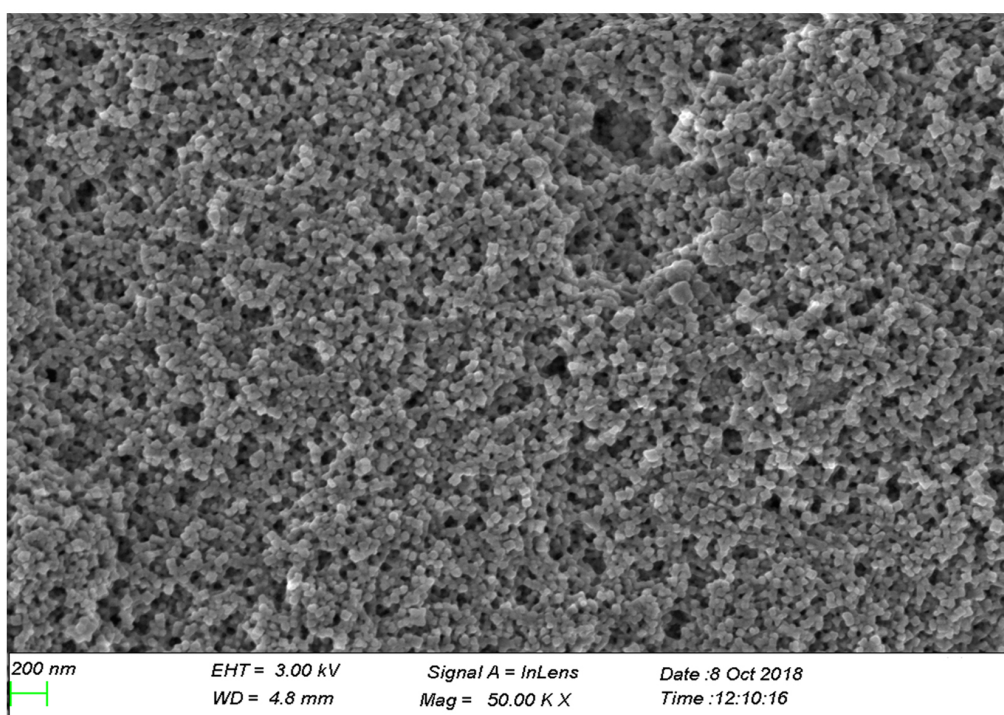
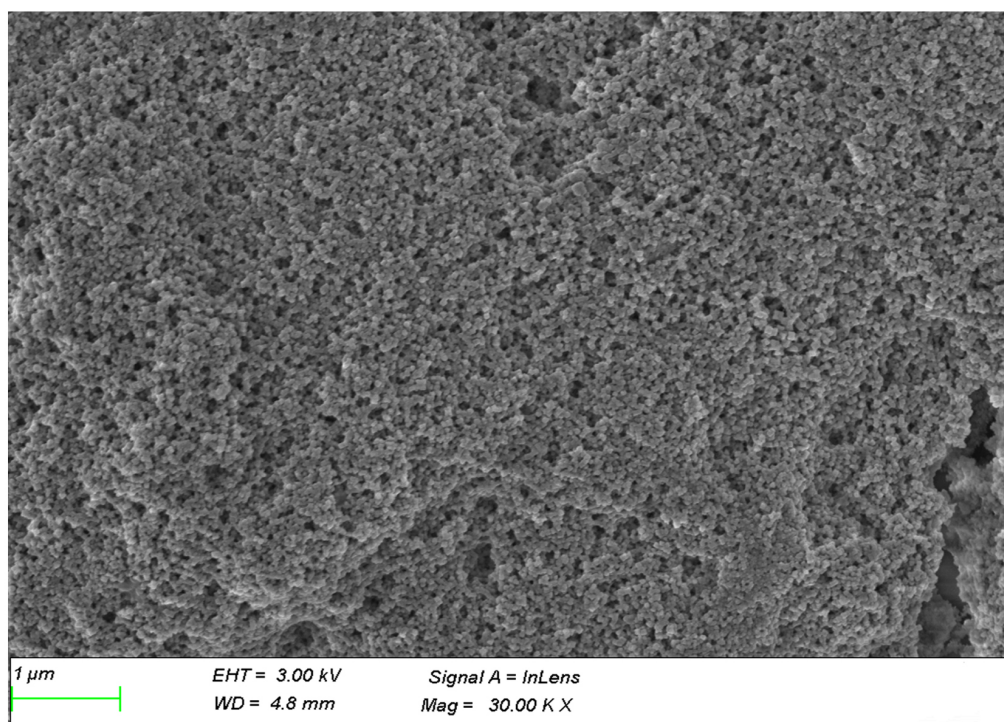
Green synthesis of silver nanoparticles. A, D, G: GHH, GHR and GE solutions before the synthesis of silver nanoparticles B, E, H: 2 mM silver nitrate solution C, F, I: GHH, GHR and GE solutions after the synthesis of silver nanoparticles. GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*

PLATE 27



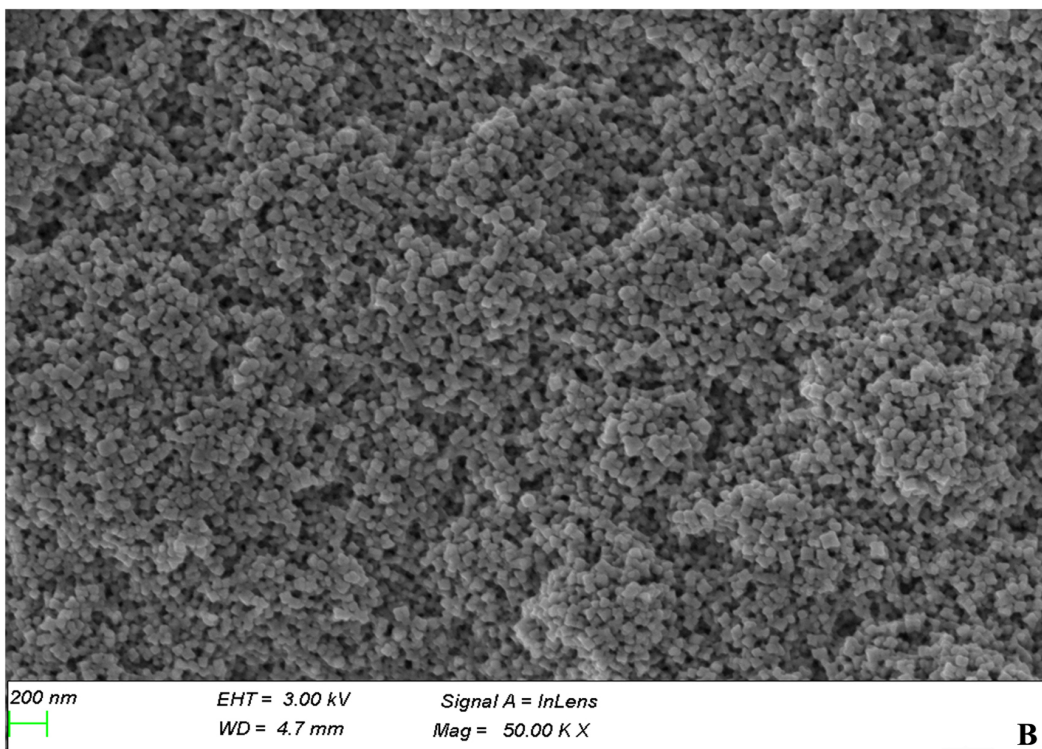
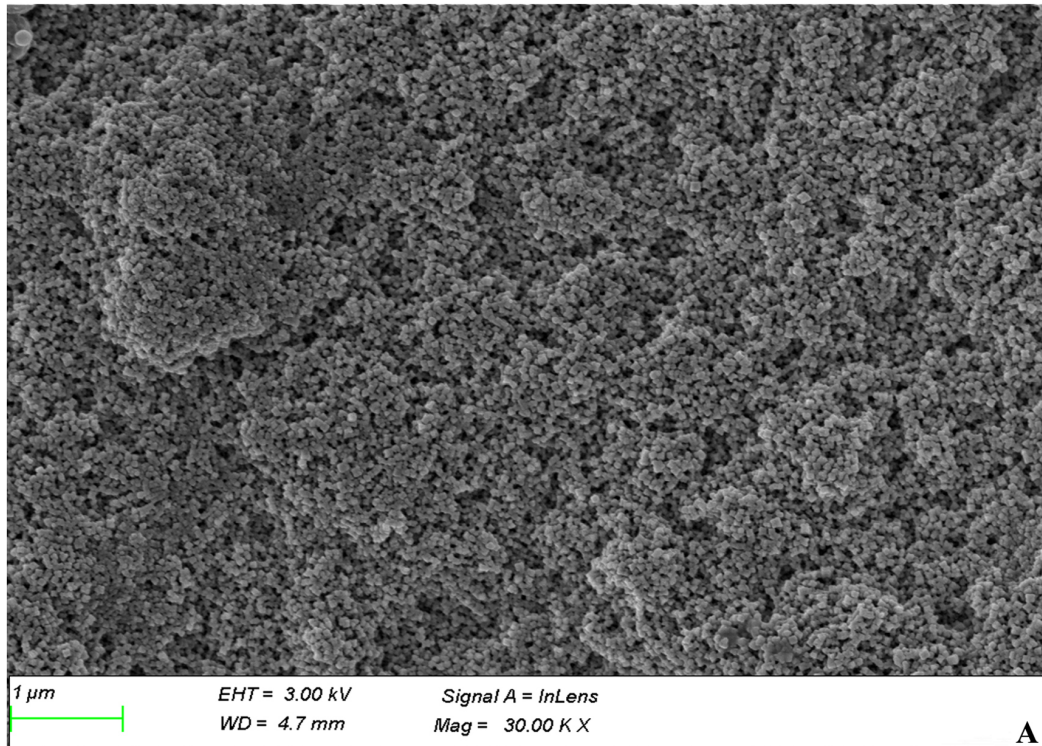
Scanning electron micrographs of silver nanoparticles synthesised by *G. heyneanum* var. *heyneanum* extract. **A:** Low magnification view **B:** High magnification view

PLATE 28



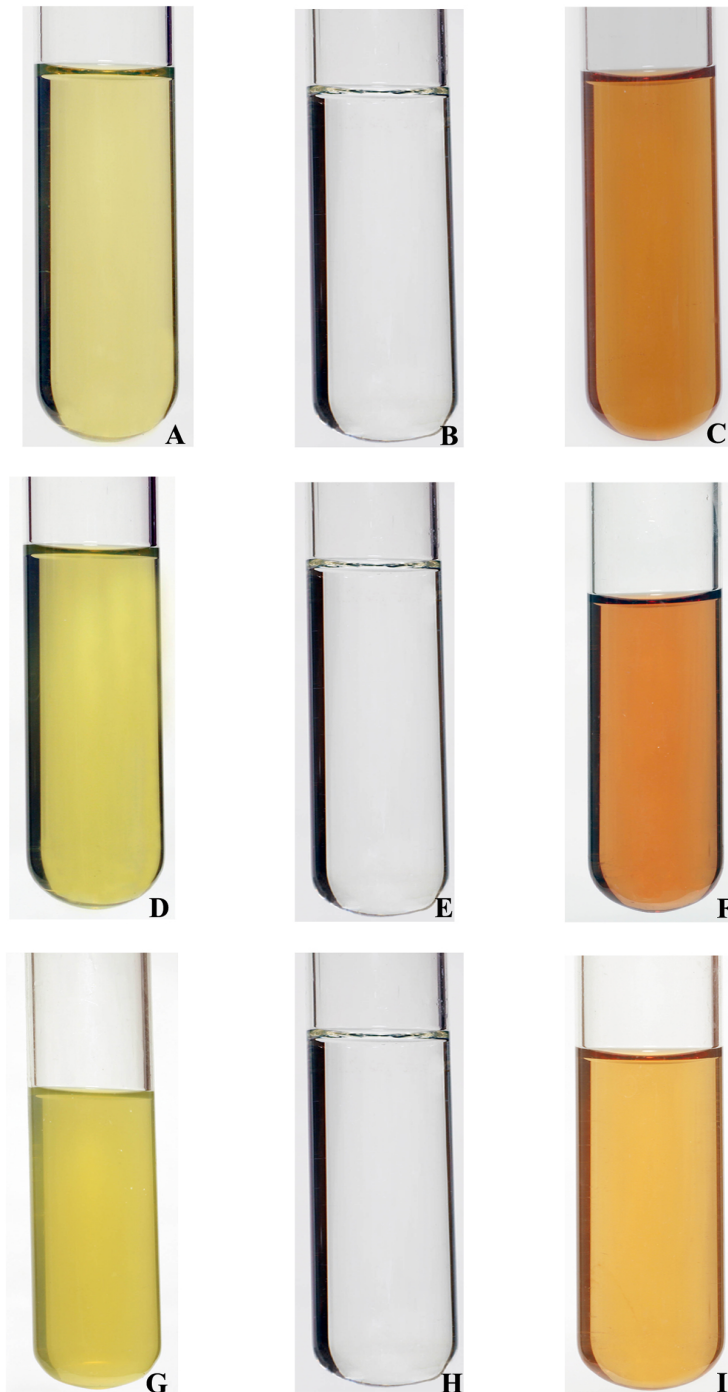
Scanning electron micrographs of silver nanoparticles synthesised by *G. heyneanum* var. *rotleri* extract. **A:** Low magnification view **B:** High magnification view

PLATE 29



Scanning electron micrographs of silver nanoparticles synthesised by *G. eriocarpum* extract. **A:** Low magnification view **B:** High magnification view

PLATE 26



Green synthesis of silver nanoparticles. A, D, G: GHH, GHR and GE solutions before the synthesis of silver nanoparticles B, E, H: 2 mM silver nitrate solution C, F, I: GHH, GHR and GE solutions after the synthesis of silver nanoparticles.
GHH - *G. heyneanum* var. *heyneanum*; GHR - *G. heyneanum* var. *rottleri*; GE - *G. eriocarpum*