In Vitro Callogenesis, Comparative Analysis of Phytochemical Constituents & Antioxidant Activity of Callus and Wild Plants of *Cyanthillium Cinereum* (L.) H. Rob

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CONTENTS

List of figures:

List of tables:

List of graphs:

ABSTRACT

Cyanthillium cinereum is a plant known for its medicinal properties in the traditional systems of medicine such as Ayurveda. It is used for the treatment of asthma, cholera, malaria, diarrhoea, and urinary bladder infections and is proven to possess antibacterial, antifungal, anthelminthic, anti-inflammatory, anticancer and antioxidant properties. Tissue culture of medicinal plants can be employed for propagation as well as the invitro production of secondary metabolites from callus cultures.

The present study is focused on the invitro callogenesis from leaf, nodes and internodes of *C. cinereum* through tissue culture and the assessment of the phytochemical composition and antioxidant activity of the callus with that of wild plants.MS media with varying concentrations of auxins NAA and 2,4 D are used. After incubation for 3 to 4 weeks, callus formation was observed in media containing 5 mg/l NAA, 2.5 mg/l NAA and 2 mg/l 2,4 D. Internodal explants showed better response compared to nodal and leaf explants. Root development from the nodal and internodal explants was also observed in media supplemented with NAA. Comparative phytochemical analysis of callus with stem and leaves revealed the presence of flavonoids, alkaloids, phenols and glycosides in all three tissues. Antioxidant activity of callus, leaves and stem were compared by DPPH assay and Phosphomolybdate assay where callus shows antioxidant activity comparable to the stem and leaf extracts of wild plants. Tissue culture of *C. cinereum* can be employed for secondary metabolite production for drug development.

INTRODUCTION

Plants and their extracts have been used in treating diseases since the earliest human civilizations. Various cultures have their records of useful herbs and their applications in human life. these plant-based medicine systems, using plants within a local area, produced the well-known traditional medicine systems, the Ayurvedic and Unani of the Indian subcontinent, the Chinese and Tibetan in other parts of Asia, the Native American of North America, the Amazonian of South America, and several local systems within Africa. Approximately 70-80% of the primary health care throughout the world is mainly based on plant materials. (Mamedov *et. al*., 2012)

Plants are the source of various phytochemicals; metabolites are used in medicinal and environmental sectors as well as being widely used in commercial and pharmaceutical products. Although they produce several medicinal products, either already on the market or under trial, the amounts obtained from plant sources are very minute or difficult to synthesize at an industrial level due to the complex chemical composition and chirality exhibited by these compounds. However, plant cell cultures offer a good alternative for the consistent production of desired secondary metabolites under the influence of precursors and elicitors. (Twaij *et. al.*, 2022)

1.1 Plant Secondary Metabolites

Plant secondary metabolites (PSM) are a large group of compounds that are synthesized within the plants that are not essential for the basic metabolism of the plant but as a response to stress or disease. The primary functions of these compounds in the plants in which they occur include defence against herbivores, bacteria, fungi and viruses. Other functions involve antioxidant activity, UV protection, facilitation of pollination and seed dispersal, signalling etc. they contain reactive functional groups in their chemical structures that form covalent bonds with other biological compounds such as proteins, peptides and sometimes DNA. plant secondary metabolites are primarily organic compounds and can simply be grouped into three major classes, terpenes: volatiles, cardiac glycosides, carotenoids and sterols; phenolics: phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignin; nitrogen-containing compounds: alkaloids and glucosinolates. (Hussein *et. al*., 2019)

- Flavonoids

Flavonoids are widely distributed in plants, fulfilling many functions. They are the most important plant pigments for flower colouration, producing yellow or red/blue pigmentation in petals designed to attract pollinator animals. In higher plants, they are involved in UV

filtration, symbiotic nitrogen fixation, and floral pigmentation. They may also act as chemical messengers, physiological regulators, and cell cycle inhibitors. In addition, some flavonoids have inhibitory activity against organisms that cause plant diseases, e.g., *Fusarium oxysporum*.

- Coumarins

Coumarins are a family of plant-derived secondary metabolites that are produced via the phenylpropanoid pathway. Besides their role in iron uptake, coumarins have been extensively studied for their potential to fight infections in both plants and animals. Coumarin's activities range from antimicrobial and antiviral to anticoagulant and anticancer. (Stringlis *et. al*., 2019)

Coumarins are used in medicine as an anticoagulant. it can also be used as a rodenticide precursor, manufacturing laser dyes, perfumes and aromatizers.

- Terpenes and terpenoids

Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. In plants, terpenes and terpenoids are important mediators of ecological interactions. For example, they play a role in plant defence against herbivory, disease resistance, the attraction of mutualists such as pollinators, as well as potentially plant-plant communication. They appear to play roles as antifeedants. Other functions of terpenoids include cell growth modulation and plant elongation, light harvesting and photoprotection, and membrane permeability and fluidity control.

- Phenolics

Phenolic compounds are produced by plants and microorganisms. Plants sometimes synthesize phenolic compounds in response to ecological pressures such as pathogen and insect attacks, UV radiation and wounding. As they are present in food consumed in human diets and plants used in the traditional medicine of several cultures, their role in human health and disease is a subject of research. Some phenols are germicidal and are used in formulating disinfectants.

- Alkaloids

Alkaloids are produced by a large variety of organisms including bacteria, fungi, plants, and animals.

Alkaloids have a wide range of pharmacological activities,
including antimalarial (*e.g.* quinine), antiasthma(*e.g.* ephedrine), anticancer (*e.g.* homoharring tonine),cholinomimetic (*e.g.* galantamine), vasodilatory (*e.g.* vincamine), antiarrhythmic (*e.g* . quinidine), analgesic (*e.g.* morphine), antibacterial (*e.g.* chelerythrine),
and antihyperglycemic activities (*e.g.* piperine). Many have found use
in traditional or modern medicine, or as starting points for drug discovery. Other alkaloids possess psychotropic (*e.g.* psilocin) and stimulant activities
(*e.g.* cocaine, caffeine, nicotine, theobromine), and have been used in entheogenic rituals or as recreational drugs. Alkaloids can be toxic too (*e.g.* atropine, tubocurarine).

The pharmacological activities of plant extracts are essentially due to the presence of secondary metabolites. Their mode of action may be direct or indirect. Sometimes multiple metabolites are contributing to the effect. This is probably why several plant extracts or recipes may not be reproduced by the isolated purified chemical constituents of the herb or recipe. Various secondary compounds elicit different kinds of activities in man, animal models and cell cultures. In many instances, the degree of activities of the active secondary metabolites may vary depending on many factors such as the selected plant species, parts of the plant, geographic origin, time of collection, method of preparation, the amount ingested, and so on.

Due to their diverse biological and physio-chemical properties, secondary metabolites are of great interest to man and impart uses as drugs, oils, waxes, perfumes, flavouring agents, dyes and many other commercially important materials. (Ahmed et. al., 2017)

Medicinal plants have been playing a pivotal role in the design and development of potent therapeutic agents. Medicinal plants have been playing an important role in developing various therapeutic agents. Due to the increasing awareness about drug-resistant pathogen strains and side effects associated with the use of synthetic drugs, the demand for plant-derived products has increased worldwide. Medicinal plants have great economic potential due to the various valuable products they provide. (Rungsung et. al., 2015)

However, due to climate change, pollution and habitat destruction many plant species face the risk of extinction. This increases the need to identify and conserve potentially therapeutic plants through phytochemical studies.

1.2 Phytochemical Analysis

Phytochemical studies involve the collection, extraction, and qualitative and quantitative analysis of phytochemicals. Through various separation and purification techniques, useful chemicals can be isolated from plant materials. Since phytochemicals when used as medicine cause little to no side effects compared to synthetic drugs, they are safer to use.

Plants under consideration may be collected either from wild forests or from herbariums. After the plants are collected from the wild or herbarium, they have to be processed for cleaning to prevent the deterioration of the phytochemicals present in plants. The cleaning process may involve the following steps. Cleaning, washing, peeling or stripping leaves from stems. Plants have to be dried immediately as soon as the plant's collection or this will lead to spoilage of plant materials. After complete drying of plants, they have to be powdered well for further analysis.

Plant extracts are made after tissue homogenization by methods like serial exhaustive extraction, Soxhlet extraction, maceration, decoction, infusion, digestion, percolation and sonication. (Banu et. al., 2015)

Qualitative analysis of various secondary and primary metabolites is carried out by chemical tests. Quantitative studies are done using techniques like spectroscopy, chromatography etc.

Medicinal herbs have been used in one form or another under indigenous systems of medicine. Dubey, et. al. (2004) mentioned that complete phytochemical investigations of medicinal plants in India should be carried out because these secondary metabolites are responsible for the medicinal activity of the plant. (Savithramma et. al., 2011)

2. Tissue culture

Tissue culture (TC) is the cultivation of plant cells, tissues, or organs on specially formulated nutrient media. It is seen as an important technology for developing countries for the production of disease-free, high-quality planting material and the rapid production of many uniform plants. (isaaa.org/ ,2006).

Plant tissue culture is an important technique in agriculture, horticulture, and plant biotechnology. It is an area of applied science that involves the aseptic culture of cells, tissues, organs, and their components under defined chemical and physical *in vitro* conditions. This science follows a basic concept in which the plant body or organ or any

tissue can be dissected into smaller parts called “explants” and any explants can be further developed into a whole plant. This concept led to the development of an effective technique called *in vitro* propagation. Plant regeneration forms the basis of *in vitro* propagation.

Totipotency and genetic and cellular machinery information of the plant cell is essentially required to generate the whole plant. Therefore, this *in vitro* science includes several concepts and techniques that can be utilized to produce a higher number of plants that are genetically similar to a parent plant as well as to one another. To understand the regeneration potential of plant cell culture an in-depth knowledge of plasticity and totipotency is required.

Additionally, plants themselves hold certain key features that favour their growth and development. Features like a longer life span and the sessile nature of plant cells enhance plant tolerance power and adaptability to extreme conditions. Thus, processes that are involved in the growth and development of the plant cell always run parallel to the environmental conditions. When plant cells or tissues are exposed to *in vitro* conditions most of the cells generally exhibit a very high degree of plasticity, which allows one type of organ or tissue to be initiated from another type. Following this principle, the whole plant can be regenerated. Thus, totipotency is the regeneration process acquired by the plant to preserve its genetic potential.

Furthermore, artificial nutrient medium plays a vital role in regeneration by supplementing organic and inorganic nutrients. The success of the culture is much dependent on the appropriate composition of the medium. Manipulation of the culture media is a usual practice to check the growth pattern of implanted explants. The mixture of salts, organic supplements, and sucrose (source of fixed carbon) concentration varies from media to media. Each medium has its different composition, which can be later manipulated according to the designed study.

Supplementation of the appropriate concentration of hormones (auxin and cytokinin) always decides the growth pattern of the implanted explants. The selection of an organ or tissue segment called the explant, which can potentially induce *in vitro* propagation of the explants on a supporting solidified nutrient medium under sterile conditions, is also an important step to begin the desired full *in vitro* propagation in tissue culture studies. Culturing of such explants in an appropriate medium gives rise to an unorganized, growing, and dividing the mass of cells called callus. During callus formation, a degree of dedifferentiation happens both in morphology and metabolism.

One of the major consequences of dedifferentiation is that most plant cultures lose their ability to perform photosynthesis. The addition of other components such as carbon and vitamins to the culture media, apart from the unusual mineral nutrients, enhances the cell's photosynthetic and regeneration power. All these processes include various basic concepts and techniques that lead to the successive development of callus and finally the establishment of the desired full cell line of the plant, which could be experimented on again for various research purposes. (Bhatia et.al.,2015).

Plant cell culture technologies were introduced at the end of the 1960s as a possible tool for studying and producing plant secondary metabolites. The usage of an in-vitro system has been extensively studied to improve the production of plant chemicals. Cell suspension culture could be used for large-scale culturing of plant cells from which secondary metabolites could be extracted. The advantage of this method is to provide a continuous, reliable source of natural products (Vanisree et al., 2004). Plant tissue culture plays a vital role in the search for an alternative to the production of desirable medical compounds (Rao and Ravishankar, 2002). Plant tissue culture can be utilized for the conservation of plants with pharmaceutical, industrial, or ecological value and large-scale and environment-friendly production of beneficial secondary metabolites. Conservation of genetic material of many threatened species of medicinal plants is practised by employing different culturing techniques.

3. Antioxidant activity

Atoms or molecules containing one or more unpaired electrons are termed free radicals which are accountable for tissue degeneration through DNA and protein damage and lipid peroxidation. Oxidative stress associated with free radicals is involved in the pathophysiology of ageing and various age-related ailments such as cataracts, atherosclerosis, diabetes, Alzheimer's disease, and so forth. The extent of damage caused by free radicals might be mitigated through supplementation with one or more antioxidants. Diverse medicinal plants have been screened and assessed for properties in antagonism to free-radical-induced oxidative stress.

Description of the selected plant species

Cyanthillium cinereum (L.) H. Rob (Synonym: *Vernonia cinerea*)

Figure 1: Habit of Cyanthillium cinereum (L.) H. Rob.

Common name: Ironweed (Poovamkurunnila in Malayalam)

Kingdom: Plantae

Subkingdom: Tracheobionta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Asterales

Family: Asteraceae

Genus: *Cyanthillium*

Species: *cinereum*

Morphology: Annual or perennial herb with erect stems covered with scabridulous hairs. Leaves are elliptic and are arranged alternating on the stem. The plant bears numerous capitula with pink or purplish florets. Fruits are achenes with white pappus. (Beentje *et al.*,2005)

Cyanthillium cinereum (Less.) H. Rob. (Synonym: *Vernonia cinerea* (Linn.) Less.), commonly known as little ironweed, is a common annual weed (Asteraceae) with a wide range of geographical distribution. The plant has great medicinal value in diverse traditional usage in different nations, and also gets recognition in the *Ayurveda*. The whole plant is used in decoction or infusion to treat fever. It provides a remedy for spasms of the urinary bladder and strangury and is often combined with quinine to treat malaria. Sesquiterpene lactones, which possess antimalarial activity, have been isolated from the plant. *Cyanthillium cinereum* has therapeutic potential against asthma, cancer, cholera, colic pain, cough, diarrhoea, dysentery, impotency and night blindness. The seeds are used as a source of alexipharmic and anthelmintic drugs, and as an alterative in leprosy and chronic skin diseases. *Cyanthillium cinereum* leaves have analgesic, antipyretic and anti-inflammatory effects. Paste of stem/bark is used to heal cuts, while flowers are traditionally used to treat conjunctivitis, arthritis and rheumatism. A root infusion is used as an antidote to scorpion sting and snake venom. (Guha et.al.,2011)

AIMS AND OBJECTIVES

The present study aims in standardizing a protocol for developing callus in vitro from leaf, nodal and internodal explants of *Cyanthillium cinereum* and also to study the effect of auxins in the initiation and maintenance of culture. The present study also aims at the quantitative and qualitative analysis of phytochemical constituents from stem and leaves from the wild plants and in vitro generated callus using fresh extracts. Another aim is the analysis of the antioxidant potential of leaf and stem extracts and their comparison with that of callus.

The objectives of the present investigation are the following

- To standardize a protocol for developing callus from nodes, internodes and leaf explants of *Cyanthillium cinereum*
- Preliminary phytochemical analysis of fresh extracts of stem and leaves using different solvents. and ethanolic extract of callus.
- Comparative quantitative analysis of selected phytochemical constituents present in the in-vitro generated callus, leaves and stem of *Cyanthillium cinereum.*
- Comparative analysis of the antioxidant potential of in-vitro generated callus with that of leaf and stem extracts.

REVIEW OF LITERATURE

This plant is commonly called ash-coloured fleabane belonging to the family Asteraceae. The chief constituents are the triterpenes. Parts that were used include the flower (treatment of conjunctivitis), seeds (used as anthelmintic), root (dropsy), and juice (piles). The whole plant is also considered to promote perspiration in febrile conditions. The plant is anthelmintic, antibacterial, antiviral, antifungal, anti-inflammatory, diuretic, and stomachic. The plant is used as an anticancer, febrifuge, diaphoretic (infusion of the herb, combined with quinine, is used against malaria. The Ayurvedic Pharmacopoeia of India recommends the plant in intermittent fever, filariasis, pityriasis versicolour (tinea versicolor), blisters, boils, vaginal discharges and in cases of psychoneurosis. (Shelar, D. *et.al.*,2014).

Goggi, A. *et.al.,* (2017) analysed the antioxidant activities of root, stem and leaves of *Vernonia cinerea*. Radical scavenging assays such as DPPH and ABTS along with phosphomolybdate assay were conducted. All plant parts showed varying antioxidant powers in different kinds of solvents.

Petacci, F. *et.al*., (2012)., quantified the polyphenols in ethanol extracts from leaves of 12 species of Asteraceae weeds collected in Diamantina, Minas Gerais State, Brazil. The screening of Asteraceae extracts revealed the presence of tannins, steroids, triterpenes, anthocyanins, and flavonoids.

Antioxidant Activity and Phytochemical Screening of Some Asteraceae Plants was conducted by Bakar, F. *et.al*. (2015). Antioxidant activities were investigated by using 1,1- diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay and measuring malondialdehyde (MDA) levels. All the tested extracts exhibited antioxidant activity in DPPH radical scavenging test.

A Review on the Phytoconstituents and Related Medicinal Properties of Plants in the Asteraceae Family by Achika *et.al*., (2014). 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.

Aliyu *et.al.* (2011) conducted phytochemical screening and antibacterial activities of three species of Vernonia (Asteraceae). The MIC of 1.25 mg/mL exhibited by the chloroform fractions on both Gram-positive and negative bacteria indicates broad spectrum activity of the Vernonia species being studied. The overall results indicate that the extracts are potent antibacterial preparations at least in vitro.

Soxhlet extraction of phenolic compounds from *Vernonia cinerea* leaves and its antioxidant activity was studied where the effects of extraction time, feed-to-solvent and ethanol concentration on the yield of extract, total phenolic content (TPC) and total flavonoid content (TFC) were examined. (Alara, O. R. *et.al.*,2018).

Phytochemical screening of Vernonia cinerea (Family: Asteraceae) showed the presence of steroids, glycosides, triterpenoids & esters in the methanolic extract of stem bark and leaves of the plant. The presence of these compounds indicates different medicinal properties of V. cinerea. NMR data also confirmed the presence of Lupeol, 12-oleanen-3-of-3ß-acetate, Stigmasterol, and ß-sitosterol in the n-hexane portion. (Haque, M. A *et.al*., 2012).

Danish Rizvi, S. M. *et.al*. (2011) studied the In-vitro antibacterial and antioxidant potential of leaf and flower extracts of Vernonia cinerea and their phytochemical constituents. Preliminary phytochemical analysis of methanol extract of leaf and flower indicated the presence of alkaloids, phenols, tannin, saponins and flavonoids. The antibacterial activity of different extracts (hexane, petroleum ether, chloroform and methanol) of leaf and flower of Vernonia cinerea were tested separately against both gram-positive and gram-negative bacteria using the agar well diffusion method. Maximum antioxidant potential using DPPH radical scavenging capacity was noticed in the methanol leaf extract.

A pharmacognostical study of *Vernonia cinerea* Less (Asteraceae) and evaluation of anti-inflammatory and antibacterial activities of the stem were conducted by Singh, A. *et.al.* (2014). The antibacterial assay indicated a zone of inhibition with alcoholic and chloroform extracts of *V. cinerea* leaf and stem. Diclofenac sodium and chloroform extract showed inhibition of inflammation, whereas higher inhibition was observed with alcoholic and hydroalcoholic extracts.

In the study titled Therapeutic Potential of Polar and Non-Polar Extracts of *Cyanthillium cinereum* In Vitro by Guha, G. *et.al.*, (2011), several parameters including free-radical ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, H_2O_2 and $^{\bullet}OH$) scavenging, reducing power, protection of DNA against oxidative damage, cytotoxicity, inhibition of oxidative haemolysis in erythrocytes, total phenolic content and inhibition of lipid peroxidation were examined. All extracts showed potential free radical scavenging activity of varying magnitudes.

The medicinal plants can be conserved through a biotechnological approach in tissue culture, micropropagation, synthetic seed, somatic embryogenesis. the demonstration of hormonal regulation of growth, differentiation and organ

formation in plants (Skoog and Miller, 1957), the regeneration of plantlets from callus culture (Reinert, 1958) and plantlet regeneration from cell suspension culture (Steward et. al, 1958).

Total Polyphenol Content, in vitro antifungal and antioxidant activities of callus cultures from *Inula crithmoides*, was studied by Bucchini, A. *et.al*. (2013). Callus cultures were initiated from leaf sections, on initial culture MS basal medium supplemented with various concentrations of 2,4-D (2,4-dichlorophenoxyacetic acid), NAA (1-naphthaleneacetic acid) and IBA (indole-3-butyric acid) and a 72% survival were achieved. Significant differences between the various auxins used as phytohormones on callus growth were found. Maximum callusing was noticed on the leaf explants grown on MS basal medium supplemented with 1 mg L^{-1} 2,4-D.

Nurokhman *et.al*. (2019) studied the effect of plant growth regulators and explant types on in vitro callus induction of *Gynura procumbens* (Lour.) Merr. The explants used were leaves, stem node, stem internode and petiole while the plant growth regulator used were 2,4-D and BAP, 2,4-D and Kinetin, NAA and BAP, and 2,4-D and IAA. The results of this study indicated that the treatment of 0.5 mg/L NAA and 0.5 mg/L BAP on the petiole explants was the best combination of plant growth regulators to produce the highest callus fresh and dry weights.

A Study on the Effect of Different Concentration of Plant Hormones (BAP, NAA, 2, 4-D, and Kinetin) on Callus Induction in *Brassica Napus* was conducted by Borjian, L. & Arak, H. (2013). MS complemented with BAP, NAA, and 2,4-D hormones were used. The highest callus production was obtained from a 5-day-old seedling in the first medium. Thus, the results showed that interactions between BAP, NAA, and 2,4-D hormones were the most effective factor for callus induction and production. The lowest amount of callus was just from a medium with 2,4-D.

Micropropagation of *Stevia rebacediana* Bertoni (Asteraceae) was done where shoot tips and nodal segments were used as explants. Shoot tips on MS medium with 1 mg/l kinetin showed the best results after 3 weeks of culture. Shoot elongation and rooting was successfully in MS medium 2 weeks later. Mass propagation of shoots was carried out in a temporary immersion bioreactor (Norazlina *et. al.*, 2012).

Amin *et.al.*, (2013), had done an extensive review regarding members of Asteraceae using various media and methods for micropropagation of genera *Saussurea*,

Inula, Atractylodes, Artemisia, Echinacea, Helichrysum, Vernonia etc. In most cases, MS medium with different concentrations of auxins and cytokinins either alone or in different combinations was used.

Invitro plant regeneration and clonal micropropagation of *Leontopodium nivalae* (Asteraceae). Callus induction was obtained from cotyledons of In Vitro Germinated seeds on MS with 2,4-D. Regeneration of shoots achieved by 6- BAP concentrations ranging from 0.5 to 2.0 mg/l. (Pace *et.al.,* 2009)

Cascado *et.al*., (2001) conducted a study on Micropropagation of *Santolina canescens*, a member of Asteraceae, in vitro volatiles production of shoot explants using MS medium containing different concentrations of BAP and Kinetin, for Rooting phase IAA, IBA and NAA are supplemented. The best axillary bud proliferation by using MS Medium containing 1.33 mg/l BA and 0.32 mg/l NAA.

Efficient plant regeneration protocol through callus for *Saussurea obvallata* (DC) Edgew (Asteraceae). All the explants are used with MS medium containing BA and NAA. The best result is observed in leaf explants. 100% callusing was achieved in MS medium supplemented with 2.5 µM BA and 1.0 µM NAA. Invitro rooting of shoots was achieved by MS medium supplemented with 2.5 µM IAA. (U. Dhar and M. Joshi, 2005).

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

Micropropagation of *Eclipta alba* (Asteraceae) using nodal segments as explants for organogenesis. Multiple shoots are obtained by using MS medium with various concentrations of BAP and Kn alone or in combination with NAA and IAA. A maximum number of multiple shoots is supplemented with 1.0 mg/l BAP and 0. l mg/l NAA. In further studies, different concentrations of IBA, IAA and NAA are used. MS medium with 0.1 mg/l IBA shows the best results. (Yesmin *et. al,* 2015).

Wani, M. *et.al.* (2010) conducted callus induction studies in *Tridax procumbens* L. The sterilized leaf, internode and shoot apical bud explants were inoculated in MS media containing various combinations of auxins such as 2, 4, dichloro phenoxy acetic acid (2, 4-D)

and naphthalene acetic acid (NAA) and cytokinins such as kinetin and 6 benzyl amino purine (BAP). Leaf and apical bud explants showed early and profuse callus induction whereas internodal explants showed comparatively delayed but profuse callus induction. Leaf and apical bud explants showed a maximum response in terms of callus by using MS media with the combination of 2, 4-D 0.5mg/lit and BAP 0.5mg/lit. Whereas internodal explants showed maximum callus induction by using a hormonal concentration of 2 mg/lit 2,4 D and 0.5 mg/lit BAP. In vitro generated callus can be used as a source for the isolation of secondary metabolites from the Tridax plant.

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

Che Mei-Yin and Hamsawi Sani, (2007), conducted a study on *V. amygdalina* for callus induction. Nodal explants are used in this study. Different combinations of BAP and NAA are used for the best results.

Khalafalla *et al*., (2007) conducted a study on in vitro multiple shoot regeneration in nodal explants of *Vernonia amygdalina*. They used MS medium supplemented with BAP alone or in a combination with NAA alone or a combination with 2,4 -D. A maximum number of shoots was observed on the medium containing 0.5mg/l BAP with 0.5 mg/I NAA. Regenerated shoots were rooted on MS supplemented with 2 mg/l NAA.

Invitro shoot regeneration from leaf and nodal explants of Vernonia cinerea on MS medium supplemented with different concentrations of BAP. Shoot multiplication was achieved by MS supplemented with 2.0mg/l BAP and 1.5 mg/l NAA. 1.5 mg/l TAA shows rhizogenesis (Seetharam, Y.N *et. al.*, 2007).

Plants were regenerated from the shoot tips of *C. cinereum* in MS supplemented with BA and kn. A maximum number of shoots in BA (13.32mg/) and roots in IBA (7.38mg/l) developed. The rooted plantlets are established in the field. (Maharajan *et.al;* 2010).

Sundar and Jawahar (2011) developed an efficient protocol for inducing direct organogenesis using shoot tip explants of *C. cinerea* on MS medium supplemented with different

concentrations of BAP (2.22 to22.22 µM/l) and KN (2.42 to 23.20 µM/1) for direct shoot induction. The frequency of multiple shoot induction and proliferation increased with increasing concentration of BAP and KN at optimum level. The high frequency of multiple shoot proliferation was observed on MS medium containing 13.32 µM/l BAP and 13.92 µM KN. The regenerated shoots were successfully rooted on MS medium with IBA (7.38 µM).

Vernonia Cinerea is an important medicinal plant which is frequently used in the traditional system of medicine. However, due to over-exploitation, its availability in the natural habitat has become limited. To overcome this problem, a viable protocol for large-scale multiplication of the species through callus induction and regeneration and somatic embryogenesis has been standardized (Reny *et.al*, 2013).

A study was conducted on in-vitro micropropagation of *Vernonia cinerea* on MS medium supplemented with BAP (1.0-3.0 mg/l), GA: (1.0- 5 mg/l) and KN (0.5 mg/1) for direct shoot induction. A maximum number of shoots were obtained from nodal and leaf explants at BAP (2.5 mg/), KN (0.5 mg/) and GAs (2.0 mg/1) respectively. Multiple shoot elongation and their development were better in MS medium with 1.5 mg/l BAP and 3.0 mg/l GA3. (Ayyandurai and K. Ramar, 2019).

Organogenesis, shoot regeneration, and flowering response of *Vernonia cinerea* to different auxin/cytokinin combinations were studied by Maheshwari, P., & Kumar, A. (2006). Lateral buds on nodal explants grew into shoots within 2 weeks of culture in Murashige and Skoog (MS) basal medium supplemented with 20.9 µ*M* BA. Similarly, leaf, nodal, and internodal explants were cultured on an MS basal medium supplemented with different concentrations of BA, NAA, and IAA either alone or in combinations for callus induction and organogenesis.

Maheshwari, P. *et.al.* (2007) studied alkaloid production in *Vernonia cinerea* callus, cell suspension and root cultures. Maximum biomass of callus, cell suspension and root cultures were obtained in the medium supplemented with 1 mg/L α-naphthaleneacetic acid (NAA) and 5 mg/L benzyl amino purine (BA), 1.0 mg/L NAA and 0.1 mg/L BA and 1.5 mg/L NAA, respectively. The 5-week-old callus cultures resulted in maximum biomass and alkaloid contents (750 µg/g). Cell suspension growth and alkaloid contents were maximal in 20-day-old cultures and alkaloid contents were 1.15 mg/g. Maximum alkaloid contents were obtained in root cultures *in vitro* compared to all others including the alkaloid content of *in*

vivo obtained with aerial parts and roots (800 µg/g and 1.2 mg/g dry weight, respectively) of *V. cinerea.*

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

MATERIALS AND METHODS

INVITRO PROPAGATION OF *Cyanthillium cinereum*

In-vitro propagation of the plant was carried out by following standard plant tissue culture procedure at the Tissue culture laboratory, [redacted]

Source and choice of plant material

Young and healthy plant parts of *Cyanthillium cinereum* were collected from [redacted] Stem and leaves of the plant were used as explant materials for the current study.

MATERIALS REQUIRED

Glassware

In the plant tissue culture laboratory glassware such as beakers, conical flasks, standard flasks, culture tubes, culture vessels, Petri dishes, pipettes, measuring cylinders, glass rods, etc., were used. Conical flasks, thermostable glass vessels and culture tubes were used for the establishment of culture. Standard flasks, measuring cylinders and beakers are used for media preparation.

All the glassware in use was cleaned with distilled water before use to avoid contamination. Glassware was first thoroughly washed in running tap water using detergent. All the cleaned glassware was placed in a hot air oven for 2 hours at 100°C to make them dry. Once used culture vessels were first autoclaved and washed following the earlier procedure. Culture tubes and vessels were soaked in 20% hydrochloric acid overnight and dried in a hot air oven after each wash. In addition to glassware, certain instruments like scalpels, forceps, and knives used which are made of stainless steel and were sterilized every time before use.

Equipment

The machines used in the tissue culture laboratory included Laminar Air Flow Cabinet, Hot Air Oven, Microwave Oven, Refrigerator, Electronic balance, Digital Electronic pH Meter, Autoclave, Steel Racks, Air Conditioner, and Rotary shaker.

Preparation of stock solutions

Standard procedures were followed for the preparation of M S media. The media strength, composition and other supplements are depicted in Table 1. For the present study 500 ml of each stock, the solution is prepared. Stock solutions of macronutrients were prepared at 20X

(Stock 1), $CaCl_2$.$2H_2O$ stock is prepared at 20X (Stock 1A), and micronutrients were prepared at 100X (Stock 2). Iron source stock solution was made at 50X (Stock 3) and vitamin stock solutions were made at mg/ml concentrations.

The stock solutions of nutrients, vitamins and growth regulators were stored in amber-coloured bottles under 4^{o}C in the refrigerator. Chemicals such as sucrose, Myo-inositol and agar, were weighed and added freshly to the culture media.

The plant growth regulators were used and their concentrations are presented in Table 2. Stock solutions of growth regulators made at mg/ml solution. It was prepared by dissolving an appropriate amount of chemicals in distilled water. The growth regulators that are not soluble in water are dissolved in small amounts of 1 N sodium hydroxide and made to final volume with distilled water.

CONSTITUENTS	**CONCENTRATION IN MEDIUM** (Mg L^{-1})	**CONSTITUENTS IN STOCK** (Mg L^{-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
$Na_2MoO_4.2H_2O$	0.25	25	
$CuSO_4.5H_2O$	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		Added freshly	
Myo-inositol	100	50	
Nicotinic acid	0.5	50	
Pyridoxine-HCl	0.5	10	100
Thiamine-HCl	0.1	200	
Glycine	2		
CARBON SOURCE		Added freshly	
Sucrose	3%		

Table 1: constituents of medium, concentration and volume of the stock solution of M S medium used for the present study.

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

Plant growth regulators	Concentration (Mg L^{-1})
NAA	2.5 3 4 5
2,4 D	0.1 0.5 1 2 5

MEDIA PREPARATION

From the stock solutions required quantity of solution was added into a standard flask based on the need of the amount of medium prepared. 3% sucrose (for 100 ml medium) and myo-inositol are weighed as per the specification of media and were added and dissolved in the media. Plant growth regulators for the different concentrations were added to the media from a stock solution of hormones. After adding nutrients, vitamins and hormones make up the media to the required volume by using distilled water.

The pH of the medium was adjusted between 5.6 and 5.8 using 1 N NaOH or 1 N HCl with the help of an electronic pH meter. Then 0.8% bacteriological agar was weighed and added to the solution for the preparation of a solid medium. The solution was mixed well and heated in a microwave oven till the agar is dissolved uniformly. The medium was then poured into pre-sterilized culture vessels. 15 ml medium was poured into the culture tubes, 50 ml was taken in conical flasks (250ml) and 25 ml medium was poured into culture vessels. The culture

tubes and conical flask containing nutrient medium were plugged tightly with non-absorbent cotton wool plugs. Media can also be stored in a 250 /500 ml conical flask for further use.

Sterilization

The sterilization of the culture medium was carried out in an autoclave for 15 minutes at 121^{o}C (250F) and 15 lbs pressure. After sterilization, the culture tubes, vessels and flasks were stored in an airconditioned culture room until further use. All Petri plates and beakers used in the inoculation cabinet were sterilized in an autoclave at 121^{o}C and 15 lbs pressure for 20 minutes. Scalpels, forceps, scalpel blades etc., used were also flame sterilized after dipping in alcohol.

Explants preparation and surface sterilization

Cyanthillium cinereum, the study material is collected from [redacted] In the present study leaf and internodes were used. The plants were brought to the laboratory, and leaves and internodes were isolated and collected from the source plants and washed in running tap water for 15 minutes. The explants were washed for 30 minutes in disinfectant solution and then washed in distilled water 3-4 times and dipped in distilled water. The explants were transferred to the Laminar Air Flow cabinet where further sterilisation was performed. All operations should be done under, a Laminar Air Flow cabinet to avoid contamination.

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 before use. All the operations were carried out using pre-sterilized instruments and glassware. The explants after washing with distilled water were brought into the Laminar Air Flow Cabinet. Then plants are cut into small pieces for easy handling. For surface sterilization, the materials were transferred into a sterilized beaker containing 0.1% Mercuric Chloride solution, and then continuously stirred to ensure maximum exposure of Mercuric Chloride to all parts (Mercuric Chloride solution was prepared by adding 1g of Mercuric Chloride in 1L sterilized distilled water), for 5 minutes. After decanting the mercuric chloride, the explants were rinsed with sterilized distilled water several times, decanted and allowed to dry. Then the explants were transferred to the pre-sterilized Petri plates with the help of sterile forceps.

Inoculation

The internode explants were cut into small sizes, approximately lcm with help of a sterile scalpel blade. Then small wounds are made on the surface of the internode with a scalpel

blade. With the help of forceps, the explant transferred into the culture media. The leaf explants were cut, each including midrib to the appropriate size (1x1 cm) by using a sterile scalpel blade. Wounds are created on the lower surface of the leaf explants. Then explants were inoculated into the surface of solidified culture medium After the inoculation the mouth of culture tubes were flamed quickly and tightly plugged. The tubes were labelled properly and were transferred to the growth room.

Incubation

The cultures were maintained in the culture room at 25+2^{o}C under a 16:8 light and dark regime. The cultures were given illumination by white fluorescent light.

Callus subculture

After 3-4 weeks, the old callus was collected and subcultured on a fresh medium with the same growth regulators.

PHYTOCHEMICAL ANALYSIS

For phytochemical analysis, fresh leaves and stems of the plant and also the callus developed from the in vitro culture were used.

Preparation of plant samples

For preparing fresh extracts, the selected plant parts as well as the callus were excised and washed thoroughly in distilled water. 3g of plant samples were weighed out, and ground using mortar and pestle. The plant samples were soaked in 30ml of polar solvents like ethanol, chloroform, distilled water and nonpolar solvent like benzene for 2 days. The extracts were collected and filtered by using Whatman No.1 filter paper. Then the extract was transferred into Petri plates and was oven dried at 50^{o}C for one day. Then residues are collected and redissolved in the respective solvents into 20ml. All the samples were stored at 4°C.

Qualitative analysis

The stem and leaf extracts prepared using solvents benzene, chloroform, ethanol, and distilled water were screened for the presence of the phytochemical constituents by using the methodology of Sofowora (1982), Kepm (1986), and Harborne (1973). Ethanolic extracts of invitro-generated callus were also analysed for the presence of phenolics and alkaloids, which are responsible for many medicinal effects of plants including antioxidant effects.

1. Tests for 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 a few drops of lead acetate solution. The formation of a yellow colour precipitate indicates the presence of flavonoids.

2. Tests for saponins

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

b) Foam Test: 0.5 gm of the extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

3. Tests for phenols

a) Ferric Chloride Test: Extracts were treated with 3-4 drops of aqueous 5% ferric chloride solution. The formation of bluish-black colour indicates the presence of phenols.

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 phenolic compounds.

4. Tests for alkaloids

Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

a) Dragendorff's Test: Filtrates were treated with Dragendorff's reagent (solution of Potassium Bismuth Iodide). The formation of a reddish-orange precipitate indicates the presence of alkaloids.

b) Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). The presence of alkaloids is confirmed by the formation of a yellow-coloured precipitate.

5. Tests for carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

a) Molisch's Test: Filtrates were treated with 2 drops of alcoholic a-naphthol solution in a test tube. The formation of the violet ring at the junction indicates the presence of Carbohydrates.

b) Benedict's test: Filtrates were treated with Benedict's reagent and heated gently. Orange-red precipitate indicates the presence of reducing sugar.

c) Fehling's Test: a small portion of various filtrates heated with Fehling's A & B solutions. A colour change indicates the presence of reducing sugars.

6. Tests for tannins

a) Gelatin Test: To the extract, when treated with gelatin solution give white precipitate indicates the presence of tannins.

5 ml of the various extracts was dissolved in a minimum amount of water separately, filtered and filtrates were then subjected to the following test.

b) Ferric chloride Test: To the above filtrate, a few drops of ferric chloride 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 were added. The formation of a reddish-brown precipitate indicates the presence of tannins.

7. Tests for proteins and amino acids

a) Biuret test: 1 ml of each of the various extracts was warmed gently with 10% sodium hydroxide solution and a drop of dilute copper sulphate solution. The formation of reddish violet colour indicates the presence of protein and amino acid.

b) Ninhydrin Test: 2 drops of ninhydrin solution (10 mg in 200ml acetone) were added to 2ml of aqueous filtrate. The formation of purple colour indicates the presence of proteins and amino acids.

8. Tests for diterpenes

Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. The formation of emerald green colour indicates the presence of diterpenes.

9. Tests for glycosides

a) Bromine water test: test solution was dissolved in bromine water and the formation of a yellow-coloured precipitate indicates the presence of glycosides.

b) Keller Kiliani test: crude extract was mixed with 2ml of glacial acetic acid containing 1-2drops of 2% solution of ferric chloride. The mixture was then poured into another test tube

containing 2ml of concentrated sulfuric acid. A brown ring at the interphase indicates the presence of cardiac glycosides.

10. Tests for carotenoids

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

11. Tests for coumarins

1ml of each alcoholic extract was treated with a 10% sodium hydroxide solution. Production of dark yellow colour indicates the presence of coumarins.

Quantitative Analysis

Analysis of total phenol content and flavonoid content of ethanolic extracts of stem, leaves and callus was conducted.

1. Total phenol content

Total phenol content was estimated by Malick and Singh, (1980). Pipette out 0.5ml of sample into test tubes. Makeup volume in each test tube to 3ml with distilled water. Add 0.5ml of Folin-Ciocaltueau reagent. After 3 minutes, add 2ml of 20% sodium carbonate solution to each test tube. Mix each thoroughly. Place the 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. Total flavonoid content

Total flavonoid content was estimated using the procedure described by Zhishen et al. (1999). 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. The mixture was shaken well and left 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}.

ANALYSIS OF ANTIOXIDANT ACTIVITY

Total antioxidant capacity was assayed by DPPH assay and Phosphomolybdate assay.

1. DPPH radical scavenging assay

DPPH radical scavenging assay is used in this study to analyse the free radical scavenging potential of ethanolic extracts of leaf, stem and callus of the plant. The assay was carried out according to Blois (1958). 0.1 mM DPPH (2, 2 - Diphenyl- 1- Picrylhydrazyl) was prepared. 1 ml of this solution is added to 3ml of ethanolic extracts of varying concentrations (50 µg/ml, 100µg/ml, 150µg/ml, 200µg/ml and 250µg/ml). 3 ml ethanol was used as blank.

The mixture was incubated for 30 minutes in dark. After incubation, the absorption of reduced DPPH was measured at 517 nm (UV-VIS spectrophotometry). Ethanolic DPPH was used as control. The Radical scavenging activity (RSA) was calculated in percentage by the following formula.

RSA (%) = (Abs control - Abs sample /Abs control) *100

The results were also reported as IC50 which is the amount of antioxidant required to decrease the DPPH concentration by 50%. IC50 values were calculated from regression analysis and expressed as µg dry weight equivalents per ml sample. (Goggi, A. *et.al.*,2017).

2. Phosphomolybdate Assay

The phosphomolybdate assay proposed by (Prieto et al;1999) was used to find the total antioxidant capacity of plant extracts. 1ml of ethanolic plant extract with a concentration of 250ug/ml is prepared. 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. Phosphomolybdate reagent was prepared by mixing 100ml 0.6M Sulphuric acid, 100ml 28Mm Sodium phosphate and 100ml 4mM Ammonium molybdate solutions. Within a test tube, 300ul plant extract was mixed with 3 mL phosphomolybdate reagent. 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 then the absorbance was read at 765 nm. Blank was made 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 present study aims to standardize a protocol to develop callus and comparative phytochemical evaluation of fresh extract and callus extract of the plant. The work also aims at evaluating the effect of different concentrations of growth regulators on the behaviour of callus during culture. In this study auxins such as naphthalene acetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) were used for inducing callus from leaf, node and internodal explants. In this study, quantitative and qualitative evaluation of different phytochemical constituents from fresh extracts of the plant and also from the callus were conducted. The study also involves a comparative study of the antioxidant potential of ethanolic extract of the derived callus with that of fresh stem and leaf extracts.

1. Development of callus

MS medium is used as the basal medium for callus culture in which *Cyanthillium cinereum* showed a positive response. Different concentrations of two auxins viz. NAA and 2,4-D were used. The response of various explants towards each Plant Growth Regulator (PGR) is shown in Table 3. Visual grading based on the performance of explant on callus development was done and it is represented in the table with '+' and '– 'signs.

Within 7 days of inoculation, callus proliferation began from cut surfaces of internodal explants inoculated on the medium with 5 mg/L NAA. Some of the nodal explants inoculated in 5 mg/L NAA medium showed signs of callus development within 14 days while others showed root development. Callus initiation was found in internodal explants inoculated on a medium with 2 mg/L 2,4-D within 14 days. Root generation was observed from the calluses at 16-20 days in the medium containing 5mg/L NAA. Callus development was observed from internodal explants in 2.5 mg/L NAA containing medium, and root proliferation was observed after 7-8 days. Calluses obtained were subcultured into fresh media of same concentrations after 2 weeks.

Table 3: Effect of PGRs on internodal, nodal and leaf explants of *Cyanthillium cinereum*

PGRs	Concentration (mg/L)	Internodal explant			Nodal explant		
		shoot	callus	root	shoot	callus	root
NAA	2.5	-	+	++	-	-	-
	3	-	-	-	-	-	-
	4	-	-	-	-	-	-
	5	-	+++	++	-	+	+
2,4-D	0.1	-	-	-	-	-	-
	0.5	-	-	-	-	-	-
	1	-	-	-	-	-	-
	2	-	++	-	-	-	-
	5	-	-	-	-	-	-

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

C. cinereum showed a higher response towards M S medium supplemented with NAA, compared to 2,4-D medium. Medium with NAA concentration 5 mg/L showed the best response where prolific callogenesis was observed within the first week. Medium supplemented with 2.5 mg/L NAA and 2 mg/L 2,4-D showed a moderate response where callus developed within 14-16 days. Numerous roots developed from the callus in the two weeks old culture of internodal explant in medium supplemented with 5 mg/L NAA. When it was subcultured in a 2.5 mg/L NAA medium, reduced root growth and increased callus growth resulted. Both callus and roots developed when internodal explant was inoculated in a 2.5 mg/L NAA medium. Nodal explants inoculated in the medium containing 5 mg/L NAA showed a feeble response.

Internodal explants inoculated in M S medium supplemented with 2mg/L 2,4-D showed characteristic swelling within 7 days, and callus proliferation was observed after about 16 days of inoculation. In all cases, internodal explants showed better response compared to leaf and nodal explants.

Figure 2: Development of callus on medium with 5 mg/L NAA from the internodal explant.

Figure 3: Development of callus on medium with 2.5 mg/L NAA from internodal explant.

Figure 4: Development of callus on medium with 2mg/L 2,4-D from internodal explant

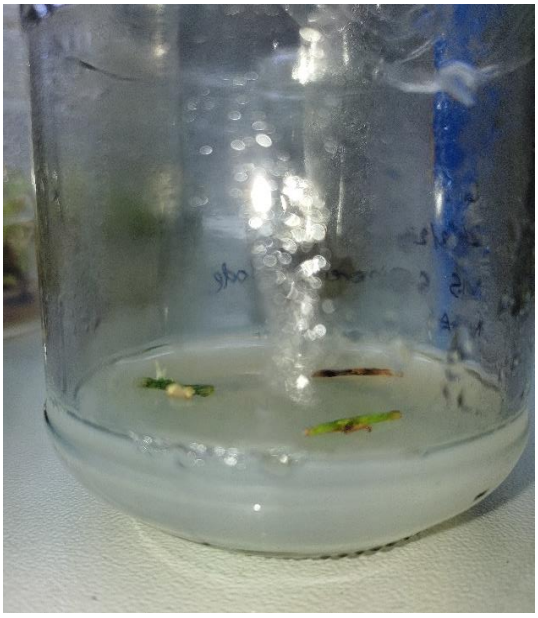


Figure 5: Development of callus on medium with 5 mg/L NAA from the nodal explant.

Figure 6: Development of roots on medium with 5 mg/L NAA from internodal explant.

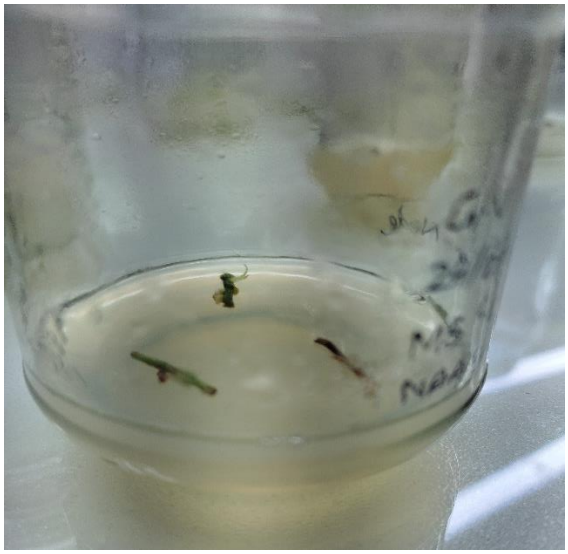
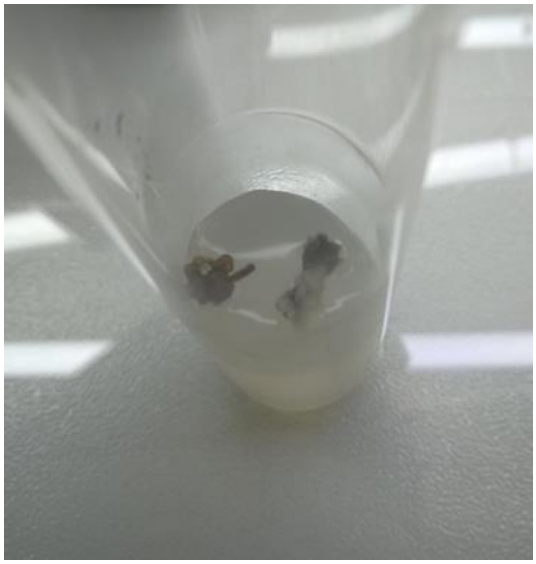


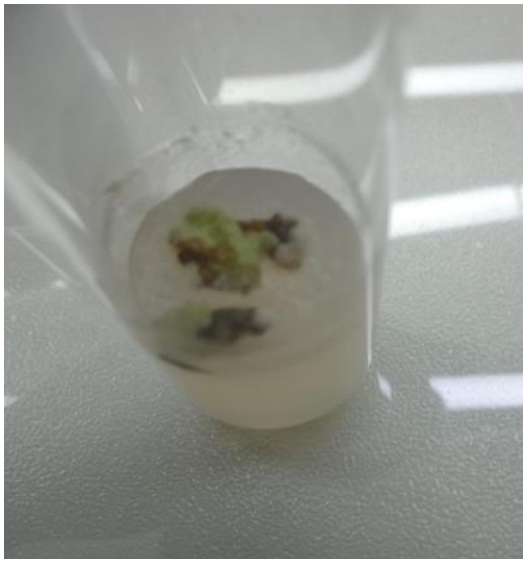
Figure 7: Development of roots on medium with 5 mg/L NAA from the nodal explant.

Subculturing of callus

A

B





C **D**

Figure 8a- 8d: Subculturing of callus

2. Phytochemical Analysis

Preliminary phytochemical analysis of ethanol, benzene, chloroform and distilled water extracts of leaf and stem was conducted (Tables 4 and 5) and the results were compared with that conducted in the ethanolic extract of the callus tissue. (Table 6)

Benzene extract of fresh stem shows the presence of alkaloids, saponins, carboxylic acids, quinones, coumarins and sugars. Chloroform extract shows the presence of alkaloids, carbohydrates, carboxylic acids, quinones, coumarins and sugars. Ethanol extract shows the presence of flavonoids, alkaloids, carbohydrates, diterpenes, glycosides, phenol, carotenoids, coumarins and sugars. Distilled water extract shows the presence of alkaloids, diterpenes, proteins, quinones, glycosides, coumarins and sugars. (Table 4)

Table 4: Qualitative analysis of different extracts of stem of *C. cinereum*

Phytochemical compounds	Benzene extract	Chloroform extract	Ethanol extract	Distilled water extract
Flavonoids	-	-	+	-
Alkaloids	+	+	+	+
Carbohydrates	-	+	+	-
Saponins	+	-	-	-
Phenol	-	-	+	-
Tannins	-	-	-	-
Proteins & amino acids	-	-	-	+
Diterpenes	-	-	+	+
Glycosides	-	-	+	+
Carotenoids	-	-	+	-
Coumarins	+	+	+	+

'+' present and '– 'absent

Benzene extract of leaves shows the presence of alkaloids, saponins, carboxylic acid, coumarins and sugars. Chloroform extract shows the presence of alkaloids, carboxylic acid, glycosides, coumarins and sugars. Ethanol extract shows the presence of flavonoids, alkaloids, carbohydrates, saponins, phenol, diterpenes, quinone, glycosides, carotenoids, coumarins and sugars. Distilled water extracts showed the presence of alkaloids, carbohydrates, proteins, diterpenes and sugars. (Table 5)

Table 5: Qualitative analysis of different extracts of leaves of *C. cinereum*

Phytochemical compounds	Benzene extract	Chloroform extract	Ethanol extract	Distilled water extract
Flavonoids	-	-	+	-
Alkaloids	+	+	+	+
Carbohydrates	-	-	+	+
Saponins	+	-	+	-
Phenol	-	-	+	-
Tannins	-	-	-	-
Proteins & amino acids	-	-	-	+
Diterpenes	-	-	+	+
Glycosides	-	+	+	-
Carotenoids	-	-	+	-
Coumarins	+	+	+	-

'+ 'present and '– 'absent

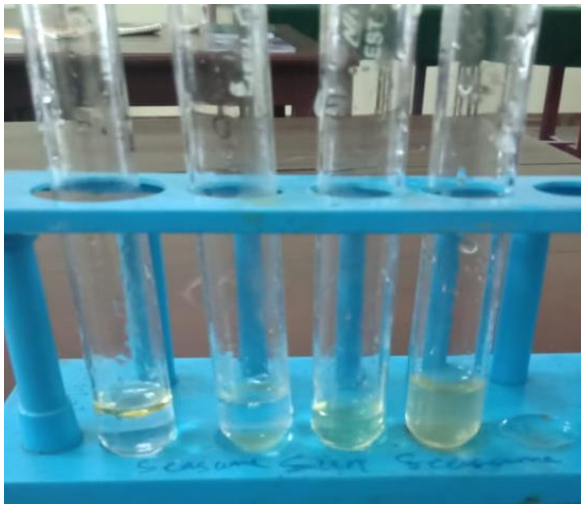


Figure 9: Test for saponins

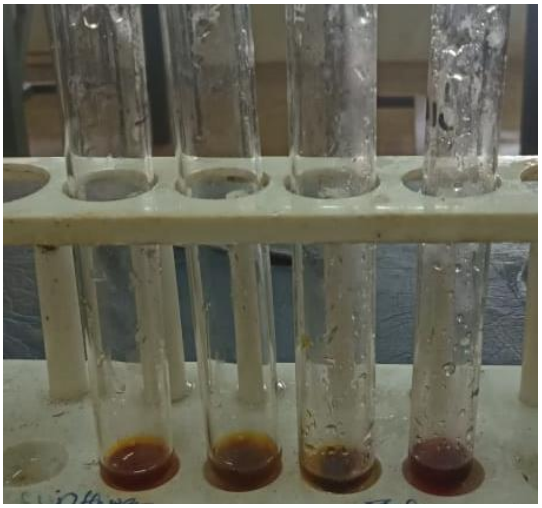


Figure 10: Test for alkaloids

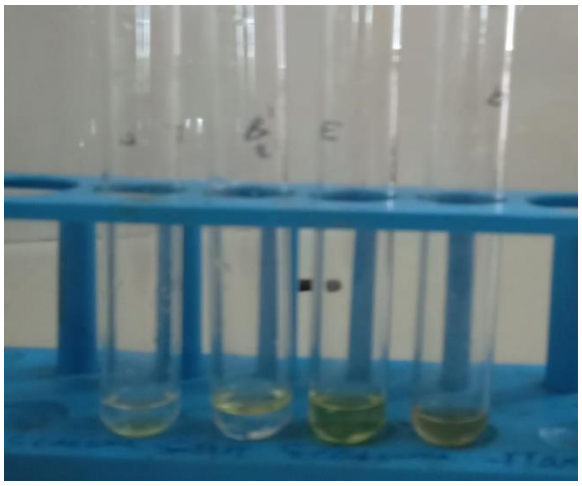


Figure 11: Test for flavonoids

Comparative Qualitative Analysis of Leaves, Callus and Stem

Ethanolic extract of the invitro generated callus shows the presence of flavonoids, phenol, glycosides and alkaloids. On comparison, all three extracts show the presence of flavonoids, alkaloids, phenol and glycosides in common. (Table 6)

Table 6: Comparative qualitative tests for the presence of various phytochemicals in leaves, callus and stem of *C. cinereum*

Phytochemicals	Leaves	Callus	Stem
Flavonoids	+	+	+
Alkaloids	+	+	+
Carbohydrates	+	-	+
Saponins	+	-	-
Phenol	+	+	+
Tannins	-	-	-
Proteins and amino acids	-	-	-
Diterpenes	+	-	+
Glycosides	+	+	+
Carotenoids	+	-	+
Coumarins	+	-	+

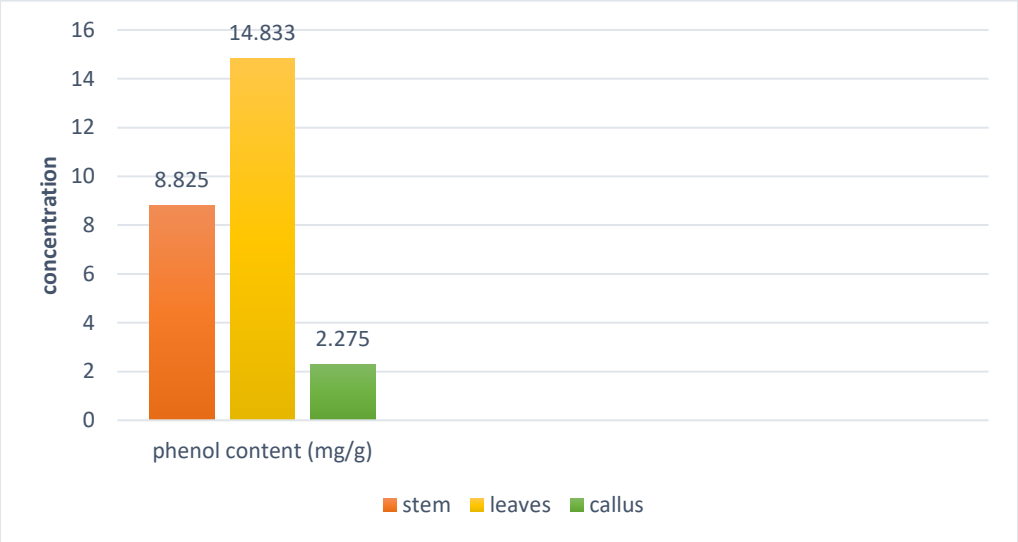
Comparative Quantitative Analysis of Leaves, Callus and Stem

a) Total phenol content

Total phenol content was quantified in ethanolic extracts of leaf, stem and callus and it was observed that maximum phenolic content was found in leaf extract (14.833 mg/g). (Table 7)

Table 7: Phenol content of leaves, stem and callus of *C. cinereum.*

Part of the plant	Total phenol content (mg/g)
Stem	8.825
Leaves	14.833
Callus	2.275



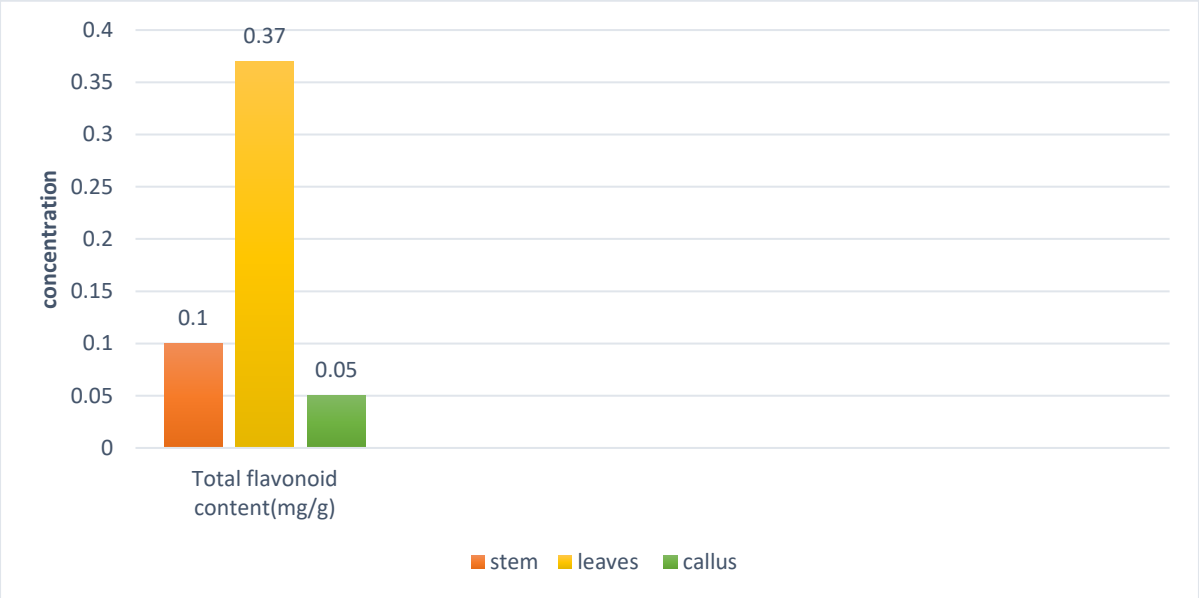
Graph 1: Total phenol content (mg/g) from leaves, stem and callus of *C. cinereum* plant.

b) Total flavonoid content

Total flavonoid content was estimated in ethanolic extracts of leaf, stem and callus. Higher flavonoid content was observed in leaves (0.37 mg/g) compared to the others. (Table 8)

Table 8: Flavonoid content of leaves, stem and callus of *C. cinereum.*

Part of the plant	Total flavonoid content (mg/g)
Stem	0.1
Leaves	0.37
Callus	0.05



Graph 2: Total flavonoid content (mg/g) from leaves, stem and callus of *C. cinereum.*

3. Comparative analysis of Antioxidant activity

Antioxidant activity of ethanolic extracts of leaf, stem and callus of C. cinereum was compared and the results are given below:

3.1 DPPH Radical Scavenging Assay

Free radical scavenging potential of leaves, stem and callus at different concentrations was assayed by DPPH assay and the results are given below.

Table 9: Antioxidant activity determined by DPPH assay

The concentration of the plant extract (µg/ml)	% Antioxidant activity		
	Callus	Leaves	Stem
50	17.75	37.85	43.45
100	21.69	39.25	44.39
150	22.42	48.59	46.72
200	27.42	58.87	49.53
250	28.36	66.82	52.80

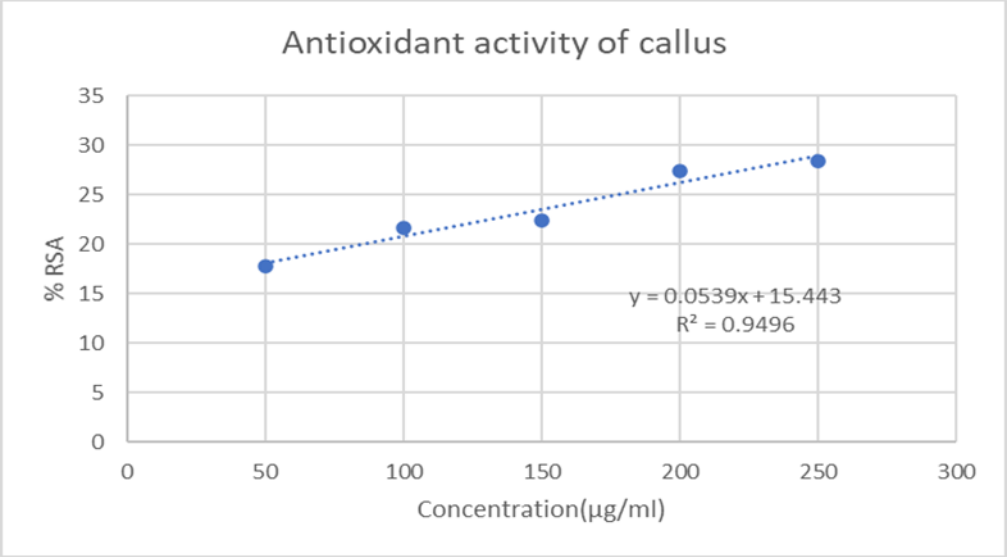
The concentrations were plotted against the percentage of radical scavenging activity from which the IC50 value of the extracts was calculated based on the equation y= mx + c.

x is the concentration of the extract at which 50% radical scavenging activity is observed, also called the IC 50 value of the extract. This value is inversely proportional to the antioxidant capacity.

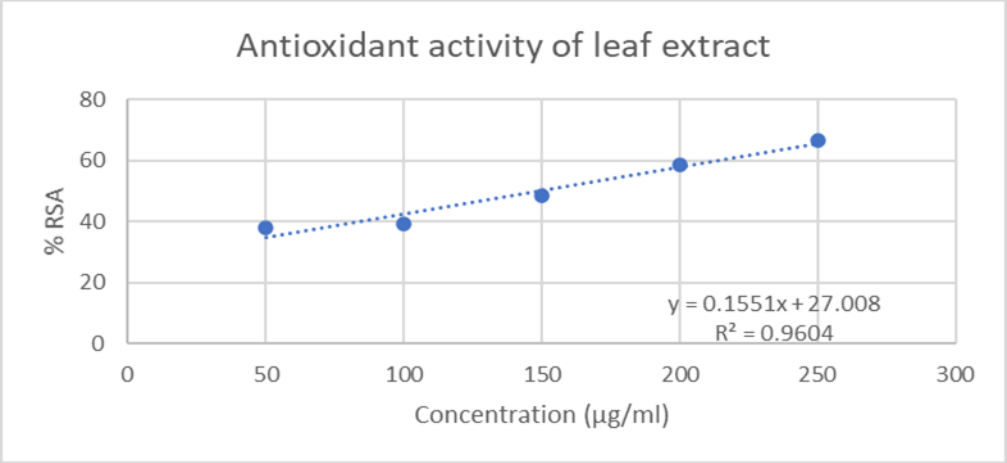
Table 10: IC 50 values of callus, leaves and stem of *C. cinereum.*

Plant extract	IC 50 concentration (µg/ml)
Callus	641.13
Leaf	148.23
Stem	200.84

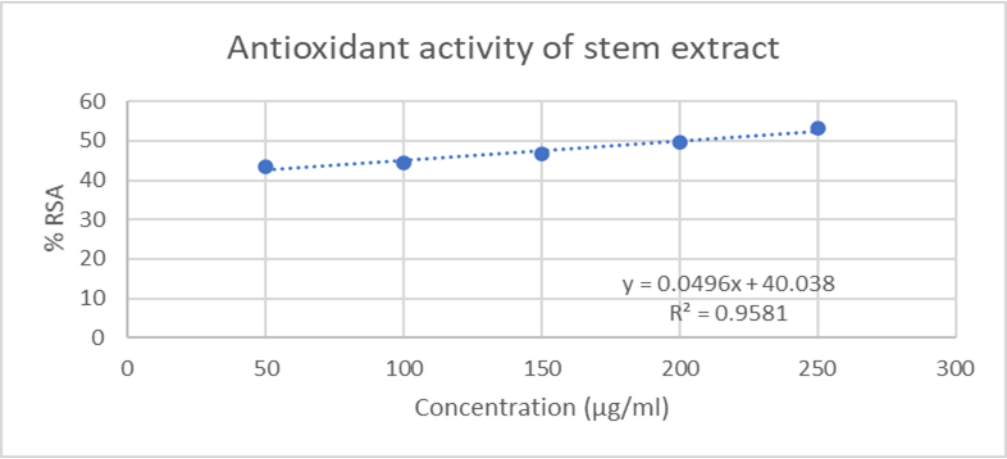
All extracts showed antioxidant potential which increases with an increase in the concentration of the extract. The highest activity was shown by leaf extract, followed by stem and callus extracts.



Graph 3: Antioxidant activity of callus by DPPH assay.



Graph 4: Antioxidant activity of leaves by DPPH assay.



Graph 5: Antioxidant activity of stem by DPPH assay.

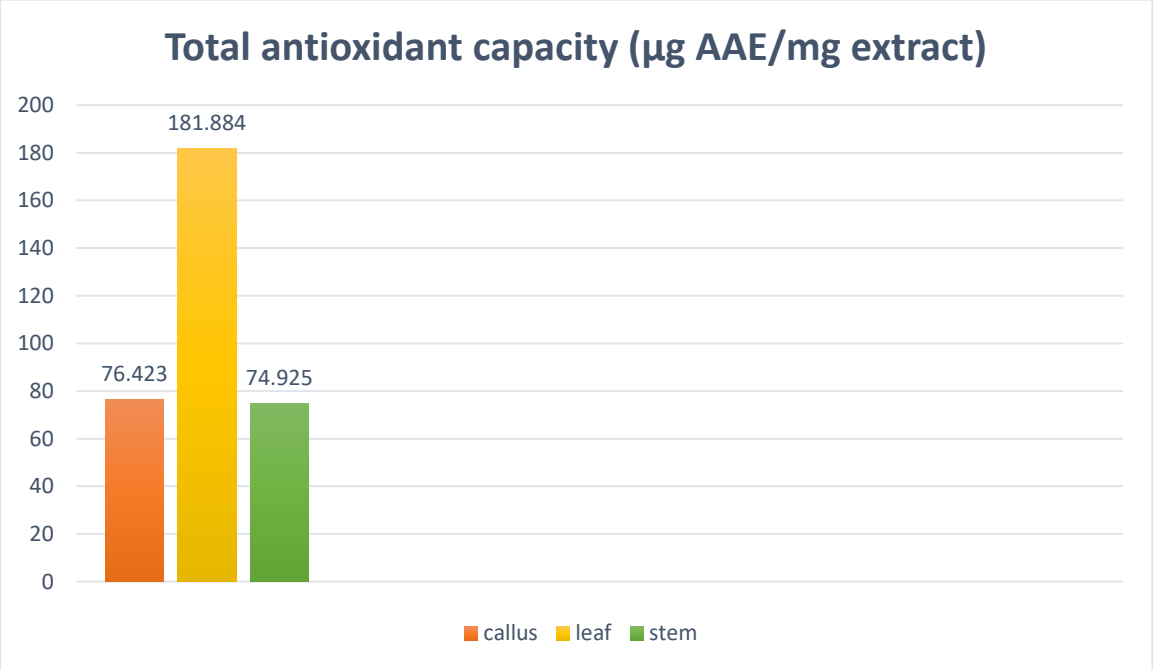
3.2 Phosphomolybdate assay

The reduction of phosphomolybdate ion by ethanolic extracts of leaves, stem and callus were estimated by phosphomolybdate assay and the results are given below.

Table 11: Total antioxidant capacity determined by phosphomolybdate assay

Plant extract	Total antioxidant capacity (µg AAE/mg of extract)
Callus	76.423
Leaves	181.884
Stem	74.925

Extract of leaves shows higher antioxidant capacity, followed by callus and stem extracts. Callus extract showed a slightly higher antioxidant capacity than stem extract in the assay.



Graph 6: Total antioxidant capacity (µg AAE/mg extract) of callus, leaves and stem of *C. cinereum* by phosphomolybdate assay.

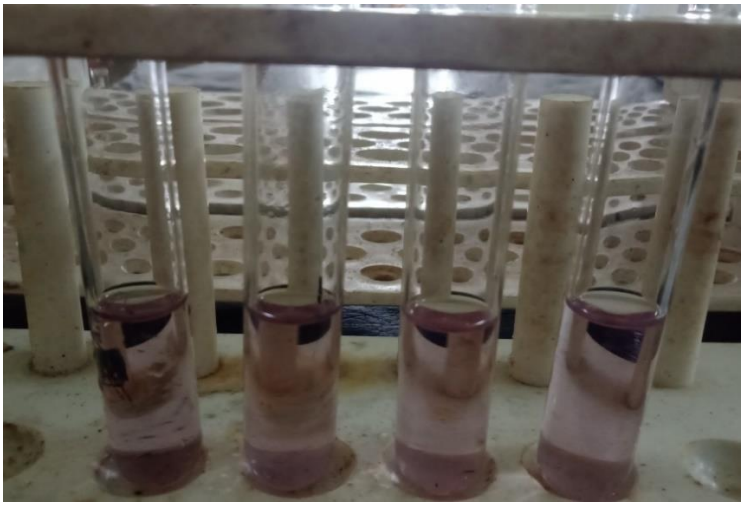


Figure 12: DPPH Assay

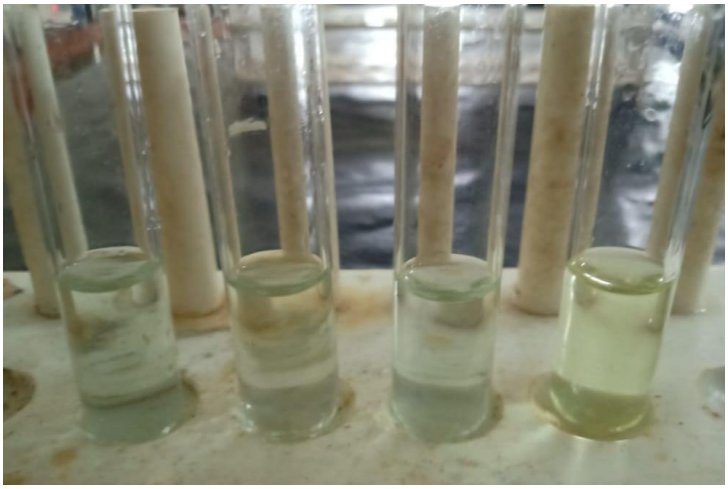


Figure 13: Phosphomolybdate Assay

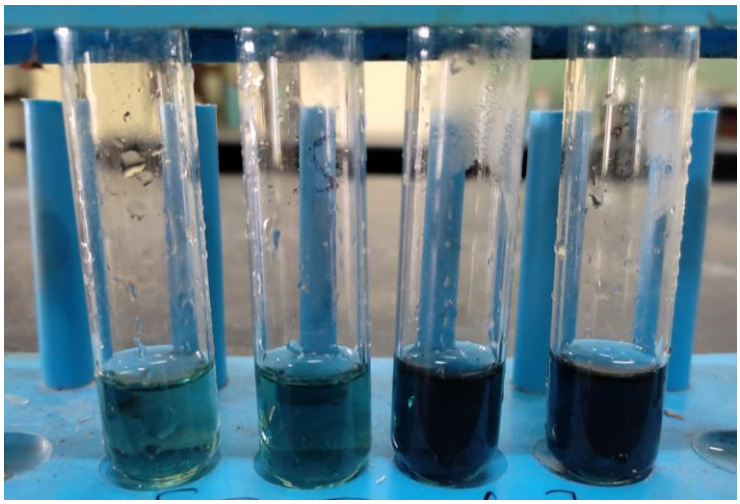


Figure 14: Standard graph preparation using Ascorbic acid

DISCUSSION

Many plants in our environment possess medicinal properties that are recognised by traditional systems of medicine. This therapeutic potential is due to the presence of certain chemical substances called secondary metabolites. Valuable bioactive components from plants are identified by phytochemical studies which are then used for developing various drugs after further studies. After identification, the compounds are to be isolated, quantified and studied for determining their mode of action, dosages as well as synergistic effect with other compounds.

Phytochemicals include carbohydrates, proteins etc. which are primary metabolites or secondary metabolites which include phenolics and alkaloids. Secondary metabolites are mostly responsible for the therapeutic value of a plant. They are known to possess antimicrobial, antioxidant and anticancer effects.

Cyanthillium cinereum is a plant with many useful phytochemicals. The current study revealed the presence of flavonoids, alkaloids, diterpenes, phenol, glycosides, carotenoids, coumarins, and saponins in the leaf extracts during preliminary phytochemical analysis.

Stem extracts showed the presence of flavonoids, alkaloids, phenol, diterpenes, glycosides, carotenoids, and coumarins. Phytochemical analysis of the *in vitro* generated callus obtained by tissue culture was identified to possess various secondary metabolites such as flavonoids, alkaloids, and glycosides.

The literature survey also agreed with the current observations. The presence of alkaloids, phenols, saponins, glycosides, coumarins and quinones in leaves of *C. cinereum* agrees with the findings of Chudasama *et.al.,* (2018). The present study also agrees with the findings of Fanou *et.al*., (2022) and Roy, S. *et.al*., (2019).

Plant tissue culture is a technique for regenerating plants through the development of callus or by direct organogenesis or somatic embryo development. It can be applied for multiplying plants in-vitro reducing the pressure on nature and for conserving plants of medicinal value. Callus cultures of medicinal plants can be exploited for isolating various phytochemicals such as alkaloids (Maheswari *et.al.,* 2007), isoflavonoids (Gueven, A. *et.al*., 2011), flavonoids (Indu, S. *et.al.,* 2013) etc. By providing an appropriate medium with suitable concentration and type of plant growth regulators, the development of the explant tissue can be manipulated to attain the required outcome.

In the present study, leaf, stem and nodal explants of *C. cinereum* were inoculated in media containing varying concentrations of auxins NAA and 2,4D. NAA (naphthalene acetic acid) and 2, 4-D (2, 4-dichlorophenoxyacetic) are synthetic auxins which induce cell division, cell elongation and development of callus in cultured plant tissues. The response of cultures is determined mainly by the type and concentration of growth regulators or hormones and also by cultural conditions, duration, genetics and physiological nature of the particular plant. In this study, using auxins solely produced better results.

M S medium supplemented with 5 mg/L NAA induced fast generation of callus from both nodal and internodal explants, the response being better in internodal explants. Media containing 2 mg/L 2,4 D also showed good response in internodal explants. This result can be compared with the findings of Shylesh *et.al*., (2001) in *Emilia sonchifolia* where a combination of 2,4 D and KN was used for callus induction from internodal explants.

Direct root development was observed in internodal and nodal explants inoculated in media containing 5 mg/l and 2.5 mg/l NAA. The root-inducing effect of high concentrations of NAA is reported in *Citrus grandis* by Tao, H. *et.al*., (2002).

The comparative phytochemical analysis of callus with leaves and stem revealed the presence of multiple secondary metabolites in the callus such as flavonoids, alkaloids, phenol and glycosides. The phytochemical profile of the callus is comparable to that of the stem and leaves. Comparative quantitative analysis of total phenolic content and flavonoid content of callus, leaves and stem was also conducted where leaf shows maximum content of both phenols and flavonoids.

The present study also conducted a comparative study of the antioxidant potential of ethanolic extracts of the callus, leaf and stem. The in-vitro generated callus of C. cinereum contains secondary metabolites like phenol and flavonoids which imparts antioxidant properties to its extract. Phenol and flavonoid content were found to be highest in leaf extract (14.833 mg/g and 0.37 mg/g) followed by stem and callus extracts. Whitaker *et al.,* 1986 also observed that cultured plant cells tend to produce secondary metabolites in lesser quantities when compared with intact plant parts.

Total antioxidant capacity was analysed by DPPH assay and Phosphomolybdate assay. In the DPPH assay, leaves showed the highest antioxidant potential followed by callus and stem. A concentration-dependent increase in the percentage of radical scavenging activity was

observed in all three extracts. This increased activity complies with the findings of Fanou *et.al*., (2022).

In the phosphomolybdate assay in ethanolic extracts of callus, leaves and stem, the highest total antioxidant capacity was detected in the leaf extract. The activity of callus extract showed a greater activity compared to stem extract in the assay. This may be because the reduction of different chemicals governed by different components and concentrations of extracts.

This is the first attempt to analyse the antioxidant property of callus tissue of *Cyanthillium cinereum* compared to that of leaves and stem. The antioxidant action can be due to the presence of various secondary metabolites in the callus like flavonoids, alkaloids, phenol and glycosides. Further interventions in the cultural conditions and techniques can be used to improve the production of antioxidants in cultured callus. The accumulation of active principles in cultured cells at a higher level than those in native plants through optimization of cultural conditions has been observed in *Panax ginseng* (Ushiyama, 1991). Rosmarinic acid by *Colleus blumei* (Ulbrich et al., 1985), shikonin by *Lithospermum erythrorhizon* (Takahashi and Fujita, 1991), diosgenin by *Dioscorea* (Rokem *et al*., 1984), ubiquinone-10 by *Nicotiana tabacum* (Matsumoto, 1980) were accumulated in much higher levels in cultured cells than in the intact plants. (Janarthanam *et.al*., 2010).

SUMMARY AND CONCLUSION

Many plants with medicinal properties are used in traditional systems of medicine for treating various ailments. Modern medicine is also shifting its attention to herbal sources of therapeutic drugs. This is mainly due to the side effects caused by the usage of synthetic drugs and the increasing case of drug-resistant strains of pathogens. To meet this increasing need for fresh plant materials, *in vitro* multiplication of plants through micropropagation and development of callus cultures with enhanced production of bioactive compounds is gaining more and more importance.

The present work focuses on *in vitro* callogenesis and comparative analysis of phytochemistry and antioxidant properties of *Cyanthillium cinereum*. In this study, the first step was to standardise a protocol for the development of callus. M S medium supplemented with different auxin concentrations was used to inoculate leaf, internodes and nodes as explant materials. Medium containing 5 mg/l NAA showed callus and root formation from nodal and internodal explants within 14 days. Medium supplemented with 2.5 mg/l NAA showed callus and root development from internodal explants within 21 days of culture. Medium supplemented with 2 mg/l 2,4 D also showed callus development in 3-4 weeks of incubation.

Comparative phytochemical analysis of callus with leaves and stem revealed the presence of secondary metabolites such as flavonoids, alkaloids, phenol and glycosides in both *in vitro* callus extract and *in vivo* plant parts.

Comparative analysis of antioxidant activity shows that leaves possess greater antioxidant activity compared to callus and stem. It was observed that the callus of *C. cinereum* possesses antioxidant components in fair quantities compared to the intact plant.

Through suitable tissue culture practices and cultural manipulations, the synthesis of antioxidant compounds in callus can be improved and thus serve as a valuable source of secondary metabolites throughout the year. The study observed the presence of several bioactive compounds which may help in finding therapeutic uses for the plant.

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