Melatonin-mediated Elicitation in TDZ Induced Callus Cultures of *Echinacea purpurea*

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In the Name of Allah, the Entirely Merciful, the Especially Merciful
Al-Fatihah [1: 1], Nobel Quran

DECLARATION

I, Tehreem Mahmood D/O Mahmood Ahmad Warraich, Registration no 02272113008, MPhil Biotechnology scholar, Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan, hereby declare that the quoted data in the thesis entitled "**Melatonin-mediated elicitation in TDZ induced Callus Cultures of *Echinacea purpurea***" is based on genuine work carried under the supervision of Dr. Bilal Haider Abbasi and has not been submitted or published somewhere else. Additionally, I am familiar with the concepts of 'plagiarism' and 'copy right'. Even after the degree has been awarded, the institution reserves the right to take any action if plagiarism is found at any level. Furthermore, Turnitin software was used to verify this thesis for plagiarism.

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Tehreem Mahmood

Dated: _____

DEDICATION

I dedicate this thesis to my beloved father who has been the strongest pillar of my life, and to my mother whose constant love and prayers keep elevating my determination amidst hard times.

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All praise is to Almighty Allah, the omnipotent and the most compassionate, on whom we ultimately depend for sustenance and guidance. Peace be upon the Prophet Muhammad ﷺ, the most perfect among all who have walked the surface of the earth, serving as an everlasting source of guidance and knowledge for humanity.

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I humbly pray that Allah bestows eternal happiness and success upon all of you. May His blessings be with you. Ameen.

(Tehreem Mahmood)

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LIST OF ABBREVIATIONS

µg/mg	microgram/milligram-Dry Weight
AAE	Ascorbic acid equivalent
BAP	Benzylaminopurine
DPPH	2,2-Diphenyl-1-picrylhydrazyl
CIF	Callus Induction Frequency
FCR	Folin–Ciocalteu reagent
GAE	Gallic acid equivalent
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
mg/L	milligram/liter
mL	milliliter
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
NaOH	Sodium Hydroxide
PGR	Plant Growth Regulator
PTC	Plant Tissue Culture
QE	Quercetin equivalent
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
SMs	Secondary Metabolites
TAC	Total Antioxidant Capacity
TDZ	Thidiazuron
TFC	Total Flavonoid Content
TFP	Total Flavonoid Production
TPC	Total Phenolic Content
TPP	Total Phenolic Production
w/v	weight by volume

Abstract

Echinacea purpurea (L.) Moench, is an important medicinal herb of the Asteraceae family. The plant is highly enriched with antioxidant and anti-inflammatory compounds, greatly benefiting the pharmaceutical industries. The *in vitro* cultures enable the efficient and sustainable production of plant metabolites that is usually enhanced by the addition of elicitor in culture medium. In the current study, TDZ was efficiently employed for the optimum induction of *E. purpurea* callus cultures. Furthermore, the impact of varying doses of melatonin on biomass accumulation, antioxidant potential, and secondary metabolites production was assessed in callus cultures of *E. purpurea.* Among all the tested concentrations of TDZ and inoculated explants, stem-derived callus developed using 3 mg/L TDZ showed 100% callus induction frequency and maximum biomass accumulation (FW: 222.08 g/L, DW: 21.625 g/L). This callus of friable nature was used in further experiments because of the fast proliferation of the segregated cells. Exposing the established callus culture with varying concentrations of melatonin showed promising results. The maximum biomass accumulation (FW: 208.62 g/L, DW: 11.87 g/L), maximum total phenolic production (TPP: 132.8 mg GAE/L) and total flavonoid production (TFP: 585.9 mg QE/L) was observed in callus treated with 25µM melatonin. The optimal DPPH radical scavenging activity (DPPH-FRSA: 92.7%), and total antioxidant capacity (TAC: 63.2 ug AAE /mg) was also recorded in callus maintained at 25µM melatonin. A positive correlation was found between these parameters and biomass. HPLC analysis further revealed the maximum synthesis of majority of compounds at this concentration. However, a peak production of amino acids was observed at lowest concentration of melatonin (1 µM). Our research contributes to a better understanding of TDZ as an effective plant growth regulator having a potential for callus induction and biomass accumulation. The outcomes of this study revealed that elicitation with melatonin might prove to be an effective strategy for increasing biomass, phenolic compounds, flavonoids, and antioxidant potential in *E. purpurea* callus cultures.

Keywords: *E. purpurea;* Melatonin; Elicitation, Thidiazuron; Callus Cultures; Phytochemicals

CHAPTER 1
INTRODUCTION

1. Introduction:

The profound impact of medicinal plants on our society cannot be underestimated. Plants have extensively contributed to the evolution of medicines, mainly because of their capacity to produce secondary metabolites exhibiting biological activities of potential significance. In ancient medicinal practices, a variety of methods involved the utilization of botanical elements to address a diverse spectrum of health conditions. From the socio-economic perspective, medicinal plants provide a critical source of income for many rural populations. Thus, in addition to serving medical purposes, they also have economic importance. The study of medicinal plants has thus captured the attention of the scientific community since it is thought that they represent an essential reservoir of bioactive chemicals with therapeutic potential (Bari et al., 2017; Khesht et al., 2021).

Compositae or Asteraceae family is a family of flowering plants in which more than 23,600 species, 1620 genera, and 13 subfamilies are currently recognized. Various plant species, like coreopsis, chicory, lettuce, sunflower, dahlias, and daisies, belong to this botanical family. Additionally, this plant family encompasses several valuable medicinal plants, including chamomile, wormwood, and dandelion. Many of them are spices that are utilized in traditional medicine and have shown a variety of pharmacological effects. Their pharmacological activities can be attributed to the phytochemical components they contain, which include essential oils, polyphenolic compounds, lignans, saponins, phenolic acids, sterols, and polysaccharides (Rolnik & Olas, 2021). Several diseases have long been cured by the plants belonging to the Asteraceae family due to their anti-inflammatory, antimicrobial, analgesic, antiproliferative, antiviral, and antioxidant properties (Momtaz & Abdollahi, 2008). A few species have also been involved in the treatment of tumors, hepatotoxicity, epilepsy, indigestion, sleeping sickness, and swollen lips (Bahar et al., 2016). Indeed, because of their exceptional medicinal characteristics, plants in the Compositae family require more scientific attention.

Echinacea purpurea (L.) Moench (purple coneflower), is a North American species of flowering plant belonging to the family Asteraceae. *Echinacea purpurea* is a perennial herb with a characteristic height of 100–150 cm. The stem of the plant ascends from the roots which are branched and fibrous. It simply develops a rosette of leaves in the initial year of cultivation; the second year is when it blooms. As a general characteristic of the Asteraceae family, the head unit

of the plant is an assembly of several small-sized florets. The droopy outer (ray) florets have teeth at their ends and encircle the spine-tipped inner (disc) florets. The floral heads of the plants are spiny, and the cone-shaped receptacle is high (Mistr\'\iková & Vaverková, 2007).

In *Echinacea purpurea*, the frequently encountered phytochemicals encompass lipoproteins, sesquiterpenes, polyacetylene, echinacoside, chicoric acid, alkamides, polysaccharides, and betaine. These kinds of bioactive chemicals are responsible for numerous biological functions, which also rely on the section of the plant that is being used (Coelho et al., 2020). *E. purpurea* is among the frequently employed medicinal plants that offer treatment against a range of diseases, including snake bites and wound infections. Additionally, it is well known for having anti-inflammatory, antioxidant, and anticancer effects (Oláh et al., 2017; Barnes et al., 2005: Aarland et al., 2017). Extracts of *Echinacea purpurea* have also been involved in enhancing monocytes and natural killer cells, serving as the initial guardians against infections within the body's defense system (Elshahawy et al., 2022).

Plants like *Echinacea purpurea* are regarded as chemical factories because of their potential to synthesize phytochemicals of therapeutic and industrial significance (M. B. Ali et al., 2006). However, the use of wild plants often results in an inconsistent supply of phytoconstituents due to overexploitation, geographical and seasonal constraints, and problems encountered during purification. Other factors that limit the production of phytochemicals in wild plants include high costs and the employment of suitable screening bioassays (Halder et al., 2019). *In vitro* plant tissue culture represents an innovative approach for producing valuable plant components, as by negating the necessity of depending on natural flora, it provides independence from geographic circumstances (Espinosa-Leal et al., 2018).

Due to the endless possibilities for crop manipulation and enhancement, *in vitro* plant tissue culture is a crucial practice for the ongoing production of chemicals that are active in plants, such as secondary metabolites and synthetic molecules (B. Zhang et al., 2012). Throughout all stages of a plant's *in vitro* growth and development, Plant Growth Regulators (PGRs) are necessary. The role of plant growth regulators in embryo development, root formation, callus establishment, shoot induction, micropropagation, and morphogenesis is the subject of every research using plant cell cultures. When administered at a certain dose and under certain conditions, several PGRs have

been demonstrated to function as both cytokinin and auxin. Among these, Thidiazuron (TDZ) is the most intriguing and significant one. TDZ can improve cytokinin activity in callus cultures and can promote a variety of biochemical and physiological processes, including the induction of new calluses (Thomas & Katterman, 1986; Yadav et al., 2010). The development of transgenic plants with improved traits, such as resistance to drought, high temperatures, salt stress, microbial assault, and other difficulties, depends crucially on callus cultures. Due to its potential uses in industry, agriculture, and medicine, secondary metabolite generation in callus cultures is of great interest (Fazili et al., 2022). However, under the conditions of conventional tissue culture, the generation of secondary metabolites is often minimal (Efferth, 2019). Several forms of *in vitro* approaches (suspension culture system, elicitation, hairy root culture system, etc.) have been substantially utilized for improving the production of secondary metabolites in plants.

Elicitation is one of the most efficient and often used biotechnological methods for synthesizing new compounds or improving the accumulation of secondary metabolites in *in vitro* plant tissue cultures (Akula & Ravishankar, 2011; Doran, 2013). Diverse routes in plant cell are activated in response to various biotic and abiotic triggers, including fluxes of ions across the cell membrane, the production of reactive oxygen species (ROS), and the proteins' phosphorylation/dephosphorylation. These are all potential parts of signal transduction pathways that trigger defense reactions in response to elicitors (B. Zhang et al., 2012). Several parameters such as the type and concentrations of elicitor used, duration of exposure, culture type, the composition of medium, and the type of growth regulator determine the efficiency of elicitation techniques (Kumar, 2018; Shanker & Shanker, 2016). The synthesis of secondary metabolites has been reported to be increased in callus cultures of many plant species by using elicitors such as salicylic acid, methyl jasmonate, chitosan, melatonin, and heavy metals (DiCosmo and Misawa 1985).

Melatonin, known chemically as 5-methoxy-N-acetyl tryptamine, is categorized as an indole derivative and is produced from the precursor tryptophan. It is a natural indolamine that controls a variety of physiological processes in both plants and animals. (Iqbal & Khan, 2022). Melatonin is referred to as a biostimulator of growth in plants, particularly in stressful environmental conditions. It serves a variety of purposes in plants, including osmoregulation,

metabolic regulation under stressful circumstances, the mobilization of hazardous metals, increased uptake of sulfur and nitrogen, and the control of antioxidant properties (Dawood, 2022). Plants are responsive to the external treatment of melatonin and store it in their organs in addition to the synthesis of endogenous melatonin. Exogenous melatonin administration boosts photosynthetic carbon uptake, encourages the production of cold-responsive genes, enhances plant disease resistance, promotes tolerance against salinity, and reduces redox imbalance (L. Y. Wang et al., 2016). Numerous investigations have demonstrated the successful implementation of melatonin as an elicitor for plant cell culture, suggesting the necessity for additional investigation in this area (Coskun et al., 2019; Sheshadri et al., 2022).

The main aim of this study is to establish callus cultures of *Echinacea purpurea* utilizing TDZ as a plant growth regulator and to investigate the influence of melatonin on biomass, antioxidant capacity, and secondary metabolite production within the callus cultures of plants. To the best of our knowledge, no scientific study has yet explored the effects of melatonin on the TDZ-induced callus cultures of *Echinacea purpurea*.

1.1 Aims and Objectives

- To develop an efficient protocol for the optimum induction of callus cultures of *Echinacea purpurea* by using TDZ.
- To investigate the effect of exogenously administered melatonin on the biomass accumulation and secondary metabolism of *E. purpurea* callus cultures.
- To determine the impact of different melatonin concentrations on antioxidant potential of *E. purpurea.*
- To perform the phytochemical analysis to assess the level of bioactive compounds within the callus cultures of *E. purpurea* under the influence of melatonin.

CHAPTER 2
LITERATURE REVIEW

2. Literature Review

Herbal plants have long captivated our fascination for their multifaceted healing properties and natural therapeutic potential. *Echinacea purpurea* (L.) Moench distinguishes among these botanical miracles because it possesses a variety of distinctive qualities. Because of its vibrant petals and potent phytochemical composition, *Echinacea purpurea* has carved out a special place for itself in both traditional medicine and modern herbal remedies. This interesting plant, valued for its ability to increase immune function and general well-being, stands out among herbal remedies because it displays the best example of nature's creativity.

2.1 Botanical description of *E. purpurea*

The "echinacea" originates from the Greek word "echinos" (which also means sea urchin or hedgehog). The flower likely received its name due to the prickly spikes on its head (Mistríková & Vaverková, 2007a). *Echinacea purpurea* (L.) Moench is a strong, perennial herb that grows to a height of 100 to 150 cm. The surface of the cylindrical roots exhibits a brownish-gray hue, contrasting with the white interior. The aerial stem takes on the appearance of a shrub due to its branching structure, rough hair covering, and the presence of reddish-brown markings. The leaves of this plant are lance-shaped, coarsely toothed, and dark green in color. Both sides of the leaves are covered in hair. In the first year of cultivation, it only produces a rosette of leaves and then blooms in the following year in June and July (Awang & Kindack, 1991; McGregor, 1968; Mistríková & Vaverková, 2007b).

The central cone (achene), which produces the yellowish-brown seeds that germinate irregularly in 10 to 21 days, grows more pointed and pricklier as the flower matures. Birds, particularly finches, feed on cones when they are fully seeded (Burlou-Nagy et al., 2022). The flowers are grouped in inflorescences, and sharp bracts are present at the base of inflorescence (Figure 2.1). These bracts undergo maturation and lignification and eventually become spiky. The ligules of the pink sterile ray flowers are 5-7 cm in length and 0.5 cm in width, and these flowers are placed on the edge of the inflorescence. Inside the inflorescence are the bisexual, tubular, orange-brown disc flowers (Keller, 1962; Mistríková & Vaverková, 2007a; Upton et al., 2016). Weed invasion is a frequent issue for Echinacea crops. Herbicides stand out as highly efficient

tools in the chemical management of weeds. Nevertheless, their application has the potential to influence the physiological and morphological aspects of Echinacea plants (Karimmojeni et al., 2022).

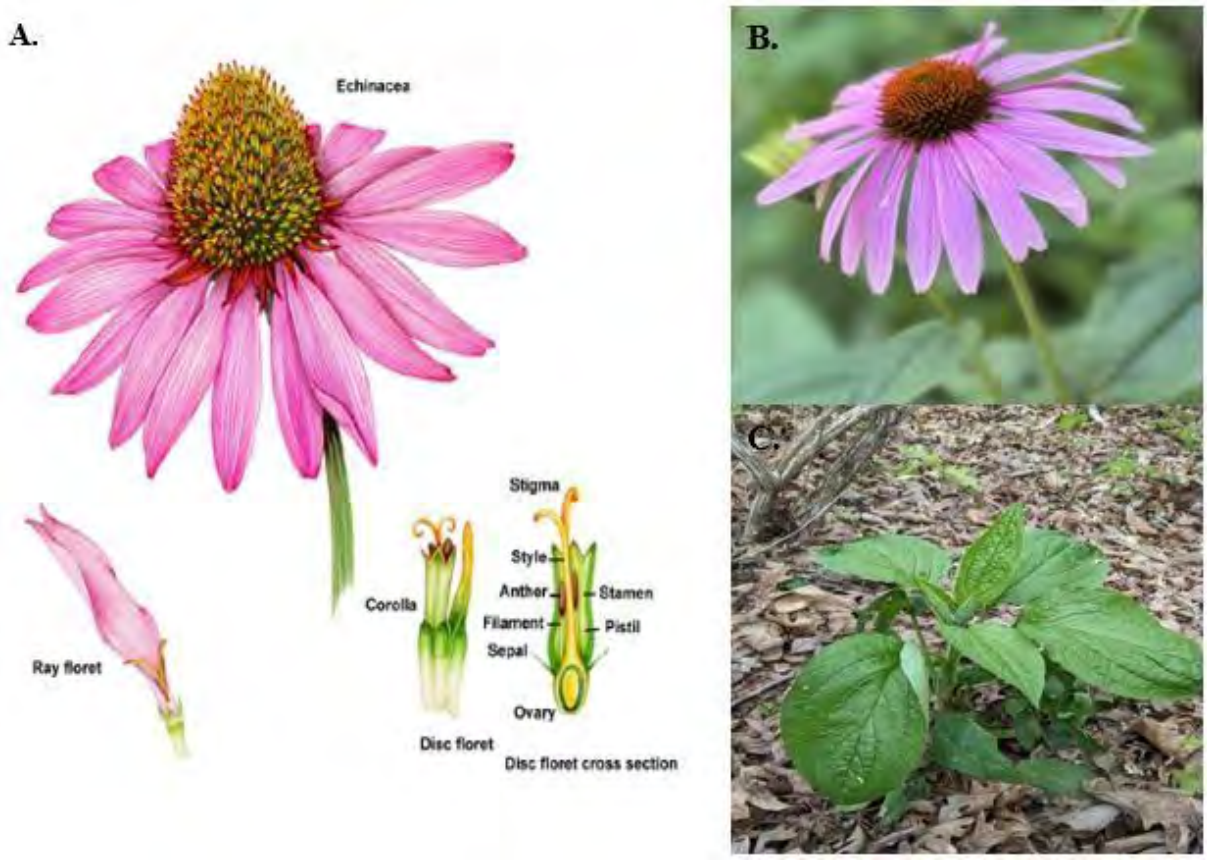

Figure 2.1: A. Sterile ray and disc floret of *E. purpurea* (Balick, 2014), **B.** Flower showing the central cone **C.** Leaves of *E. purpurea* (iNaturalist, 2023)

2.2 Taxonomy of *E. purpurea*

Linnaeus gave the plant its first name, *Rudbeckia purpurea*. Conrad Moench changed the name to *Echinacea purpurea* in 1794, though. Despite this modification, botanical books continued to use the *Rudbeckia* designation long into the nineteenth century. In 1848, Asa Gray finally decided to use the name *Echinacea*. Dr. Asahel Clapp in the mid-1800s is also credited for favoring *Echinacea* over *Rudbeckia* in medical botany. Although *Echinacea* is the herb of choice today, historians

researching the herb's therapeutic history should also consider mentions made before the American Civil War under the *Rudbeckia* genus. In 2002, Binns and his coworkers revealed a mistake in using the name *Echinacea purpurea* for the plant correctly known as *Echinacea serotina* in 1836. To avoid uncertainty amongst gardeners and herbalists, the authors recommended keeping the names as they are (Binns et al., 2001; Flannery, 1999: Felger, 2007) . The scientific classification of the plant is given in Table 2.1.

Table 2.1: Scientific classification of *E. purpurea* (Kindscher, 2016)

Kingdom	Plantae
Phylum	Anthophyta
Class	Dicotyledoneae
Order	Asterales
Family	Asteraceae
Genus	Echinacea
Species	*Echinacea purpurea*

2.3 Alternate names for *Echinacea purpurea*

Some of the common names and synonyms that refer to *Echinacea purpurea* include Purple Coneflower, Eastern Coneflower, Missouri Snakeroot, Sampson Root, Purple Echinacea, Purple Rudbeckia, *Rudbeckia purpurea*, and Hedge Coneflower (Kindscher, 2016; Farms, n.d.).

2.4 Geographical distribution of *Echinacea purpurea*

Although *E. purpurea* has an extremely wide natural range, it is an ecotone species in the wild, favoring the shaded borders of savannas and glades, and open forests with some sun exposure. Currently, the *Echinacea* genus can be found from southern Alberta, Canada, to Texas, close to the Gulf of Mexico shore (Urbatsch et al., 2005). *Echinacea purpurea*, which has a typical discontinuous distribution, is found in isolated prairie remnants in the southern states of Louisiana and North Carolina, to the foothills of the Rocky Mountains as well as in the Midwest states of Ohio, Kentucky, and Tennessee. The deep lavender ligules contrast with the bright orange-tipped

paleae and make the wild populations of *E. purpurea* in Louisiana and Mississippi particularly stunning in terms of coloring. Any genetic component underlying the coloration of these populations ought to be the subject of selection for ornamental value (McKeown, 1999).

2.4 Cultivation and Ecology

Purple coneflower can be cultivated from seeds, nursery stock, root cuttings, and plant divisions. A period of cold stratification is necessary for seeds to help with germination, and they can be introduced indoors four to six weeks before the last day of frost. Before planting, seeds can be prepared by storing them in the refrigerator for a few weeks (Marinelli, 2008). Although it may grow in a variety of soil types, this plant does best in well-drained, damp loam. Once established, it can thrive in both full sun and moderate shade and is drought tolerant. It can be simply propagated by seed and will naturally reseed itself in the garden and is also resistant to heat, humidity, and salt. *Echinacea purpurea* should be planted in soil with a pH of 6 to 8 (Hall et al., 2003).

2.5 Phytoconstituents of *Echinacea purpurea*

From *Echinacea* species, several noteworthy families of bioactive chemicals have been identified. The main groups of phytochemicals found in *Echinacea purpurea* are reported in Table 2.2.

Glycoproteins, alkylamides, polysaccharides, flavonoids, phenolic chemicals, caffeic acid derivatives like caftaric acid, cynarin, chlorogenic acid, chicoric acid, and echinacoside are the most crucial elements of *Echinacea purpurea* (Attarzadeh et al., 2020; Shahrezaei & Swain, 2008), and their amounts depend upon the sections of plant (Harborne & Williams, 2004).

Glycoproteins, polysaccharides, and alkylamides are the chemical components that give purple coneflower roots their immunomodulatory properties (Balciunaite et al., 2020). Carbohydrates and protein chains called glycoproteins play a part in several biological processes, such as immunology. In addition to having a high bioavailability, the alkylamides present in the genus Echinacea (Asteraceae) have been proven to have immunomodulatory effects. They share an amine bond as a structural component and often contain a short-chain amine coupled to an aliphatic chain of polyunsaturated fatty acids. Complex carbohydrate polymers known as polysaccharides are made up of more than two monosaccharides. Pectins, arabinogalactans, and

inulin are significant polysaccharides found in the Asteraceae family. A portion of the traditional usage of *Echinacea purpurea* may be supported by their bioactive polysaccharides.

Table 2.2: Phytochemical components of *Echinacea purpurea* (Megha et al., 2012).

Class of constituents	Examples
Glycosides	Echinacoside, Echinacin
Phenyl propanoids	Luteolin, kaempferol, quercetin, apigenin and isorhamnetin.
Flavonoids	Caffeic acid and its derivatives
Terpenoids	Borneol, germacrene D, Caryophyllene epoxide and palmitic acid.
Nitrogenous compounds	Alkamides, alkaloids like glycine betaine, pyrrozolidine, alkaloids like tussilagine and isotussilagine
Others	Polysaccharides, phytosterols, polyacetylenes, sugars, metal salts like potassium, aluminum calcium, magnesium, ferric chloride, carbonates, silicates, and sulphates. Ascorbic acid is also present.

To obtain plant extracts that are abundant in bioactive components, an *in vitro* investigation was conducted. Dihydroxybenzoic acid hexoside, dicaffeoylquinic acid, chlorogenic acid, chicoric acid isomers, and caftaric acid were all found to be phenolic acids. Rutin, a flavonol glycoside, was also discovered in the roots (Maggini et al., 2019).

Within the costituents of *Echinacea purpurea*, chicoric acid is regarded as the truly important derivative of caffeic acid and is the primary constituent of the root and petiole (Erkoyuncu & Yorgancilar, 2021; Liu et al., 2006). The antioxidant and antibacterial properties of caffeic acid derivatives might assist the immune system of the body to function better. Nevertheless, the quantities of caffeic acid derivatives will differ depending on the variety of plant, organ type, growth circumstances, and environmental factors.

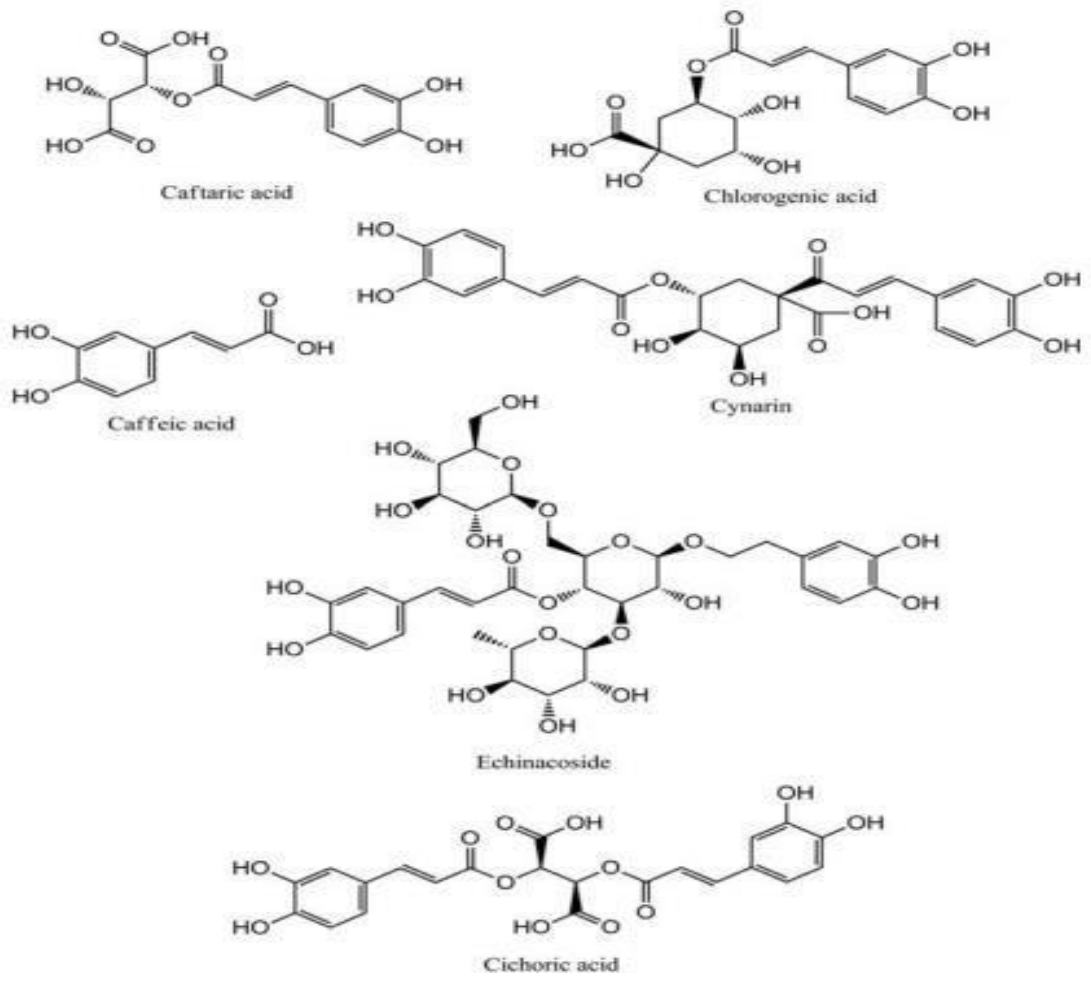

Figure 2.2: Molecular structures of the common phytochemicals in *Echinacea purpurea*

A caffeic acid derivative echinacoside with a 1.45% concentration can be discovered in the flower. Echinacoside has been reported to provide several pharmacologically significant advantages for human health, especially cardiovascular and neuroprotective properties (Geng et al., 2007; Tabar et al., 2019). Along with these components, *Echinacea* species have other flavonoids, polyacetylenes, and alkaloids (Bauer, 1996; Hou et al., 2020).

Concerning age and year of collection, the presence of ascorbic acid (commonly known as vitamin C) in freshly harvested *Echinacea purpurea* was examined. Ascorbic acid accumulated greatest in the leaves and least in the stems. Results from a two-year investigation showed that fresh pulp had a small amount of vitamin C as compared to the fresh plants (Zagumennikov et al., 2015).

Phylloxanthobilins are components that were isolated from the leaves of *Echinacea purpurea* and are of significant value. These organic tetrapyrrole compounds are produced naturally during the degradation of chlorophyll. About ten years ago, in the leaves of deciduous trees, phylloxanthobilins were found. They are currently thought to be a family of compounds with significant bioactivity potential that needs further investigation. But until recently, no examples of phylloxanthobilins being found in plant based formulations have been stated (Karg et al., 2020: Karg et al., 2019).

In one investigation utilizing gas chromatography/mass spectrometry, it was shown that α-phellandrene was abundant in the roots of *Echinacea purpurea*. In a research project by Mirjalili et al. (2006), the essential oil of *Echinacea purpurea* flowerheads was obtained by hydrodistillation. GC and GC-MS were used to evaluate the essential oils. Most of the chemicals in *E. purpurea* belonged to the sesquiterpene hydrocarbon class. The oil of *E. purpurea* contains monoterpene hydrocarbons, notably α -phellandrene (6.4 percent).

Moreover, all the varieties of purple coneflower contain acetaldehyde, camphene, dimethyl sulfide, α-pinene, and limonene. Dimethyl sulfide has been found in minor quantities in the leaves, stems, and flowers; nevertheless, it is the highly prevalent compound in the *E. purpurea* roots. Aldehydes, butanals, and propanols, account for 6–14% of the headspace among the bloom and the stem tissue, 19–29% of the headspace in the leaf tissue, and 41.5–57% of the headspace in the root tissue (Long et al., 2014; Thomsen et al., 2012).

In another study, n-hexane extract of *Echinacea purpurea* was also found to consist of sterols (13.9 %), fatty acids (25.8%) encompassing linoleic acid, palmitic acid, stearic acid, and long-chain hydrocarbons (14.6 %) (Coelho et al., 2020). The most often used herbal remedies in Europe and the US are preparations having *E. purpurea* active ingredients. (Billah et al., 2019; Cupp & Cupp, 2000). It is important to remember that enzymatic reactions may degrade bioactive compounds during preservation because of prolonged storage from collection to commercialization, which could result in compositional alterations.

2.6 Therapeutic potential of *Echinacea purpurea*

The most significant application of *Echinacea purpurea* lies in its traditional use as a remedy for both handling and stopping upper respiratory tract infections, including the common cold in traditional medicine. This herbal remedy has been relied upon for generations to combat these

ailments effectively (Weber et al., 2005). *Echinacea purpurea* offers a diverse range of medicinal properties, making it a versatile and valuable herbal plant in traditional medicine.

2.6.1 Immunomodulatory activity

The most well-known herbal medications with immunomodulatory effects are *Echinacea* preparations. It has been demonstrated that purified polysaccharides made from the root and herb of *E. purpurea* strongly stimulate macrophages, causing them to produce pronounced extracellular cytotoxicity against tumor targets without the assistance of lymphocytes. After being activated with polysaccharides, macrophages produce and secrete more oxygen radicals and interleukin-1 (IL-1), which increases their cytotoxicity toward cancerous and infectious cells. B lymphocyte, however, are moderately affected (Stimpel et al., 1984). Spence (2002) evaluated the immunomodulatory effects of fractions derived from *Echinacea purpurea*. The young mice treated with *Echinacea purpurea* showed substantial increases in mean interleukin-12 titre. In mice given oral doses of the phenolic, polysaccharide, or alkylamide fractions of *Echinacea purpurea* and the dead *Salmonella typhimurium* vaccination, interferon-gamma levels rose. Additionally, it was proposed that the activity was caused by oxidized alkylamide derivatives formed by the extract's metabolism. Dried powder of *Echinacea purpurea* roots (including 1.5 percent total polyphenols, estimated as chlorogenic acid) was given via the oral route at a dose of 30 or 100 mg/kg per day for fourteen days, and it boosted the splenic lymphocytes' resistance to apoptosis (Hovater et al., 2006).

2.6.2 Anti-microbial activity

Anti-microbial properties have also been discovered in the herb *Echinacea purpurea*. Candida species' growth was impeded by the extracts from the roots. This activity demonstrated antifungal action since it was caused by ketoalkenes and ketoalkynes (Binns et al., 2000; Merali et al., 2003). When cultured on mice fibroblasts for 24 hours, the root extract of *Echinacea purpurea,* prepared in methanol and distilled water, demonstrated a resistant reaction towards influenza A2, herpes, and vesicular stomatitis infection as well as against Herpes simplex virus (HSV). It was discovered that the activity was caused by a high molecular weight fraction of *E. purpurea* that contained polysaccharides and glycoproteins. Moreover, when n- Hexane root extract of *E. purpurea* was

subjected to visible and UV-A light, HSV Type I was inhibited. It was reported that the action was due to the presence of alkenes and amides of the plant (Binns et al., 2002).

2.6.3 Anti-inflammatory activity

The roots of *E. purpurea* contain alkamides, particularly polyunsaturated isobutylamides, which have anti-inflammatory properties because they inhibit the enzyme 5-LOX (5- Lipo-oxygenase) (Müller-Jakic et al., 1994; Wagner et al., 1989). Mice with carrageenan-induced paw oedema responded favorably to the anti-inflammatory influences of *E. purpurea* (dry root powder). Several constituents of the *E. purpurea* were found to be in charge of these activities. Alkamides reduced NO and iNOS generation, which are the agents that cause inflammation, and the polar caffeic acid fraction increased arginase activity, a key player in anti-inflammatory action (Zhai et al., 2009).

E. purpurea essential oils have anti-inflammatory properties as well. Using several animal models, including xylene-induced mouse ear edoema, egg-white-induced rat paw edoema, and cotton-induced granuloma tissue proliferating inflammation in mice, the ability to reduce inflammation was evaluated *in vivo*. Blood count of pro-inflammatory cytokines IL-2, IL-6 and TNF-α was decreased in the groups that were treated with the plant extract, confirming the anti-inflammatory properties of herb (Yu et al., 2013). Six sesquiterpenes, namely purpureaterpenes, were obtained and detected from the aerial section of *E. purpurea* in a study directed by Cheng et al. (2020). All of the isolated chemicals were found to be engaged in the NF-kappaB signaling pathway that is reported to be involved in inflammation. These purpureaterpenes exhibited the property of inhibiting this pathway, thus acting as an anti-inflammatory agent.

2.6.4 Antioxidant activity

Chlorogenic acid, cichoric acid, caffeic acid, and cynarine can scavenge hydroxyl radicals, as well as the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and the ABTS radical. Additionally, they have been shown to lengthen the lag phase of soybean liposome peroxidation and hinder the synthesis of conjugated diene hydroperoxide caused via heat breakdown of 2,2'-azobis (2-amidinopropane) dihydrochloride (Hu & Kitts, 2000). H. Ding et al. (2019) showed that *Echinacea purpurea*'s cichoric acid could greatly lessen the histological alterations of LPS-induced acute lung damage. It has been shown that cichoric acid has the capacity to reduce the amounts of reactive

oxygen species (ROS), malondialdehyde (MDA), and the depletion of glutathione (GSH) and superoxide dismutase (SOD) that is brought on by LPS challenge. Karg et al. (2019) showed that human endothelium kidney cells can absorb phylloxanthobilins, which were produced from the leaf extracts of *Echinacea purpurea*. A significant *in vitro* anti-oxidative activity of this natural product was found, and total cellular glutathione levels showed that phylloxanthobilins have the ability to scavenge intracellular ROS and prevent oxidative stress.

2.6.5 Anti–androgenic activity

The influence of *Echinacea purpurea* on the sexual glands has also been reported in several studies. The change in weight of rat prostates, rat epididymis, and rat testicles, as well as changes in histology, revealed the anti-androgenic efficacy of *Echinacea purpurea* root extract. Rats that underwent the treatment demonstrated a noteworthy decrease in the weight of the prostate gland and efficient enhancement in the production of lymphocytes (Skaudickas et al., 2003). In another study, *E. purpurea* root extract was given to animals with benign prostatic hyperplasia and consequently, all the degenerative changes in the animals were reversed. The findings of the study declare that the extract is effective at halting the growth of benign prostatic hyperplasia (Skaudickas et al., 2009).

2.6.6 Cannabinoidomimetic activity

A novel class of cannabinomimetics is alkylamides derived from echinacea. This type of activity is demonstrated by the alkylamides named as dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide and dodeca-2E,4E-dienoic acid isobutylamide. They have a stronger affinity for cannabinoid type (CB) receptors (CB2 and CB1 receptors) than the endogenous cannabinoids (Raduner et al., 2006).

2.6.7 Radioprotective activity

Radiation-induced metabolic abnormalities and tissue damage are reported to be lessened by *Echinacea purpurea* extract (Ahmed et al., 2017). The effects of the *E. purpurea* extract were measured in gamma-irradiated mice. The findings showed that gamma-rays had a negative impact on levels of haemoglobin (Hb) in blood levels, red blood cell counts, various white blood cell counts, and bone marrow cell counts. The decrease in the level of red blood cells and haemoglobin seen in the irradiated groups at all three-time points was attributed to impaired cell division,

damaged blood-forming organs, digestive system disturbances, depletion of components necessary for the differentiation of erythroblasts, ability of bone marrow to release reticulocyte, the exodus of cells from circulation because of haemorrhage or capillary wall leakage, and/or direct cellular apoptosis. RBC count and Hb level both showed signs of recovery in both the protection and recovered groups. However, *E. purpurea* had a greater radio-protection efficiency than radio-recovery efficiency (Mishima et al., 2004).

2.6.8 Antitumor activity

It was discovered that the hexanic root extract of the plant had cytotoxic and proapoptotic qualities. It has a concentration- and time-dependent effect on cell viability reduction. These findings serve as the foundation for establishing logical proof of the potential contribution of *Echinacea* species to medical oncology. Driggins (2005) studied E. *purpurea's* anti-carcinogenic characteristics and led to the conclusion that the plant could be employed as a breast cancer treatment alternative. A study led by Ren et al., (2023) also revealed that a homogenous polysaccharide extract from *E. purpurea* has anti-tumor effectiveness because it activates the inflammasome in M1 macrophages through a phagocytosis-mediated endocytosis mechanism.

2.6.9 Anti-aging potential

Herbal plants are widely recognized for their potential to be used as active components in cosmeceutical items as well as their promising skin benefits. Research led by Chaiyana et al. (2021) evaluated the anti-aging capabilities of 16 edible flowering plants, and found that the extract of *Echinacea purpurea* had the strongest anti-collagenase (78.5%), anti-elastase (69.0%), and anti-hyaluronidase (64.2%) effects. These herbal extracts possess potential for usage as active components in skin-care items due to their beneficial impact on the skin. The collagenase, elastase, and tyrosinase repressing activity of the *E. purpurea* extracts was remarkable, demonstrating their antiaging characteristics. Even small amounts of plant extract (as low as 2.5 mL extract/mL) have the ability to stimulate the closure of scratch in keratinocyte monolayers and to inhibit hyaluronidase (IC50 values lower than 30 L μ extract/mL), thus showing the promising benefits of *E. purpurea* on wound healing (Ciganović et al., 2023).

2.6.10 Anti-diabetic activity

Echinacea purpurea ethanolic extracts have been demonstrated to have anti-diabetic properties; for instance, they stimulate peroxisome proliferator-activated receptor γ (PPARγ) and boost insulin-stimulated glucose absorption (Shin et al., 2014). Chiou et al. (2017) also investigated the *in vitro* inhibitory properties of 50% ethanolic extracts of *E. purpurea* and caffeic acid derivatives on α-amylase, α-glucosidase, and angiotensin-converting enzyme (ACE) related to type 2 diabetes. The activities of α-amylase, α-glucosidase, and ACE were diminished by the extract and CAD in a concentration-dependent mode. The *in vitro* findings imply that CAD and *E. purpurea* extract have excellent potential for treating hyperglycemia, that can be further explored to plan a considerable therapy for diabetes.

2.7 Plant tissue culture

All around the world, humans have used plants for their medicinal benefits since ancient times. Their pharmacological actions rely on the phytochemical constituents of plants, notably the secondary metabolites. Plants respond to many forms of stress by producing secondary metabolites that serve several therapeutic purposes. They have complicated chemical compositions and are applied in the food and beverage, cosmetics, pharmaceutical, and dietary supplement industries. Because of the extensive industrial use of these metabolites, research has to be focused on boosting production utilizing plant tissue culture (PTC) methods and enhancing mass-scale synthesis by employing bioreactors (Chandran et al., 2020).

In 1902, Gottlieb Haberlandt published his theory on the totipotency principle, the innate potential of separate plant cells for independent life. It was the beginning of *in vitro* plant cell and tissue culture research (Haberlandt, 1902). In 1930s, the development of maintenance and long-term growth of cultured plant tissues took place, which gave experimental support for this idea. The phytohormones auxin and cytokinin were found to be necessary for *in vitro* cell proliferation. Additionally, it was found that the morphogenetic trajectory of *in vitro* cultured tissue is governed by the proportion of these hormones. Higher and lower ratios of cytokinin to auxin favor shoot and root regeneration, respectively. Conversely, more balanced quantities result in the disorganized proliferation of cell masses (Skoog, 1957). Murashige & Skoog (1962) developed a specified mineral nutrient composition based on their investigation into the chemical composition of tobacco

leaves. This mixture successfully encouraged the growth and division of tobacco cells and tissues. The resultant MS medium was crucial in developing plant tissue culture, and its contribution is still valued in modern plant research.

Traditional methods take a long time since it takes a plant a year to develop and achieve the step where the required compound is produced. To allow the accumulation of phytochemicals effectively and quickly for commercial use, *in vitro* PTC techniques offer an alternative (Kolewe et al., 2008; Verpoorte et al., 2002). It permits large-scale plant propagation under regulated environmental conditions without regard to the seasons (Murthy et al., 2014; Ochoa-Villarreal et al., 2016). Therefore, despite all obstacles, *in vitro* tissue cultures are a crucial tool for both basic research and commercial applications.

2.8 Callus cultures

In order to apply biotechnology to harness plant products for human benefit, callus culture is one of the essential ways. It entails the development of an undifferentiated mass from plant tissues. Callus can be engineered to produce useful goods for food, agriculture, cosmetics, and pharmaceutical industries. The advancement of callus culture as a biological engineering method for medicinal chemicals has attracted significant attention in recent years (Benjamin et al., 2019). It has been a vital system for studying cells, comprehending various systems, and controlling plant cells to produce important metabolites (Bhatia et al., 2015).

Mixtures of auxin and cytokinin are frequently employed for callus induction. Callus cultures exhibit the potential to produce therapeutically important secondary metabolites commercially (Ogita, 2015; Wu et al., 2016). For extracting medicinal compounds, callus culture has repeatedly proven to be extra dependable than gathering wild plants. They can be employed to establish single-cell suspension cultures utilizing batch or continuous fermentation to make the desired secondary metabolites, as well as for the micropropagation of numerous clones of plants (Fischer et al., 1999; Xu et al., 2011). Callus and suspension cultures can therefore produce secondary metabolites, and this makes it possible to alter the production of those SMs. Callus cultures have been reported to be employed for the accumulation of resveratrol, serpentine,

alkaloids, paclitaxel, α-tocopherol, flavonoids, ajmaline, reserpine, scopolamine, stilbene, and anthocyanins (Efferth, 2019).

2.9 In vitro cultures of *Echinacea purpurea*

Different studies have reported the development of *Echinacea purpurea in vitro* cultures. Coker & Camper (2000) tested different protocols for the propagation of *Echinacea purpurea*. On 2,4-D-kinetin combination medium, extensive callus and few shoots and roots developed. Explants on medium with NAA-kinetin resulted in mature plants with blooms that were true to type in terms of shape and color. In a study led by Koroch et al. (2002), effective regeneration of plants was attained via organogenesis from callus cultures induced from leaflets of *E. purpurea*. Leaf explants were used to initiate the growth of proliferating shoot cultures on Murashige and Skoog (MS) medium supplemented with a combination of BAP (4.44 μM) and NAA (0.054 μM). Another study investigated the effects of thidiazuron on the morphogenesis of *E. purpurea*. On leaf explants cultivated on a medium comprising 2,4-dichlorophenoxyacetic acid or dicamba, callus growth and root organogenesis were seen, but no plantlets were regenerated. Regenerable callus cultures were created when TDZ was the only growth regulator added to the MS media (Jones et al., 2007). Erkoyuncu & Yorgancilar (2021) tested several mediums supplemented with various PGRs utilizing leaf, petiole, cotyledon and root as the explants. The MS medium containing 1.0 mg l−1 2,4-D + 2.0 mg l−1 BAP, 1.0 mg l−1 NAA + 0.5 mg l−1 TDZ, 2.0 mg l−1 NAA + 1.0 mg l−1 TDZ and 0.5 mg l−1 NAA + 0.5 mg l−1 BAP in showed the best callus development by using leaf, petiole, cotyledon, and root explants, respectively. However, to a greater extent, the establishment of callus cultures by using different plant growth regulators is reliant on the laboratory conditions and the type of explant used.

2.10 TDZ as a plant growth regulator

TDZ ($C_9H_8N_4OS$ or 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea) is a substituted phenyl urea molecule that acts as a hormone by inducing defoliation and regeneration in plant tissues (Ferrante et al., 2002). The use of TDZ results in a broad range of cultural reactions, from the development of somatic embryos to the development of callus. It was primarily made to process cotton bolls automatically (Suttle, 1985). Phenyl and thiadiazol are the two functional groups of TDZ (Sharma et al., 2013). It is dispersible in water and several organic solvents, such as acetone, methanol,

dichloromethane, hexane, and toluene (B. N. S. Murthy et al., 1998). The process of forming an entire plant from explants begins with callogenesis. The development of the roots and shoots comes after callogenesis (Gill & Ozias-Akins, 1999). Callus production is usually commenced by a balanced ratio of auxin to cytokinin (Centeno et al., 1996). The influence of auxin and cytokinin on the development and specialization of cultured explants closely resembles the effects elicited by thidiazuron (TDZ) (Nakano et al., 2018). It has proved to be more effective than other cytokinins, including zeatin, kinetoplastin, and even auxins (Kanyand et al., 1994).

It is believed that TDZ plays a crucial function in callogenesis (Ahmad et al., 2011). In callus cultures that depend on cytokinin, TDZ can carry out stronger cytokinin activity (Yadav et al., 2010), due to its ability to breakdown cytokinin oxidase/dehydrogenase (CKX), an enzyme that facilitates the breakdown of cytokinines (Nisler et al., 2021). Callus induction is one of the many biochemical and physiological processes that TDZ can improve in cells (H. M. Ali et al., 2022). When used alone, TDZ has diverse impacts on callogenesis for various species and cultures under various circumstances. TDZ-containing media frequently exhibit improved callus initiation; for instance, TDZ-containing media have demonstrated to be most effective for the improved regeneration and efficient callus induction of wheat and barley plants. Depending on the type of plant, the explants, and the length of exposure, the ideal TDZ concentration may change (Yadav et al., 2010).

2.11 Elicitation

Many secondary metabolites are only synthesized in small amounts, during the exponential development phase of PTCs, as their basic precursor metabolites are essential for the synthesis of biomass. There is proof that stationary growth phase is the best time to induce the formation of secondary metabolites from primary substances. For this reason, establishing a two-stage culture which permits the cells to be initially kept in an ideal medium for biomass development and then transferred to an ideal synthesis medium, that promotes the production of secondary metabolites, is a viable approach for a plant cell factory. Elicitors and biosynthetic precursors can be introduced to this system during the moment of highest yield, which is in the second phase of culture (Malik et al., 2011)**.** One of the best methods now employed for increasing biotechnology output of secondary metabolites is elicitation. Elicitors are substances that activate all plant defence

mechanisms, boosting secondary metabolism to safeguard the cell and the plant. Physical elements can elicit a cell culture, but the main technique utilized in biotechnological cell cultures is the addition of elicitors, either biotic or abiotic, to the culture medium (Baenas et al., 2014; Klarzynski & Fritig, 2001; Zhao et al., 2005).

Numerous elicitors have been observed to dramatically increase the secondary metabolism of *Echinacea purpurea*. For instance, Wu et al. (2007) used 100 μM sodium nitroprusside (SNP), which exogenously generates nitric oxide, to elicit the adventitious roots of *E. purpurea*, and the results showed that the phenolics, flavonoids, and caffeic acid derivatives were accumulated in greater amounts as compared to control. In one research by Ramezannezhad et al. (2019), silver nanoparticle treatment increased the formation of cichoric acid derivatives in cell suspension cultures of *E. purpurea*. Elicitation has been demonstrated to be a useful technique for enhancing the capacity of the plant *in vitro* cultures for secondary metabolism and thus, needs to be further explored.

2.12 Melatonin as an elicitor

Since the 1995 discovery of melatonin in higher plants, information regarding melatonin's existence and potential function has steadily accumulated (Arnao & Hernández-Ruiz, 2015). It has been established that plants produce a sizable quantity of it internally. Because of the wide range of biological uses for melatonin, it is referred to as a multiregulatory molecule.

Plants are also responsive to the exogenous use of melatonin in addition to natural melatonin's production, and they can store it in their organs as a result (N. Zhang et al., 2015). Exogenously administered melatonin boosts cold-responsive gene expression and photosynthetic carbon absorption (F. Ding et al., 2017), increases plant disease resistance (Chen et al., 2019), combats resistance to salinity and reduces oxidative stress (L. Y. Wang et al., 2016). Crops including rice, maize, corn, wheat, barley, and oats, as well as fruits such as tomato and apple, and vegetables like okra and cabbage, have all been proven to be impacted by melatonin. Exogenous melatonin, for instance, delayed the senescence of apple leaves, increasing the amount of apples that might be produced, according to an indirect influence on apples (P. Wang et al., 2012).

In callus cultures of different plants, exogenous melatonin is essential for triggering and controlling cell growth and development (Bano et al., 2022; Fazal et al., 2018). This has primarily been attributed to melatonin's function as an anti-stressor (Arnao & Hernández-Ruiz, 2015). When different melatonin doses are applied to growth media, this usage of exogenous melatonin to *in vitro* plant cultures greatly enhances biomass output. Sheshadri et al. (2022) investigated how melatonin affects the callus cultures of *Catharanthus roseus*. According to the research, exogenous melatonin boosted cell wall invertase transcription and activity as well as terpenoid indole alkaloids synthesis. By using a 100 µM concentration of melatonin, it has been observed that the generation of terpenes and ketones is increased in callus cultures of *Salvia officinalis* (Kilic et al., 2023). In another study of Khan et al., (2019), the callus cultures of *Fagonia indica*, grown over a TDZ-containing media, were exposed to different doses of melatonin. The outcomes showed that application of melatonin enhances the biomass accumulation in a direct correlation with their phenolic and flavonoid content, as well as their antioxidant capacity. However, there are specific circumstances when exogenous melatonin treatment has been demonstrated to reduce callus induction in particular plants, like sweet basil (*Ocimum basilicum*). It was reported by Duran et al. (2019) that callus culture formation was inhibited by 200 µM melatonin. In *O. basilicum* callus cultures, the decline has coincided with an enhancement in the synthesis of rosmarinic acid and phenolic constituents.

CHAPTER 3
MATERIALS AND METHODS

3. Material and Methods:

All the conducted experiments were devised and carefully executed within the Plant Cell Culture Lab (PCCL), Department of Biotechnology, Quaid-i-Azam University, Islamabad, under the supervision of Prof. Dr. Bilal Haider Abbasi.

3.1 Chemicals and equipment

Ethanol, hydrochloric acid, sodium hydroxide, mercuric chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, FC reagent, aluminum chloride, sulfuric acid, sodium phosphate, ammonium molybdate, sodium carbonate, potassium acetate, ascorbic acid, quercetin, gallic acid, plant growth regulators, and distilled water are some of the chemicals used in this study.

Equipment used includes filter paper, forceps, micropipettes, petri dishes, spatula, blades, vortex mixer, mortar and pestle (for grinding plant samples), UV-visible spectrophotometer, Sonicator, Incubator, pH meter by Jenway 3305, Centrifuge, Electrical balance (GF-300), autoclave (KP-30L, ALP Tokyo Japan), laminar flow transfer cabinet (ESCO), and glassware (petri dish, glass beaker, measuring cylinder, Erlenmeyer flask).

3.2 Media preparation

Following the protocol formerly adopted by Abbasi et al. (2010), Murashige and Skoog basal media was prepared for the germination of *Echinacea purpurea* seeds. 30 g of sucrose and 4.4 g of MS medium were weighed and allowed to mix in distilled water and the volume was made up to 1 liter. The acidity of the solution was regulated within a range of 5.6 ± 0.2 through the utilization of sodium hydroxide (1.0 N) and hydrochloric acid (1.0 N). After setting pH, to solidify the media, 8 g of agar was added and dissolved by heating. Each 100 mL of Erlenmeyer flask was then filled with 40 mL of prepared media. Cotton plugs and aluminum foils were used to properly seal the flasks. To avoid contamination, the flasks were then autoclaved for 20 minutes at 121°C and 15-psi pressure, and then left overnight to allow proper solidification.

3.3 Surface sterilization

All the glassware and utensils employed during the research were properly cleansed with running water and detergent to remove any settled dust particles. All the equipment was then dried, wrapped, and was allowed to be sterilized in an autoclave for 20 minutes at a temperature of 121°C and 15-psi pressure.

3.4 Seed germination

Echinacea purpurea seeds were collected from the University of Tours, France, by Dr. Bilal Haider Abbasi in March 2022. Seeds were inoculated within a sterilized setting of laminar flow hood. All the autoclaved instruments including forceps, petri dishes, blades, and beakers were placed in the laminar flow cabinet along with the media-containing autoclaved flasks, distilled water, and ethanol. To minimize any chance of contamination, each piece of equipment was again sterilized by using 70% ethanol. The laminar flow hood was closed, and the UV (GKL-511, 50 Hz, 19w) light was turned on for twenty minutes. Once the UV light was turned off, the hood was opened, and fans were switched on. Hands were sterilized by using 70% ethanol and the spirit lamp was turned on. The initial step involved rinsing the seeds using distilled water. Subsequently, they were immersed in a solution containing 0.1% (w/v) Mercuric chloride for a duration of one minute. Afterward, washing was done with 70% EtOH for nearly 40 seconds. Finally, three rinses using distilled water were done to remove any remaining dust particles. The Whatman filter paper was used for drying the seed surface. Each flask was then inoculated with two to four seeds. The flasks were then placed in a growth chamber at 25°C. A 16/8-hour (light/dark) photoperiod was maintained at a regular intensity of 40 μmol $m^{-2}s^{-1}$ (Philips TLD) from fluorescent light bulbs.

3.5 Establishment of Callus Cultures

For callus establishment, explants from 60 days old *in vitro* grown *E. purpurea* plants were used. Leaf and stem explants (1.5 cm) were inoculated on MS media having sucrose (30 g/L), agar (8 g/L), and varying amounts of thidiazuron (TDZ) (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mg/L), α-naphthaleneacetic acid (NAA) (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mg/L), and Benzylaminopurine (BAP) (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mg/L). After autoclaving, inoculated flasks were put in a growth room at 25°C with a 16/8 photoperiod of light/dark and a light intensity of 40 μmol $m^{-2}s^{-1}$. The entire

procedure was conducted three times, with three sets of replicates for each concentration. On the 40th day, callus cultures were collected, and after determining the fresh weight, they were dried in an oven. Every three weeks, the callus was subcultured on the appropriate hormonal medium. Callus induction frequency of TDZ-induced calli was determined by using the following formula.

$$\% \textbf{ Frequency of Callus Induction} = \frac{\textbf{Number of responding explants}}{\textbf{Total inoculated explant}} \times \mathbf{100}$$

3.6 Preparation of Elicitor

The stock solution of melatonin was prepared by weighing 50 mg melatonin and dissolving it in 50 mL of distilled water. Continuous stirring for one hour was done to achieve adequate mixing.

3.7 Callus establishment with elicitors

On MS medium enriched with varying dosages of melatonin (0, 1, 5, 10, 25, and 50 µM) as well 3.0 mg/L of TDZ, 1 g of fresh callus from the previously subcultured callus was inoculated. The control group consisted of the callus and the MS medium that had been supplemented solely with TDZ (3.0 mg/L). The cultures were kept at a temperature of 25°C in a growth room with a photoperiod of 16 hours light/8 hours dark, and 40 µmol $m^{-2}s^{-1}$ of light intensity. The calli were gathered after a period of 35 days of growth and were subsequently delicately positioned on absorbent filter paper for separating the media and removing any water content prior the determination of fresh weight. Each callus obtained was then grounded for further phytochemical study after being oven dried to determine their dry weight.

3.8 Sample extraction

Following the procedure outlined in a study by M. Ali et al. (2013), samples were extracted for phytochemical analysis, specifically to evaluate their total phenolic contents (TPCs), total flavonoid contents (TFCs), and antioxidant potential. 50 mg of dried samples were mixed with 500 µL of 80% (v/v) methanol and vortexed for 10 minutes. Sonication (20 minutes) was done three times with a 30-minute break in between. The mixtures were then centrifuged (10,000 rpm, 10 min). The pellets were discarded, and supernatants were kept at 4 °C for further analysis.

3.9 Evaluation of TPC and TFC

Following the technique of Velioglu et al. (1998), total phenolic content (TPC) was assessed using the Folin-Ciocalteu reagent (FCR) method. The amount of TPC was calculated by mixing the Folin-Ciocalteu reagent (90 µL) with the samples (20 µL) present in each well of 96-well microplate. After then, the combination was permitted to react. Then, 90 µL of sodium carbonate was included to each sample combination and gently spun around. After allowing the mixture to incubate at room temperature for a duration of 5 minutes, it was proceeded to measure the absorbance at a wavelength of 630 nm using a microplate reader. Gallic acid (0-50 µg/mL) was employed as standard to plot the calibration curve (R^2= 0.968). The formula shown below was employed to determine total phenolic production, which was then represented in mg GAE/L.

Total phenolic production (mg/L) = DW (g/L) ×TPC (µg/mg)

For evaluating the total flavonoid content, the aluminum chloride colorimetric method described by Shahriar et al. (2012) was followed with minor modification. 10 µL Aluminum trichloride solution and 10 µL of 1M potassium acetate solution were added to a 96-well plate that already had 20 µL of the samples in the reaction wells. The total reaction volume was increased to 200 µL by the addition of 160 µL of distilled water. The samples were then left at room temperature for 30 minutes. The wavelength of 415 nm was used to determine the absorbance. As a standard, Quercetin was employed to plot the calibration curve (0-60 µg/mL, R^2 = 0.998). The formula given below was employed to evaluate the total flavonoid production.

Total flavonoid production (mg/L) = Dry weight (g/L) × Total Flavonoid Contents (µg/mg)

3.10 Antioxidant assays

3.10.1 DPPH scavenging activity

The capability of callus extracts to scavenge free radicals was investigated by following the protocol of Khan et al. (2018). Briefly, the (DPPH) reagent (180 µL) was added to each of the wells in the row containing 20 µL of samples. Following an hour of incubation at room temperature in dark, a microplate reader was employed to assess the absorbance at 517 nm. In this case, ascorbic

acid was employed as a control. The calculation of FRSA (Free Radical Scavenging Activity) involved the utilization of the subsequent equation:

% FRSA = 100 × (1-Ac/As)

Where As represents the DPPH solution absorbance with no additions, and Ac represents the sample absorbance at 517 nm after the addition of methanolic callus extract.

3.10.2 Total Antioxidant capacity

To estimate total antioxidant activity of the samples, slightly modified protocol of Zia et al. (2021) was employed. In microplate wells, 120 µL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was combined with 20 µL of test samples, and the mixture was then incubated for 90 min at 95 °C. Utilizing a microplate reader, absorbance at 695 nm was measured after cooling at room temperature. DMSO and ascorbic acid were used as negative and positive controls, respectively. The unit of measurement employed for TAC is µg ascorbic acid equivalent per mg extract (µg AAE/mg extract).

3.11 HPLC Analysis of Phytochemicals

Analyses were carried out on a Waters Acquity UPLC system (Waters Co., Milford, MA, USA) coupled to a Waters Synapt G2-Si HDMS quadrupole time-of-flight mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with an electrospray ionization interface (ESI). Chromatographic separations were performed on a Waters Acquity UPLC BEH C18 (100*2.1 mm, 1.7 µm) column, and the temperature was kept at 40°C. Mixtures of H_2O (A) and ACN (B) were eluted as flow rate of 0.4 mL/min with a gradient as follow: 10-60% B (0-12 min), 100% B (12-16 min), 10% B (16-20 min). Fast data-dependent acquisition (fDDA) mode was used to analyze the samples (1 µL injection volume) for 19 compounds. This mode included a full MS survey scan in the m/z 50/1200 Da range (scan time = 0.2 ms) followed by MS/MS scans for the three most intense ions (m/z 100-1200 Da; scan time = 0.05 ms). Low-mass collision energy gradients were established at 10–40 V and high-mass collision energy gradients at 40–90 V. The samples (1µL injection volume) were also analyzed in MS mode consisting of full MS survey scan in the m/z 100/1200 Da range to acquire high-resolution MS1 data. In order to compare metabolites abundance between samples, peak areas were divided by the ratio dry weight of plant/ extract

weight obtained for each sample. The standard solution of each sample was prepared by placing the powdered plant materials (50 mg dry weight for each sample) in a glass tube with 1 ml of methanol with 0.1 % formic acid and sonicated for 1h. Supernatants were separated from the residuals powder by 15 min centrifugation at 4000 G at 4°C, followed by a second identical centrifugation process before being evaporated in a speed-vac. Extracts were re-suspended in MeOH at 0.01 mg/mL prior to LC/MS analysis.

3.12 Statistical analysis

Every experiment was performed thrice, in triplicates. Average values of each experiment were calculated, and standard error was determined using Microsoft Excel program. The representation of data was done as mean and ± Standard error mean. Graphical representation was done by using software Origin pro-2018.

CHAPTER 4
RESULTS AND DISCUSSION

4. Results and Discussion

4.1 Hormonal Effect on Callogenesis and its Morphology

The level of PGRs is a crucial factor affecting the callus induction, progression, and synthesis of essential plant bioactive ingredients in culture medium. Moreover, the PGRs type and concentration strongly depend on the type of explant and endogenous hormones present in them (Rao & Ravishankar, 2002). Explants (Figure 4.1) were obtained after approximately two months of seed inoculation and were used for callus establishment. Induction of callus from leaf and stem explants started to become visible after an average of 28 days of inoculation on medium containing different PGRs treatments (NAA, BAP, and TDZ).

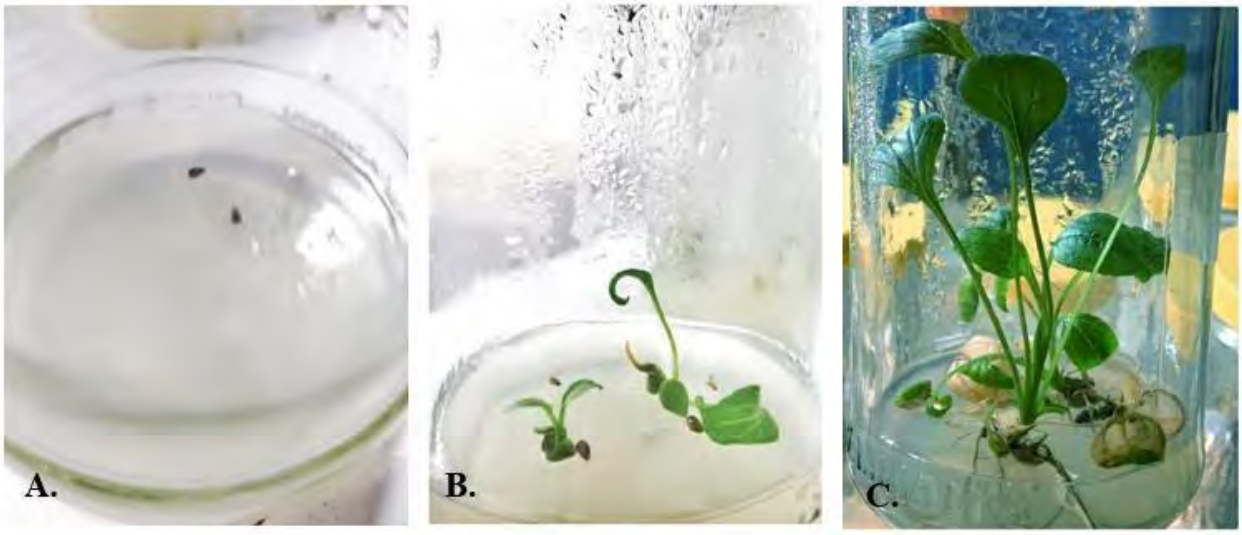

Figure 4.1: A. Seeds inoculated on MS basal media, **B.** *in vitro* Seedlings, **C.** *in vitro* grown plant of *E. purpurea.*

Negligible amount of callus was observed in media containing BAP and adventitious rooting was induced in NAA containing media. On the other hand, TDZ treatment led to an efficient callus induction and biomass accumulation (Figure 4.2). This might be due to the reason that promotion of adventitious root formation has been considered as one of the characteristics of NAA (Rout, 2006). In a study by Fazal et al., (2014), the optimum production of adventitious roots from leaf explants in *P. vulgaris* culture was stimulated by NAA, that further supported our results. The results were also in agreement with the research of Koroch et al., (2002) where NAA alone induced adventitious root system formed by indirect origin via callus of *E. purpurea*. Moreover,

several studies have reported that TDZ is more efficient in plant regeneration as compared to BAP (Lazić & Ružić, 2007). Corresponding to study conducted by Khan et al., (2016), the ability of BAP to induce callus production was exceptionally low in contrast to TDZ.

As reported by Jones et al., (2007), the callus growth of *E. purpurea* is improved by adding TDZ to the culture medium. The finding that TDZ can replace both the auxin and cytokinin needs is in line with prior findings using many regeneration systems, where it was shown that employing TDZ as the only growth regulator caused a high frequency of callogenesis (Murthy et al., 1998; Visser et al., 1992). Analysis of current research demonstrated that the highest CIF (100%) was noted on MS medium incubated with 3 mg/L TDZ using the stem explant, followed by CIF (91.6%) on MS medium incubated with 3 mg/L TDZ using the leaf explant (Table 4.1). These findings are in agreement with Srivastava et al., (2017), where the maximum CIF (100%) was observed in the *Brassica oleracea*, utilizing MS medium containing TDZ (9.1 µM) alone. Similary, Pourebad et al., (2015) also reported the efficient callus induction and phytochemical production of *Lallemantia iberica* by using TDZ in contrast to BAP and NAA.

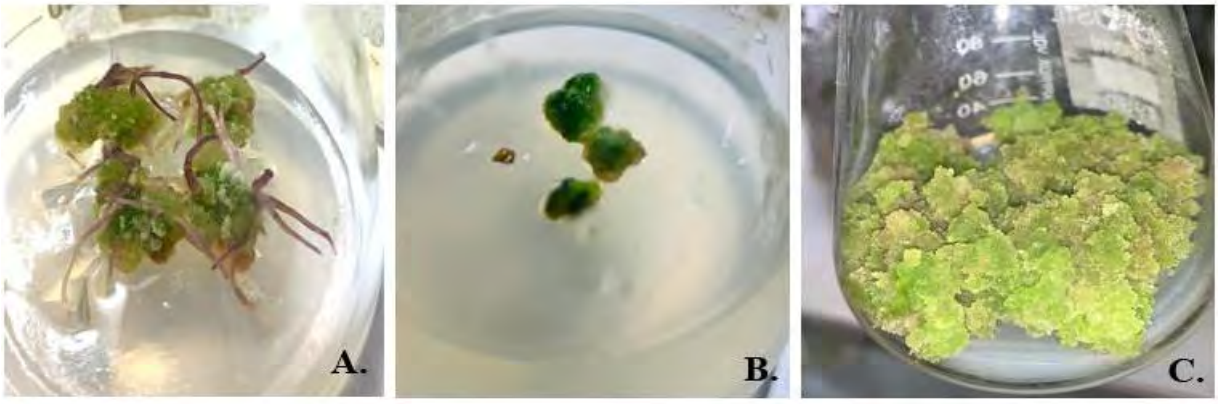

Figure 4.2: A. Rooting and minimal callus induction observed at NAA, **B.** Negligible Callus induction at BAP, **C.** Optimum callus induction at 3 mg/L TDZ (stem-derived)

The maximum FW (222.08 g/L) was observed at 3 mg/L TDZ using stem explant followed by 4 mg/L TDZ using stem explant (FW: 190 g/L). On the other hand, maximum DW (21.625 g/L) was also observed at 3 mg/L of TDZ concentration (by stem explant). The minimum biomass accumulation (FW: 108.58 g/L, DW: 5.93 g/L) was witnessed in MS medium encompassing 1 mg/L of TDZ using the leaf explant (Figure 4.3). These results are supported by the study of Khan et al., (2016), where stem explants resulted in a highest callus biomass (17.50 g FW/ flask) at 3

mg/L of TDZ treatment in *Fagonia indica*. Furthermore, in another study by Khurshid et al., (2018), stem explants of *Eclipta alba* produced the best callogenic response as compared to leaf explants in terms of both dry weight and fresh weight.

Table 4.1: Effect of different TDZ concentrations on the callus morphology and induction frequency

Callus Frequency					
Explant	**TDZ treatment (mg/L)**	**Callus Induction Started (Day)**	**Callus Color**	**Callus Texture**	**Callus induction frequency (%)**
Stem	0.5	28	Dark Green	Friable	66.6
	1	27	Dark Green	Friable	83.3
	2	27	Yellowish Green	Friable	83.3
	3	26	Yellowish Green	Friable	100
	4	28	Dark Green	Friable	86.6
	5	27	Yellowish Green	Friable	75
Leaf	0.5	29	Whitish Green	Friable	66.6
	1	28	Whitish Green	Compact	50
	2	27	Yellowish Green	Compact	75
	3	28	Yellowish Green	Friable	91.6
	4	28	Whitish Brown	Compact	75
	5	29	Whitish Green	compact	75

Morphological differences have also been observed in *E. purpurea* callus cultures maintained on different concentrations of TDZ, depicting their varied effects on callogenesis. Leaf explants treated with TDZ exhibited different color ranges such as greenish white, brownish white, and yellowish green, while the stem explants resulted in either dark green or yellowish green callus. Friable calli were observed from all stem explants. However, the leaf derived callus was compact for 1 mg/L, 2 mg/L 4 mg/L, and 5 mg/L concentration of TDZ. The results depicting the friable morphology of *E. purpurea* callus cultures contradict with Erkoyuncu & Yorgancilar, (2021), where the callus texture was compact in most of the PGRs concentrations in *E. purpurea* callus cultures. This might be related to the fact that we employed leaf and stem explants while Erkoyuncu & Yorgancilar, (2021) used cotyledonary, petiole, leaf, and root explants instead of only using stem explants for callus production. In our study, all the TDZ concentrations were capable

of inducing callus with stem derived callus giving the best response on MS media supplemented with 3mg/L of TDZ.

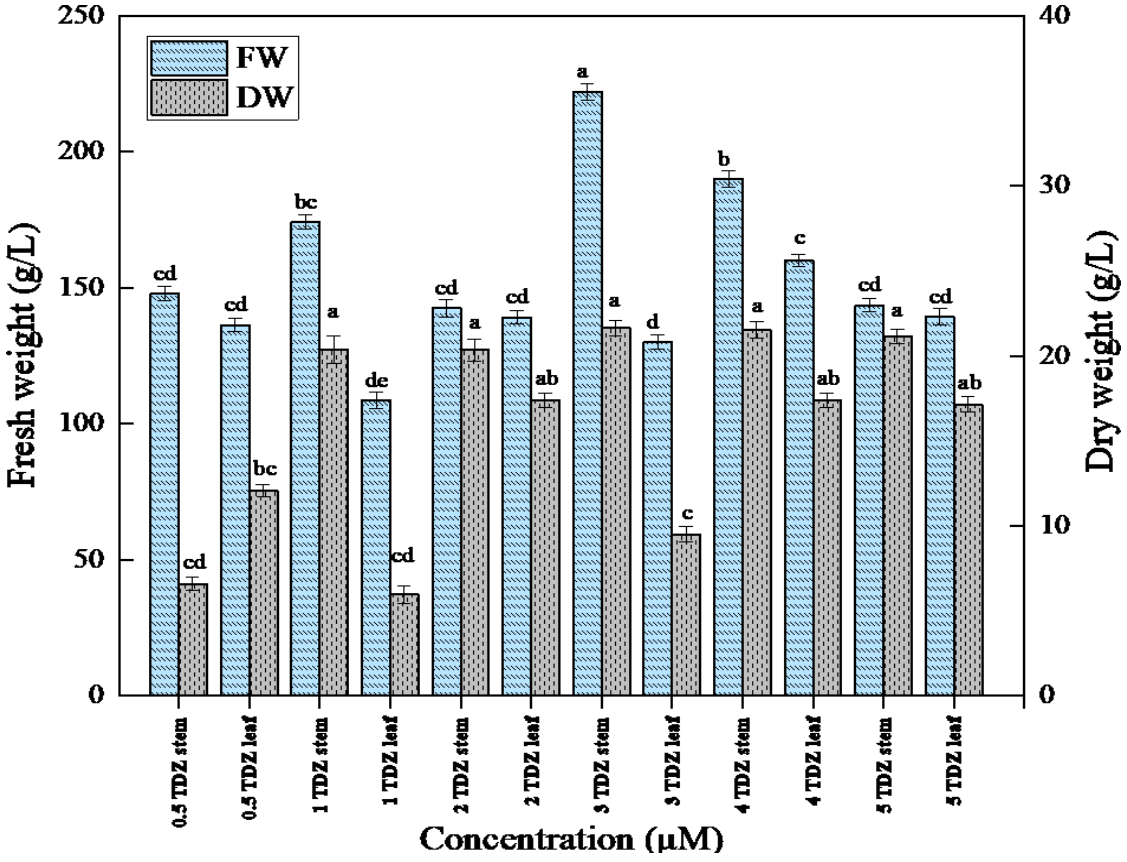

Figure 4.3: Fresh weigh (FW) and dry weight (DW) accumulation in callus cultures at different concentrations of TDZ using different explants (stem and leaf)

4.2 Effect of Melatonin on callogenesis of *E. purpurea*

According to our knowledge, no prior research has been done on the application of various doses of melatonin in *E. purpurea* callus culture. Exogenous melatonin is known to be engaged in modifying plant growth and development by functioning as an antioxidant, stabilizing the membrane, and controlling the up and down regulation of genes expression. Melatonin may also play the crucial role as a stress-response messenger in plants (Nawaz et al., 2016; Ramakrishna & Gill, 2018).

E. purpurea callus obtained from the 3 mg/L TDZ concentration (employing the stem explant) was used in this study. It was chosen because of its friable nature, fast growth rate, 100% CIF, and maximum biomass accumulation (FW, DW) among all concentrations. Friable callus is favored because it quickly disaggregates into single cells and tiny clusters, making it ideal for establishing cell suspension cultures in the future studies. The fast proliferation of segregated cells is aided by the availability of nutrients from all directions, making it more ideal for biochemical experiments (Bhatia, 2015).

The morphological variations in *E. purpurea* callus cultures in response to different concentrations of melatonin have been shown in Figure 4.5. All doses of melatonin resulted in enhanced biomass production as compared to control (FW: 92.7 g/L, DW: 3.5 g/L). Among the elicited cultures, good callogenic response was seen at 25 µM melatonin and the maximum biomass (FW = 208.625 ± 1.8 g/L, DW = 11.87 ± 0.21 g/L) was accumulated at this concentration, followed by 10 µM (FW:166.25 g/L, DW: 8.675 g/L. It demonstrates that in the current trials, 25 µM melatonin was quite successful at inducing callus and increasing biomass. The lowest biomass accumulation (FW: 132.5 g/L, DW: 6.25 g/L) was observed at 50 µM melatonin. With an increase in melatonin content up to a certain point (25 µM), the production of biomass steadily increased (Figure 4.4). Following that concentration, biomass began to decline as melatonin levels rose. 50 µM melatonin caused an inhibition in the biomass production as well as the darkening of callus. The discovered results are related to a prior work where a comparable tendency was seen in the biomass accumulation of *Prunella vulgaris* tissue culture, where increasing the concentration of melatonin up to a certain limit led to a decrease in biomass production (Fazal et al., 2018). The same trend of biomass accumulation under melatonin treatment was observed in the callus cultures of *Fagonia indica* (Khan et al., 2019). In agreement with our results, another study by Duran et al., (2019), reported that greater concentrations of melatonin caused a negative impact on the biomass accumulation of *Ocimum basilicum* callus cultures.

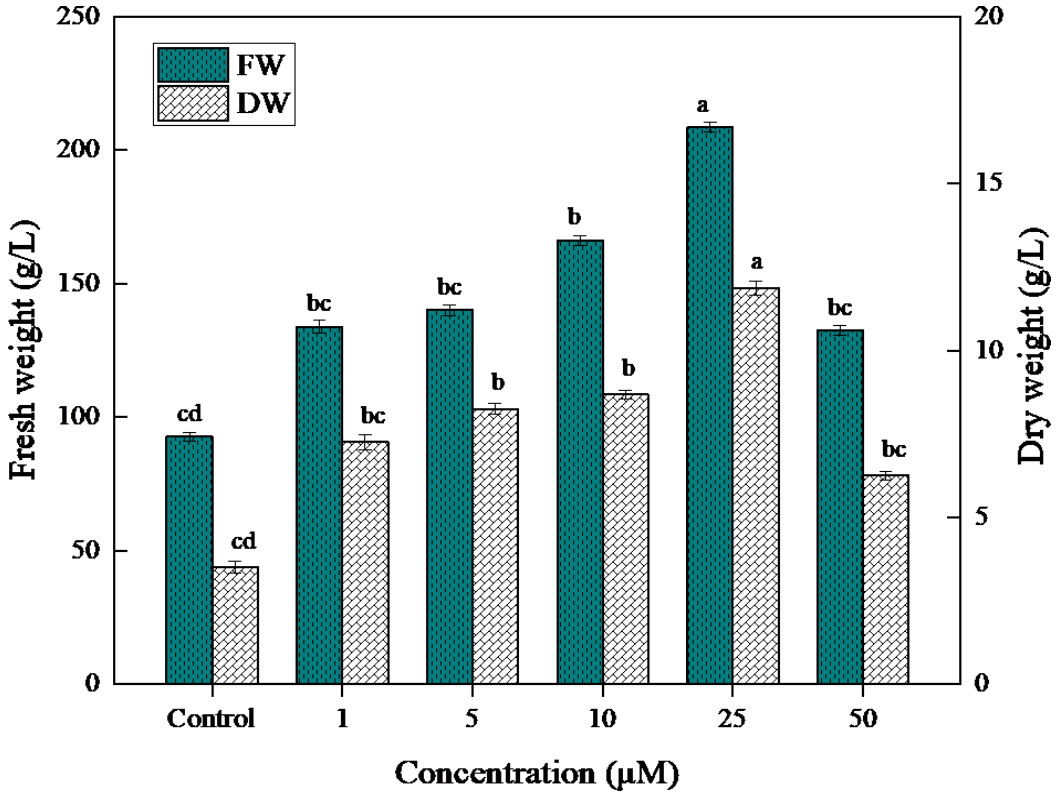

Figure 4.4: Growth kinetic curve of fresh weights (FW) and dry weights (DW) of *E. purpurea* callus cultures elicited with different concentrations of melatonin

In addition to serving as a stress-relieving agent under biotic and abiotic stress, melatonin also fosters plant growth, facilitates seed germination, enhances biomass production, and amplifies plant yield (Qiao et al., 2019). The proliferation of callus was observed to be inhibited by greater melatonin doses. This could be the result of stress brought on by increased ROS generation and concentration, which stop cells from proliferating and cause them to die (Fazal et al., 2016).

4.3 Trends in TPC, TFC, TPP, and TFP

Phytochemical analysis reveals the existence of several significant phenolic, polyphenolic, and flavonoid compounds in *E. purpurea* callus cultures. TPC and TFC were studied in *E. purpurea* callus cultures developed under various melatonin doses. Figure 4.6 and Figure 4.7 demonstrate the effect of melatonin treatments on TPC and TFC.

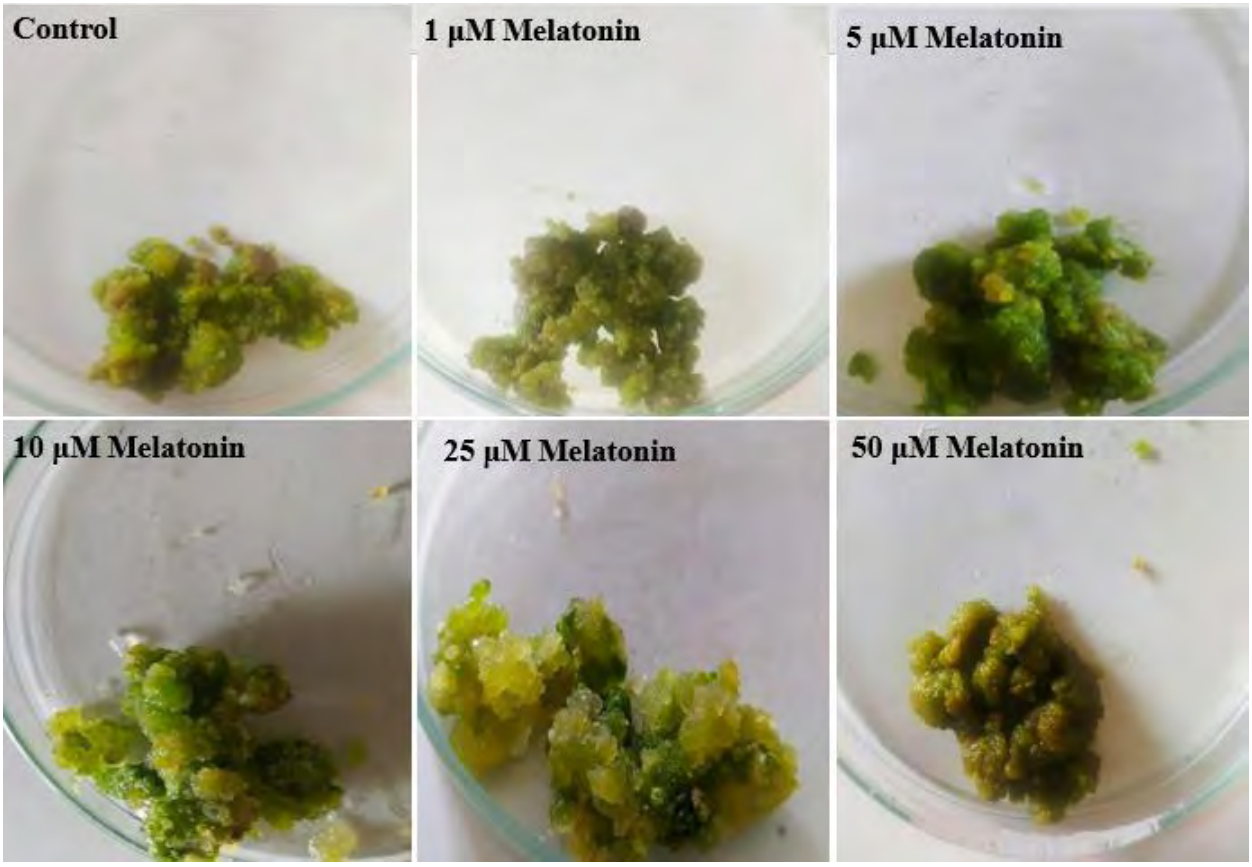

Figure 4.5: Melatonin-induced changes in biomass accumulation of *E. purpurea* Callus Cultures

4.3.1 Influence of Melatonin on Total Phenolic Content

In contrast to control, all melatonin doses led to an increase in TPC. Among all melatonin treatments, the highest TPC (11.26 µg GAE/mg DW) was observed on 25 µM melatonin, followed by 9.59 µg GAE/mg DW at 10 µM. Whereas, the lowest TPC (7.21 µg GAE/mg DW) was observed in callus grown on MS medium containing 50 µM melatonin.

The TPC in wild *E. purpurea* leaves was found to be about 22.3 mg GAE/g (Lee et al., 2010), which was significantly higher as compared to the phenolic content of *E. purpurea* callus cultures as reported by Koroch et al., (2002). Polyphenols, the major group of plant-specific phytochemicals, are well known molecules that serve important physiological functions in plants, including stress protection (Šamec et al., 2021). It is widely known that the phenylpropanoid pathway is triggered in response to unfavorable ecological circumstances such as salt, drought,

metal pollution, UV rays, and severe temperatures, which causes the buildup of various phenolic compounds (Linić et al., 2019; Sharma et al., 2019). Thus, callus cultures frequently have lesser quantities of phenolic compounds than wild plants because of the intense stress conditions in the wild as opposed to *in vitro* environments. Several studies also have reported higher TPC in wild plants compared to their callus cultures. The content of phenolic diterpenes was found to be higher in wild plants as compared to the callus cultures of *Solidago chilensis* (Schmeda-Hirschmann et al., 2005). Similarly, the TPC in wild *Trifolium pretense* methanolic extract was higher than the callus and *in vitro* grown plants (Khorasani Esmaeili et al., 2015).

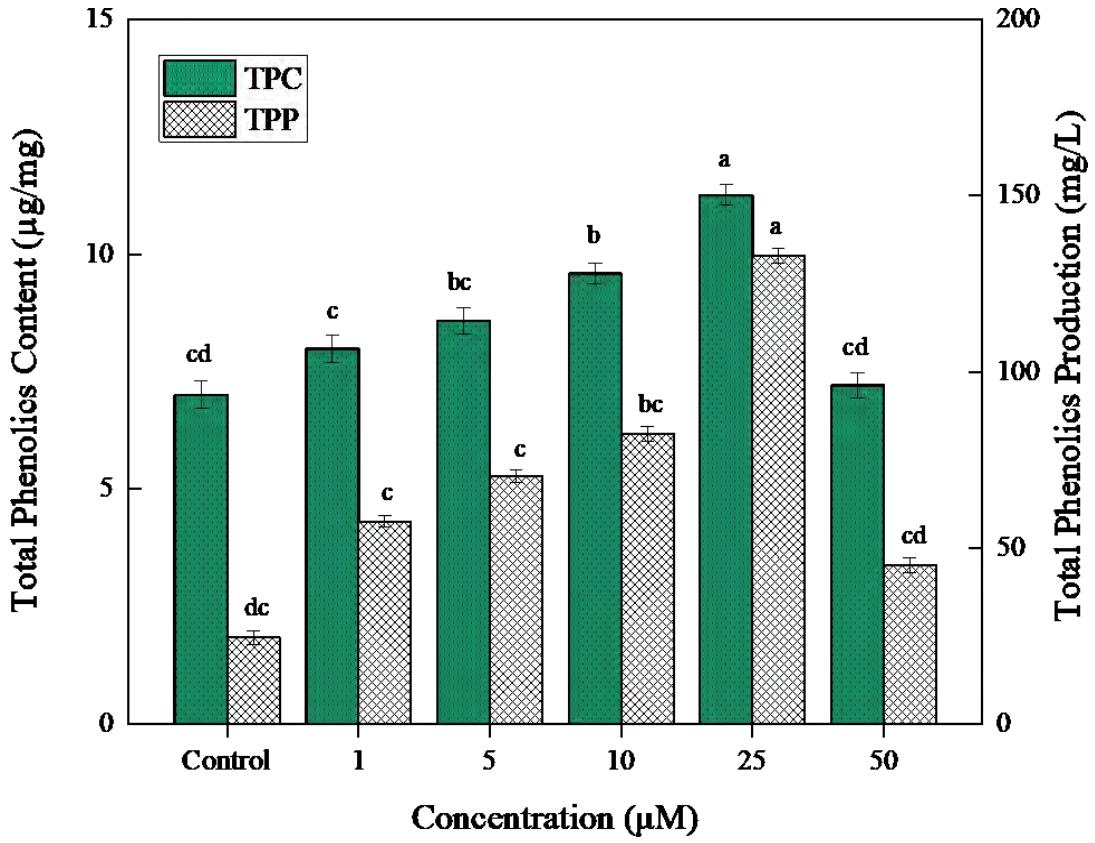

Figure 4.6: Total phenolic contents (TPC) and Total phenolic production (TPP) accumulated in callus cultures of *E. purpurea* elicited with various concentrations of melatonin. Values signify means ± standard deviations from triple replicates

4.3.2 Influence of Melatonin on Total Flavonoid Content

Flavonoids are a group of bioactive compounds and fall under the category of polyphenolic compounds. Flavonoids play a variety of roles in plants, including those related to anti-oxidative stress, UV radiation damage management, phytopathogen defense, legume nodulation, male fertility, visual signaling, and auxin transport regulation (Wang et al., 2022). Among all the melatonin concentrations employed in the current study, maximum TFC (49.66 µg QE/mg DW) was observed in callus grown in MS medium supplemented with 25 µM Melatonin, followed by 10 µM Melatonin (TFC: 46.4 µg QE/mg DW), and the lowest TFC (32.99 µg QE/mg DW) was obtained at the highest melatonin concentration (50 µM melatonin). However, all treatments were capable of enhancing flavonoid content in contrast to the control. The variation in TFC in callus cultures might be ascribed to the main enzyme activation, i.e., phenylalanine ammonia lyase (PAL) that is responsible for the production of flavonoids (Guo et al., 2007). Melatonin treatment has been described to enhance the antioxidant action and PAL activity in the roots of *Lolium perenne* L. (Wei et al., 2023).

A previous study determined the TFC in *E. purpurea* wild plant (TFC: 86.0 mg QE/g), which is comparatively higher than the flavonoid content of callus cultures (Lee et al., 2010). In comparison to their wild plants, callus cultures show comparatively lower amounts of flavonoids. The controlled laboratory setting where the plants are grown, which is distinguished by uniform and constant circumstances, is mainly responsible for this divergence. Plants have developed defense systems, such as the increased production of SMs, to survive in harsh ecological conditions of their native environments (Razavizadeh et al., 2017).

Our primary focus is on the analysis of TPP and TFP. It was observed that among all the melatonin concentrations, 25 µM of melatonin showed the highest TPP (132.8 mg GAE/L) and TFP (585.9 mg QE/L) in *E. purpurea* callus cultures. Whereas 50 µM of melatonin exhibited minimum TPP (45.06 mg GAE/L DW) and TFP (206.2 mg QE/L DW).

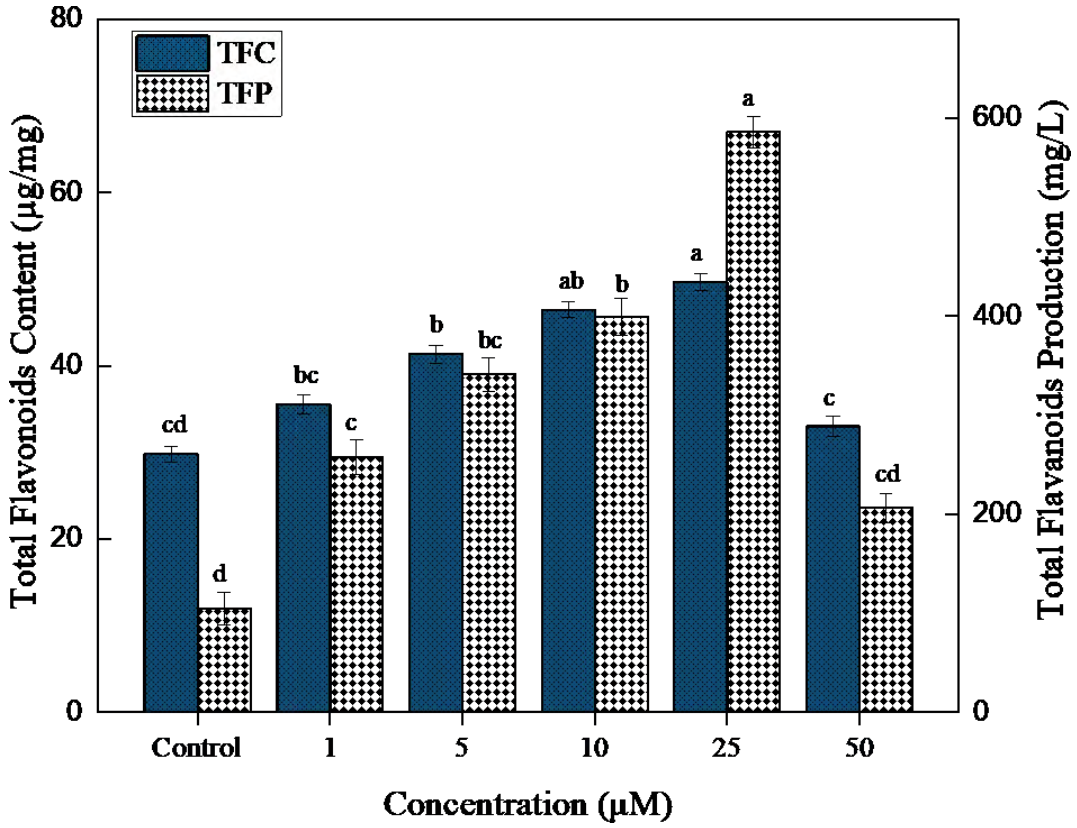

Figure 4.7: Total flavonoid content (TFC) and Total flavonoid production (TFP) accumulated in callus cultures of E. purpurea elicited with various concentrations of melatonin. Values signify means ± standard deviations from triple replicates.

Even though it is usually recognized that phenolic content is greater than flavonoid content in most cases, we found an opposite trend in our research. It might be due to the polarity of the extracting solvents, which could provide a wide range of polyphenolic concentration in the extract. Therefore, the low phenolic content of our extracts might be explained by the fact that ethanol extraction does not release bound phenolic chemicals from the callus cells. In addition, a study showed that many nonphenolic compounds do indeed show considerable reactivity towards the F-C reagent (Everette et al., 2010). Despite the fact that different compounds respond to the Folin-Ciocalteu reagent differently, it is a rapid and widely used method for identifying TPC (Kähkönen et al., 1999). In agreement with our findings, Park et al., (2020) noticed a higher flavonoids

production as compared to phenolic production in methanolic extracts of the differently induced callus cultures of *Sophora flavescens*. Another research reported that *Clitorea ternatea* flower extracts yielded higher TFC (187.05 mg/g DW) than TPC (51.6 mg/g DW) (Jaafar et al., 2020).

The secondary metabolite production in our study was found to be dependent on biomass, and a direct correlation was observed in phenolic and flavonoid production as well as with biomass accumulation. These findings are coherent with those of Khan et al., (2019), where they observed positive correlation among biomass production, TFC, and TPC under the influence of melatonin. Melatonin has a role in a variety of gene expressions that control the production of secondary metabolites and aid plants in stress defense (Sarrou et al., 2015). The increased output could result from melatonin's interaction with catalases, which enhances H_2O_2 buildup and the generation of ROS to further activate secondary metabolic pathways (Li et al., 2012).

4.4 Effect of Melatonin on Antioxidant Potential of *E. purpurea*

A normal cellular homeostatic condition requires a delicate balance between ROS production and ROS elimination. When this balance gets disturbed under various environmental stress circumstances like salinity, drought, cold, heavy metals, UV irradiation etc., great amounts of ROS accumulate in plant and cause oxidative damage (Das & Roychoudhury, 2014). To counteract the negative consequences of oxidative stress, plants have a built-in mechanism of defense. Plants produce a number of SMs including phenols, terpenoids, and flavonoids that exhibit antioxidant properties and can scavenge the ROS and protect the plant cells from oxidative damage (Mittler, 2002).

The current study used two *in vitro* antioxidant assays, i.e., DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and phosphomolybdate method to evaluate antioxidant potential of *E. purpurea* callus cultures in response to different melatonin concentrations.

4.4.1 DPPH-Free Radical Scavenging Activity

DPPH is a stable organic, paramagnetic free radical with a prominent absorption band at 517 nm. It forms a purple-colored solution in methanol at room temperature. After accepting an electron or a free radical species, and becoming a stable diamagnetic molecule, it no longer acts as a free radical. Consequently, it transforms color from purple to yellow. This technique is simple, and

sensitive so that it can quickly analyze the free radical scavenging ability of antioxidants existing in the sample (Bajwa et al., 2023). Formerly, the DPPH assay showed 93.6% FRSA of ethanolic extracts of *E. purpurea* wild plant (Stanisavljević et al., 2009). A study done by Elshahawy et al., (2022) has reported the 79.02% antioxidant potential of *E. purpurea* unelicited callus cultures and 79.02 –89.51% for the elicited ones.

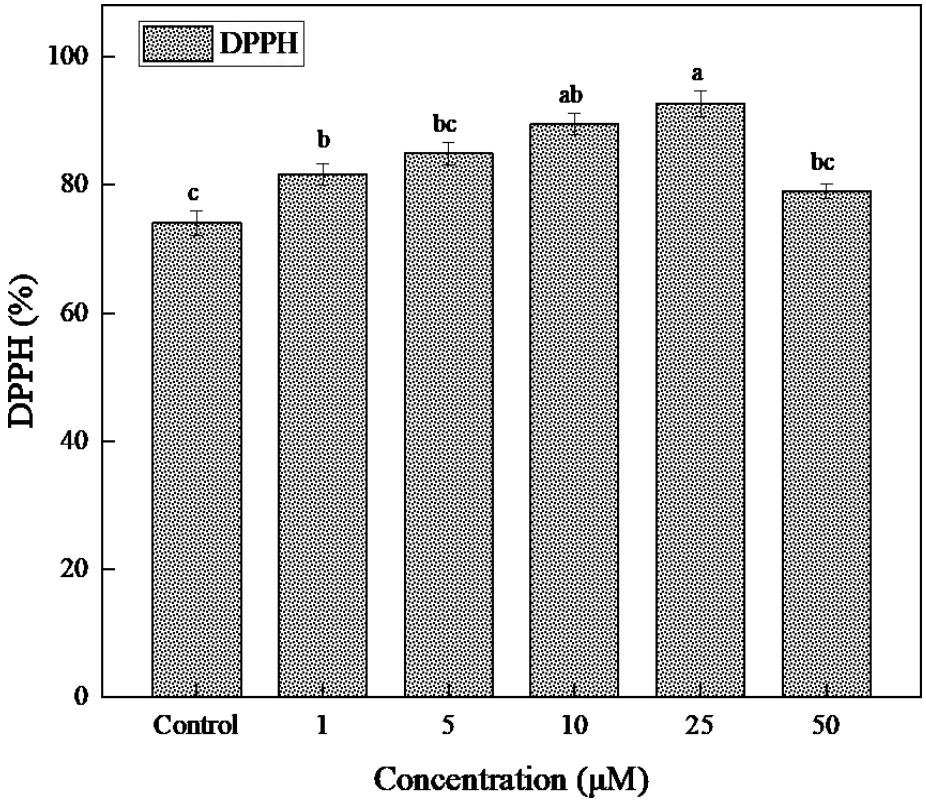

Figure 4.8: Antioxidant potential (FRSA) in *E. purpurea* callus cultures exposed to various melatonin doses. Presented values indicate means ± standard errors obtained from triplicate samples.

In this study, melatonin elicited cultures displayed enhanced FRSA activity than control (74%). The highest DPPH-FRSA (92.7%) was recorded on callus grown at 25 µM Melatonin, followed by 10 µM Melatonin (DPPH-FRSA: 89.5%). The lowest DPPH-FRSA (79.02 %) was observed in callus grown at 50 µM Melatonin. The highest concentration of Melatonin led to the reduction of antioxidant potential (Figure 4.8). Our findings are consistent with the study of Esmaeili et al., (2023), where antioxidant potential raised quicky after control and after reaching a

certain point, exposure to greatest concentrations of melatonin decreased the antioxidant activity of *Linum album* cells.

Increased levels of biosynthesized flavonoids and phenolics, that are primarily accountable for antioxidant actions, may be the cause of the enhanced FRSA. Furthermore, melatonin itself is a powerful and well-known scavenger for hydroxyl radicals, ROS, and nitrogen. One product of these processes, cyclic-3-hydroxymelatonin, is likewise highly effective in scavenging free radicals (Hernández-Ruiz & Arnao, 2018; Sarropoulou et al., 2012).

4.4.2 Total Antioxidant Capacity

Plant extracts have been frequently assessed for their total antioxidant capacity using the phosphomolybdate technique where Mo (VI) is reduced to Mo (V) in the presence of extracts having antioxidant potential, forming a green phosphomolybdenum V complex with a maximum absorbance at 695 nm (Prasad et al., 2009; Prieto et al., 1999). In a previous study by Mark, (2020), the highest TAC of *Echinacea purpurea* leaves extract was found to be 49.23 mg AAE/g extract. However, no study has yet reported the employment of phosphomolybdate assay for determining the antioxidant capacity of *E. purpurea* callus cultures. In our current research, all the doses of Melatonin led to an increase of TAC from control (39.6 ug AAE/mg), except for the 50 µM melatonin (Figure 4.9). The highest value (63.22 ug AAE/mg) of TAC was obtained at 25 µM melatonin and the lowest value (34.16 ug AAE/mg) was found at 50 µM melatonin. Our results are supported by the study of Neamah et al., (2022), where moderate treatment with melatonin was found to be potent for increased secondary metabolism and antioxidant capacity of *Hyoscyamus pusillus*. The highest values of TAC might be attributed to the presence of greater amounts of secondary metabolites that can reduce Mo (VI) to Mo (V), by donating an electron to the Mo (VI) molecule and giving increased intensity of the blue color which is proportional to the TAC of the sample. Melatonin itself exhibits antioxidant potential and is capable of neutralizing reactive species by donating an electron (Rusanova et al., 2019).

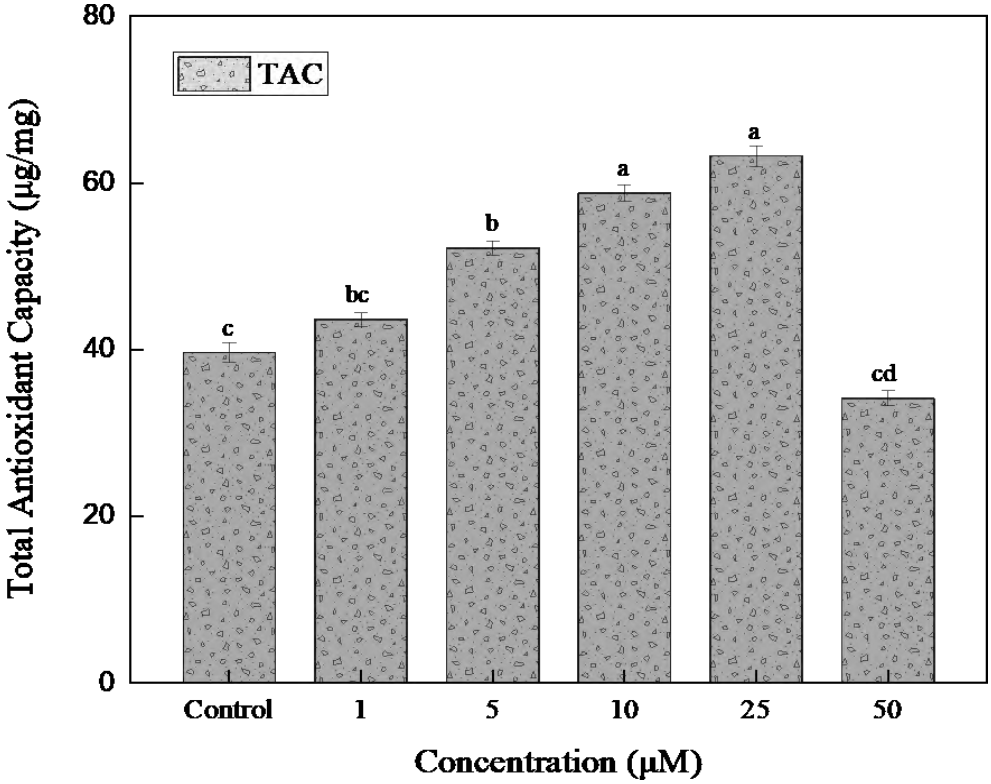

Figure 4.9: Total antioxidant capacity (TAC) of the callus cultures of E. purpurea elicited with various concentrations of melatonin. Values signify means ± standard deviations from triple replicates.

Furthermore, the results of both assays showed direct correlation in accumulation of phenolics, flavonoids, FRSA, and Total antioxidant capacity. The current results agree with the reports of Canadanovic-Brunet et al., (2005), where it was discovered that antioxidant activities in *A. absinthium* were positively correlated with its high TPC and TFC levels. The study of Fazal et al., (2016) further supports our result by showing a positive correlation between dry biomass, TPC, TFC, and antioxidant potential of *Prunella vulgaris* cell suspension cultures.

4.5 HPLC Analysis

The impact of various melatonin doses on the production of 14 phytochemicals and 5 amino acids in the callus cultures of *Echinacea purpurea* was investigated using HPLC-based studies. It's vital to note that numbers are stated in arbitrary units (Table 4.2), and that the findings have been

converted into 10^{-3} units for clarity (AU x 10^{-3}). All alkaloids and amino acids had substantial variations in their biosynthesis during the course of the tested melatonin concentrations. It shows complex relationships between melatonin and the plant metabolic pathways regulating the chemical production of each compound.

In our study, several compounds exhibited distinct trends in response to increasing melatonin concentration. A number of compounds, including neochlorogenic acid, hydroxyferulic acid hexoside, 3,4-dicaffeoylquinic acid, 1,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, and quercetin 3-O-(6-O-malonyl-glucoside) showed the highest production in the callus cultures treated with 25 µM melatonin. Some compounds namely quercetin 3-O-rutinoside, 3,5-dicaffeoylquinic acid, caffeic acid, caftaric acid, chlorogenic acid, and chicoric acid were found to be highly produced in callus cultures elicited with 50 µM melatonin. This pattern suggests a potential biphasic response, where optimal compound production occurs within a certain melatonin range. Our results are supported by the study of Ullah et al., (2019), where HPLC results showed that maximum productivity of SMs was obtained under a certain range of Melatonin (20-50µM) within the callus cultures of *Lepidium sativum* L. Thus, melatonin may serve as a stimulant for increased synthesis of phytochemicals with significant economic value. Another study by Duran et al. (2019) showed that the highest concentration was found to be inhibitory for biomass production, yet it was the most promising for the accumulation of specific phytochemicals.

Echinacoside, a natural phenylethanoid glycosides, was found to be produced in maximum amount in callus cultures that were not treated with melatonin. Melatonin might selectively activate transcription factors and signaling pathways responsible for enhancing the production of other compounds, while not significantly impacting the regulatory elements governing echinacoside biosynthesis (Xiong et al., 2019). This discrepancy could arise from variations in promoter structures, transcription factor binding, and epigenetic modifications that render the genes associated with the other compounds more responsive to melatonin-induced signaling (Czajka et al., 2021). Further investigation into these regulatory mechanisms is required to shed light on the specific genetic pathways driving this observed pattern.

Biosynthesis of amino acids under the influence of melatonin showed a contrasting trend in callus cultures of *Echinacea purpurea*. Usually, the main metabolism of amino acids, lipids, and

carbohydrates produces secondary metabolite (Malerba & Cerana, 2019). Our findings showed that callus cultures treated with the lowest melatonin concentration—1 μM —exhibited the highest amounts of glucogenic amino acids, and further increasing melatonin does lead to a decrease in amino acid productivity. It might be attributed to the ability of melatonin to metabolize glucogenic amino acids in gluconeogenesis pathway. These results can be supported by the study of Kobylińska et al., (2018), where high melatonin dose shifted the Tobacco cells metabolism on gluconeogenesis pathway and allowed for synthesis of carbohydrates by degrading glucogenic amino acids.

The observed variations in phytochemical production highlight the necessity to investigate individual compounds when evaluating the effects of melatonin treatment. Our knowledge of melatonin's effects on the formation of alkaloids and amino acids in *Echinacea purpurea* is improved by the integrated phytochemical study, which also raises the possibility of using melatonin to synthesize specific compounds. These findings call for more proteomic and transcriptomic research into the molecular mechanisms involved. The ramifications extend to a variety of fields, including medicine, plant biotechnology, and the search for natural products.

Table 4.2: HPLC-analysis of bioactive compounds and amino acids

Melatonin Treatment (µM)						
Compounds (AU x 10^{-3})	**Control**	**1**	**5**	**10**	**25**	**50**
Caffeic acid	27.51	49.12	28.23	21.38	17.56	**49.62**
Caftaric acid	124.16	114.5	114.14	108.85	75.19	**156.18**
1,3-dicaffeoylquinic acid	124.75	59.98	103.80	61.90	114.25	**128.70**
Chlorogenic acid	256.5	165.87	22.123	126.51	227.4	**263.24**
Chicoric acid	945.22	797.7	940.19	815.97	611.9	**1034.79**
Hydroxyferulic acid hexoside	35.64	35.23	28.73	41.05	**41.62**	39.08
Neochlorogenic acid	9.70	6.75	10.12	6.89	**12.37**	11.13
3,4-dicaffeoylquinic acid	113.25	78.29	119.01	69.62	**150.50**	107.74
1,4-dicaffeoylquinic acid	27.21	20.17	39.99	17.90	**44.214**	28.80
4,5-dicaffeoylquinic acid	284.02	205.30	312.72	178.64	**336.96**	295.02
Quercetin 3-O-(6-O-malonyl-glucoside)	17.45	17.05	28.42	22.60	**30.15**	27.763
Echinacoside	**0.871**	0.76	0.76	0.78	0.64	
Quercetin 3-O-rutinoside	1.04	5.79	5.82	3.77	7.96	**8.03**
3,5-dicaffeoylquinic acid	341.88	209.64	308.783	206.28	362.65	**380.571**
Leucine	2556.26	**3371.53**	1914.43	2248.57	1568.61	2256.09
Isoleucine	1934.09	**2522.92**	1590.26	1741.19	1237.66	1811.09
Phenylalanine	3783.26	**6035.41**	4137.29	4200.13	2063.36	4140.7
Tyrosine	**206.93**	194.12	158.67	126.58	167.34	166.52
Tryptophan	462.73	**506.49**	466.68	430.67	290.36	293.72

CHAPTER 5
CONCLUSION

5. Conclusion

In this research, an efficient protocol was established for the callus induction of *E. purpurea* and the impact of varying melatonin doses on biomass accumulation, secondary metabolism, and antioxidant potential of callus cultures was determined. Among all the PGRs, 3 mg/L TDZ, using the stem explants, was shown to be most efficient in callus induction (100% CIF), maximum biomass accumulation (FW, DW), and fast growth rate. The employment of melatonin as an elicitor yielded promising outcomes, demonstrating amplified biomass production, and heightened secondary metabolism within the callus cultures. Remarkably, melatonin as an elicitor resulted in increased levels of phenolics, flavonoids, and antioxidants. Additionally, HPLC analyses unveiled a substantial accumulation of vital medicinal compounds within the elicited cultures. 25 µM melatonin concentration was found to be most efficient of all treatments and resulted in significant enhancement of secondary metabolism. The current study also showed a positive correlation between biomass accumulation, phenolic and flavonoid production, as well as antioxidant potential. These collective findings not only highlight the potential of TDZ in efficiently inducing the callus production but also emphasize the potential of melatonin in facilitating the sustainable and improved synthesis of both phytochemicals and biomass within *E. purpurea* callus cultures. The findings of this study underscore the need for further research at both pilot and commercial scales to validate and amplify these promising results.

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