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Authors
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PREFACE

Medicinal orchids are rich source of health-promoting phytochemicals and often used in pharmaceutical industries. Many orchid species have been documented as medicinally important in the traditional health care system. With the global inclination towards natural product medicines, collection and exploitation of such medicinal species has been accelerated in recent times. Therefore, it has become imperative to document and conserve such natural resources for sustainable utilization. At the same time, the traditional knowledge system also needs to be scientifically validated for its safety and efficacy.

Towards this endeavor, six medicinal orchids *viz. Aerides odorata* Lour., *Bulbophyllum odoratissimum* Lindl., *Cephalantheropsis obcordata* (Lindl.) Ormerod, *Crepidium acuminatum* (D.Don) Szlach. (syn. *Malaxis acuminata* D.Don), *Dendrobium chrysotoxum* Lindl. and *Dendrobium nobile* Lindl. were studied for conservation through *in-vitro* propagation methods; evaluation of secondary metabolites in different plant parts; characterization of phytochemical compounds; and quantitative estimation (through HPLC Chromatograms) of phytoconstituents. The results have been presented in the e-book "Micropropagation and Screening Based on Polyphenol and Antioxidant Potential of Six Medicinal Orchids in Meghalaya".

The various aspects included in this study include - standardization of protocol for micropropagation and mass multiplication; comparative study between different parts of mother and *in-vitro* raised plants based on Total Phenolic Content (TPC), Total Flavonoid Content (TFC), reducing power plus antioxidant activities and HPLC quantification of phytochemicals. As a result, the protocol for *in-vitro* propagation was developed and large number of seedlings could be produced which can be used for reintroduction in the suitable habitats. It could be established that, this *in-vitro* generated plants can meet the demand of phytochemicals and in turn, reduce the pressure of harvesting from the wild. All these species were found as rich in Total Phenolic Content, Total Flavonoid Content and with high antioxidant properties. A comprehensive list of important secondary metabolites found in different plant parts through HPLC profiling could be determined.

It is hoped that the e-book "Micropropagation and Screening Based on Polyphenol and Antioxidant Potential of Six Medicinal Orchids in Meghalaya" will act as a reference material for similar studies on other species and will be useful for the researchers, students, stakeholders, academicians and conservation enthusiasts. Any suggestion and constructive criticism is welcome.

(Authors)

Contents

ABBREVIATIONS

MS	Murashige and Skoog's medium
PGRs	Plant growth regulators
BA	Benzyladenine
2-iP	Isopentenyl adenine
GA	Gibberellic acid
IBA	Indole-3-butyric acid
CW	Coconut water
AC	Activated charcoal
l	litre
mgl^{-1}	Miligram(s)/litre
PLBs	Protocorm like bodies
DAI	Days after inoculation
Fig.	Figure
Sl no.	Serial number
no.	Number
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
NaOCl	Sodium hypochlorite
RH	Relative humidity
g	gram(s)
cm	centimeter
mm	millimeter
%	percentage
sp.	Species
ANOVA	Analysis of variance
SD	Standard deviation
SEM	Standard error mean
N	Normality

µg	Micro gram
µl	Micro liter
µM	Micro Molar
TPC	Total phenolic content
TFC	Total flavonoid content
RP	Reducing power
ABTS	2, 20- azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
DPPH	1,1-Diphenyl-2-picrylhydrazyl
IC50	Half-maximal inhibitory concentration
OD	Optical density
t_R	Retention time
GAE	Gallic acid equivalents
RE	Rutin equivalents
AAE	Ascorbic acid equivalent
ext.	Extract
HPLC	High-performance liquid chromatography
mg	milligram
min	minute(s)
ml	milliliter
mM	Millimolar
mm	millimeter
nm	nanometer

PLATE 1

Fig 1. Seed germination study of *A. odorata* **A.** Plant bearing seed pod **B.** Inoculation of seed on 10% banana medium **C.** Swelling of inoculated seed **D.** PLBs initiated after 37 days of inoculation. **E.** Development of leaves from PLBs. **F.** Fully developed seedlings (seed germinated plants) **G.** *In vitro* raised seedlings with healthy roots

PLATE 2

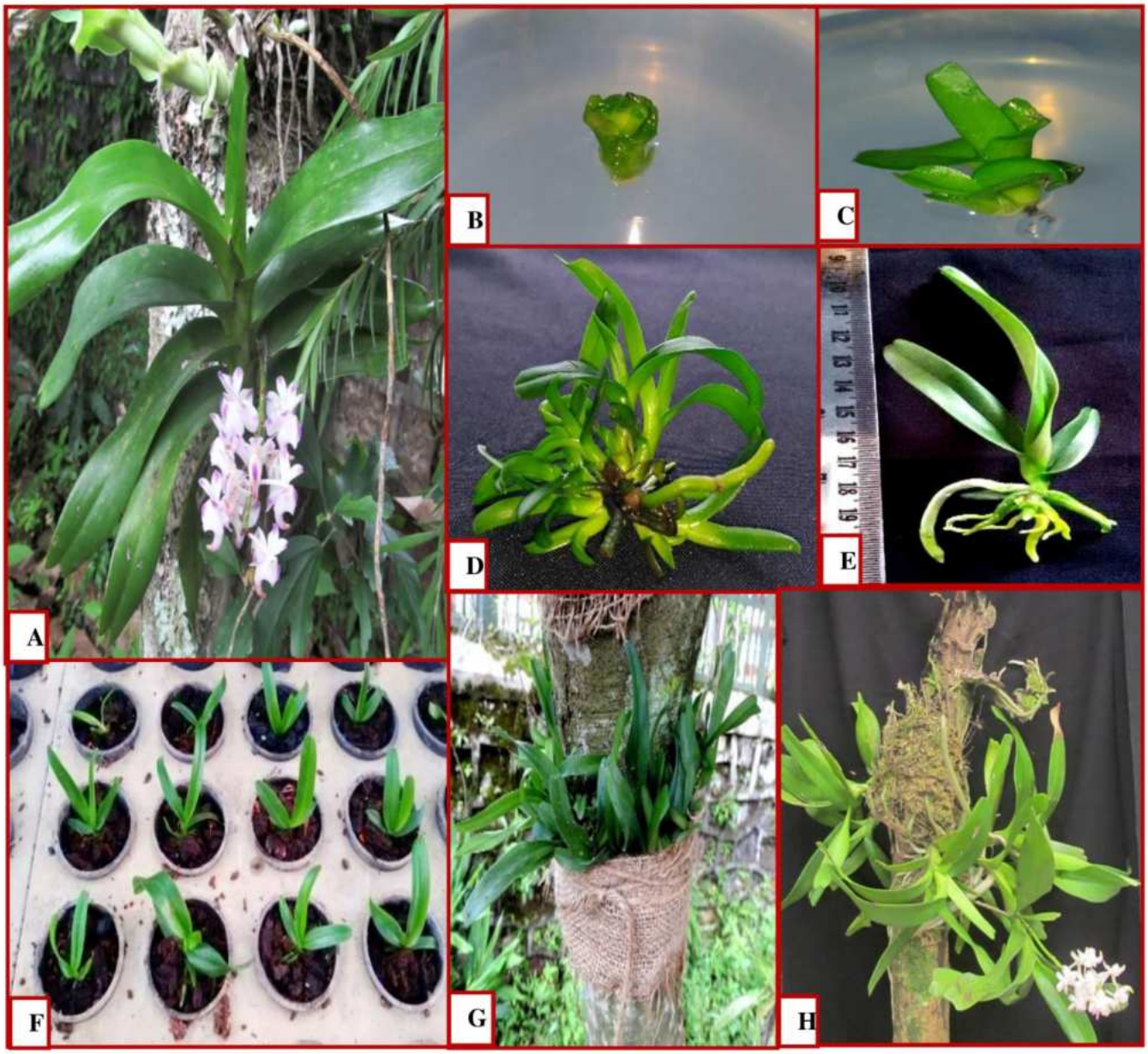

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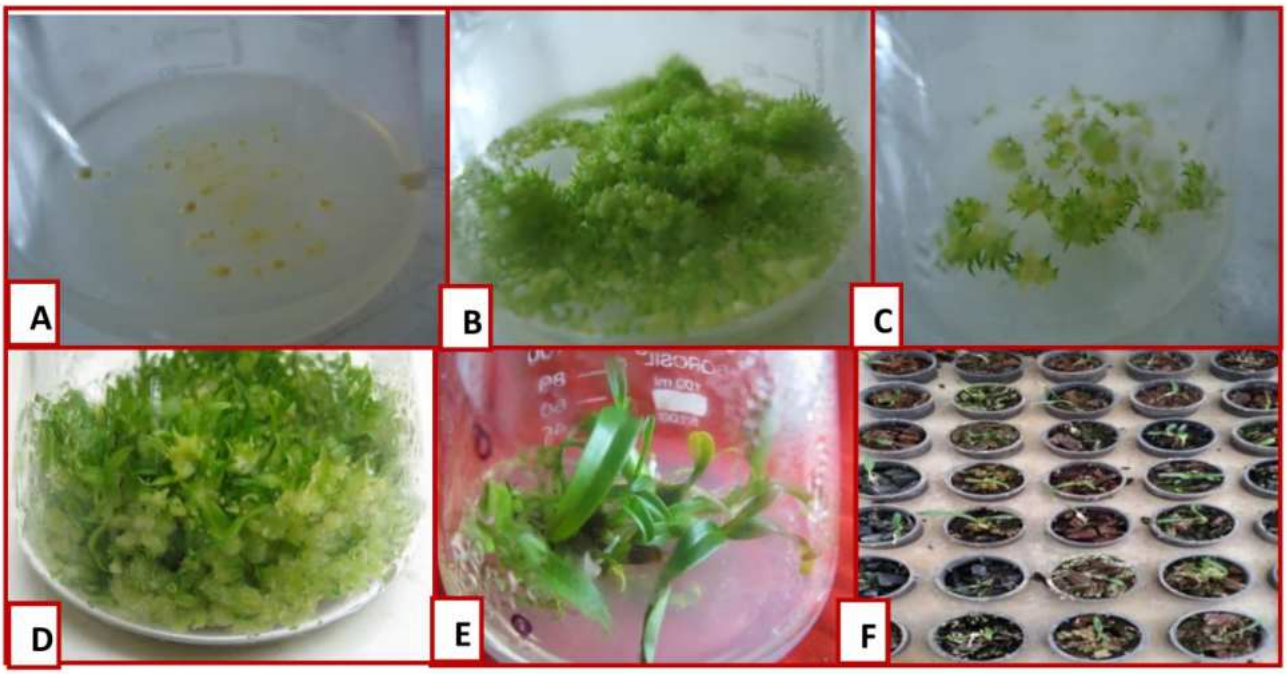
PLATE 3



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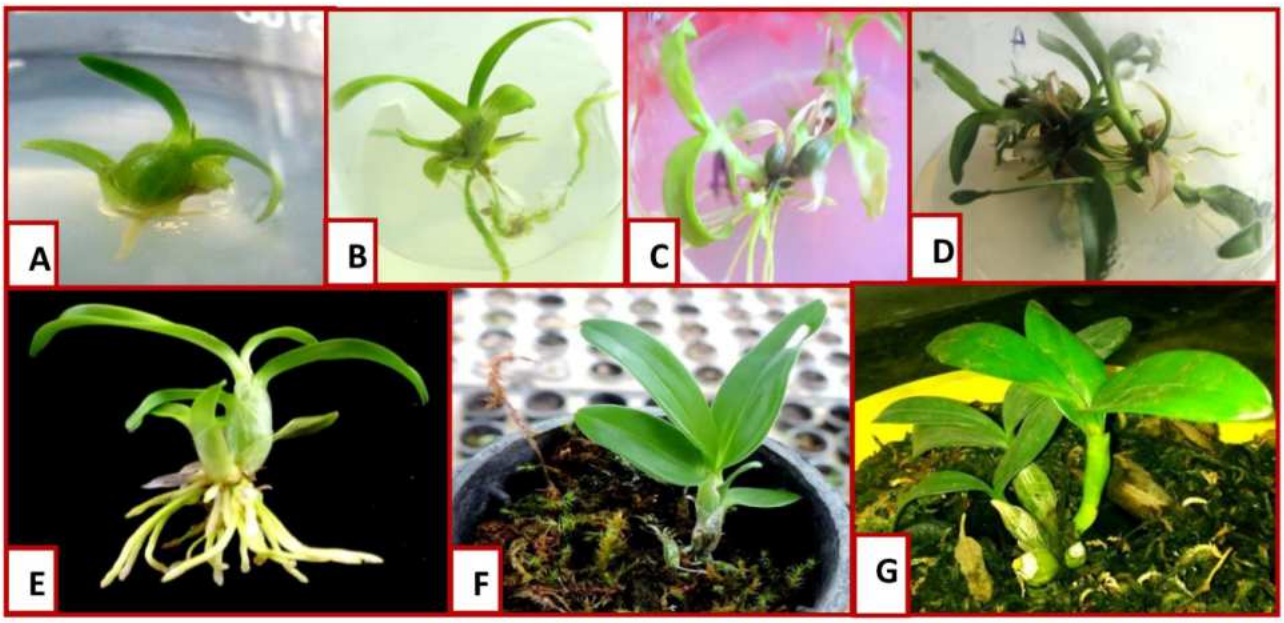

Fig 4. Micropropagation of *D. chrysotoxum* A-D. Developmental stages of multiple shoot induction studies of *D. chrysotoxum*. **E.** Rooted plantlets **F**. plantlet maintained in greenhouse

PLATE 4

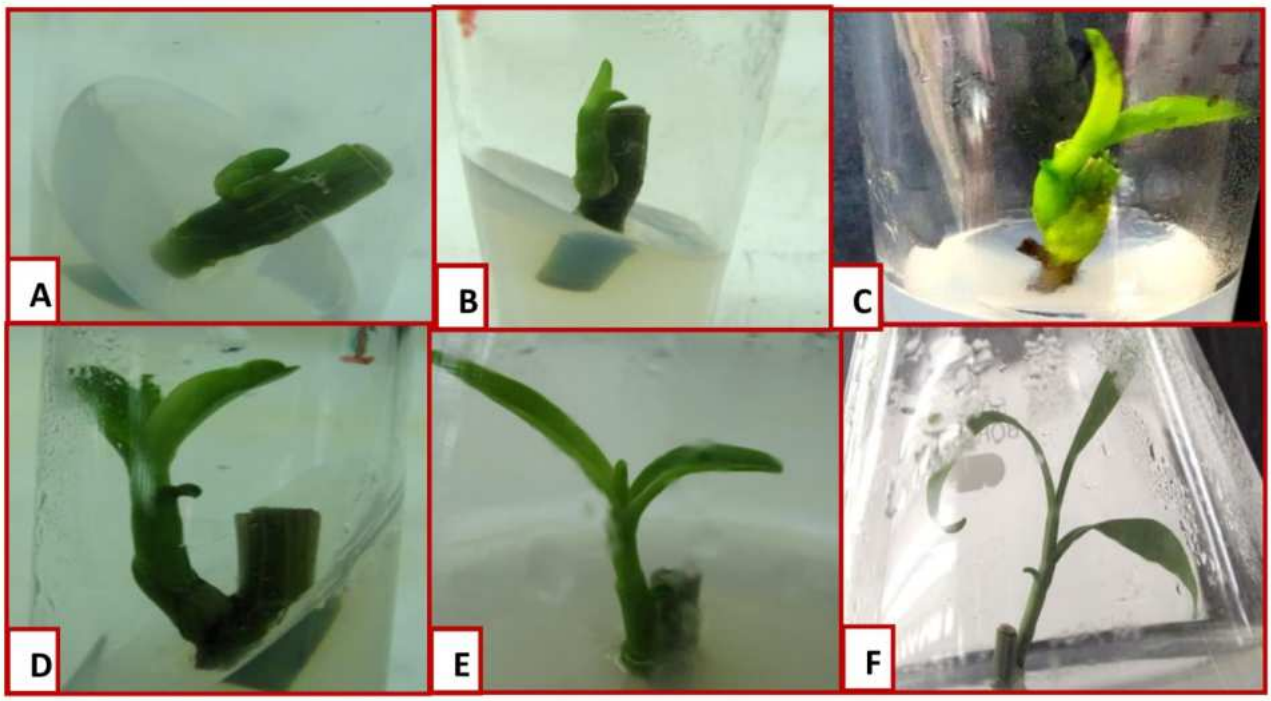

Fig 5. Micropropagation of *D. nobile* from nodal segments of wild plant A-F.
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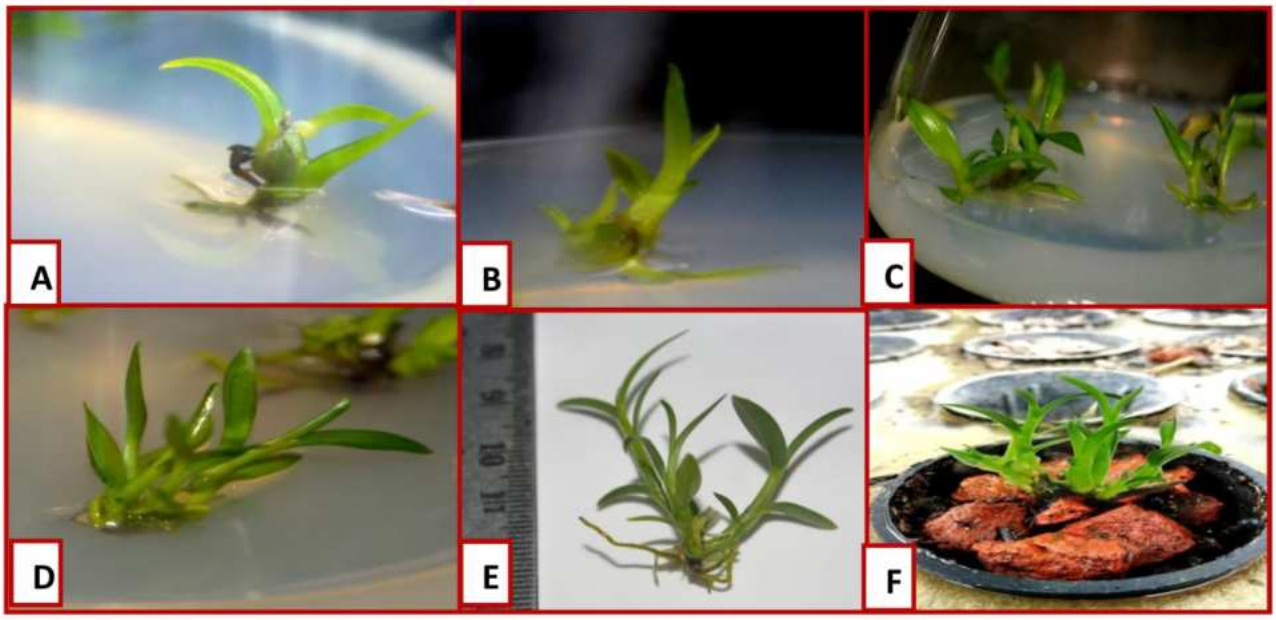

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PLATE 5

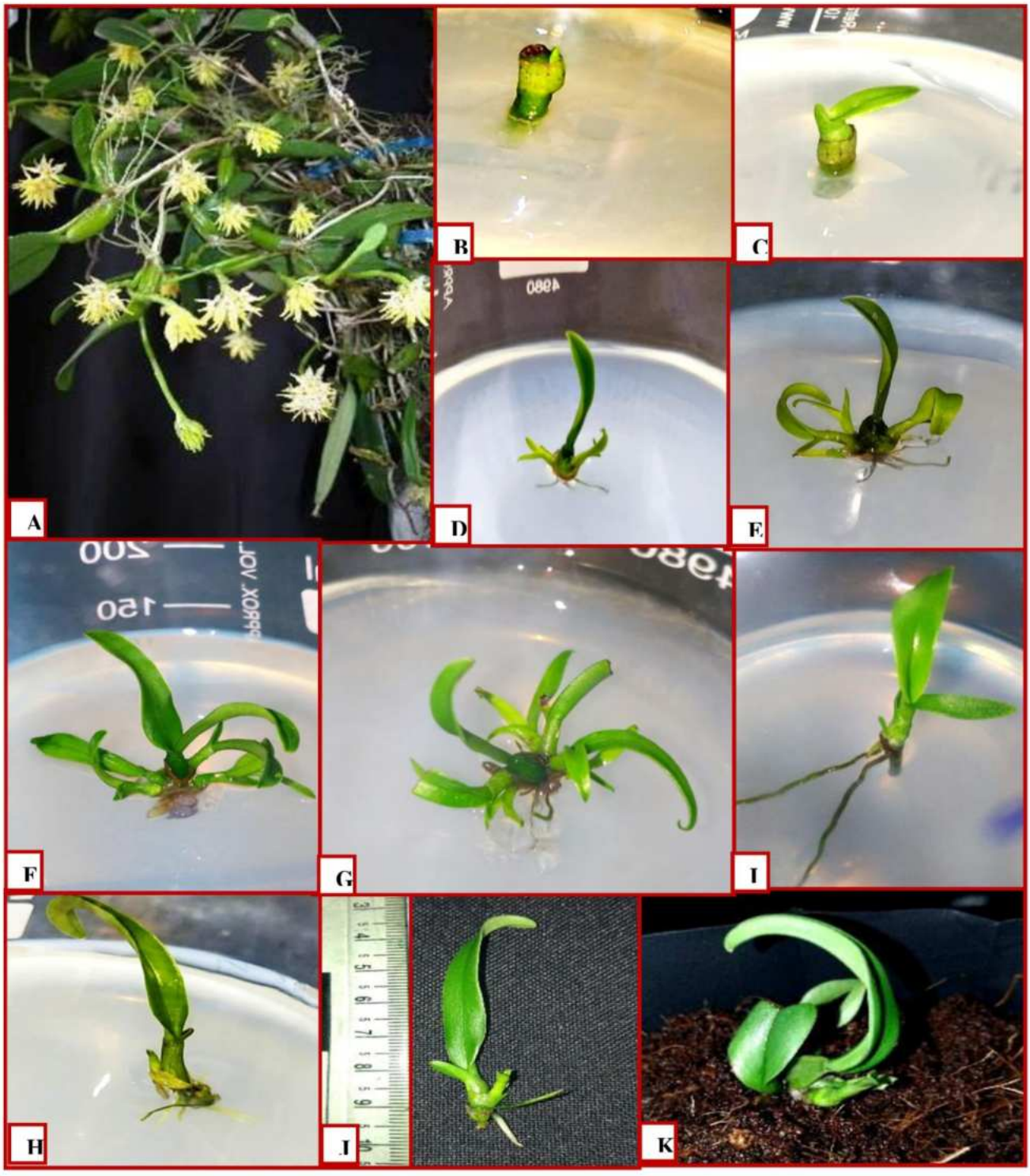

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PLATE 6

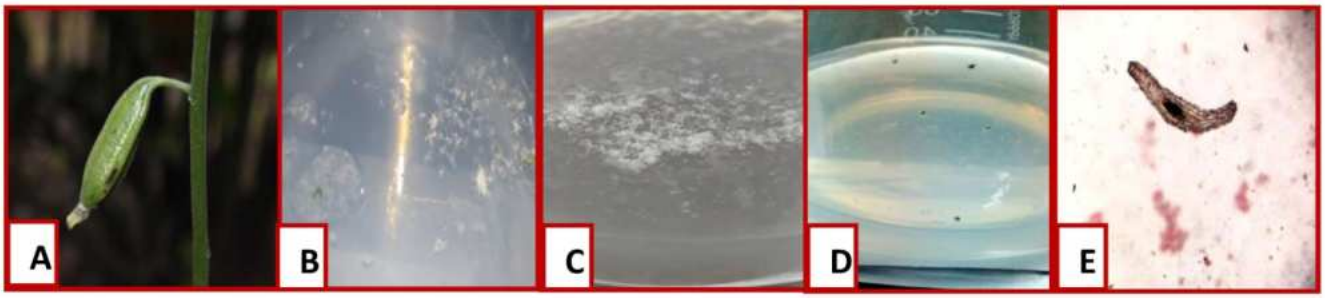


Fig 8. Seed germination study of *C. obcordata* A. Plant bearing seed pod **B.** Seed inoculated on control **C.** 10% banana **D.** 10% coconut water **E.** An undifferentiated embryo

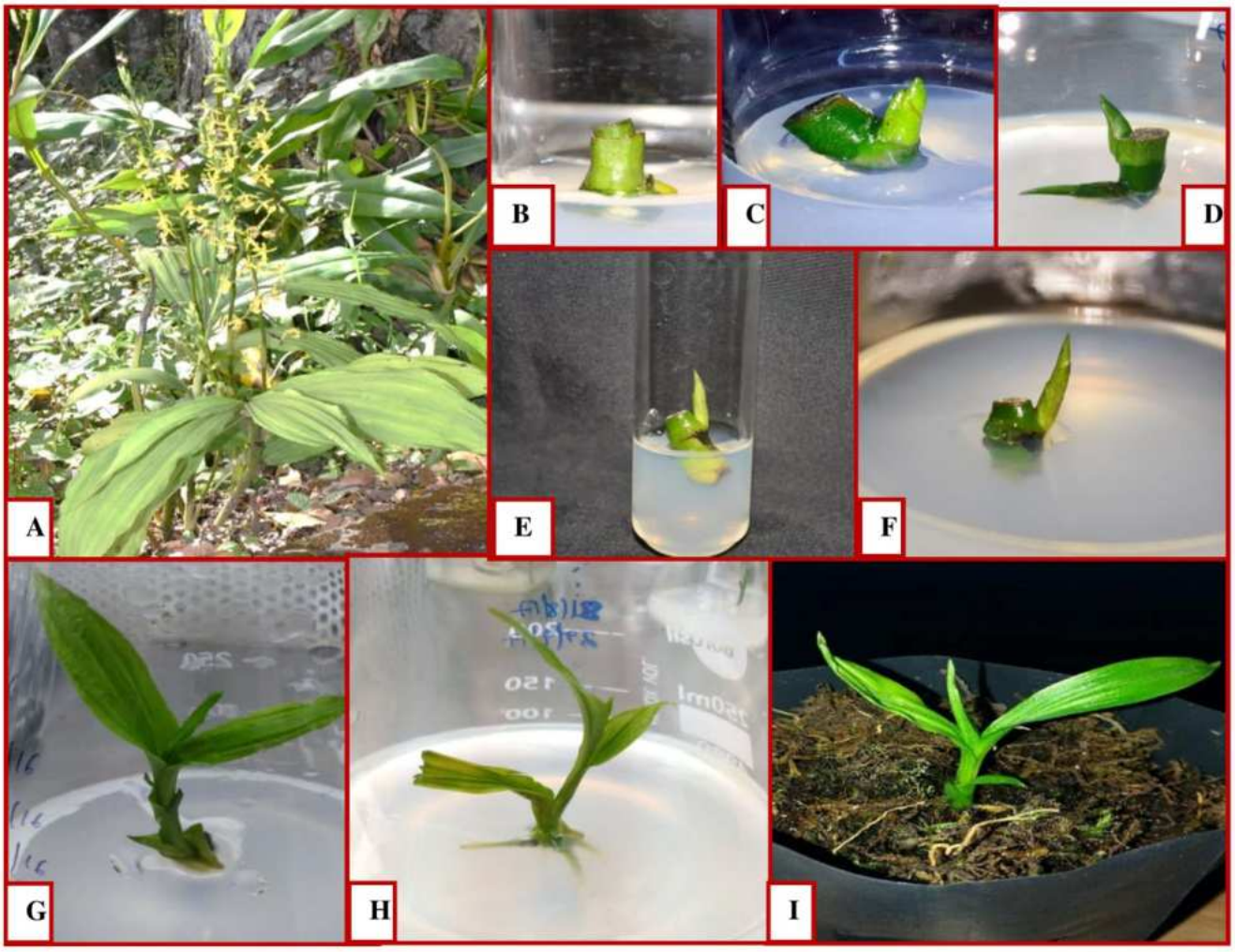
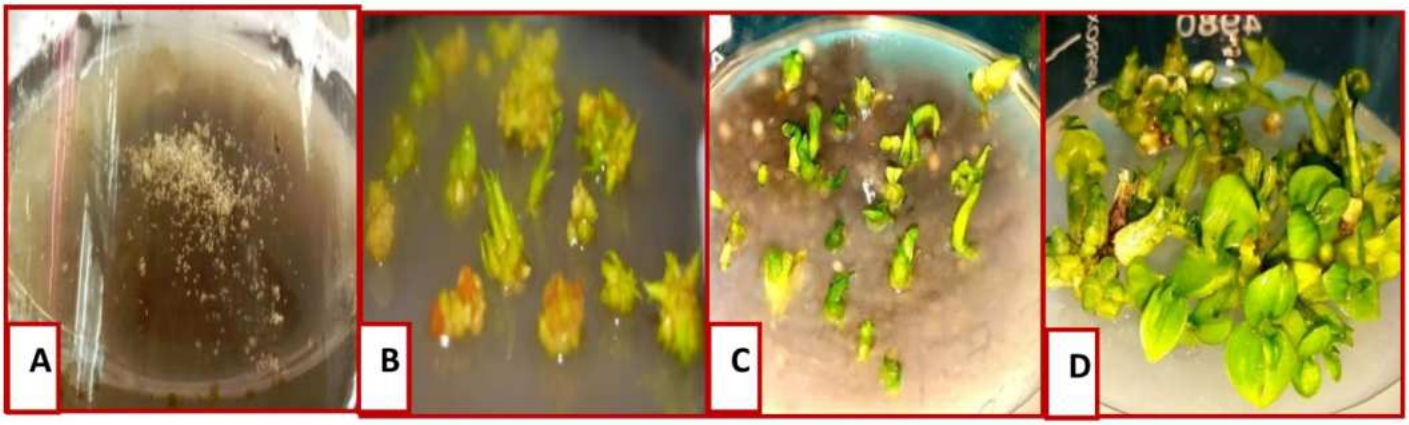


Fig 9. Micropropagation of *C. obcordata* from nodal segments A. *C. obcordata* in its natural habitat **B.** Initiation of new shoot from nodal segment **C.** Proliferation of newly grown shoot **D.** One shoot developed from each node **E-G.** Developmental stages of *in vitro* regenerated shoot of *C. obcordata* **H.** *In vitro* regenerated plantlet with healthy roots. **I.** *In vitro* raised plants maintained in greenhouse

PLATE 7



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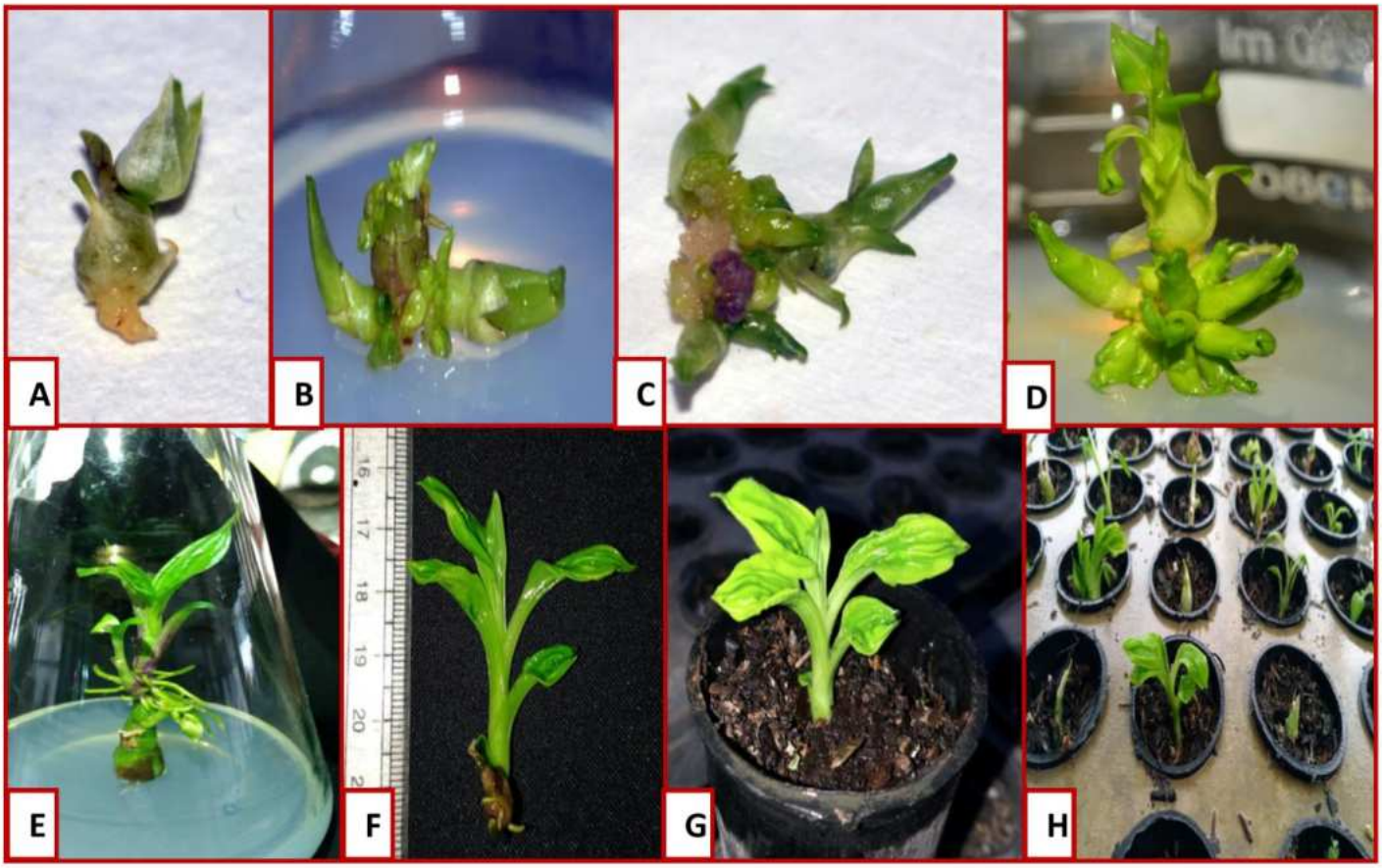

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PLATE 8

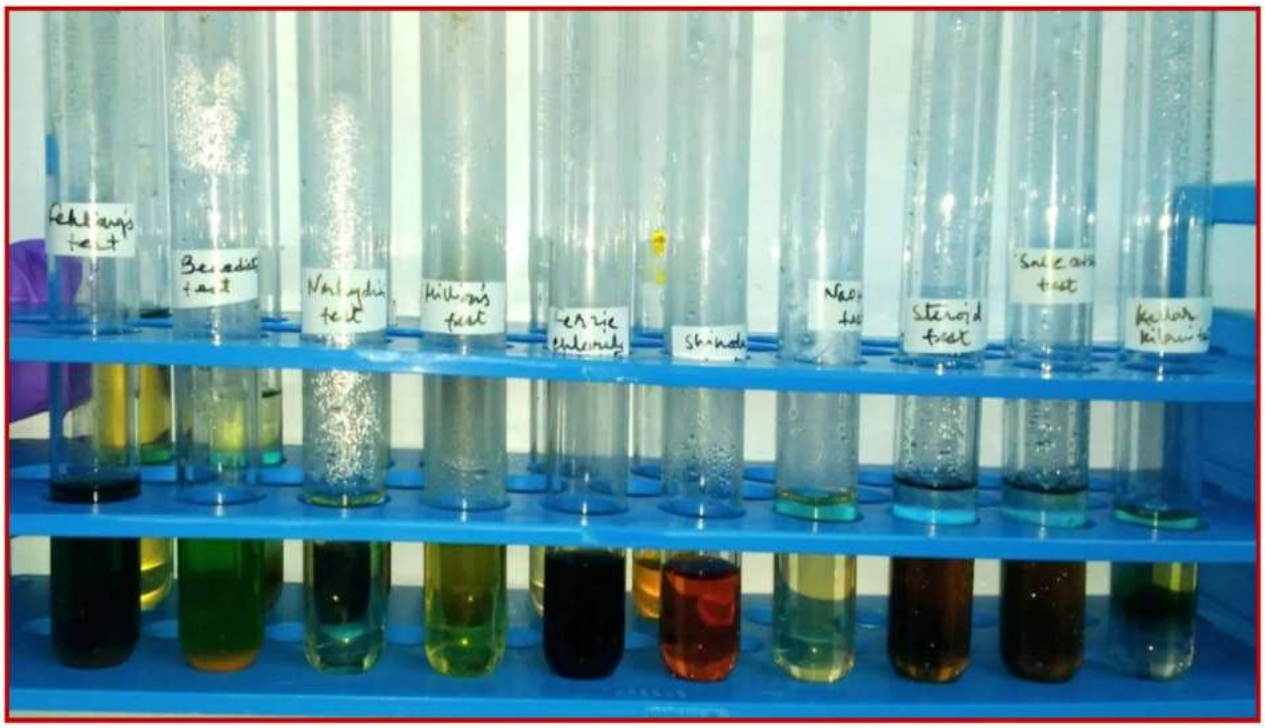


Fig 12.Qualitative phytochemical test of orchids (Phenol test, flavonoid test, saponin test, glycoside test, steroid test, terpenoid test, alkaloid test)

Chapter I
INTRODUCTION

Orchidaceae is one of the largest families amongst the flowering plants and it occupies about 7% of the total species of angiosperms as well as almost 40% of monocotyledons (Dangat & Gurav, 2014)**.** The Royal Botanical Garden, Kew documented a total of 25,000 species under 880 genera, of which about 300 to 350 genera are from tropical America, 250 to 300 genera from tropical Asia, 125 to 250 genera from tropical Africa, 50 to 70 genera from Oceania, 40 to 60 genera from Europe and temperate Asia and 20 to 30 genera from North America respectively (De & Debnath, 2019). The genus *Bulbophyllum* Thouars is one of the largest genera of Orchidaceae containing approximately 2000 species distributed in the tropical and subtropical zone of the World and the highest diversity was found in the tropics of Africa and Asia (Maisak & Anh, 2018), followed by *Epidendrum* (1500 species), *Dendrobium* (1400 species) and *Pleurothallis* (1000 species) **(**Linthoingambi & al., 2014).

India is enriched with orchid flora with a total number of 1300 species belonging to 160 genera (De & Debnath, 2019) with a wide distributional range from sea level to snow peak excluding Northwestern part due to dry and hot weather with scanty rain (Hegde, 1997). Orchids are distributed throughout the different parts of the country *i.e.* Western Himalayas harbors 288 species under 75 genera, Eastern Himalayas and North-Eastern India are enriched with 870 species under 159 genera, Peninsular India (Madhya Pradesh, parts of Orissa, Andhra Pradesh, Gujarat, extra peninsular region of Central India and Gangetic plains along with Eastern and Western Ghats) constitute about 379 species under 89 genera and Andaman and Nicobar Islands contains 115 species under 53 genera (De, 2020).

Northeast India (NEI), a hotspot of biodiversity, comprises eight states (Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim, and Tripura) which are very rich in orchid flora, and consist of 900 species of which as many as 34 species of orchids from NEI are listed as threatened plants of India (De & Singh, 2017). The state of Arunachal Pradesh is recorded with the highest number of orchid species (622) followed by Sikkim (543 species), Meghalaya (389 species), Assam (290 species), Nagaland (246 species), Mizoram (234), Manipur (215) and Tripura (57 species) respectively (De & Singh., 2015). Most of the orchid species are distributed mainly in the Himalayan regions especially the North Eastern Himalayas (Medhi & al., 2012). The Meghalaya is rich in biodiversity and is considered an important reservoir of orchids (352 species under 98 genera) due to altitude variation, topographical features, soil characteristics, and favorable microclimatic condition that supports the profuse growth of orchid flora in this region (Medhi & Chakrabarti, 2009). This state harbor a wide variety of important orchids such as *Paphiopedilum insigne, P. venustum, Rynchostylis retusa, Coelogyne corymbosa, Dendrobium devonianum, Cymbidium elegans and Vanda coerulea* (Nayar & Sastry 1987, 1988, 1990; Haridasan & Rao, 1985).

Uses and Importance of Orchids

Orchids are extensively used for ornamental purposes, potted plants, herbal medicine, aromatic products, food, and source of phytochemicals**.** Orchids are considered as most beautiful flowering plants distributed throughout the world. In floriculture, they are in great demand due to the splendid beauty of its flower. It occupies around 10% of the international cut flower market and covers a significant position in the global floriculture industry owing to eye-catching color combination, long-lasting qualities, ease in packing, and transportation (De & Medhi, 2014). Presently, the orchids hold sixth position among the top ten cut flowers of the world and orchids contribute 10% share in international trade (De & al., 2011). Primarily orchids are important for their commercial value as flower has exotic beauty and long lasting flowering period. Besides that, many orchids are used as traditional folk medicine by many different tribes in the different parts of world (Kasulo & al., 2009). Traditional medicines are widely used for human wellness since ancient times. Orchids are being useful in treating different diseases and ailments including tuberculosis, paralysis, stomach disorders, chest pain, arthritis, syphilis, jaundice, cholera, acidity, eczema, tumor, piles, boils, inflammations, menstrual disorder, spermatorrhea, leucoderma, diarrhea, muscular pain, blood dysentery, hepatitis, dyspepsia, bone fractures, rheumatism, asthma, malaria, earache, sexually transmitted diseases, wounds and sores (Hossain, 2011).

Conservation of Orchid

Orchids are highly heterozygous and their vegetative propagation also takes long time. This kind of complexity in nature drives the orchid community to extinction. The rate of vegetative propagation is very slow and the seed germination in nature is very poor, i.e. only 2 to 5% of seed can germinate (Rao, 1997) even if they do it so, but germination process is very slow and it takes longer time for their germination. Association with a specific fungal partner is a pre-requisite for orchid seed germination. The symbiotic relationship of mycorrhizal fungi to orchid for nutrient transfer has already been established (Cameron & al., 2006; Dearnaley & al., 2017). Due to lack of endosperm in seeds, most orchids are associated with mycorrhizal fungi that provide nutrients to the seeds necessary for germination. Thus, orchid species are myco-heterotrophic (MH) during germination (Arditti, 1992; Merckx, 2013). Fungal mycelia decompose the organic matter, such as bark to provide nutrients to epiphytes (Smith & al., 1997). It has been reported that mycorrhiza have found in terrestrial orchids and also present in many epiphytes (Hadley & al., 1972; Benzing & al., 1981; Ramsay & al., 1986; Currah & al., 1997).

Climate change due to global warming which leads to increase habitat loss and destruction, invasive species and it might have a severe impact on the ecosystem. Like all plants, orchid species are affected by environmental factors as they are interacted with pollinators, mycorrhizal fungi and host trees. Rapid urbanization, industrialization and economic development may be a further threat to make the ornamentally and medicinally important species endangered. Thus, orchids have to face greater challenges than many other plant groups. World Orchid Conference held in Miami in 1984, it was proposed that the orchid community should start banking orchid seed as an insurance against possible losses of species from their habitats in the wild (De & al., 2011). The rapid depletion of orchids from their natural habitats is due to many factors such as their complex life cycle, habitat alteration or destruction due to logging, fire, road construction and the expansion of forest plantations and agriculture, over-exploitation for medicinal uses and floral business (Gale & al., 2018; De, 2020). Xiao & al. (2012) reported in their study that the growth rate of *Cephalantheropsis obcordata* population is in descending order due to changes in rainfall patterns in Luofu Mountain, China.

Orchids are at greater risk due to their complex life cycle as they require a specialized mode of living, specific pollinator for pollination, and a lack of reserved food material in the seed which depends on mycorrhizal fungi for seed germination (De, 2020). A comprehensive list of orchid species that undergo major threats of various categories and require immediate attention has been enlisted in the Red data book. Many international plant conservation groups including the Orchid Specialist Group of the IUCN Species Survival Commission are using orchids as flagship species in the conservation debate and play a vital role in the prevention of biodiversity by promoting effective orchid conservation, improving networking and technology transfer, interacting with decision-makers and by educating the orchid community (Cribb & al., 2003). All the orchid species were placed on the appendices of the Convention on International Trade in Endangered Species (CITES) (e.g., Cribb & al., 2003), to ensure the check on illegal smuggling of orchids in which orchids account for > 70% of the species listed on CITES (Fay, 2018). Despite the conservation strategies, there is a possibility of to complete disappearance of the orchid species as the absence of pollinators due to overuse of pesticides, fragmented habitats, or other modifications of the biome. Hence, micropropagation is an efficient strategy to replenish its population by regenerating new plantlets. This technique is an economically viable option for obtaining large-scale multiplication within short time.

***In vitro* propagation**

In vitro germination of seeds is an important aspect in the orchid multiplication and conservation program since the dust like tiny seeds have the capability of developing into complete seedlings without any fungal aid. An efficient seed germination protocol has been standardized to focus on propagation of orchid seedling in huge numbers which will help in the conservation of endangered or threatened orchid species. It was reported in many literature that addition of organic additives to orchid seed germination medium promotes seed germination, accelerates PLBs formation and ultimately produce ample number of seedlings. Coconut water (CW) is the colorless liquid endosperm of green coconuts (*Cocos nucifera* L.), which is rich in soluble sugars as a natural source of carbon, amino acids, phenols, fiber and vitamins, moreover, it also contains diphenyl urea which functions as cytokinin that can enhance the explant growth and regeneration by inducing cell division (Texeira da Silva & al.,

2006; Gnasekaran & al., 2010). Another important additive is crushed banana (10%). It was revealed that banana fruit pulp contains different carbohydrates, minerals, amino acids, fatty acids, niacin, vitamins, cellulose, polyols, and sterols (Tamura, 1970). These organic additives contain a natural source of carbohydrates, inorganic ions, amino acids, vitamins and phytohormones which are responsible for orchid propagation by promoting growth and morphogenesis in asymbiotic seed cultures. An efficient regeneration system for micropropagation is shoot tip culture for the production of large number of plantlets in a short period of time. It has also been proved to be economically viable option for biodiversity and gene pool conservation.

Acclimatization

In tissue culture, the quality of *in vitro* raised plants directly depends upon the acclimatization stage (Diaz & al., 2010). The *ex vitro* establishment of regenerated plantlets plays an important role in the large-scale propagation of various orchids. Regenerated plants under *in vitro* conditions have weak root systems and poorly developed cuticles and are susceptible to a high percentage of damage and loss (Ortega-loeza & al., 2011; Mathur & al., 2008) which requires a hardening process to get sturdy for better survival before *ex vitro* transplantation. Different substratum may be used singly or in combination based on orchid species to achieve maximum survival rate and successful establishment of regenerated plants. Potting mixture plays an important role in sustainability of the *in vitro* raised plants. The potting mixture is important for water holding capacity, proper aeration, and draining out of excess water for the proper establishment of plantlets (Diaz & al., 2010; Kang & al., 2020). Potting mixture may consist of small brick chips (~ 5–7 mm in size), charcoal pieces (~ 5–7 mm in size), coco peat, decayed bark pieces and sphagnum moss in a particular ratio.

Phytochemical screening

Phytochemicals are bioactive chemical compounds naturally found in plants that build up as defense system to deal with environmental stress. These biologically active compounds are known as secondary metabolites that have attained increasing attraction for their medicinal importance on human health (Tripathi & al., 2012). The phytochemicals are of two types *i.e*., primary and secondary metabolites. The primary metabolites play an important role in normal growth, development, and reproduction of plants. However, various environmental factors enhanced secondary metabolites production in plant cells. The production of secondary metabolites in plants is directly influenced by different factors such as genotype, physiology, environment, and pathogen during their developmental stages (Gonçalves & Romano, 2018; Isah, 2019). This secondary metabolite possesses a wide range of potent biological and pharmacological activities for human health benefits *i.e.,* antioxidative activity, free radical scavenging capacity, cardioprotective, antidiabetic, anti-inflammatory, anti-allergic, antiviral, anticancer activity (Pietta, 2000; Karak, 2019).

Recently, the natural antioxidants of plants become of major industrial importance due to their health-promoting capacity. Antioxidants are important compounds that help in reducing the oxidation process in the cell or lipid peroxidation for protecting the human biological system from oxidative stress induced by overproduction of oxygen radicals which may cause induction of various life-threatening diseases including cancer, atherosclerosis, gastric ulcer, and arthritis (Behera & al., 2018). Flavonoids are shown to have higher antioxidant capacity due to the double bond present in C-ring (Loganayaki & al., 2013). The antioxidant compound has the efficacy to break the initiation or proliferation of free radical chain reactions. Phenolic or aromatic rings enable the antioxidants to donate hydrogen to scavenge the free radicals produced by the oxidation process (Tan & al., 2018). Phenolic compounds are known to be responsible for the plant to be potent in natural antioxidants (Silva & al., 2010; do Nascimento & al., 2018; Reddy & al., 2020) which play a vital role in the broad spectrum of biological and pharmacological activities (Baskaran & al., 2014; Wink, 2015; Ahmed & al., 2016; Tungmunnithum & al., 2018).

The first written records on the medicinal uses of plants appeared in about 2600 BC from the Sumerians and Akkaidians (Samuelson & al., 1999). Orchids contain different natural compounds mainly phenols, diterpenoids, steroids, flavonoids, cardenolides, triterpenoids, coumarins, isocoumarins and many more which exhibit interesting biological activities such as anti-rheumatic, anti-inflammatory, antiviral, anti-carcinogenic, anticonvulsive, diuretic,

neuro protective, relaxation, anti-aging, wound healing, hypoglycemic, antitumor, anti-cancer, antimicrobial, antibacterial, antioxidant, anti-diarrheal properties (Ghanaksh & al., 1999; Moin & al., 2012; Pant & al., 2013; Islam & al., 2013; Bhattacharjee & al., 2014; Rokaya & al., 2014; Dalara & al., 2015). Other medicinal properties of orchids were reported such as tonic in hysteria, spasm, madness, and epilepsy, treatments of rheumatism, tuberculosis, body ache, eczema, headache and fever, aphrodisiac, and cardiac, respiratory, and nervous disorders (Pant & al., 2013).

Plants and its derived products are backbone of traditional medicines around the world. The phytochemical compound present in the plant such as terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains and other olites which are rich in antioxidant activity which is responsible for medicinal properties. Antioxidants may help our body to protect itself against various types of oxidative damage caused by reactive oxygen species (ROS) are major reasons for several human disorders including cancer, diabetes, shock, arthritis and acceleration of the ageing process. They may act by decreasing oxygen concentration, intercepting singlet oxygen, preventing first chain initiation by scavenging initial radicals, binding metal ion catalysts, decomposing primary products to non-radical compounds and chain-breaking to prevent continued hydrogen abstraction from substrates (Zheng & al., 2001).

As it is known that synthetic drugs are prepared by using different types of chemicals and hence it may be unfavorable for our health. Natural phytochemical compound found in plants can be used as substitute drug. Ethnomedicine is the origin of all modern drugs and recently the importance of traditional medicine has been increased throughout the world (Singh & al., 2002). Such medicinal plants become the base for the development of a medicine, a natural blueprint for the development of new drugs (Iwu & al., 1999). The phytochemical compounds of plants have importance not only for drug discovery; it derives such economic materials as tannins, oils, gums, flavonoids, saponins, essential oils. These are the precursors for the synthesis of complex chemical substances (Basker & al., 2012).

So far very little research has been done for phytochemical analysis in medicinal orchids of North East India. In this context, the proposed study was confined to the state of Meghalaya. The study focused on the screening of polyphenols which can be utilized for the welfare of the people of India as well as the mankind. Apart from this, the present study focused on protocol standardization and successful micropropagation of six important medicinal orchids of Meghalaya. The important medicinal orchids in which the study focused are as follows: *Aerides odorata* Lour., *Dendrobium chrysotoxum* Lindl., *Dendrobium nobile* Lindl., *Bulbophyllum odoratissimum* (Sm.) Lindl. ex Wall., *Cephalantheropsis obcordata* (Lindl.) Ormerod., and *Malaxis accuminata* D. Don.

Aerides odorata Lour.

The leaf extract of *Aerides odorata* is used for the treatment of wounds by the people of Nepal (Mohanty & al., 2015) and the aqueous extract of this species is reported to have good antimicrobial activity against the three strains of *E. coli*. (Paul & al., 2013). The Dongaria Kandha tribes of Orissa used the leaf juice to heal the boils in the ears, nose and to cure tuberculosis (Dash & al., 2008).

Dendrobium chrysotoxum Lindl.

Erianin, a natural product isolated from *D. chrysotoxum,* caused moderate growth delay in xenografted human hepatoma Bel7402 and melanoma A375. The published literature states that Erianin has the therapeutic potential to inhibit angiogenesis Donorand *in vitro* (Gong & al., 2004). A new compound, 1,4,5-trihydroxy-7-methoxy-9H-fluoren-9-one, has been isolated from the whole plant of *D. chrysotoxum* which displayed selective cytotoxicity (Chen & al., 2008).

Dendrobium nobile Lindl.

Lee & al. (1995) reported the isolation of two phenanthrenes from the aerial part of *D. nobile* Lindl. and these two compounds were found to be cytotoxic against A549 (human lung carcinoma), SK-OV-3 (human ovary adenocarcinoma), and HL-60 (human promyelocytic leukemia) cell lines. Zhao & al. (2001) reported three new

sesquiterpene glycosides isolated from the stems of *D. nobile* Zhang & al. (2007) reported that ethanol extract of the stems of *D. nobile* led to the isolation of two new bibenzyl derivatives, nobilin D(1) and nobilin E(2), and a new fluorenone, nobilone(3) which possess significant antioxidant activity.

In addition, fresh and dried stem is aphrodisiac and analgesic. Pseudobulb extract cures eye infections and soothe burns.

Bulbophyllum odoratissimum Lindl.

Two new dimeric phenanthrenes derivatives of *B. odoratissimum* namely, bulbophythrins A (1) and B (2) showed cytotoxic activity against human cancer cell lines such as human leukemia cell lines K562 and HL-60, human hepatoma BEL-7402, human lung adenocarcinoma A549 and human stomach cancer cell lines SGC-7901 (Xu & al., 2009).

Moreover, this orchid is well known for its importance as folk medicine in different places. It has been reported that powdered form of whole plant (known as Thurjo) used as a traditional medicine to cure tuberculosis, chronic inflammation and fractures by the people of Nepal (Mohanty & al., 2015) and also used by the Chinese community to treat phthisis and rheumatism (Zhang & al., 2007).

Cephalanceropsis obcordata (Lindl.) Ormerod.

Crude methanol extract of rhizomes of *C. obcordata* showed significant cytotoxicity against human breast carcinoma (MCF-7), lung carcinoma (NCI-H460), and central nervous system carcinoma (SF-268) cell lines (Wu & al., 2006).

Gutiérrez (2010) reported ethnomedical use of *Cephalantheropsis obcordata* as anticancer agent in Taiwan, China.

Malaxis acuminata D. Don.

Metal content and volatile constituents in *M. acuminata* collected from Uttarakhand were analyzed by Atomic Absorption Spectrophotometer (AAS) and GC and GC-MS respectively. Therefore, more useful work for purification, isolation and characterization on bio-active compounds of this plant is required (Lohani & al., 2013).

Since ancient times *M. acuminata* has been used as traditional medicine. It is used in the preparation of Chyawanprash (Caius, 1986) and also used to increase the quantity of semen or to stimulate the production of semen (Chauhan, 1999). Its swollen stem is sweet, refrigerant, aphrodiasiac, styptic, antidysenteric, febrifuge and tonic. It is used in condition of sterility, vitiated condition of pitta and vata, seminal weakness, internal and external haemorrhages, dysentery, fever, emaciation, burning sensation and general debility (Jadhav, 2008). Paste of pseudobulb can be applied externally in case of insect bites, and when mixed with other plants are used in the treatment of rheumatism (Chakarvarty, 1976).

The most efficient technique for isolation of secondary metabolites is plant tissue culture yield higher productivity within short time as compared to the extraction from wild plant (Gonçalves & Romano, 2018). The *in vitro* culture is an attractive alternative approach for viable production of secondary metabolites irrespective of geographical and climatic conditions throughout the year. In some cases, *in vitro* grown plantlets produce higher secondary metabolites as compared to mother plant (Giri & al., 2012; Bhattacharyya & al., 2014; 2015; 2016; Chavan & al., 2014). Various approaches include the use of different medium strengths, plant growth regulators (PGRs), elicitors, and different additives to stimulate the production of secondary metabolites (Muthukrishnan & al., 2018), however, maximum accumulation of secondary metabolites is reported to be present in the wild plant by several researchers (Joshi & al., 2009; Muthusamy & al., 2015).

High-performance liquid chromatography (HPLC) is a versatile, reproducible column chromatographic technique used in the estimation of secondary metabolites/bioactive compounds (Malviya, 2010). It is an important qualitative and quantitative technique used for isolation, and quantification of the individual components of the

mixture in phytochemical and analytical chemistry (Boligon & Athayde, 2014). Among other analytical techniques, HPLC is a more effective method for quality control of herbal plants (Fan & al., 2006; Boligon & Athayde, 2014). This is a powerful approach for the detailed characterization of a sample by analyzing its chemical property. The integral components that are biologically active are often present in minor bits in an extract. The HPLC is ideally built on both analytical and preparative scales for the rapid processing of such multicomponent samples. These days HPLC is modular in design and is equipped with a solvent delivery pump, a sample introduction device such as an auto-sampler or manual injection valve, an analytical column, a guard column, a detector, and a recorder or a printer (Sasidharan & al., 2011). In HPLC, chemical compounds were separated based on C18 reverse-phase columns and a binary solvent gradient. Generally, the mobile phases contain an aqueous solution of acid and a less polar organic solvent (acetonitrile or methanol). Application of HPLC preferably focuses to characterize and quantify secondary metabolites present in plant extracts like phenol compounds, steroids, flavonoids, alkaloids (Boligon & Athayde, 2014).

Objectives:

1. Collection of selected medicinal orchid plants *viz. Dendrobium nobile, Dendrobium chrysotoxum, Malaxis acuminata, Bulbophyllum odoratissimum, Aerides odorata, Cephalantheropsis obcordata.*
2. Standardization of micropropagation protocol/*in vitro* seed germination for the selected orchid plants *viz. Dendrobium nobile, Dendrobium chrysotoxum, Malaxis acuminata, Bulbophyllum odoratissimum, Aerides odorata, Cephalantheropsis obcordata.*
3. Sequential extraction of plant parts using appropriate solvents for phytochemical profiling of those selected orchids.
4. Qualitative and quantitative analysis of bioactive compounds from the plant extracts of *Dendrobium nobile, Dendrobium chrysotoxum, Malaxis acuminata, Bulbophyllum odoratissimum, Aerides odorata, Cephalantheropsis obcordata.*
5. Comparative studies of its antioxidant properties both *in vitro* and mother plants of these selected orchids.

Chapter II

REVIEW OF LITERATURE

Plant tissue culture

According to Schleiden and Schwann (1838), each cell is capable enough to regenerate into a whole new plant provided a suitable environmental condition prevails for the purpose. Gottlieb Haberlandt (1902), a German physiologist first attempted to culture isolated single palisade cells from the leaves of the plant and was successful with his pioneering experimentation which the explant survived for 3-4 weeks which became the foundation for plant tissue culture techniques and for which he was recognized as the father of plant tissue culture (Hussain & al., 2012). Later, Gautheret (1934, 1935) tried with cambial tissue of *Acer pseudoplatanus* and successfully developed a healthy callus on the explant (Thorpe, 2007). Skoog and Miller (1957) discovered, the ratio of auxin to cytokinin in nutrient media greatly affects the morphological development of roots and shoots in plant tissue culture. Murashige and Skoog (MS) (1962) developed a medium that contains a nutrient blend of inorganic salts, vitamins and amino acids which became the standard formulation and widely used for micropropagation, organ culture, callus culture and suspension culture. The mutual relationship between orchids and fungi was discovered as early as 1824 whereas the role of the mycorrhizal association in orchid seed germination was established in 1899 and the asymbiotic seed germination of orchid was established in 1921 (Yam & Arditti, 2009). The seeds of an Orchid contain an undifferentiated embryo and are devoid of endosperm because of which their germination rate in nature is very poor. The orchids are highly heterozygous, and few plants are produced in a period of five to six years as their vegetative propagation through division, splitting of shoots and kiekis are very slow (Sarmah & al., 2017). On the other hand, the plant tissue culture is the process of growing isolated plant cells or organs in an artificial nutrient media to develop thousands of genetically identical plants from one single parent plant known as soma clones, and this process is known as micropropagation. This method offers an advantage over other methods as it can be used to develop disease-free plants from disease-rode plants by using their meristems (apical and axillary) as explants. The most notable example of the application of micropropagation was observed in the farming of orchids as it rose exponentially due to the availability of millions of plantlets due to tissue culture methods. Plant tissue culture has been proved to be an economically viable option for healthy and disease-free large-scale multiplication of orchids within short span of time (Debnath & al., 2006). Panwar & al. (2012) developed a very efficient *in vitro* propagation protocol for *Eulophia nuda* Lindl. an endangered orchid of Aravalli hills using the tuber (1 cm cut pieces) used as explants and shoot multiplication on MS medium containing BA (8.88 µM), Kin (4.68 µM) with additives and demonstrated the shoots with the tubers successfully rooted *in vitro* and *ex vitro* mixed with IBA (2.46 mM) in the medium. Devi & al. (2013) studied *Aerides odorata* Lour. an endemic orchid of Manipur for a rapid clonal propagation using both the leaf base cuttings and the shoot tip as the explants and subsequently observed the callus formation from the explants in the medium containing NAA and promote direct shoot regeneration due to the synergistic effect of cytokinin and auxin. They found a higher number of shoots (4.80±0.18) corresponding to higher concentrations of NAA (2.0 mgl^{-1}) and BAP (4.0 mgl^{-1}) with maximum root proliferation in ½ MS medium fortified with NAA (0.5 mgl^{-1}). Kabir & al. (2013) reported Phytamax (PM) as the most effective medium for seed germination of *Dendrobium fimbriatum* and recorded a 100% rate seed germination. They observed the multiple shoot (4.35/explants) formation on MS medium mixed with BAP (1.0 mgl^{-1}) and Picloram (0.5 mgl^{-1}); Effective shoot elongation in supplement with 6-benzylaminopurine (BAP) (2.0 mgl^{-1}) and indole-3-butyric acid (IBA) (0.01 mgl^{-1}) and maximum rooting in media with indole-3-acetic acid (IAA) (1.0 mgl^{-1}). Bhattacharjee & Islam (2014) worked on multiple shoots regeneration of *Vanda tessellata* (Roxb.) Hook. ex G. Don an endangered medicinal orchid using shoot segments as explants in four different culture media *viz.,* MS, ½MS, B5, PM, and found MS medium the most effective with an 80% rate of seed germination. They used twenty (20) different combinations of plant growth regulators (PGRs) for rapid mass multiplication and subsequently the differentiation of shoot occurred after 28 days of culture in MS media mixed with BAP, NAA, IAA, and Kin concentration (0.5-1.5 mgl^{-1}). The maximum shoots (7.52 shoots per single shoots) were obtained from the shoot bud *in vitro* growing seedlings on MS

media with NAA (1.0 mgl^{-1}) and BAP (1.0 mgl^{-1}) and the highest number of root (6.1) and length (4.0 cm) was recorded in ½MS medium with IAA (1.0 mgl^{-1}). Baker & al. (2014) developed a high-frequency shoot proliferation protocol from the protocorms of an endangered orchid, *Orchis catasetum* and reported a maximum number of PLBs regeneration (20.40 $plantlet^{-1}$), largest number roots (7.16 $plantlet^{-1}$), leaf (10.10 $plantlet^{-1}$), maximum plant height (114.20 mm $plantlet^{-1}$) and root length (193.40 mm $plantlet^{-1}$) in MS medium with BA (0.5 mgl^{-1}) and NAA (0.5 mgl^{-1}). Hongthongkham & Bunnag (2014) developed an efficient protocol for *in vitro* propagation and cryopreservation of *Aerides odorata* using leaf segments as explants and observed initiation of protocorm-like bodies (PLBs) in the ND media with BA (1.0– 3.0 mgl^{-1}) and NAA (0.5-1.0 mgl^{-1}) and shoot development at high concentration of BA (5.0 mgl^{-1}). The leaf segment-derived encapsulated PLBs were used for cryopreservation purposes with 2% Na-alginate combined with 2 M glycerol and 0.4 M sucrose as the survival rate was found to be higher. The encapsulated PLBs were pre-cultured and assess the genetic stability by flow cytometry. Sherif & al. (2016) worked on *Anoectochilus elatus* Lindl. and established an indirect organogenesis protocol via callus formation using node, internode, and leaf as explants. During the process, they observed the rate of organogenic callus proliferation from three different parts of the explants were recorded with internode (77.8%), node (69.7%) & leaf (64.2%) on a mixed Mitra medium with TDZ (1.0 mgl^{-1}) & NAA (0.5 mgl^{-1}). Besides, the higher number of shoots per explants was recorded from the internodal cuttings with an average of 41.8 shoots per explants and length 2.5cm on the mixed media with BA (1.0 mgl^{-1}), NAA (0.5 mgl^{-1}), coconut water (10%) and highest rooting frequency (2.1 cm long) in the media amended with $AgNO_3$ (1.0 mgl^{-1}). Mahendran & Bai (2016) worked on *Aphyllorchis Montana* Rchb.f. a saprophytic achlorophyllous orchid for its seed germination. The germination rate was found best in the BM-1-terrestrial orchid medium and the maximum number of shoots (17.24 shoots) and effective for shoot regeneration in media supplemented with thidiazuron (TDZ) (6.8 mM) and organic additives. Bhowmik & Rahman (2017) studied *Calanthe densiflora* Lindl. a terrestrial orchid from Bangladesh regarding *in vitro* seed germination and using rhizome segments of aseptic seedlings as explants for micropropagation protocol. They observed a higher rate of seed germination in MS media mixed with sucrose, maximum multiple shoot buds (6.45±0.41/segment) produce from a rhizome as a result of the direct organogenesis in the media mixed with 3%(w/v) sucrose + IAA (1.0 mgl^{-1}) + BAP (2.0 mgl^{-1}) and obtained the best root system (strong and stout) in the medium supplemented with sucrose (3%w/v) and IBA (0.5 mgl^{-1}). Sherif & al. (2017) reported several factors such as lack of suitable pollinators, low fruit development, symbiotic association with mycorrhizal fungus and indiscriminate collection are responsible for the declining of *Anoectochilus elatus* Lindl., an endangered terrestrial orchid in its habitat. To protect and regenerate this important species they developed the micropropagation protocol for eco-restoration programs and recorded proliferation of multiple shoots in MS medium supplemented with TDZ (1.5 mgl^{-1}) and peptone (50 mgl^{-1}) in which the axillary bud (17.2 shoots/explant) shows a better explant than shoot tip (14 shoots/explant). In the subsequent year (Sherif & al., 2018) they established the somatic embryogenesis protocol (direct and indirect) for *Anoectochilus elatus* Lindl., with direct somatic embryogenesis from nodal explants recorded at the rate of 7.4 embryos (per explants) and 63.4% successful responses on mixed Mitra medium with Morel vitamins, thidiazuron (4.54 µM) and ∞-naphthaleneacetic acid (2.69 µM). On the other hand, the indirect somatic embryogenesis using the internodal explant was successful in the same media mixed with peptone (50 mgl^{-1}) & coconut water (5%), besides, the regenerated plantlets were established successfully during the significant heterotrophic to a photoautotrophic stage. Decruse & Gangaprasad (2018) regenerated *Smithsonia maculata* (Dalzell.J.Saldanha an endemic orchid of the Western Ghats by seed germination and *in vitro* propagation technique. The eight-month-old seed of this orchid was successfully germinated at the rate of 70% in Mitra medium (1976) supplemented with organic additives and vigorous growth of protocorms with deep green pigmentation promoted by coconut water (20%). Further, the development of protocorms into seedlings with roots and leaves within a 2-3 months period by mixing the media with coconut water (20%) or casein hydrolysate (0.05%) respectively. Also, the leaf base of aseptically grown seedlings produces an average of 11.25 shoots/leaf in 6-9 months on M medium fortified with BAP (10 mgl^{-1}) and IAA (1.0 mgl^{-1}) and root proliferation was successful in woody plant medium (Lloyed and McCown, 1980; WPM) enriched with banana pulp (5%). Kunakhonnuruk & al. (2018) reported *Epipactis flava* Seidenf., an endangered Thai rheophytic orchid on the verge of extinction due to habitat destruction and to conserve this orchid, an asymbiotic seed germination was done with 2, 4, 6, & 8 weeks old seed capsule after its pollination on a semi-solid VW medium supplemented with coconut water (150 mL/L) & potato extract (50 g/L)

along with the highest seed germination rate of 79.2% recorded from 6-weeks old cross-pollination capsules and high shoot number obtained in liquid MS medium. Park & al. (2018) work on *Cymbidium goeringii* (Rchb.f.) Rchb.f. and explored the influence of different compounds *viz*., 2,4-dichlorophenoxyacetic acid (2,4-D), benzyladenine (BA), and thidiazuron (TDZ) on direct rhizome induction and shoot formation using rhizome as the explants and observed 100% rhizome formation in MS media with of 2,4-dichlorophenoxyacetic acid/2,4D (20 µM), formation of adventitious shoots with either BA or TDZ, highest shoot buds (21.8±1.8) per rhizome segment in media with 2,4-D (20 µM) and TDZ (2µM) and 100% root induction and formation of roots (5.3±1.1) per shoot in ½ MS medium with α-naphthaleneacetic acid (2 µM). Maharjan & al. (2020) study on *Dendrobium chryseum* Rolfe on its immature seed germination obtained from the plant and recorded its successful germination and protocorms were produced from *in vitro* propagation and its seedlings inoculated successfully in ½ MS medium mixed with Kinetin (Kn), 6-Benzylaminopurine (BAP), and Gibberellic Acid (GA_3) in three concentrations (0.5 mgl^{-1}, 1.0 mgl^{-1} & 2.0 mgl^{-1}) either coconut water (CW) in 5% or 10% concentrations and maximum shoot proliferation observed in ½ - MS medium with Kn (2.0 mgl^{-1}) & CW (10%) and rooting development in mixed with IAA (1.5 mgl^{-1}). Ma & al. (2020) developed a standard surface sterilization protocol with sodium hypochlorite (20%) for about 15 mins (sterilization period) for regeneration of *Dendrobium aurantiacum* (F. Muell.) F. Muell from the shoot explants and observed 83.33±5.8% survival rate even after one month of its applications and highest callus induction and proliferation were obtained achieved in a Murashige and Skoog medium (MS medium) supplemented with 2,4-D (10 mgl^{-1}). Kashem & al. (2020) worked on direct organogenesis in *Eria tomentosa* (J.Koenig) Hook.f. using stem and leaf segments of *in vitro* grown seedlings of an important medicinal orchid of Bangladesh which observed the shoot primordial-like structures (SPSs) proliferated in MS medium mixed with BAP (1.5-3.0 mgl^{-1}) + Kn or in combination with 0.5- IAA (1.0 mgl^{-1}) + NAA and SPSs were induced effectively in liquid MS + sucrose (3% w/v) + Pic (0.5 mgl^{-1}) + BAP (1.0 mgl^{-1}) in less time than agar solidified PM. Lal & al. (2020) developed the mass propagation protocol (*in vitro*) for maximal plantlet conversion of two orchids *viz*., *Aerides multiflora* Roxb. and *Rhynchostylis retusa* (L.) Blume using immature seeds as explants found in the Himalayan region which are widely used both for ornamental and medicinal purposes. They observed the effective inducing shoots (3.33) and roots (3.67 per protocorm) of *A. multiflora* in Mitra medium supplemented with BAP (4.44 µM) along with NAA (5.38 µM) and the plantlet regeneration from protocorms of *R. retusa* in Mitra medium mixed with BAP (4.44 µM). Gantait & al. (2020) studied *Calanthe odora* Griff., a lithophyte terrestrial orchid, which is found both in the North West and the North-Eastern part of India for mass propagation using germination of seed technique and observed the most efficient multiplication of protocorm and its plantlet development in MS mixed media in combination with Kinetin (1.0 mgl^{-1}) and NAA (1.0 mgl^{-1}) along with a maximum number of roots per plant (6.33) in the media with Kinetin (1.0 mgl^{-1}) and NAA (1.0 mgl^{-1}). Bhowmik & Rahman (2020) developed a micropropagation protocol for mass propagation of an aromatic orchid *Aerides multiflora* Roxb. using nodal segments and leaves as explants obtained from *in vitro* raised plants and recorded nodal explants more responsive to MS medium mixed with NAA (1.0 mgl^{-1}) + BAP (2.0 mgl^{-1}) which reported the maximum multiple shoot buds formations (8.83±0.45/segment), whereas, greenish PLBs were produced from the leaf explants on the media with IAA (1.0 mgl^{-1}) replacing NAA and efficient development of shoot length (4.32 ± 0.24 cm/shoot bud) and roots proliferation (3.19 ± 0.22 /shoot bud) were observed on MS (agar solidified) mixed with IBA (1.0 mgl^{-1}). Manokari & al. (2021) studied on *in vitro* regeneration of *Vanda tessellata* (Roxb.) Hook. ex G. Don., for large-scale production due to its importance in horticulture, medicinal, phytochemistry and pharmacology. They observed 100% seed germination in MS medium mixed with IBA (1.5 mgl^{-1}) and the protocorms proliferated into multiple shoots (24.8 ± 0.52 shoots & 5.2 ± 0.35 cm length) on the medium augmented with BAP & IAA (0.5 mgl^{-1}) + AC (100 mgl^{-1}) after the 4th sub-culture and the highest rooting frequency observed in the media with IBA (1.0 mgl^{-1}). Tikendra & al. (2021) reported for the first time the successful *in vitro* propagation of *Dendrobium fimbriatum* Hook., an ornamental and medicinal orchid using Mitra medium (MM) supplemented with different growth regulators and effective shoot formation recorded in mixed medium with KN alone or in combination with IBA or NAA along with root proliferation in IBA either in combination with BAP or KN respectively. Bhowmik & Rahman (2020) worked on *Dendrobium palpebrae* Lindl, an epiphytic fragrant orchid for mass propagation using the upper and lower portion of the pseudobulb segments of *in vitro* growing plantlets used as explants inoculated in MS medium mixed with auxins (IAA, IBA, NAA,

Picloram) and cytokinins (BAP, Kinetin) and observed the maximum number of multiple shoot buds in both lower part (8.21 ± 0.44) and upper part (6.43±0.40) segments in the media mixed with NAA (1.0 mgl^{-1}) + BAP (2.0 mgl^{-1}), whereas, a lesser record of shoot buds formation in both the lower part (7.24 ± 0.41) and upper part (5.96±0.37) segments in the media supplemented with Picloram (1.0 mgl^{-1}) + BAP (2.0 mgl^{-1}) with increased in length (3.76±0.14 cm) of individual shoot bud in MS medium (agar solidified) with Picloram (1.0 mgl^{-1}) + BAP (1.0 mgl^{-1}) and slightly lesser length (3.11±0.12 cm) recorded in the liquid media with NAA (0.5 mgl^{-1}) + BAP (1.0 mgl^{-1}). The highest number of roots formation (4.82±0.22 cm/shoot bud in lower segment and 2.75±0.17 cm /shoot bud in upper segment) was observed in MS media (Agar solidified) with NAA (0.5 mgl^{-1}).

Acclimatization

The survivability and successful establishment of the regenerated plantlets depend solely upon the hardening process and the types of substrata used (Deb and Imchen, 2010). The main characteristics of an appropriate potting mixture are water holding capacity, helps in aeration, and draining out of excess water for the proper establishment of plantlets (Diaz & al. 2010; Kang & al. 2020). The potting mixture plays an important role in the sustainability of the *in vitro* raised plants and different substrata were reported to be found as suitable potting media in several studies on micropropagation of orchids. Poobathy & al. (2019) observed the survival rate of *in vitro* raised plantlets obtained from 1-2 cm tall explant was 73% and the plantlets produced from the inner leave tissue of *Ludisia discolor* (Ker Gawl.) A.Rich. (Jewel orchid) was 79% which was successfully acclimatized under low light conditions using coconut coir, coconut husk, and peat moss. Lal & al. (2020), reported an 80% survival rate of seedlings of both *Aerides multiflora* Roxb. and *Rhynchostylis retusa* (L.) Bl. when the plantlets were raised from *in vitro* conditions was transplanted to polyhouse and the field respectively. Manokari & al. (2021) reported an effective potting mixture for acclimatization of *Vanda tessellata* (Roxb.) regenerated plantlets were using soilrite® + cocopeat + coconut husk complex in the greenhouse for 4 weeks which enhanced the survival percentage (98%) for hardened plants under relatively increased temperature and low humidity for 4–5 weeks. Similarly, the survival percentage (90%) of *Spathoglottis plicata* Blume *in vitro* regenerated plantlets were observed in the greenhouse using potting mixtures of soilrite® and vermicompost. Gantait & al. (2020) recorded the maximum percentage survival (86.7%) of *in vitro* raised plantlets of *Calanthe odora* Griff during the hardening process on vermiculite media in polyhouse after four weeks of its transfer. Asa and Kaviani (2020) recorded about 90% survived of *in vitro* plantlets of *Phalaenopsis amabilis* (L.) Blume var. *jawa* while transferring to pots consisting of coco chips and sphagnum moss in a proportionate ratio (70:30).

Phytochemical Screening on Medicinal Plants

Phytochemistry is the study of chemicals derived from plants, particularly the important secondary metabolites (Egbuna & al., 2018). Sohag & al. (2017) investigated on a rare medicinal orchid *Luisia zeylanica* Lindl. for its phytoconstituents and antioxidant activity on different parts of the plant (leaf, stem and root) in different solvent extraction like Methanol, n-Hexane, Butanol, and Dichloromethane (DCM) and reported the presence of alkaloids and secondary metabolites *viz.,* flavonoid, steroid, saponin, phlobatannin, terpenoid, tannin, glycoside, anthraquinone, quinine, and coumarin. They observed the leaves extraction contained more secondary metabolites and the stem and root extracts possessed a superior antioxidant potential over the leaf extracts. Sinha & Biswas (2020) reported phytochemical profiling of *Dendrobium fimbriatum* Hook. in different extraction media and confirmed the presence of alkaloids, terpenoids, flavonoids, tannins, and glycosides in the plant along with the highest percentage yield in warm ethanol extraction with 12.6%, followed by ethyl acetate and lowest in cold ethanol. Akter & al. (2020) investigated the phytochemical components, antioxidant activity and anti-inflammatory activity of one medicinal orchid *Eria tomentosa* (J.Koenig) Hook.f. using different solvents like hexane, dichloromethane (DCM), methanol, and butanol fractions for extraction on dried samples of leaf, bulb, and root of the plant. They reported the presence of flavonoids, tannins, and quinine in high quantity and saponins, steroids, coumarin in moderate in the leaf extracts. Similarly, the bulb extract was rich in saponins, tannins, steroids, glycosides, and coumarin. Also, they observed the presence of phlobatannins, tannins, terpenoids, steroids, glycosides, quinine, and coumarin in roots extract and at the same time the roots contain more disease-resisting secondary metabolites with the leaf extracts possessed the most potential compounds for the antioxidant activity.

Antioxidant activity and HPLC screening

Giri & al. (2012) worked on the production of phenolic compounds from the callus suspension cultures of *Habenaria edgeworthii* Hook.f. ex Collett and observed the effects of BA in containing the total phenolic content ranging from 10.33 to14.30 mg gallic acid equivalent (GAE) per gram dry weight (DW) with maximum antioxidant activity in the callus and the analysis of High-performance liquid chromatography (HPLC) of the callus rich in high gallic acid content (143.63mg/100 g DW) as compared to the wild tuber (5.5 mg/100 g DW). Bhattacharyya & al. (2014) analysis the phytochemical compositions in the stem and leaf of both the mother and the micropropagated plants of *Dendrobium nobile* Lindl. and reported the presence of phenolic and flavonoid contents in both the plants (mother and micropropagated) of which the micropropagated plant exhibited higher phenolic content in the stem extract (methanolic) along with higher DPPH activity and the donor plant revealed the lowest content of phenolic in the leaf extract (chloroform). Chavan & al. (2014) worked on the phytochemical contents in the tuber, stem, and leaf) of the mother and the *in vitro* raised plants (both direct shoot organogenesis, DSO and indirect shoot organogenesis, ISO) of *Ceropegia santapaui* Wadhwa & Ansari using four different solvents (methanol, ethanol, acetone, and water). They observed the ISO-derived plants showed a higher level of total phenolic content with antioxidant activity as compared to DSO-derived and mother tissues. Similarly, Bhattacharyya & al. (2015) studied the phytochemical constituents on both the mother and the *in vitro* micropropagated plant of *Dendrobium thyrsiflorum* B.S.Williams and reported both the ISO- and DSO-derived plants possess higher antioxidant potentials, higher yield of secondary metabolites as compare to the mother plant and ISO-derived plants were more phytochemically enriched as compared to the DSO plants. Minh & al. (2016) investigated on phytochemical compounds using ethanol extracts of the leaves and roots of six commercial hybrids of *Phalaenopsis* spp. and reported that high amounts of phenolic compounds was found in the extracts prepared from leaves and roots which exhibit strong antioxidant activities. Eleven phenolic compounds (protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, vanillin, ferulic acid, sinapic acid, p-coumaric acid, benzoic acid, and ellagic acid. Ferulic, p-coumaric and sinapic acids) were identified in these extracts. (Mention the 11 phenolic compounds). Of these, ferulic acid, p-coumaric acid, and sinapic acid are found in large quantity in the roots as compared to the leaves. In conclusion, the root extracts of Phal. orchid hybrids are potentially important sources of natural antioxidants. Mahendran & Bai (2016) worked on a saprophytic achlorophyllous orchid, *Aphyllorchis montana* Rchb.f. and determine the antioxidant, antimicrobial activity, total phenolics and flavonoid content using methanolic extracts both in wild-grown and micropropagated plants and reported the higher antioxidant, antimicrobial activity, total phenolics, and flavonoid content in wild-grown plants than *in vitro* propagated plants. Suja & Williams (2016) documented on phytochemical screening and antioxidant potential of a wild epiphytic orchid *Acampe praemorsa* (Roxb.) Blatt. & McCann. Phytochemical analysis of *A. praemorsa* showed the presence of alkaloid, flavanoids, phenol, terpenoid and steroid as well as it contains strong DPPH radical scavenging activity. Singh & Babbar (2016) carried out the chemical profiling of *Herminium lanceum* (Thunb. ex Sw.) Vuijk, a medicinally important orchid and makes a comparative evaluation of chemical profiling between *in vivo* and seed germinated plants by using High performance liquid chromatographic (HPLC). The chromatogram revealed medicinally important phenolic acids like gallic acid (GA), p-hydroxybenzoic acid (HBA), syringic acid (SA) and caffeic acid (CA) identified in leaves and tubers of both *in vitro* and *in vivo* plants. Of these three phenolic acids, GA, HBA and SA, were concentrated more in the leaves of both *in vitro* and *in vivo* plants than in their respective tubers. In conclusion, secondary metabolites were found to be higher in *in vitro* raised plants than natural plants. The reason could be their differences in their age and the climatic conditions in which they grew. Szopa & al. (2017) studied on the phytochemical components of an Asian medicinal plant *Schisandra chinensis* (Turcz.) Baill. from the lyophilized biomass of the plant in the growth media and observed the highest total amounts of phenolic acids (71.48 mg/100g DW) and flavonoids (29.36 mg/100g DW) and detected the rich presence of protocatechuic acid (max. 35.69 mg/100g DW), chlorogenic acid (max. 13.05 mg/100g DW), and quercitrin (max. 27.43 mg/100g DW) in the plant. Bose & al. (2017) worked on an orchid *Malaxis acuminata* D. Don., and reported the presence of antioxidant potential, secondary metabolites, biological activities against skin-aging-related enzymes (anti-collagenase, anti-elastase, anti-tyrosinase, and xanthine oxidase) and anti-inflammatory activity (5-lipoxygenase and hyaluronidase) in different parts both in wild and *in vitro*-derived plants. They recorded the higher antioxidant

potential (DPPH, metal chelating, and ABTS) *in vitro* regenerated plants than the wild plants. On the other hand, they observed the photoprotective activity against UV-B and UV-A radiations with higher sun protection factor (SPF) in the leaves and stems methanolic extracts of both wild and *in vitro* derived plants. Similarly, the Gas Chromatography-Mass Spectrometry (GC–MS) analysis of both the leaves and the stems revealed many bioactive metabolites such as dietary fatty acids, α-hydroxy acids, phenolic acids, sterols, amino acids, sugars, and glycosides. Giri & al. (2017) investigated the phytochemical compositions of eight herbs in Ashtvarga an Ayurvedic rejuvenating tonics like Chyvanprash and reported the highest total phenolic content in *Polygonatum cirrhifolium* (Wall.) Royle., and significantly high content of tannin and flavonoid with minimum TPC in *Roscoea procera* Wall. The different antioxidant activities *in vitro* assay *viz.*, 2,2-azinobis (3- ethylbenzothiazoline-6-sulphonic acid) radical scavenging (ABTS), 2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH), ferric reducing antioxidant power (FRAP), superoxide scavenging activity and HPLC analysis concluded the Ashtvarga plant extracts expressed strong antioxidant potential and can prevent DNA damage from oxidative stress. Mishra & al. (2018) evaluated the phytochemical constituents in *Satyrium nepalense* D. Don., like total phenolic and flavonoid contents and reported the methanol extracts of the plant contained higher levels of phenolics (19.2 mg GAE/g) and flavonoids (11.20 mg QE/g) with strong antioxidant (IC50= 30.79 µg/mL and 24.53 µg/mL for DPPH and ABTS, respectively), antibacterial activities (MIC 71.5 to >100 µg/mL), rich in gallic acid (19.04 mg/g) and quercetin (23.4 mg/g). Paudel & al. (2018) worked on the phytochemical components of *Dendrobium moniliforme* (L.) Sw. and reported the high content of TPC in chloroform extracts (DMC) and TFC content in acetone extract (DMC) respectively with the highest DPPH radical scavenging activity (94.48%) recorded in hexane extract (DMH) and observed the cytotoxic effect both in methanol extract (DMM) and ethanol extract (DME). Bhattacharyya & al. (2018) assessed the phytochemicals compositions between the wild mother and micropropagated plant of *Dendrobium aphyllum* (Roxb.) C.E.C.Fisch. based on DPPH and FRAP assays and observed a significant higher antioxidant potential in the *in vitro* regenerated plants along with higher degree of antioxidant activity in the methanolic extracts and lowest in chloroform extracts of the leaves of micropropagated plants as compared to the wild ones. Kotiloğlu & al. (2020) studied on the tubers of *Dactylorhiza romana* subsp. *georgica* (Klinge) Soó ex Renz & Taubenheim orchid used as food and traditional medicine in Anatolia by using five solvents with different polarities (hexane, ethyl acetate, chloroform, ethanol, or methanol) for plant tuber extraction to determine total phenolic content (Folin–Cicalteu assay), total antioxidant capacity (Phosphomolybdate method), antioxidant (ABTS and DPPH assays) and enzyme inhibition effects of tuber extracts were determined along with HPLC profiling and reported the total phenolic content and antioxidant properties of ethyl acetate extract were considerably higher than the others. In addition, benzoic acid was found abundantly in identified phenolic standards. Paul & Kumaria (2020) studied the effects of different acids *viz.*, caffeic acid, P-coumaric acid and ferulic acid added in the growth medium of a medicinal orchid *Dendrobium fimbriatum* Hook. and reported the addition of caffeic acid (CA) enhances the secondary metabolites production and maximised the rate of total phenolic content (50.73 mg GAE/gm DW at 0.5 mM CA) and flavonoid content (5.73 mg QE/gm DW at 1.0 mM CA) and recorded higher antioxidant activity in the cultures treated with 0.5 mM caffeic acid. On the other hand, the addition of P-coumaric acid (4.0 mM) and ferulic acid (2.0 mM) benefitted the content of tannin (14.77 mg TAE/gm DW) and alkaloids (317.82 mg ATP/gm DW). Song & al. (2020) studied a comparative phytochemical component between the shoot cultures and the callus of *Mammillaria herrerae* Werderm., a critically endangered ornamental cactus and reported higher yield of phytochemicals in shoot extracts as compared to callus cultures and higher total flavonoid content in the shoot (0.96 mg RE/g extract) than callus (0.41 mg RE/g extract). Abdulhafiz & al. (2020) assessed the antioxidant properties of ethanolic extracts on different parts (callus, fruit and petiole) of *Alocasia longiloba* Miq. between the field-grown, *in vitro* propagated, and *in vitro*-derived callus of the plant and reported the highest total phenolic content in *in vitro* derived callus extract (538 mg GAE) followed by the extracts of fruit and petiole of the field-grown plant (504 and 300 mg GAE) and recorded the least in the petiole extracts of *in vitro* derived greenhouse-grown plant (98 mg GAE) along with maximum flavonoids content recorded in the petiole extract. The DPPH-radical-scavenging activity showed better responses in the fruit extract (IC50: 0.088 mg mL^{-1}) as compared to the *in vitro*-derived callus (IC50: 0.113 mg mL^{-1}). Similarly, Rajput & Agrawal (2020) make a comparative evaluation of phytochemicals between the mother plant and *in vitro* raised plant by indirect organogenesis using root-derived callus of a critically endangered therapeutic herb, *Atropa acuminata* Royle ex Lindl. and reported a high yield of phenols and flavonoids with strong

antioxidant activities (DPPH and phosphomolybdenum assay) in regenerated plants and more alkaloids on the leaves extract. Kumar & al. (2020) documented the phytochemical components of an important green leafy vegetable vine, *Basella rubra* using HPLC-UV profiling method and reported the rich presence of important phenolics and flavonoids like gallic acid, trans-cinnamic, quercetin, protocatechuic, and rutin. Yaowachai & al. (2020) studied on the phytochemical compositions in the plantlets regenerated using bulbil as an explant both *in vitro* and the wild plants of *Globba globulifera* Gagnep. based on TPC, TFC, and FRSA methods using methanolic extraction and reported the highest value of TPC (52.28 mg GAE/g crude extract) and FRSA (93.55%) along with the lowest content of IC50 (0.46 mg/ml) in the rhizomes extracts of *in vitro* derived plantlets. Kaur & al. (2021) make a comparative study on the phenolic content, free radical scavenging activities between the field-grown mother plant, and *in vitro* plants of *Withania somnifera* (L.) Dunal, an important medicinal plant and reported higher TPC contents and Free radical scavenging activities *in vitro* plants as compared to the field-grown mother plants, whereas higher content of withanolides in the field-grown mother plants. Bisht & al. (2021) reported the leaves of *in vitro* raised plants of *Berberis asiatica* Roxb. ex DC. a medicinal shrub from Himalayan region, very rich in two alkaloids *viz*., berberine and palmatine as compared to the wild plant along with high antioxidant and antimutagenic activities in the leaves of tissue culture raised plants.

Chapter III

MATERIALS AND METHODS

A. *In vitro* seed germination/ Micropropagation

1. *Aerides odorata*

1.1 *In vitro* seed germination

Mature seed pods were collected from experimental garden of Botanical Survey of India (BSI), Eastern Regional Centre (ERC), Shillong and used for *in vitro* seed germination studies. To standardize optimum medium for seed germination different additives were used.

For seed germination studies, MS basal medium (Murashige and Skoog, 1962) was used. It contains 3% sucrose (Hi-media, India), 0.8% agar (Hi-media, India) and different additives (Table 1). MS medium devoid of additives was used as a control. About 40ml of medium were poured into 100ml conical flask (Borosil Glass Works Ltd., Mumbai, India) and autoclaved (NSW India) along with all the equipment needed for the culture at 121^{o}C and 1.05 kg/cm^{2} pressure for 20 minutes. Seed pods were washed with distilled water thoroughly and kept it in laminar air flow chamber. The pods were fully dipped in alcohol and flamed it over spirit lamp under laminar flow chamber. The pods were cut open using sterile blade. Approximately same amount of seeds using spatula were inoculated in each conical flask. The cultures were maintained in culture room under white fluorescent light at 16/8h photoperiod and 25±2ºC.

Table 1. Different additives for seed germination of *A. odorata*

Medium	Treatment
MS	Control
	10% banana
	10% coconut water

1.2 Effect of different additives on the growth pattern

To standardize the optimum medium for the growth of *A. odorata*, experiments were designed using different additives. For this experiment, 3 months old *in vitro* raised plantlets (initial size of explant ca.0.5cm) were inoculated on MS basal medium containing 3% sucrose (Hi-Media, India) and 0.8% agar (Hi-Media, India). Different additives such as 10% banana homogenate, 10% coconut water, 0.2% activated charcoal were used individually in the medium (Table 2). Banana homogenate was prepared from ripened banana purchased from market. Appropriate amount of banana pulp was weighed, ground and added to the medium. Coconut water was collected from fresh green coconuts, filtered with Whatman filter paper and poured in the medium. 0.2% Activated charcoal (Hi-Media, Mumbai, India) was used in the medium. MS medium devoid of additives was used as a control. About 40 ml of medium was poured in 100 ml flask (Borosil Glass Works Ltd., Mumbai, India) and autoclaved along with all the equipment needed for the culture at 121^{o}C for 20 minutes. Each treatment includes 15 replicates. The pH of the medium with or without additives was adjusted to 5.6.

Table 2. Different additives on growth pattern of *A. odorata*

Medium	Treatments
MS	Control
	10% banana
	10% coconut water
	0.2% activated charcoal

1.3 *In vitro* regeneration through shoot multiplication

This step deals with rapid multiplication of the regenerative system for obtaining large number of shoots. Healthy and young nodal shoot segments from *in vitro* raised plantlets used as source of explants for shoot initiation experiments.

Explant with single node was inoculated on MS medium supplemented with different concentrations (0.5, 1.0, 2.0, 4.0, 8.0 mgl^{-1}) of BA alone and the optimum concentration of BA (4.0 mgl^{-1}) combined with (0.5, 1.0 and 2.0 mgl^{-1}) of GA_3. MS medium devoid of any PGR used as control. Medium devoid of PGR served as the control. About 15ml of medium was dispensed into test tubes. Sterilization of media was carried out in 1.05 kg/cm^2 pressure at 121°C temperature for 20min using autoclave. The cultures were incubated at 25±2ºC under 16/8 hours (light /dark) photoperiod with white fluorescent light intensity.

1.4. Acclimatization

In vitro raised well rooted plantlets were carefully taken out from the culture flasks and were washed thoroughly with tap water to remove the culture medium. The plants were then transferred to root trainer containing small brick chips, charcoal pieces, decayed wood and coconut husk in the green house for hardening (25˚C, RH. 90%). These plants were always kept moist by watering on alternate day. Care was taken so that roots of the seedling passed through the space in between charcoal and brick pieces. Watering with half strength MS medium was done every 7 days interval for 2 months.

2. *Dendrobium chrysotoxum*

2.1. *In vitro* seed germination

The methodology was similar as previously mentioned for the seed germination of *A. odorata.* The additives were used for *in vitro* seed germination of *D. chrysotoxum* were 10% banana, 10% coconut water, 0.2% activated charcoal.

2.2 *In vitro* regeneration through shoot multiplication

The methodology was similar as previously mentioned for the seed germination of *A. odorata.* The plant growth regulators used for multiple shoot induction were 0.5, 1, 2 and 4 mgl^{-1} BA.

2.3 Acclimatization

Plantlets with well-developed shoots (5-6 cm) and roots (4-5 cm) were removed from the culture and washed thoroughly in running tap water. They were then transferred to root trainer containing charcoal, brick pieces and covered with sphagnum maintained in greenhouse. Potted plantlets were watered every three days with half-strength MS salt solution for two weeks. After twelve weeks, acclimatized plants were transferred to field condition.

3. *Dendrobium nobile*

3.1 *In vitro* seed germination

The methodology was similar as previously mentioned for the seed germination of *D. chrysotoxum.* The additives were used for *in vitro* seed germination were 10% banana, 10% coconut water, 0.2% activated charcoal. MS medium without any additives (control) was used for *in vitro* seed germination.

3.2 Effect of different additives on the growth pattern

Same as described in *A. odorata.* The additives were used for *in vitro* seed germination were 10% banana, 10% coconut water, 0.2% activated charcoal.

3.3. *In vitro* regeneration through shoot multiplication

Same as described in *D. chrysotoxum.*

3.4. Acclimatization

Healthy plantlets along with stout root system were removed from the culture and washed thoroughly in running tap water. They were then transferred to root trainer containing charcoal, and brick pieces maintained in greenhouse. Potted plantlets were watered every three days with half-strength MS salt solution for two weeks. After twelve weeks, acclimatized plants were transferred to field condition.

4. *Bulbophyllum odoratissimum*

4.1 *In vitro* regeneration through shoot multiplication

This step deals with rapid multiplication of the regenerative system for obtaining large number of shoots. Explant with single node was inoculated on MS basal medium containing 3% sucrose, 0.8% agar and different concentration of BA (0.5, 1, 2, 4, 8 mg/L). Medium devoid of PGR served as a control. About 15ml of media was dispensed into test tubes. Sterilization of media was carried out in 1.05 kg/cm^2 pressure at 121°C temperature for 20 min using autoclave. The cultures were incubated at 25±2ºC under 16/8 hours (light /dark) photoperiod with white fluorescent light intensity.

4.2. Acclimatization

The *in vitro* regenerated microshoots with strong and stout root systems were removed from the culture vessels followed by rinsing with plain water to remove adhering agar. Plants were transferred to perforated black plastic pots containing small brick chips, charcoal pieces and coco peat (1:1:1) for hardening. The regenerated plants were sprinkled with water regularly and MS liquid medium devoid of sucrose and vitamins was provided every alternate day.

5. *Cephalantheropsis obcordata*

5.1 *In vitro* seed germination

Same as described in *A. odorata*

5.2. *In vitro* regeneration through shoot multiplication

Healthy shoot segments (3-4 nodes) were collected from the experimental garden of BSI, ERC, Shillong during morning hours from newly grown branches of *C. obcordata*. The explants were treated with standard method with some modification. After washing with sterile distilled water 3-4 times under laminar airflow the explants were aseptically transferred to 20×150mm test tube containing MS nutrient medium supplemented with sucrose 3% and different concentrations (0.5, 1.0, 2.0 and 4.0 mgl^{-1}) of BA, 2iP alone. The cultures were maintained in culture room under warm fluorescent light at 16h photoperiod at 25± 2ºC.

5.3. Acclimatization

Same as described in *B. odoratissimum.* The substratum used for potting mixture was small brick chips, charcoal pieces, decayed wood and mulched with sphagnum moss (1:1:1)

6. *Malaxis acuminata*

6.1 *In vitro* seed germination

The methodology is same as described in the section *A. odorata.* Only control and 10% banana medium were used for seed germination.

6.2. *In vitro* regeneration through pseudobulb culture

In shoot induction studies, pseudobulbs of *M. acuminata* were collected from the experimental garden of BSI, ERC, Shillong and used as explants. The explants were inoculated on MS basal medium containing 0.8% Difco-bacto agar, 3% sucrose. The pH of the media was adjusted to 5.6 before autoclaving. Each of 15 ml medium was dispensed into test tube. All the equipments required for inoculation along with test tubes were sterilized using autoclave at 121^{o}C for 20 minutes.

Pseudobulbs were treated with Labdet-05 solution for 10 minutes and continuously stirred to remove dust or soil particle from the surface area. Then the explants were kept under running water for 1 hour. The materials were then disinfected with 10% (v/v) sodium hypochlorite solution with 2-3 drops of Tween-80 per 100 ml for 30 minutes followed by washing with sterile distilled water 3-4 times under laminar airflow.The explants were cut with blade and made segments of 5mm in size. These excised explants were inoculated aseptically on sterilized MS medium. The cultures were maintained in culture room under warm fluorescent light at 16h photoperiod at 25 ± 2ºC.

6.3. *In vitro* regeneration through shoot multiplication

Seed germinated plantlets (6 months old) were inoculated on MS basal medium containing 3% sucrose, 0.8% agar and fortified with supplemented with different concentrations (0, 0.5, 1, 2, and 4 mgl^{-1}) of 2ip (2-isopentenyladenine) and BA (6-Benzyladenine) alone has been set up for multiple shoot induction. The pH of the medium was adjusted to 5.7 before autoclaving. Observation for shoot initiation and number of shoots produced were recorded in weekly intervals.

6.4. Acclimatization

The methodology is same as described in the section *A. odorata* but as potting mixture soil, sand, brick pieces and decayed wood (1:1:1:1) were used.

Data analysis

For *in vitro* propagation study, culture vessels were under observation and morphogenetic responses were noted at regular intervals. Data were recorded for each parameter and were statistically evaluated using one-way analysis of variance (ANOVA) in JMP version 7.0.1 (SAS Institute, Cary, NC, USA) and significance level was tested at 0.05. Each treatment contains minimum of 15 replicates for each set and experiment was carried out in triplicate. Mean values of each parameter were compared using Tukey's Honestly Significant Difference (HSD) test. Results were expressed as mean ± SD.

B. Preliminary Phytochemical Screening in the Selected Orchids

Phytochemical Analysis

The present phytochemical study deals with six medicinal orchids *viz*., *Aerides odorata, Dendrobium chrysotoxum, Dendrobium nobile, Bulbophyllum odoratissimum*, *Cephalantheropsis obcordata* and *Malaxis acuminata* and the whole or different parts of the plant were used for various phytochemical analysis. The different methods employed to determine the different phytochemical constituents of the six medicinal orchids are described below.

Preliminary Screening of Phytochemicals

The plant materials of these orchids were obtained from the mother plant which was grown in the experimental garden as well as from the acclimatized *in vitro* raised plants. The different chemical reagents which were used for the qualitative test were $Fecl_3$, Magnesium ribbon, HCL, NaOH, Chloroform, Acetic acid, H_2SO_4, Mayer's and Wagner's reagents

Method

The preliminary phytochemical screening of the six medicinal orchids was carried out following the method of Yadav & al. (2011). For this study, the whole plant was collected from both the regenerated plants and the wild mother plants. The plant parts were washed thoroughly several times with running tap water and finally washed with double distilled water. The plant samples were cut into smaller pieces and kept for drying in a hot air oven (50˚C) and finally grounded into a powder. One gram of each coarse powdered plant material was taken and extracted with 80% ethanol with agitation for about 18-24 hrs at ambient temperature. The final extracts were filtered through Whatman No.1 filter paper and diluted to 25 ml and aliquots were subjected to qualitative tests for the identification

of various plant constituents such as flavonoids, terpenoids, steroids, glycosides, phenols, and saponins by using the following standard methods.

Test for phenols

Add 2 ml of $FeCl_3$ solution (2%) to the ethanolic plant extract due to which if the formation of a blue-green or black coloration appears in the solution it indicated the presence of phenols.

Test for flavonoids (Shinoda test)

Add a few fragments of magnesium ribbon into the crude plant extract and to this add a few drops of concentrated HCl. The appearance of pink scarlet color in the solution indicated the presence of flavonoids.

Alkaline reagent test

2 ml of 2% solution of NaOH was added to the plant extract, and an intense yellow color was formed which later on turned colorless on the addition of a few drops of diluted HCl acid confirming the presence of flavonoids.

Test for saponins

Add 5 ml of distilled water to the ethanolic plant extract and shake vigorously for about 30 seconds and stable foam formation indicated the presence of saponins.

Test for glycosides (Liebermann's test)

Add 2 ml of chloroform and 2 ml of acetic acid to the crude plant extract and allowed the mixture to cool in the ice. After cooling down, add concentrated H_2SO_4 into the mixtures and if the mixture color changes from violet to blue to green it indicates the presence of a steroidal nucleus, i.e., glycone portion of a glycoside.

Salkowski's test

Add 2 ml of chloroform along with 2ml of concentrated H_2SO_4 in the crude plant extract and due to which a reddish-brown color steroidal ring appears indicating the presence of glycone, a portion of the glycoside.

Keller-kilani test

Add 1-2 drops of 2% solution of $FeCl_3$ to the crude plant extract and mixed with 2ml of glacial acetic acid. Add 2ml of concentrated H_2SO_4 to the mixture solution and if a brown ring appears at the interphase, it indicated the presence of cardiac glycosides.

Test for steroids

The **c**rude plant extract was mixed with 2 ml of chloroform in a test tube and a few drops of concentrated H_2SO_4 were added sidewise and the appearance of red color in the lower chloroform layer indicated the presence of steroids.

Test for terpenoids

Add 2 ml of chloroform into ethanolic plant extract and the mixture was placed in a small beaker and evaporated to dryness. The solution was transferred into a dry test tube and mixed with 2 ml of concentrated H_2SO_4 and heated for about 2 minutes. A greyish color if appeared is considered as evidence of the presence of terpenoids.

Test for alkaloids

Add 2 ml of 1% HCL into a crude plant extract and heat on a steam bath and Mayer's and Wagner's reagents were added to the mixture and the formation of precipitation if occurred denoted the presence of the alkaloids.

Table 3. Different plant parts used for qualitative phytochemical analysis

Sl. No.	Name of the plant	Parts used	Mother & *in vitro*
1.	*Aerides odorata*	Whole plant	Mother & *in vitro*

2.	*Dendrobium chrysotoxum*	Whole plant	Mother & *in vitro*
3.	*Dendrobium nobile*	Whole plant	Mother & *in vitro*
4.	*Bulbophyllum odoratissimum*	Whole plant	Mother & *in vitro*
5.	*Cephalantheropsis obcordata*	Whole plant	Mother & *in vitro*
6.	*Malaxis acuminata*	Whole plant	Mother & *in vitro*

C. Antioxidant Activities and HPLC study of Selected Six Medicinal Orchids

The Antioxidant activities of the six medicinal orchids were carried out and a comparative analysis between the mother plant and the regenerated plants was done. The different parts of the plants (shown in the Table 9) and their extracts were used for different antioxidant activity studies using various methods and were described accordingly below.

Table 4. Different plant parts used for phytochemical screening

Sl. No.	Name of the plant	Parts used	Mother & *in vitro*
1.	*Aerides odorata*	Leaves	Mother
2.	*Aerides odorata*	Leaves	*in vitro*
3.	*Aerides odorata*	Root	Mother
4.	*Aerides odorata*	Root	*in vitro*
5.	*Dendrobium chrysotoxum*	Leaves	Mother
6.	*Dendrobium chrysotoxum*	Leaves	*in vitro*
7.	*Dendrobium chrysotoxum*	Pseudobulb	Mother
8.	*Dendrobium chrysotoxum*	Pseudobulb	*in vitro*
9.	*Dendrobium nobile*	Leaves	Mother
10.	*Dendrobium nobile*	Leaves	*in vitro*
11.	*Dendrobium nobile*	Pseudobulb	Mother
12.	*Dendrobium nobile*	Pseudobulb	*in vitro*
13.	*Bulbophyllum odoratissimum*	Leaves	Mother
14.	*Bulbophyllum odoratissimum*	Leaves	*in vitro*
15.	*Bulbophyllum odoratissimum*	Pseudobulb	Mother
16.	*Bulbophyllum odoratissimum*	Pseudobulb	*in vitro*
17.	*Bulbophyllum odoratissimum*	Root	Mother
18.	*Bulbophyllum odoratissimum*	Root	*in vitro*
19.	*Cephalantheropsis obcordata*	Leaves	Mother
20.	*Cephalantheropsis obcordata*	Leaves	*in vitro*
21.	*Cephalantheropsis obcordata*	Stem	Mother
22.	*Cephalantheropsis obcordata*	Stem	*in vitro*
23.	*Cephalantheropsis obcordata*	Root	Mother
24.	*Cephalantheropsis obcordata*	Root	*in vitro*
25.	*Malaxis acuminata*	Leaves	Mother
26.	*Malaxis acuminata*	Leaves	*in vitro*
27.	*Malaxis acuminata*	Pseudobulb	Mother
28.	*Malaxis acuminata*	Pseudobulb	*in vitro*

Chemicals: The different chemical reagents and solvents that were used for the analysis of antioxidant activities in the six medicinal Orchids are as follows

Folin-Ciocalteus's phenol reagent, potassium ferricyanide, potassium persulphate, aluminum chloride, $FeCl_3$, sodium carbonate, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

(ABTS) and, butylated hydroxytoluene (BHT), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chemicals used as solvents were of analytical grade. Standards of phenolic acids (gallic, caffeic, syringic, p-coumaric, ferulic, and sinapic) and, flavonoids (catechin, rutin, myricetin, quercetin, naringin, apigenin and kaempferol) were purchased from Sigma-Aldrich (Germany). HPLC grade solvents such as ethanol, chloroform, methanol and acetic acid were purchased from Merck (Germany).

Preparation of sample for Antioxidant Analysis

The different plant parts of six medicinal orchids were collected from both the mother (grown in the experimental garden, Botanical survey of India, Shillong) and the *in vitro* raised plants (acclimatized regenerated plants). The different parts of the plants were rinsed thoroughly with tap water and cut into small pieces and air-dried at room temperature. The plant material was grounded into a fine powder and one gram of coarse powder of each material was extracted with 80% aqueous ethanol and kept on a mechanical shaker for 18-24 h at room temperature. The extracts were filtered through Whatman filter paper and diluted to 25 ml using the same solvent. Extracted solutions were stored at 4°C for further analysis.

Determination of total phenolic content

The total phenolic content (TPC) of crude extracts was determined by using the Folin- Ciocalteu procedure (Singleton & al. 1965). 1.0 ml of Folin- Ciocalteu reagent and 0.8 ml of sodium carbonate (7.5%) were added to 20 - 100 µl of each extract to prepare the reaction mixture and allowed to incubate for 30 min. The absorbance of mixture was measured at 765 nm using a UV spectrophotometer (UV-visible spectrophotometer Shimadzu UV 1800). The total phenolic content was estimated by the equation of calibration curve; $y = 0.0013x + 0.0498$, $R^2 = 0.999$ where y was the absorbance and x was the Gallic acid equivalent (GAE) in mg/g.

Determination of total flavonoid content

The amount of total flavonoids was estimated using the aluminum chloride method (Ordonez & al. 2006). The reaction mixture was prepared by mixing 0.5 ml plant extract with 0.5 ml of 2% $AlCl_3$ solution and incubating it for 1 hour at room temperature. The absorbance of the reaction mixture was measured at 420 nm (UV visible spectrophotometer Shimadzu UV 1800). Total flavonoid contents were calculated based on the calibration curve: $y = 0.0182x - 0.0222$, $R^2 = 0.9962$, where y was the absorbance and x was the rutin equivalent in mg/gm and expressed in terms of Rutin equivalent (RE) in (mg/g) of dry extract.

Determination of Reducing Power Activity

The reducing capacity of each plant extract was determined according to Oyaizu (1965). In this study, reaction mixture was prepared by mixing 100 µl of plant extracts with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5 ml) and kept for 20 min at 50°C. Then, Aliquots of 2.5 ml trichloroacetic acid (10%) were added to reaction mixture followed by centrifuging at 3000 rpm for 10 min. Finally, the upper layer solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml of freshly prepared ferric chloride solution (0.1%). The absorbance was recorded at 700 nm. An increase in the absorbance of reaction mixture indicates higher reducing power. The reducing power of the plant extracts was calculated based on the calibration curve; $y = 0.0023x - 0.0063$, $R^2 = 0.9955$ where y was the absorbance and x was the ascorbic acid equivalent (AAE) in mg/g.

DPPH free radical scavenging activity

The antioxidant capacity of each plant extract was determined using DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging activity (Blois, 1958) and butylated hydroxyl toluene (BHT) as positive control. In this assay, 3.9 ml of freshly prepared DPPH solution (25 mgL^{-1}) in methanol was added to each test tube containing 20 – 100 µl of test sample and mixed thoroughly. The reaction mixture was kept for 30 min. The degree of discoloration (gradual changes of purple color to clear solution) is directly proportionate to the antioxidant level of test samples. Absorbance was measured at 517 nm (UV-visible spectrophotometer, Shimadzu UV 1800). The scavenging activity of each extract was calculated based on the ability to quench the DPPH radical, using the following equation: DPPH scavenged (%) = {(Ac – At)/Ac} x 100 Where Ac is the absorbance of the control and At is the absorbance of plant extracts. The antioxidant activity of the extract was expressed as IC_{50}. The concentration (mg /ml dry extract) of

plant sample that can inhibit 50% DPPH radical (IC_{50} value) in reaction mixture was calculated and revealed that lower IC_{50} value indicates greater antioxidant activity of the sample.

ABTS radical scavenging activity

The ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] radical scavenging activity was carried out to assess antioxidant activity using a spectrophotometric method by Re *& al* (1999). ABTS was dissolved in deionized water to 7 mM concentration. The ABTS radicals were generated by adding 2.45 mM potassium persulphate to ABTS and allowed the mixture to stand in the dark for 12–16 hours at room temperature. The resultant $ABTS^+$ solution was diluted with ethanol to attain its absorbance at 734 nm to 0.70 ± 0.02 and 10 µl of plant extract was mixed with 1 ml of diluted $ABTS^+$ working solution and shaken thoroughly. The absorbance at 734 nm was measured after 6 min of the initial mixing, using ethanol as the blank and the inhibition percentage was calculated by the equation:

ABTS scavenged (%) = {(Ac - At) / Ac} x100

Where Ac is absorbance of control and At is absorbance of test sample at 734nm

HPLC analysis for the quantification of phenolic acids and flavonoids

The HPLC analyses were performed with the help of Dionex Ultimate 3000 liquid chromatography including a diode array detector (DAD) with a 5 cm flow cell and Chromeleon system manager as a data processor. For the quantification study of the sample, a 20 μL of the sample (plant extracts) was injected into the HPLC and separation of the compounds was achieved by a reversed-phase Acclaim C18 column (5-micron particle size, 250 x 4.6 mm).

Preparation of standard solutions for HPLC analysis

To prepare 1 mg/ml concentration stock solution 1 mg gallic acid was dissolved in 0.5 ml HPLC-grade methanol followed by sonication for 10 min and then make up the final volume (1ml) with mobile phase solvent (acetonitrile and 1% aq. acetic acid 1:9). Phenolic acids and the flavonoids standard stock solutions were prepared using the same procedure. Now, the stock solution was diluted with the same solvent to prepare working solution for further study. Solutions were filtered through 0.45µm PVDF-syringe filter and before injecting the solution, the mobile phase was degassed.

Methods for HPLC analysis

HPLC analyses were carried out according to the method as described by Datta & al. (2019) with minor modifications using Dionex Ultimate 3000 liquid chromatograph equipped with a diode array detector (DAD), a reversed-phase Acclaim C18 column (5-micron particle size, 250 x 4.6 mm) and Chromeleon system manager as a data processor. The method was validated according to the USP and ICH guidelines. The mobile phase was composed of methanol (Solvent A) and 1% aqueous acetic acid solution (Solvent B), the column was thermostatically controlled at 28°C and the injection volume was kept at 20µl. Gradient elution was performed by varying the proportion of solvent A to solvent B. The gradient elution was 10% to 20% A and 90% to 80% B with a flow rate of 1 ml/min in 28 min, 20% A with the same flow rate for another 17 min, solvent A changed from 20 to 45% with the same flow rate in 55 min and allowed to run for 17 min, solvent A changed from 45 to 65% in 2 min and allowed to run for 8 min. The mobile phase composition back to the initial condition (solvent A: solvent B: 10: 90) in 81 min and allowed to run for another 4min, before the injection of another sample. The total analysis time per sample was 85min. The phytochemical compounds were detected by their retention time and confirmed by comparing them with the individual standard. The extracts were filtered through a 0.45 µm polyvinyl difluoride (PVDF) membrane for HPLC analysis.

Data analysis

All the experiments were done using triplicate samples. Results were represented as value± standard error mean (SEM). Experimental results were subjected to univariate analysis of variance (ANOVA), followed by the Tukey test ($p \leq 0.05$) using the statistical package for the social sciences (SPSS version 7.5).

Chapter IV
RESULTS

A. *In vitro* seed germination/ Micropropagation

1. *Aerides odorata*

1.1 *In vitro* seed germination

Natural additives such as 10% banana and 10% CW besides the control are used in the medium to obtain maximum response in seed germination. The highest percentage of the seed germination (93.06%) was observed in the medium containing the 10% Banana (B) as an additive followed by 68.22% in coconut water (CW) and only 44.92% of seed germination in the control medium respectively. The inoculated seeds began to germinate after four weeks of inoculation in the medium supplemented with two additives *viz*., 10% banana and 10% coconut water respectively. The germinated seed grew in size and transformed into protocorm-like bodies (PLBs). Subsequently, the first leaf-like structure appeared from the PLBs after nine weeks and a complete seedling developed after fourteen weeks of inoculation irrespective of the medium used.

1.2 Effect of different additives on the growth pattern of *A. odorata*

The effect of four different treatments *viz*., control, 10% banana pulp, 10% coconut water (CW), 0.2% activated charcoal (AC) on different morphogenesis (shoot growth and development, rooting) in *in vitro* seedling of *A. odorata* was carried out and observed the MS medium supplemented with 10% CW produces maximum leaf number (4.46±0.15) and root number (5.96±0.24) in the seedlings along with increasing in shoot length (2.20±0.10 cm) and root length (2.21±0.09 cm) (Table 5). In contrast the MS media containing 0.2% activated charcoal showed the lowest number of leaves (1.53±0.11) and roots (0.50±0.11) along with stunted shoot and root growth even after the 12 weeks of its culture. MS containing 10% coconut water was the most effective condition for induction of strong and stout root system among the all additives.

Table 5. Effect of different additives on the development of seedlings of *A. odorata*

Treatments	Shoot length(cm)	Leaf no.	Root no.	Root length (cm)
Control	1.39 ± 0.08^{b}	2.33 ± 0.17^{c}	1.63 ± 0.20^{c}	0.82 ± 0.09^{b}
10% B	1.46 ± 0.11^{b}	3.70 ± 0.13^{b}	2.73 ± 0.26^{b}	1.71 ± 0.22^{a}
0.2% AC	1.02 ± 0.03^{c}	1.53 ± 0.11^{d}	0.50 ± 0.11^{d}	0.41 ± 0.08^{b}
10% CW	2.20 ± 0.10^{a}	4.46 ± 0.15^{a}	5.96 ± 0.24^{a}	2.21 ± 0.09^{a}

#Values expressed as mean ± SD (Standard deviation). Means followed by different letters within columns indicate significant differences at $p \leq 0.05$

1.3 *In vitro* regeneration through shoot multiplication

In the present study, shoot tips were cultured on MS medium supplemented with different concentration of BA (0.5-8.0 mgl^{-1}). The MS medium supplemented with a low concentration (0.5 mgl^{-1}) of BA did not favor shoot development, and the regenerated shoots were found not healthy. The optimum concentration (4.0 mgl^{-1}) of BA alone showed only 80% of explant response along with 3.37±0.13 shoots/explant (Table 7). As the concentration of BA increased in the medium, the number of regenerated shoots also increased significantly ($p<0.05$) but a high concentration (8.0 mgl^{-1}) of BA in the medium inhibited the shoot multiplication (2.48±0.07).

The optimum concentration (4.0 mgl^{-1}) of BA was combined with different concentrations (0.5, 1.0, 2.0 mgl^{-1}) of GA_3 to enhance the growth and multiplication and subsequently, the new shoot initiated as a small green protuberance after three weeks of its culture in MS medium supplemented with BA (4.0 mgl^{-1}) + GA_3 (0.5 mgl^{-1}) and BA (4.0 mgl^{-1}) + GA_3 (1.0 mgl^{-1}) respectively. The highest explant response (100%) along with the maximum number (4.48±0.07 shoots/ explant) of proliferated shoots was recorded in MS medium supplemented with BA (4.0 mgl^{-1}) + GA_3 (1.0 mgl^{-1}) (Table 6).

Table 6. Effect of PGRs on multiple shoot induction of *A. odorata*

Treatment (mgl^{-1})		Regeneration percentage (%)	Mean shoot no.
BA	GA_3		
0	0	0	0.00 ± 0.00^{g}
0.5	0	53.33	1.64 ± 0.07^{f}
1.0	0	62.22	2.15 ± 0.08^{e}
2.0	0	71.11	2.86 ± 0.11^{d}
4.0	0	80	3.37 ± 0.13^{bc}
8.0	0	68.88	2.48 ± 0.07^{e}
4.0	0.5	91.11	3.68 ± 0.09^{b}
4.0	**1.0**	**100**	**4.48 ± 0.07^{a}**
4.0	2.0	100	3.24 ± 0.13^{cd}

#Values expressed as mean ± SD (Standard deviation). Means followed by different letters within columns indicate significant differences at $p \leq 0.05$

1.4. Acclimatization

The combination of small brick chips (~5–7 mm in size), charcoal pieces (~5–7 mm in size), decayed wood, and coconut husk (1:1:1:1) were found to be the most suitable potting mixture for the growth of *A. odorata* with the highest survival rate (97.05%) of acclimatized plantlets. Finally, acclimatized plantlets that showed the best growth were reintroduced into tree trunks and their roots were covered with sphagnum moss and subsequently, they bloomed after about 5 years of replanting.

2. *Dendrobium chrysotoxum*

2.1. *In vitro* seed germination

In vitro seed germination started within 2 weeks and protocorm like bodies (PLBs) initiated within 3 weeks. From PLBs, leaf developed within 7 weeks. This seed germination studies showed higher percentage of germination in all treatments. Subculture of the plantlets had to be done regularly after four weeks for better growth.

2.2 *In vitro* regeneration through shoot multiplication

Shoots initiated from nodal segment within 3rd week in MS media supplemented with BA ($2mgl^{-1}$). Healthy shoots with 2-3 leaves developed in 8-9 weeks. On an average 3 (three) no. of shoots were produced in the concentration range at $2mgl^{-1}$ BA and 100% multiple shoot formation was calculated for the same. They were subcultured on the same medium for further shoot development. The experiments were repeated thrice. The observations were made regularly and data recorded accordingly. The results were tested using the one-way ANOVA test and were analyzed using Tukey Multiple Comparison using SPSS (Version 11.5) software package.

Table 7. Effect of BA on multiple shoot induction of *D. chrysotoxum*

BA (mgl^{-1})	No. of shoots/Explant	Shoot length	Root No.
0	0.70 ± 0.13^{d}	0.71 ± 0.13^{c}	1.42 ± 0.27^{d}
0.5	1.50 ± 0.21^{c}	1.66 ± 0.21^{ab}	2.75 ± 0.37^{bc}
1.0	2.20 ± 0.19^{b}	1.81 ± 0.17^{a}	3.97 ± 0.33^{b}
2.0	**3.05 ± 0.14^{a}**	**1.86 ± 0.07^{a}**	**5.72 ± 0.23^{a}**
4.0	1.10 ± 0.19^{cd}	1.07 ± 0.16^{bc}	2.20 ± 0.38^{cd}

#Values expressed as mean ± SD (Standard deviation). Means followed by different letters within columns indicate significant differences at $p \leq 0.05$

2.3 Acclimatization

Plantlets with 4-6 fully expanded leaves and well developed roots (4-5cm) were transferred to green house for acclimatization. After hardening in a potting mixture of brick, charcoal (1:1) and covered with sphagnum for twelve

weeks, survival rate of regenerated plantlet was over 95%. During acclimatization, original leaves turned brown and senesced and new leaves and roots developed.

3. *Dendrobium nobile*

3.1. *In vitro* seed germination

The seed germinated within seven weeks of inoculation in MS medium. The protocorms developed into a complete seedling after twelve weeks of culture.

3.2. Effect of different additives on the growth pattern

After 12 weeks, it was observed that MS containing 10% banana showed highest number of shoots and it was found to be more effective for the growth of plantlets. Explants inoculated on medium supplemented with 0.2% activated charcoal and 10% coconut water was initially green and after 1 month started turning light brownish in color.

Table 8. Average length (in cm) of plantlets of *D. nobile* grown on different additives

Treatments	No. of shoots	Shoot length
Control	1.50 ± 0.36^{b}	0.80 ± 0.19^{a}
10% B	$\mathbf{3.30\pm0.33^{a}}$	$\mathbf{1.31\pm0.17^{a}}$
0.2% AC	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}
10% CW	0.10 ± 0.05^{c}	0.11 ± 0.06^{b}

#Values expressed as mean ± SD (Standard deviation). Means followed by different letters within columns indicate significant differences at $p \leq 0.05$

3.2. *In vitro* regeneration through shoot multiplication

The number and length of shoots varied significantly with different concentrations of BA. Nodal segments cultured on MS medium supplemented with 2mg/l BA gave the best (97%) explant establishment and highest number of shoots (shoot number 2.77; shoot length 2.56 cm). It was observed that without any PGRs in the medium, shoot formation was very slow. While increase in the concentration of BA has a promoting effect for both number and length of shoots up to a certain level and higher concentrations (4mg/l) in the medium were found to inhibit shoot multiplication.

Table 9. Effect of BA on multiple shoot induction of *D. nobile*

BA (mgl^{-1})	No. of shoots	Shoot length(cm)	Root no.	Root length(cm)
0	0.45 ± 0.07^{c}	0.55 ± 0.11^{b}	0.95 ± 0.17^{d}	1.17 ± 0.21^{c}
0.5	1.42 ± 0.22^{b}	1.13 ± 0.16^{b}	3.12 ± 0.46^{bc}	1.52 ± 0.22^{cd}
1.0	1.82 ± 0.17^{b}	2.21 ± 0.17^{a}	4.15 ± 0.34^{ab}	2.89 ± 0.17^{ab}
2.0	$\mathbf{2.77\pm0.16^{a}}$	2.56 ± 0.11^{a}	5.27 ± 0.24^{a}	3.28 ± 0.11^{a}
4.0	1.67 ± 0.20^{b}	2.06 ± 0.20^{a}	2.82 ± 0.30^{c}	1.22 ± 0.21^{bc}

#Values expressed as mean ± SD (Standard deviation). Means followed by different letters within columns indicate significant differences at $p \leq 0.05$

3.3 Acclimatization

The *in vitro* derived plants when transferred to community potting mixture containing charcoal, and brick pieces at 1:1 ratio showed about 75% survival of plantlets after twelve weeks.

4. *Bulbophyllum odoratissimum*

4.1 *In vitro* regeneration through shoot multiplication

Explant sterilization is an essential step to diminish contamination in an aseptic culture of orchids. The highest (93.33%) survival percentage of explant with minimum tissue injury was obtained when treated with 30% (v/v) NaOCl for 15 minutes. In the present study, it was observed that the percentage of explant response varied significantly with different concentrations of BA used in the medium. BA alone at 4.0 mgl^{-1} induced direct shoot

without any callus formation after the 5th week of inoculation and young shoot also developed in MS medium supplemented with BA (8.0 mgl^{-1}). Comparatively, the medium containing BA (4.0 mgl^{-1}) was found to be optimum as the highest (77.77%) regeneration frequency in this concentration and it produced 2.08±0.55 shoots from a single explant (Table 10).

As the regeneration percentage of the explant was low and the development of a new shoot took longer time in the earlier study, a further experiment was designed to overcome the difficulties. In this approach, the optimum concentration (4.0 mgl^{-1}) of BA was used in amalgamation with various concentrations (0.25, 0.5, 1.0, 2.0 mgl^{-1}) of NAA, IBA and IAA. The addition of IBA in the medium indicate more pronounced in the shoot proliferation than the effect of NAA and IAA. The shoot started to develop after the 4th week of culture in MS media supplemented with BA (4.0 mgl^{-1}) and IBA (0.5 mgl^{-1}) and on the 12 weeks it produced higher number of new shoots (5.31±0.46) with maximum shoot length (3.04±0.60) when compared to BA (4.0 mgl^{-1}) alone (Table 11). The present study revealed the addition of BA and IBA in the media produces the highest number of shoots (5.31±0.46) and maximum shoots elongation (3.04±0.60), whereas BA combination with NAA and IAA was less effective than IBA for shoot proliferation. BA combined with IAA did not appear to have good effect on shoot multiplication (0.97±0.14).

Table 10. Effect of BA on multiple shoot induction of *B. odoratissimum*

BA (mgl^{-1})	Shoot induction (%)	Mean shoot number	Mean shoot length (cm)
0	0	0.00	0.00
0.5	40	0.80 ± 0.40^{d}	1.04 ± 0.47^{e}
1.0	53.33	1.20 ± 0.78^{c}	1.80 ± 0.40^{d}
2.0	64.44	1.68 ± 0.79^{b}	2.11 ± 0.38^{c}
4.0	**77.77**	**2.08 ± 0.55^{a}**	**2.77 ± 0.55^{a}**
8.0	71.6	1.55 ± 0.62^{bc}	2.48 ± 0.62^{b}

Table 11. Effect of BA (4.0 mgl^{-1}) and auxin combinations (NAA, IAA, IBA) on multiple shoot formation of *B. odoratissimum*

Plant growth regulators (mgl^{-1})				Mean shoot number	Mean shoot length(cm)
BA	NAA	IBA	IAA		
4.0	0.25	-	-	1.24 ± 0.57^{g}	1.04 ± 0.52^{e}
	0.5	-	-	2.02 ± 0.14^{f}	1.46 ± 0.50^{d}
	1.0	-	-	2.97 ± 0.49^{d}	1.93 ± 0.25^{c}
	2.0	-	-	2.40 ± 0.49^{e}	1.51 ± 0.54^{d}
	-	0.25	-	3.44 ± 0.57^{c}	1.91 ± 0.41^{c}
	-	**0.5**	-	**5.31 ± 0.46^{a}**	**3.04 ± 0.60^{a}**
	-	1.0	-	4.17 ± 0.71^{b}	2.57 ± 0.51^{ab}
	-	2.0	-	2.68 ± 0.55^{de}	1.55 ± 0.78
	-	-	0.25	0.97 ± 0.14^{gh}	0.55 ± 0.72^{f}
	-	-	0.5	0.75 ± 0.43^{h}	0.31 ± 0.70^{fg}
	-	-	1.0	0.00	0.00
			2.0	0.00	0.00

#Values expressed as mean ± SD (Standard deviation). Means followed by different letters within columns indicate significant differences at $p \leq 0.05$

4.2. Acclimatization

The survival rate (91.66%) of the plantlets was observed in potting medium comprised of three substratum mixtures in an equal ratio of small brick chips, charcoal pieces and coco peat (1:1:1). During the successive phases of the acclimatization of *B. odoratissimum* plantlets, it was observed the newly formed root grew and bound to the charcoal surface which indicates the charcoal chips help in anchoring the plantlets for its further growth and development.

5. *Cephalantheropsis obcordata*

5.1. Seed germination study

There were no significant changes were observed in the seeds of *C. obcordata* in any of the treatments in the medium which indicated the mixing of additives and PGRs doesn't have any effect on its seed germination (Fig 4.2). To determine the seed viability after 4 months of inoculation, few seeds from the flask were scraped out with a spatula and Tetrazolium assay was performed. Tetrazolium (TZ) assay is the fast evaluation for seed viability and alternative quick method for seed's germinability (Porter & al., 1947). For each species/treatment combination three replicates were used to determine staining percentages.

1% TTC solution was prepared by dissolving 1g of 2, 3, 5-TTC in 100mL of sterile deionized water. The pH was adjusted to 7 with 1M NaOH. The incubation time varies with seed type and morphology (Verma & al., 2013). Seeds were observed using a stereozoom microscope. Seeds were evaluated on the basis of staining pattern and colour intensity. Among stained seeds, seeds with bright red staining are completely viable while partially stained seeds may produce either normal or abnormal seedlings. Pink or greyish red stain indicates dead tissue. Completely unstained seeds are non-viable. The observed seeds were bright red with significant seed coat. It was observed that oval shaped undifferentiated embryo did not swell and no spherule was formed.

5.2. *In vitro* regeneration through shoot multiplication

Sterilization of explants is an essential step in reducing contamination during orchid cultures. The survival percentage (84%) of explant was maximum when treated with 30% (v/v) NaOCl for 15 minutes. In the present study, young and healthy nodal segments were used as explants for the recovery of *C. obcordata*.

The present study revealed that the percentage of morphogenetic response was found significantly different in each treatment however, responded explant produced only 1.00 number of shoot in all treatments including the control. Initiation of a new shoot was first triggered at the axillary meristem regions of the nodes in a period of 20 to 30 days irrespective of the medium used. The percentage of explant response differs with the concentrations of BA and the highest frequency (84.4%) along with maximum shoot elongation (2.14±0.15 cm) was achieved at an optimum concentration of 2.0 mgl^{-1} BA.

The addition of 2iP to MS basal medium produced a maximum (71.1%) percentage of shoot induction at 1.0 mgl^{-1}. At this concentration, the highest shoot length was 2.02±0.10 cm which is not significantly different from the maximum shoot length (2.14±0.15 cm) observed in the optimum concentration of BA. Reduction of shoot length was observed with an increasing concentration of 2iP although, the percentage was not significantly different from other concentrations.

The *in vitro* raised plantlets were excised from the cultured explant while the plantlets consist of 2-3 nodes. Therefore, each node of the regenerated plants was cut out and recultured individually to freshly prepared medium for further multiplication of axillary shoots. Accordingly, subcultures of *in vitro* raised nodal segments generated 100% growth at all times.

Table 12. Effect of PGRs on shoot regeneration study of *C. obcordata*

Plant growth regulators (mgl^{-1})		Mean shoot length (cm)
BA	**2iP**	
0	-	0.70 ± 0.11^{g}
0.5	-	1.13 ± 0.13^{f}
1.0	-	1.55 ± 0.16^{d}
2.0	**-**	$\mathbf{2.14\pm0.15^{a}}$
4.0	-	1.79 ± 0.16^{cd}
	0.5	1.34 ± 0.08^{e}
-	1.0	2.02 ± 0.10^{ab}
-	2.0	1.89 ± 0.22^{c}
-	4.0	1.56 ± 0.09^{d}

#Values expressed as mean ± SD (Standard deviation). Means followed by different letters within columns indicate significant differences at $p \leq 0.05$

5.3. Acclimatization

The rooted plantlets were successfully established in the potting materials comprising small brick chips, charcoal pieces and decayed wood in the ratio 1:1:1 and mulched with sphagnum moss with 89.09% survival in greenhouse. The acclimatized plants showed normal growth and were morphologically similar to the mother plant. When transfer to external environment the rate was decreased as some plants became brown. But it was observed that plantlets were gradually acclimatized in natural environment and produce new shoots.

6. *Malaxis acuminata*

6.1. *In vitro* seed germination

PLBs developed within 20 weeks of culture followed by shoot formation in 24 weeks. Seeds exhibited higher percentage of germination in MS medium without additives.

6.2 *In vitro* regeneration through pseudobulb culture

Explants on MS medium containing BA and 2iP alone showed different developments of new shoots. BA (1mg/l) gave the highest number of new shoots (3.6 shoots per explant) with the average length of 1.2cm whereas explants in 2iP (1mg/l) gave 2.4 shoots per explant with an average length of 1.3cm. In this study, BA was superior to 2iP in giving more shoots per explant when same concentrations of the two plant growth regulators were compared.

Table 13. Effect of BA and 2iP on multiple shoot induction of *M. acuminata*

Plant growth regulators (mgl^{-1})		Shoot no.	Shoot length	Root no.	Root length
BA	2iP				
0	0	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
0.5	0	2.00 ± 0.16^{bcd}	1.19 ± 0.36^{abc}	3.03 ± 0.13^{ab}	1.32 ± 0.36^{c}
1	0	**3.60 ± 0.38^{a}**	1.22 ± 0.36^{b}	3.16 ± 0.41^{ab}	3.13 ± 0.22^{a}
2	0	3.40 ± 0.64^{ab}	2.03 ± 0.36^{a}	4.06 ± 0.39^{a}	2.61 ± 0.23^{ab}
4	0	2.60 ± 0.53^{abc}	1.76 ± 0.34^{a}	2.83 ± 0.45^{b}	1.96 ± 0.39^{bc}
0	0.5	0.73 ± 0.21^{de}	0.51 ± 0.14^{cd}	0.90 ± 0.26^{d}	0.92 ± 0.22^{cd}
0	1	2.43 ± 0.34^{abc}	1.31 ± 0.03^{abc}	2.76 ± 0.17^{b}	2.03 ± 0.11^{abc}
0	2	1.16 ± 0.13^{cde}	1.49 ± 0.17^{ab}	2.23 ± 0.27^{bc}	2.66 ± 0.27^{ab}
0	4	1.06 ± 0.26^{cde}	0.71 ± 0.16^{bcd}	1.23 ± 0.29^{cd}	1.69 ± 0.38^{bc}

#Values expressed as mean ± SD (Standard deviation). Means followed by different letters within columns indicate significant differences at $p \leq 0.05$

6.3 Acclimatization

The well-developed rooted plantlets were successfully transplanted in the potting materials comprising soil, sand, brick pieces and decayed wood in the ratio 1:1:1:1. The percentage survival of the plantlets was 90% after transplantation to potting mixture. After twelve months, it was observed that plantlets were successfully adapted to greenhouse conditions.

B. Phytochemical Screening in the Selected Orchids

Qualitative Phytochemical Screening of Six Medicinal Orchids

The qualitative assessment of phytochemicals *viz.*, phenols, flavonoids, steroids, saponin, terpenoids, glycosides, and alkaloids was carried out in three medicinal orchids (*viz*., *A. odorata, B. odoratissimum, C. obcordata*) using 80% ethanol as the solvent and observed that, all the three whole plant extracts were rich in a wide range of

phytochemical constituents. The secondary metabolites such as phenol, flavonoids, glycosides, terpenoids, steroids and alkaloids except saponins were present in all the three medicinal orchids *viz*., *A. odorata, B. odoratissimum* and *C. obcordata,* both in the mother and the regenerated plants. In qualitative phytochemical analysis of *D. nobile* and *D. chrysotoxum*, flavonoids was present whereas glycosides, terpenoids, steroids and alkaloids were absent. In *M. acuminata*, phenol, flavonoids, glycosides, terpenoids, were present whereas steroids and alkaloids were absent (Table 14).

Table 14. Phytochemical constituents in three medicinal orchids

Name of the Plants	Mother/ *In vitro*	Phenol test	Flavonoids test	Saponin test	Glycosides test	Steroid test	Terpenoid test	Alkaloid test
A. odorata	Mother	P	P	A	P	P	P	P
	In vitro	P	P	A	P	P	P	P
B. odoratissimum	Mother	P	P	A	P	P	P	P
	In vitro	P	P	A	P	P	P	P
C. obcordata	Mother	P	P	A	P	P	P	P
	In vitro	P	P	A	P	P	P	P
D. nobile	Mother	A	P	A	A	A	A	A
	In vitro	A	P	A	A	A	A	A
D. chrysotoxum	Mother	A	P	A	A	A	A	A
	In vitro	A	P	A	A	A	A	A
M. acuminata	Mother	P	P	A	P	A	P	A
	In vitro	P	P	A	P	A	P	A

P = Present; A= Absent

Comparative Evaluation of Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Reducing Power and Antioxidant activities between the mother plant and *in vitro* raised plants.

I. Extractive value

The extractive value of the tested plants with 80% ethanol solvents are depicted in Table 14 & Fig. 1.

Table 15. Extractive value of the plants

Sl. No.	Name of the plant	Parts used	*in vitro*/ mother	Extractive value in mg/gm
1.	*Aerides odorata*	Leaves	mother	244.02
2.	*Aerides odorata*	Leaves	*in vitro*	193.12
3.	*Aerides odorata*	Root	mother	255.20
4.	*Aerides odorata*	Root	*in vitro*	162.63
5.	*Dendrobium chrysotoxum*	Leaves	mother	115
6.	*Dendrobium chrysotoxum*	Leaves	*in vitro*	124
7.	*Dendrobium chrysotoxum*	Pseudobulb	mother	82.33
8.	*Dendrobium chrysotoxum*	Pseudobulb	*in vitro*	40
9.	*Dendrobium nobile*	Leaves	mother	194.61
10.	*Dendrobium nobile*	Leaves	*in vitro*	40

11.	*Dendrobium nobile*	Pseudobulb	mother	234.29
12.	*Dendrobium nobile*	Pseudobulb	*in vitro*	60
13.	*Bulbophyllum odoratissimum*	Leaves	mother	151.25
14.	*Bulbophyllum odoratissimum*	Leaves	*in vitro*	300
15.	*Bulbophyllum odoratissimum*	Pseudobulb	mother	82.5
16.	*Bulbophyllum odoratissimum*	Pseudobulb	*in vitro*	245
17.	*Bulbophyllum odoratissimum*	Root	mother	60
18.	*Bulbophyllum odoratissimum*	Root	*in vitro*	55
19.	*Cephalantheropsis obcordata*	Leaves	mother	170
20.	*Cephalantheropsis obcordata*	Leaves	*in vitro*	220
21.	*Cephalantheropsis obcordata*	Stem	mother	180
22.	*Cephalantheropsis obcordata*	Stem	*in vitro*	140
23.	*Cephalantheropsis obcordata*	Root	mother	145
24.	*Cephalantheropsis obcordata*	Root	*in vitro*	200
25.	*Malaxis acuminata*	Leaves	mother	110
26.	*Malaxis acuminata*	Leaves	*in vitro*	36
27.	*Malaxis acuminata*	Pseudobulb	mother	30
28.	*Malaxis acuminata*	Pseudobulb	*in vitro*	10

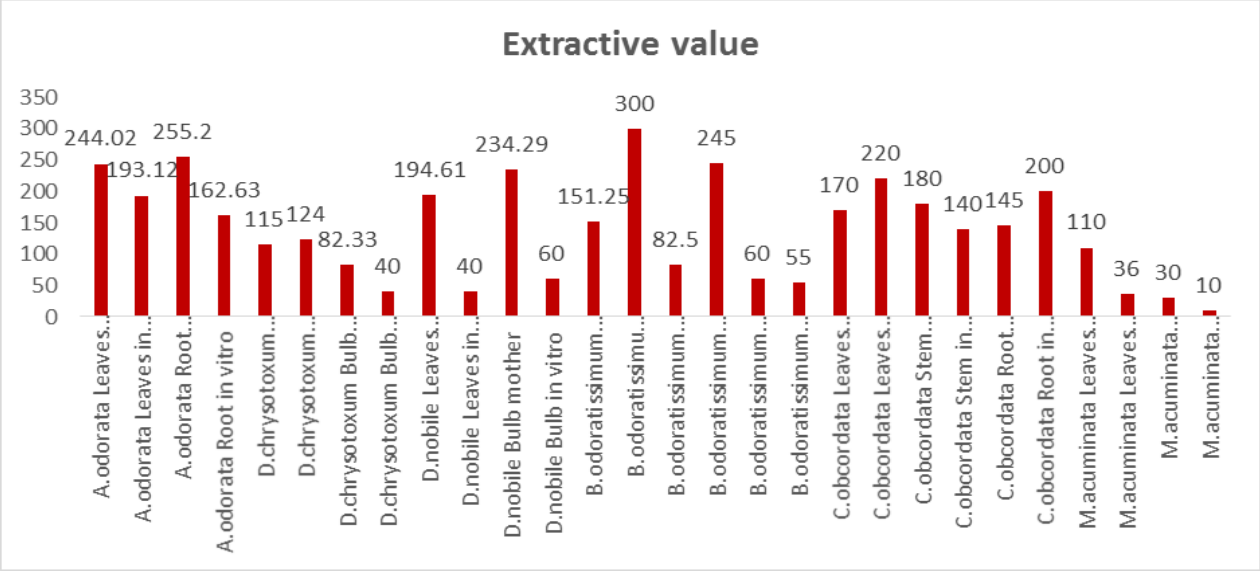


Fig. 1. Extractive value of the plant

II. Estimation of total phenolic content

The amount of total phenolics in the plant samples was measured according to Folin-Ciocalteu procedure and 20-100µl of the tested samples were introduced into test tubes. 1.0ml of Folin-Ciocalteu reagent and 0.8 ml of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured (UV-visible spectrophotometer Shimadzu UV 1800). The total phenolic content was expressed as gallic acid equivalents (GAE) in miligram per gram (mg/g) of extract using the following equation based on the calibration curve $y = 0.0013x + 0.0498$, $R^2 = 0.999$ where y was the absorbance and x was the Gallic acid equivalent (mg/g).

Table 16. Total phenolic content (GAE mg/gm dry extract) in the plant material

Sl. No.	Name of the plant	Parts used	*in vitro*/ Mother	Total phenolic content mg/gm dry ext (Mean ± SEM)
1.	*Aerides odorata*	Leaves	Mother	111.62±2.40
2.	*Aerides odorata*	Leaves	*in vitro*	159.52±1.52
3.	*Aerides odorata*	Root	Mother	86.03±1.55
4.	*Aerides odorata*	Root	*in vitro*	189.65±3.75
5.	*Dendrobium chrysotoxum*	Leaves	Mother	170.35±3.10
6.	*Dendrobium chrysotoxum*	Leaves	*in vitro*	104.42±2.06
7.	*Dendrobium chrysotoxum*	Pseudobulb	Mother	151.20±3.89
8.	*Dendrobium chrysotoxum*	Pseudobulb	*in vitro*	130.96±6.54
9.	*Dendrobium nobile*	Leaves	Mother	82.05±5.22
10.	*Dendrobium nobile*	Leaves	*in vitro*	108.52±1.15
11.	*Dendrobium nobile*	Pseudobulb	Mother	67.81±4.73
12.	*Dendrobium nobile*	Pseudobulb	*in vitro*	102.05±1.95
13.	*Bulbophyllum odoratissimum*	Leaves	Mother	177.41±3.69
14.	*Bulbophyllum odoratissimum*	Leaves	*in vitro*	75.6±2.26
15.	*Bulbophyllum odoratissimum*	Pseudobulb	Mother	158.20±2.06
16.	*Bulbophyllum odoratissimum*	Pseudobulb	*in vitro*	82.63±1.50
17.	*Bulbophyllum odoratissimum*	Root	Mother	134.58±1.49
18.	*Bulbophyllum odoratissimum*	Root	*in vitro*	171.80±1.86
19.	*Cephalantheropsis obcordata*	Leaves	Mother	225.34±3.64
20.	*Cephalantheropsis obcordata*	Leaves	*in vitro*	90.10±2.85
21.	*Cephalantheropsis obcordata*	Stem	Mother	82.35±2.64
22.	*Cephalantheropsis obcordata*	Stem	*in vitro*	19.32±0.32
23.	*Cephalantheropsis obcordata*	Root	Mother	84.05±1.50
24.	*Cephalantheropsis obcordata*	Root	*in vitro*	56.93±0.40
25.	*Malaxis acuminata*	Leaves	Mother	206.83±1.11
26.	*Malaxis acuminata*	Leaves	*in vitro*	57.09±1.25
27.	*Malaxis acuminata*	Pseudobulb	Mother	47.23±2.27
28.	*Malaxis acuminata*	Pseudobulb	*in vitro*	70.80±0.66

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

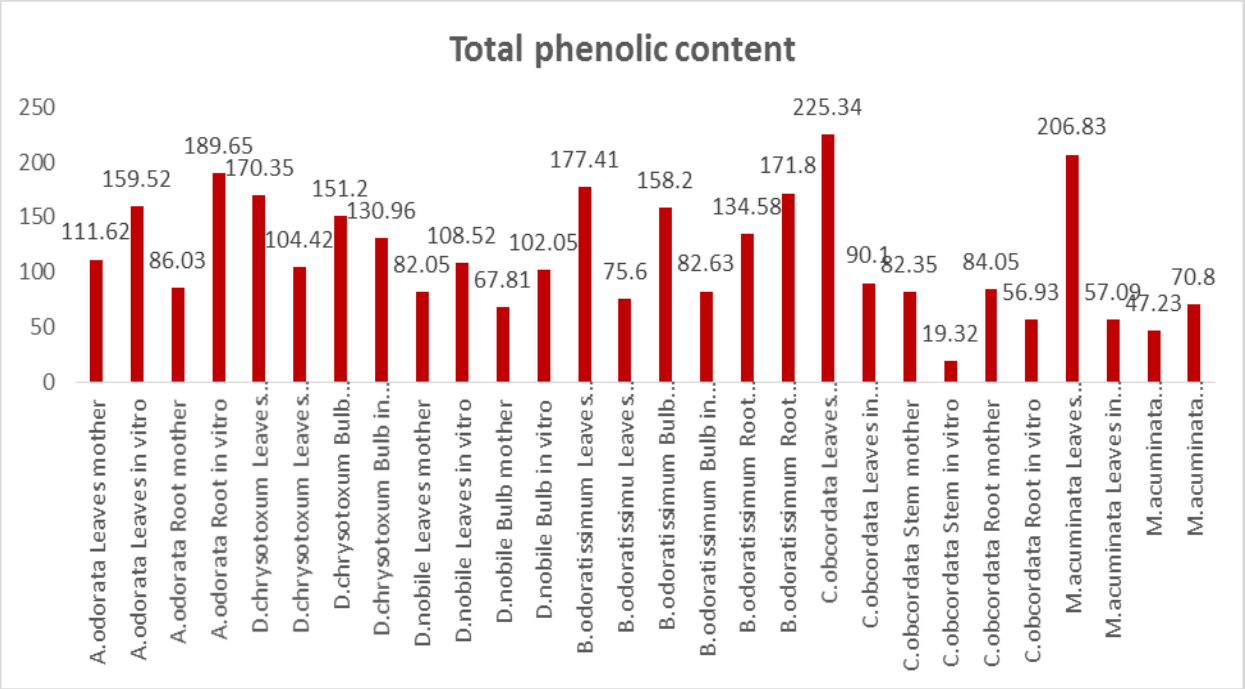


Fig. 2. Total phenolic content (GAE mg/100 gm dry extract) in the plant material

III. Estimation of total flavonoids

Total flavonoids were estimated using the method of Ordonez *& al* (2006). To 2.0 ml of sample, 2.0ml of 2% $AlCl_3$ ethanol solution was added. After one hour, at room temperature, the absorbance was measured at 420 nm (UV-visible spectrophotometer Shimadzu UV 1800). A yellow color indicated the presence of flavonoids. Total flavonoid contents were calculated as rutin (mg/g) using the following equation based on the calibration curve: $y = 0.0182x - 0.0222$, $R^2 = 0.9962$, where y was the absorbance and x was the Rutin equivalent (mg/g).

Table 17. Total flavonoid content (Rutin equivalent mg/gm dry extract) in the plant material

Sl. No.	**Name of the plant**	**Parts used**	***in vitro*/ Mother**	**Total flavonoid content mg/gm dry ext** (**Mean** ± SEM)
1.	*Aerides odorata*	Leaves	Mother	20.59±0.19
2.	*Aerides odorata*	Leaves	*in vitro*	22.61±0.23
3.	*Aerides odorata*	Root	Mother	16.46±0.02
4.	*Aerides odorata*	Root	*in vitro*	25.78±0.05
5.	*Dendrobium chrysotoxum*	Leaves	Mother	40.68±0.50
6.	*Dendrobium chrysotoxum*	Leaves	*in vitro*	19.75±0.10
7.	*Dendrobium chrysotoxum*	Pseudobulb	Mother	22.62±0.20
8.	*Dendrobium chrysotoxum*	Pseudobulb	*in vitro*	19.24±0.040
9.	*Dendrobium nobile*	Leaves	Mother	21.69±0.16
10.	*Dendrobium nobile*	Leaves	*in vitro*	64.94±0.18
11.	*Dendrobium nobile*	Pseudobulb	Mother	10.71±0.03
12.	*Dendrobium nobile*	Pseudobulb	*in vitro*	40.48±0.34
13.	*Bulbophyllum odoratissimum*	Leaves	Mother	74.16±0.97
14.	*Bulbophyllum odoratissimum*	Leaves	*in vitro*	13.45±0.07
15.	*Bulbophyllum odoratissimum*	Pseudobulb	Mother	26.56±0.15
16.	*Bulbophyllum odoratissimum*	Pseudobulb	*in vitro*	15.23±0.01
17.	*Bulbophyllum odoratissimum*	Root	Mother	24.19±0.06
18.	*Bulbophyllum odoratissimum*	Root	*in vitro*	33.51±0.15
19.	*Cephalantheropsis obcordata*	Leaves	Mother	39.82±0.14
20.	*Cephalantheropsis obcordata*	Leaves	*in vitro*	19.66±0.03
21.	*Cephalantheropsis obcordata*	Stem	Mother	20.68±0.04
22.	*Cephalantheropsis obcordata*	Stem	*in vitro*	3.63±0.02
23.	*Cephalantheropsis obcordata*	Root	Mother	17.40±0.04
24.	*Cephalantheropsis obcordata*	Root	*in vitro*	10.11±0.02
25.	*Malaxis acuminata*	Leaves	Mother	59.41±0.31
26.	*Malaxis acuminata*	Leaves	*in vitro*	14.59±0.11
27.	*Malaxis acuminata*	Pseudobulb	Mother	7.89±0.37
28.	*Malaxis acuminata*	Pseudobulb	*in vitro*	10.86±0.20

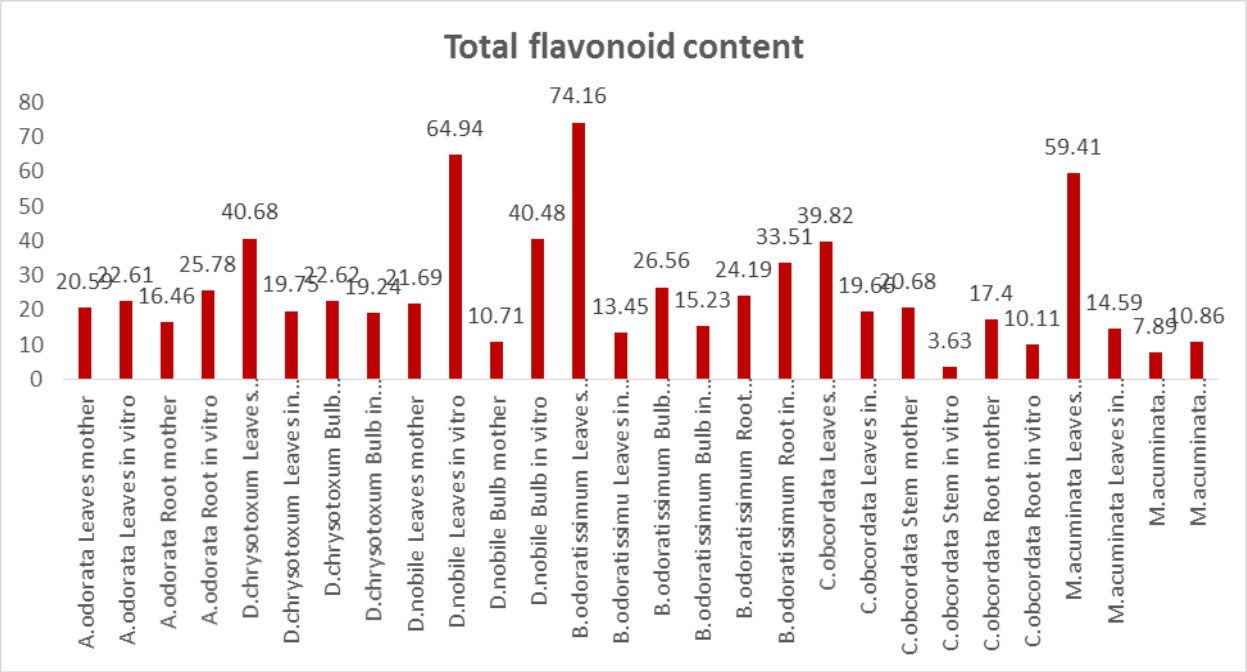


Fig. 3. Total flavonoid content in the plant material

IV. Measurement of reducing power

The reducing power of the extracts was determined according to the method of Oyaizu (1986). Extracts (100 µl) of plant extracts were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50°C for 20 min. Aliquots of 10% trichloroacetic acid (2.5 ml) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm. Reducing power is given in ascorbic acid equivalent (AAE) in milligram per gram (mg/g) of dry material using the following equation based on the calibration curve: $y = 0.0023x - 0.0063$, $R^2 = 0.9955$ where y was the absorbance and x was the ascorbic acid equivalent (mg/g).

Table 18. Reducing power (Ascorbic acid equivalent mg/gm dry extract) of the plant material)

Sl. No.	Name of the plant	Parts used	*in vitro*/ Mother	Reducing power mg/gm dry ext (Mean ± SEM)
1.	*Aerides odorata*	Leaves	Mother	52.04±0.92
2.	*Aerides odorata*	Leaves	*in vitro*	56.07±1.04
3.	*Aerides odorata*	Root	Mother	46.70±1.09
4.	*Aerides odorata*	Root	*in vitro*	83.71±0.96
5.	*Dendrobium chrysotoxum*	Leaves	Mother	99.21±2.52
6.	*Dendrobium chrysotoxum*	Leaves	*in vitro*	82.41±1.00
7.	*Dendrobium chrysotoxum*	Pseudobulb	Mother	124.24±1.52
8.	*Dendrobium chrysotoxum*	Pseudobulb	*in vitro*	39.65±0.95
9.	*Dendrobium nobile*	Leaves	Mother	54.97±1.65
10.	*Dendrobium nobile*	Leaves	*in vitro*	71.72±1.30
11.	*Dendrobium nobile*	Pseudobulb	Mother	44.85±0.67
12.	*Dendrobium nobile*	Pseudobulb	*in vitro*	51.07±0.67
13.	*Bulbophyllum odoratissimum*	Leaves	Mother	97.47±2.70
14.	*Bulbophyllum odoratissimum*	Leaves	*in vitro*	19.42±0.01
15.	*Bulbophyllum odoratissimum*	Pseudobulb	Mother	146.64±2.74
16.	*Bulbophyllum odoratissimum*	Pseudobulb	*in vitro*	24.87± 0.09
17.	*Bulbophyllum odoratissimum*	Root	Mother	13.57±0.12
18.	*Bulbophyllum odoratissimum*	Root	*in vitro*	15.38±0.18
19.	*Cephalantheropsis obcordata*	Leaves	Mother	96.31±1.49
20.	*Cephalantheropsis obcordata*	Leaves	*in vitro*	19.14±0.06
21.	*Cephalantheropsis obcordata*	Stem	Mother	7.69±0.07
22.	*Cephalantheropsis obcordata*	Stem	*in vitro*	6.80±0.09
23.	*Cephalantheropsis obcordata*	Root	Mother	7.89±0.13
24.	*Cephalantheropsis obcordata*	Root	*in vitro*	5.37±0.03
25.	*Malaxis acuminata*	Leaves	Mother	16.39±0.46
26.	*Malaxis acuminata*	Leaves	*in vitro*	10.74±0.14
27.	*Malaxis acuminata*	Pseudobulb	Mother	21.08±0.13
28.	*Malaxis acuminata*	Pseudobulb	*in vitro*	26.59±0.10

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

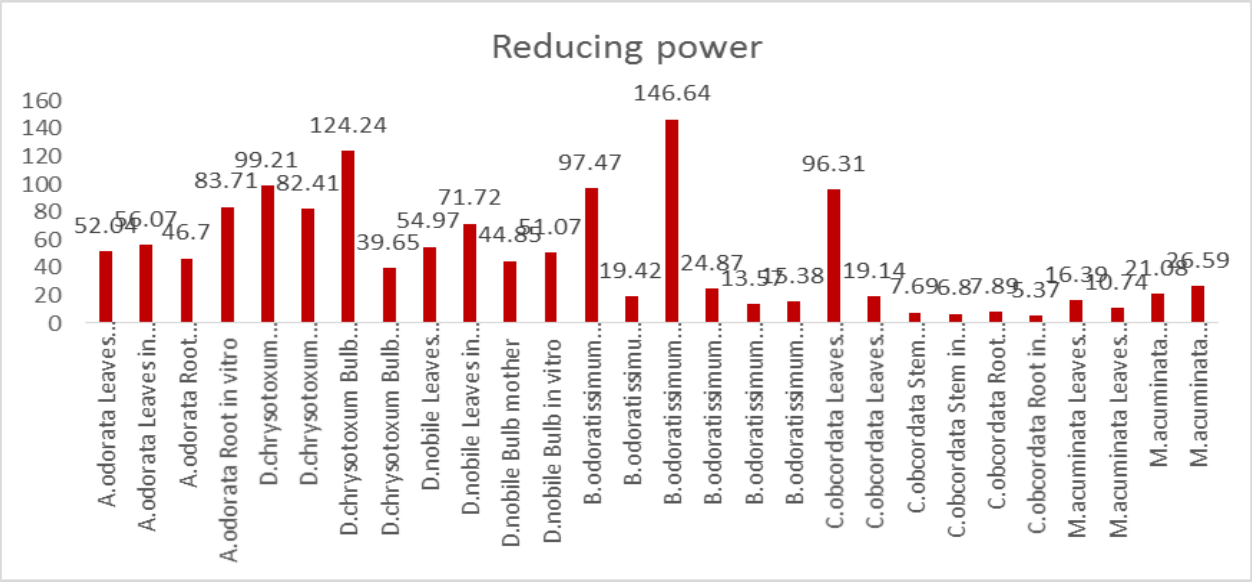


Fig. 4. Reducing power of the plant material

V. Determination of DPPH free radical scavenging activity

The free radical scavenging activity of the plant samples and butylated hydroxyl toluene (BHT) as positive control was determined using the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl). Aliquots 100µl of the tested sample were placed in test tubes and 3.9 ml of freshly prepared DPPH solution (25 mg L^{-1}) in methanol was added in each test tube and mixed. After 30 minutes, the absorbance was measured at 517 nm (UV-visible spectrophotometer Shimadzu UV 1800). The capability to scavenge the DPPH radical was calculated, using the following equation:

DPPH scavenged (%) = {(Ac – At)/Ac} x 100

Where Ac is the absorbance of the control reaction and At is the absorbance in presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration in mg of dry material that inhibits the formation of DPPH radicals by 50%. Each value was determined from regression equation.

Table 19. Free radical scavenging ability of the plants by the use of a stable DPPH radical (antioxidant activity expressed as IC_{50} mg / g dry extracts)

Sl. No.	Name of the plant	Parts used	*in vitro*/ Mother	DPPH radical scavenging activity (IC_{50} mg/gm dry material) (Mean ± SEM)
1.	*Aerides odorata*	Leaves	Mother	0.47±0.014
2.	*Aerides odorata*	Leaves	*in vitro*	0.44±0.015
3.	*Aerides odorata*	Root	Mother	0.59±0.045
4.	*Aerides odorata*	Root	*in vitro*	0.22±0.007
5.	*Dendrobium chrysotoxum*	Leaves	Mother	0.25±0.002
6.	*Dendrobium chrysotoxum*	Leaves	*in vitro*	0.47±0.03
7.	*Dendrobium chrysotoxum*	Pseudobulb	Mother	0.32±0.02
8.	*Dendrobium chrysotoxum*	Pseudobulb	*in vitro*	0.79±0.08

9.	*Dendrobium nobile*	Leaves	Mother	0.47±0.01
10.	*Dendrobium nobile*	Leaves	*in vitro*	0.29±0.006
11.	*Dendrobium nobile*	Pseudobulb	Mother	0.58±0.03
12.	*Dendrobium nobile*	Pseudobulb	*in vitro*	0.47±0.01
13.	*Bulbophyllum odoratissimum*	Leaves	Mother	0.11±0.003
14.	*Bulbophyllum odoratissimum*	Leaves	*in vitro*	0.87±0.001
15.	*Bulbophyllum odoratissimum*	Pseudobulb	Mother	0.24±0.02
16.	*Bulbophyllum odoratissimum*	Pseudobulb	*in vitro*	0.71±0.0004
17.	*Bulbophyllum odoratissimum*	Root	Mother	0.34±0.01
18.	*Bulbophyllum odoratissimum*	Root	*in vitro*	0.25±0.002
19.	*Cephalantheropsis obcordata*	Leaves	Mother	0.17±0.005
20.	*Cephalantheropsis obcordata*	Leaves	*in vitro*	0.79±0.006
21.	*Cephalantheropsis obcordata*	Stem	Mother	0.83±0.005
22.	*Cephalantheropsis obcordata*	Stem	*in vitro*	2.83±0.18
23.	*Cephalantheropsis obcordata*	Root	Mother	0.78±0.005
24.	*Cephalantheropsis obcordata*	Root	*in vitro*	1.46±0.008
25.	*Malaxis acuminata*	Leaves	Mother	0.44±0.018
26.	*Malaxis acuminata*	Leaves	*in vitro*	1.32±0.13
27.	*Malaxis acuminata*	Pseudobulb	Mother	3.56±0.18
28.	*Malaxis acuminata*	Pseudobulb	*in vitro*	1.76±0.07

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

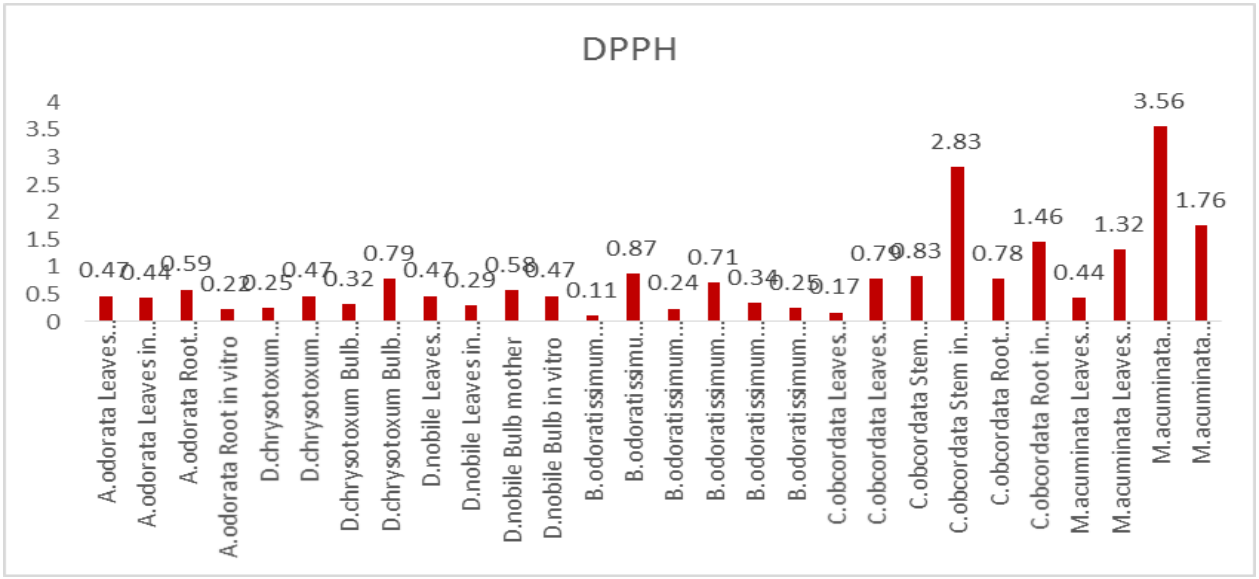


Fig. 5. Free radical scavenging ability of plants by the use of a stable DPPH radical

VI. Scavenging activity of ABTS radical cation

The 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation ($ABTS^{\cdot+}$)-scavenging activity was measured according to the method described by Re & al.(1999) ABTS was dissolved in water to a 7mM concentration. The ABTS radicals were produced by adding 2.45 mM potassium persulphate (final concentration). The completion of radical generation was obtained in the dark at room temperature for 12–16 h. This solution was then diluted with ethanol to adjust its absorbance at 734 nm to 0.70±0.02. To determine the scavenging activity, 1 ml

of diluted $ABTS^{\cdot+}$ solution was added to 10µl of plant extract, and the absorbance at 734 nm was measured 6 min after the initial mixing, using ethanol as the blank. The percentage of inhibition was calculated by the equation:

ABTS scavenged (%) = $(A_{cont} - A_{test}) / A_{cont} \times 100$

where A_c and A_s are the absorbencies of the control and of the test sample, respectively. From a plot of concentration against % inhibition, a linear regression analysis was performed to determine the IC_{50} value of the sample.

Table 20. Free radical scavenging ability of the plants by the use of an ABTS radical cation (antioxidant activity expressed as IC_{50} mg /g dry extracts)

Sl. No.	Name of the plant	Parts used	*in vitro*/ Mother	ABTS radical scavenging activity (IC_{50} mg/gm dry material) (Mean ± SEM)
1.	*Aerides odorata*	Leaves	Mother	0.20±0.002
2.	*Aerides odorata*	Leaves	*in vitro*	0.13±0.001
3.	*Aerides odorata*	Root	Mother	0.17±0.002
4.	*Aerides odorata*	Root	*in vitro*	0.09±0.001
5.	*Dendrobium chrysotoxum*	Leaves	Mother	0.11±0.001
6.	*Dendrobium chrysotoxum*	Leaves	*in vitro*	0.22±0.05
7.	*Dendrobium chrysotoxum*	Pseudobulb	Mother	0.23±0.006
8.	*Dendrobium chrysotoxum*	Pseudobulb	*in vitro*	0.29±0.006
9.	*Dendrobium nobile*	Leaves	Mother	0.39±0.01
10.	*Dendrobium nobile*	Leaves	*in vitro*	0.15±0.006
11.	*Dendrobium nobile*	Pseudobulb	Mother	0.34±0.006
12.	*Dendrobium nobile*	Pseudobulb	*in vitro*	0.16±0.003
13.	*Bulbophyllum odoratissimum*	Leaves	Mother	0.09±0.0008
14.	*Bulbophyllum odoratissimum*	Leaves	*in vitro*	0.84±0.002
15.	*Bulbophyllum odoratissimum*	Pseudobulb	Mother	0.14±0.001
16.	*Bulbophyllum odoratissimum*	Pseudobulb	*in vitro*	0.70±0.002
17.	*Bulbophyllum odoratissimum*	Root	Mother	0.30±0.001
18.	*Bulbophyllum odoratissimum*	Root	*in vitro*	0.21±0.0008
19.	*Cephalantheropsis obcordata*	Leaves	Mother	0.09±0.0008
20.	*Cephalantheropsis obcordata*	Leaves	*in vitro*	0.77±0.004
21.	*Cephalantheropsis obcordata*	Stem	Mother	0.78±0.002
22.	*Cephalantheropsis obcordata*	Stem	*in vitro*	0.81±0.005
23.	*Cephalantheropsis obcordata*	Root	Mother	0.76±0.004
24.	*Cephalanceropsis obcordata*	Root	*in vitro*	1.46±0.009
25.	*Malaxis acuminata*	Leaves	Mother	0.35±0.004
26.	*Malaxis acuminata*	Leaves	*in vitro*	1.02±0.05
27.	*Malaxis acuminata*	Pseudobulb	Mother	0.61±0.02
28.	*Malaxis acuminata*	Pseudobulb	*in vitro*	0.33±0.02

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

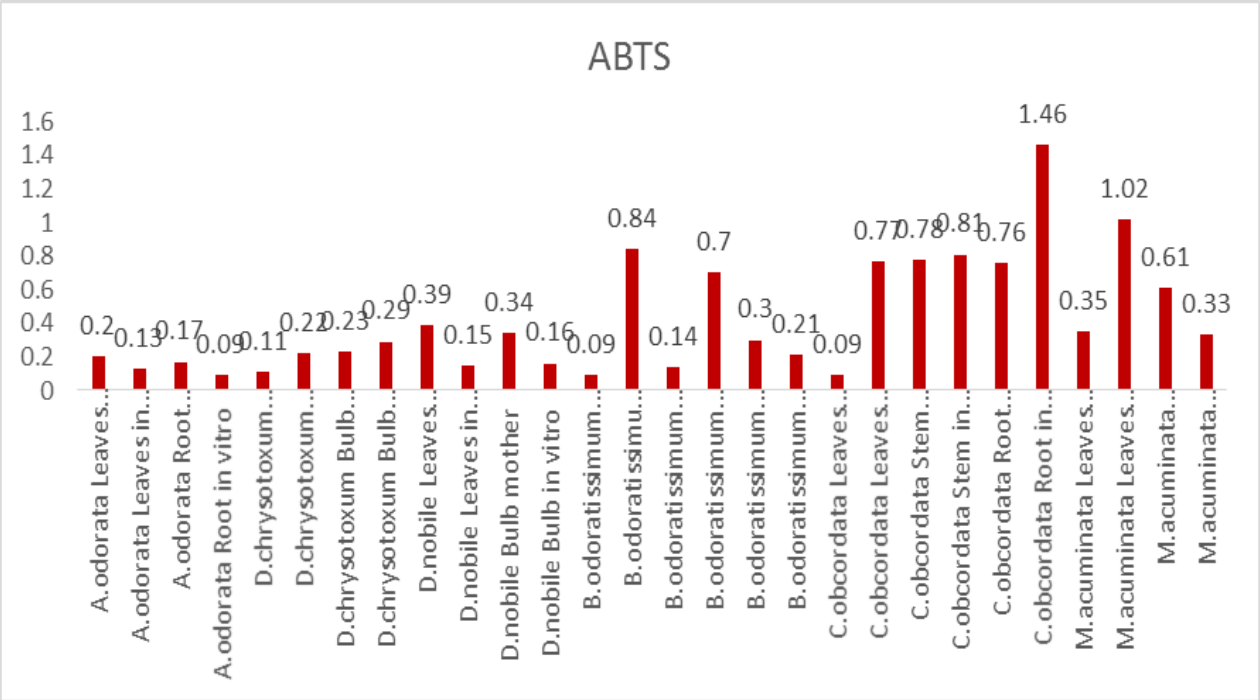


Fig. 6. Free radical scavenging ability of the plants by the use of ABTS radical cation

Comparative account of antioxidant activities of different orchid species (Mother and *in vitro*) based on phenolic content, flavonoid content, reducing power and radical scavenging activities (ABTS and DPPH)

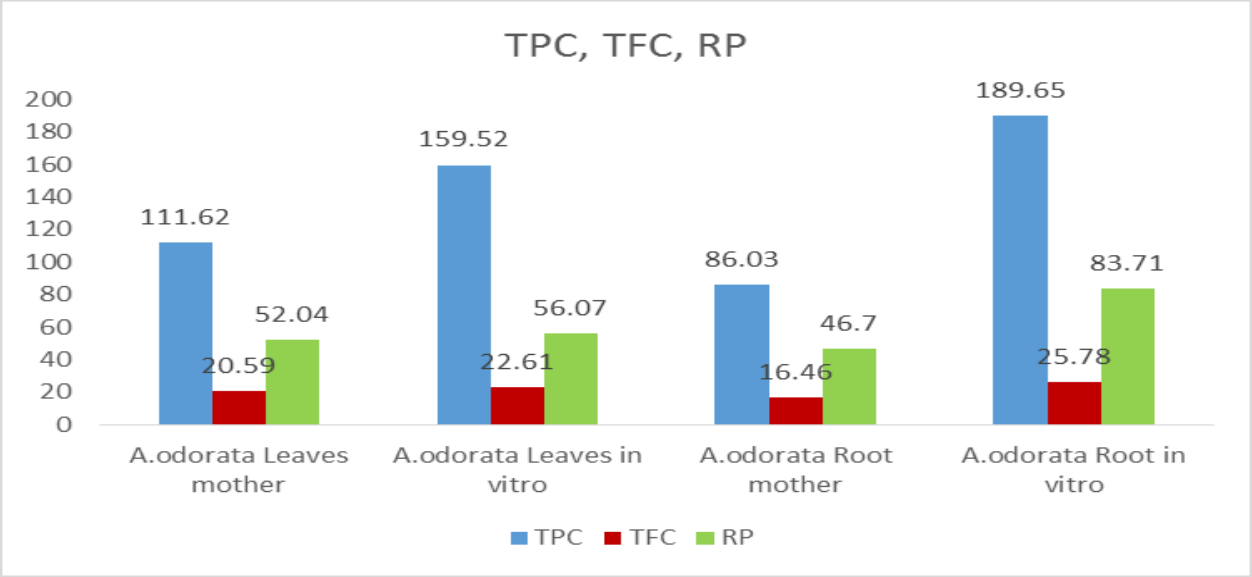


Fig. 7. Comparative antioxidant activities of *A. odorata* (Leaves and root: *in vitro* and Mother) based on phenolic content, flavonoid content and reducing power

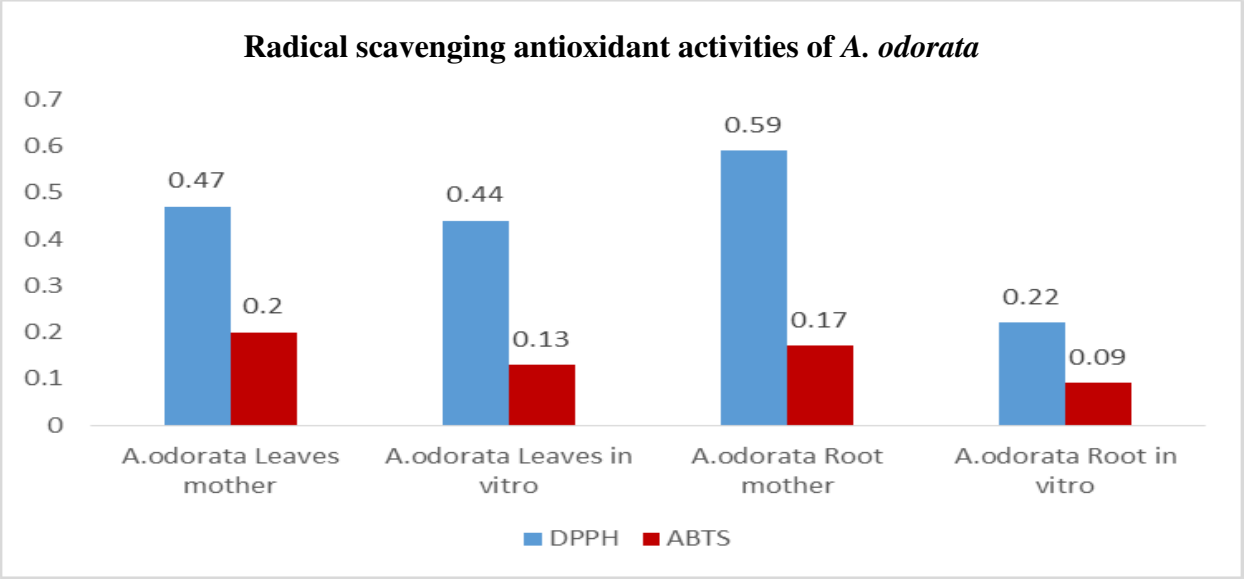


Fig. 8. Comparative antioxidant activities of *A. odorata* species (Leaves and root: *in vitro* and Mother) based on radical scavenging activities (ABTS and DPPH)

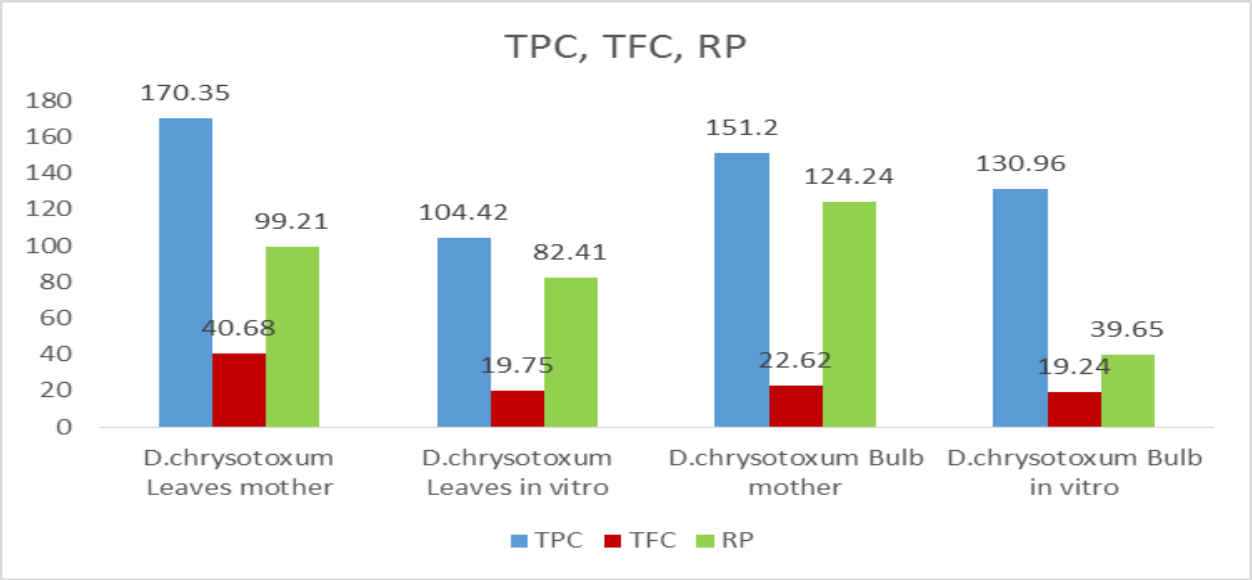


Fig. 9. Comparative antioxidant activities of *D. chrysotoxum* (Leaves and pseudobulb) (*in vitro* and Mother) based on phenolic content, flavonoid content and reducing power

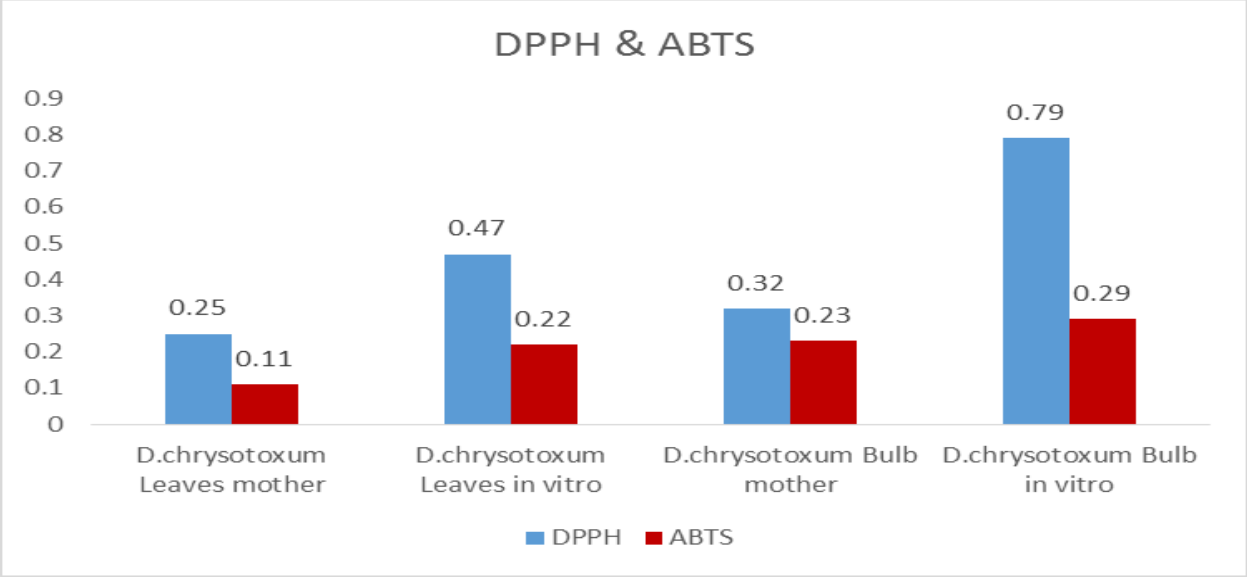


Fig. 10. Comparative antioxidant activities of *D. chrysotoxum* species (Leaves and pseudobulb) (*in vitro* and Mother) based on radical scavenging activities (ABTS and DPPH)

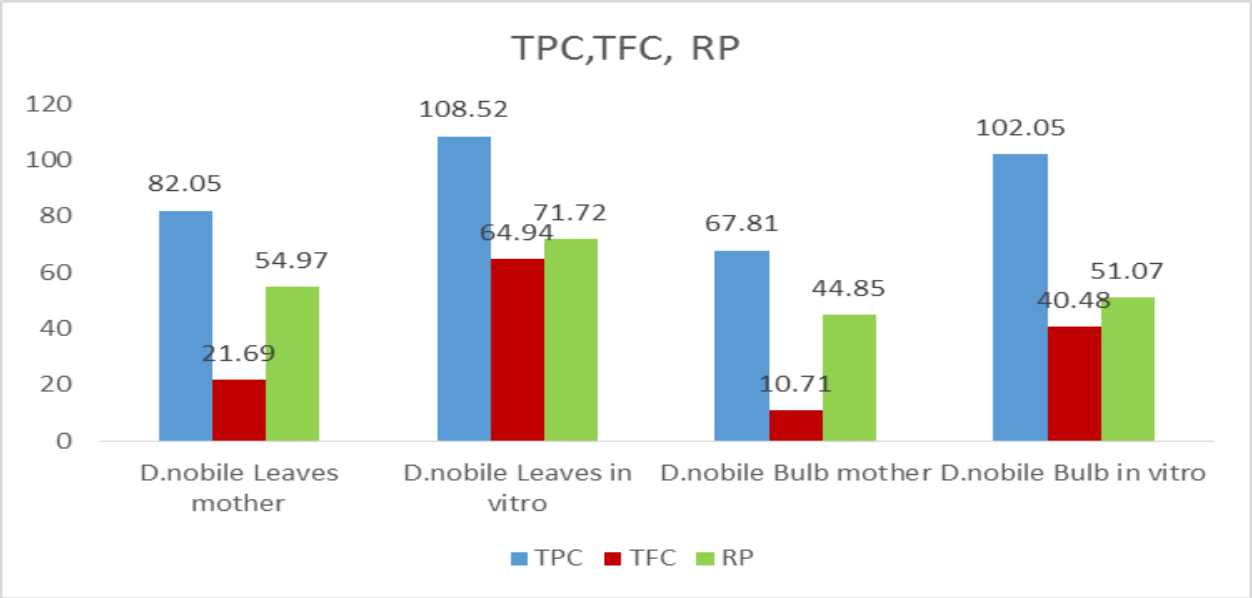


Fig. 11. Comparative antioxidant activities of *D. nobile* (Leaves and pseudobulb) (*in vitro* and Mother) based on phenolic content, flavonoid content and reducing power

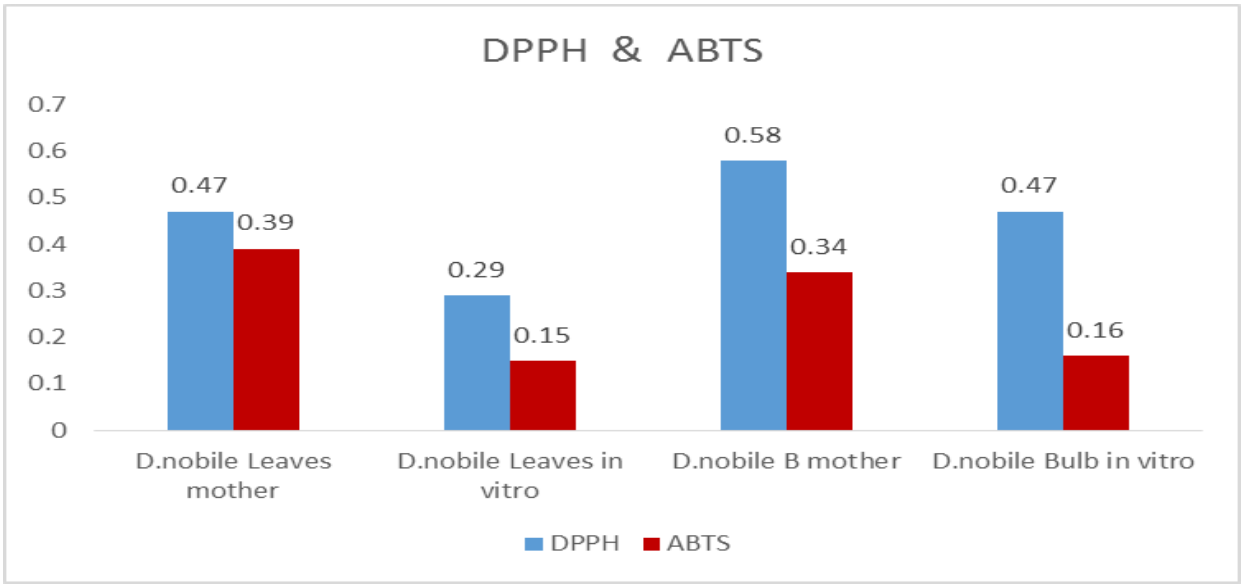


Fig. 12. Comparative antioxidant activities of *D. nobile* (Leaves and pseudobulb) (*in vitro* and Mother) based on radical scavenging activities (ABTS and DPPH)

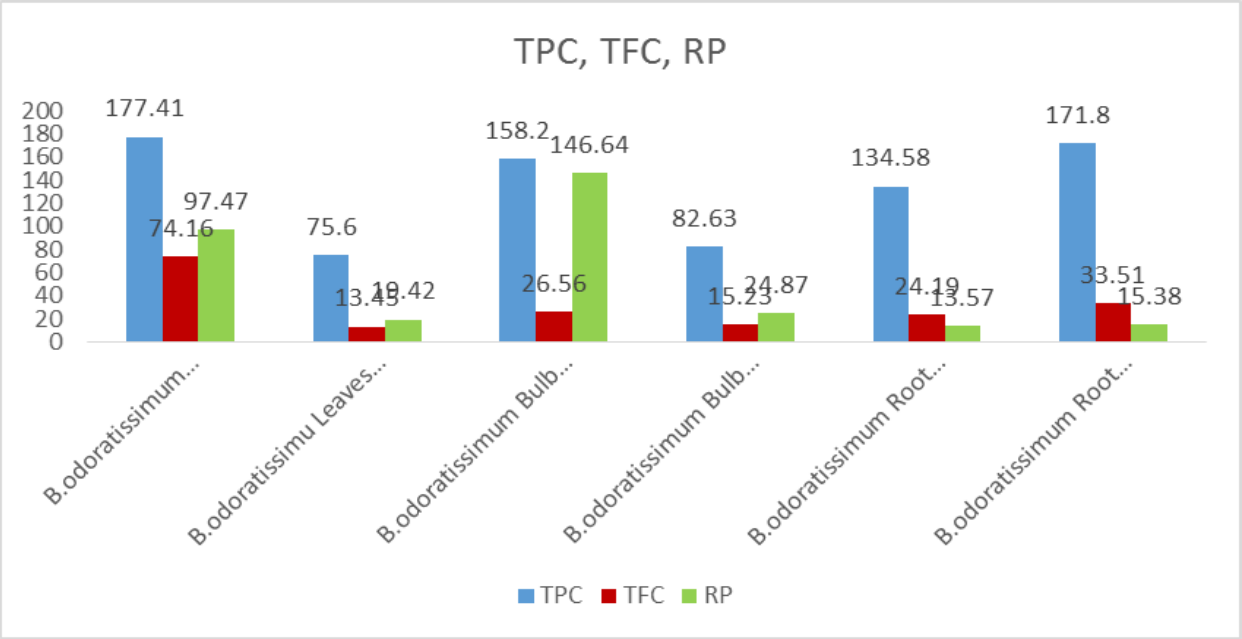


Fig. 13. Comparative antioxidant activities of *B. odoratissimum* (Leaves and pseudobulb) (*in vitro* and Mother) based on phenolic content, flavonoid content and reducing power

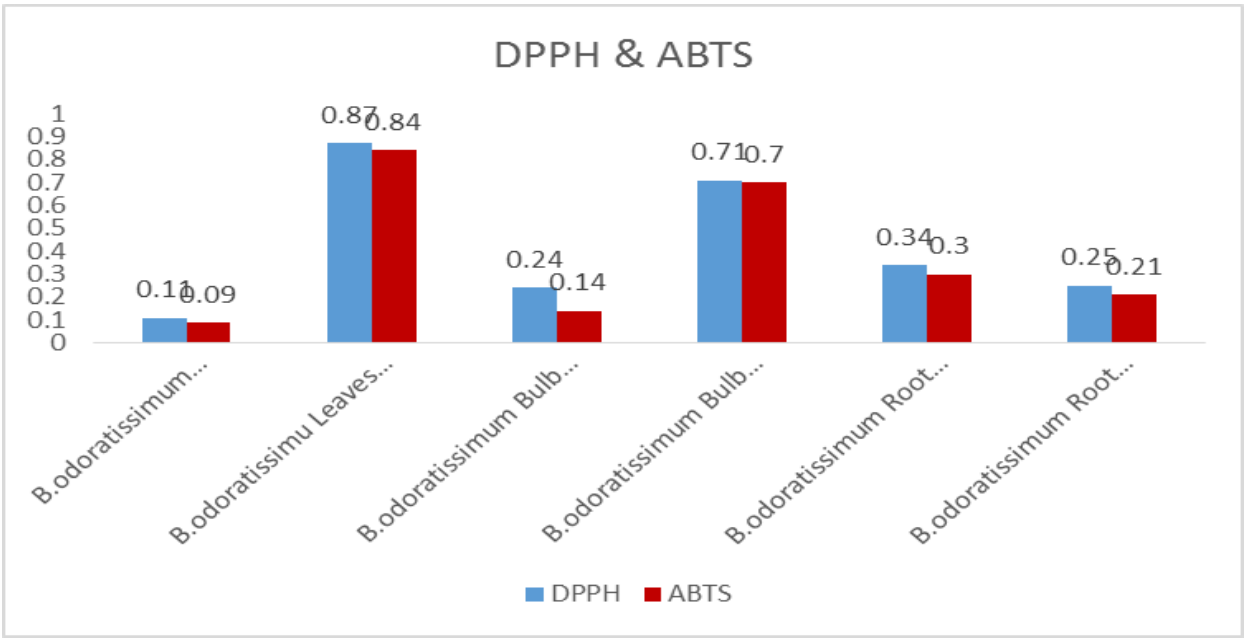


Fig. 14. Comparative antioxidant activities of *B. odoratissimum* (Leaves and pseudobulb) (*in vitro* and Mother) based on radical scavenging activities (ABTS and DPPH)

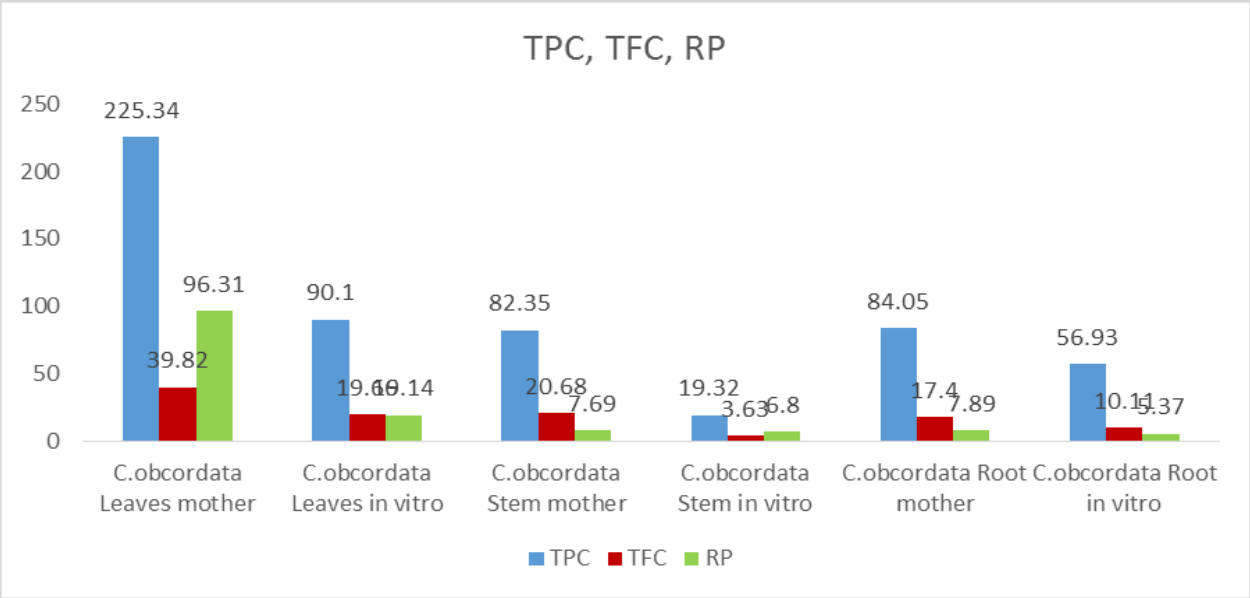


Fig. 15. Comparative antioxidant activities of *C. obcordata* (Leaves and stem) (*in vitro* and Mother) based on phenolic content, flavonoid content and reducing power

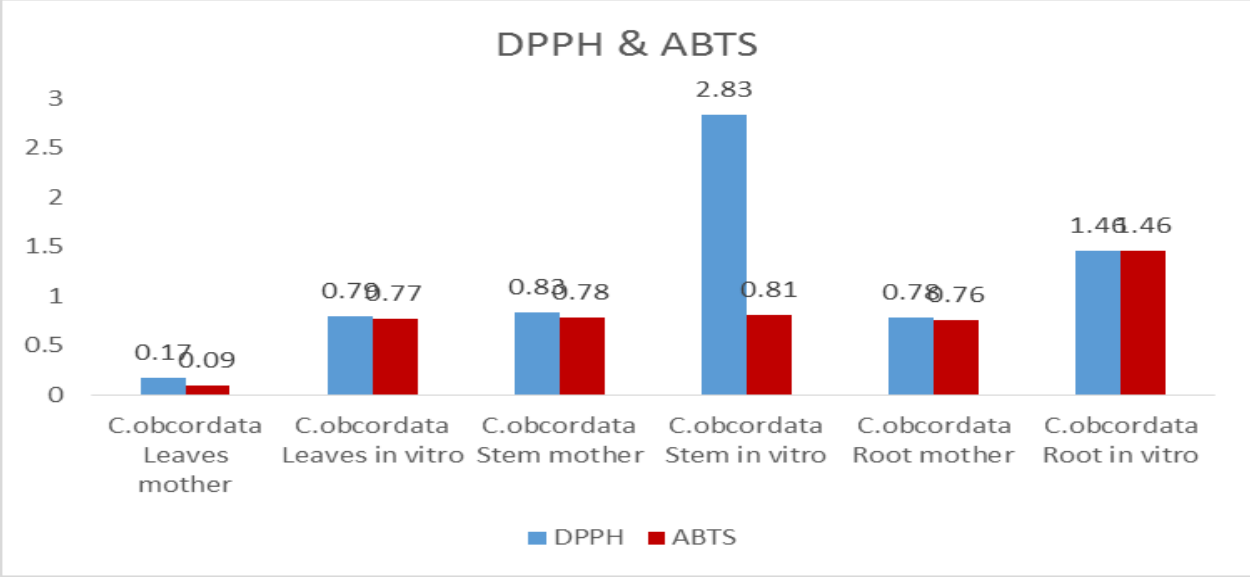


Fig. 16. Comparative antioxidant activities of *C. obcordata* (Leaves and stem) (*in vitro* and Mother) based on radical scavenging activities (ABTS and DPPH)

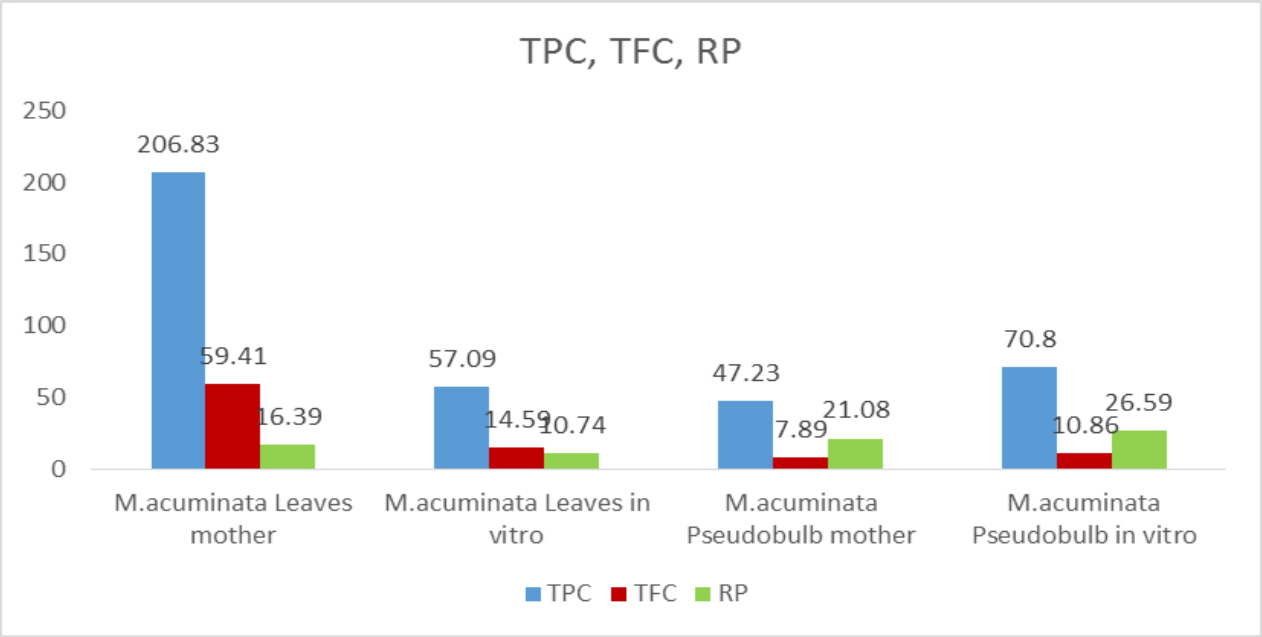


Fig. 17. Comparative antioxidant activities of *M. acuminata* (Leaves and pseudobulb) (*in vitro* and Mother) based on phenolic content, flavonoid content and reducing power

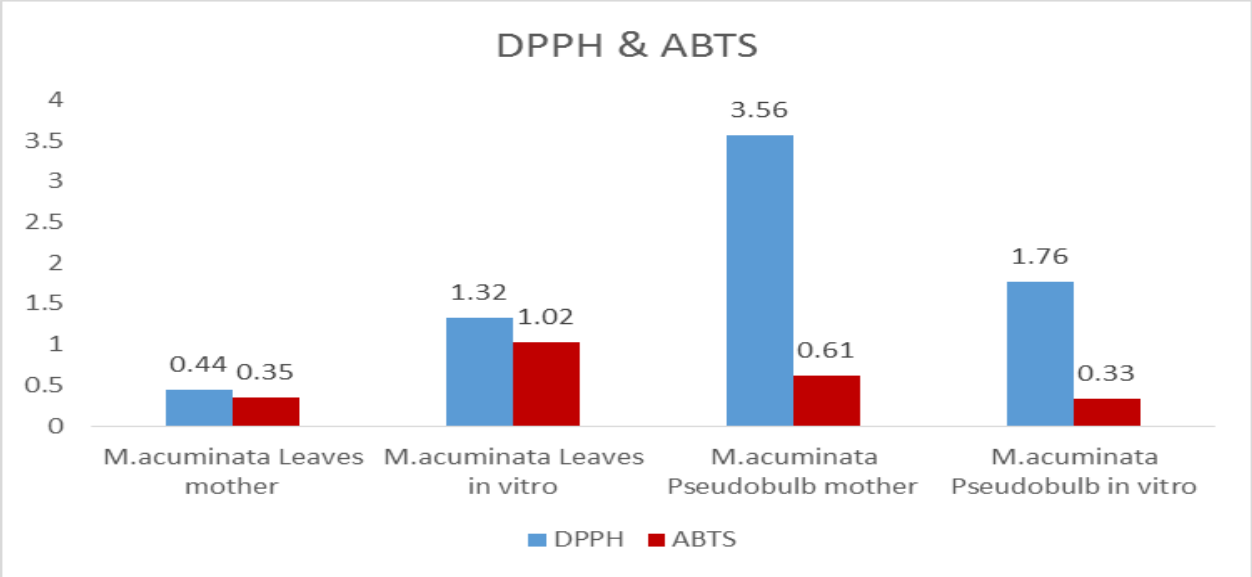
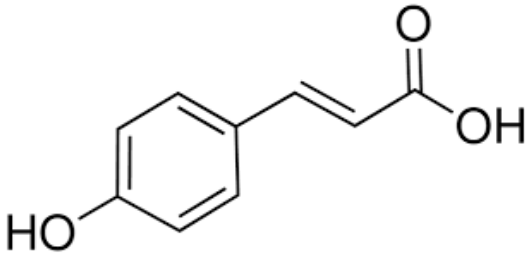


Fig. 18. Comparative antioxidant activities of *M. acuminata* (Leaves and pseudobulb) (*in vitro* and Mother) based on radical scavenging activities (ABTS and DPPH)

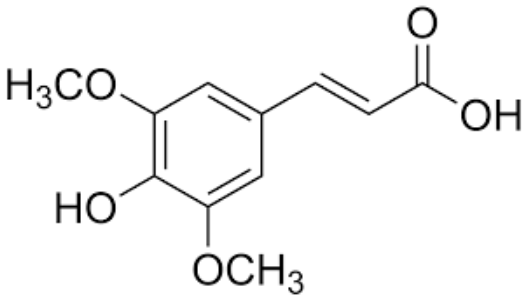
Phytochemicals responsible for the antioxidant activities of the plant

Free phenolic acids

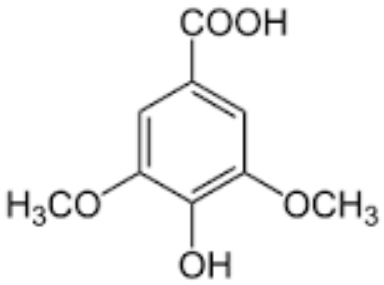
Gallic acid, Methyl gallate, Caffeic acid, Syringic acid, Ferulic acid, p-coumaric acid, Sinapic acid Phenolic acids are powerful antioxidants and have been reported to demonstrate antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasodilatory actions. So the identification and quantification of these compounds can give vital information relating to antioxidant properties, food quality, and potential health benefits of the plants.



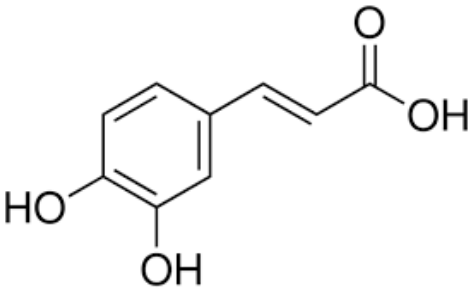
p-Coumaric acid



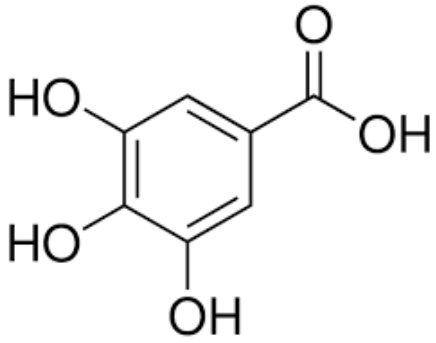
Sinapic acid



Syringic acid



Caffeic acid

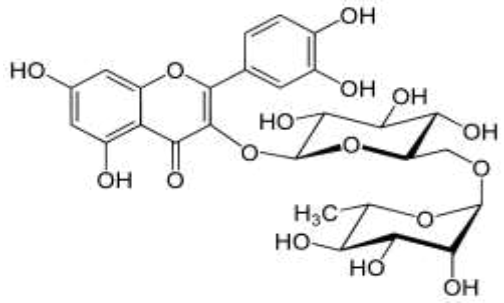


Gallic acid

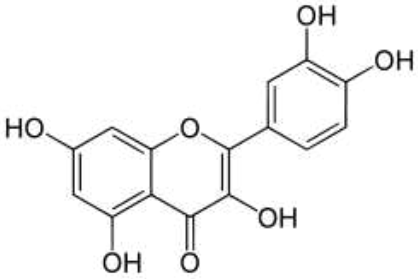
Flavonoids

Catechin, Rutin, Quercetin, Myrecetin,Apigenin, Kaempferol

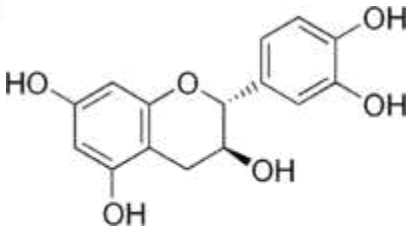
Flavonoids are considered to be important components in the human diet. Flavonoids like Rutin, kaempferol, quercetin, apigenin etc are known for its anti-inflammatory, anti-allergic, antithrombitic, hepatoprotective, antispasmodic and anticancer properties.



Rutin



Quercetin



Catechin

C. <u>Quantitative analysis of phenolic acids and flavonoids and ascorbic acid content in the plants under investigation by HPLC</u>

HPLC equipment

HPLC analyses were performed using Dionex Ultimate 3000 liquid chromatography including a diode array detector (DAD) with 5 cm flow cell and with Chromeleon system manager as data processor. Separation was achieved by a reversed-phase Acclaim C18 column (5 micron particle size, 250 x 4.6 mm). 20 μL of sample was introduced into the HPLC.

1. HPLC chromatogram of *A. odorata*

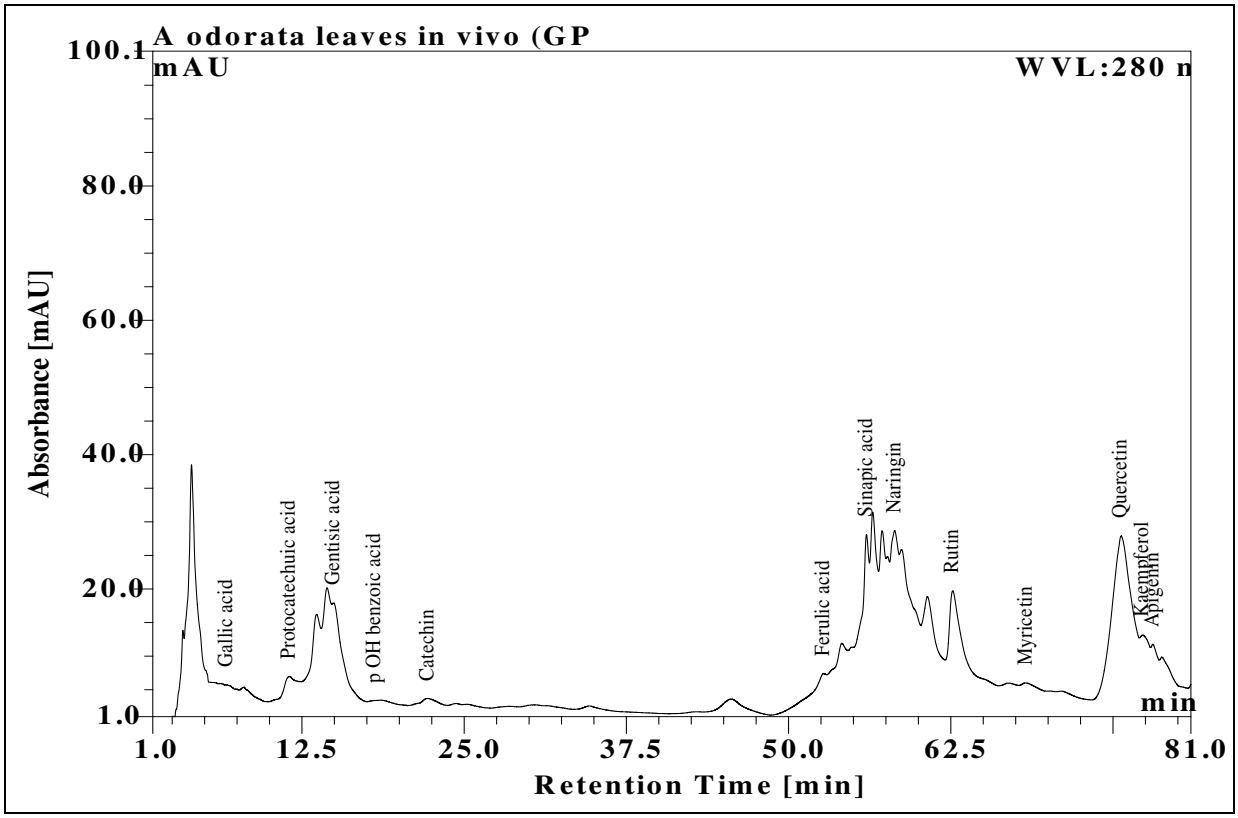


Fig. 19. HPLC chromatogram of 80% ethanolic extract of *A. odorata* leaves (Mother plant)

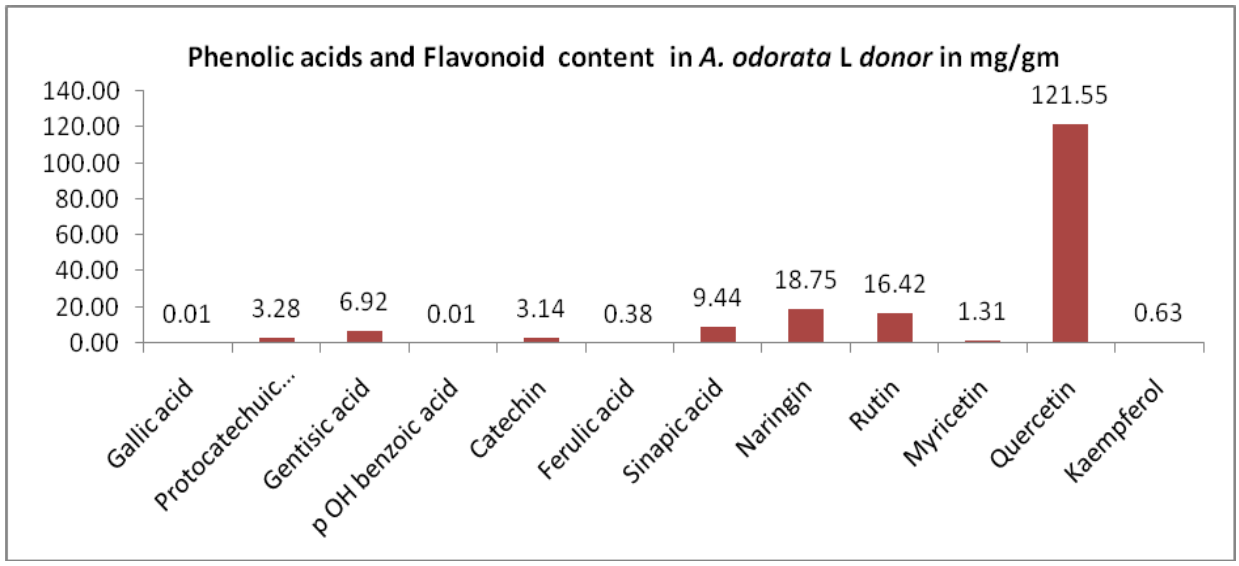


Fig. 20. Phenolic acids and Flavonoid content in *A. odorata* leaves (Mother plant)

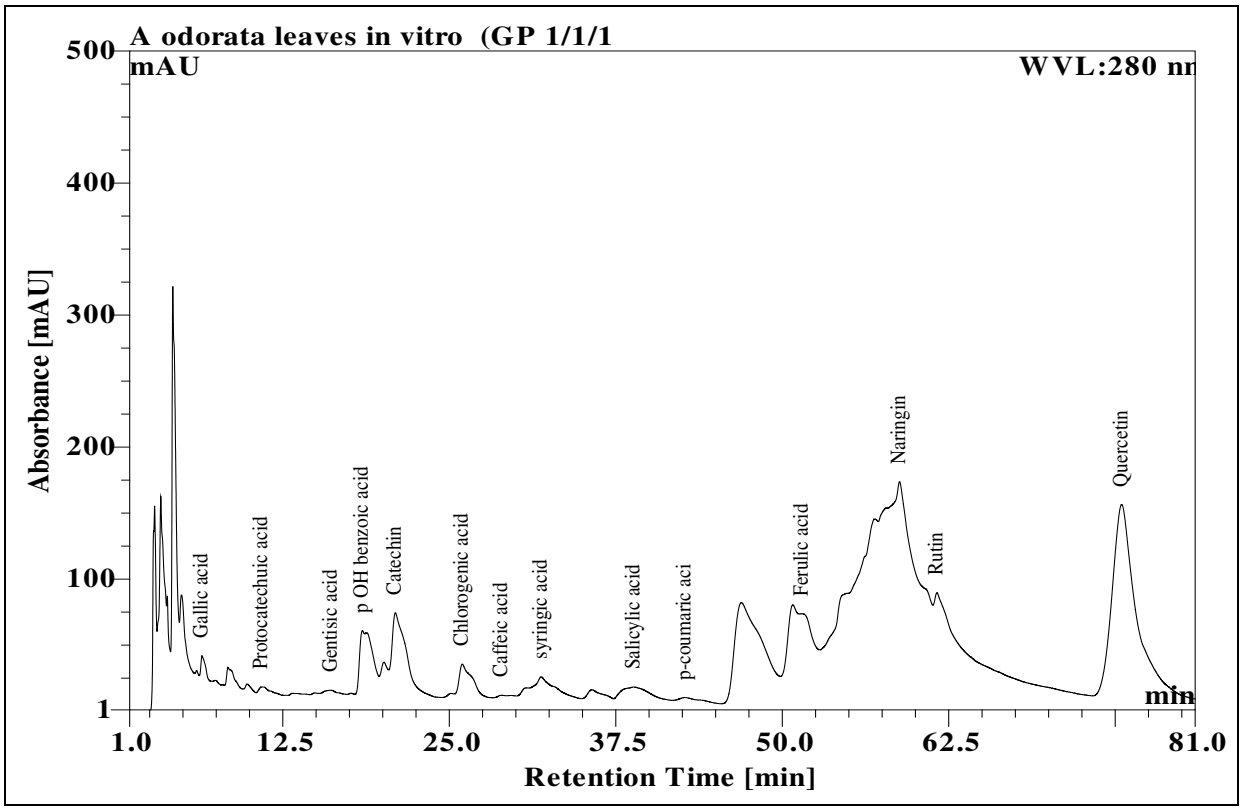


Fig. 21. HPLC chromatogram of 80% ethanolic extract of *A. odorata* leaves (*in vitro*)

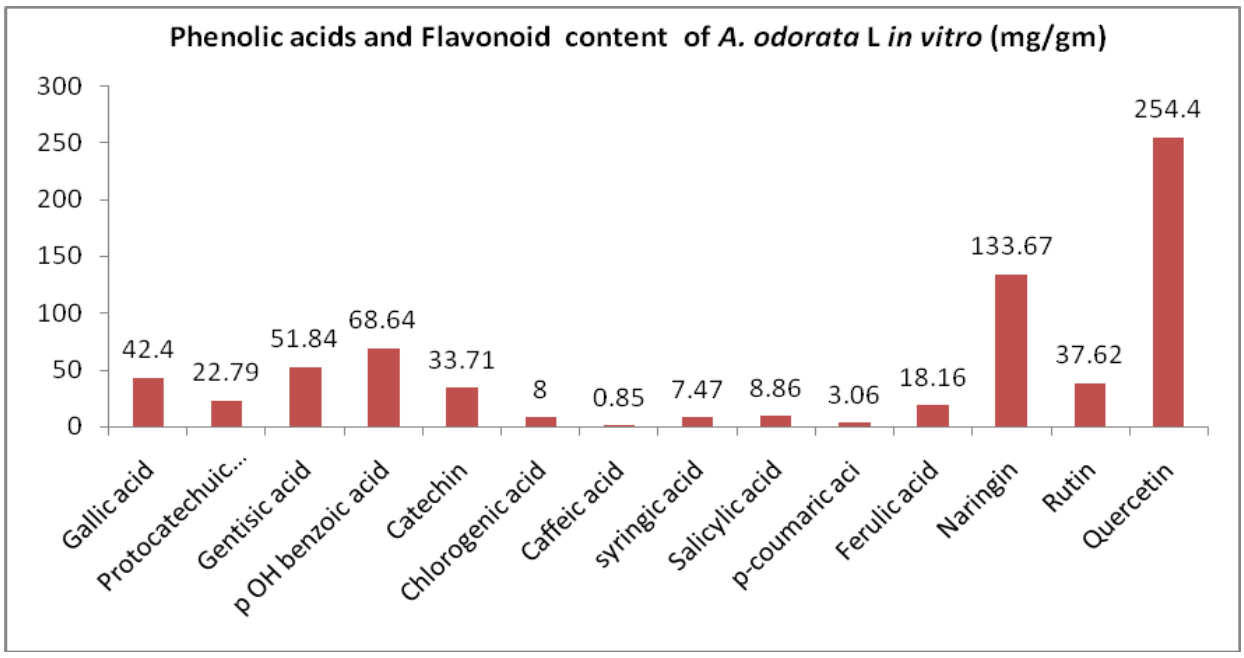


Fig. 22. Phenolic acids and flavonoid content in *A. odorata* leaves (*in vitro*)

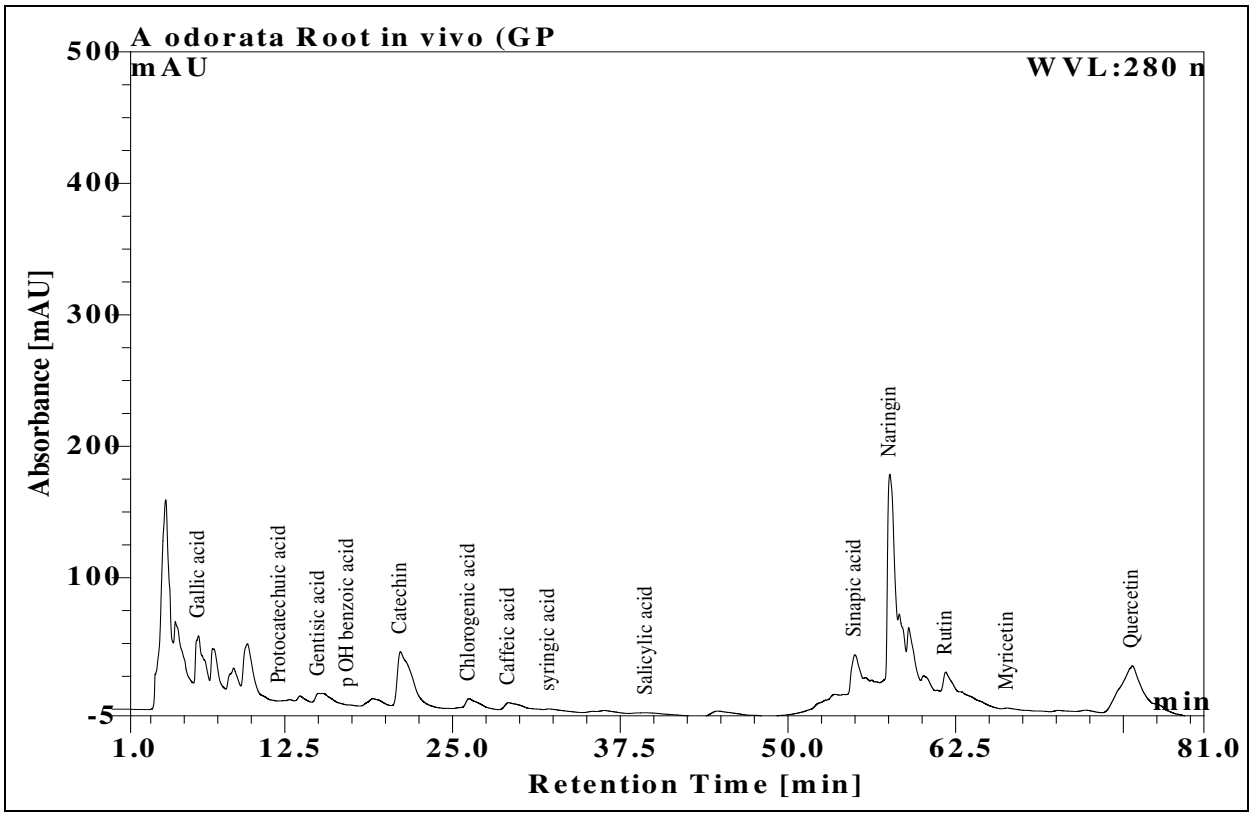


Fig. 23. HPLC chromatogram of 80% ethanolic extract of *A. odorata* root (mother plant)

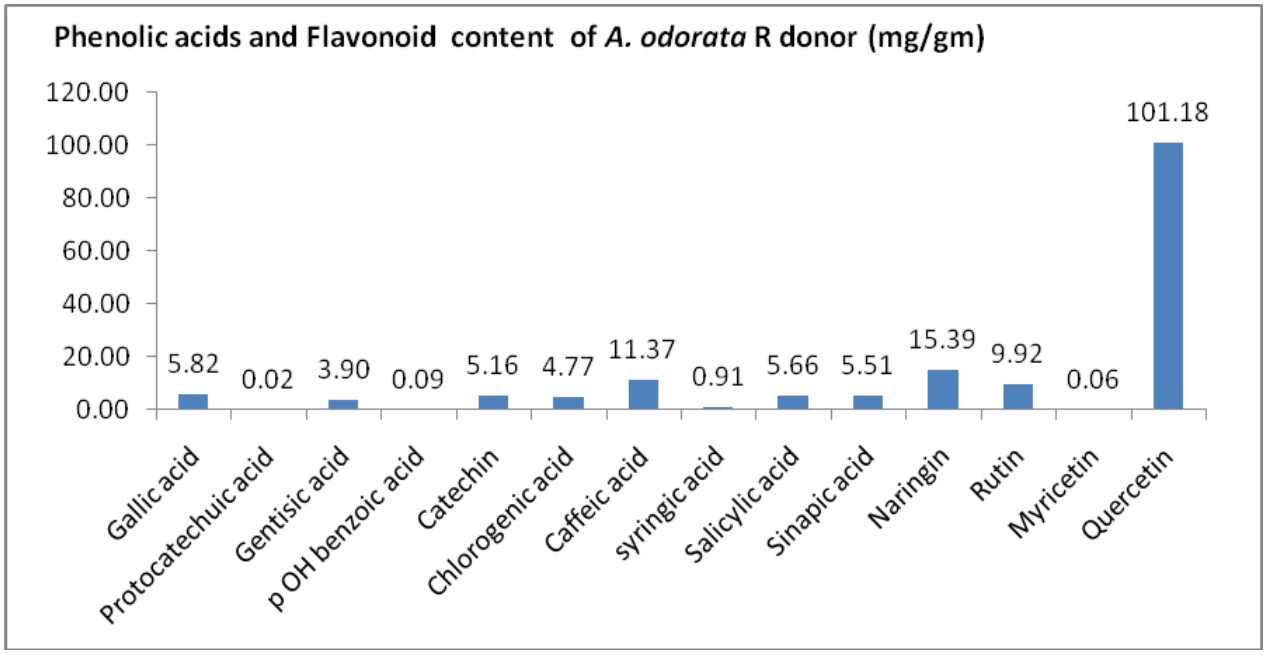


Fig. 24. Phenolic acids and flavonoid content in *A. odorata* root (mother plant)

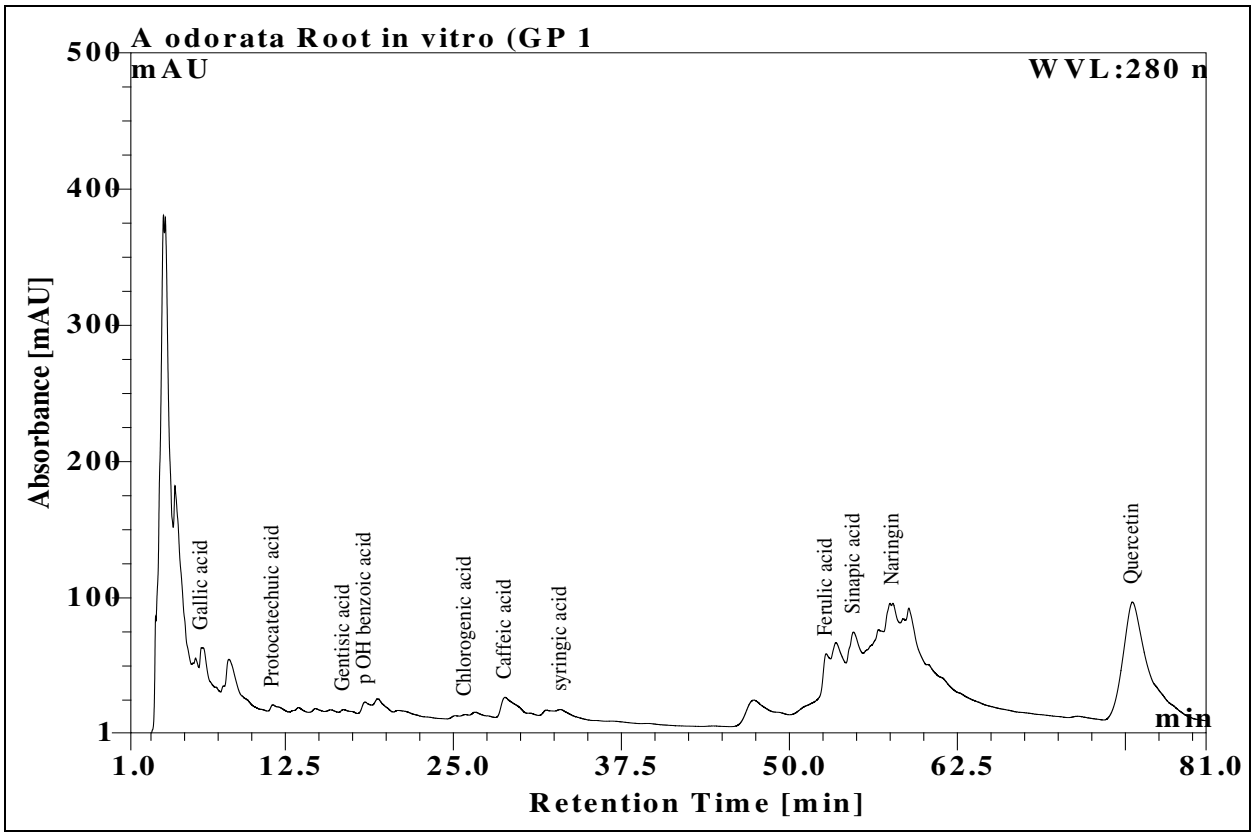


Fig. 25. HPLC chromatogram of 80% ethanolic extract of *A. odorata* root (*in vitro*)

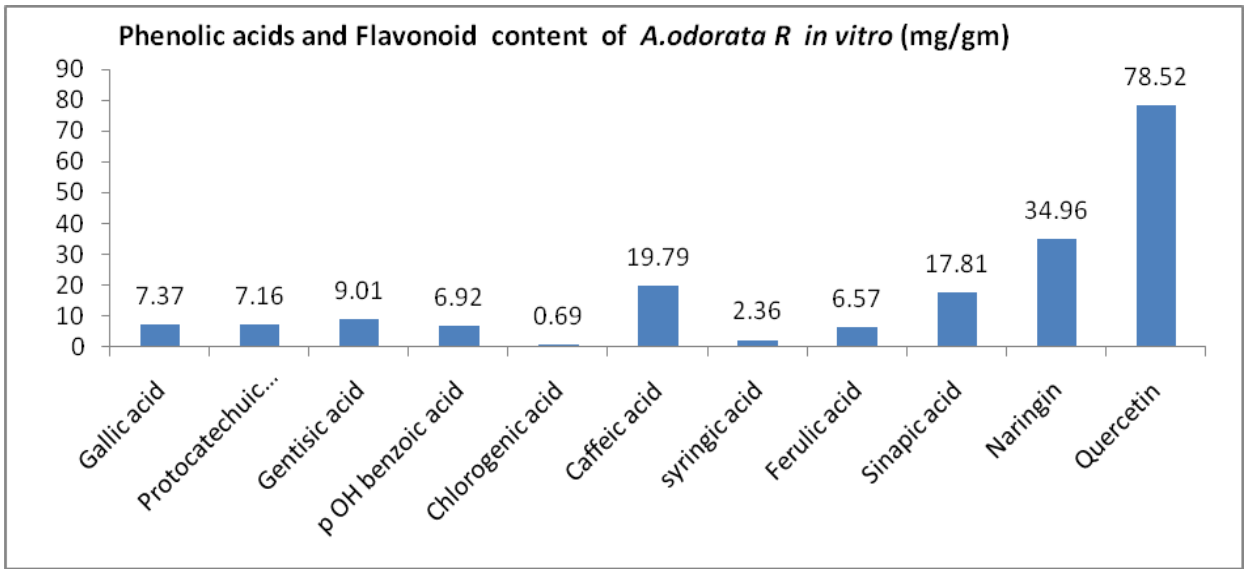


Fig. 26. Phenolic acids and flavonoid content in *A. odorata* Root (in *vitro*)

2. HPLC chromatogram of *D. chrysotoxum*

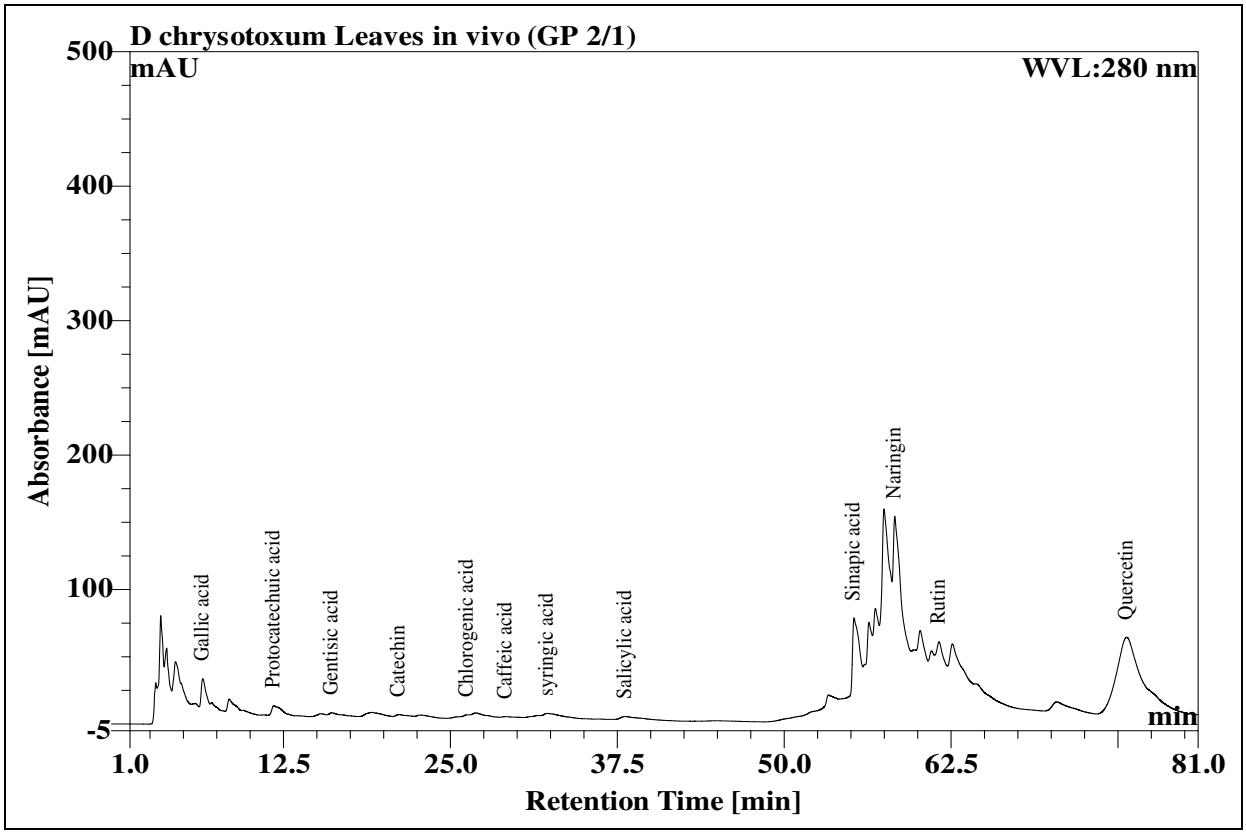


Fig. 27. HPLC chromatogram of 80% ethanolic extract of *D. chrysotoxum* leaf (Mother plant)

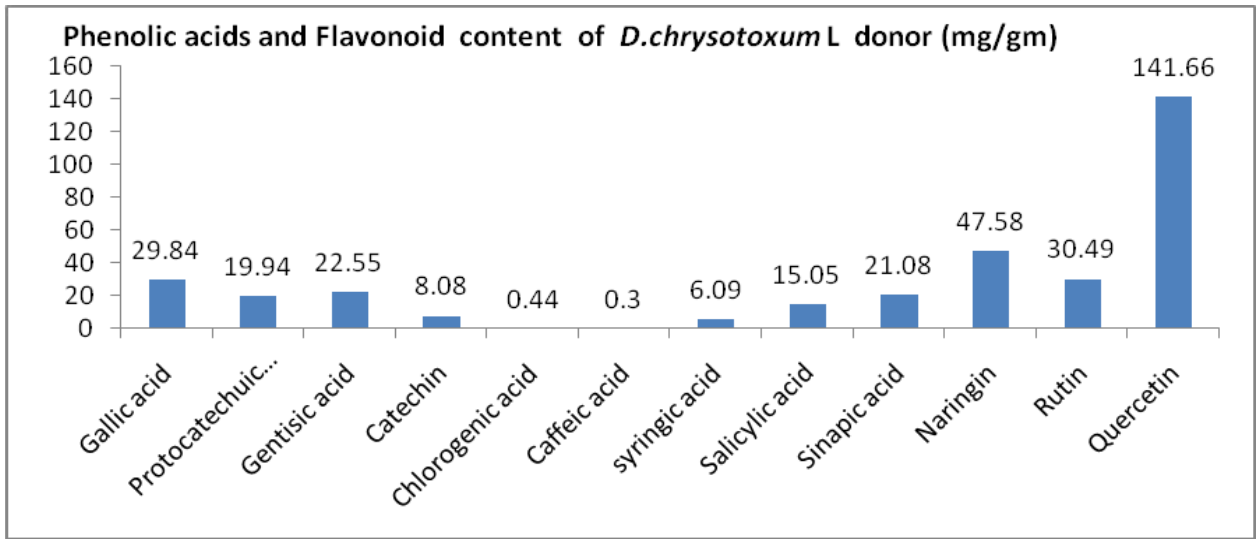


Fig. 28. Phenolic acids and flavonoid content of *D. chrysotoxum* L (Mother plant)

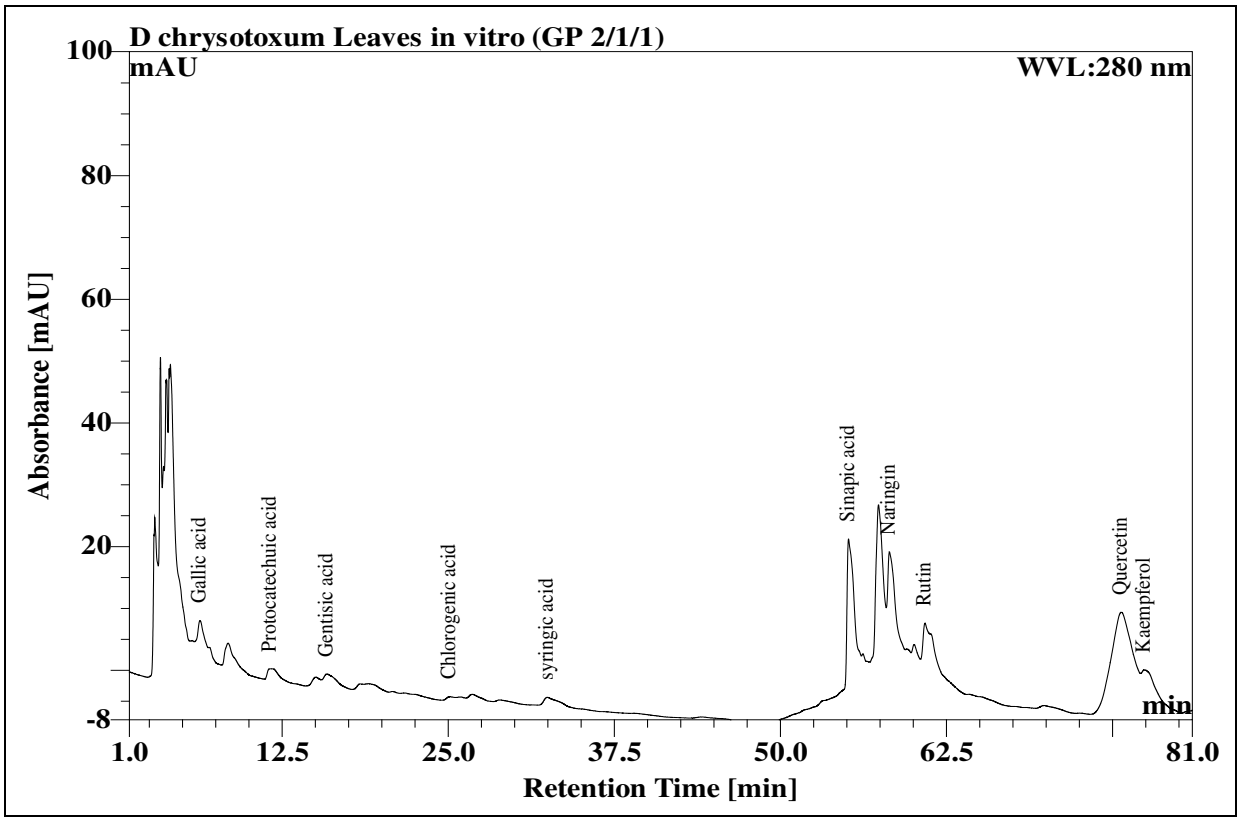


Fig. 29. HPLC chromatogram of 80% ethanolic extract of *D. chrysotoxum* leaf (*in vitro*)

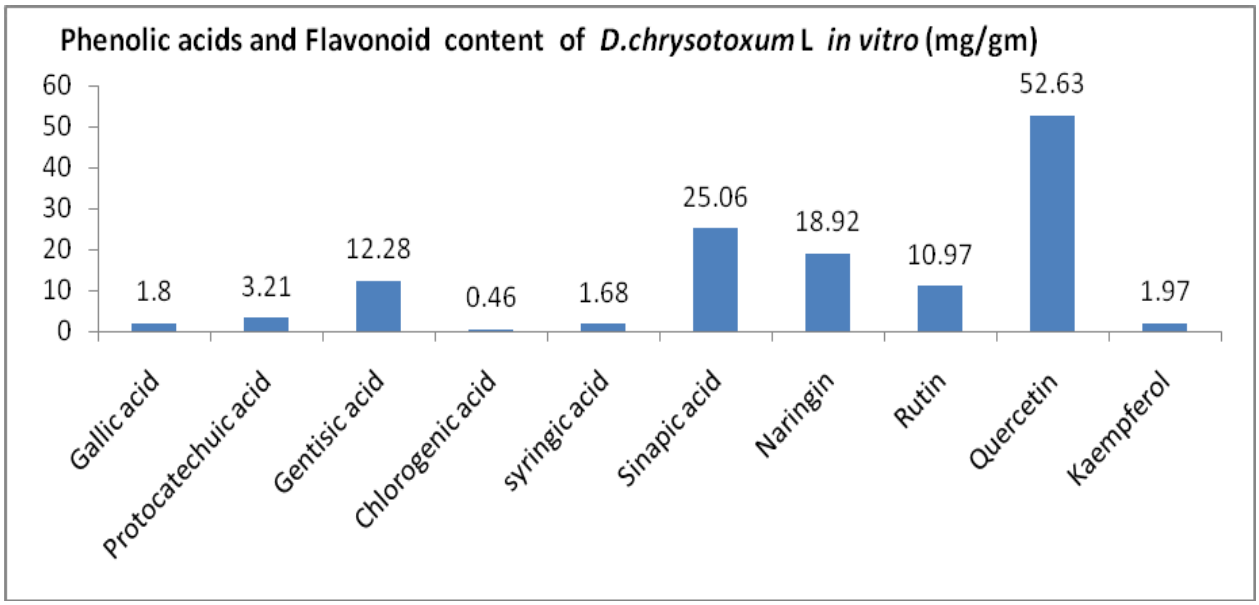


Fig. 30. Phenolic acids and flavonoid content of *D. chrysotoxum* L (*in vitro*)

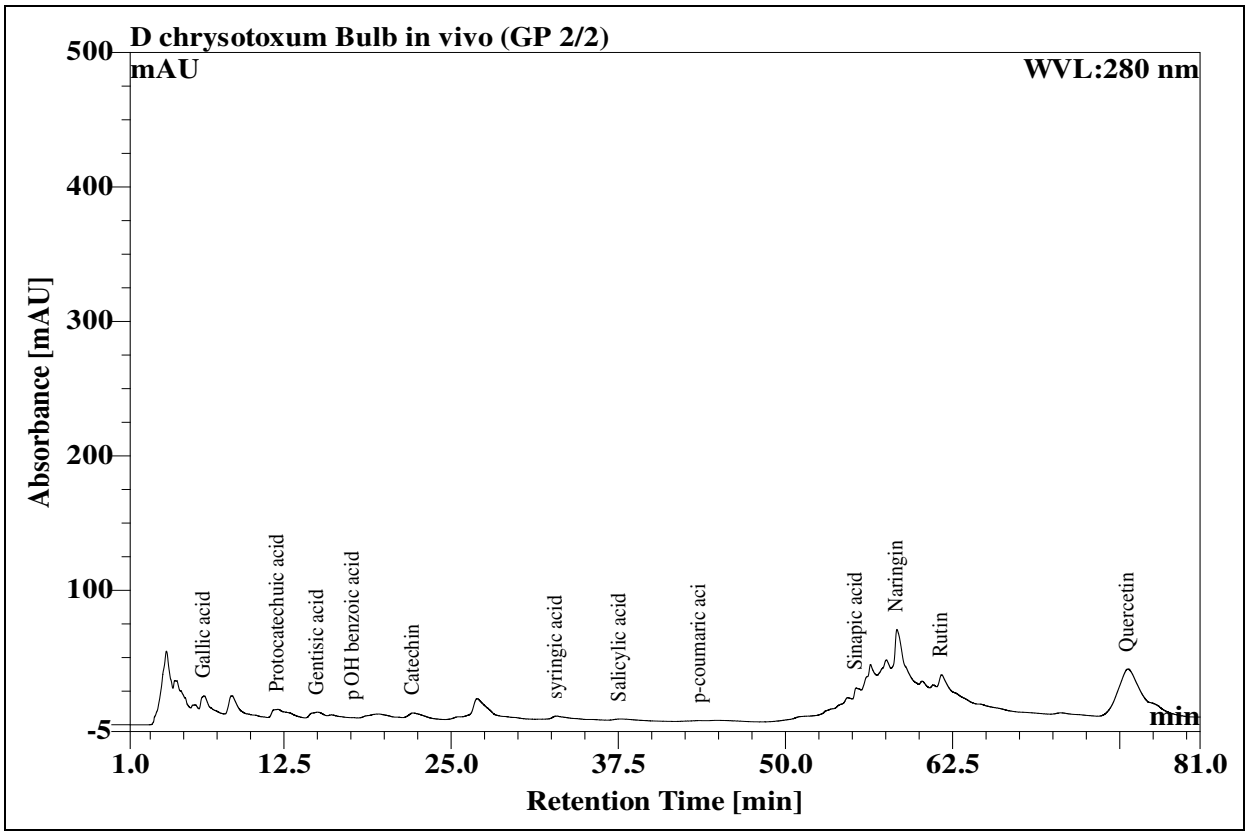


Fig. 31. HPLC chromatogram of 80% ethanolic extract of *D. chrysotoxum* pseudobulb (Mother plant)

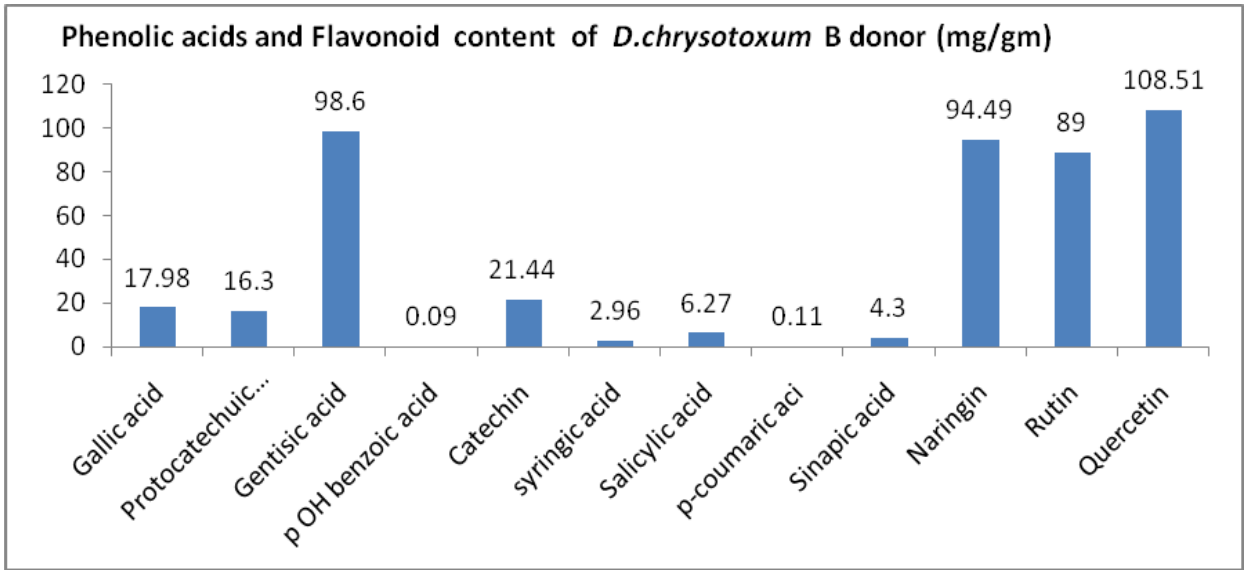


Fig. 32. Phenolic acids and flavonoid content of *D. chrysotoxum* pseudobulb (Mother plant)

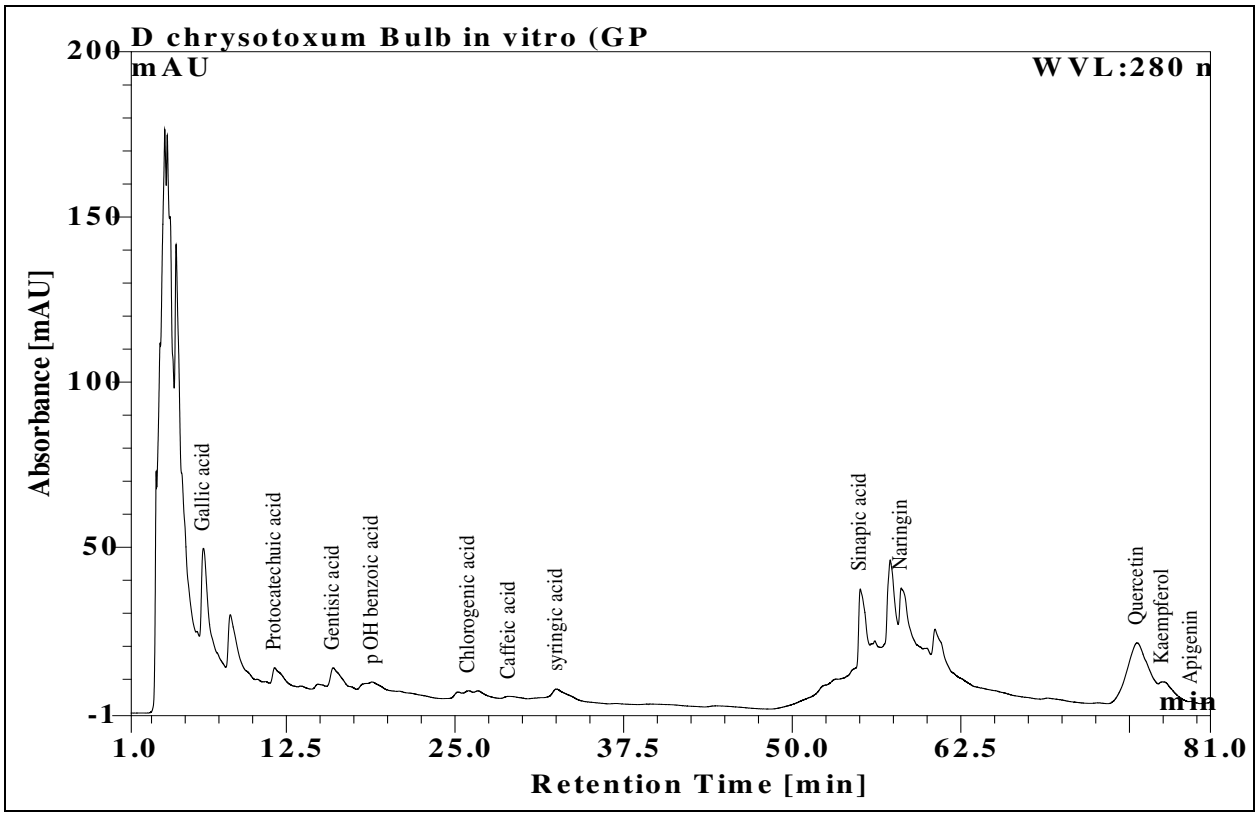


Fig. 33. HPLC chromatogram of 80% ethanolic extract of *D. chrysotoxum* pseudobulb (*in vitro*)

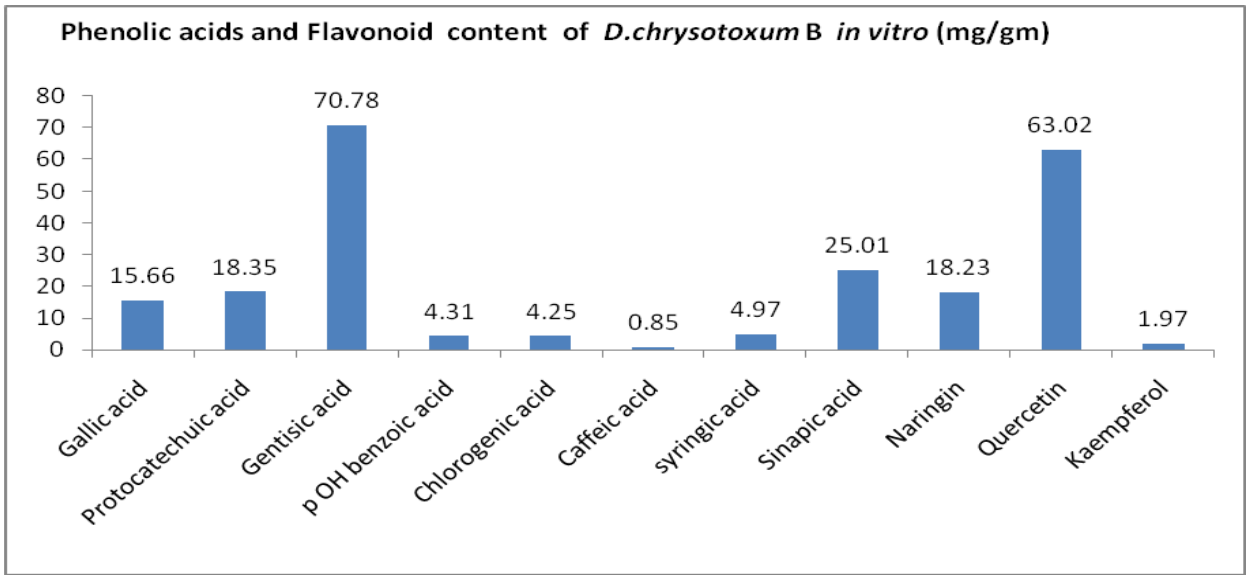


Fig. 34. Phenolic acids and Flavonoid content of *D. chrysotoxum* pseudobulb (*in vitro*)

HPLC chromatograph of *D. nobile*

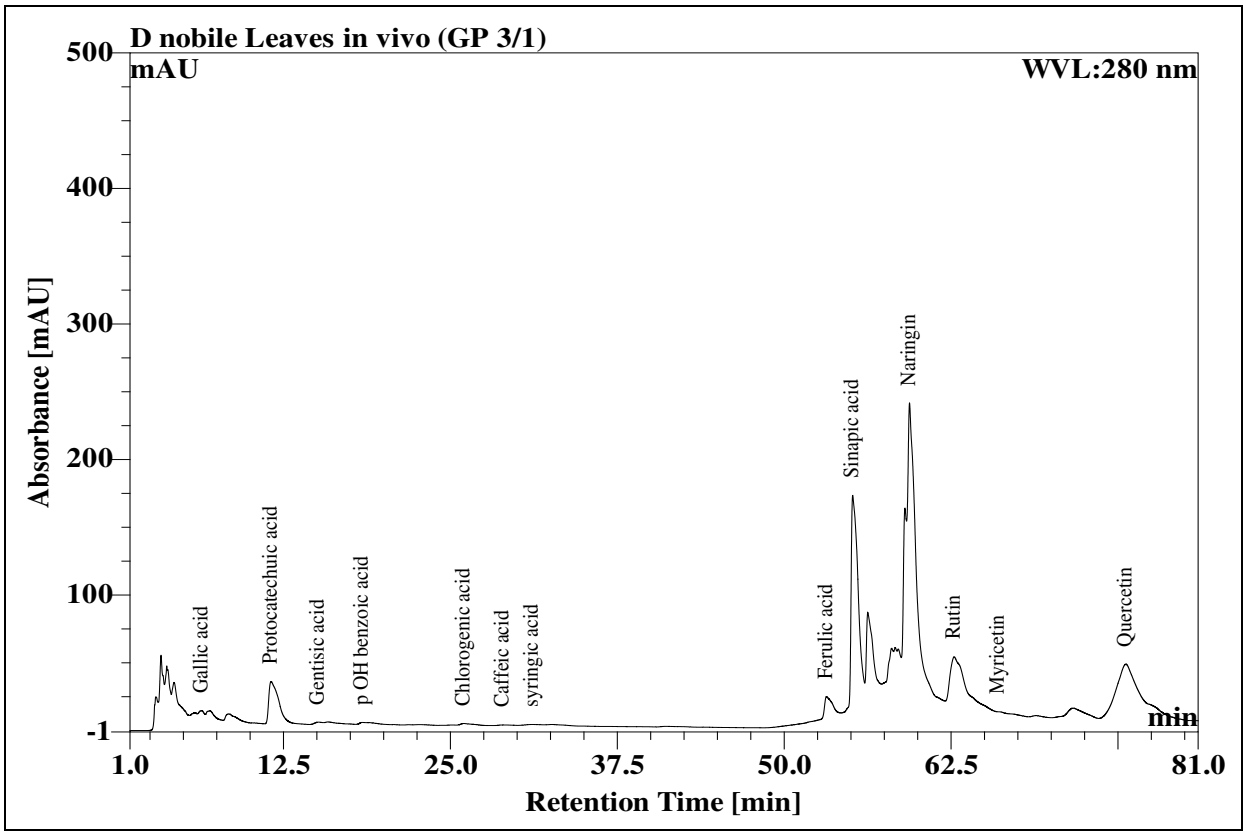


Fig. 35. HPLC chromatogram of 80% ethanolic extract of *D. nobile* leaf (Mother plant)

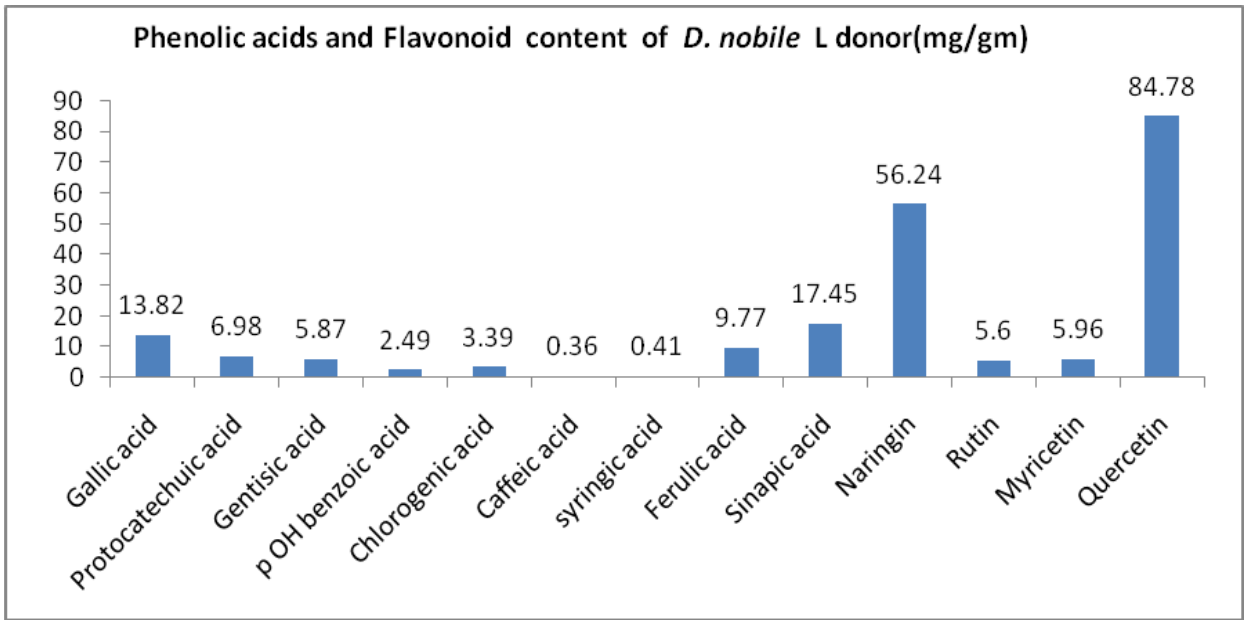


Fig. 36. Phenolic acids and flavonoid content of *D. nobile* L (Mother plant)

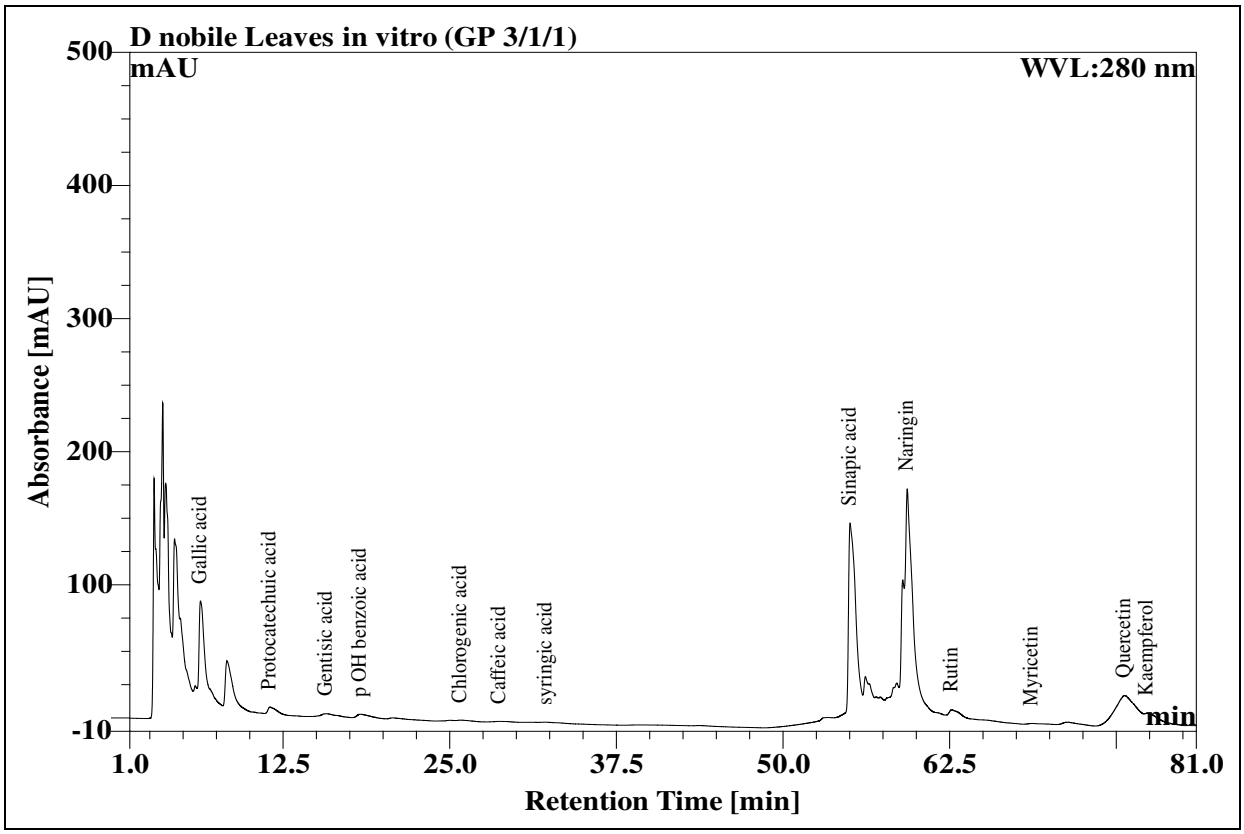


Fig. 37. HPLC chromatogram of 80% ethanolic extract of *D. nobile* Leaf (*in vitro*)

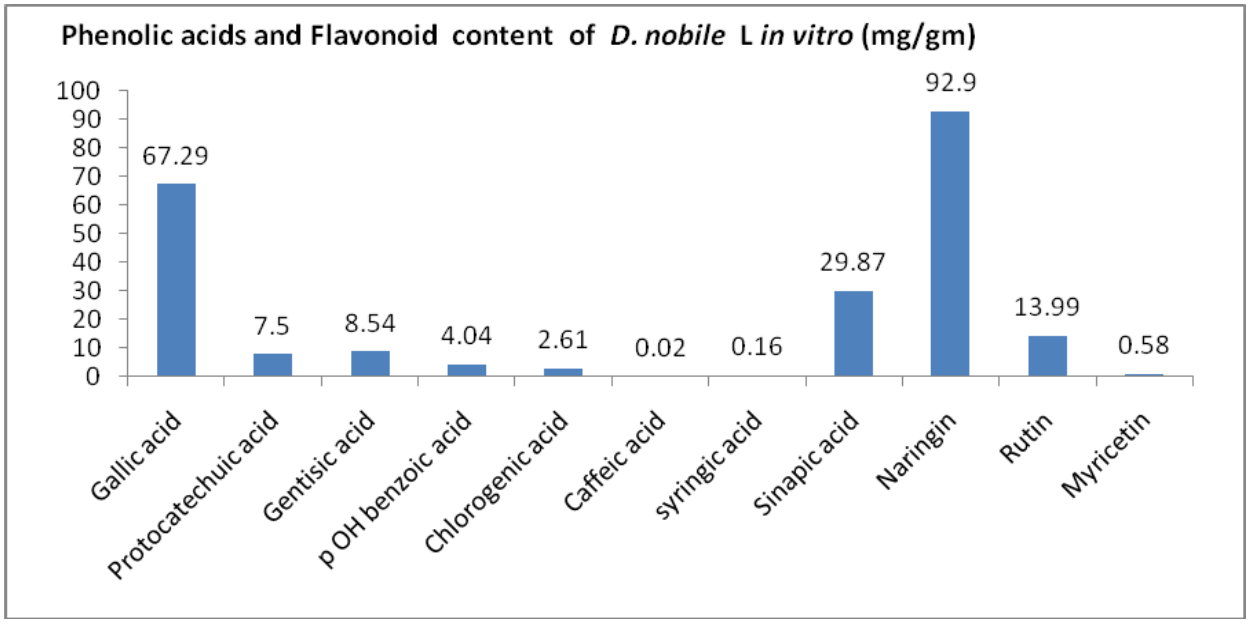


Fig. 38. Phenolic acids and flavonoid content of *D. nobile* leaf (*in vitro*)

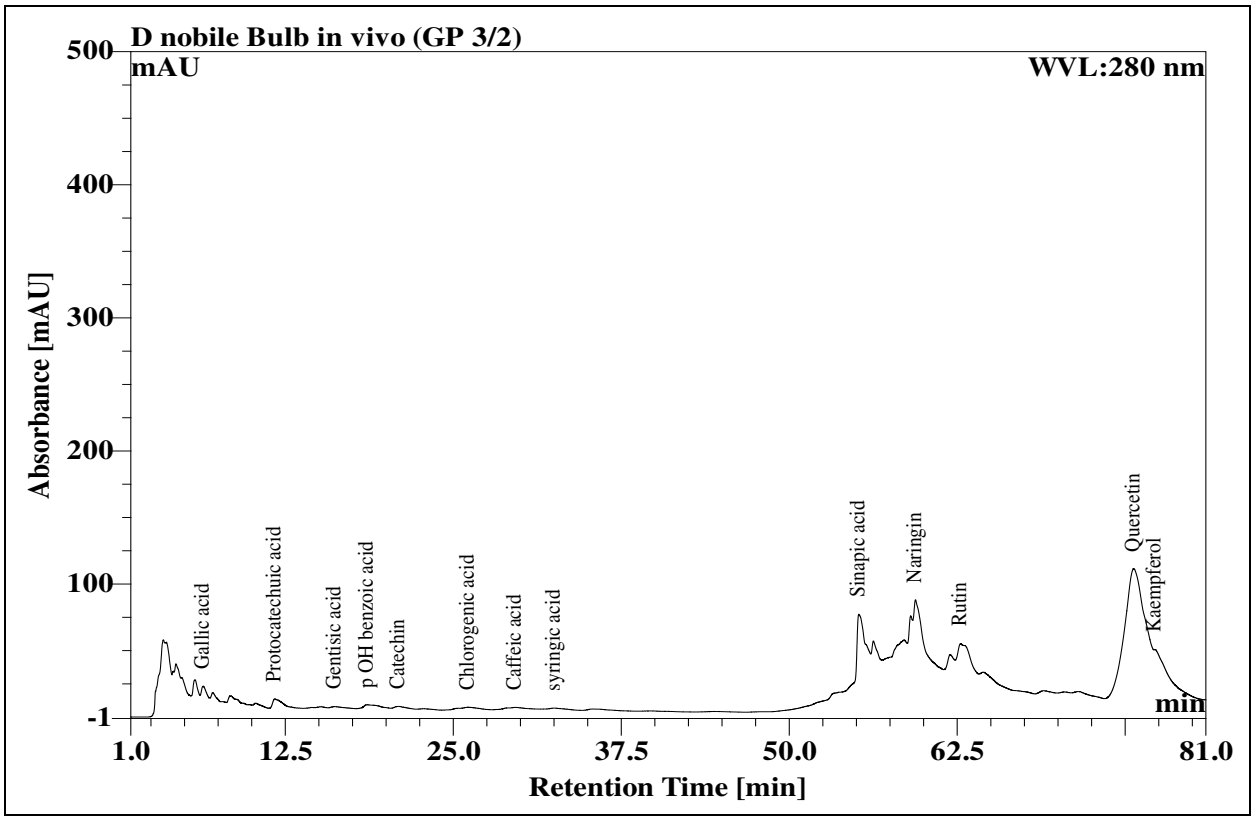


Fig. 39. HPLC chromatogram of 80% ethanolic extract of *D. nobile* pseudobulb (Mother plant)

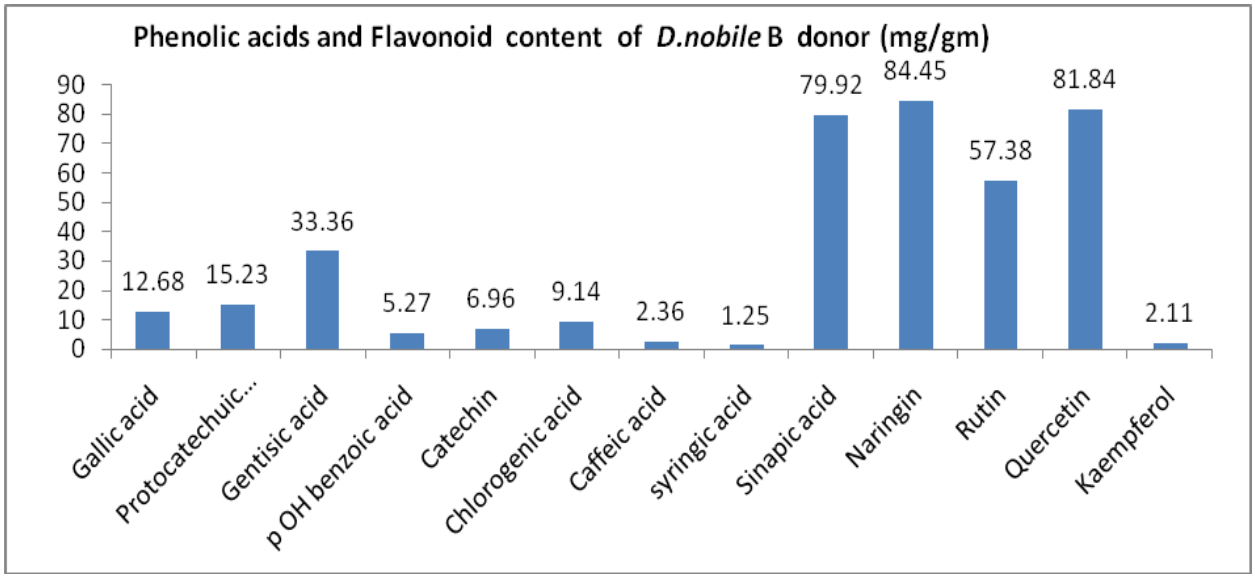


Fig. 40. Phenolic acids and flavonoid content of *D. nobile* pseudobulb (Mother plant)

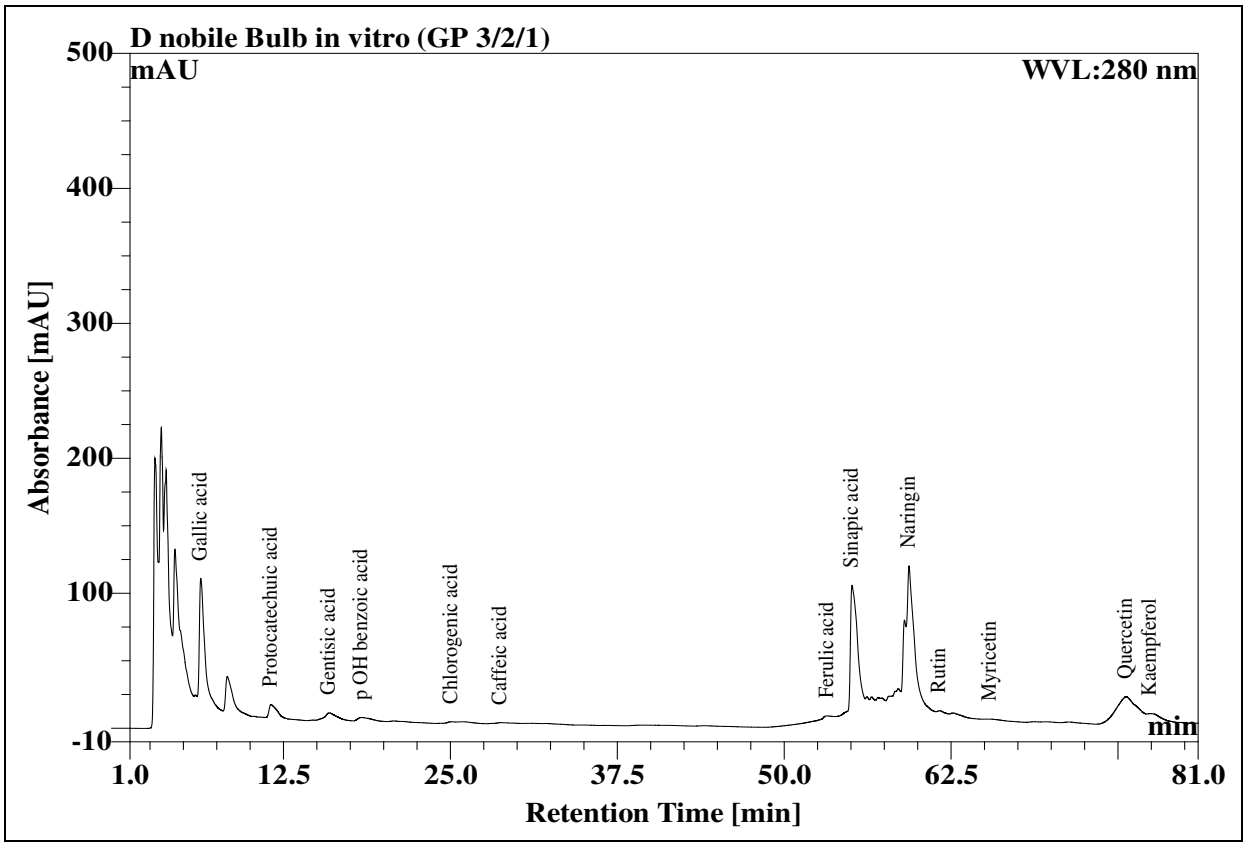


Fig. 41. HPLC chromatogram of 80% ethanolic extract of *D. nobile* pseudobulb (*in vitro*)

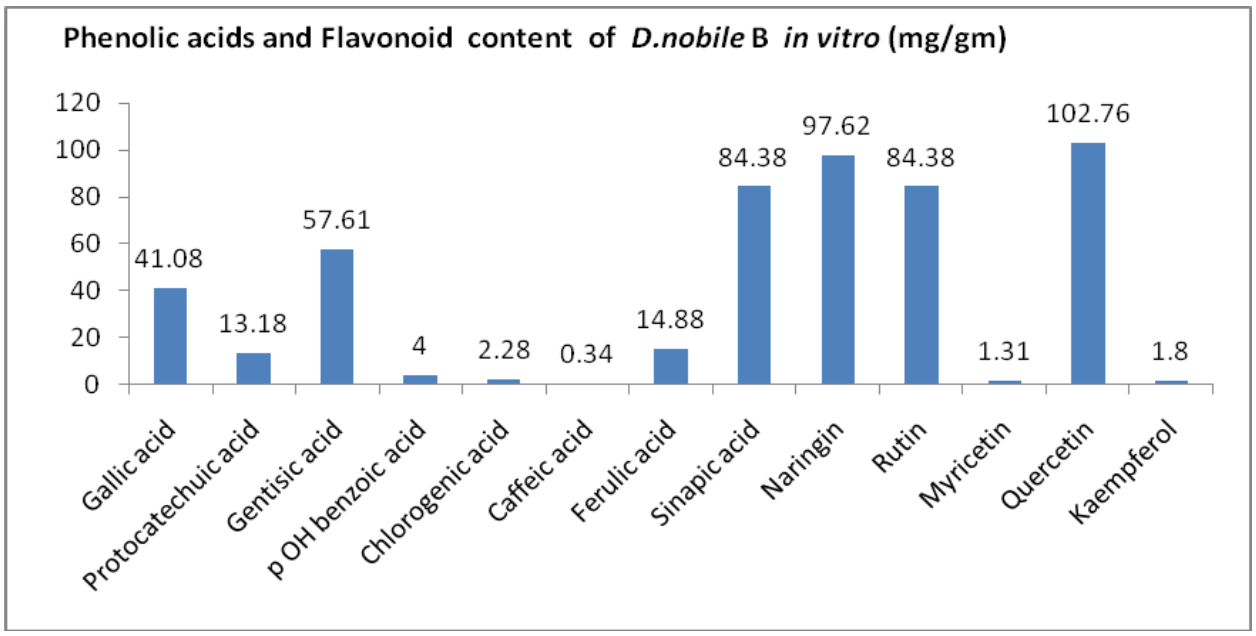


Fig. 42. Phenolic acid and flavonoid content in *D. nobile* pseudobulb (*in vitro*)

HPLC chromatogram of *B. odoratissimum*

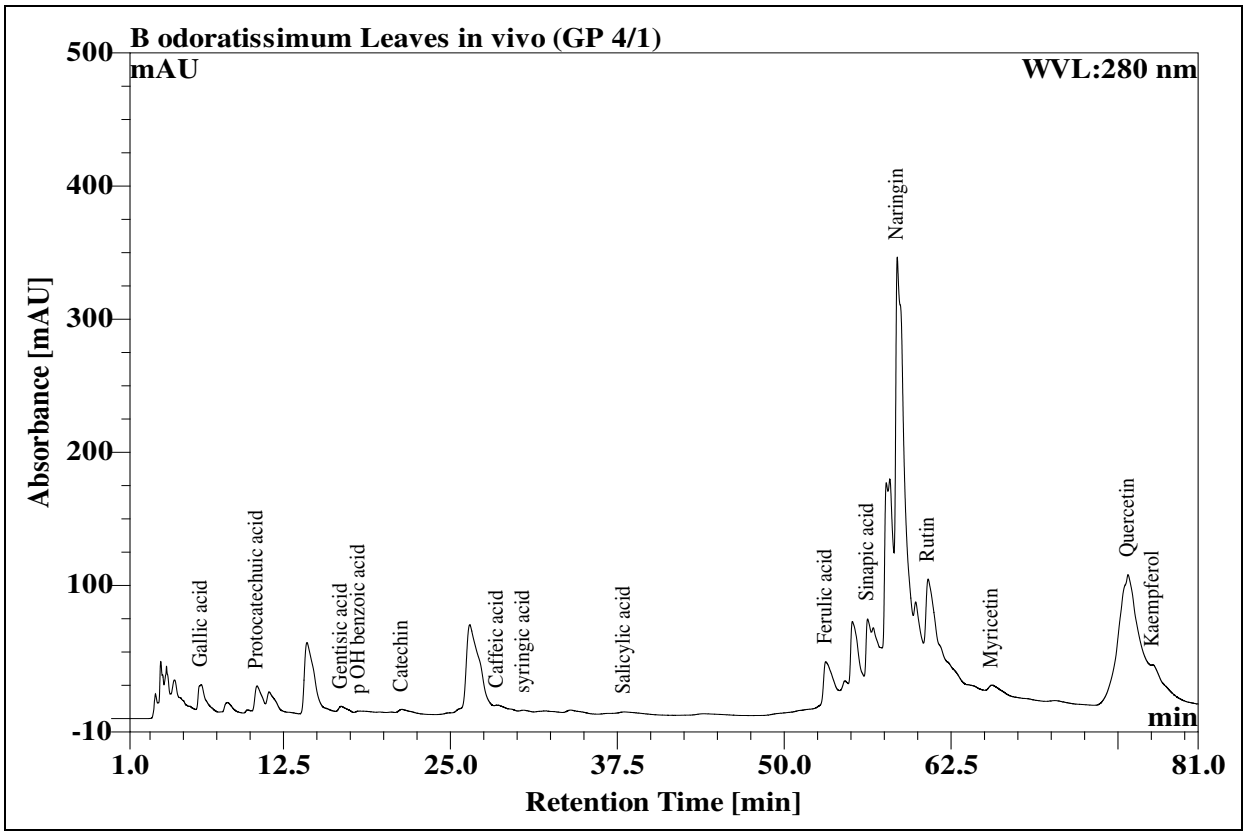


Fig. 43. HPLC chromatogram of 80% ethanolic extract of *B. odoratissimum* leaf (mother plant)

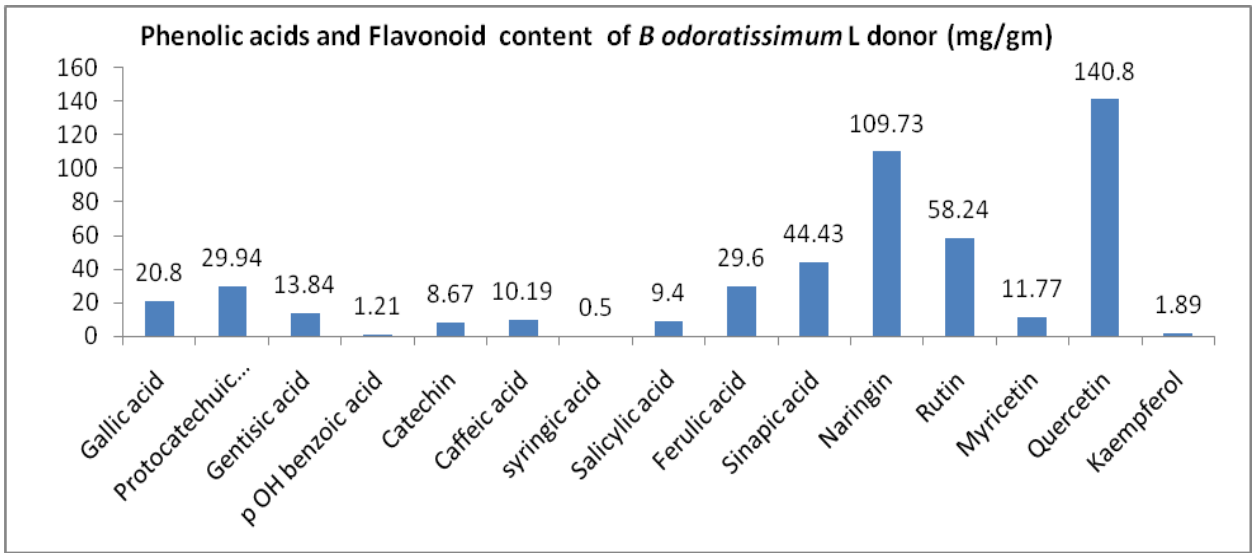


Fig. 44. Phenolic acid and flavonoids content in *B. odoratissimum* leaf (mother plant)

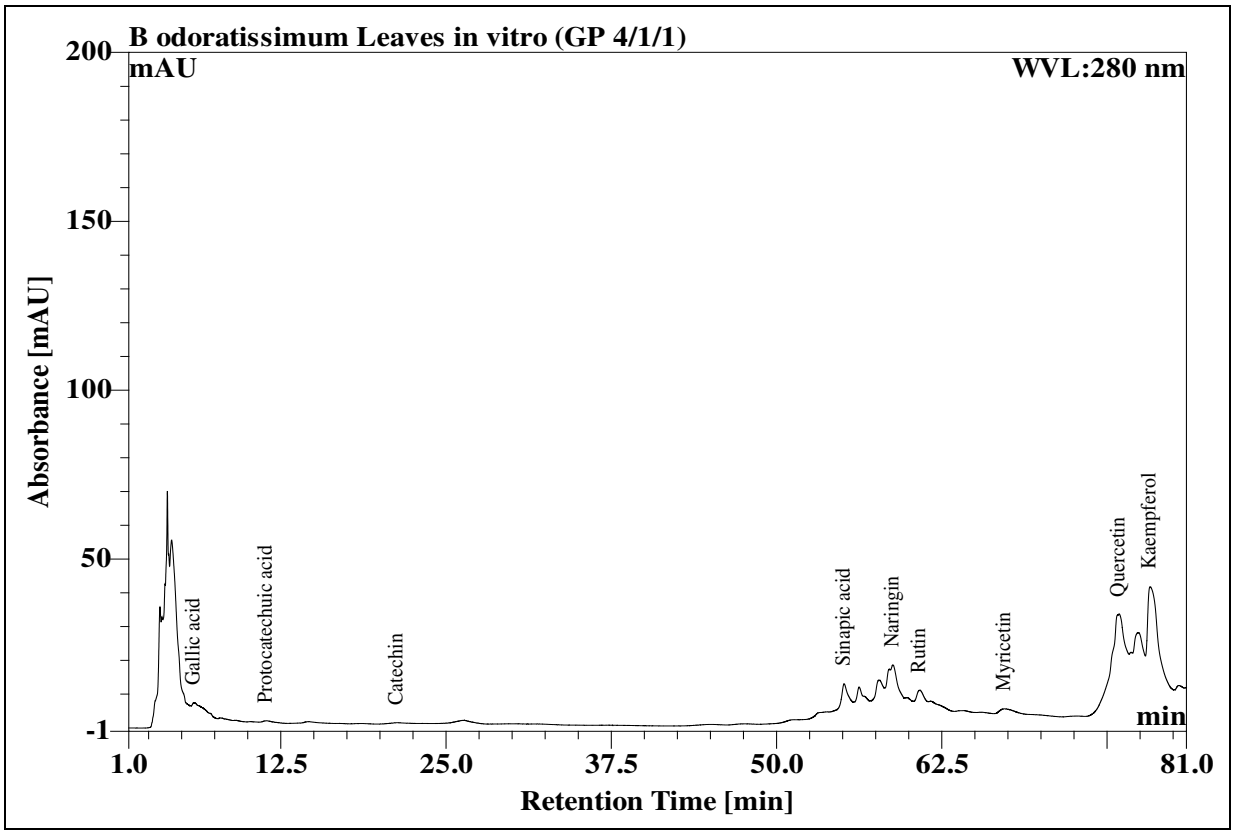


Fig. 45. HPLC chromatogram of 80% ethanolic extract of *B. odoratissimum* leaf (*in vitro*)

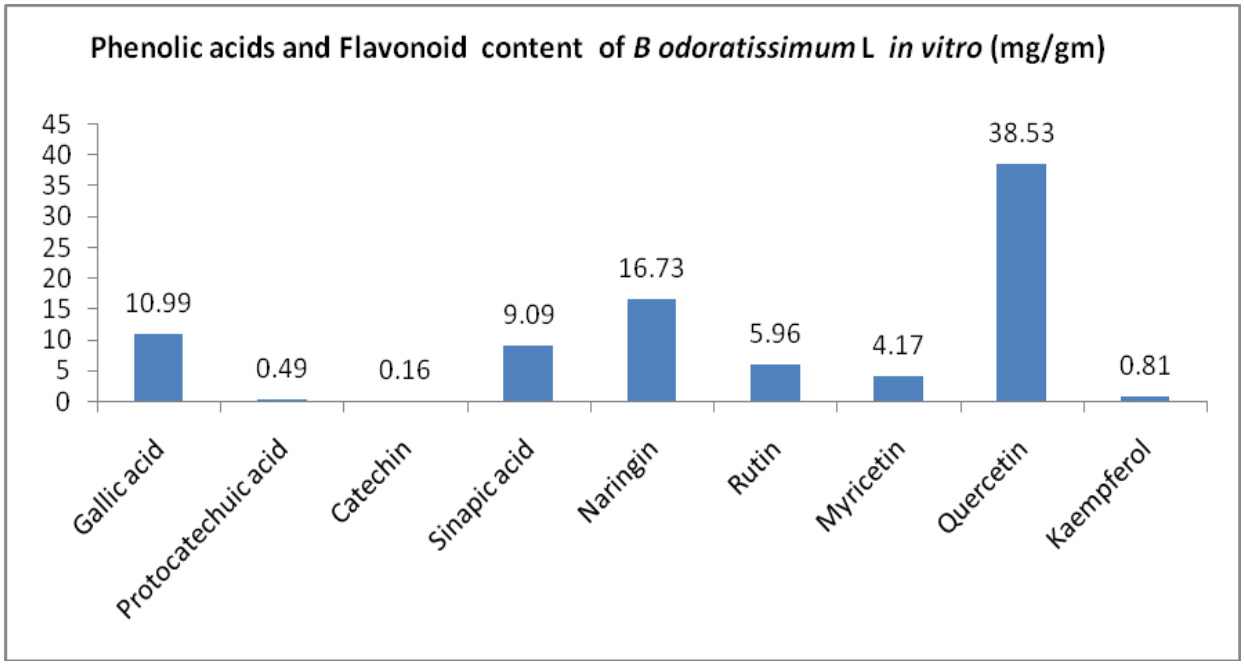


Fig. 46. Phenolic acid and flavonoid content in *B. odoratissimum* leaf (*in vitro*)

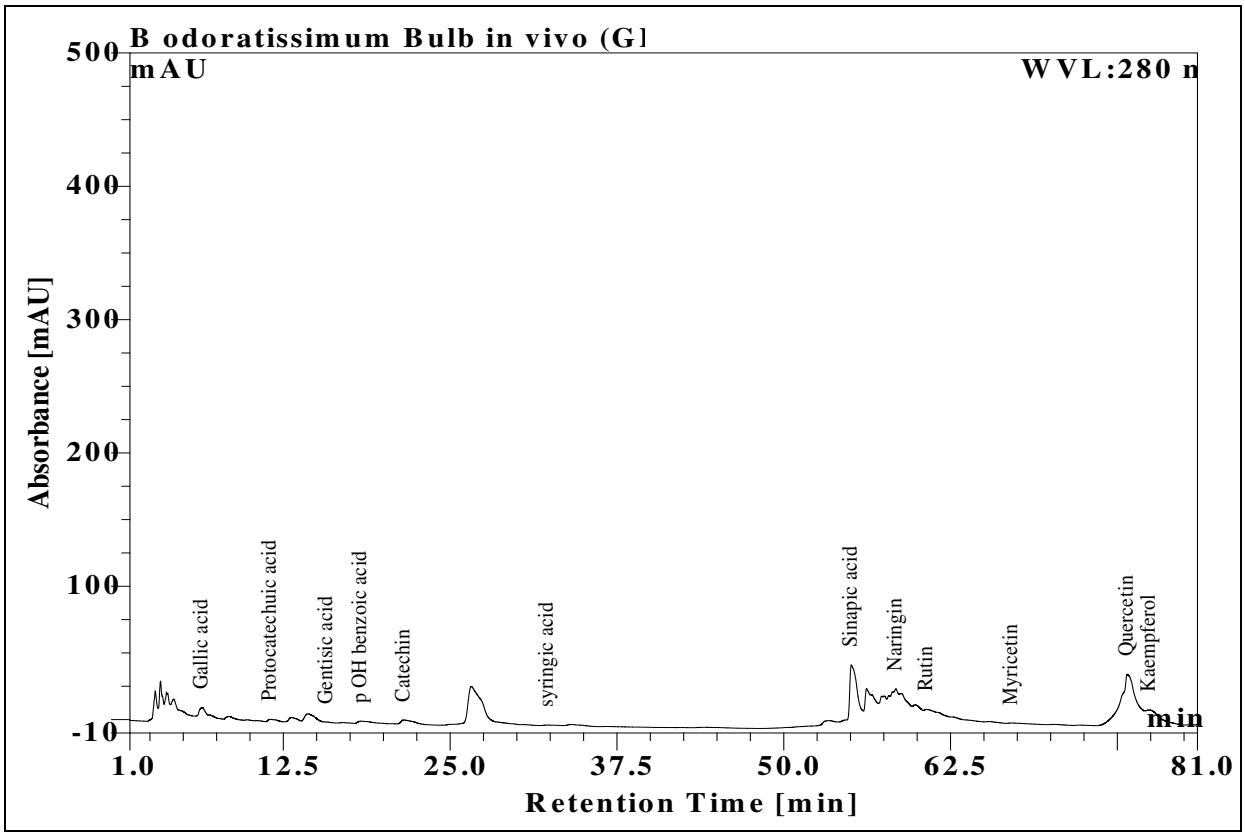


Fig. 47. HPLC chromatogram of 80% ethanolic extract of *B. odoratissimum* pseudobulb (mother plant)

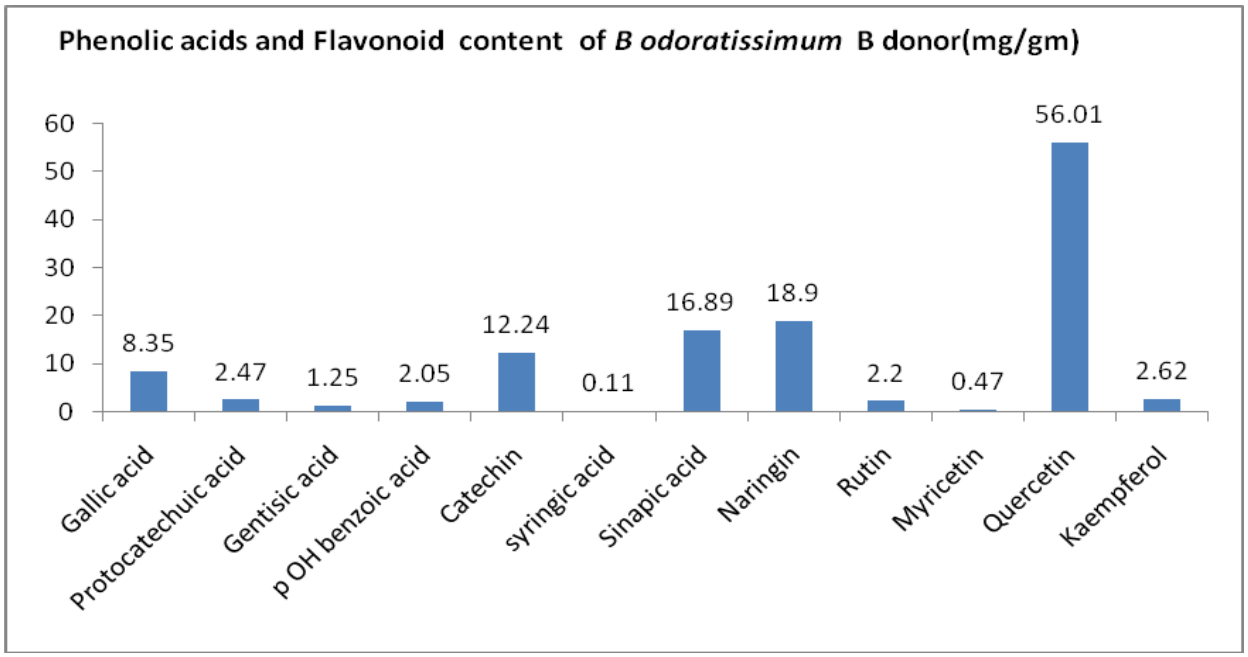


Fig. 48. Phenolic acid and flavonoid content in *B. odoratissimum* pseudobulb (mother plant)

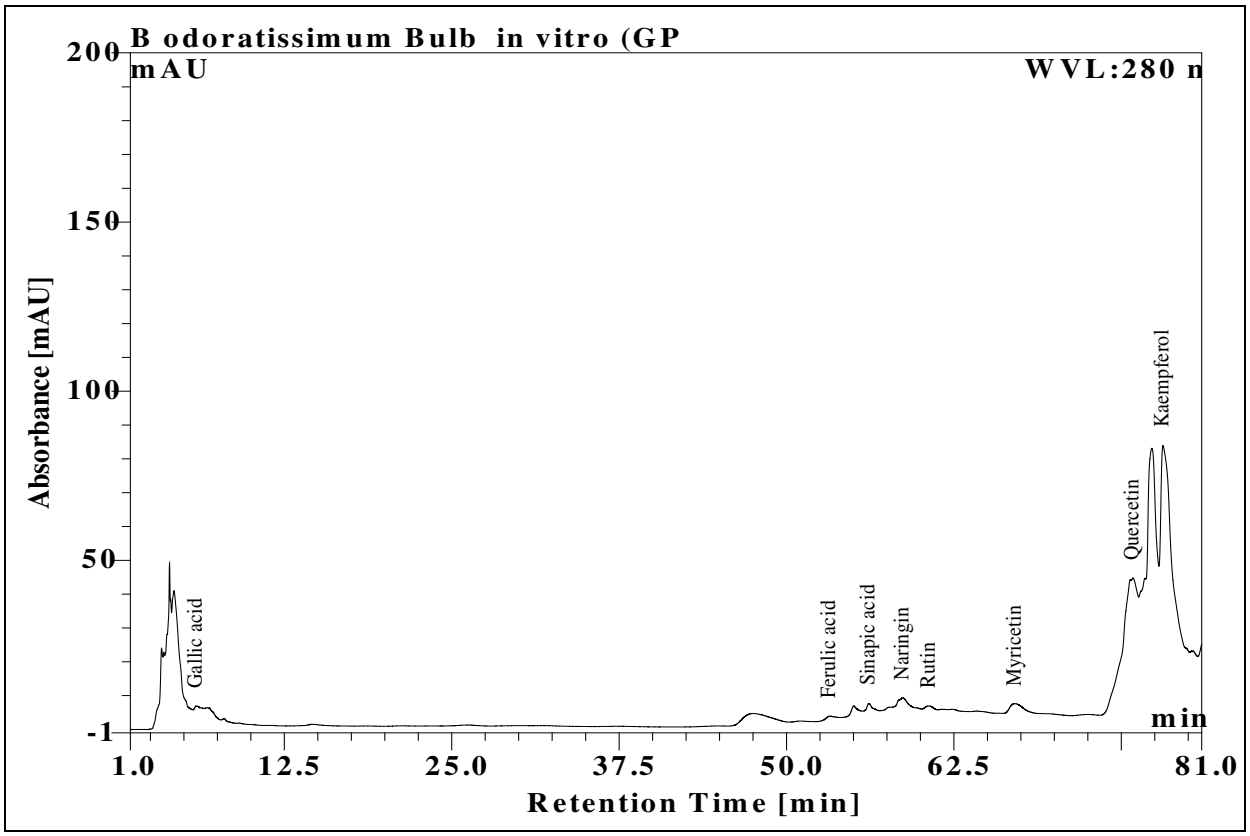


Fig. 49. HPLC chromatogram of 80% ethanolic extract of *B. odoratissimum* pseudobulb (*in vitro*)

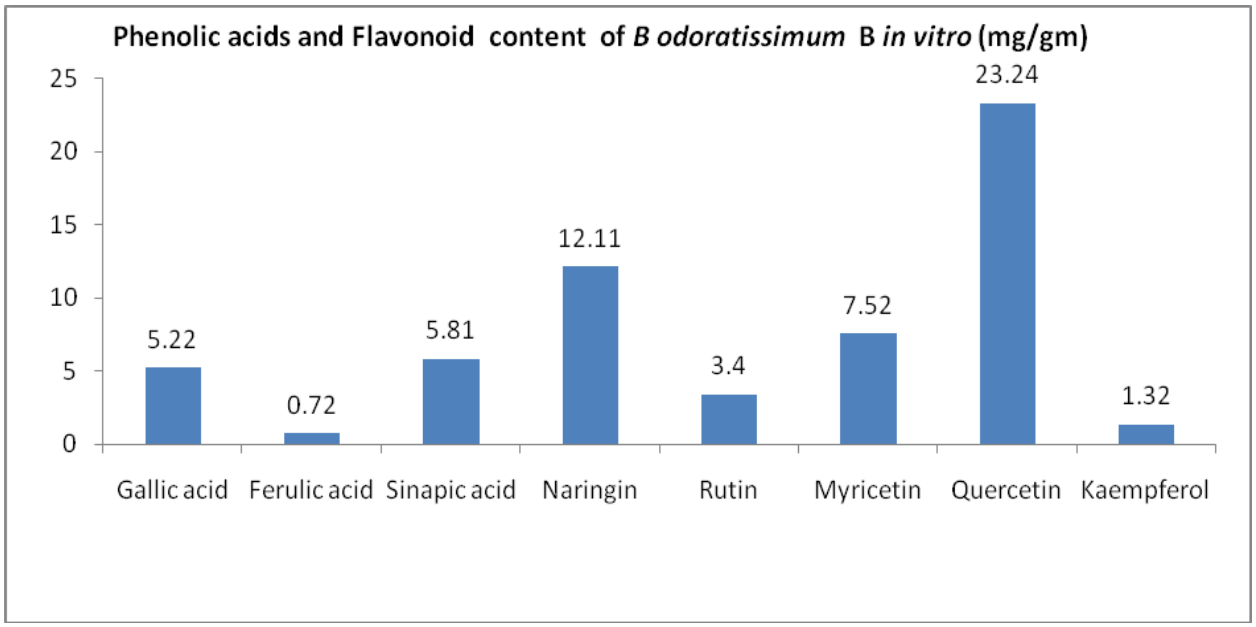


Fig. 50. Phenolic acid and flavonoid content in *B. odoratissimum* pseudobulb (*in vitro*)

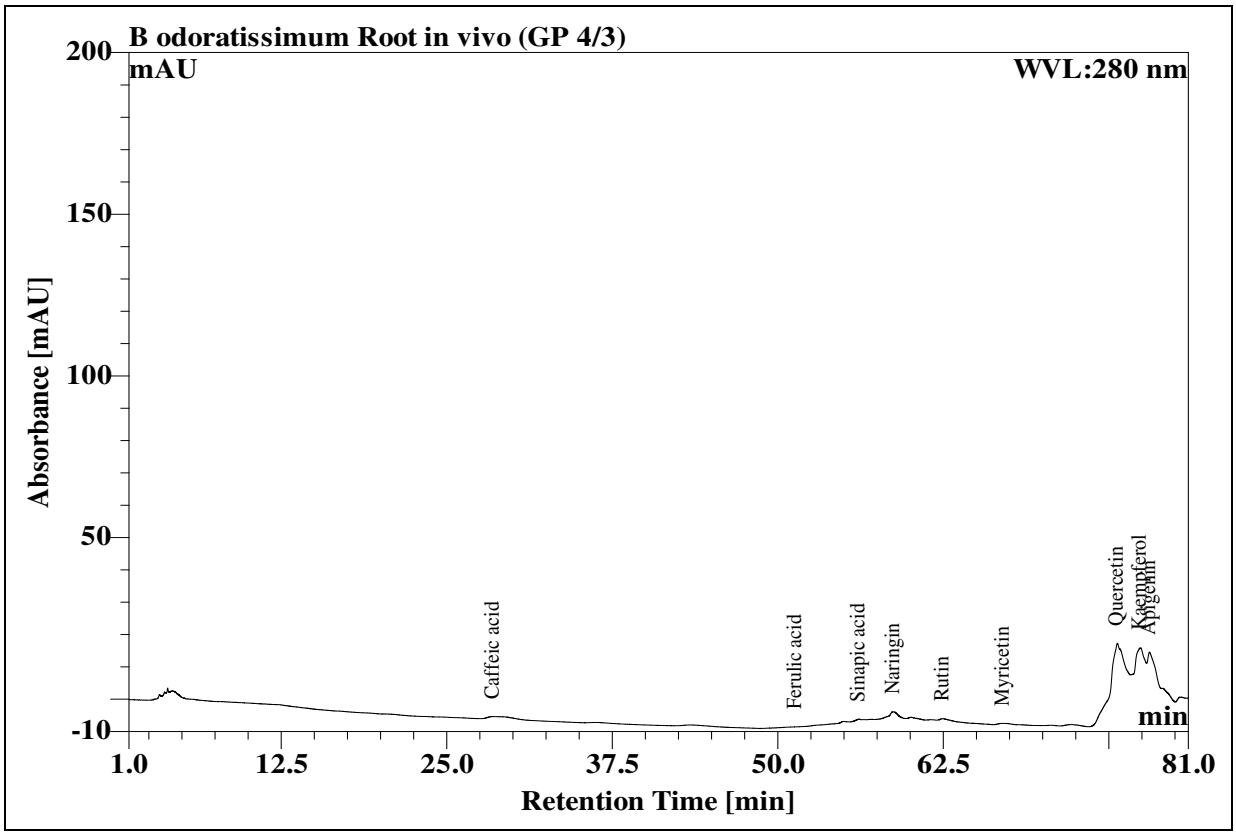


Fig. 51. HPLC chromatogram of 80 % ethanolic extract of *B. odoratissimum* root (mother plant)

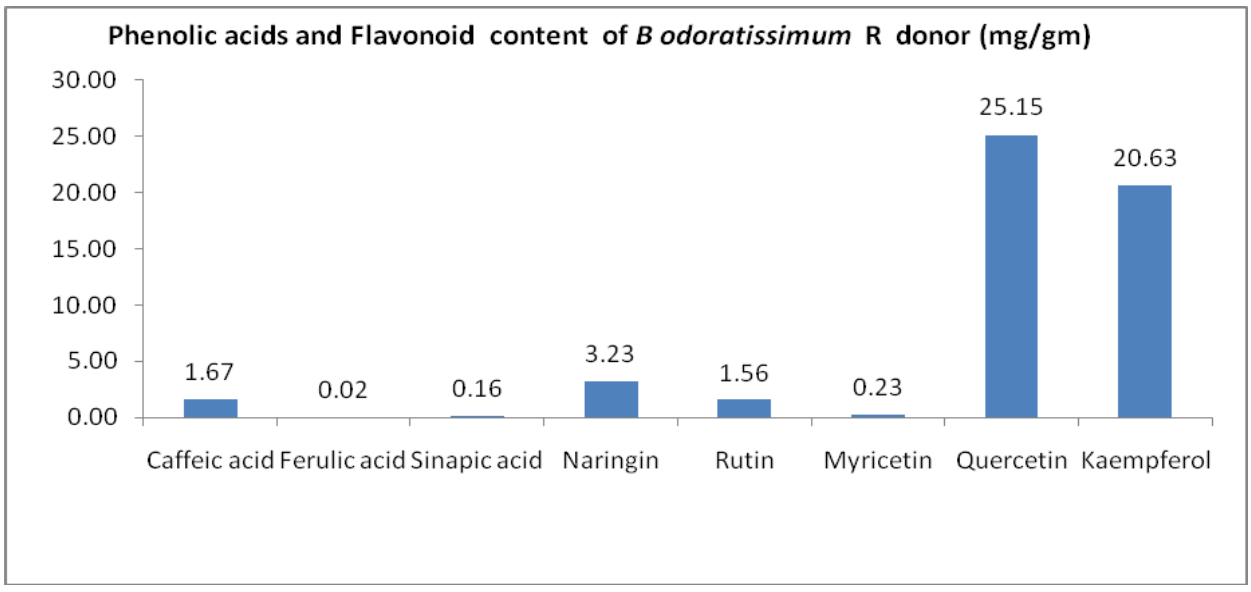


Fig. 52. Phenolic acid and flavonoid content in *B. odoratissimum* root (mother plant)

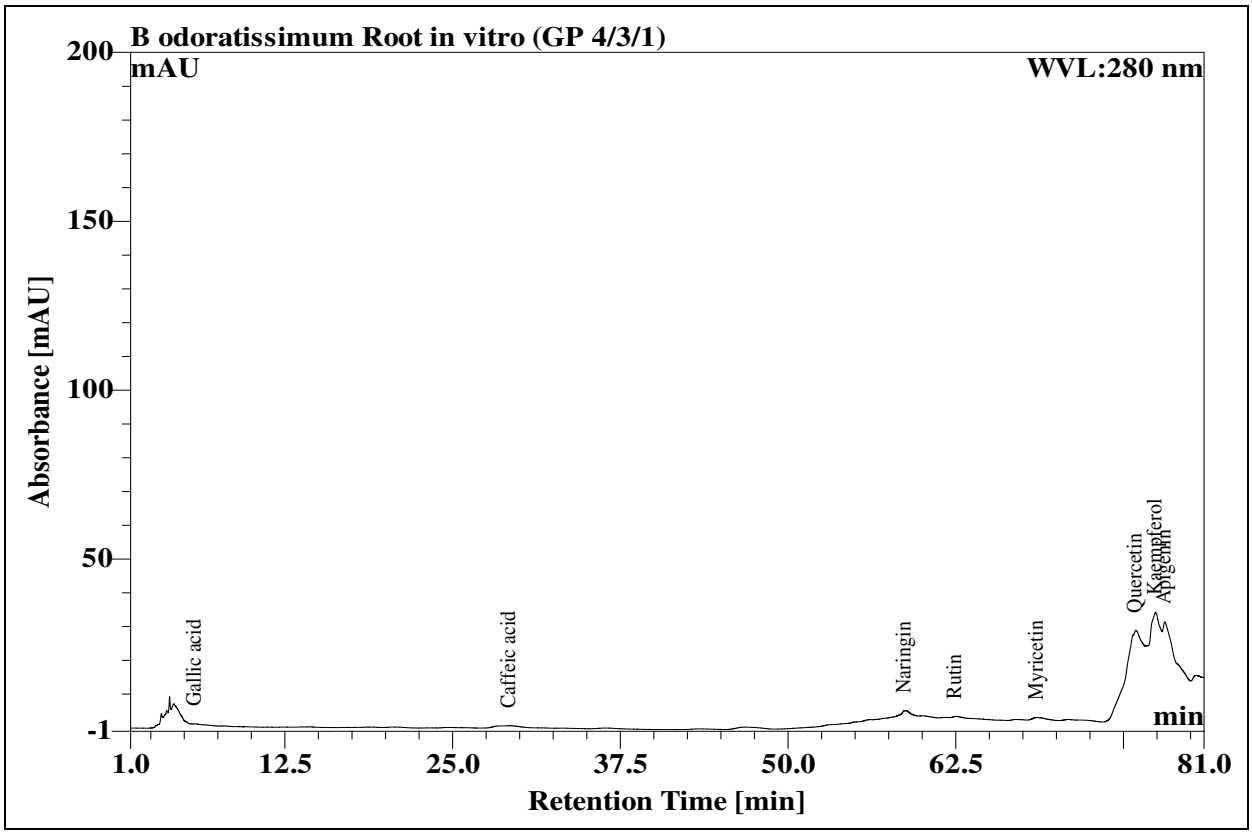


Fig. 53. HPLC chromatogram of 80% ethanolic extract of *B. odoratissimum* root (*in vitro*)

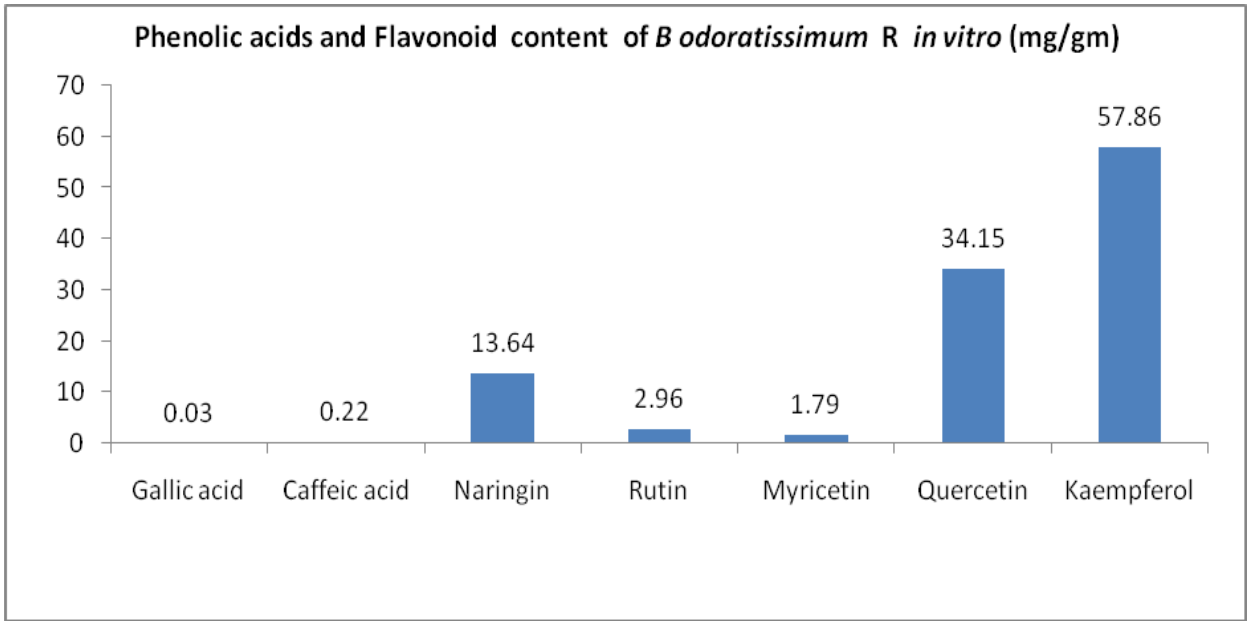


Fig. 54. Phenolic acid and flavonoid content of *B. odoratissimum* root (*in vitro*)

3. HPLC chromatogram of *C. obcordata*

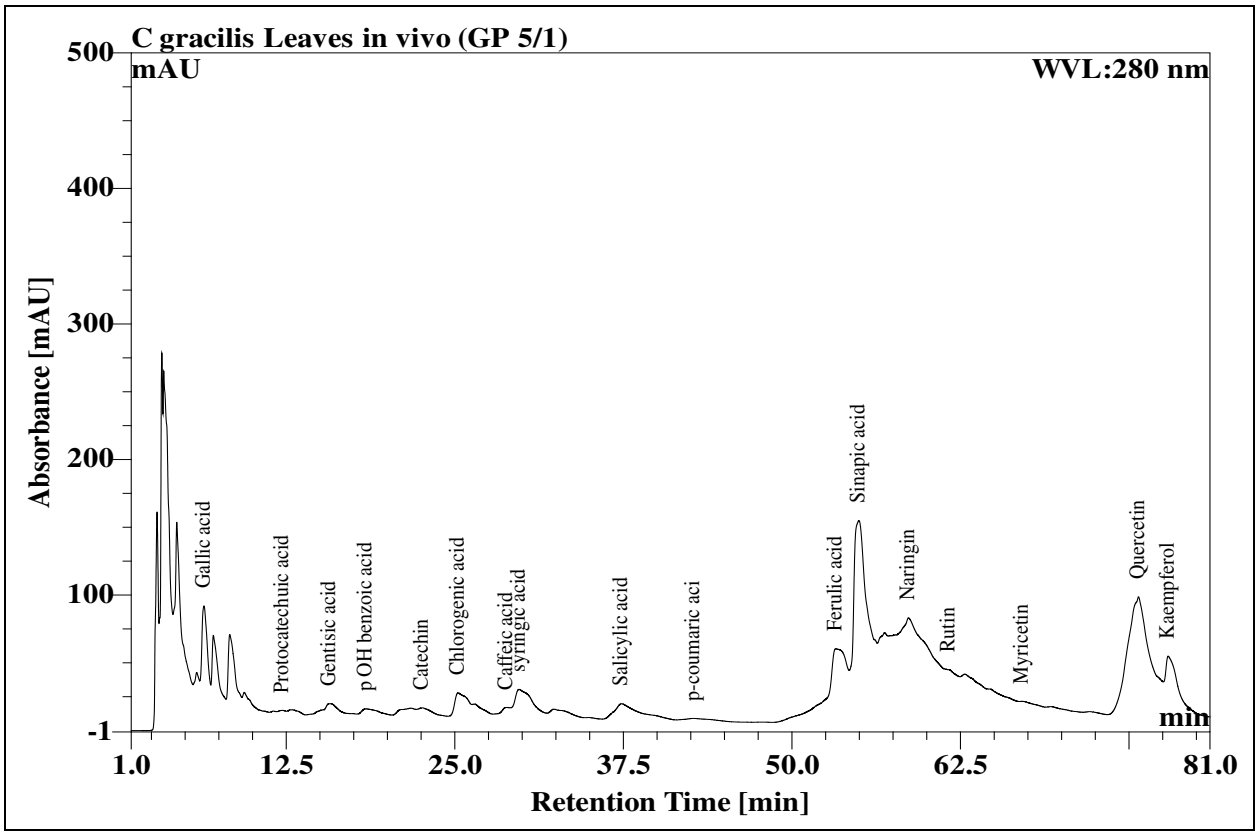


Fig. 55. HPLC chromatogram of 80% ethanolic extract of *C. obcordata* leaf (mother plant)

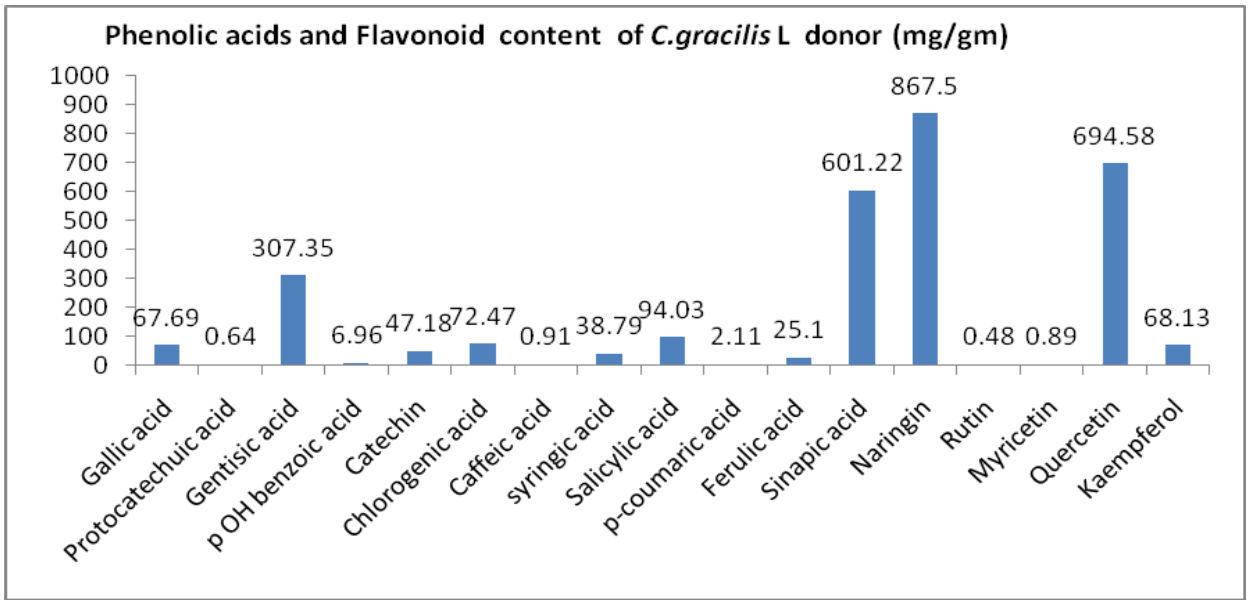


Fig. 56. Phenolic acids and flavonoid content in *C. obcordata* leaf (mother plant)

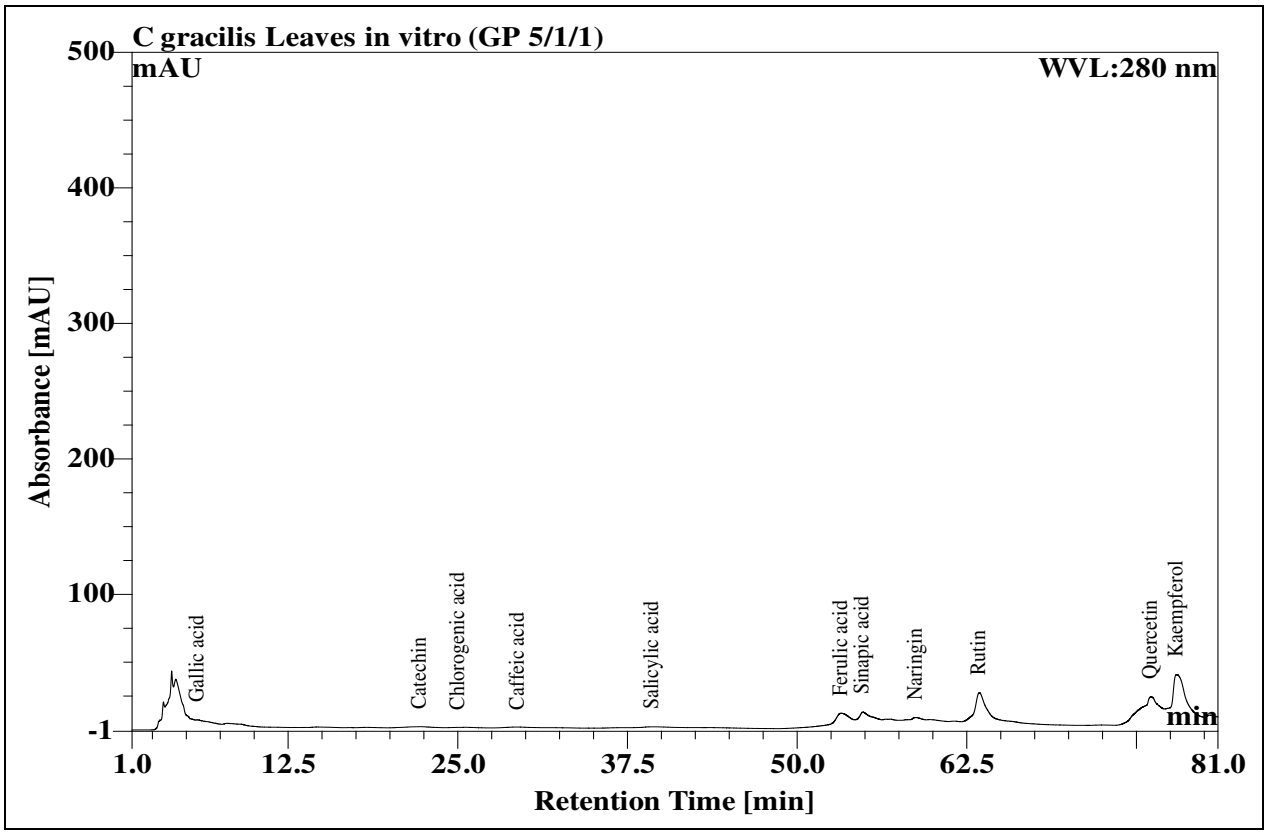


Fig. 57. HPLC chromatogram of 80% ethanolic extract of *C. obcordata* leaf (*in vitro*)

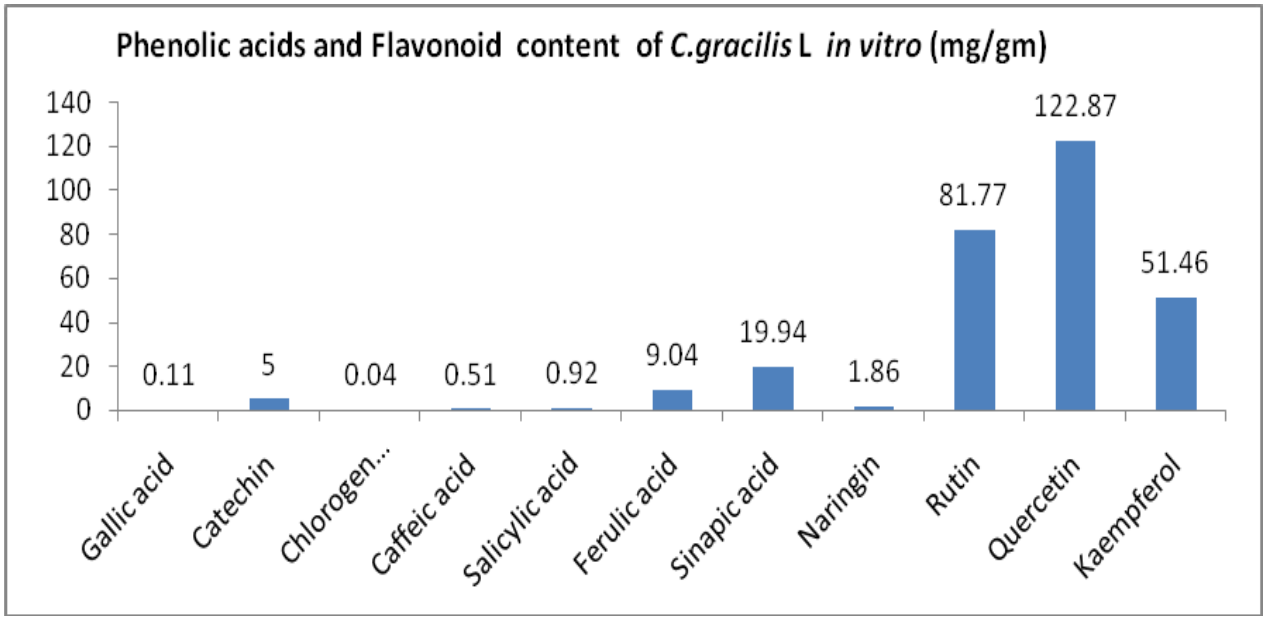


Fig. 58. Phenolic acids and flavonoid content in *C. obcordata* leaf (*in vitro*)

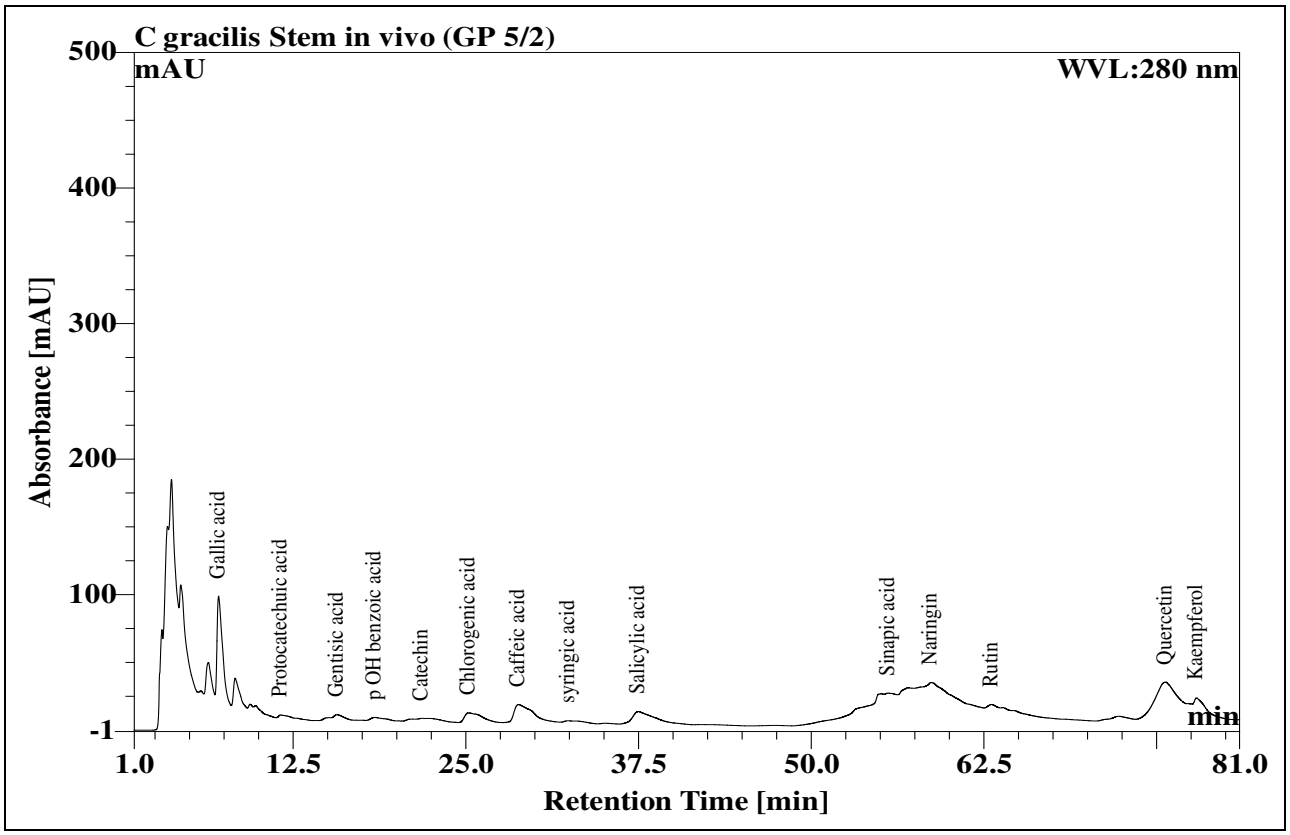


Fig. 59. HPLC chromatogram of 80% ethanolic extract of *C. obcordata* stem (mother plant)

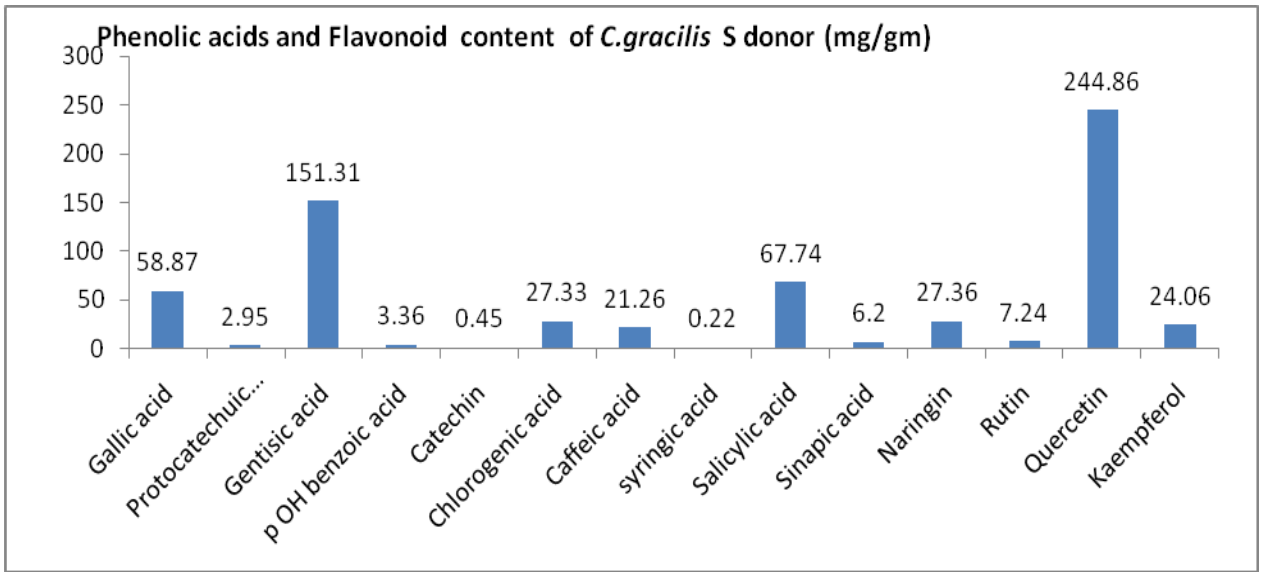


Fig. 60. Phenolic acids and flavonoid content in *C. obcordata* stem (mother plant)

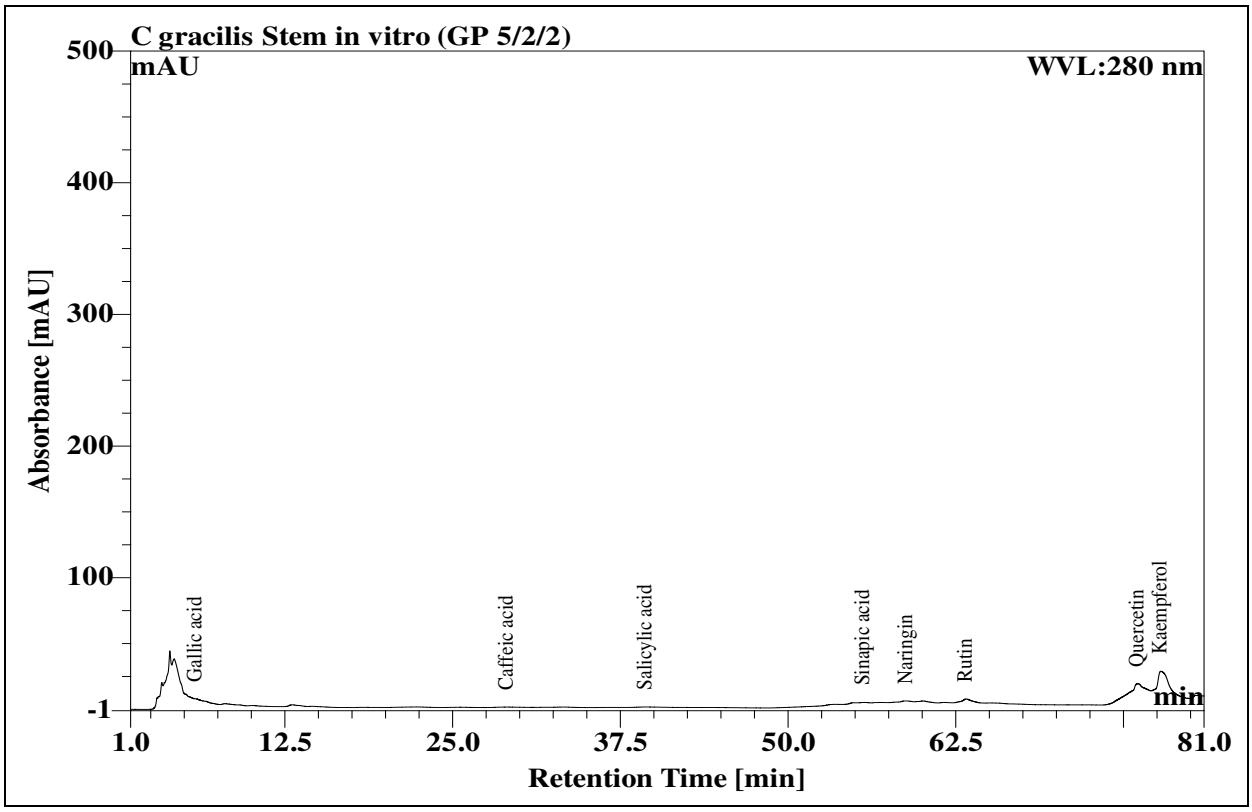


Fig. 61. HPLC chromatogram of 80% ethanolic extract of *C. obcordata* stem (*in vitro*)

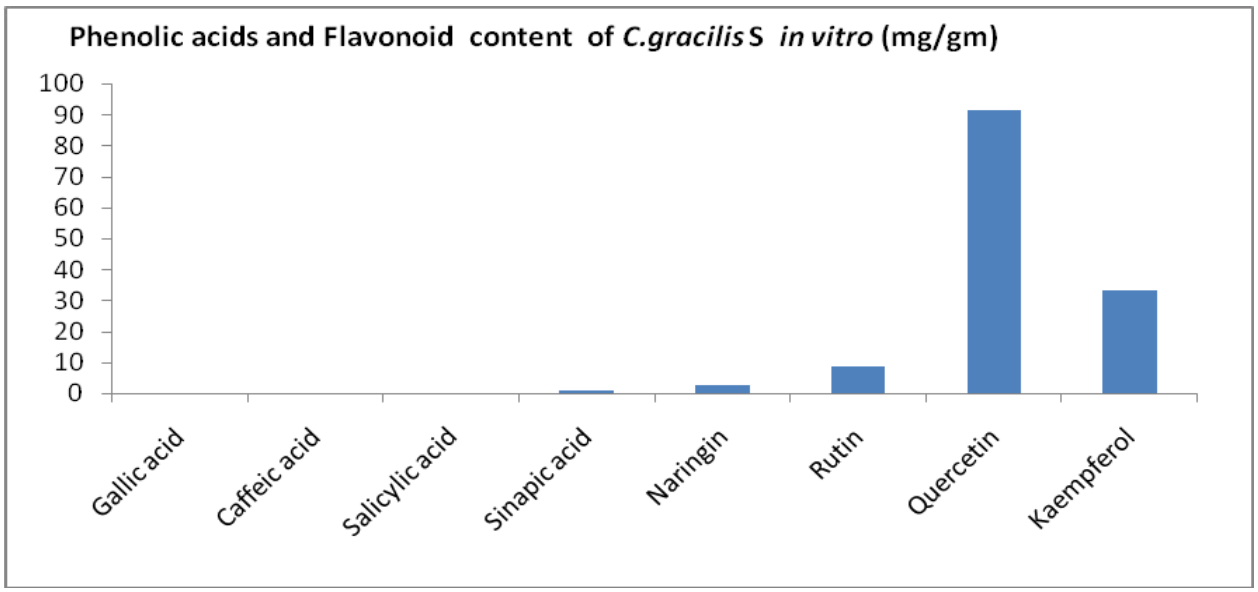


Fig. 62. Phenolic acids and flavonoid content in *C. obcordata* stem (*in vitro*)

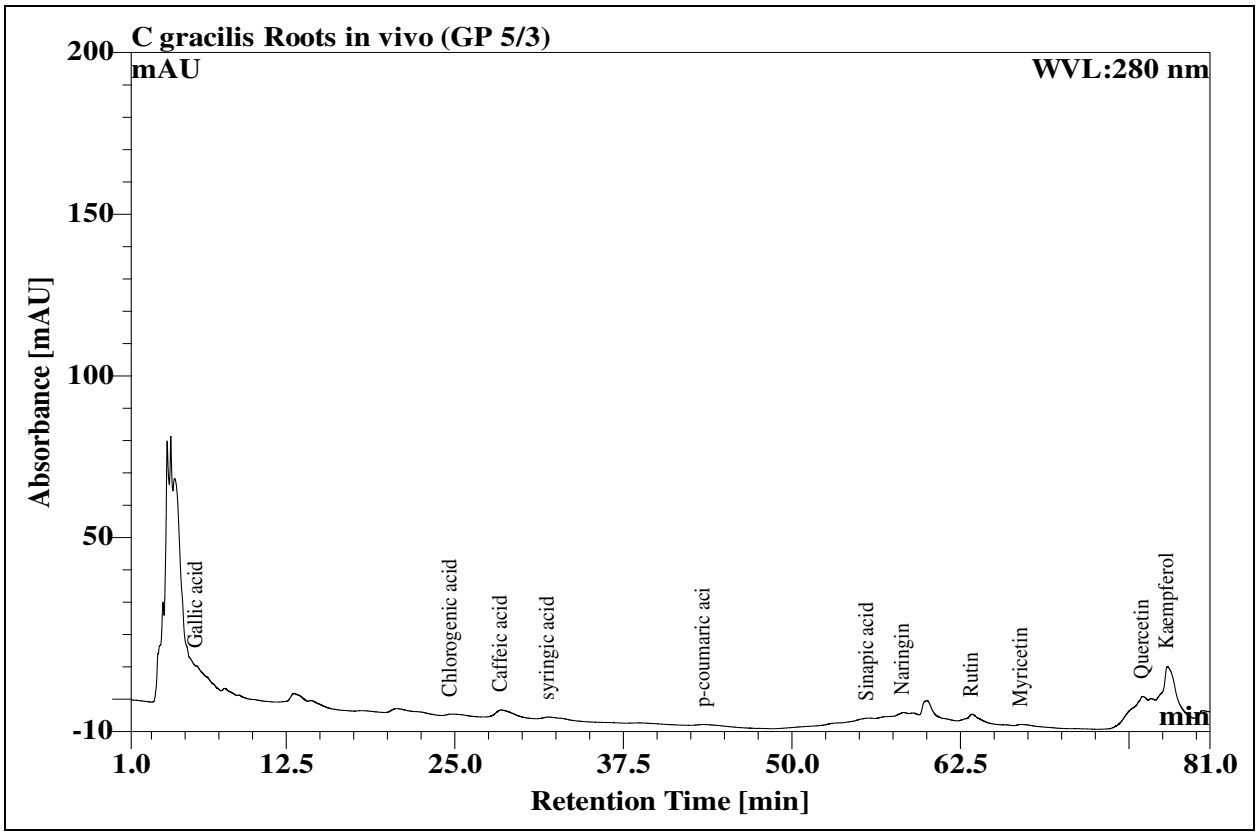


Fig. 63. HPLC chromatogram of 80% ethanolic extract of *C. obcordata* root (mother plant)

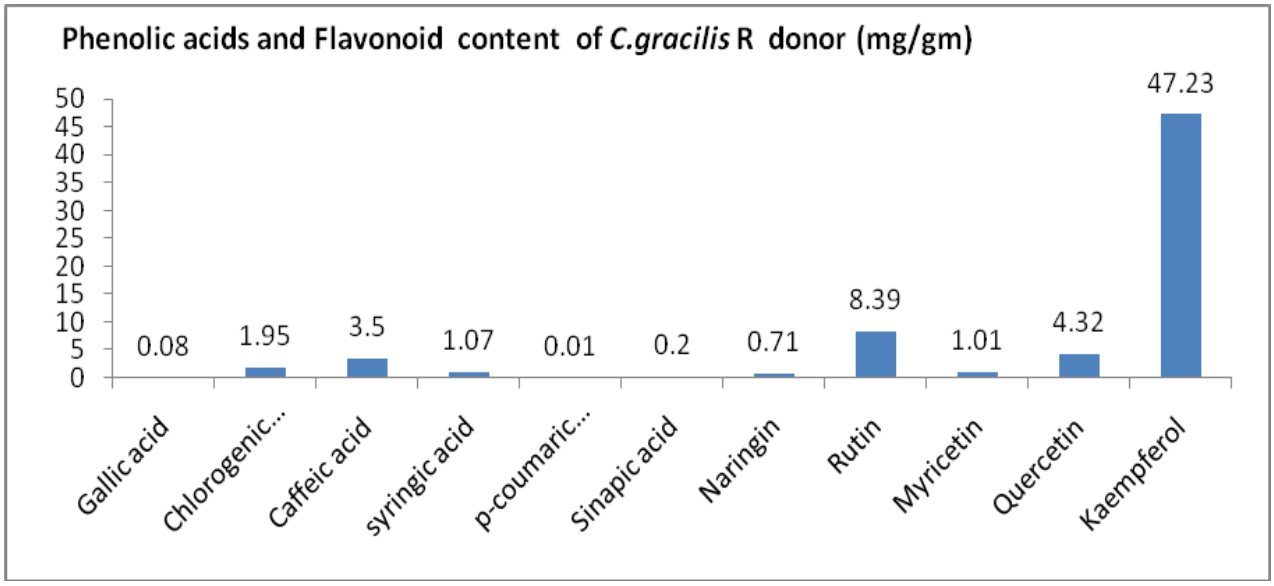


Fig. 64. Phenolic acids and flavonoid content in *C. obcordata* root (mother plant)

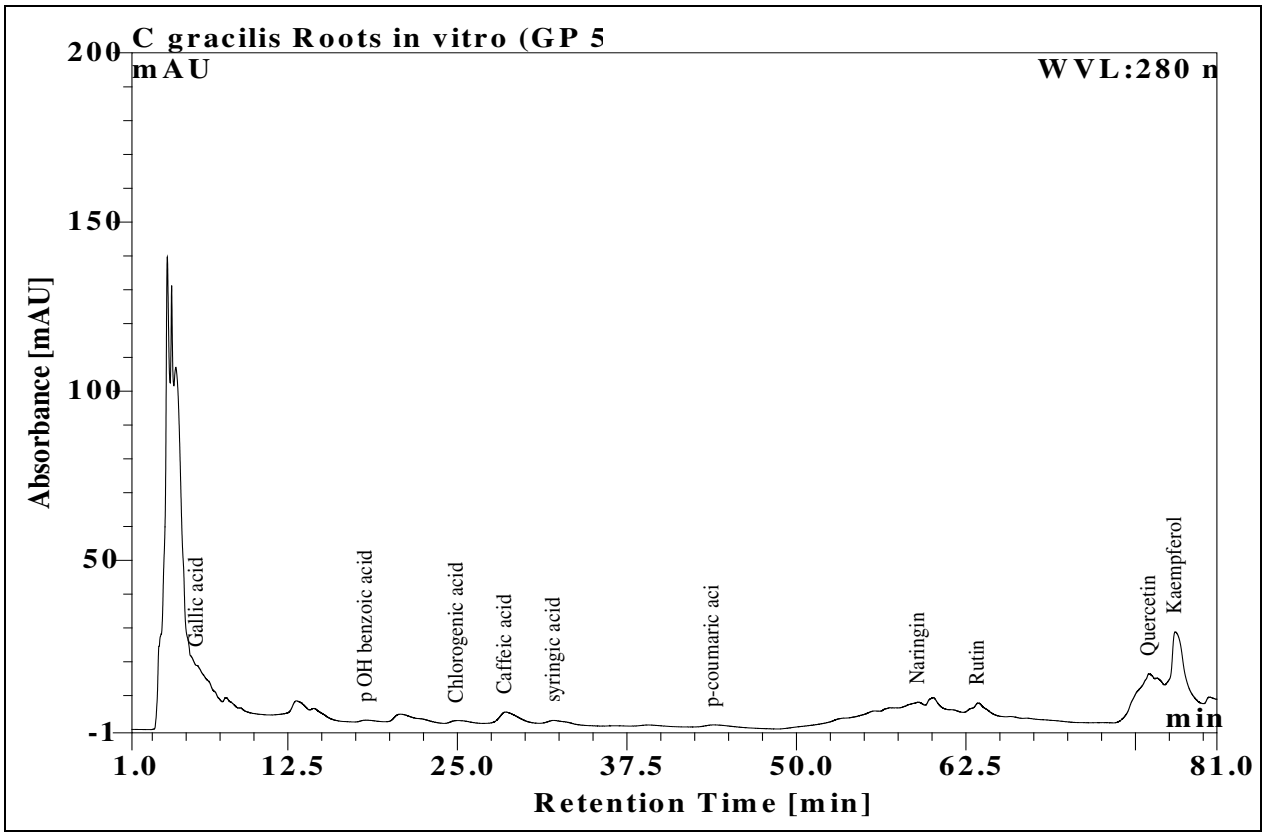


Fig. 65. HPLC chromatogram of 80% ethanolic extract of *C. obcordata* root (*in vitro*)

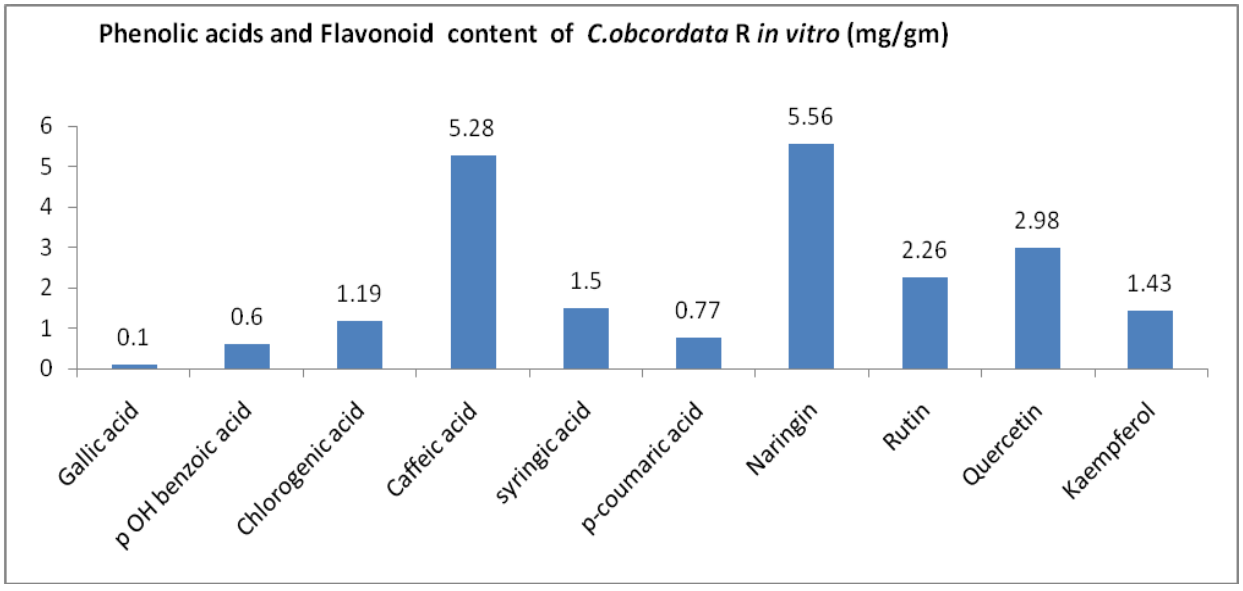


Fig. 66. Phenolic acids and flavonoid content in *C. obcordata* root (*in vitro*)

HPLC chromatogram of *M. acuminata*

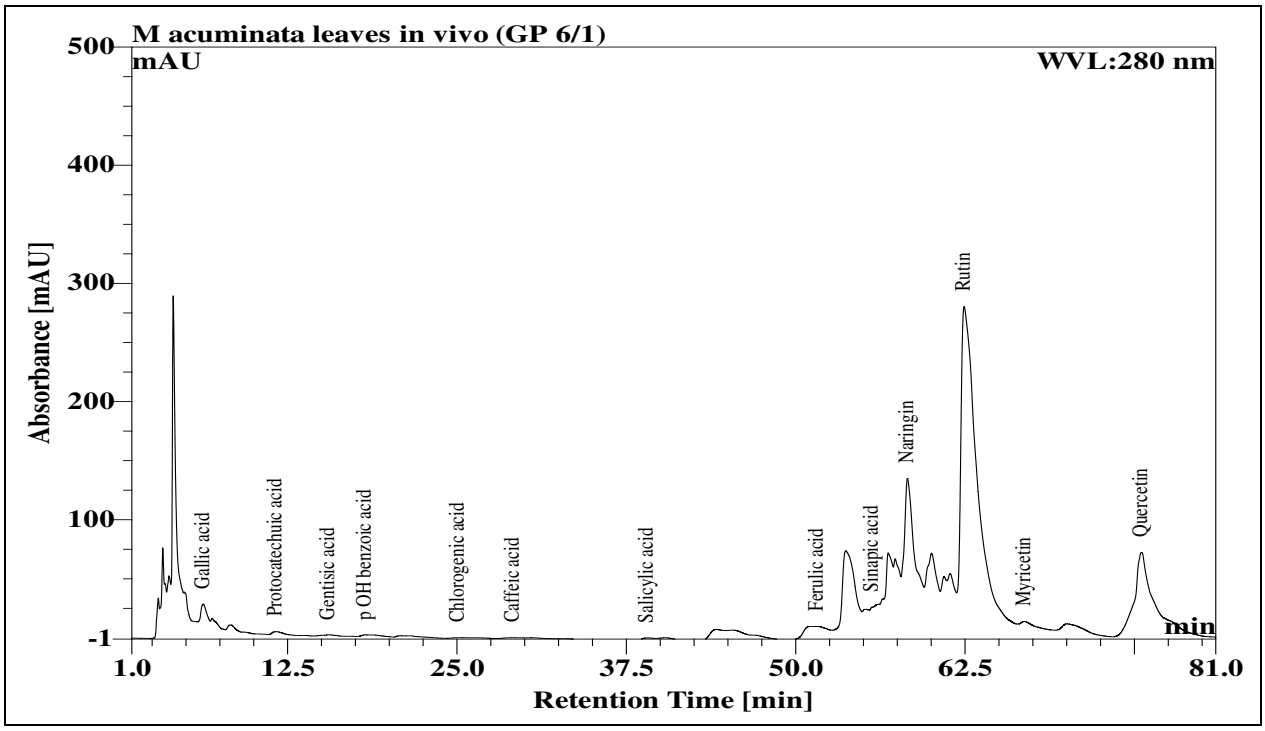


Fig. 67. HPLC chromatogram of 80% ethanolic extract of *M. acuminata* L (mother plant)

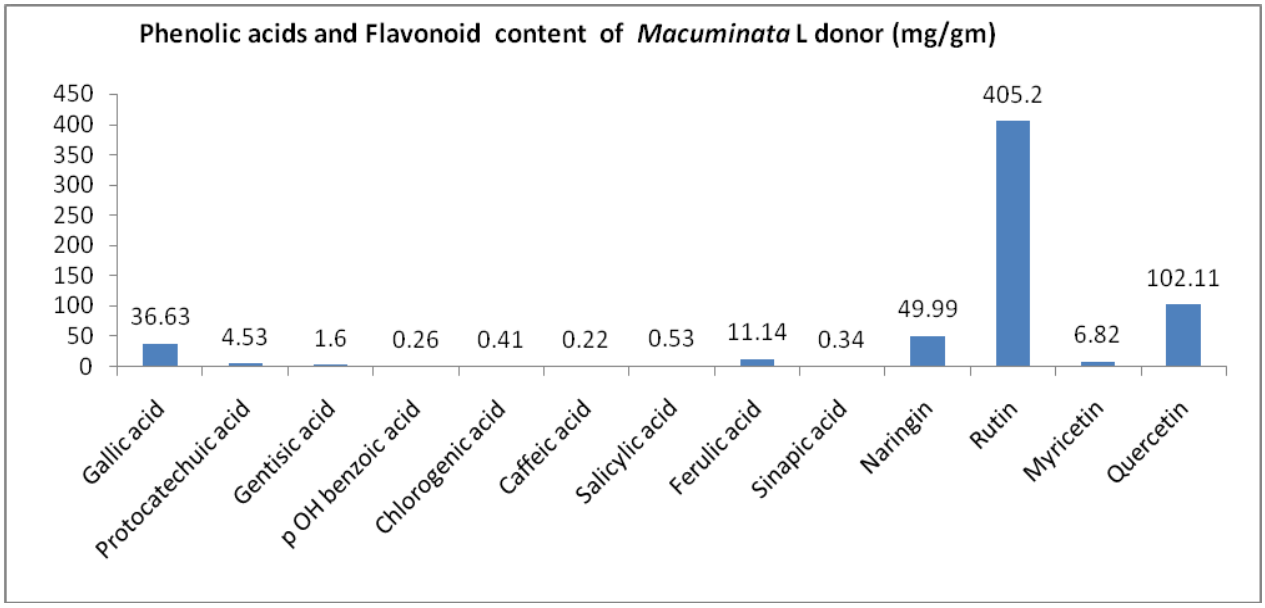


Fig. 68. Phenolic acids and flavonoid content in *M. acuminata* L (mother plant)

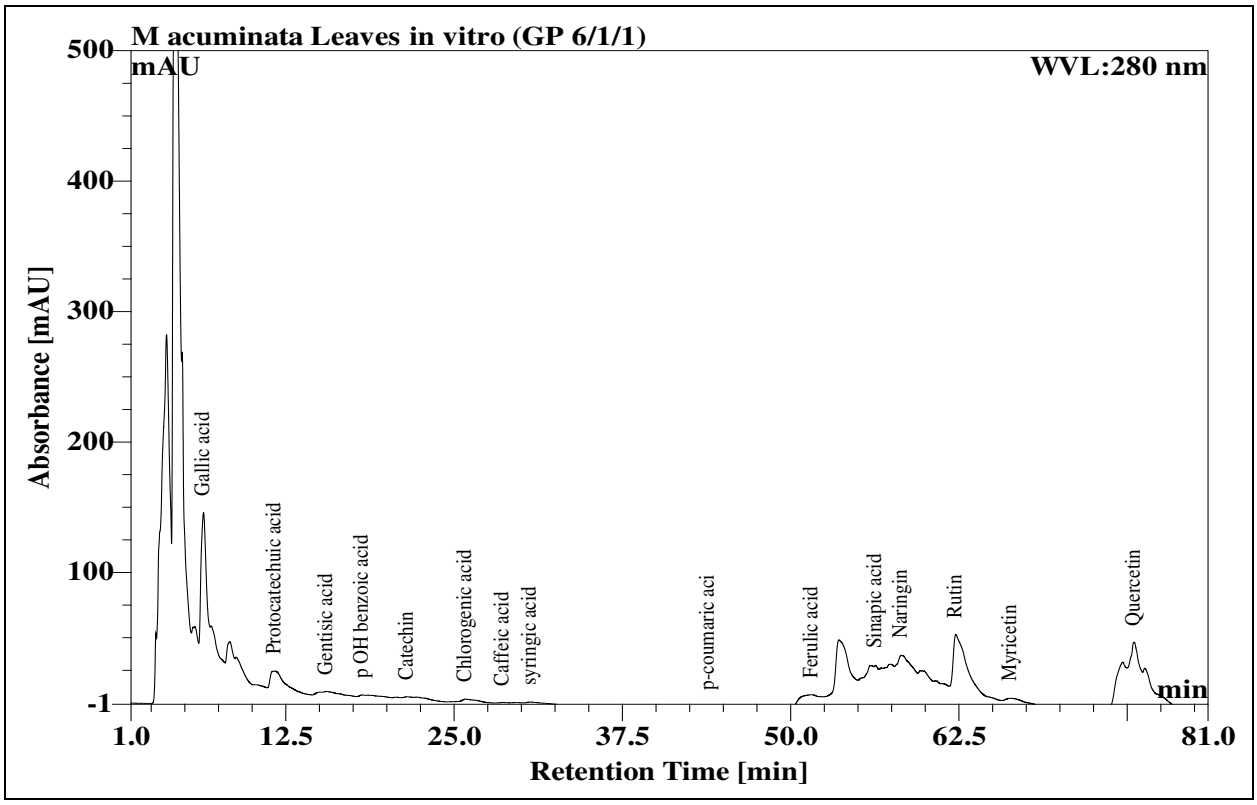


Fig. 69. HPLC chromatogram of 80% ethanolic extract of *M. acuminata* L (*in vitro*)

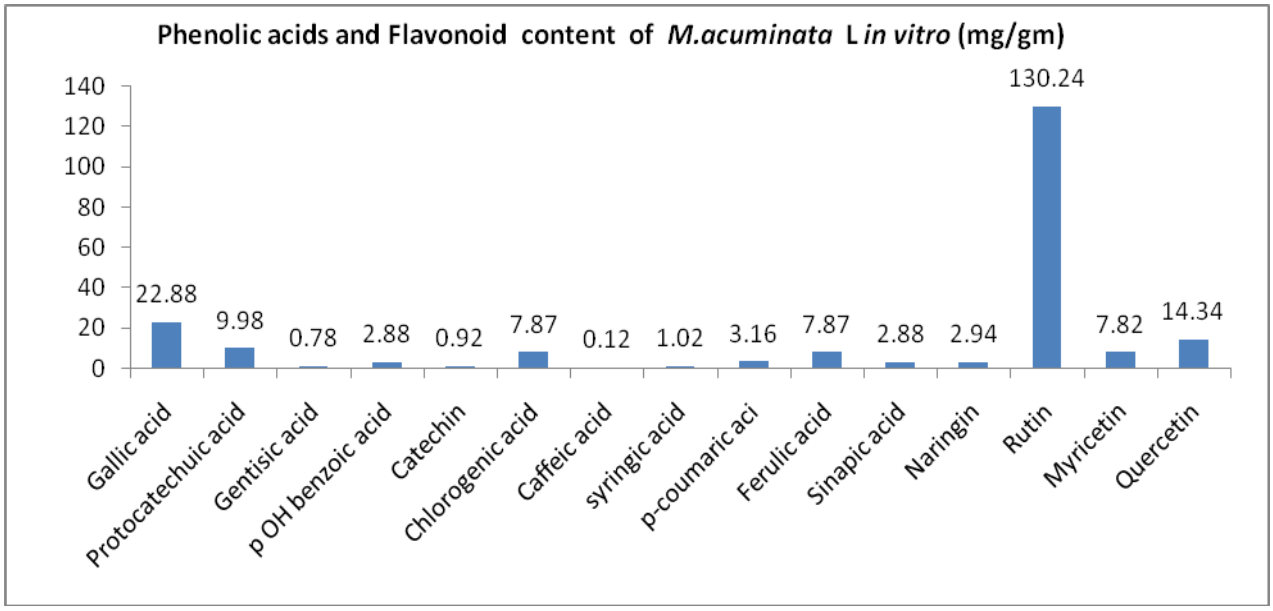


Fig. 70. Phenolic acids and flavonoid content in *M. acuminata* L (*in vitro*)

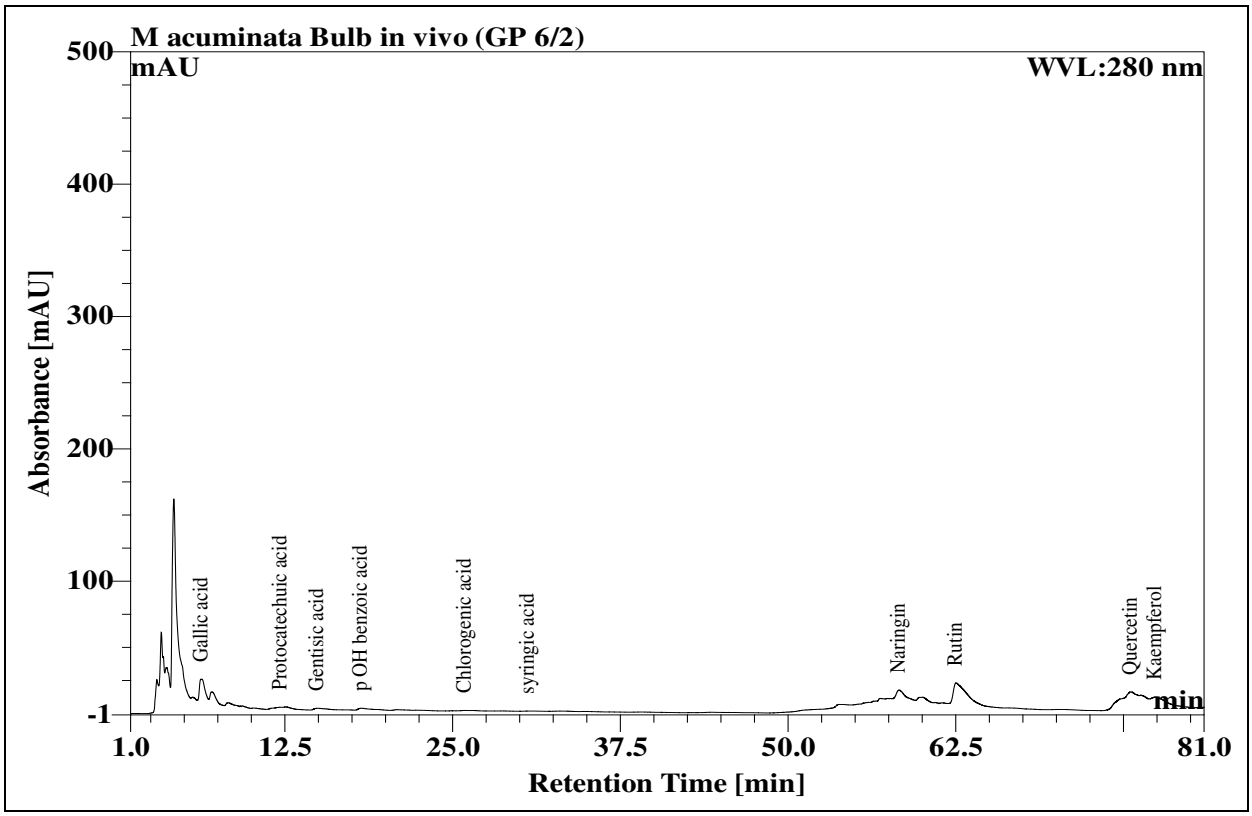


Fig. 71. HPLC chromatogram of 80% ethanolic extract of *M. acuminata* pseudobulb (mother plant)

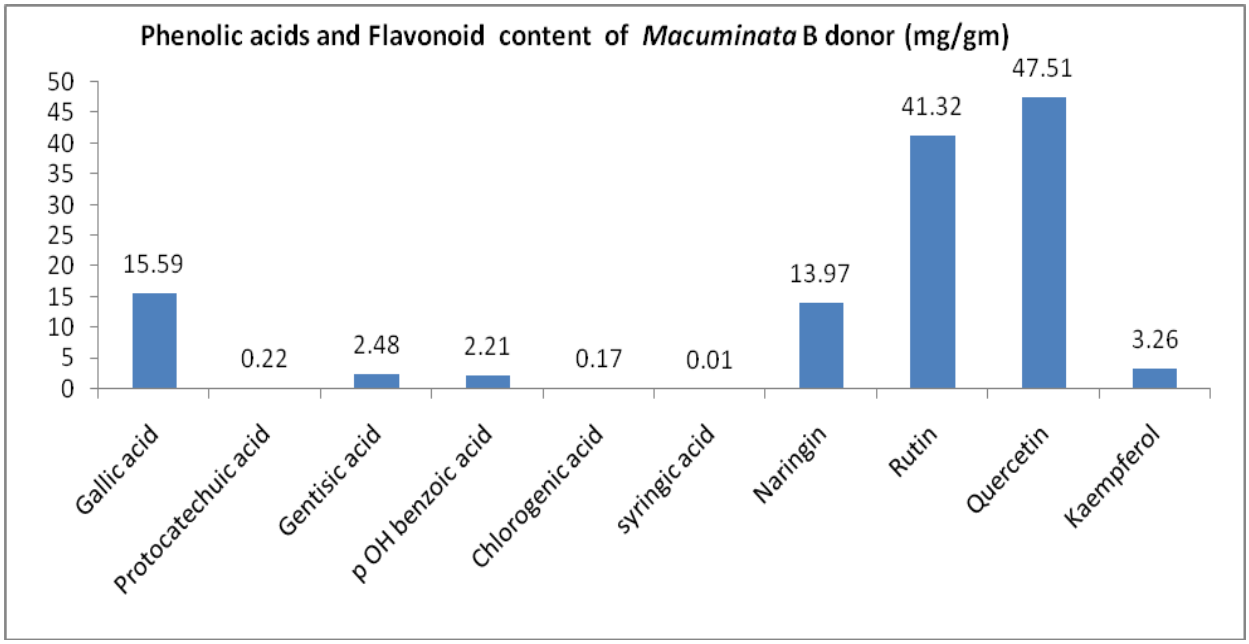


Fig. 72. Phenolic acids and flavonoid content in *M. acuminata* pseudobulb (mother plant)

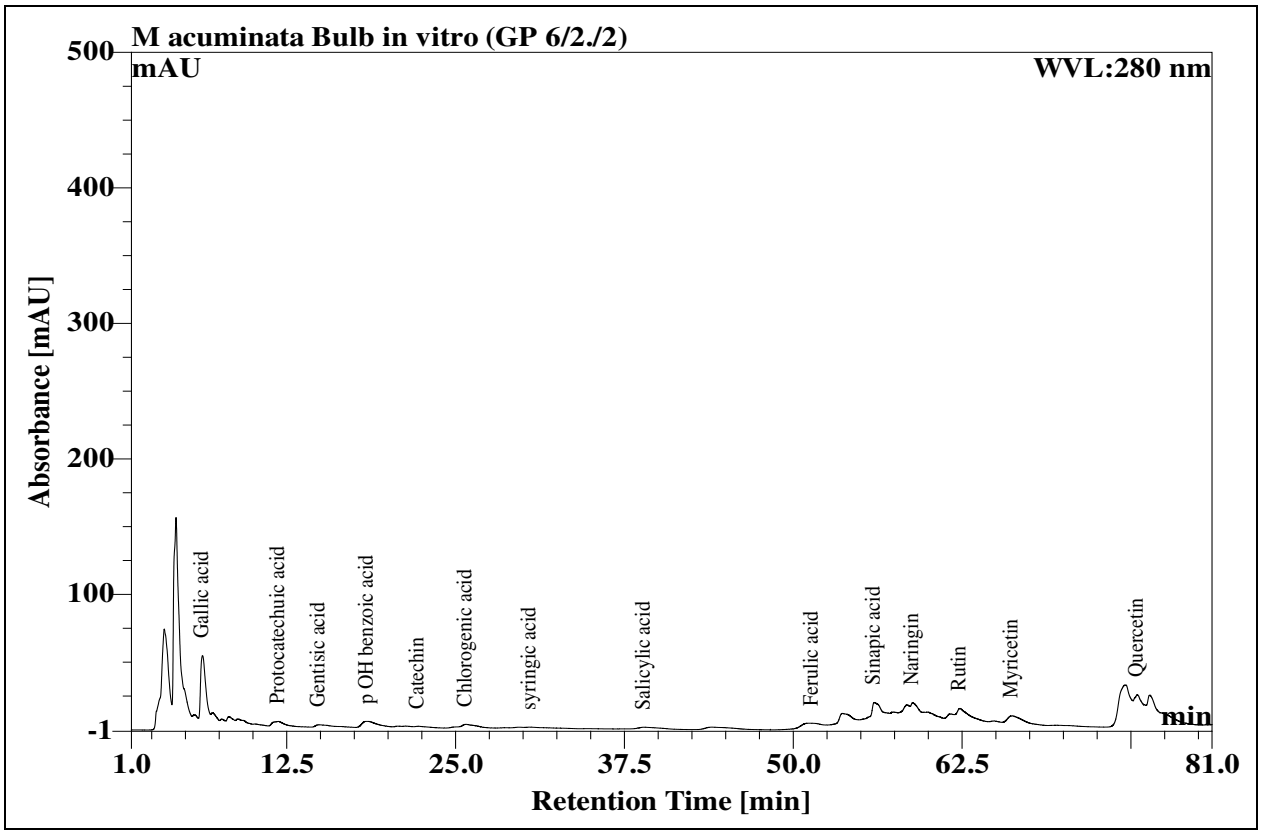


Fig. 73. HPLC chromatogram of 80% ethanolic extract of *M. acuminata* pseudobulb (*in vitro*)

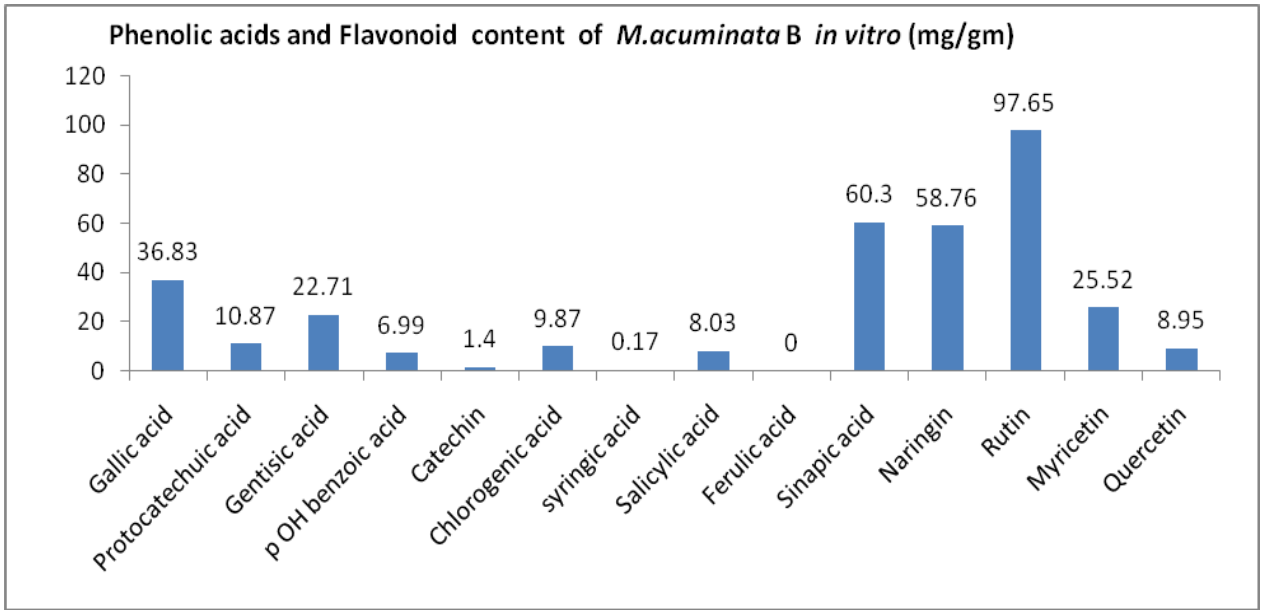


Fig. 74. Phenolic acids and flavonoid content in *M. acuminata* pseudobulb (*in vitro*)

Chapter V

DISCUSSION

Orchids are one of the most exquisite flowering plants which are distributed throughout the world from the tropical to the high alpine region (White and Sharma, 2000). Since ancient times, orchids are used as traditional medicine by different tribes due to their possible pharmacological values. Most herbal practitioners are highly dependent on natural products collected from ecosystems to meet their demand for raw materials. Due to excessive collection from its natural habitat and deforestation its populations have been reduced to a great extent and become a rare sight in the wild. Conservation of endangered orchid species is of utmost importance to stop the depletion of these plant resources. Micropropagation is an *in vitro* technology for mass multiplication of elite plants by using the latest tissue culture techniques. This technique is widely used in agro, horticultural, forestry industries, and medicinal crops (Moraes & al., 2022). The present study was designed to develop a sustainable regeneration protocol for each orchid that can effectively produce *in vitro* progenies at a large scale enriched with phytochemical content.

Seed germination studies

In *A. odorata*, the highest percentage (93.06%) of *in vitro* seed germination was observed in the MS medium supplemented with 10% banana (B) followed by 68.22% of *in vitro* seed germination in 10% coconut water (CW) supplement medium and the least percentage 44.92% of seed germination rate in the control medium. In case of *D. chrysotoxum*, high percentage of germination was observed in all the treatments and in *M. acuminata* seeds exhibited higher percentage of germination in MS medium without additives. Withner (1953) reported banana homogenate (BH) shows stimulatory role in the germination of *Paphiopedilum* seeds. Ernst (1982) observed that effect of BH plays as growth promoter on *Paphiopedilum* seedlings. It is reported that banana fruit pulp contains different carbohydrates, minerals, amino acids, fatty acids, niacin, vitamins, cellulose, polyols, and sterols (Tamura, 1970). Similar type of results has also been observed in *Anoectochilus formosanus* (Shiau & al., 2002), *Cattleya* (Islam & al., 2000), *Hetaeria cristata* (Yam & al., 1990) and *Phalaenopsis* (Ernst, 1986).

Role of organic additives on seedling growth

Addition of various organic supplements and plant extracts can promote growth and morphogenesis of plant tissues (Fonnesbech, 1972). Some such common additives are 10% banana pulp, 0.2% activated charcoal, 10% coconut water.

Among all additives used, MS containing 10% coconut water showed highest number of leaves and roots in *A. odorata* and in case of *D. nobile* 10% banana found to be more effective for the growth of plantlets. It is reported that coconut water is rich in magnesium, phosphate and contains high amounts of sugar around 2.5% (w/v). It has several organic compounds and mineral nutrients which plays important role in plant development. Besides that, coconut water has high levels of nitrogen in the form of amino acids and phytohormones in an adequate balance for plant requirements (Souza & al., 2013). These statements indicated that the use of coconut water as a component of culture medium plays an important role in its development and growth. Nasib & al. (2008) reported that addition of 20% (v/v) coconut water combined with BAP during *in vitro* propagation of Kiwifruit indicated by the best shoot length and number of leaves and concluded that the use of coconut water help to prolong the subculturing time and produced highly robust plants which were more able to survive in greenhouse condition.

In vitro shoot multiplication

In vitro propagation is an effective method for large-scale propagation and conservation of endangered orchid species with a diminished population. The type of explants and concentrations of plant growth regulators used to determine the regeneration response of shoot tip and nodal explants in shoot regeneration study. The plant growth regulator combination induces direct organogenesis in the shoot regeneration experiment of each orchid.

In *A. odorata*, the highest explant response (100%) along with the maximum number (4.48±0.07 shoots/ explant) of proliferated shoots was recorded in MS medium supplemented with BA (4.0 mgl^{-1}) + GA_3 (1.0 mgl^{-1}). The

synergistic effect of BA and GA_3 enhanced the number of shoot proliferation (4.48±0.07) as compared to the treatment with BA alone (3.37±0.13). George & al. (2008) reported that Gibberellic acid (GA_3) alone can induce adventitious shoot, or it can be used as an auxin substitute in shoot formation study. Influence of GA_3 was observed in other plants (Gonbad & al., 2014) where the addition of GA_3 with cytokinins enhanced shoot multiplication and elongation of Tea Clone Iran 100 (*Camellia sinensis* (L.) O. Kuntze). A combination of BA and GA_3 was also found to be beneficial for the shoot multiplication rate of wild roses (Pawłowska, 2011).

In case of *B. odoratissimum,* the medium containing BA (4.0 mgl^{-1}) was found to be optimum as the highest (77.77%) regeneration frequency in this concentration and it produced 2.08±0.55 shoots from a single explant. MS media supplemented with BA (4.0 mgl^{-1}) and IBA (0.5 mgl^{-1}) produced higher number of new shoots (5.31±0.46) with maximum shoot length (3.04±0.60) when compared to BA (4.0 mgl^{-1}) alone. Faisal & al., (2018) reported the synergistic effect of high levels of cytokinin along with low levels of auxins facilitated the shoot proliferation.

In multiple shoot induction studies of *M. acuminata*, *D. nobile* and *D. chrysotoxum,* BA was found to be most effective for shoot regeneration. This finding is in agreement with the results of Pant & al. (2011) where MS media supplemented with BA (0.5 mg/l) showed best medium for shoot multiplication in *Cymbidium iridioides*. As a growth regulator, BA was found to be most suitable for shoot regeneration in other orchids *viz*., *Anoectochilus sikkimensis and Anoectochilus regalis* (Gangaprasad & al., 2000), *Habenaria bractescens* (Medina & al., 2009), *Phaius Tancarvilleae* (Pant & Shrestha, 2011), *Eulophia cullenii* (Decruse & al., 2013), *Eulophia dabia* (Panwar & al., 2022).

In *C. obcordata*, the percentage of explant response differs with the concentrations of BA and the highest frequency (84.4%) along with maximum shoot elongation (2.14±0.15 cm) was achieved at an optimum concentration of 2.0 mgl^{-1} BA. The beneficial effect of BA was found to be most suitable for shoot regeneration in other terrestrial orchids *viz*., *Anoectochilus sikkimensis and Anoectochilus regalis* (Gangaprasad & al., 2000), *Habenaria bractescens* (Medina & al., 2009). The addition of 2iP to MS basal medium produced a maximum (71.1%) percentage of shoot induction at 1.0 mgl^{-1}. At this concentration, the highest shoot length was 2.02±0.10 cm which is not significantly different from the maximum shoot length (2.14±0.15 cm) observed in the optimum concentration of BA. In *Dendrobium aqueum*, the maximum number of shoots was observed on the medium containing 2iP (Parthibhan & al., 2015*)*. In addition, 2iP was found to be better in *Dactylorhiza incarnata* ssp. *incarnate* (Wotavová-Novotná & al., 2007) and *Orchis coriophora* (Bektas & al., 2013).

Preliminary phytochemical screening

The plants produce both the primary and secondary metabolites that are involved in a broad range of activities (Zwenger & Basu, 2008). The primary metabolites are associated with the nutrition and reproduction of plants, whereas the secondary metabolites though not directly involved in the cellular process but play an essential role to provide plant protection against biotics (microorganisms like bacteria, nematodes, fungi, and insects or animals grazing) and abiotic stresses (unfavorable climate such as higher temperature and moisture, shading, injury or presence of heavy metals) (Pagare & al., 2015). Plant-derived secondary metabolites are often known as plant natural products which play an important beneficial role to human beings in various ways (Zwenger & Basu, 2008) such as drugs, flavors, fragrances, insecticides, and dyes. Some metabolites like terpenoids, alkaloids, and flavonoids are involved in the development of a potent drug to cure various diseases or used in dietary supplements to decrease the risk of diseases (Pagare & al., 2015). Phenolics are widely found in the plant kingdom and are therapeutically significant plant substances (Cosme & al., 2020). Flavonoids are the low molecular weight compound (Panche & al., 2016), which are reported to possess numerous biological activities such as antioxidative activity, free radical scavenging capacity, cardioprotective, antidiabetic, anti-inflammatory, anti-allergic (Karak, 2019). Plant steroids possess a wide range of medicinal, pharmaceutical, and agrochemical properties such as anti-tumor, immunosuppressive, hepatoprotective, antibacterial, plant growth hormone regulator, sex hormone, antihelminthic, cytotoxic, and cardiotonic activity (Patel & Savjani, 2015). Alkaloids are generally involved in defense mechanisms in plants (Matsuura, & Fett-Neto, 2015).

The qualitative assessment of phytochemicals *viz*., phenols, flavonoids, steroids, saponin, terpenoids, glycosides, and alkaloids was carried out in three medicinal orchids (*viz*., *A. odorata, B. odoratissimum, C. obcordata*) using 80% ethanol as the solvent and observed that, all the three whole plant extracts were rich in a wide range of phytochemical constituents. The secondary metabolites such as phenol, flavonoids, glycosides, terpenoids, steroids and alkaloids except saponins were present in all the three medicinal orchids *viz*., *A. odorata, B. odoratissimum* and *C. obcordata,* both in the mother and the regenerated plants (Table 14). In qualitative phytochemical analysis of *D. nobile* and *D. chrysotoxum*, flavonoids was present whereas glycosides, terpenoids, steroids and alkaloids were absent. In *M. acuminata*, phenol, flavonoids, glycosides, terpenoids, were present whereas steroids and alkaloids were absent. Similarly, the different phytochemicals in other orchids were reported by several workers in the past. Johnson & Janakiraman (2013) reported the presence of steroid, terpenoid, alkaloids, tannins, phenols, and flavonoids in *Dendrobium panduratum*. Bhattacharjee & al., (2015) revealed the presence of alkaloid, terpenoid, flavonoids, phenols, tannins, steroids, and glycosides in *Vanda tessellata* during their preliminary phytochemical study. Theng & Korpenwar (2014) reported the presence of alkaloid, glycoside, steroids, saponins, tannin and flavonoids in the leaf extracts of *Geodorum densiflorum*. Harshitha & al., (2013) observed similar phytochemicals *viz*., alkaloids, tannin, phenol, and reducing sugar in *Bulbophyllum neilgherrense* and only alkaloids in *Luisia zeylanica* (Sohag & al., 2017). Shubha and Chowdappa (2016) reported the presence of alkaloids, flavonoids, phenols, quinine, coumarin, saponins, tannins, etc. in leaf extract of *Cymbidium aloifolium* and similar phytochemicals in *Rhynchostylis retusa* (Radhika & al., 2013) except phenols and quinine.

TPC, TFC, reducing power, antioxidant activity and HPLC study

screening of ethanolic extract of the six orchids revealed that there was a wide variation in the amount of total phenolics ranging from 19.32±0.32 to 225.34±3.64 mg GAE/g dry material. Out of six medicinal orchids studied, the leaves of *C. obcordata* recorded the maximum amount of phenolic acids while pseudobulbs of *D. nobile* possess the minimum amount. The phenols contain hydroxyls that are responsible for the radical scavenging effect is mainly due to redox properties (Rice-Evans & al., 1997). From HPLC chromatogram, gallic acid is found to be high in roots of *in vitro* plants of *D. chrysotoxum* (1.43 mg/gm).

The flavonoid content of the extracts in terms of quercetin equivalent was 10.71±0.03 to 74.16±0.97 mg/g dry material. Highest amount of flavonoid content was observed in leaves of mother plants of *B. odoratissimum* (74.16±0.97 mg/gm). By HPLC analysis, it is found that rutin content is high in leaves and pseudobulb of *M. acuminata* of mother and *in vitro* plantlets (405.2 mg/gm and 130.24 mg/gm respectively), leaves of *C. obcordata in vitro* plantlets (81.77 mg/gm), leaves of mother plants of *D. chrysotoxum* (89 mg/gm). Catechin content was very high in leaves of *C. obcordata* of mother plants (47.18 mg/gm). Leaves and stem of *C. obcordata* (mother) exhibited the highest quercetin content are 694.58 mg/gm and 244.86 mg/gm.

The antioxidant activities of plant extracts and the standard were assessed on the basis of the free radical scavenging effect of the stable DPPH free radical activity (Braca & al., 2002). In the present study, the highest radical scavenging activity was shown by the leaves of *B. odoratissimum* (IC50 = 0.11±0.003 mg/gm dry material), whereas pseudobulb of *M. acuminata* (mother) showed lowest activity (IC50 = 3.56±0.18 mg/gm dry material. The high radical scavenging property may be due to the hydroxyl groups existing in the phenolic compounds chemical structure that can provide the necessary component as a radical scavenger. The results of the DPPH free radical scavenging assay suggest that different parts of all orchid species have potent antioxidant property of scavenging free radicals.

In case of *A. odorata* total antioxidant activity tissue culture plantlets were comparatively higher than mother plants. Nagesh & al. (2011) have also noticed that the The phytochemical analysis was carried out using 80% ethanolic extracts of different plant parts of six medicinal orchids. A comparative evaluation of total phenolic content (TPC), total flavonoid content (TFC), reducing power and antioxidant properties (DPPH and ABTS) in various parts of the mother and the tissue cultured plants were done to determine the differences in chemical constituents.

Phytochemical antioxidant activity of natural leaf extracts of *Mollugo nudicaulis* was comparatively lower than *in vitro* derived leaf extracts. Similarly, Sawsan & al. (2010) noticed that total phenolic and antioxidant activity in

the micropropagated *Gardinia jasminoides* are correlated with each other and it was observed that the phenolic compounds were the important contributor of the antioxidant activity. It was found that, when the MS medium supplemented with the BA and GA_3, it induce the shoot with high phenolic contents and great antioxidant activity. Nagesh & al. (2011) observed that any increase in the total phenolics in the plant will lead to the increase in the antioxidant activity.

In ABTS free radical scavenging activity assay result showed that leaves of *C. obcordata* (mother), leaves of *B. odoratissimum* (mother) and roots of *in vitro* plants of *A. odorata* (IC50 = 0.09±0.0008, 0.09±0.0008 and 0.09±0.001 mg/gm dry material respectively) gives higher free radical scavenging activity followed by leaves of *D. nobile* (*in vitro*) (IC50 = 0.15±0.006mg/gm dry material), leaves of mother plants of *D. chrysotoxum* (IC50 = 0.11±0.001mg/gm dry material), leaves of *in vitro* raised plants of *A. odorata* (IC50 = 0.13±0.001 mg/gm dry material) and pseudobulb of *B. odoratissimum* (IC50 = 0.14±0.001mg/gm dry material). Valyova & al. (2012) reported that ABTS free radical scavenging activity of ethanolic extract of *Tagetes erecta* L. is IC50 = 0.8±0.2 TEAC.

According to the present study, the high phenolic content in different parts of orchids can explain its high free radical scavenging activity. This study reveals that tested plant materials have moderate to significant antioxidant activity and free radical scavenging activity. The result of the present study suggests that selected plants can be used as a source of antioxidants for pharmacological preparations.

Purification of $CHCl_3$ and EtOAc solubles of MeOH extract of *Cephalanceropsis obcordata* showed significant cytotoxicity (Wu & al., 2006). By HPLC analysis, it is found that rutin content is high in leaves of *C. obcordata* (81.77mg/gm). Dixit (2014) reported that the beneficial action of rutin is probably due to its ability to stimulate the antioxidant enzymes in the cells. This increases in enzyme activity effectively down-regulate the generation of reactive oxygen species (ROS) and lipid peroxidation (LPO) in the skin and thus might reduce the incidence of skin papillomas on the treated areas.

Powder of *B. odoratissimum* leaves are used in treating tuberculosis (Mohanty & al., 2015). Lin & al. (2002) showed that flavonoids are having therapeutical activity against *Mycobacterium tuberculosis*. It was found that leaves of *B. odoratissimum* contain different flavonoids like rutin (58.24mg/gm), kaempherol (1.89mg/gm), naringin (109.73mg/gm), quercetin (140.8mg/gm), myrecetin (11.77mg/gm), catechin (8.67mg/gm). The presence of flavonoids in high amount supports the traditional medicinal use of this plant in the treatment of tuberculosis.

Paste of leaves of *A. odorata* used externally to treat wounds (Mohanty & al., 2015). From the present study, it was found that leaves of *A. odorata* contain different flavonoids like rutin (37.62 mg/gm), quercetin (254.4mg/gm), naringin (133.67mg/gm) and catechin (33.71mg/gm). According to Ramachandran & al. (2009), phytochemically the plant has been reported to contain rutin as main constituent together with crotsparinine, crotosparine and its methyl derivatives aphorbol which play a key role in wound healing. The efficacy of this plant in wound healing may be due to its chemical constituent rutin and antioxidant enzymes, thereby justifying the traditional claim. It is also reported that it is having antibacterial properties (Paul & al., 2013). Flavonoids have been recognised as having a protective effect in plants against microbial invasion by plant pathogens (Horborne & al., 2000). Isolated flavonoids have been shown to possess a host of important biological activities, including antifungal and antibacterial activities (Sathiamoorthy & al., 2007; Alarcón & al., 2008; Galeotti & al., 2008). Manimozhi & al. (2012) reported that flavonoids have excellent antibacterial activity against several pathogenic bacteria like β-lactamase positive *Staphyloccus aureus* and gram negative bacteria. From this study it may be said that wound healing property of *A. odorata* could be due to the presence of flavonoids which has antioxidant and antibacterial activities.

In case of *Dendrobium chrysotoxum,* whole plant extract serves as antitumorous and anticancerous (Sood & al., 2006). Present study of HPLC chromatogram, it was found that rutin and gallic acid is found to be high in leaves (30.49 mg/gm and 29.84 mg/gm respectively). Gallic acid can inhibit tumor development by several mechanisms, such as the inhibition of metastasis (Ohno & al., 2001); the suppression of angiogenesis (Liu & al., 2014); the induction of apoptosis and/ornecrosis (You & al., 2010); the inhibition of cell viability, proliferation, invasion and tube formation (Lu & al., 2010); and the inhibition of migration and invasion (Liao & al., 2012). The presence of gallic acid in high amount supports the traditional medicinal use of this plant in the treatment of cancer.

Present study revealed that leaves and pseudobulb of *M. acuminata* contain higher amount of phenolic and flavonoids (*i.e.* rutin 405.2mg/gm, quercetin 102.11mg/gm, naringin 49.99 mg/gm, gallic acid 36.63mg/gm) has been implicated as anticarcinogenic, antimicrobial, antimutagenic, antiangiogenic and anti-inflammatory agents.

It is reported that various active compounds like dendrobine, moscatilin, gigantol, denbinobine, nobiline and dendrophenol are present in the stems and leaves of *D. nobile* which has strong antimutagenic properties and has been found to be anti-carcinogenic against lung carcinoma, ovary adenocarcinoma and promyelocytic leukemia (Lee & al., 1995). Similarly, our findings reveal that the leaves and pseudostem of *D. nobile* also contain a wide array of phytochemical entities (*i.e.* rutin 57.38mg/gm, quercetin 81.84mg/gm, naringin 84.45 mg/gm, gallic acid 13.82 mg/gm).

Total phenolic content and flavonoid content in these wild and micropropagated orchid species collected were analyzed. The *in vitro* raised plants of *A. odorata* and *D. nobile* contained higher amount of phenolic and flavonoid contents as compared to those of mother plants. The result of the present study is in conformity with previous findings in other Orchids where the production of phenolic compounds is more *in vitro* raised plants of *Habenaria edgeworthii*, *Dendrobium nobile, D. thyrsiflorum* and *Ceropegia santapaui* (Giri & al., 2012; Bhattacharyya & al., 2014; 2015; Chavan & al., 2014). Similar finding had been reported by Nagesh & al.(2011) where *in vitro* propagated leaves of *Mollugo nudicaulis* exhibited a higher total phenolic content than the native leaves extract. Several authors had also reported that that micropropagated plants contain higher amount of secondary metabolites than that of field grown plants (Dias & al., 2000; Romero & al., 2009; Shinde & al., 2010). Gupta & al. (2017) reported that contents of phenolic and flavonoid were higher in *in vitro* raised plant in compared to wild plant extracts of *Lysimachia laxa*. The quantity variation in the phenolic and flavonoid contents in mother and micropropagated plants might be due to the hormonal content, specific metabolic as well as endogenous physiological changes taking place in culture. Stress conditions during *in vitro* cultivation may have stimulated polyphenol production might have been responsible. Many authors reported that PGRs and media could be the reason for enrichment of secondary metabolite in cell, tissue, organ culture or entire plant (Baskaran & al., 2014; Thiruvengadam & al., 2014). Dakah & al. (2014) showed that the radical scavenging ability of micropropagated plant extracts of *Ziziphora tenuior* was higher than aqueous and methanol extracts of wild plants. During *in vitro* propagation, stress conditions may have enhanced polyphenol production and plant growth regulator treatment might have been responsible for this. The formation rate of some phenolic compounds depends on the growth rates of the cultured tissue (Barz, 1977), and on auxin/cytokinin levels into the medium (Sargent & al., 1960).

But other orchid species (*D. chrysotoxum, B. odoratissimum, C. obcordata, M. acuminata*) did not follow the same trend and micropropagated plants have lower amount of bioactive compounds compared to mother plants with some exceptions. The present findings of TPC and TFC in *B. odoratissimum* are consistent with the previous study on a saprophytic orchid, *Aphyllorchis montana* wherein *in vitro* raised plants possess a lower amount of total phenolic and flavonoid content than the wild plants (Mahendran and Narmatha Bai, 2016). The present findings of TPC and TFC in these orchids are consistent with the previous study on a saprophytic orchid, *Aphyllorchis montana* wherein *in vitro* raised plants possess a lower amount of total phenolic and flavonoid content than the wild plants (Mahendran and Narmatha Bai, 2016). The present study revealed that the regenerated plants of *D. chrysotoxum, B. odoratissimum, C. obcordata, M. acuminata* have equal biosynthetic potential in terms of secondary metabolite synthesis as to the wild mother plant.

Govarthanan & al. (2015) reported that metabolites found in *in vitro* propagated plants of *Centella asiatica* have been decreased than that of wild species. Several reasons may explain the differences *i.e.* genetic diversity, environmental conditions, and nutrients present in the soil/culture medium may change the metabolism of plants. Besides that, climatic conditions, bioavailability of nutrients, may modify the growth pattern of explants which can affect plant metabolic activity (Bourgaud & al., 2001).

Goyali & al. (2013) also demonstrated that higher amount of phenolic and flavonoids were found in the leaves of conventional softwood cutting plants than in those of tissue cultured plants. It may be due to difference in nutritional levels of both propagation methods. The most abundant class of secondary phenolic compounds in plants is derived from phenylalanine through the secondary metabolic pathway. The reaction catalyzed by phenylalanine ammonia lyase is an important regulatory step in the formation of phenolic compounds. The activity of phenylalanine

ammonia lyase L is increased by environmental factors, such as low nutrient levels and light (Taiz and Zeiger, 2006). Nutritional stresses, for example deficiency in nitrogen, phosphate and iron levels in soil, enhanced the phenolic accumulation in plants [reviewed by Dixon and Paiva, 1995; Zhao & al., 2006]. However, increasing nitrogen application seems to decrease the level of phenolic compounds (Bourn and Prescott, 2002).

Chapter VI
SUMMARY & CONCLUSIONS

In *A. odorata*, the highest percentage (93.06%) of *in vitro* seed germination was observed in the MS medium supplemented with 10% banana (B) followed by 68.22% of *in vitro* seed germination in 10% coconut water (CW) supplement medium and the least percentage 44.92% of seed germination rate in the control medium. Among all additives used for this experiment, MS containing 10% coconut water showed highest number of leaves and roots and it was found to be more effective for the growth of plantlets. In shoot induction experiments, new shoot formation was observed after three weeks in MS medium fortified with of BA (4mg/L). The highest efficiency (100%) of shoot formation along with the maximum number (4.48±0.07 shoots/ explant) of proliferated shoots after 12 weeks occurred in the MS medium supplemented with BA (4.0 mgl^{-1}) + GA_3 (1.0 mgl^{-1}) and BA alone was not effective for induction of multiple shoots. Qualitative phytochemical analysis using the leaf and root samples of *A. odorata* was done and it was found that as phytochemical constituents *viz.* phenols, flavonoids, glycosides, steroids, terpenoids, alkaloids were present and saponin was absent. In quantitative analysis it was investigated that leaf and root of *in vitro* raised plants of *A. odorata* showed higher content of phenolic acid and flavonoid than that of *in vivo* plants. The *in vitro* raised plants exhibited a higher degree of free radical scavenging activity than the mother plant.

In *D. chrysotoxum*, seed germination started within 2 weeks and protocorm like bodies (PLBs) initiated within 3 weeks. From PLBs, leaf developed within 7 weeks. This seed germination studies showed high percentage of germination in all treatments (10% banana, 10% coconut water, 0.2% activated charcoal). In shoot induction experiments, shoots initiated from nodal segment within 3rd week in MS media supplemented with BA (2mg/L). Healthy shoots with 2-3 leaves developed in 8-9 weeks. On an average 3 (three) no. of shoots were produced in the concentration range at 2mg/l BA and 100% multiple shoot formation was calculated for the same. In qualitative phytochemical analysis flavonoids was present whereas glycosides, terpenoids, steroids and alkaloids were absent. Both the roots and leaves of *D. chrysotoxum* (*in vivo and in vitro*) showed higher content of phenolic acid, flavonoids in quantitative screening test.

In *D. nobile*, MS containing 10% banana showed highest number of shoots and it was found to be more effective for the growth of plantlets. In multiple shoot induction, MS medium supplemented with 2mg/l BA showed good response and plantlets attained a height of 3cm after 12 weeks. In qualitative phytochemical analysis flavonoids was present whereas glycosides, terpenoids, steroids and alkaloids were absent. The quantative phytochemical studies revealed that the contents of various secondary metabolites *viz.* phenolic acid, flavonoids in leaf and while pseudobulbs possess the minimum amount.

In *B. odoratissimum*, the medium containing BA (4.0 mgl^{-1}) was found to be optimum as the highest (77.77%) regeneration frequency in this concentration and it produced 2.08±0.55 shoots from a single explant. MS media supplemented with BA (4.0 mgl^{-1}) and IBA (0.5 mgl^{-1}) produced higher number of new shoots (5.31±0.46) with maximum shoot length (3.04±0.60) when compared to BA (4.0 mgl^{-1}) alone. In qualitative phytochemical analysis, phenols, flavonoids, glycosides, steroids, terpenoids, alkaloids were present and saponin was absent. In quantitative analysis, phenolic acid and flavonoids were found to be present in leaf showed the highest concentration than that of pseudobulb whereas the reducing power activity of the leaf showed the lower concentration. Leaf and pseudobulb of *In vitro* raised plants possess lower amount of total phenolic and flavonoid content than the wild plants. In contrast, root of micropropagated plants contain higher phenolic and flavonoid content than those of mother plant.

In *C. obcordata*, no changes were observed in the seed germination study. In multiple shoot induction study, the percentage of explant response differs with the concentrations of BA and the highest frequency (84.4%) along with maximum shoot elongation (2.14±0.15 cm) was achieved at an optimum concentration of 2.0 mgl^{-1} BA. The addition of 2iP to MS basal medium produced a maximum (71.1%) percentage of shoot induction at 1.0 mgl^{-1}. At this concentration, the highest shoot length was 2.02±0.10 cm which is not significantly different from the maximum shoot length (2.14±0.15 cm) observed in the optimum concentration of BA. Increasing the 2ip concentration had a detrimental effect on *in vitro* proliferation, evident especially in terms of mean shoot number. In qualitative phytochemical analysis, phenols, flavonoids, glycosides, steroids, terpenoids, alkaloids were present and saponin

was absent. In quantitative analysis, it was observed that leaves of *C. obcordata* showed higher phenolic acid content than that of stem. By HPLC analysis, it is found that rutin content is high in leaves of *C. obcordata. In vitro* raised plants possess lower amount of total phenolic and flavonoid content than the wild plants.

In *M. acuminata*, seeds exhibited higher percentage of germination in MS medium without additives. In multiple shoot induction study, BA was superior to 2iP in giving more shoots per explant when same concentrations of the two plant growth regulators were compared. In qualitative phytochemical analysis phenol, flavonoids, glycosides, terpenoids, were present whereas steroids and alkaloids were absent. Present study revealed that leaves and pseudobulb of *M. acuminata* contain higher amount of phenolic and flavonoids.

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About the authors

GARGI PRASAD, Senior Project Fellow, worked under 'Flora of India' project regarding plant tissue culture and phytochemistry. She has published two papers both in national and international journals

DEEPU VIJAYAN, Scientist-D, has been working in plant tissue culture, molecular biology and phytochemistry for the past 20 years. He has published more than 40 research papers both in national and international journals, 2 Books and completed seven projects.

ASHIHO ASOSII MAO, Scientist- G & Director, has been working in plant tissue culture and allied techniques, in micropropagation of RET, Orchids, Rhododendrons, Medicinal plants, ethnobotany & floristic study especially of northeast India. He has published around 150 research papers both in national and international journals, 4 Books and completed many Environmental assessment reports. He is also recipient of UGC- NET (1989), Indian Overseas Scholarship (1990), Schlich Prize (2010), and Dr. S.K. Jain Award.

About the book

This book mainly focuses on effective conservation strategies along with phytochemical screening including HPLC profiling of six medicinal orchids found in Meghalaya. This publication will be highly helpful to the students, future researchers, and sustainable utilization of this endangered orchid.

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Botanical Survey of India
CGO Complex, 3rd MSO Building,
Block F (5th and 6th Floor), DF Block,
Sector I, Salt Lake City, Kolkata - 700 064