Discussion

Plants can be used as therapeutic resources in several ways. They can be used as crude extracts or as fractions in pharmaceutical industries, when they are considered as herbal medicine. Finally, plants can be subjected to standardization, extraction and purification process to isolate the compounds which can themselves be active and used directly as a drug. Quality assessment of herbal formulations is of paramount importance in order to justify their acceptability in modern system of medicine (Madhavi *et al*., 2011). National Center for Complementary and Alternative Medicine (NCCAM) accentuates the need to ensure quality and safety of herbal medicine by modern techniques and applying suitable standards and has proposed guidelines for development of standard herbal medicine (Ariyanathan *et al*., 2010).

Several pharmacopoeia containing monographs of the plant materials describe only the physicochemical parameters. Modern techniques are useful in identification and quantification of active constituents present in the plant materials (Banu and Cathrine, 2015). Hence, to standardize the crude drug in the present investigation, three different species of *Eupatorium* namely *E. glandulosum*, *E. odoratum* and *E. triplinerve* were subjected to screen for their pharmacognostical, phytochemical, antioxidant and antimicrobial properties.

Pharmacognostical studies

Pharmacognosy is concerned with the thorough description and identification of plant drugs in whole state or in powdered form for establishing the herbal drug quality. The quality of secondary metabolites was affected by various environmental factors. Hence a systematic study of crude drug is important for correct identification and quality evaluation.There are number of pharmacognostical techniques are available to evaluate crude drugs (Trease and Evans, 2005).

In the present investigations, the pharmacognostic tools like physicochemical, antioxidant, antimicrobial and heavy metal studies were carried out in the selected *Eupatorium* species. The physicochemical parameters like loss on drying, ash value, extractive value and fluorescence analysis are useful to measure the quality of the plant powder. Moisture is one of the major factors responsible for the self deterioration of the drugs and affecting their shelf life (Tripathi *et al.,* 2013). From the result of current investigation, the loss on drying was found to be higher in *E. odoratum* followed by *E. glandulsoum* which indicates the biomass of the plant source.

Determination of ash value provides criteria for judging the purity of the drug (Kuete, 2017). A high ash value is an indicator of contamination, substitution or adulteration. In the present investigation, a very low ash value was recorded in all the selected plants. The rate of acid insoluble ash content was also observed between 0.3-0.4% in all the three plants. These values are very low in comparison to the earlier report available in the leaves of *Sesbania grandiflora* and *Wedelia trilobata* (Momin and Kadam, 2011; Karthika and Manivannan, 2018) which conform the purity of the selected plant powders.

Extractive value plays an important role in evaluation of crude drugs. Less extractive value indicates addition of exhausted material, adulteration or incorrect processing during drying, storage or formulation (Chandel *et al.,* 2011). In the present investigation, the extractive values were observed to be better in the polar solvents than the non polar solvents. The highest percentage of extractive value was observed in ethanol extract of *E. odoratum* (3.99%) and *E. glandulosum* (3.21%) indicated the quality of the drugs. The higher extractive value of *E. odoratum* and *E. glandulosum* is due to its higher biomass content and it is reported that ethanol is the suitable solvent for extraction of maximum phytoconstituents from the selected plants (Mamtha, 2011).

One of the physicochemical parameters like fluorescence analysis is sensitive and enables the precise and accurate determination of drug. The fluorescence colour is specific for each compound. This method was widely used as an identification tool to identify the quality or adulteration in crude drug (Trease and Evans, 2005; Kokate *et al.,* 2009). The colour of the selected plant powders treated with different organic and inorganic solvents revealed no colour variation among the species under ordinary and UV light. But *E. odoratum* showed distinct variation (Reddish brown colour) under UV light when treated with ethanol. This may be due to the presence of inorganic compounds.

Phytochemical studies

After the standardization of the physicochemical character of selected plants, they were subjected to screen for the detection of secondary metabolites. Phytoconstituents present in plants have been a part of herbal medicine, play a vital role in the prevention and treatment of various diseases and have largely contributed in all existing prevention strategies (Sofowora *et al.,* 2013). As per the current observation, the three selected plants confirmed the existence of the important secondary metabolites like alkaloids, glycosides, flavonoids, saponins, tannins and phenols in ethanol and water extracts. This result confirmed the earlier reports available in the leaves of *E. glandulosum* (Negi *et al.,* 2010: Rajeswary and Govindarajan, 2013); *E. odoratum* (Okwu, 2004, Liu, 2004 and Afolabi, 2007) and *E.triplinerve* (Sugumar *et al.,* 2014). All the phytochemical constituents reported in the selected plants are responsible for curing various ailments of human and animals.

Regarding medicinal importance of the secondary metabolites, alkaloids possess various pharmacological activities such as anticancer, anti-HIV, antiparasitic and antimalarial activity (Cunha *et al.,* 2005; Bouayad *et al.,* 2011). Glycosides serve as a defense mechanism against predation by many microbes (De *et al.,* 1999). Plants enriched with flavonoid content showed

various pharmacological effects including antioxidant, antiinflammation, antiplatelet, antiallergic, anti cancer and heart disease (Asif and Khodadadi, 2013). Tannins are medicinally used as astringents, diuretics, antitumours, antiinflammatory, antiseptic, antioxidant and haemostatic pharmaceuticals (Bruyne *et al.*, 1999; Dolara *et al.*, 2005). Besides, saponins were also used in hypercholesterolemia, hyperglycaemia, antioxidant, anticancer, anti-inflammatory and weight loss (De-lucca *et al.*, 2005).

From the preliminary phytochemical studies of the selected plants, confirmed the presence of most of the important secondary metabolites in ethanol and water extracts. Hence, these two extracts were taken for GCMS and HPTLC studies. GCMS is an important technique for quantitative analysis of unknown samples. It is fast and susceptible method which determinates the thermally stable and volatile compounds. The leaf extracts of *E. glandulosum* and *E. triplinerve* revealed the presence of 20 compounds and *E.odoratum* showed 21 compounds. The compounds identified in selected plants possess various biological properties which were confirmed by earlier reports. Among the reported compounds, n-Hexadecanoic acid, Phytol and Diisooctyl phthalate were common in all the selected plants. But the other compounds vary according to the species which shows variation in their chemical character. The major compound of *E. glandulosum* was 2(3H) - Naphthalenone4, 4a, 5, 6, 7, 8-hexahydro-4a-methyl which possess anti-inflammatory activity (Amudha and Rani, 2014). The compounds like Cycloprop[e]indene-1a, 2(1H)-dicarboxaldehyde, 3a,4,5,6,6a,6b-hexahydro-5,5,6b-trimethyl-, (1aa, 3aá,6aá, 6ba) present in *E. odoratum* has shown the highest peak area percentage which possesses antitumour property (Doskotch *et al.*, 1972). The major compound reported in the leaf extract of *E. triplinerve* was 1H-2-Indenone,2,4,5,6,7,7a-hexahydro-3-(1-methylethyl)-7a-methyl, which possess Hepatoprotective and Hypercholesterolemic activity and also act as HIV-RT-inhibitor (Selvi and Murugesh, 2017).

Besides these, other compounds such as n-Hexadecanoic acid, Phytol, Squalene, Di-isooctyl phthalate, 8-Pentadecanone, Dasycarpidan-1-methanol, acetate (ester) were also reported in the selected plants. They were differing in their pharmacological properties. According to Rowshanul and Rezaul (2009) Di-isooctyl phthalate possess antimicrobial and cytotoxic activity. The compounds n-Hexadecanoic acid, Phytol and Squalene possess antioxidant, hypocholesterolemic nematicide, lubricant, antiandrogenic, antimicrobial and anticancer activity (Arunkumar and Muthuselvam, 2009; Sermakkani and Thangapandian, 2012; Gunes, 2013; Sheeja *et al.*, 2016). 8-Pentadecanone was used in the treatment of Demyelination Conjunctivitis and also showed hepatotoxic activity (Sunita *et al.* (2017). Dasycarpidan-1-methanol, acetate (ester) contains inflammatory, anti-bacterial, anti-fungal, anti-diabetic and anti-cancer properties (Rubaye *et al.*, 2017).

Quercetin is a flavonoid compound and also a potent antioxidant, possesses biological and therapeutic properties including anticancer, antioxidant, antimicrobial, anti-inflammatory, antiviral, cardioprotective and hepatoprotective activities (Harwood *et al.*, 2007; Hernandez *et al.*, 2012; Andrea, 2015). Hence, considering the importance of the medicinal compound quercetin, the methanol and water extracts of leaf powder of *E. glandulosum*, *E. odoratum* and *E. triplinerve* were screened for quercetin content quantitatively using HPTLC. Among the selected three plants, the ethanol extract of *E. glandulosum* showed the maximum amount of quercetin (17.44mg/g) followed by *E. odoratum* (13.40mg/g) and *E. triplinerve* (9.29mg/g). As per the earlier report, *E. glandulosum* contains 4.96% of quercetin content (Mukherjee *et al.*, 2001). But in the present study the quantity of quercetin content is four times higher than previous report. It is due to the seasonal and geographical variation. Other than quercetin, the leaf extracts also revealed many number of peaks at different Rf values shows the presence of other unknown compounds.

Heavy metal studies

Plant species greatly differ in their ability to uptake, accumulate and tolerate heavy metals (Kursat*et al.*, 2010). Heavy metal ions in contaminated soils may easily enter the human and animal food chain through crop plants (Peris *et al.*, 2007). Accumulation of heavy metal causes serious health problems like high blood pressure, hyperactivity of the nervous system and kidney problems (Iqbal and Lajberkhan, 2010). As per the norm of WHO, the medicinal plants which form raw materials for the finished products may be checked for the presence of heavy metals (WHO, 1998). Hence in the present study, selected plant materials along with the soil from the habitat were subjected to analyze for heavy metals like lead, nickel, cadmium, chromium and zinc and the results revealed that all the selected plant materials were possess the permissible level of heavy metals prescribed by WHO. From this result, it is confirmed that the plant powders are safe to use.

Antimicrobial studies

Generally, diseases are mainly caused by microbes and all the microbial infections are treated by the allopathic medicine. Meanwhile, prolonged use of antibiotic leads to develop resistance in microbes. Plants have the phytoconstituents which are capable to act as an antibiotic. It contains secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, terpenoids, steroids, glycosides and phenols which are found to have antimicrobial properties (Cowan, 1999). Human pathogens such as virus, bacteria and fungus cause many diseases and affect the immune system of our body. Bacterial pathogens cause diseases such as typhoid, tetanus, diphtheria, anthrax, skin infections, inflammatory bowel diseases, bacterial vaginosis, white pox disease etc. These are mainly caused by *Streptococcus, Staphylococcus, Klebsiella* and *Pseudomonas* species. Fungal pathogens also have an enormous impact on human health. The pathogens such as *Trichophyton, Microsporum, Candida, Aspergillus* and *Cryptococcus* causes

life threatening infections in humans (Havlickova *et al.*, 2008; Brown *et al.*, 2012). Therefore, in the present study, the the leaf extracts of *E. glandulosum*, *E. odoratum* and *E. triplinerve* were tested for their antimicrobial activity against human pathogens, which was collected from the PSG Institute of Medical Sciences & Research, Coimbatore.

The result of antimicrobial studies revealed that ethanol extract of *E. glandulosum* and *E. odoratum* shows good inhibitory effect against all the selected bacterial pathogens. The result of MIC studies confirmed that the leaf extract of *E. glandulosum* at very low concentration (10-18 μg/ml) is capable of inhibiting the growth of the selected bacterial pathogens effectively. The leaf extract of *E. glandulosum* showed good inhibitory effect against *Trichophyton rubrum E. odoratum* control the growth of *Candida tropicalis* and *Aspergillus flavus*. *E. triplinerve* inhibits the growth of *Microsporum canis* and *Aspergillus niger*. Thus the result of MIC studies reveals that 14-18 μg of *E. odoratum* leaf extracts are enough to inhibit the selected fungal pathogens.

Among the two solvents (ethanol and water) tested, the ethanol extracts showed good antimicrobial activity than water extract. This is due to the maximum extractive value of the solvent ethanol. The antimicrobial property of the plants is also mainly due to the presence of secondary metabolites such as alkaloids, flavonoids, tannins, phenols and saponins (Bonjar *et al.*, 2004). In the present investigation, the preliminary phytochemical screening of the selected plants showed the presence of all the important secondary metabolites.

The GCMS analysis of the *E. glandulosum* and *E. odoratum* leaf extracts showed the presence of the compounds Phytol, and Di-isooctyl phthalate which possess antimicrobial property. The antimicrobial property of these compounds was confirmed by the earlier reports (Rowshanul and Rezaul, 2009; Sermakkani and Thangapandian, 2012). As per the earlier reports, the leaves of *E. glandulosum* inhibited the growth of *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella*

pneumonia and *Escherichia coli* (Sasikumar *et al.,* 2005: Desingh *et al.,* 2014). Dichloromethane butanol extract of *E. odoratum* leaves also showed best antibacterial activity against *Klebsiella oxytoca, Salmonella enterica, Shigella sonnei* and *Vibrio cholerae* (Menonve *et al.,* 2013). In addition to that essential oil obtained from leaves of *E.odoratum* showed antifungal activity against *Candida albicans, Asperigillus niger, Asperigillus flavus, Asperigillus terreus,* and *Penicillium notatum* (Moses *et al.,* 2010; Velliangiri*et al.,* 2011). The volatile oil extracted from the leaves of *E. glandulosum* showed good antifungal activities against *Fusarium moniliformae, F. eroliferum, F. proliferatum, F. Oxysporum, Sclerotium rolfsii, Macrophomina phaseolina and Rhizoctonia solani* (Tian *et al.*,2007; Sobrinho *et al* ., 2017).

Antioxidant activity

Medicinal plants with antioxidant properties play a vital role in exhibiting beneficial effects and employed as an alternative source of medicine to mitigate the disease associated with oxidative stress. Antioxidants protect the body against the damaging effects of free radicals produced naturally within the body. These free radicals production could cause damage to proteins, DNA and the genetic material within the cells (Weisburger, 2000).

In the present study, ethanol and water extracts of leaf powder of selected plants shows good antioxidant activity. Among the three plants, ethanol extracts of *E. odoratum* (81.35%) and *E. glandulosum* (75.87%) revealed maximum antioxidant activity tested by DPPH method at the concentration of 100µg/ml. The reason for the better antioxidant property is due to the presence of the flavonoid quercetin which was confirmed by the HPTLC analysis. The quercetin content was found to be maximum in *E. glandulosum* followed by *E. odoratum*. The earlier researchers stated that the flavonoid has the ability to scavenge the free radicals and acts as a potent antioxidant (Pietta, 2000; Amarowicz and Pegg, 2008). In the present investigation, the GCMS analysis of the *E. glandulosum* and *E. odoratum*

possess hexadeconic acid which possess antioxidant property (Sermakkani and Thangapandian, 2012).

According to the earlier reports, the leaf extracts of *E. odoratum* exhibited 87.93% inhibition at the concentration of 100µg/ml (Amatya and Tuladhar, 2011). However in the present study, the scavenging activity is lesser (81.35%) than the earlier report. Iyeswarya *et al* (2013) reported that 100mg/ml of *E. glandulosum* leaf extract showed 66.8% of scavenging activity, whereas in the present study, the leaves of *E. glandulosum* exhibited 75.87% of scavenging activity. These variations in the results were due to the age, seasonal and geographical variation of the plants.

Genetic variation studies

Random Amplified Polymorphic DNA (RAPD) analysis was made to identify the molecular variations among the selected three plants. This technique is an effective method for analysing genetic variability. It is a reliable method for characterizing variation among species, within a species and among populations. Polymorphism is a certain mutations in the genotype of the plants.

In the current investigation, initially nine RAPD primers were used for RAPD analysis. However 6 Operon series primers did not show amplification of bands in all the samples. But, the primers OPA-13, OPA-18 and OPA-19 showed amplification in the samples. Hence these three primers were screened for further analysis. Totally 63 different randomly amplified DNA fragments from 3 species of *Eupatorium* were detected consistently with three primers. The bands amplified by different primers showed diversity within and among the provenances. On the whole, out of 63 amplified fragments, 33 fragments were found polymorphic (52.39%). The highest polymorphism was seen in *E. glandulosum* with primer OPA-19, and least polymorphism (40%) was obtained in *E. odoratum* by the primers OPA-18 and OPA-19. The

Polymorphism between the plants is due to the biodiversity, adaptation and extent of variation from the ancestral origin of the plants. *E. glandulosum* grows on high altitude while the other two species grown on plains. Hence this may also be a reason for the variation of the plant species.

Earlier, the genetic diversity was studied in fifteen populations of three species of *Anthemis melampodina, Anthemis, pseudocotula and Anthemis, bornmuelleri* using different RAPD primers. A close genetic relation was observed between *A. melampodina* and *A. Pseudocotula* whereas *A. Bornmuelleri* showed wide variation compare to other two species (Hasan and Qari, 2017). This is the first report on the analysis of genetic variability among the three selected *Eupatorium* species using RAPD technique.

In vitro cytotoxic activity

Cancer is a dangerous disease which is associated with a wide range of increasing effects at molecular and cellular levels. Therefore it seems that chemoprevention is the best to cure immediately. Chemotherapy is associated with high cytotoxic loads and invasive procedures (Bertram, 2001; Amin and Mousa, 2007). Food and Drug Administration (FDA) approved tamoxifen as chemopreventive agent for reducing the risk of cancer. This agent was found to reduce the cancer incidence by 50% at high risk. But it causes serious side effects such as uterine cancer, blood clots, ocular disturbances, hypercalcemia, and stroke. Later, FDA approved chemopreventive drugs is an issue of particular concern when considering long-term administration of a drug. This clearly indicates the need for alternative medicine, which is safe and efficacious in preventing cancer. Hence, there is a need for development of natural drugs which lead a huge demand for producing herbal drugs for effective and safe treatment (Anand *et al.*, 2008).

In the present study, the leaf extracts of the *E. glandulosum, E. odoratum* and *E. triplinerve* were tested under *in vitro* condition against colon cells (HT-29) and monitored by using MTT, SRB and LDH assays. The result of these two assays revealed the same results in ethanol extracts of *E. glandulosum* with the inhibition rate of 62.97% in MTT assay and 62.43% in SRB assay. All the plant extracts inhibited the growth of cells and the inhibitory rate was directly proportional to the concentration of the plant extracts. There is no growth inhibition was observed in untreated cells (Control).

Leakage of cytoplasmic located enzyme (LDH) into the extracellular medium is measured in lactate dehydrogenase assay. The increase inlactose dehydrogenase (LDH) release into the medium is proportional to the number of lysed cells. In the present study there was a dose dependent increase in the LDH release observed at increasing concentrations. The ethanol extract of selected three plants. *E. glandulosum* showed maximum amount of LDH release (5346.01 U/L) followed by *E. odoratum* (4470.77 U/L). The LDH leakage under control was found to be 3260.50 U/L which shows the good anticancer effect of *E. glandulosum*. This is due to the presence of maximum quercetin content in the leaves of *E. glandulsoum* which possesses anticancer property (Harwood *et al.,* 2007; Hernandez *et al.,* 2012; Andrea, 2015). The GCMS study was also confirmed the presence of anticancerous compounds such as n-Hexadecanoic acid, Phytol, and Di-isooctyl phthalate in *E. glandulosum*. The earlier reports were also confirms the anticancer property of above said compounds in *Aloe vera*, *Calotropis gigantea*, *Cassia italic* and *Gracilaria edulis* (Arunkumar and Muthuselvam, 2009; Rowshanul and rezaul, 2009; Sermakkani and Thangapandian, 2012; Gunes, 2013; Sheeja *et al.*, 2016; Rubaye *et al.*, 2017).

Many reports are available regarding the anticancer activity of *E. glandulosum* and *E. odoratum* against human breast adenocarcinoma (MCF7), hepatocarcinoma (HepG2), and cervix adenocarcinoma (HeLa) (Bipransh *et al*., 2015). Among that, *E. glandulosum* showed 61% inhibition and *E. odoratum* showed 53.11% inhibition against HeLa (Breast cancer) cell lines. Similarly, in the present investigation, *E. glandulosum* and *E. odoratum* exhibited 62.97% and 33.35% of inhibition against colon cancer cell line (HT-29).

Tissue culture studies

In vitro propagation is generally used for the production and multiplication of economically important rare and endangered medicinal plants. In other hand, advances in the area of tissue culture is for the production of secondary metabolites by callus culture have made it possible for increasing the yield of secondary metabolites used in pharmaceuticals such as alkaloids, terpenoids, steroids, saponins, phenols, and flavonoids (Ramachandra and Ravishankar, 2002). Based on the current reports, it is confirmed that all the selected three plants were possess maximum number of secondary metabolites with good antioxidant, antimicrobial and anticancer properties. Therefore, by considering the medicinal importance of the plants, tissue culture technique has been adopted to standardize a protocol for mass production of callus and shoots.

Effect of growth regulators on explant culture

The node, internode and leaf explant of *E. glandulosum, E. odoratum and E. triplinerve* were cultured in MS basal medium supplemented with different concentration of IAA, BA, Kinetin and 2, 4-D. The callus formation was observed only in the medium containing 2, 4-D which is considered as the most potent auxin to stimulate callus induction (Murashige, 1974). The leaf explants, took six days to initiate callus and stem explants took eight to nine

days to produce callus under *in vitro* condition. The callus produced from the leaf explants was green and friable, whereas the stem explants produced friable yellow coloured callus. The frequency of callusing and augmentation of callus was recorded maximum in *E. glandulosum* (85.71%) at the concentration of 2mg/l of 2, 4-D. Among the different concentrations of hormones tested, medium with 2mg/l of 2,4-D is optimum for callus production. The effect of 2,4-D at higher concentration decreases the rate of callus production. From this result, it is observed that, the leaf explants of *E. glandulosum* showed best response in callus production compare to *E. odoratum* and *E. triplinerve*. Similarly maximum callus formation was reported in Ginger (Samsudeen *et al.,* 2000), *Curcuma amada* (Prakash *et al.,* 2004), *Saccharum officinarum* (Tahir *et al.,* 2011), *Citrullus colocynthis* (Shasthree *et al.,* 2012), *Ocimum sanctum* (Sharma *et al.,* 2015; Wongsen *et al.,* 2015), *Crataegus azarolus* (Chaabani *et al.,* 2015), *Stevia rebaudiana* (Sharma *et al.,* 2015) *Stachys cretica*, (Ozdemir *et al.*,2017) and *Bacopa monnieri* (Meenashree *et al.*, 2017) in MS medium with 2, 4-D.

Browning of callus was observed in nodal explants of *E. odoratum* in medium supplemented with 3mg/l IAA and 1mg/l BA. After 3 weeks, it turns into brown colour. The browning of callus is due to the phenolic compounds present in the cells. The accumulation of the phenolics in the cytoplasm undergoes oxidation and polymerization and the oxidized products appear brown in colour (Lukas *et al.,* 2000). Similar results were reported in leaf explants of *Ipomea aquatica* (Prasad *et al.,* 2006) cultured in the medium supplemented with various concentrations of 2, 4-D.

Effect of factorial combinations of IAA and BA on shoot bud induction in nodal explants of *E. glandulosum*

From the preliminary studies, it is confirmed that the leaves of *E. glandulosum* yield maximum amount of phytoconstituents, quantity of callus and quercetin content. Besides, that it also possesses maximum antimicrobial,

antioxidant and anticancer properties. Hence, *E. glandulosum* has been selected for further tissue culture studies to standardize a protocol for mass propagation. From the literature survey, it is understood that *E. odoratum* is a commonly available plant; many tissue culture reports are available in *E. triplinerve* (Martin, 2003; Samydurai *et al.,* 2012; Usha and Karpagam, 2017). But *E. glandulosum* is found only on higher elevations (Hilly regions) and there is limited informations are available regarding tissue culture studies in this plant. Hence, *E. glandulosum* is selected for the mass propagation under *in vitro* condition.

To produce multiple shoots, fresh and healthy leaf, internode and nodal explants of *E. glandulosum* were selected and cultured in MS medium supplemented with various concentrations of auxin and cytokinin. Nodal explants cultured in medium with various concentrations and combination of IAA and BA respond for shoot proliferation. Among the hormonal concentrations used, maximum frequency of shoot forming explants (83.3%) and the number of shoots (64.50± 3.76) per explants was observed in MS medium enriched with combination of 0.5mg/l of IAA and 3mg/l of BA. But the length of the shoots is increased by the addition of BA in to the medium. Similarly maximum number of shoots was reported in *Solanum nigrum* at the concentration of 3 mg/l of BAP with 0.5 mg/l of IAA (Sridhar and Naidu, 2011) and *Solanum lycopersicum* at the concentration of 0.1mg/l IAA and 3.0 mg/l BAP (Shah *et al.,* 2013).

Influence of plant growth regulators and seasonal variations on shoot bud induction

Influence of growth regulators and seasons on multiple shoot formation in *E. glandulsoum* was studied in various seasons. Among the seasons, explants collected during the month of June-August have shown maximum number of shoots per explant (64.50± 3.76) at the concentration of 0.5mg/l of IAA with 3mg/l of BA. But the maximum shoot length was observed at the concentration

of 1mg/l IAA with 4mg/l BA in the same period. This result confirmed that the medium with less auxin and high cytokinin induces the explants to produce maximum number of shoots in the period of June-August. Similar results were reported in the nodal segments of *Embelia ribes* and *Cinnamomum tamala* collected during June-August responded for shoot induction (Dhavala and Rathor, 2010 and Deb *et al*., 2014). This is due to favourable rainfall and temperature prevailed in those months. In contrast, Mudoi *et al* (2014) reported that the explants of *Bambusa nutans* collected during Sep-Aug showed maximum shoot number and shoot length. Contradictorily *Ochreinauclea missionis* collected during Dec-Jan and *Leptadenia reticulate* collected during spring and rainy seasons showed best response for shoot number and shoot length.

Induction of roots

Rooting of *in vitro* regenerated shoots is necessary to complete the growth of the whole plantlet. *In vitro* regenerated shoots were aseptically implanted on different rooting media with various concentrations of auxins such as IAA and IBA. Among the two growth regulators, IBA induced maximum number of roots per explant (32.67 ±0.89) with maximum length (6.1 ± 0.12 cm) at the concentration of 4mg/l and 3mg/l respectively. Similar results were reported in *Cunila galoides* (Fracaro *et al.,* 2001), *Clitoria ternatea* (Shahzad *et al.,* 2007), *Gentiana lutea* (Petrova *et al.,* 2011), *Musa acuminata* (Arun *et al.,* 2012), *Mentha viridis* (Senthil and Kamaraj, 2012), *Psoralea corylifolia* (Pandey *et al.,* 2013) and *Plectranthus amboinicus* (Rahman *et al.,* 2015).

Acclimatization and Hardening

The success of any micropropagation is depends on the establishment of regenerated plantlets under *ex vitro* field conditions with high survival rate (Torres *et al.,* 2006; Deb and Imchen, 2010; Yadav *et al.,* 2015). The *In vitro* raised plantlets will be very sensitive to the external environment. Therefore, a

gradual shifting of the plantlets from medium to the pots was done with great care for their adaptation to environmental changes.

In the present study, the *in vitro* grown rooted plantlets of *E. glandulosum* were transferred to pots containing various potting mixture. They were regularly irrigated with half strength MS medium for 2 weeks. The effect of various potting mixtures on the shoot length of the plantlets was measured at different intervals of 7^{th}, 14^{th}, 21^{st} and 49^{th} day. Among the different potting mixtures tested, the pot contained soil, sand and cocopeat at the ratio of 1:1:1 showed the best growth with 100% survival rate. Similar results were reported in *Psoralea corylifolia* with 100% survival rate in potting mixture containing vermiculite, soil and sand at the ratio of 1:2:1 (Pandey *et al.,* 2013). But *Plectranthus bourneae* showed 82.5% survival in the potting mixture containing sterile sand, garden soil and vermiculite at the ratio of 1:1:1 (Rajaram *et al*., 2015).

Screening of *in vitro* grown materials for secondary metabolites

The GCMS analysis of leaves collected from the mother plant in wild condition confirmed the presence of important phytochemicals. The name of chemicals and their medicinal values were discussed in detail in chapter I under the heading phytochemicals. As per the result, the leaves collected from *E. glandulosum* and *E. triplinerve* showed 20 compounds each and *E. odoratum* confirmed 21 compounds. To ensure the existence of phytochemicals in *in vitro* grown materials, the callus obtained from the leaf explants of selected three plants was extracted with ethanol and subjected to GCMS analysis. The results confirmed the presence of 16 compounds in *E. glandulosum*, 12 compounds in *E. odoratum* and 17 in *E.triplinerve*. When comparing the phytochemical constituents of callus with wild leaves, the number of phytochemical compounds reported in callus was lesser than the mother plant. In *E. glandulosum* 10 compounds are common for both mother plant and callus. The peak area percentage of some compounds is found to be

higher in callus extract than mother plant. For example, the peak area of Bicyclo (5.1.0) octan-2-one, 4, 6-diisopropulidene-d, e-dimethyl showed 14.42% in leaf and 32.26% in callus extracts which proved that the callus produced high amount of secondary metabolites than mothe plant. Similarly, the GCMS analysis of *Ampelocissus latifolia* revealed high amount of Beta-sitosterol and 5-Hydroxymethyfurfural in callus than wild mother plant (Anand *et al*., 2018) and *Piper longum* produced high amount of piperin content in callus than leaf extract of mother plant (Siddique *et al*., 2019).

In *E. odoratum*, there are about 7 compounds were reported to be common in leaves of wild mother plant and callus obtained from the leaves. But it is interesting thing to be noted that the callus extract produce one new compounds namely, Ethyl-N-(o-anisyl) formimidate with highest peak percentage (34.3%), which was absent in mother plant. Ths is due to the changes in the culture environment which leads to the alteration of metabolites in the callus.

The above observation made an interest to investigate chemical compounds, in *in vitro* grown plant. Hence, a pilot study was made on the leaf materials of *E. glandulosum,* collected from the *in vitro* propagated hardened plants, which was maintained in the green house. The leaves were collected from the two year old plant, dried and the leaf powder was extracted with ethanol and screened for phytochemicals by GCMS analysis. Interestingly, the leaf extract confirmed the presence of 26 compounds which was higher than the leaves from wild mother plant (20 compounds). Similar reports were available in *Merremia aegyptia* (Joshi *et al.*, 2018) and the report showed that the *in vitro* grown shoots confirmed 34 compounds which were higher than the wild plant (32 compounds). Thus the results proved that *in vitro* grown plant produced unique compounds which were not present in the wild. This change is due to the culture conditions and the effect of growth hormones supplied

externally in the medium which leads to the changes in their phytochemical pathways (Umesh and Thoppil, 2014).

Estimation of quercetin in *in vitro* grown materials

The fresh callus obtained from the leaf explant of *E. glandulosum*, *E. odoratum* and *E. triplinerve* were extracted with ethanol and water and analyzed for quercetin content by HPTLC finger printing method. The ethanol extracts showed maximum amount of quercetin content than water extract. Among the three plants, leaf callus from *E. glandulosum* showed maximum quercetin content. Comparing the callus with the leaf extract of the wild plant, the leaves from wild plant showed maximum quercetin content. This may be due to the influence of season and age of the plant. Callus being an undifferentiated meristematic tissue accumulates only minimum quantity of plant secondary metabolites. Thus majority of callus and suspension cultures produce lesser quantities of secondary metabolites. This is mainly due to the lack of fully differentiated cells in the cultures (Sudha bai *et al.,* 2018).

The quercetin content was also estimated quantitatively in the leaves of *in vitro* grown hardened plants (two years old) and found 13.51mg/g of quercetin. But the wild leaves showed 17.44 mg/g of quercetin content which was higher than the hardened plants. This variation could be due to age of the plant and the ambient conditions prevailing in the wild.