In vitro Propagation and Comparative Phytochemical & Antioxidant activity of Callus and Wild plants of *Emilia sonchofolia* (L.) DC

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Introduction

Plants are really important for the planet and for all living things, including human beings. The plant kingdom serves as a reservoir of structurally diverse bioactive molecules. Most of the best plant medicines are the sum of their constituents. Plants are potent biochemists and have been used as components of phytomedicine since time immemorial. Over 50% of all modern clinical drugs are originated from natural products and they play an important role in the drug development programme of the pharmaceutical industry. Plant-based natural constituents can be derived from any part of a plant like stem, bark, leaves, flowers, roots, fruits, seeds, etc. That is, any part of the plant may contain active components (*Gorden and David* 2001).

Phytochemistry is the study of the chemicals produced by plants, particularly the secondary metabolites which are synthesized as a measure for self-defence against insects, pests, pathogens, herbivores, UV exposure and environmental hazards. In this field, various techniques have been developed, ranging from the preparation of the plant tissue sample to sophisticated techniques for the elucidation of organic structures. The beneficial medicinal effects of plant materials typically result from the combination of products such as alkaloids, phenols, proteins and amino acids, carboxylic acid, coumarins, flavonoids, quinines, resins, saponins, sterols, tannins, xanthoproteins, sugars, etc. presents in the plant. The chemicals present in the plants are analysed quantitatively and qualitatively. Presence or absence of a chemical will give the criteria to evaluate the drug or to standardise the drug.

Plants have economic values and are not only used for mere satisfaction or sustenance of hunger but also for the maintenance of good health (Alter, *et al*., 2003). The screening of plants for bioactive agents is one of the most intensive areas of natural products research currently, the field is far from being exhausted. over three-quarters of the world population

relies mainly on plants and plant extracts for their health care needs. According to WHO, around 21,000 plant species have the potential for being used as medicinal plants. But, only around ten percent of plants have been investigated (Sandberg and Bruhn, 1979).

People have used plants as sources of materials other than nutrients for almost as long as they have been eating the plants themselves. They can be regarded as natural chemical factories due to their extraordinary ability to produce various secondary metabolites many of which are endowed with biological activities of pharmaceutical, nutraceutical or cosmeceutical interest and can be successfully used to protect human health and well-being.

The use of plant-derived medicines, poisons and narcotics is still common in many cultures, and herbal medicines in particular are becoming increasingly popular. Plants provided a good source of a wide variety of compounds, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids and some other secondary metabolites, which are rich in valuable bioactivities like antioxidants, anti-inflammatory, anti-tumour, antimutagenic, anti-carcinogenic, anti-bacterial or anti-viral activities (Maridass and Britto, 2008). Unlike other pharmaceutical chemicals, those derived from plants has less side effects. So, the phytochemicals cure diseases without causing much harm to human beings (Banu and Cathrine, 2015).

In biological systems, certain DNA reactive chemicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS) can oxidize lipids and proteins, which can cause ageing, cancers etc. Many studies have shown that free radicals can cause severe damage to nearby tissues. Radical scavenging antioxidants are particularly important in antioxidative defence in protecting cells from the injury of free radical (Youwei *et al*., 2008). Antioxidants can act as scavengers of such reactive oxygen species. They are molecules which help to neutralize harmful free radicals; thereby preventing or delaying cell damage.

Plants can be regarded as a natural source of antioxidants. Numerous antioxidants identified as active oxygen scavengers or free radicals, obtained naturally from the plant sources are used in food, cosmetic and remedial purposes proved to be brilliant alternatives for man-made antioxidants because of their inexpensiveness, and have no any harmful effect on human body (Brown, and Rice-Evans, 1998). Phytochemicals like polyphenols and carotenoids are reported to provide maximum antioxidant activity. Extracting these compounds can substitute for synthetic antioxidants, which have many side effects. They have the ability to prevent antiviral, anticancer, anti-ageing and so on, to an extent. The medicinal plants that possess antioxidant activities are screened by means of various *in vitro* assays like nitric oxide scavenging assay, DPPH radical scavenging assay, total phenolic content, reducing power assay; and via various *in vivo* animal models using rats and mice (Essien *et al*., 2020).

Since the early days of mankind, plants with the secondary metabolites have been used by humans to treat infections, health disorders and illness (Wyk and Wink, 2004). Only during the last 100 years, natural products has been partly replaced by synthetic drugs. Discovery of new plant derived chemicals can open a new way in pharmaceutical industries since it can cure many diseases where the synthetic drugs fail. The search for new plant derived chemicals should thus be a priority in current and future efforts towards sustainable conservation and rational utilization of biodiversity (Philipson, 1990).

Plant tissue culture or *in vitro* propagation is the technique of maintaining and growing plant cells or tissues aseptically in controlled conditions on artificial medium in suitable containers. It can be used as an important biotechnological tool for basic and applied purposes ranging from investigation on plant developmental processes, functional gene studies, commercial plant micropropagation, generation of transgenic plants with specific industrial and agronomical traits, plant breeding and crop improvement etc. Additionally, the continuous production of active compounds including secondary metabolites of industrial and

pharmaceutical interest. Tissue culture relies on the principle of totipotency which is defined as the ability of plant cells to divide and de-differentiate into meristematic state and then re-differentiate into fully differentiated plants. The disorganized or undifferentiated proliferated mass of meristematic tissue is called callus. Callus formation is vital to many investigative and applied tissue culture procedures.

The idea of experimenting with the tissues and organs of plants in isolation under controlled laboratory conditions arose during the latter part of the nineteenth century, finding its focus in the work of the great German plant physiologist Gottlieb Haberlandt, who is known as the 'father of tissue culture'. The production of chemicals from plant tissue culture was first detailed by Routien and Nickel1 in 1956.

Micropropagation is one of the most popular technique of tissue culture. It is the practice of rapidly multiplying stock plant material to produce large number of progenies which are the exact copies of their donor plant. Success of tissue culture relies on several factors such as concentration of hormones, composition of nutrient media, light intensity, humidity etc.

The portion of plant body, which has been taken from the plant to establish a culture is called explant. Explant can be obtained from plants, which are grown in controlled environmental conditions. Such plants will be usually free from pathogens and are homozygous in nature. Explant may be taken from any part of the plant like root, stem, leaf or meristematic tissue like cambium, floral parts like anthers, stamens etc. Age of the explant is also an important factor in callus production. Young tissues are more suitable than mature tissues. Exogenous application of auxin and cytokinin induces callus in various plant species. The cells of callus are parenchymatous, amorphous and undifferentiated. They are actively dividing when level of hormones, especially auxin and cytokinin, are optimum. An intermediate ratio

of auxin and cytokinin promotes callus induction, while a high ratio of auxin-to-cytokinin or cytokinin-to-auxin induces root and shoot regeneration, respectively (Skoog and Miller, 1957).

Plant tissue culture techniques being independent of climatic and geographical conditions will provide an incessant, sustainable, economical and viable production of secondary metabolites on a continuous year-round basis. At least in some cases, the yield may exceed than conventional propagules. When a valuable product is found in wild or rare plant species, intensive cell culture is a practical alternative to produce the same. The production of secondary metabolites was also found to be influenced by light, temperature, pH and oxygen concentration. However, it is difficult to predict how variations of these parameters affect phytochemical synthesis in different plant species.

Extraction from the *in vitro* tissue is much simpler than extraction from organized, complex tissues of a plant. Research has succeeded in producing a wide range of valuable secondary phytochemical in unorganized callus or suspension cultures (Dornenberg and Knorr, 1997). This situation often occurs when the metabolite of interest is only produced in specialised plant tissue or glands in the parent.

DESCRIPTION OF SELECTED PLANT SPECIES

Figure 1: Habit of *Emilia sonchifolia* (L.) DC

Scientific classification

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Asteridae
Order	:	Asterales
Family	:	Asteraceae
Genus	:	Emilia
Species	:	sonchifolia

Emilia sonchifolia (L.) DC commonly known as lilac tassel flower is a branching annual herb believed to be native to China and South-East Asia. They are fast growing weeds and can thrive under a wide range of conditions. They can grow in most soil types. Since spreading from its natural range, *E.sonchifolia* now has a pan-tropical distribution and is naturalized elsewhere in Asia, as well as in Australia, the Pacific Islands, Africa and the Americas. Currently it is listed as invasive in India, Mexico, Brazil, Costa Rica, the Galapagos, Puerto Rico, the Virgin Islands and on many other islands in the Pacific Ocean.

The plant is erect or prostrate at base and up to 10-150 cm tall. It often branches from very base, usually purplish green and deep rooting. Stem is cylindrical and pubescent. The leaves are lyrate-pinnatilobed, up to 10 cm long and 4 cm broad, sometimes becoming purplish as they get old. They are sessile or often with narrowly winged petiole, with alternate arrangement, dark green above and lighter green or tinged with purple beneath, and more or less irregularly coarsely dentate. On upper end of the stem leaves are lanceolate with their base encircling the stem, and those on lower end are oval or round.

The inflorescence is terminal head and few together in slender corymbs or rarely solitary. They are flat-topped with 3–6 stalked flower heads. The flower head is urn-shaped and consists of 30–60 florets per head. The flowers are usually pink, purple, white, red or orange in colour. The fruit is a dry one seeded achene, approximately 0.2 inches long and brown in colour. The fruit has white pappus hairs, which are up to 0.3 inches long. They help in wind dispersal of seeds.

The plant has been reported as a weed for a number of crops and has been shown to reduce yields and act as a reservoir for crop pathogens. It has certain effects on individual crops such as it decreases the weight of lettuce by 70% and mustard cabbage by 30% and also decrease yield of tomato by 18% (Floresca, 1976). It is a host of Xanthomonas campetris which

causes bacterial infection of beans in Brazil and Cuba (Rodriguez *et al*., 1991; Valarini and Spadotto, 1995).

The plant is documented in ethno medicine to possess medicinal benefits in treating diarrhoea, night blindness and sore throat (Ghani, 1998; Ahmed *et al*., 2009), rashes, measles, inflammatory diseases, eye and ear ailments (Shen *et al*., 2012), fever (Brando *et al*., 1985; Shen *et al*., 2012), stomach tumour (Mohiduddin *et al*., 2012), Malaria (Kohler *et al*., 2002), asthma (Panthong *et al*., 1986; Jain *et al*., 1994; Zani *et al*., 1995), liver disease (Yangf *et al*., 1987), eye inflammation (Singh *et al*., 1980), earache (Manandhar, 1994), chest pain (Parkash *et al*., 2008) etc. In China, the leaves are used for the treatment of dysentery and roundworm infestations, wounds and abscesses, influenza, burns and snake bites (Josh, 1998; Galinato *et al*., 1999). *E.sonchifolia* is also used by traditional midwives and other birth attendants in Africa during child delivery. Africans usually eat the leaves as vegetables for its laxative property and it has been documented in the Nigerian folk medicine for the treatment of epilepsy in infants.

In addition to several traditional uses, *Emila sonchifolia* is also listed in Ayurveda and Siddha systems of medicine. Ayurveda recommends the plant for treatment of gastropathy, diarrhoea, ophthalmia, nyctalopia, cuts and wounds, intermittent fevers, pharyngodyma and asthma. In Siddha system of medicine, the plant is recommended for treating intestinal worms and bleeding piles. It is one among the "Ten Sacred Flowers of Kerala collectively known as Dasapushpam. A study carried out on the plants showed that the aqueous leaf extract of *Emilia sonchifolia* has anti-inflammatory and analgesic activity (Essien *et al*., 2020). Along with these, the plant also possesses antioxidant activity, so that it can act as oxygen scavengers in biological systems. The present study was undertaken to compare the antioxidant properties of fresh plant parts with callus.

Aims and Objectives

In vitro development of callus offers a possibility of obtaining desirable compounds as well as ensuring sustainable conservation of economically important plants. As *Emilia sonchifolia* is considered as an important medicinal plant, its conservation is essential for its existence.

The main aim of this project was to standardize a protocol for the *in vitro* callus induction from leaf and internode explants of *Emilia sonchifolia* and to compare the effect of different plant growth regulators singly and in combination for callus induction.

The present study was also designated to investigate quantitative and qualitative analysis of various phytochemicals. Also, evaluating the antioxidant activity of the ethanolic extract of fresh stem and leaves of *Emilia sonchifolia*, and to compare it with callus.

The objectives of present study are;

- To standardize a protocol for developing callus from leaf and internode explants of *Emilia sonchifolia*
- To study the effect of different plant hormones on developing callus
- Callus culture using standardized medium
- Quantitative and qualitative phytochemical analysis of fresh samples of stem and leaves using different solvents
- Analysis of antioxidant activities of fresh stem and leaves
- Comparison of antioxidant properties of fresh plant extracts with callus extracts

Review of Literature

Advancement in the field of phytochemistry and molecular biology clearly demonstrated that secondary products play a major role in the adaptation of plant to their environment. These molecules largely contribute to plant fitness by interacting with the ecosystems. They have been described as being antibiotic, antifungal and antiviral, and therefore able to protect plants from pathogens (phytoalexins), and also anti-germinative or toxic for other plants (allelopathy) (Bourgaud, 2001).

The family Asteraceae is widely distributed and many of the plants in this family are medicinally important. It is because of the presence of chemical compounds in them. They had been used since ancient time all over the world as antioxidants, astringents, antipyretic, anti-inflammatory, diaphoretic in fevers, smooth muscle relaxant, nerve tonics, laxative and for treatment of wounds, bleedings, headache, pains, haemorrhoids etc. Phytochemical investigations of the Asteraceae family have revealed that many components from this family are highly bioactive.

Studies on chemical constituents of *Emila sonchifolia* was done by Gao *et.al* (1993) and five known compounds were obtained from the whole plant of by means of chemical and spectral methods. They were identified to be simiral, beta-sitosterol, stigmasterol, palmitic acid and honey acid.

Study on the anticancer activity of secondary metabolites from selected medicinal plants employing cell and tissue culture techniques were carried out by B.S Shylesh in 2000. Internode leaf disc and seed embryo were selected as explants and cultured in MS medium, whites medium and woody plant medium with different concentrations of phytohormones.

Emila sonchifilia showed maximum performance of shoot generation from MS medium supplemented with IAA 2mg$L^{-1}$and IBA 1mgL^{-1}.

Shylesh (2001) reported that callus induction is done through the culture of various parts of the *Emilia sonchifolia* viz. Shoot tips, leaf disc, petiole which is served as explants. For callus initiation MS medium supplemented with different combinations and concentrations of growth regulators were tested. Callus formation was noticed maximum in hormonal combination of 2, 4-D (2 mgL-1) and KN (0.5 mg L-1) in all explants.

Phytochemical studies indicated that the aerial parts of *Emilia sonchifolia* contain alkaloids (Cheng and Roder, 1986), flavonoids and terpernes (Srinivasan and Subramanian, 1980; Fu *et al.,* 2002; Lija *et al*., 2006). The preliminary phytochemical studies revealed the presence of flavonoids (Sharma *et al.*, 1996; Nwafor *et al*., 2003).

The pharmacological studies on *Emilia sonchifolia* have shown that the plant possess numerous notable biological activities such as antioxidant activities (Shyur *et al*., 2005; Guha *et al*., 2011; Sophia *et al*., 2011; Raj, 2012), analgesic and anti-inflammatory activities (Essien *et al*., 2009 and Rahman *et al*., 2012), anticancer activities (Shylesh and Padikkala, 2000; Jiny *et al*., 2010), anti-cataract activities (Lija *et al*., 2006; Patel *et al*., 2011; Singh *et al*., 2012), antimicrobial activity (Yoga *et al*., 2009), anti-diabetic (Monago *et al*., 2010), anti-fever (Ediriweera, 2007; Adebajo *et al*., 2012).

Micropropagation of *Emilia zeylanica* using explants of inflorescence rachis by C.B. Clarke *et.al* (2005). The highest shoot proliferation was obtained on Murashige and Skoog medium supplemented with 0.05 mg L^{-1} BAP.

Philip *et al.,* (2009) described a protocol for somatic embryogenesis of *Emilia zeylanica* (Asteraceae) a rare medicinal plant species, using stem explants. Highest frequency of embryogenic callus formation obtained from stem explants on MS media supplemented with

KIN (0.50 mgL-1) and 2, 4- D (0.10 mgL-1). Fast growing greenish yellow, nodular callus lines containing somatic embryos were established on initiation medium containing KIN (0.50 mgL-1) and 2, 4- D (0.50 mgl·l). The well-developed embryos germinated into complete plantlets on MS medium containing BAP (0.05 mgL-1) and ABA (0.10 mgL-1).

The preliminary phytochemical screening of methanol extract of leaf of *E. sonchifolia* revealed the presence of terpines, alkaloids, flavoniods, tannins and saponins in the extract (Essien *et al.,* 2009).

Hussein *et al*., (2010) reported that callus was obtained from hypocotyl explants of *Brassica nigra* (L.) *in vitro* under both dark and light incubation conditions separately using MS media enriched with sucrose, benzyladenine (BA), Naphthalene Acetic Acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4 D). The obtained calli were subjected to phytochemical screening. Results of their study have revealed that calli were rich in secondary metabolites as they gave positive results for testes of volatile oils, anthraquinones, flavonoids and tannins. The total phenolic content was higher in calli obtained under light incubation conditions than calli obtained under dark incubation conditions or the mother plant parts from which calli were induced. It is also observed that older calli accumulated more amounts of total phenolics.

Preliminary phytochemical screening of methanolic extract of *E. sonchifolia* revealed that carbohydrates, cardioglycosides, saponins, oils and fats, alkaloids, flavonoids, tannins and phenolic compounds, amino acids and quinones were present in the plant (Thenmozhi *et al*., 2011).

The phytochemical analysis and antibacterial activity of leaf and callus of *Centella asiatica* was conducted by Arumugam *et al* in 2011. Leaf explants were cultured on MS medium and maximum callus was achieved in medium supplemented with 4.0 mg/L BAP and 2.0 mg/L 2,4-D. In the preliminary phytochemical screening, alkaloids, glycosides, terpenoids,

steroids, flavonoids, tannins, saponins and reducing sugars were present in most of the tested extracts of leaf and *in vitro* grown callus of *C. asiatica*.

Sophia *et al*., (2011) conducted *in vitro* antioxidant activity and HPTLC determination of n-hexane extract of *Emilia sonchifolia* and the results of the study indicate that the n-hexane extract of the whole plant of *Emilia sonchifolia* possess a significant scavenging effect with increasing concentrations probably due to its antioxidant potential.

Shen *et al*., (2012) done a recent study on searching for bioactive natural products from the ethanol extracts of the aerial parts of *E. sonchifolia* obtained emiline which is a peculiar alkaloid.

In vitro callus production and preliminary phytochemical analysis of *Bacopa monnieri* was conducted by Kumar, (2012). The leaf callus of *B. monnieri* was initiated on MS medium supplemented with BAP (0.5) + NAA (1.0, 2.0mgL-1), 2, 4-D (2.0) + BAP (0.5,1.0 mgL1). Phytochemical tests on the leaf callus ethanolic and aqueous extract revealed the presence of Tannins, flavonoids, glycosides, terpenoids, saponins, and steroids.

Dominic Sophia *et al.,* (2012) investigate the effect of *n*-hexane extract of the herb, *Emilia sonchifolia* for the recovery from oxidative damage, biochemical and histopathological changes in the pancreas of the Wistar rats which was fed high protein diet.

Amin *et al*., (2013) had done an extensive review regarding the medicinally important plants of family Asteraceae that have been successfully micro propagated through tissue culture. In this study, they highlighted various methods and media used for the micro propagation of the genera Saussurea, Inula, Atractylodes, Artemisia, Echinacea, Helichrysum, Calocephalus, Vernonia, etc. In most of the cases, MS medium with different concentrations of auxins and cytokinins either alone or in different combinations was used. In some cases, other growth media like, Gamborg B5, Braun medium, Nitsch (N6) medium, Linsmair-Skoog

medium and growth adjuvants like TDZ, Zeatin (ZEA), casein hydrolysate (CH), tyrosine, glutamine, AgNO3, 2-ip, Malt Extract (ME), maize extract have also been used which proved very effective.

Arya and Patni (2013) conducted a preliminary phytochemical investigation in *in vivo* (leaf, stem and root) and *in vitro* (callus) plant parts of *Pluchea lanceolata*. Callus was produced on MS medium consisting of basal salts and vitamins with 3% (w/v) sucrose and 0.8% agar with NAA (1.0 mg/l) and BAP (0.5 mg/1) using leaf explants. Results of phytochemical evaluation revealed the presence of alkaloids, flavonoids, proteins, carbohydrates, tannins, phenols, glycosides, and terpenoids.

Supe *et al*., (2014) presented a review on successful methods for the micro propagation and phytochemical analysis of callus and leaf extracts of *Momordica charantia.* Callus was formed on 2, 4-D, with profuse callusing at 2mgl-1 of 2,4-D. A combination of NAA+BAP+2,4-D was most effective for callus formation with best response in 2mgL-1 NAA + 0.5 mg L-1 BAP+2 mgL·1+2,4-D. Qualitative phytochemical analysis of *M. charantia* confirms the presence of phytochemicals like flavonoids, saponins, terpenoids, coumarins, emodins, alkaloids, proteins, cardiac glycosides, anthraquinones, anthocyanins, steroids etc.

Chandra *et al*., (2014) reported the phytochemical analysis of callus extracts of *Biophytum sensitivum*. Callus is formed from the leaf explants of this species on Murashige and Skoog basal medium supplemented with various concentrations of BA and NAA. BA 1.0 mgL-1 with NAA 1.0 mgL-1 is the best concentration for optimal results. The callus extracted sequentially with hexane, chloroform, ethyl acetate and methanol.

Achika *et al*., (2014) done a review on the medicinal properties alongside with peculiar phytoconstituents of various plants belonging to the Asteraceae family. Various medicinal effects of these plants may be due to the presence of a broad range of secondary

bioactive metabolites such as flavonoids, phenolic acids, coumarins, terpenoids (monoterpenes, sesquiterpenes, diterpenes and triterpenes) and sterols which have been frequently reported from the Asteraceae family.

The phytochemical examination of *E. sonchifolia* revealed presence of a wide range of chemical constituents that are responsible for its promising multidimensional pharmacological activities, such as antimicrobial, analgesic, anti-inflammatory, antioxidant, anticancer, antidiabetic, hepatoprotective, antianxiety, anticataract and anticonvulsant activities. A few phytoconstituents have been isolated and the plant is rich with flavonoids. (Dash Gouri Kumar *et al.,* 2015).

C.H Hsieh *et al.,* (2015) analysed hepatotoxic pyrrolizidine alkaloids in *Emilia sonchifolia* from Taiwan. 11 pyrrolizidine alkaloids (PAs) identified in *Emilia sonchifolia*, for 1st time. PA content level for different tissues are analysed in this study and observed high level of toxic PA content.

The micro propagation and phytochemical analysis of *Scrophularia kakudensis* was carried out by Manivannan *et al*., (2015). For phytochemical analysis the methanol extracts of *in vitro* plants cultured in MS medium without PGRs, callus induced by 3.0 mgL^{-1} BA and greenhouse grown *in vivo* plants (separated into shoot and root) were taken. The total phenol and flavonoid contents of *in vitro* shoot extract, *in vitro* root extract, callus extract, *in vivo* shoot extract, and *in vivo* root extract were estimated. Among the tissues, shoot contained larger amount of total phenol and flavonoid contents than root extracts.

Qualitative assay for the presence of phytochemical constituents revealed the presence of constituents such as alkaloids, saponins, flavonoids, tannins, cardiac glycosides and anthraquinones in ethanolic extracts of leaves of *E. sonchifolia* (Edagha *et al.,* 2015).

The phytochemical screening on Hexane leaf extract of *Emilia sonchifolia* showed the presence of flavonoids, tannins and saponins (Okey and Asuqwo, 2016).

The phytochemical contents of some milled Nigerian softwood chips were carried out by Chukwuma S. Ezeonu *et.al* (2016) in a quest to evaluate their potentials as sources of alternative medicine and other industrial applications. The qualitative and quantitative analysis for different phytochemicals were ascertained.

Rohela *et al.,* (2016) conducted phytochemical screening of leaf, stem and root extracts and their callus. Alkaloids, tannins and phenolic compounds are observed in leaf, stem and root extracts, while alkaloids are observed only in root callus extracts. Flavonoids are present in both stem and leaf extracts, while they are observed only in leaf callus extracts.

Godwin Michael *et.al*., (2017) evaluated the efficacy of *Emilia sonchifolia* as an antibacterial agent and as an effective herbal therapy for the treatment of diarrhoea and other diseases associated with causative pathogenic organisms. The results indicates that leaf extracts of *Emilia sonchifolia* can be harnessed and used in the formulation of medicinal therapy for the treatment of diarrhoea.

Manasa *et al.,* (2017) reported the rapid *in vitro* callogenesis and phytocmemical analysis of Mussaenda frondosa. The leaf explants were inoculated on to Murashige and Skoog medium supplemented with varying concentrations of growth regulators such as 2, 4-D, NAA, BAP, KIN for the induction of callus. Pale green, healthy, friable, and fast-growing callus were obtained on the medium enriched with NAA (2 mgL-1) + KIN (4 mgL-1). Phenolics, flavonoids, alkaloids and glycosides detected in leaf, stem and callus extracts. Chloroform extract of leaf, stem, and callus showed a maximum number of phytochemicals tested.

Rajesh K Joshi (2018) studied volatile compounds of *Emilia sonchifolia* from India. The volatile constituents were isolated by hydro-distillation and were analysed for the first time

by using GC-FID and GCMS methods. Forty-three compounds were identified, representing 96.3% of the total oil. The oil was found to be rich in sesquiterpene hydrocarbon (67.6%) type constituents.

Phytochemical screening and antioxidant activity of *Emilia sonchifolia* was done by Neethu Vijaykumar *et al*., in 2018. Phytochemical screening showed the presence of alkaloids, terpenoids, carotenoids, flavonoids and tannins. Antioxidant activity of the methanolic extract of was E. sonchifolia 65.28% for 1mg/ml. The results obtained in this study confirms antioxidant potential of E. sonchifolia.

Akintayo L. Ogundajo *et al.,* (2021) extracted essential oils from the leaves of sesquiterpenoid rich *L.egregia* and *E.sonchifolia* by hydrodistillation and screened for antimicrobial activity against a panel of bacteria and fungi. Based on these results, either *L. egregia* or *E. sonchifolia* essential oil may be recommended for exploration as complementary antibacterial or antifungal agents.

Comparative analysis of the antibacterial effects of *Emilia sonchifolia* and selected antibiotics on ocular bacteria *in vitro* showed that the effect of *Emilia* doesn't differ significantly from the antibiotics. This shows that *Emilia sonchifolia* could become a promising natural antibacterial agent with potential application in pharmaceutical industries for the production of plant based ocular drugs. (Ugvoke, G.I *et al*.,2021).

Ushie *et al*., (2022) studied antioxidant activities of *Emilia sonchifolia* using chloroform, acetone and methanol leaf extracts and the DPPH radical scavenging assay showed that extracts of methanol and acetone have a good scavenging activity among all the extracts.

From this extensive literature survey, the relevance of the present study is very well emphasised.

Materials and Methods

IN VITRO PROPAGATION OF *Emilia sonchifolia*

In vitro propagation protocols in *Emilia sonchifolia* were carried out through standard techniques at the Plant Tissue Culture Laboratory.

Source and choice of plant material

Emilia sonchifolia plants were collected from [redacted] The plants were brought to the laboratory and planted in pots and maintained in green house of the college. Leaf and internode explants were used for the present investigation.

Establishment of cultures

Glassware and instruments

Glassware such as beakers, conical flasks, petridishes, standard flasks, pipettes, measuring cylinders etc., used were of Borosil. Jam bottles and culture tubes were used for the establishment of culture.

All the glassware in use was regularly cleaned to ensure no contamination. Glassware was thoroughly washed in running water using Teepol detergent. All the cleaned glassware were placed in hot air oven for 2 hours at 100°C to make them dry. Culture vessels were reused after proper washing and sterilization.

Accessories like scalpels, forceps, and knives, made of stainless steel, were sterilized every time before use. The equipment and instruments used in laboratory include Laminar Air Flow Cabinet, Hot Air Oven, Microwave Oven, Refrigerator, Electronic balance, Digital Electronic pH Meter, Autoclave, Steel Racks, Air Conditioner etc.

Basal Media

Preparation of culture media

Standard procedures were followed for the preparation of media. The media strength, plant growth regulators and other supplements used is depicted in Table 1. Stock solutions of macronutrients and micronutrients were prepared at 20X and 100X concentrations respectively. Iron source stock solution and vitamin stock solution were made as 20X and 100X concentrations.

The concentration and combinations of growth regulators used are presented in Table 2. All stock solutions were prepared in distilled water. The growth regulators that are not soluble in water are dissolved in small amounts of 1N sodium hydroxide and made to final volume with distilled water.

The stock solutions in the required quantity were measured out into a standard flask. 3% sucrose and 10mgL^{-1} myo-inositol were added and dissolved in the media. All plant growth regulators were added before the making up of media. Then it is made up to required volume by using distilled water. The pH of the medium was adjusted between 5.6 and 5.8 using in NaOH or 0.1N HCl. 0.9% bacteriological agar was then added to the solution as solidifying agent. The solution was mixed well and heated in microwave oven till the agar is dissolved uniformly. The medium was then poured in to pre-sterilized culture vessels. 15 ml medium was poured in the culture tubes and 50 ml was taken in jam bottles (250ml). The culture tubes and bottles containing nutrient medium were plugged tightly with non-absorbent cotton wool plugs and cling film to prevent entry of air contaminants.

CONSTITUENTS	CONCENTRATION IN MEDIUM (mgL^{-1})	CONSTITUENTS IN STOCK (mgL^{-1})	VOLUME OF STOCK PER LITRE OF MEDIUM (ml)
MACRONUTRIENTS			
NH_4NO_3	1650	33000	
KNO_3	1900	38000	
$CaCl_2.2H_2O$	440	8800	20
$MgSO_4.7H_2O$	370	7400	
KH_2PO_4	170	3400	
MICRONUTRIENTS			
KI	0.83	88	
H_3BO_3	6.2	620	
$MnSO_4.4H_2O$	22.3	2230	
$ZnSO_4.7H_2O$	8.6	860	100
$Na2MoO4.2H_2O$	0.25	25	
$CuSO4.5H_2O$	0.025	2.5	
$CoCl_2.6H_2O$	0.025	2.5	
IRON SOURCE			
$FeSO_4.7H_2O$	27.8	556	
$Na_2EDTA.2H_2O$	37.3	746	20
VITAMINS			
Myo-inositol	100	Added freshly	
Nicotinic acid	0.5	50	
Pyridoxine-HCL	0.5	50	
Thiamine- HCL	0.1	10	20
Glycine	2	200	
CARBON SOURCE			
Sucrose	3%	Added freshly	

Table 1: Constituents of the medium, concentration and volume of the stock of M.S media prepared for the present study

Plant growth regulators	Concentrations (mgL^{-1})
IAA	2.5 2 11 1.1 0.2
2,4-D	3 5 7 8
NAA	2 3 5
IAA + 2,4-D	1, 5 2, 5 1, 1 2, 2
2,4-D + NAA	5, 1 5, 2 1, 1
2,4-D + BAP	0.5, 0.5 2, 0.5
NAA + BAP	1, 2 4, 0.5 5, 5

Table 2: Plant growth regulators and their concentrations in the present study

Sterilization

The sterilization of the culture medium was carried out in an autoclave for at 121°C and 15 lbs pressure. After sterilization, the culture tubes and bottles were stored in an air-conditioned culture room until further use.

All petriplates and beakers used in the inoculation cabinet were sterilized in autoclave at 121°C and 15 lbs pressure for 20 minutes. Scalpels, forceps and other essential instruments used were also flame sterilized after dipping in 70% alcohol.

Explant preparation and Surface Sterilization

The explants collected from the source plants were washed in running tap water for 15 minutes. The explants were washed for 30 minutes in Tween-20 solution. Then washed in distilled water for 3-4 times and dipped in distilled water.

To avoid contamination, a Laminar Air Flow cabinet was used for all sterile operations. The chamber was made sterile by exposure to UV rays for half an hour before use. The work surface and hands were swabbed down every time with 70% alcohol prior to use. All the operations were carried out using pre sterilized instruments and glassware.

The explants were brought in to the Laminar Air Flow Cabinet and transferred to sterilized beaker containing 0.5% Mercuric Chloride solution. Mercuric Chloride solution was prepared by adding 0.5g of Mercuric Chloride in 100ml sterilized distilled water. Explants were sterilized for 5 minutes. The explants were rinsed with sterilized distilled water several times, decanted and allowed to become surface dry. Then the explants were transferred to the presterilized petriplates with the help of sterile forceps.

Inoculation and incubation

The leaf explants were trimmed, including the midrib to appropriate size (1x1cm) by using sterile scalpel blade. Wounds are created on the lower surface of the leaf explants. Then explants were inoculated in to the solidified culture medium using sterile forceps. After the inoculation the mouth of culture tubes were flamed quickly and tightly plugged.

After proper labelling clearly mentioning hormone concentration, date of inoculation etc., the culture tubes were transferred to culture room.

The cultures were maintained in the culture room at 25°C under 16:8 light and dark regime. The cultures were given illumination by white fluorescent light.

Callus subculture

After 3-4 weeks, the callus developed was sub cultured on to the fresh medium with same concentration of growth regulators.

PHYTOCHEMICAL ANALYSIS

For phytochemical analysis, fresh intact plant parts like stem and leaves were used. For extraction, two polar (butanol and distilled water) and non-polar solvents (benzene and chloroform) were selected.

Preparation of plant samples

Fresh plant parts of the selected plant were excised and washed thoroughly in distilled water. 5g of plant samples were weighed out and grinded using mortar and pestle. The grounded plant samples were soaked in 50ml of benzene, butanol and chloroform for 72 h. The extracts were collected and filtered by using cheese cloth and Whatsman No.1 filter paper. Distilled water extracts were obtained by adding 50ml distilled water to the plant samples and kept at 70°C for 2h. The extracts were filtered using cheese cloth and Whatsman No.1 filter paper.

The extracts were oven dried below their respective boiling temperature. Then the residues were weighed and redissolved in 30ml of their respective solvents. All the extracts were stored in tight screw cap bottles at -4°C and used for various phytochemical analysis.

Qualitative analysis

The plant extracts (fresh stem and leaves) prepared using solvents like benzene, butanol, chloroform and distilled water were screened for the presence of the phytochemical constituents by using the standard methods of Sofowora (1982), Kepm (1986), and Harborne (1973). The results were compared with ethanolic extracts of callus.

1. Detection of flavonoids

a) **Alkaline reagent Test:** An aqueous solution of the extract treated with 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavonoids.

b) **Lead acetate Test:** Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

2. Detection of alkaloids

Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

a) **Dragendorff's Test -** Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth iodide). Formation of reddish orange precipitate indicates the presence of alkaloids.

b) **Hager's Test -** Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.

3. Detection of Coumarins

1 ml each of alcoholic extracts was treated with alcoholic 10% NaOH solution. Production of dark yellow colour indicated the presence of coumarins.

4. Detection of Phenols

a) **Ferric chloride Test -** A fraction of the extracts was treated with aqueous 5% ferric chloride and observed for formation of deep blue or black colour.

b) **Lead acetate test -** extracts dissolved in distilled water and to this 3ml of 10% lead acetate solution is added. A bulky precipitate indicates the presence of phenols.

5. Detection of protein and amino acids

5 ml each of the various extracts were dissolved in 5 ml of water separately and were subjected to the following tests.

a) **Biuret test -** 1 ml of each of the various extracts was warmed gently with 10% NaOH solution and a drop of diluted $CuSO_4$, solution. Formation of reddish violet colour indicated the presence of proteins and amino acids.

b) **Ninhydrin test-** 1 ml each of the various extracts was separately treated with a few drops of ninhydrin solution (10mg in 200ml acetone), Change in colour showed the presence of proteins and amino acids.

6. Detection of Saponins

a) **Froth Test -** Extracts were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

b) **Foam Test -** 0.5 gm of extracts was shaken with 2 ml of water. If foam produced persists for few minutes indicates the presence of Saponins.

7. Detection of Tannins

a) **Gelatin Test -** Test solution when treated with gelatin solution would give white precipitate indicating the presence of tannins.

5 ml each of the various extracts was dissolved in minimum amount of water separately filtered add filtrate were then subjected to the following test.

b) **Ferric chloride Test-** To the above filtrate a few drops of $FeCl_2$ solution were added. The colour change indicates the presence of tannins

c) **Basic lead acetate test-** To the filtrate a few drops of aqueous basic lead acetate solution are added. Formation of reddish-brown precipitate indicated the presence of tannins.

8. Detection of Diterpenes

a) **Copper acetate test -** Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of Diterpenes.

9. Detection of Glycosides

a) **Keller Killani Test -** Crude extract was mixed with 2 ml of glacial acetic acid containing 1-2 drops of ferric chloride solution. The mixture was then poured into another test tube containing 2 ml of conc. H_2SO_4. A brown ring at the interphase indicates the presence of cardiac glycosides.

b) **Bromine water Test -** Test solution was dissolved in bromine water and observed for the formation of yellow precipitates to show a positive result of the presence of glycosides.

10. Detection of Carotenoids

1g of each sample was extracted with 10 ml of chloroform in a test tube with vigorous shaking. The resulting mixture was filtered and 85% H_2SO_4 was added. A blue colour at the interphase showed the presence of carotenoids.

11.Detection of Sugars

Each of the various extracts were dissolved separately in 5ml distilled water, filtered and then subjected to the following tests.

a) **Molisch's Test-** To the filtrate a few drops of alcoholic alpha- naphthol and 2 ml of conc. H_2SO_4 were added slowly through the sides of the test tube. Formation of reddish-brown precipitate indicated the presence of sugars.

b) **Fehling's Test -** A small portion of the various filtrate were treated with 1 ml of Fehling's solution A and B and then heated gently, change in colour indicated the presence of sugars.

c) **Benedict's Test –** Filtrates were treated with Benedict's reagent and gently heated. Formation of orange red precipitate indicates the presence of sugars.

Quantitative analysis

Quantitative analysis was performed using distilled water extracts and using this, the following tests were conducted.

1. Estimation of total phenol content

Total phenol content was estimated by Malick and Singh, (1980). 0.5ml of sample is pipette out into test tubes and made up to 3ml using distilled water. Add 0.5ml of Folin- Ciocaltueau regent and after 3 minutes, add 2ml of 20% sodium carbonate solution to each test tube. Mix thoroughly. Place the test tubes in boiling water for exactly 1 minute. Cool and measure the absorbance at 650 nm against a reagent blank. The total phenol content was determined using the regression equation **x = 0.576y +**

0.057 (y is the OD of the sample) based on catechol and expressed the content in mg g^{-1}.

2. Estimation of total flavonoid content

Total flavonoid content was estimated using the procedure described by Zhishen *et al*. A total of 1ml of plant extract was diluted with 200µl of distilled water separately followed by the addition of 150µl of sodium nitrite (5%) solution. This mixture was incubated for 5 minutes and then 150µl aluminium chloride (10%) solution was added and allowed to stand for 6 minutes. Then 2ml of sodium hydroxide (4%) solution was added and made up to 5ml with distilled water. Mixture was shaken well and left it for 15 minutes at room temperature. The absorbance was measured at 510 nm. Total flavonoid content was quantified using the regression equation **x = 0.3865y - 0.0082** (y is the OD of the sample) and expressed the content in mg g^{-1}.

Estimation of Antioxidant Activity

Comparative antioxidant activity of callus and fresh plant parts of *Emilia sonchifolia* was estimated by DPPH free radical scavenging assay using the method of Blois (1958) and phosphomolybdate assay proposed by Prieto *et al*., (1999). Qualitative estimation of alkaloids, phenols, glycosides, flavonoids and carotenoids; and quantitative analysis of total flavonoid and phenolic content were estimated using the methods given above. For estimation, ethanolic extracts were used.

Collection and preparation of plant material

The 3-4week old callus was taken from the medium, washed in distilled water and used for analysis. Fresh stem and leaves from the intact plants were used for the comparison.

For extraction, 0.5g of callus and fresh plant parts were taken and grinded in 5ml ethanol using mortar and pestle. The extracts were filtered using cheese cloth. It was centrifuged and the supernatant was collected. For dilution, 10ml ethanol was added to 200 µl of extract to make it 1mg/ml. From this sample, different concentrations were taken for DPPH assay.

DPPH Scavenging Assay

DPPH (2,2-diphenyl -1-picrylhydarzil) was prepared in ethanol solution. To 1ml DPPH, 3ml of extract was added separately in a range of concentration 50 µg/ml to 250 µg/ml (50, 100, 150, 200 and 250 µg/ml). The mixture was incubated for 30 minutes in dark. After dilution, the absorption of reduced DPPH was measured at 517nm using spectrophotometer. Ethanolic DPPH was used as control.

Lesser values of absorbance of the reaction mixture indicate higher free radical scavenging activity. The Radical scavenging activity (RSA) was calculated in percentage by following formula.

$$\textbf{RSA (\%)} = \frac{\textbf{absorbance of conrol} - \textbf{absorbance of test}}{\textbf{absorbance of control}} \times \textbf{100}$$

The results were also reported as IC_{50} which is amount of antioxidant required to decrease the DPPH concentration by 50%. IC_{50} values were calculated from regression analysis and expressed as µg dry weight equivalents per ml sample. The experiments were performed in triplicates.

Phosphomolybdate Assay for Total Antioxidant Capacity

Total Antioxidant Capacity (TAC) of *Emilia sonchifolia* extracts was determined as per phosphomolybdate assay proposed by (Prieto *et al*;1999).

For sample preparations, 250µg callus and fresh plant extracts were dissolved in 1 ml ethanol and kept for 5 minutes to get the homogenous mixture. Ascorbic acid was used as a standard. A stock solution of ascorbic acid (5000 mg/L) was prepared in distilled water, from which dilutions were made ranging from 50µg to 200µg. In a test tube, 300µl plant extract was mixed with 3 mL phosphomolybdate reagent (1ml of each 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The test tube was covered with aluminium foil and incubated at 95°C for 90 minutes. The mixture was then allowed to reach room temperature and absorbance was read at 765 nm. Blank was run using the same procedure but containing an equal volume of ethanol in place of plant extract. The antioxidant capacity was reported as µg of ascorbic acid equivalent.

Results and Discussion

The main aim of this project was to standardize a protocol for the *in vitro* callus induction from leaf and internode explants of *Emilia sonchifolia* and to compare the effect of different plant growth regulators singly and in combination for callus induction.

The study also aims to investigate quantitative and qualitative analysis of various phytochemicals. Also, evaluating the antioxidant activity of the ethanolic extract of fresh stem and leaves of *Emilia sonchifolia*, and to compare it with callus.

Callus induction and establishment

In the present study, MS medium (Murashige and Skoog, 1962) is used as the basal medium as it is a high salt medium and hence the plant *Emilia sonchifolia* also showed a positive response in this medium. In this study different concentrations of auxins such as Indole-3-acetic acid (IAA) 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1-Naphthalene Acetic Acid (NAA) are used. They also used in combination with each other and with cytokinin 6-Benzylaminopurine (BAP). Leaf and internodes are used as explants and the response intensity in these plant growth regulators is presented in Table 3. Visual grading is done to assess the performance and it is represented in the table with '+' and '-' signs.

Callus initiation was observed from the cut surfaces of the leaf and internode and also from the surface of explants within 7-9 days of inoculation. Leaf showed more response than internodes. Initial response of the explants is documented as the swelling of explants (figure 2). Some of them turned brown after few days. After 12-13 days of inoculation, green friable callus was observed which later turned brown within the next 5-7 days (figure 3). Callus were sub cultured after 5-6 weeks.

Table 3: Effects of various PGRs on callus induction

Plant growth regulators	Concentration (MgL^{-1})	Response of explants for callus	
		Leaf	Internode
IAA	2.5	-	-
	2	-	-
	11	-	-
	1.1	-	-
	0.2	-	-
2,4-D	3	-	-
	5	+	-
	7	++	-
	8	-	-
NAA	2	-	-
	3	+++	+
	5	-	-
IAA + 2,4-D	1, 5	-	-
	2, 5	-	-
	1, 1	-	-
	2, 2	-	-
2,4-D + NAA	5, 1	+	-
	5, 2	+	-
	1, 1	-	-
2,4-D + BAP	0.5, 0.5	-	-
	2, 0.5	-	-
NAA + BAP	1, 2	+	++
	4, 0.5	+	-
	5, 5	+++	+++

'+': feeble response, '++': moderate response, '+++': good response, '-': no response

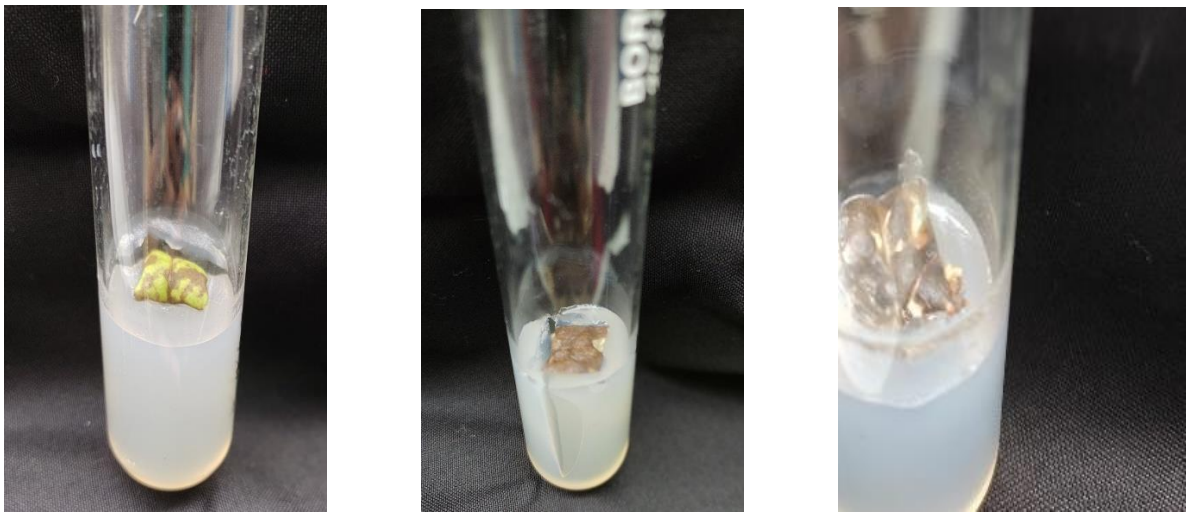


Figure 2: Initial stages of callus induction

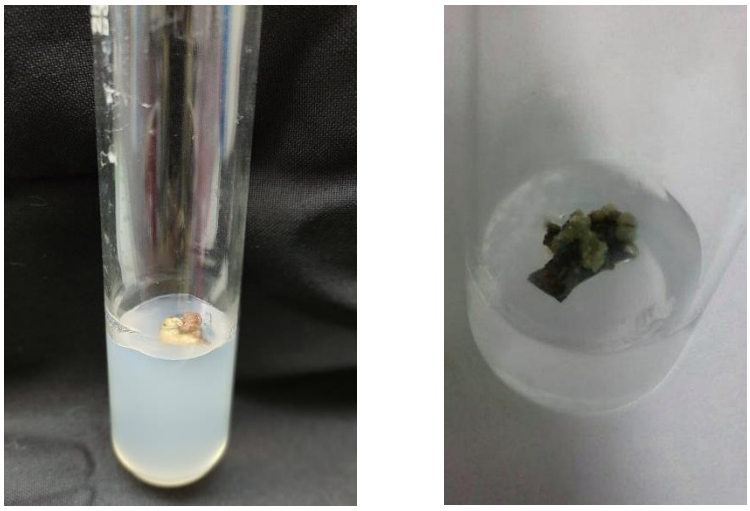


Figure 3: callus obtained from leaves and internodes

Preliminary Phytochemical Analysis

Qualitative analysis

Preliminary phytochemical analysis of different extracts of fresh stem and leaves were done (figure 4) and the results is given in the table 4 and 5 respectively. The phytochemicals were compared with ethanolic extracts of callus and is shown in Table 6.

Benzene extract of fresh stem showed the presence of alkaloids, diterpenes and glycosides. Butanol extracts showed the presence of carbohydrates, diterpines, glycosides, carotenoids and caumarins. Distilled water extracts contains flavanoids, carbohydrates, phenols and diterpenes. Chloroform extract possess flavanoids and carotenoids.

Benzene extract of fresh leaves showed the presence of alkaloids, glycosides and carotenoids. Butanol extracts possess carbohydrates, phenols, tannins, glycosides, carotenoids and caumarins. Distilled water extracts contain alkaloids, flavanoids, carbohydrates and phenols. Flavanoids, carotenoids and glycosides are present in chloroform extracts.

Ethanolic extract of callus showed the presence of alkaloids, flavonoids, carbohydrates, phenol, glycosides and carotenoids.

Phytochemical compounds	Benzene extract	Butanol extract	Chloroform extract	Distilled water extract
Alkaloids	+	-	-	-
Carbohydrates	-	+	-	+
Caroteinoids	-	+	+	-
Caumarins	-	+	-	-
Diterpenes	+	+	-	+
Flavanoids	-	-	+	+
Glycosides	+	+	-	-
Phenols	-	-	-	+
Proteins & amino acids	-	-	-	-
Saponins	-	-	-	-
Tannins	-	-	-	-

Table 4: Qualitative analysis of different extracts of stem of *Emilia sonchifolia*

"+" present

"-" absent

Phytochemical compounds	Benzene extract	Butanol extract	Chloroform extract	Distilled water extract
Alkaloids	+	-	-	+
Carbohydrates	-	+	-	+
Caroteinoids	+	+	+	-
Caumarins	-	+	-	-
Diterpenes	-	-	-	-
Flavanoids	-	-	+	+
Glycosides	+	+	+	-
Phenols	-	+	-	+
Proteins & amino acids	-	-	-	-
Saponins	-	-	-	-
Tannins	-	+	-	-

Table 5: Qualitative analysis of different extracts of leaf of *Emilia sonchifolia*

"+" present

"-" absent

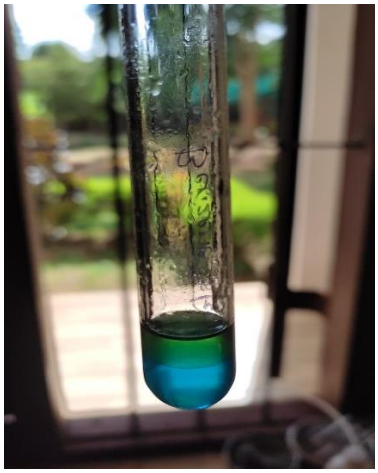
Phytochemical compounds	Stem	Leaves	Callus
Alkaloids	+	+	+
Carbohydrates	+	+	+
Caroteinoids	+	+	+
Caumarins	+	+	-
Diterpenes	+	-	-
Flavanoids	+	+	+
Glycosides	+	+	+
Phenols	+	+	+
Proteins & amino acids	-	-	-
Saponins	-	-	-
Tannins	-	+	-

Table 6: Comparative qualitative analysis of stem, leaves and callus

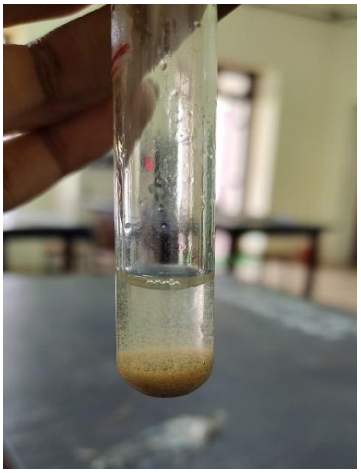
"+" present

"-" absent

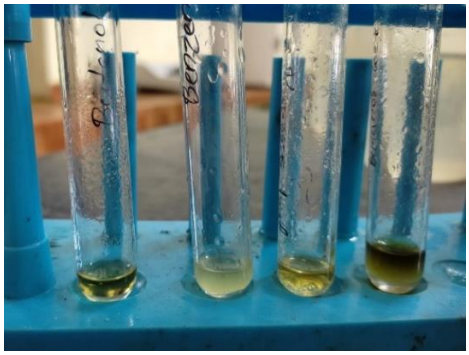
Figure 4: Different preliminary phytochemical analysis of fresh stem, leaves and callus of *E. sonchifolia*.



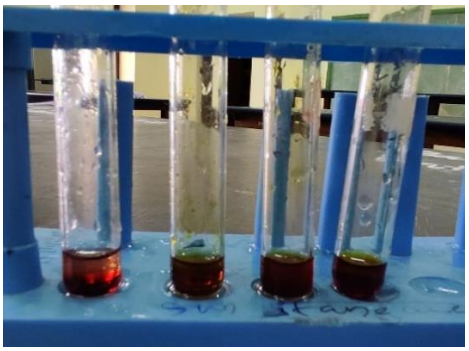
Test for diterpenes



Test for flavanoids (lead acetate test)



Test for coumarins



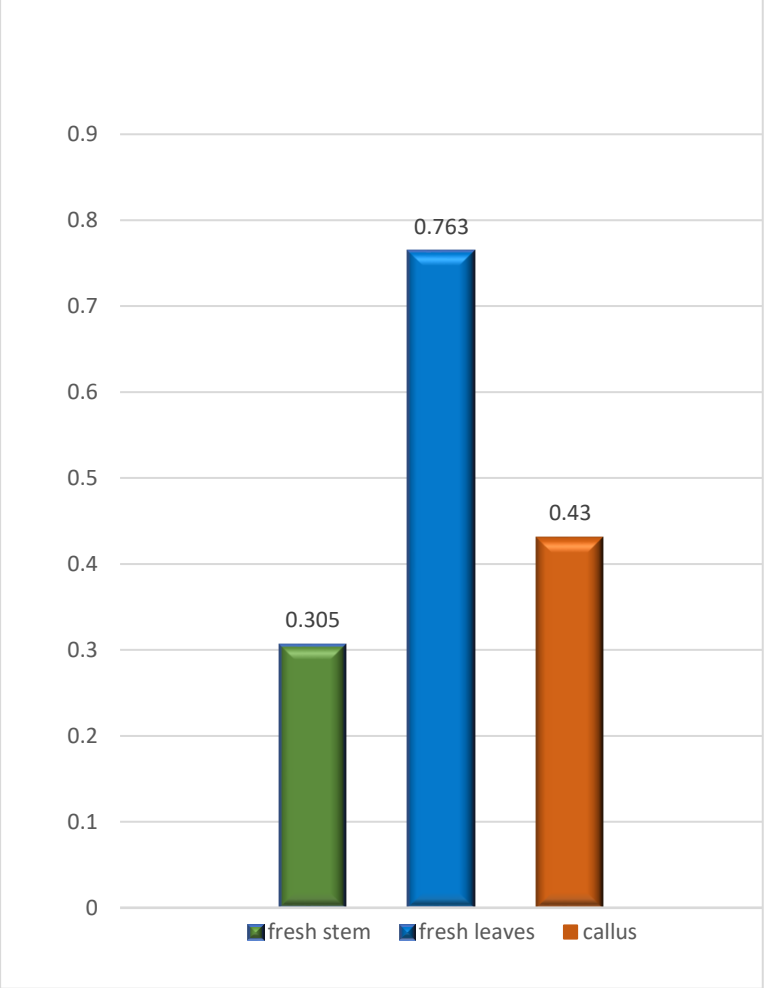
Test for alkaloids (Drangendorff's test)

Quantitative analysis

Phenol and flavonoid contents were estimated quantitatively from the distilled water extracts of fresh stem, leaves and callus samples. The results are given in the graphs below.

Total phenol content

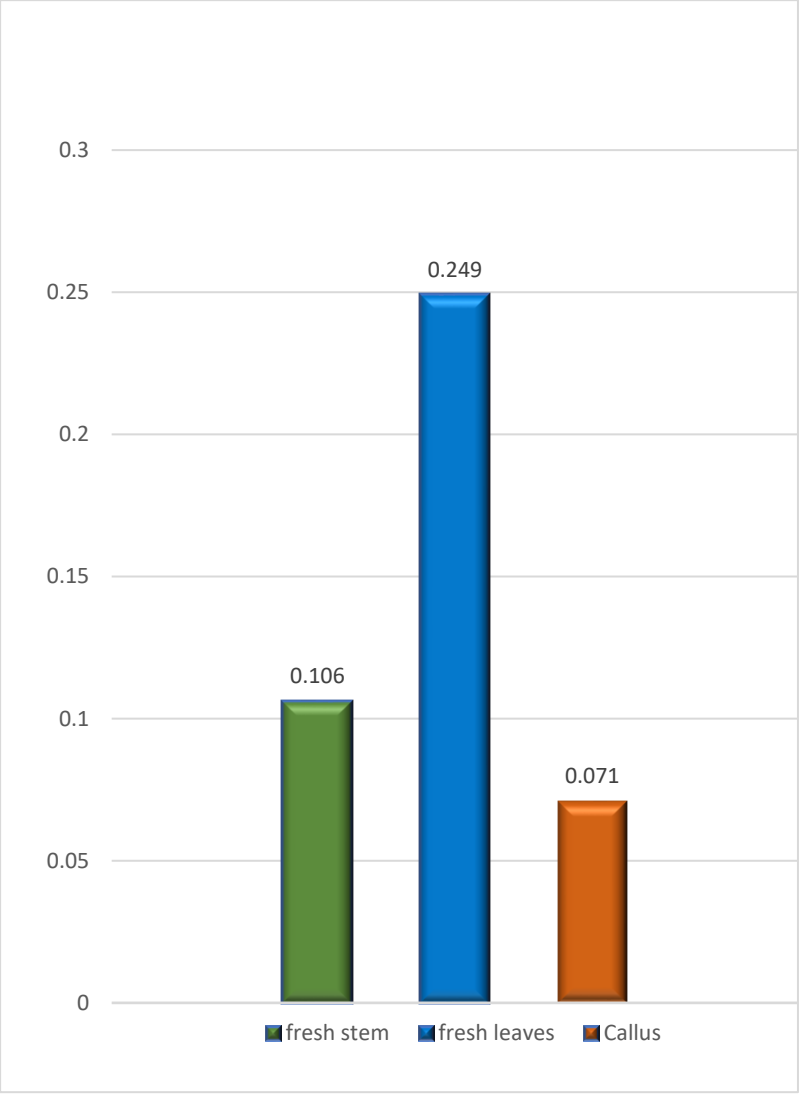
Total phenol content in fresh stem, leaves and callus is given in the graph 1. Obtained Absorbance was read at 650nm and observed O.D for stem is 0.431, for leaf is 1.226 and for callus is 0.649. The highest phenol content was showed by leaf samples and lowest was observed for stem samples.



Graph 1: Total phenol content (mg g^{-1})

Total flavonoid content

Total flavonoid content was estimated from fresh stem, leaves and callus. It is shown in the graph 2. Absorbance was measured at 510 nm and obtained the values 0.666 for leaf, 0.298 for stem and 0.206 for callus. Flavonoid content was high in leaves and low in callus.



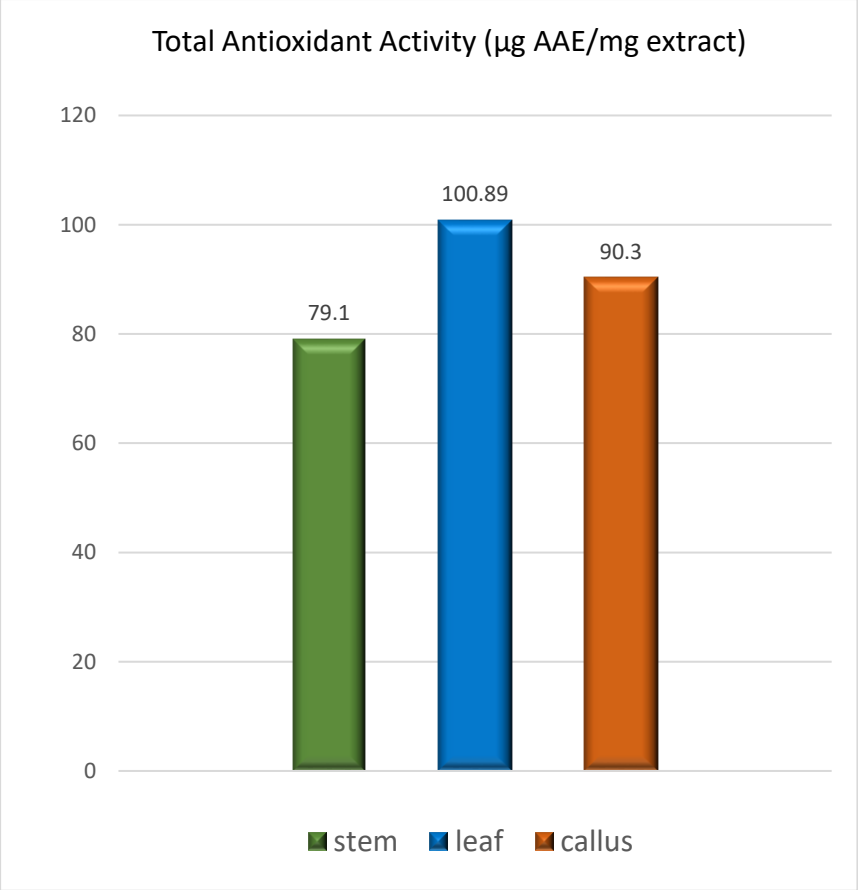
Graph 2: Total flavonoid content

Antioxidant activity

Antioxidant activity of fresh stem and leaves are compared with *in vitro* generated callus. The positive results of alkaloids, phenols, glycosides, flavonoids and carotenoids obtained from the preliminary qualitative estimation indicate the antioxidant activity of the callus and fresh plant parts. The percentage of antioxidant activity was estimated using DPPH assay and phosphomolybdate assay. The results are given below.

Phosphomolybdate assay

The phosphomolybdate method has been used to evaluate the antioxidant capacity of stem, leaves and callus extracts. In the presence of extracts, Mo (VI) is reduced to Mo (V) and forms a green coloured phosphomolybdenum (v) complex which shows maximum absorbance at 760nm (figure 5). Ascorbic Acid Equivalence (AAE) of stem, leaves and callus is calculated using the regression equation **(y= 0.0132x – 0.281)** obtained from the standard graph of ascorbic acid. In the regression equation y is the O.D of the sample. AAE is expressed as µg/mg of extract. Maximum antioxidant activity is observed for leaf extracts and minimum for stem extracts. It is shown in the graph 3.



Graph 3: Total Antioxidant Activity of stem, leaves and callus

DPPH radical scavenging assay

The DDPH assay was used to measure the antioxidant activity of the plant extracts (figure 6). The antioxidant values (percentage of inhibition) of the ethanolic extract of stem, leaves and callus were examined. The percentage of scavenging activity of DPPH radical was found to be concentration dependent. The percentage of inhibition increases with increasing concentrations. The result is shown in the table 6.

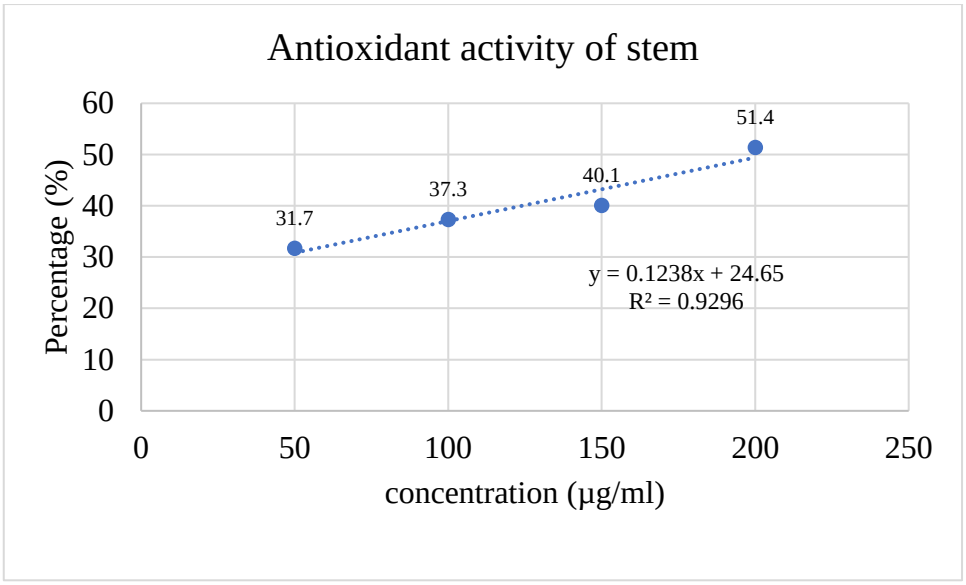
Concentration of sample (µg/ml)	Percentage of inhibition (%)		
	stem	leaves	callus
50	31.7	43	21
100	37.3	45	21.4
150	40.1	50	35.04
200	51.4	54.2	43.4

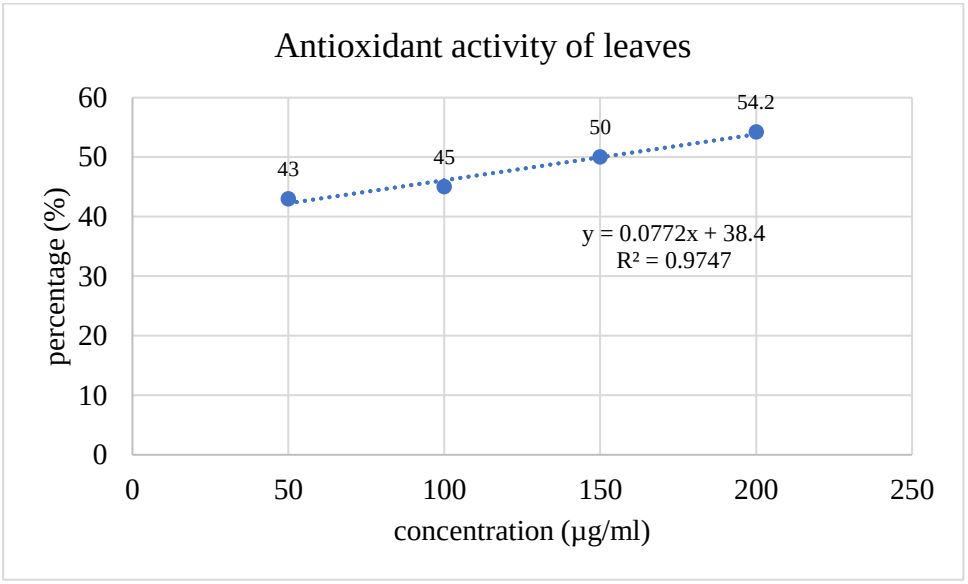
Table 6: Antioxidant properties of stem, leaves and callus of *E.sonchifolia*

When different concentrations were plotted against percentage of inhibition, a graph is obtained from which IC_{50} is calculated using the regression equation y= mx + c where, x is the IC_{50} value which determines the concentration of the sample required to inhibit 50% of radical. The lower the IC_{50} value, the higher the antioxidant activity of samples. The obtained graphs showing antioxidant activity of stem, leaves and callus are shown in the graph 6, 7 and 8 respectively. IC_{50} values obtained from the graphs are given in the table below.

Plant extract	**IC_{50} value (µg/ml)**
stem	204.7
leaves	150.25
callus	247.2

Highest value of IC_{50} is obtained for callus and lowest for leaves. It means that antioxidant activity is lower for callus and higher for leaves. Stem extracts showed intermediate activity.


Antioxidant activity of stem
Percentage (%)
60
50
40
30
20
10
0
0
50
100
150
200
250
concentration (µg/ml)
31.7
37.3
40.1
51.4
y = 0.1238x + 24.65
R² = 0.9296


Antioxidant activity of leaves
percentage (%)
60
50
40
30
20
10
0
0
50
100
150
200
250
concentration (µg/ml)
43
45
50
54.2
y = 0.0772x + 38.4
R² = 0.9747

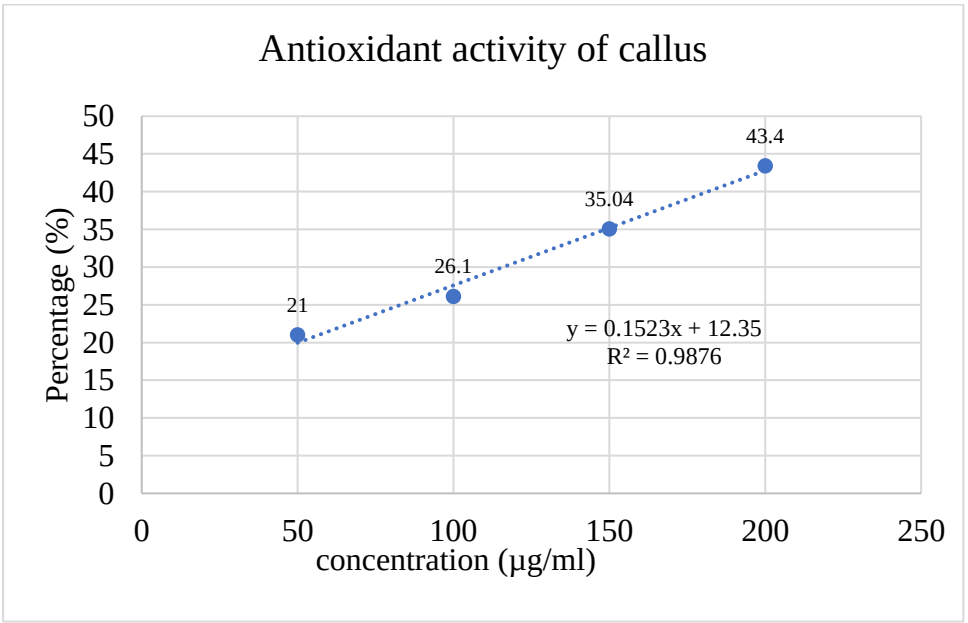
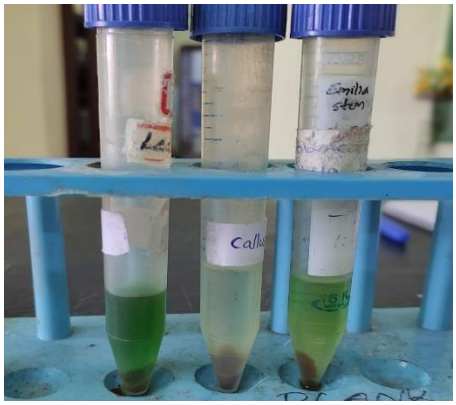
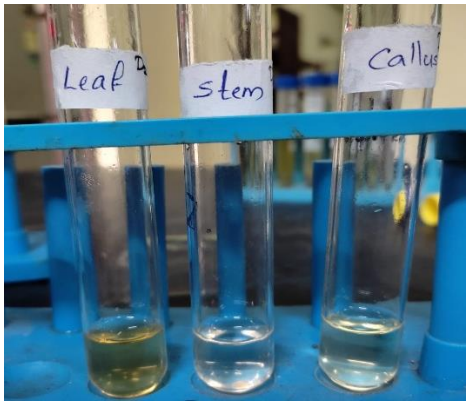
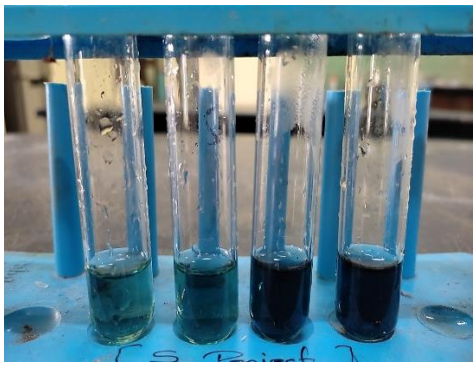

Antioxidant activity of callus
Percentage (%)
50
45
40
35
30
25
20
15
10
5
0
0
50
100
150
200
250
concentration (µg/ml)
21
26.1
35.04
43.4
y = 0.1523x + 12.35
R² = 0.9876

Figure 5: phosphomolybdate assay

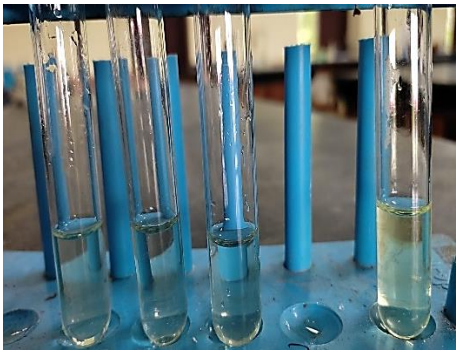




Leaves, stem and callus extracts used for the analysis.

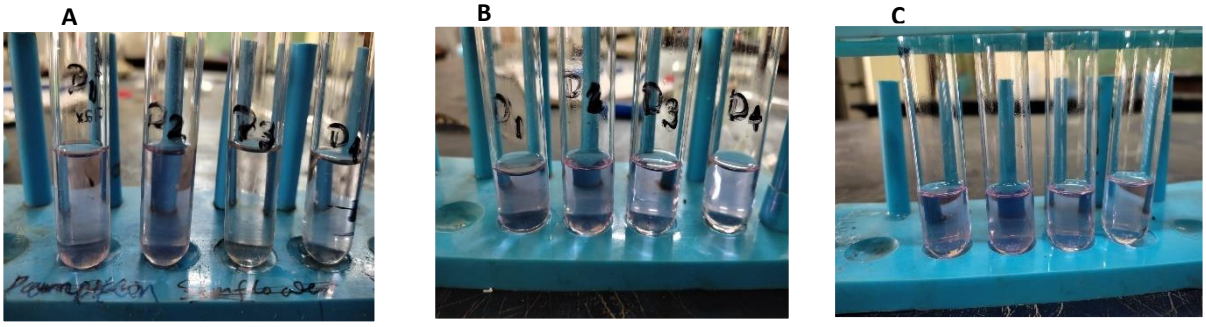


Standard ascorbic acid solutions



Test solutions of leaf, stem and callus extracts with blank.

Figure 6: DPPH assay



Different concentrations of leaves (A), stem (B) and callus (C) extracts

Discussion

Medicinal plants have bioactive compounds which are used for curing of various human diseases and also play an important role in healing. Especially in developing countries, herbal drugs play an important role in healthcare programmes. These abilities of plants are due to the presence of certain phytochemicals which occurs naturally for defence mechanisms and to protect it from various diseases.

Phytochemicals are the products of primary and secondary metabolism in plants. Primary phytochemicals include proteins and carbohydrates while secondary phytochemicals include terpenoids, alkaloids and phenolic compounds.

The members of Asteraceae family are widely used for medicinal purposes. They can be used as antipyretic, anti-inflammatory, hepato-protective, antioxidants, antibacterial etc. Phytochemical investigations of the Asteraceae family have revealed that many components from this family are highly bioactive.

Various phytochemicals are present in different parts of *Emilia sonchifolia*. In the present study, the preliminary phytochemical analysis of fresh stem revealed the presence of alkaloids, glycosides, coumarins, carotenoids, flavonoids, phenols, carbohydrates and diterpenes. In fresh leaf samples, along with these phytochemicals, tannins were also found b ut diterpenes were not found. Whereas in callus, coumarins, diterpenes and tannins were absent. The results of present study is comparable with the findings of Arumugam *et al*., (2011), Hammad *et al*., (2011) and Kumar *et al.,* (2012).

Alkaloids and flavonoids have been linked or suggested to be involved with antibacterial and antiviral activity while tannins and flavonoids are thought to be responsible for antidiarrheal activity (Enzo, 2007). This could explain the role of the plant as antimicrobial

and antidiarrheal. Glycosides can suppress and soothe irritant dry coughs. They have a helpful sedative and relaxant effect on the heart and muscles when taken in small doses. They are significantly diuretic (Sharma V. *et al*., 2011). Phenols are antiseptic and reduce inflammation when taken internally. These bioactive agents have an irritant effect when applied to the skin. Above of all, phenols have a high affinity to chelate metals and scavenge the free radicals in cells (Michalak A, 2006).

Comparative quantitative analysis for flavonoids and phenols were done. Both were found maximum in leaves, followed by callus and minimum for stem extracts.

Tissue culture is a best way to conserve economically important plants especially when they face threat of extinction. At the same time, it helps to produce more valuable phytochemicals for medicinal purposes.

In the present study, *in vitro* propagation of *E. sonchifolia* using leaf and internodal explants responded differently towards the selected plant growth regulators. Both the explants showed maximum response towards combined use of a synthetic auxin, NAA and a cytokinin, BAP. NAA alone and in combination with 2,4-D also gave positive results. Some leaf explants responded towards 2,4-D to an extent. But IAA alone or in combination with 2,4-D didn't give any response. In the study of Shylesh (2001), MS medium supplemented with 2,4-D along with kinetin showed better results.

The callus was subjected to comparative antioxidant analysis with fresh stem and leaves. Preliminary phytochemical analysis which gives positive results of alkaloids, phenols, glycosides, flavonoids and carotenoids in stem, leaves and callus indicate their antioxidant activity.

As antioxidants have been reported to prevent the oxidative damage caused by the free radical, it can interfere with the extraction process by reacting with free radicals, chelating

catalytic metals and also by acting as oxygen scavengers. Phenolic compounds and flavonoids have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic etc (Miller, 1996).

For comparing antioxidant activity of callus with fresh plant parts DPPH assay and phosphomolybdate assay were carried out. Stem, leaves and callus showed different levels of antioxidant activity. Maximum activity was observed in leaves and minimum in callus. Leaf has up to 55% activity while callus has up to 44% activity. Approximate radical scavenging power of stem is found to be 51%. This suggests that the callus is not sufficient to substitute for intact plants to be used as an antioxidant.

Results of phosphomolybdate assay differ from DPPH methods. In the former, the antioxidant activity was higher in leaves and lower in stem whereas in latter, radical scavenging activity was high in leaves but low in callus. This result indicates that the reduction of Mo depends on the types and amount of antioxidants. Both the methods suggests that the leaves are rich in antioxidants. And the whole plant of *E.sonchifilia* can be regarded as a powerful antioxidant.

Summary and Conclusion

Most plants are important in traditional medicine, since all plants produce different phytochemicals. But their composition makes it suitable for curing certain diseases efficiently. From ancient times onwards, plants have been used as medicines. Increasing knowledge of medicinal plants causes depletion of such plants in their natural habitat. Plant tissue culture is one of the solutions to this. Through plant tissue culture, we can produce large quantities of phytochemicals without affecting the diversity.

The present study aimed at developing a protocol for the *in vitro* propagation of *Emilia sonchifolia*. The leaf and internodal explants were used and callogenesis is induced by using different combinations of growth hormones. The callus obtained used for comparative study of certain phytochemicals and antioxidants. Different extracts of *Emilia sonchifolia* were studied, and presence of almost all medically important phytochemicals such as alkaloids, glycosides, coumarins, carotenoids, flavonoids, phenols, carbohydrates and diterpenes showed that this plant is very important from medicinal point of view.

Quantitative analysis of stem, leaves and callus for flavonoids and phenols were showed maximum in leaves and minimum in stem.

Comparative antioxidant activities of stem, leaves and callus are found using DPPH radical scavenging assay and phosphomolybdate assay. Both methods shows that the leaf extracts showed good scavenging activity among stem and callus extracts.

Medicinal plants are now being used as models for antimicrobial agents and it is believed that plant-based drugs cause fewer or no side effects when compared with synthetic antibiotics. Phytochemical analysis helps to identify the presence of major phytochemicals. The antioxidant study helps to analyse the activity of the plant and helps in the development of

new drugs for the treatment of various diseases. The results obtained from this study confirm the medicinal importance and antioxidant activity of *Emilia sonchifolia*, and also a protocol for its *in vitro* propagation. It needs further phytochemical exploration to isolate phytochemical constituents showing antioxidant activity.

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