Influence of Exogenous Melatonin and UV-C Exposure on the Biomass Accumulation and Phytochemistry of *Alcea rosea* Callus Cultures

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Session: 2021-2023

Department of Biotechnology

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Quaid-I-Azam University,

Islamabad, Pakistan

2023

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A thesis submitted in the partial fulfillment of the requirements for the degree

MASTER OF PHILOSOPHY

IN

BIOTECHNOLOGY

Department of Biotechnology

Faculty of Biological Sciences,

Quaid-I-Azam University,

Islamabad, Pakistan

2023

CERTIFICATE OF APPROVAL

This is to certify that the Department of Biotechnology, Faculty of Biological Sciences, Quaid-I-Azam University Islamabad, Pakistan accepts the dissertation entitled "**Influence of Exogenous Melatonin and UV-C Exposure on the Biomass Accumulation and Phytochemistry of *Alcea rosea* Callus Cultures**" submitted by **Arslan Ali Toor** in its present form as satisfying the dissertation requirement for the Degree of Master of Philosophy in **Biotechnology**.

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Date: _____

DECLARATION

I Arslan Ali Toor, hereby solemnly declare that the work presented in this thesis entitled "Influence of Exogenous Melatonin and UV-C Exposure on the Biomass Accumulation and Phytochemistry of *Alcea rosea* Callus Cultures" is original. Furthermore, I declare that this work has not been submitted for any degree or diploma to any other university/institution. I am aware of the terms copyright and plagiarism, and I will be responsible for any copyright violation found in this work.

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Arslan Ali Toor

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DEDICATION

I dedicate my dissertation work to my beloved parents who have always loved and guided me while constantly being supportive of my decisions. A special feeling of gratitude to my loving parents and siblings, whose words of encouragement and unwavering moral and emotional support have made me what I am today. I also dedicate this thesis to my respectable and honorable teachers who have supported me in developing my personality as a competent professional.

This work is also dedicated to the crazy lot of my friends from Sargodha University (Charismatics) for always being there and for contributing to my success. Furthermore, I would like to dedicate this piece of work to my future wife who apparently is so good at hide and seek that I haven't found her still. . ..

Arslan Ali Toor

ACKNOWLEDGMENT

In the name of ALLAH who is the most merciful and Almighty without the help of whom I was unable to complete my work. All respects to Holy Prophet (S.A.W) the most exalted among human being ever born on surface of earth, who is forever guidance and knowledge for humanity.

First, I would like to pay my sincere gratitude to my supervisor Professor **Dr. Bilal Haider** Abbasi, Associate Professor, Department of Biotechnology, Quaid-I-Azam University Islamabad, Pakistan for his uttermost guidance, motivation, and skilled boosting attitude during my research work. I would like to extend my deepest appreciation to those people, who helped me in one way or another during my stay at Department of Biotechnology, QAU Islamabad, Pakistan. During my M.Phil. research, I worked with a great number of people; I wish to convey my gratitude to all of them for their unique helping nature.

I would like to express my cordial thanks to my friends and lab fellows **Amen Jahangir**, **Zenab Syed**, **Fazal Rahman**, **Nawal Naveed Abbasi**, **Gohar Zaman**, **Naeem Bajwa**, **Razia Khurshid**, **Ansa** for their continuous support. I also cannot forget to pay my deepest gratitude to my dearest seniors **Hasnat Tariq**, **Zain Asif**, and **Yawar Abbas** whose continuous professional advice and help enabled thought provoking ideas during the research work.

Finally, I would like to acknowledge **Masood-Ur-Rasheed** and **Junaid-Ur-Rehman** for being the best roommates and friends. **Kinza Malik** for always being there as a friend and as a mentor. I would also like to acknowledge **Mahpara Abid** and her witty sense of humor along with my batchmates and dear friends who made this journey a very pleasant experience.

May Allah bless you all with eternal happiness and success! Ameen.

Arslan Ali Toor

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LIST OF ABBREVIATIONS

µg/g	Microgram per gram
µM	micro molar
AGEs	Advanced Glycation End products
ARF	*Alcea rosea* Flower
ARFW	*Alcea rosea* Flower Water
CAA	Cellular Antioxidant Assay
CaC_2O_4	Calcium Oxalate
CUPRAC	CUPric Reducing Antioxidant Capacity
DMSO	Dimethyl sulfoxide
DW	Dry weight
FW	Fresh weight
g /L FW/DW	Gram per liter fresh weight/ dry weight
MEL	Melatonin
mg/L	Milligram per liter
NAA	α-Naphthalene acetic acid
TDZ	Thidiazuron
ORAC	Oxygen Radical Antioxidant Capacity
PTC	Plant Tissue Culture
RNS	Reactive Nitrogen species
ROS	Reactive oxygen species
SD	Standard Deviation
SM	Secondary Metabolites
TEAC	Trolox Equivalent Antioxidant Capacity

ABSTRACT

Alcea rosea, commonly known as Hollyhock, is prized for its wide applications as antioxidant, antibacterial, and anticancer agent. Literature states that a number of biotechnological approaches are needed for the viable production of plant biomass and bioactive compounds. Elicitation has established itself as a highly efficient method for enhancing the secondary metabolites production in *in-vitro* cultures. The current study involved the exposure of callus cultures of *Alcea rosea* to various treatments of UV-C exposure and melatonin as elicitors. Enhanced biomass accumulation with higher production of phytochemical content were the main goals of this study. UV-C exposure resulted in maximum biomass accumulation (FW: 359.13 g/L, DW: 12.99 g/L) at UV-C exposure of 30 (mins). In contrast, maximum biomass accumulated (FW: 226.43 g/L, DW: 10.20 g/L). This trend was also followed in antioxidant activities with maximum ORAC (960.57 µM TEAC) and CUPRAC (556.23 µM TEAC) at UV-C 30 (mins); only the control showed highest activity for CAA (78.18 % ROS/RNS) which was followed by Callus extract at UV-C 30 (mins) with a percent inhibition (78.08) of CAA assay. The phytochemical profile through HPLC revealed 9 anthocyanins, which are responsible for imparting color to the *Alcea rosea* nigra flowers. Malvidin 3-O-malonylglucoside was the anthocyanins produced in the highest content. Maximum accumulation of phytochemicals (7.47 µg/g DW) was detected in *Alcea rosea* callus cultures under melatonin (25 mg/L) stress, which was more than 2 folds increase than the control (7.47 µg/g DW). Melatonin (3 mg/L) showed maximum tyrosinase inhibition (53.18 %) and pentosidine-like AGEs (70.3%). Whereas the maximum elastase inhibition (73.70%) and vesperlysine-like AGEs (36.8%) was exhibited by callus extracts at melatonin (25 mg/L). These higher inhibition percentages suggest role of *Alcea rosea* extract in alleviating compounds which cause age-related diseases and issues such as hyperpigmentation and elastosis, suggesting it's potential use in the cosmetics industry. Further study is needed to relate genes involved in the expression and regulation of anthocyanins involved to better incorporate *Alcea rosea* into the cosmetics industry.

CHAPTER 1

INTRODUCTION

Historically and culturally, natural products have been used in traditional remedies but the application of isolated and characterized compounds from natural products for modern pharmaceutical industry only began in the 19th century. Owning to the proven effectiveness and safety of medicinal plants more than 80% of the inhabitants of developing world are dependent on them. The importance of plants in medicine can be evaluated from the fact that even in 21st century of the 252 drugs about 11% are regarded as essential by the World Health Organization, which has flowering plants as their source (Veeresham, 2012). Plants have been an essential player in the creation of modern drugs used for the treatment of various infectious diseases. Many products, including food additives, colours, flavours, essential oils, fragrances, herbal cosmetics, insecticides, pesticides, natural rubber and gum, tannins, and waxes, are made from primary and secondary plant metabolites (Samarth *et al.*, 2017). The modern pharmaceutical industry focuses on identifying and isolating specific molecules considered to be key components in treatment of certain diseases (Phillipson, 2001).

Alcea rosea is native to South-west China but is nowadays distributed broadly in the tropical and northern-temperate regions. This short-lived perennial or biennial herbaceous plant belonging to the *Malvaceae* family can grow up to heights of 1.5m to 2m (Lim, 2014; Rakhmatova *et al.*, 2019; Tang *et al.*, 2007).

Traditionally, it has been used as an expectorant, emmenagogue, cooling and diuretic agent. The roots of the plant have been used to treat ulcer, while the roots and flowers have both been used to treat uterine and kidney inflammation. The seeds exhibit diuretic and febrifuge properties. Decoction of flowers have been employed as an anti-inflammation agent, as an antipyretic and as an astringent (Al-Snafi, 2013). Different phytochemicals have been reported for *Alcea rosea* including phenolics acid (Dudek *et al.*, 2006), kaempferol, dihydrokaempferol, quercetin and their derivatives, apigenin (Abd El-Salam *et al.*, 2016; Ammar *et al.*, 2013; Ma *et al.*, 2019), various other anthocyanins (Hosaka *et al.*, 2012) and mucilages (Al-Snafi, 2013). These mucilages constituted of rhamnose, galactose, glucuronic acid and galacturonic acid. Alkaloids, flavonoids, proteins, and minerals were also reported *Alcea rosea* (Azizov *et al.*, 2007; Classen *et al.*, 1998; Dudek *et al.*, 2006). It possesses different pharmacological activities including anticancer activity (Ahmed *et al.*, 2016), antiestrogenic activity (M. A. Papiez, 2004),

antihyperglycemic (Dar *et al.*, 2017), antimicrobial activity (Tuba *et al.*, 2010), antioxidant activity (Ammar *et al.*, 2013), antiviral activity (Asres *et al.*, 2001), chemo-preventive activity (Choi *et al.*, 2012), immunomodulatory activity (El Ghaoui *et al.*, 2008), tyrosinase inhibitory activity (Namjoyan *et al.*, 2015).

Phytocompounds with certain pharmacological applications are highly sought after in the pharma industry. Developing medicinal products using phytocompounds can be challenging due to their limited availability and significant variation in nature, as well as a lack of effective extraction methods (M. A. Khan *et al.*, 2014). In-vitro culture technology allows for an unlimited supply of phytoproducts irrespective of the environmental variations (Davies *et al.*, 2014; Matkowski, 2008). Callus culture has particularly significance in PTC, because it could be more readily employed to establish cell cultures on a laboratory and commercial scale. Callus culture have seen to produce novel phytocompounds not found in the plant's wild population (Lystvan *et al.*, 2018). Callus culture success can be assessed by measuring biomass accretion and production of bioactive compounds (Adil *et al.*, 2019).

Under stress, the plant's internal defense mechanisms are activated, prompting an elevation in secondary metabolite production. Activation of internal defense mechanisms of plant prompt increased SM production under stress due to exogenous factors. These exogenous factors capable of activating defense system of plant are simply termed as elicitors and the process elicitation. Both biotic and abiotic classifications apply to them. Using elicitors as an enhancer for SM production has gained importance since elicitors could trigger biosynthetic pathways for optimal production of commercially important phytocompounds (Angelova *et al.*, 2014; T. Khan *et al.*, 2019; Nabi *et al.*, 2021; Yang *et al.*, 2010; Yue *et al.*, 2016). The regulation of secondary plant metabolite production in low-volume cultures is achieved through the application of elicitation, which is a widely used and cost-effective technique because it reduces the required time and yield. However, variables including nutritional content, culture age, concentration, specificity, and exposure time of elicitor greatly influence elicitation (Awad *et al.*, 2014; Chodisetti *et al.*, 2015; Mulabagal *et al.*, 2004; Vasconsuelo *et al.*, 2007).

Melatonin (N-Acetyl-5-methoxytryptamine), a natural bio-stimulant is synthesized by both, plants and animals (Back *et al.*, 2016). Melatonin functions to relieve oxidative stress, provide defense against pathogens, improve plant growth, reproductive regulation in plants and help protect against abiotic stressors and have a role in plant photoprotection (Y. C. Kim *et al.*, 2021; Song *et al.*, 2022). Introduction of exogenous melatonin is an effective instrument for programming plant machinery to regulate certain

functions and to enhance biomass accumulation (Iqbal *et al.*, 2022). Ultraviolent light (UV) is defined as the radiation with wavelengths of 200 nm to 400 nm and is categorized as UV-C (200-280 nm), UV-B (280-320 nm) and UV-A (320-400 nm). UV-C exposure activates photochemical reactions by bringing structural changes in DNA, proteins, lipids and impair photosynthesis whereas low doses may show beneficial effects. It can increase SM production which then protect cell against irradiation by acting as scavengers of free radicals (Mishra *et al.*, 2020).

The study aimed to assess the influence of melatonin and UV-C elicitation on the biomass accretion of callus cultures of *Alcea rosea* and the resultant changes in their phytochemistry.

1.1 Aims and Objectives

- To assess UV-C and melatonin's impact on phytochemicals production and biomass accumulation.
- To ascertain the antioxidant activity and anti-aging activity of anthocyanins found in both, elicited and non-elicited, callus cultures of *Alcea rosea*

CHAPTER 2

LITERATURE REVIEW

2.1 Botanical description of *Alcea rosea*

Alcea rosea is a biennial or short-lived perennial flowering plant which can grow up to heights of 1.5m to 2m. The plant is overall thin, erect, and branched sparsely. The stem is terete, pubescent, and light-green in color. The petioles are 2–5 cm long and juvenile. The palmately lobed leaves with orbicular shape are found, which are 7–15 cm wide, medium green, and have crenate margins and cordate edges; petioles have a length of 2–5 cm and are pubescent. A spike-like raceme inflorescence that is axillary or terminal. Flowers appear in small groups or are solitary along the rachis. The funnel-shaped, showy, large flowers can measure 7–12 cm in diameter when completely opened. Flowers have five overlapping petals that form a funnel, greenish hairy bracts (6–9), oblong greenish sepals (6–8), and a prominent columnar structure with many stamens at the top and abundant stigmas (thread-like) below. The fruit is schizocarp and contains 15 to 20 sparse, ovoid, fat seeds (Lim, 2014). The *Alcea rosea* has kidney shaped, brownish-black seeds which are around 6 mm in diameter and have rugose and hairy margins. When immersed in water, they become mucilaginous (Fahamiya *et al.*, 2016).

2.2 Taxonomic classification

The *Malvaceae* family, which has more than 80 genera and between 1500 to 1600 species, includes *Alcea rosea* (Rakhmatova *et al.*, 2019). The genus Alcea includes over 60 species (Kumar *et al.*, 2021). *Alcea rosea's* taxonomic classification is illustrated in the figure 2.1.

2.3 Vernacular names

Alcea rosea is recognized globally by a variety of vernacular names, some of the common names in different languages are listed below:

- **Urdu:** Gul-e-Khera
- **Arabic:** Khatmae
- **Brazil:** Malva Da India
- **Chinese:** Zhu kui
- **Danish:** Stokrose, Almindelig Stokrose
- **French:** Rose Papale, Passe Rose

- **German:** Baummalve, Garten-Stockrose, Pappelrose, Stockmalve, Winterrose
- **Italian:** Malvone, Rosone, Rosoni
- **Japanese:** Tachi-Aoii
- **Norwegian:** Praktstokkrose
- **Russian:** Štockrosa
- **Spanish:** Malva De Las Indias, Malva Loc, Malva Rósea, Vara De San José (Lim, 2014).

Table 2-1 Taxonomic classification of *Alcea rosea*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Malvales
Family	Malvaceae
Sub-Family	Malvoideae
Tribe	Malveae
Genus	*Alcea L.*
Species	*Alcea rosea Linn*

2.4 Geographical distribution of *Alcea rosea*

Alcea rosea was first reported in the 15th century in China's Southwest provinces (Shaheen *et al.*, 2010; Tang *et al.*, 2007). Today, it can be found in temperate and tropical regions, including the Southern Europe, the Near East, the Middle East, Central Asia, and the Mediterranean (Al-Snafi, 2013).

2.5 Cultivation, propagation, and collection

The herbs intend to propagate well on plains throughout winter, but they do not do well if the monsoon is heavy. Under mild climatic conditions, it can be cultivated throughout the year on the plains. *Alcea rosea* generally blossoms in 4-5 months with different flowering seasons between the hills and the

lowlands. Both sandy and clayey soils that have been supplemented with organic manure work well for them. A good 60 cm to 60 cm spacing and proper watering are required (Fahamiya *et al.*, 2016).

The most common method of propagation in the plains is seeding. The seeds are sown in well-prepared and fertilized areas and watered regularly. After 30 days, the seedlings are moved to rich, and prepared-friable soils. By cutting the roots into pieces and placing crown buds and shoot-cuttings on each piece, the herbs can be cultivated on the hills. Due to the extensive cross-pollination of *Alcea rosea*, true colors and shapes might not be present in all hybrids (Fahamiya *et al.*, 2016).

2.6 Agroecology

The hollyhock is a temperate plant species capable of adapting to growth in tropical high-altitude regions. It requires fertile soil with good drainage, a loamy texture, and exposure to full sunlight for optimal growth. While light shade may be tolerated, it is not tolerant of moist soil conditions.

2.7 Phytochemical constituents of *Alcea rosea*

Six chemical isolates were reported for *Alcea rosea* flowers by utilizing bioassay-guided fractionation. Their structural features were defined using spectroscopic methods, such as 2D and 1D NMR. Dihydrokaempferol-4′-O-glucopyranoside (flavanonol glucoside) was identified in the flowers as the main constituent, exhibiting both anticancer and antioxidant effects against the HepG-2 cell line, and therefore may be regarded as a valuable marker compound. Following six structures were identified in *Alcea rosea* flowers (Abd El-Salam *et al.*, 2016; Abdel Salam *et al.*, 2018):

- Dihydrokaempferol
- Dihydrokaempferol-4'-O-β-D-glucopyranoside
- Kaempferol-3-O-α-L-rhamnopyranosyl-(1'''→6'') -β-D-glucopyranoside
- Apigenin
- Kaempferol-3-O- [6''- (E-coumaroyl)] -β-D-glucopyranoside
- Kaempferol-3-O-β-D-glucopyranoside

Astragalin, caffeic acid, ferulic acid, kaempferol, luteolin, naringenin, quercetin, Quercetin- 3- O- (6″- O- trans- p- coumaroyl) -β- D- glucopyranoside, Quercetin- 3- O-β- D- glucopyranoside, Quercetin 4′- O-β- D- glucopyranoside, and rutin were recognized in the 70% ethanolic extract of *Alcea rosea* flowers (Ma *et al.*, 2019) reported that the component with the maximum concentration was quercetin and its derivatives. She identified four distinct structural classes of NF-κB inhibitors, including

dihydroflavone, hydroxycinnamic acid, phenolic acid, and flavonoids. Furthermore, using GC-MS, the lipophilic profile of *Alcea rosea* was performed. These phytochemicals were confirmed using the Van den Dool and Kratz (I) Indices. The plant metabolite analysis revealed the presence of various types of compounds, including fatty acids and fatty acid esters, both unsaturated and saturated. In addition, hydrocarbons were detected in the n-hexane fraction. Interestingly, the fatty acid esters were newly identified through GC-MS analysis and were detected for the first time in the flowers of *Alcea rosea* (Hanif *et al.*, 2019).

Phenolic acids distribution in *Alcea rosea* var. nigra flowers was investigated using 2D-TLC and HPLC techniques. p-hydroxy phenylacetic, syringic, p-hydroxybenzoic, ferulic, vanillic, and caffeic acids were the phenolic acids identified in these fractions. The main phenolic acid contents were determined using HPLC techniques. The most prevalent phenolic acids studied were syringic acids, p-hydroxy benzoic acids, and p-coumaric acids. Hosaka (2012) isolated a luteolin 4′-O-glucoside (a flavone), 3 flavanols [(1) kaempferol 3-O-gluco-side, (2) myricetin 3-O-glucoside, and (3) kaempferol 3-O-rutinoside)] and 9 anthocyanins, [(1)cyanidin 3-O-rutinoside, (2) malvidin 3-O-malonylgluco-side, (3) malvidin 3-O-rhamnosylglucoside, (4) delphinidin 3-O-glucoside, (5) petunidin 3-O-glucoside, (6) delphinidin 3-O-rutinoside, (7) cyanidin 3-O-glucoside, (8) petunidin 3-O-rhamnosylgluco-side, and (9) malvidin 3-O-glucoside], from *Alcea rosea* 'Nigra' black flowers respectively (Hosaka *et al.*, 2012).

Quantitative researches revealed kaempferol, quercetin, and rutin in the hollyhock flavonoid fraction (Muhetaer *et al.*, 2015; M Papiez, 2001). The methanolic extract from aerial parts were analyzed and five flavonoids (kaempferol, kaempferol-3-O-β-d-glucoside, quercetin 3-O-β-d-glucuronopyranoside-8-C-β-d-glucopyranoside, kaempferol-4′-O-β-d-glucoside, and kaempherol-3-O-β-d-rutinoside) were identified (Ammar *et al.*, 2013). Several studies reported elevated level of fatty oils components in *Alcea rosea* , such as 82.197% Linoleic acid, 9.195% Oleic acid, 4.756% Palmitic acid, 2.681% Stearic acid, and 0.328% Linolenic acid (Liu *et al.*, 2006). Phytochemical evaluation of *Alcea rosea* revealed polysaccharides and gibberellin glucosides as main constituents in shoot apices (Harada *et al.*, 1970), and seed or leaf (Eskandari *et al.*, 2015). Meanwhile, phenolic acids (Dudek *et al.*, 2006) and flavonoids (Monika Papiez *et al.*, 2002) are found in flowers. Mucilages are acidic polysaccharides with a higher molecular weight (1.3-1.6 million Dalton) found in the leaves and flowers of *Alcea rosea*. The main constituents of these mucilage's are galactose, glucuronic acid, rhamnose, and galacturonic acid. Flavonoids, alkaloids, minerals, and proteins are also present (Al-Snafi, 2013).

2.8 Pharmacological properties

Alcea rosea has a myriad of medicinal properties. Some of the pharmacological properties reported in previous studies are listed below.

2.8.1 Anti-urolithiatic activity

When mice with ethylene glycol-induced renal calculi were given a hydro alcoholic extract of nigra roots, the amount of CaC_2O_4 deposition in the mouse kidneys was dramatically reduced when compared to the ethylene glycol group. Ethylene glycol caused a significant increase in renal oxalate, which the extract reduced. The plant's diuretic and anti-inflammatory properties, as well as the presence of mucilaginous polysaccharides, were linked to this impact. According to the findings, *Alcea rosea* can help prevent and eliminate calcium oxalate buildup in the mouse kidney (Ahmadi *et al.*, 2012).

2.8.2 Antiulcer activity

The study demonstrated that administration of polysaccharides extracted from the stem of *Alcea rosea* through intravenous, intraperitoneal, or enteral routes effectively reduced stomach lesions in mice induced by pylorus ligature (Barnaulov *et al*., 1985).

2.8.3 Antimicrobial activity

According to GC-MS analysis, more than 70 molecules were found in *Alcea rosea's* volatile oil with most of these compounds' beings aliphatic or aromatic. The volatile oil had a broad anti-bacterial spectrum and exhibited eminent antimicrobial activity against common infectious microbes (Muhetaer *et al.*, 2015).

Extracts from flowers and leaves of *Alcea rosea* showed good anti-microbial activity against *Listeria monocytogenes, Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Bacillus anthracis*, *Bacillus cereus*, *Escherichia coli*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. *E. coli* was the strain with the highest level of resistance (Seyyednejad *et al*., 2010). The methanolic, n-hexane, ethyl acetate, water, and ethanolic extracts of flowers of *Alcea rosea* had been shown to have antimicrobial activity against *S. aureus* (ATCC 29213, ATCC 6538), *E. coli* (ATCC 11230, ATCC 29998), *Salmonella typhimurium* (CCM 5445), *S. epidermidis* (ATCC 12228), *albicans* (ATCC 10239), and *P. aeruginosa* (ATCC 27853), utilizing the disc diffusion method. Resistance was observed in *Enterobacter cloacae* (13047), *E. coli* (25922), *Candida albicans* (10239), and *Enterococcus faecalis* (29212). There was no discernible difference in activity between extracts.

However, it was found that ceftazidime was only slightly more effective than all *Alcea rosea* extracts against the tested bacteria (Tuba *et al.*, 2010).

2.8.4 Antiviral activity

By pre-treating BALB/c mice with *Alcea rosea* extract and then infecting them with Influenza A virus (IAV) through the nasal passage, the anti-influenza properties were examined. The study results showed an improvement in the survival rate and a reduction in the virus titer in the lungs upon infection with various subtypes of Influenza A virus (IAV) following oral administration of *Alcea rosea*. This was accompanied by the induction of antiviral innate immune responses in various bodily fluids including the lungs, small intestine fluid, bronchoalveolar lavage fluid, and serum (Kim et al., 2018). Investigations into the antiviral effectiveness against Human Immunodeficiency Virus types 1 and 2 (HIV 1 and HIV 2) revealed that the methanol fraction of the plant had no discernible cytotoxic effects (Asres *et al.*, 2001).

2.8.5 Immunomodulatory activity

Polysaccharides derived from aqueous extracts of *Alcea rosea* were found to have immunomodulatory properties in a study. In vivo, the polysaccharide extracts increased B-lymphocyte polyclonal activation and antibody reactivity to egg albumin, but no effect on interferon or interleukin-4 gene transcription was observed (El Ghaoui *et al.*, 2008).

Y. C. Kim (2021) investigated the immune-stimulatory effects and mechanisms of action of the flower extract of *Alcea rosea* (ARF) on macrophages. The obtained results depicted that the water extract of ARF (ARFW) improved the production of cytokines (TNF- and IL-6) and NO in a dosage-dependent manner. Additionally, ARFW enhanced the expression of COX-2 and iNOS proteins in RAW264.7 cells, which was supported by the elevated expression of MAPK proteins (p38, JNK, ERK). ARFW also led to the phosphorylation and breakdown of IκBα, which allowed for the nuclear translocation of NF-κB. These results depicted that the immunostimulatory effects of *Alcea rosea* are arbitrated by the cytoplasmic translocation of the NFB p65 subunit into the nucleus, leading to the release of pro-inflammatory cytokines (TNF- and IL-6) and other mediators (COX-2 and iNOS) through the MAPK signaling pathway (Y. C. Kim *et al.*, 2021).

2.8.6 Anticancer activity

Scopoletin, scopoline, 1-(α-l-rhamnosyl (1 → 6)-β-D-glucopyranosyloxy)- 3,4,5-trimethoxybenzene, suberic acid, benzyl α-L-rhamnopyranosyl (1 → 6)- β-D-glucopyranoside, p-hydroxy

phenethyl trans-ferulate, and sebacic acid were identified in methanol extract of *Alcea rosea* roots. The compounds showed a weak in-vitro cytotoxic potential against cancer cell lines, ovary malignant ascites (SK-OV-3), lung carcinoma (A549), colon adenocarcinoma (HCT-15), and skin melanoma (SK-MEL-2); and had ED_{50} values above 30 µg (Kim *et al.*, 2007). Tuba *et al.* (2010) evaluated cytotoxic potential of ethyl acetate extract of *Alcea rosea* flower towards brine shrimp.

In HCT116 and SW480 cells, *Alcea rosea* seed extract inhibited colony formation and proliferation in a timely and dose-dependent way and encouraged apoptosis, as evident by decreased levels of BCL-xl protein, elevated Bax expression and PARP disruption. Moreover, AR extracts arrested cells in the G_0/G_1 phase of cell cycle with a lower level of cyclin D_1. AR extract treated cells showed reduction in colon-sphere number and size coinciding with decreased cancer cell markers (ALDH1A1 and Dclk1). In-vivo inhibition of tumor development was facilitated by AR extract owing to decreased levels of EZH2, Ki-67, Cyclin D_1, ß-catenin, and CSC markers. The findings strongly propose that AR extract/active constituent(s) could be a useful therapeutic/preventive agent for colon cancer (Ahmed *et al.*, 2016)**.**

The study, the methanolic extract of *Alcea rosea* was investigated for its potential to prevent neoplastic cell transformation in JB6 P+ mouse epidermal cells. The results indicated that the extract effectively inhibited the transformation by decreasing the kinase activity of the EGFR (epidermal growth factor receptor). Further, the extract was able to inhibit the activation of EGFR by EGF (epidermal growth factor) in EGFR-positive cells, but not in EGFR-negative cells. Additionally, the extract demonstrated inhibitory effects on the growth of EGFR-positive mouse embryonic fibroblasts stimulated by EGF.

These results suggest that using plant extracts to target EGFR may be a promising approach for chemoprevention and chemotherapy. By inhibiting the activity of EGFR, the extract could help prevent the development and growth of cancer cells. The results also indicate that the effectiveness of this approach may depend on the presence of EGFR in the cells, which highlights the importance of considering individual differences in the response to treatment (Choi *et al.*, 2012).

2.8.7 Antidiabetic activity

Dar *et al.* (2017) examined the antioxidant and antidiabetic activities of *Alcea rosea* seed extract in diabetic rats, showing a drop in blood glucose levels of 24% and 46% for the aqueous and methanol extracts, respectively, at a defined dose of 300 mg/kg.bw. The methanol and aqueous extracts also

improved the antioxidant status of the liver and pancreas by enhancing CAT, SOD, GR, and GPx levels in hyperglycemic rats.

Zhang *et al.* (2015) demonstrated that after eight weeks of oral administration, ethanolic extract of *Alcea rosea* flower can substantially reduce blood sugar and cholesterol levels in mice (KK-Ay). Meanwhile, expression of PI3K, IRS2, AKT, AMPK, and GLUT4 genes in the liver was increased dramatically. ARF generated three novel dihydroflavonol glycosides, flavanonolosides A, B, and C, as well as two previously known dihydroflavonol glycosides. In the HepG2 cell line, glucose absorption increased by 30%-40% at a dose of 20 g/ml for 1-5. Glucose metabolism regulating function of hollyhock flowers has been linked to dihydroflavonols.

2.8.8 Hepatoprotective effect

Hepatoprotective properties of hollyhock in mice against acetaminophen-induced hepatotoxicity was investigated by (Hussain *et al.*, 2014). For seven days, a methanolic aqueous extract of *Alcea rosea* was administered orally, succeeded by a toxic dosage of acetaminophen. The hepatoprotective action of *Alcea rosea* was evaluated at the end of the treatment course using hepatic enzyme indicators (aminotransferases, alkaline phosphatase, and bilirubin) along with histological examination of liver tissues. When compared to mice treated with acetaminophen, acetaminophen significantly increased blood levels of hepatic enzyme indicators, whereas *Alcea rosea* extract notably lowered blood levels of increased hepatic enzyme markers in a dosage-dependent manner. Protective effects of *Alcea rosea* on liver enzyme markers were established by histopathological analysis of liver tissues.

2.8.9 Bronchodilatory effect

Alcea rosea may contain a variety of bioactive phytocompounds that promote bronchodilation. Flowers lowered carbamylcholine and contractions stimulated by K+ (80 mM) in guinea-pig isolated trachea, upregulated isoprenaline concentration-response curves (CRCs), and suppressed Ca2+ CRCs. The researchers discovered that *Alcea rosea* promoted bronchodilation by inhibiting both the phosphodiesterase enzyme and the Ca2+ influx , implying that it has the potential to treat airway problems (Hanif *et al.*, 2019).

2.8.10 Antiestrogenic activity

A significant increase in 3-βHSD, G6PD, and NADP activities was observed in rats introduced with *Alcea rosea's* aqueous flower extract for 30 days. Leydig cells also showed an analogous increase in

Khanolkar reaction intensity (M Papiez, 2001). Leydig cells demonstrated statistically insignificant changes in G6PD and NADP activities upon exposure to the extract for 180 days; conversely, 3- βHSD activity and intensity of Khanolkar reaction increased significantly implying restorative alterations. The extract's mild antiestrogenic effect was evident from the increase in androgen levels accompanied with decreased levels of estrogen in the homogenates of group A2 testes. In addition, Monika Papiez *et al.* (2002) established that the antiestrogenic activity of the flavonoids present in hollyhock extract was primarily arbitrated by the aromatase and estrogen receptor beta in rat testicular cells, rather than by the estrogen receptor alpha.

Further investigative studies revealed a prominent increase in the δ(5)β-HSD and G6PDH activities in the Leydig cells when rats were introduced with a dosage of 100 mg/day of hollyhock's flower extract (M. A. Papiez, 2004). After hollyhock administration, no prominent variations in the height of seminiferous epithelium or the radius of seminiferous tubules were observed. Furthermore, only a minor amount of interstitial tissue hyperplasia was observed. A direct but minor effect of hollyhock's methanolic extract on rat testes was found after studying morphological and enzymatic changes of Leydig cells. There was no effect of the extract on steroidogenesis as evident from trivial changes in the testosterone (testicular) and estradiol content.

2.9 Other uses

Alcea rosea is usually grown for its ornamental value. The plant is favored for use in mixed borders and for increasing aesthetic appeal of fences. Nowadays, different ornamental races with a myriad of colors (white, mauve, rose, purple, black/red, also yellow) are cultivated. Mostly the flowers are spotted; double flower varieties can also be found.

The flowers' red anthocyanin constituent is used as a litmus test while the petals are used to make a brown dye. The dried and powdered herb mixture of hollyhock flowers was utilized as a component in the 'Quick Return' herbal compost activator, aimed at enhancing bacterial activity and expediting the composting process. A drying oil content of 12% is found in the seed. Historically , stem fibers of hollyhock have been utilized in the production of paper (Lim, 2014). *Alcea rosea* can also be utilized in the phytoremediation of cadmium-rich soils as it can act as hyperaccumulator of Cd by using chemical agents (Lim, 2014; Liu *et al.*, 2006).

2.10 Importance of plant tissue culture

Wild plants have always served as a rich source of secondary plant metabolites, but their commercial extraction is restricted due to different geographical, ecological and/or seasonal constraints. Plants can be developed through traditional cultivation techniques to the point where SMs of interest are produced by the plant, but this is laborious and time intensive work. The plant tissue culture approach helps in overcoming the constraints of traditional cultivation. Plant cells, tissues, and organs are all cultured under aseptic settings in a controlled environment in an artificial nutritional medium (Chadipiralla *et al.*, 2020). This method primarily relies on micropropagation, defined as the rapid multiplication of cells from any part of plant tissue (axillary bud, stem, leaf, and root). It is therefore widely used for industrial-scale plant multiplication. The quick production of bioactive metabolites that are relevant for use in medicine and have commercial value is made possible by this technology. Callus culture is viewed as a potential bio factory that could provide a consistent production system and a steady supply of SM products while conserving plant's natural habitat. It lacks a number of abiotic and biotic components (Rao *et al.*, 2002).

2.11 Applications of PTC

- This technique plays a vital role in the germplasm conservation of important therapeutic plants.
- Helps in improving the robustness and vitality of plants.
- Employed in the production of pharmaceutically important bioactive compounds.
- PTC is commonly used for Improving overall nutritional content of crop plants.
- PTC is utilized in the production of artificial seeds which helps propagate plant species that cannot propagate through seeds normally.

2.12 Micropropagation

Micropropagation is the in vitro asexual multiplication and clonal propagation of plants using somatic tissues or organs, performed under sterile conditions. The process leverages the totipotency of each plant cell, which has the capability to develop into an entire plant, as its underlying principle (Bhojwani *et al.*, 2013). The biotechnological method of micropropagation is critical for the preservation of threatened and extinct medicinal plant species, including selection, multiplication, and preservation of germplasm (Abbasi *et al.*, 2016). This technique was used to produce high-quality bioactive molecules for pharmaceutical development, conserve endangered plant germplasm, create disease-free plant varieties, and improve genetic traits (I. Khan *et al.*, 2020).

In vitro multiplication of essential medicinal plants has garnered a lot of interest being developed over the past two decades. A myriad of explants, including roots, shoot apex, rhizomes, cotyledons, embryo axis, petals, leaves, bud scale, have been used to develop medicinal plants. *Ajuga bracteosa*, *Rosmarinus officinalis*, *Mentha piperita*, *Lallemantia Iberica*, *Lavandula angustifolia*, and other medicinal plants, have been successfully micro propagated in vitro (A. Khan *et al.*, 2021).

2.13 Callus culture

Undifferentiated mass of cells produced from plant tissue is termed as callus; under in vitro conditions it is labelled as callus culture. It functions mainly to complement sparse plant resources. Callus cultures acts as a source of important phytochemicals which can be extracted directly from callus culture negating the need for removal of whole plants for phytochemicals thus protecting plant's natural habitat. Nutritional medium and phytohormones (auxins and/or cytokinin) are essential for callus induction. Callus is totipotent, meaning that each cell has the potential to form an entire new plant through direct regeneration or somatic embryogenesis. Under stress conditions, secondary metabolites are produced in callus culture, which can be induced by a variety of abiotic and biotic elicitors (Fehér, 2019).

Callus culture is an economically feasible approach for producing new bioactive phytochemicals that can be utilized in a variety of commercial products such as cosmetics and food. Micropropagation has the potential for utilization in multiple commercial applications, including the production of therapeutic antibodies and recombinant proteins. They can be used to regenerate agricultural and horticultural plants through callus regeneration (Benjamin *et al.*, 2019). Due to recent advances in pharmaceutical engineering, various biological engineering approaches have been employed to increase the yield of beneficial bioactive plant metabolites in callus cultures (Ogita, 2015).

2.14 In-vitro tissue culture of *Alcea rosea*

Alcea rosea callus culture was previously established by our senior lab member Hasnat Tariq *et al*., 2023. Leaflets (1.5 cm) from a month-old in vitro seed-derived plantlet was used as explant. MS media containing sucrose (30 g/L), agar (8 g/L) and a variety of PGR concentrations either in combination or alone was experimented with to find the hormone(s) giving maximum results. The optimum DW and FW was obtained by using hormonal combination of NAA (1 mg/L) and TDZ (0.1 mg/L) [Unpublished data].

2.15 Elicitation

As a part of plant's natural defense mechanism secondary metabolites are produced to combat diseases and harmful effects caused by internal or external stress. Elicitors are chemicals or bio-factors that can induce physiological changes in plants which alter the plant metabolic machinery resulting in the buildup of secondary plant metabolites in higher quantities. By nature elicitors are classified as either biotic or abiotic (Patel *et al.*, 2013).

Elicitation has been the most successful of all the approaches used to increase important plant metabolites production. This technique functions by activating various plant metabolic pathways resulting in increased regulation of targeted phytocompounds. Elicitation is a viable approach for enhancing the production of plant secondary metabolites (Yang *et al.*, 2010).

The cell membrane-bound receptors recognize the elicitors by improving secondary metabolism, and then activate the appropriate genes via the signal transduction pathway (Isah, 2019). These stress-inducing agents are collectively referred to as elicitors and fall under the abiotic or biotic categories (Baenas *et al.*, 2014). Biotic elicitors encompass plant cell wall components (such as pectin and cellulose) and microorganisms (such as yeast, fungi, and bacteria extracts), while abiotic elicitors encompass various physical and chemical stimuli, including metals, light, salinity, osmotic stress, drought, temperature, and hormonal factors (Naik *et al.*, 2016).

2.16 Melatonin as an elicitor

Nearly all living organisms synthesize melatonin (tryptophan-derived biomolecule), including plants and animals. Melatonin functions as a signaling molecule to mediate plant's natural defense mechanism, alleviating natural and chemical stressors. It also acts as a stimulant for plant development and growth (Back *et al.*, 2016; Zhao *et al.*, 2011). Currently, the presence of melatonin in diverse species have been discussed in more than 5700 reports. Leaves and flowers of medicinal plants have higher concentrations of melatonin present which can act as a possible treatment for neurological disorders (Murch *et al.*, 2002). Melatonin ratios affected in vitro plant morphogenesis as well as circadian rhythms, light/dark responses, and seasonality. Additionally, it has been suggested that melatonin controls physiological procedures like elimination of free radicals, diurnal responses, and environmental adaptations (Murch *et al.*, 2002).

2.17 UV-C as an elicitor

Ultraviolet Light has gathered a great deal of interest in the past owning to its powerful effects on medicinal plants and triggering bioactive molecules synthesis (UV C starting line). UV-C irradiation is an effective type of UV light for secondary metabolites induction in many plants (Mishra *et al.*, 2020). UV light activates plant natural defense mechanism by causing stress, resulting in the production of phytoalexin. As a part of defensive strategy, plants produce secondary metabolites, antioxidant enzymes, and cell wall changes to ameliorate the oxidative damage caused by UV-C irradiation and by scavenging ROS species (Anjum *et al.*, 2017).

Coupling elicitation procedures have improved the efficiency of plant metabolite production. Increased antioxidant and anti-inflammatory potential along with silymarin content was observed under the influence of melatonin combined with a variety of light regimes in *Silybum marianum* callus cultures (Shah *et al.*, 2019). Nazir *et al.* (2020) demonstrated that using melatonin and UV-C as elicitors enhanced the antioxidant capability and phenylpropanoid metabolite profile of *O. basilicum* callus cultures.

CHAPTER 3

MATERIALS AND METHODS

All callus culture experiments were planned and performed at Plant Cell Culture Lab (PCCL), Department of Biotechnology, Quaid-I-Azam University, Islamabad, under the guidance of Prof. Dr. Bilal Haider Abbasi.

3.1 Chemicals and equipment

Ethanol, hydrochloric acid, sodium hydroxide, plant growth regulators, and distilled water are some of the chemicals used in this study. Equipment used includes filter paper, forceps, spatula, blades, pH meter by Jenway 3305, 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

The media preparation procedure followed for the subsequent subculturing of already optimized leaf-derived callus culture of *Alcea rosea* at PCCL was provided by Hasnat et al., 2023. (Unpublished data). Murashige and Skoog medium (4.4 g) and sucrose (30 g) were weighed in an electronic balance before being combined with distilled water in an Erlenmeyer flask to make 1 liter in total. Plant growth regulators, NAA (1 ml) and TDZ (0.1 ml) were supplemented in the solution respectively and pH of the media was measured. The pH of media was maintained at 5.65 ± 0.05 by using 1.0 N HCL and NaOH respectively. The medium was later solidified by adding agar (8 g) to it. Agar was properly dissolved by heating the flasks in microwave for 5-6 minutes. Media was poured into Erlenmeyer flasks (40 ml/flask) and sealed firmly with cotton plugs and top was shielded with aluminum foil. The flasks were subjected to sterilization through autoclaving at 121°C temperature and 15-psi pressure for a duration of 20 minutes. Autoclaved media was left overnight to ensure solidification and confirming that the media was contamination free. The media preparation protocol was obtained from (Abbasi *et al.*, 2010) and the hormonal combination was reported by Hasnat et al., 2023 (unpublished data)

3.3 Elicitor preparation

Melatonin solution was made by using 50 ml of distilled water as a solvent and 50 mg melatonin along with continuous prolonged stirring ensured appropriate mixing of melatonin. The UV-C lamp (Spectro line, model ZQJ-2) with an intensity of 3 W/m^2 and a wavelength of 254 nm was utilized.

3.4 Elicitor treatment on callus cultures of *Alcea rosea*:

Callus cultures of hollyhock were subjected to UV-C exposure and exogenous melatonin in separate experiments. The method of treatment used for the elicitors is discussed below.

3.4.1 UV-C treatment

The effect of UV-C stress on *Alcea rosea* callus cultures was appraised by comparison with a control group. The UV-C lamp (Spectro line, model ZQJ-2) with an intensity of 3 W/m2 and a wavelength of 254 nm was utilized. The calli were exposed to UV-C radiation for various time durations after being inoculated onto MS media supplemented with hormones (1 mg/L NAA and 0.1 mg/L TDZ). The time durations varied from 10 to 70 minutes. The lamp was stabilized, and the entire experiment was conducted in a growth room with controlled conditions of 25 ± 2 °C and a 16h light/8 h dark period for 4 weeks. The control was not exposed to UV-C light. The harvested calli were analyzed for biomass accumulation and phytochemical production over the 4-week growth phase.

3.4.2 Melatonin treatment:

Fresh callus (0.5 g) was transferred from a previously sub cultured leaf-derived callus that was optimized with hormones (1 mg/L NAA and 0.1 mg/L TDZ) to MS media with the same hormonal combinations and various concentrations of melatonin (1 mg/L, 3 mg/L, 6 mg/L, 10 mg/L, 15 mg/L, 20 mg/L and 25 mg/L). The control group, lacking melatonin, was also included. The experiment was conducted in a growth room under monitored and controlled conditions, with a temperature of 25 ± 2 °C and a 16-hour light/8-hour dark period, for a total of four weeks. After 28 days, the calli were harvested for analysis of biomass accumulation and production of relevant phytochemicals.

3.5 Sample extraction and extract preparation

After 28 days, the calli were harvested from the cultured media to assess biomass accumulation and production of phytochemical constituents. The fresh calli were dried at room temperature for 20 minutes on Whitman filter paper to remove any residual moisture and then weighed. The dried calli were ground into a fine powder using a motor and pestle and then mixed with 0.5 ml of methanol. The mixture

was vortexed for 5 minutes and sonicated for 30 minutes with a sonicator (USC1200TH) before being centrifuged for 15 minutes at 12,000 rpm to obtain the extract (Zahir *et al.*, 2014).

3.6 ANTIOXIDANT ACTIVITIES

3.6.1 ORAC assay

As describe by Prior *et al.* (1998), The Oxygen Radical Absorbance Capacity (ORAC) assay was conducted by combining 190 μL of fluorescein (0.96 M) with 10 μL of the extracted material at concentrations ranging from 8 M to 1 mM, followed by incubation at 37°C with periodic shaking for at least 20 minutes. The fluorescence intensity was monitored using a fluorescence spectrophotometer (BioRad) with an excitation wavelength and an emission wavelength of 485 nm and 535 nm respectively, after the addition of 20 μL of 119.4 mM 2,2'-azobis-amidinopropane (ABAP). The fluorescence was monitored every five minutes for 2.5 hours at 37°C. The antioxidant capacity was measured in triplicate and reported as Trolox C equivalent antioxidant capacity (TEAC).

3.6.2 CUPRAC assay

Cupric ion reducing antioxidant capacity (CUPRAC) was utilized for the assessment of antioxidant activity, by Apak *et al.* (2004) and Benzie *et al.* (1996) with minor modifications. The CUPRAC assay was performed using a ratio of 1:1:1 (v/v/v) of the extracted material and CUPRAC solution (10 mM Cu (II), 7.5 mM neocuproine, and 1 M acetate buffer at pH 7). The reaction mixture was incubated for 15 minutes at room temperature (25 ± 2 °C) and then analyzed using a BioTek ELX800 Absorbance Microplate Reader for absorbance at 450 nm.

3.6.3 CAA (% ROS/RNS inhibition) assay

According reported by Tungmunnithum *et al.* (2020), The cellular antioxidant assay (CAA) involved determining the levels of reactive nitrogen (RNS) and reactive oxygen (ROS) species by using the fluorescent dye dihydrorhodamine-123 (DHR-123). Six hours prior to inducing oxidative stress, the extracts were evaporated under a nitrogen flow, dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 g/mL, and applied to the cells at a final concentration of 1 mg/mL with a final DMSO concentration of 1% (v/v) in the cell. The control sample had a DMSO concentration of 0.1% of the final volume. The hollyhock extracts were used to incubate yeast cells overnight, followed by two washes with PBS. The extracts were then maintained in the dark at 30°C for 10 minutes. The fluorescent signal was detected using a BioRad fluorescence spectrophotometer (emission at 535 nm, excitation at 505 nm) after multiple PBS washes.

3.7 ANTI-AGING ACTIVITIES

3.7.1 Elastase assay

The activity of porcine pancreatic elastase (from Sigma Aldrich) was determined through an elastase assay. The procedure involved the use of N-Succ-Ala-Ala-Ala-p-nitroanilide (AAAVPN) as the substrate and detection of p-nitroaniline release at 410 nm through a spectrophotometer (BioTek ELX800 from BioTek Instruments). This method was based on the work of Wittenauer *et al.* (2015). The anti-elastase activity was determined through three replicates, and the inhibition level of each extract was expressed as a percentage in comparison to the control group, which consisted of the addition of an equivalent volume of the extraction solvent.

3.7.2 Tyrosinase assay

The tyrosinase inhibitory activity was measured using the method defined by Chai *et al.* (2018). The assay consisted of combining 5 mM L-DOPA (diphenolase substrate) with 10 µL of *Alcea rosea* extract and 0.2 mg/mL of mushroom tyrosinase in 50 mM sodium phosphate buffer (pH 6.8) to create a final volume of 200 µL. A control was performed by replacing the extract with an equal volume of extraction solvent. The reaction was monitored using a BioTek ELX800 microplate reader (BioTek Instruments) at a wavelength of 475 nm. The tyrosinase inhibitory effect of each extract was expressed as a percentage of inhibition relative to the corresponding control.

3.7.3 Anti-AGE formation activity

The inhibitory capacity of advanced glycation end product (AGE) formation was determined using a method described by Kaewseejan *et al.* (2015). The *Alcea rosea* extracts were combined with 20 mg/mL bovine serum albumin (BSA) from Sigma Aldrich, 0.5 M glucose from Sigma Aldrich, and a 0.1 M phosphate buffer (pH 7.4) solution. The mixture was incubated for five days in the dark at 37 °C using a Versa Fluor fluorometer from Bio-Rad with a 330 nm excitation wavelength and a 410 nm emission wavelength. The inhibitory effect on AGE formation for each extract was expressed as a percentage of inhibition compared to the corresponding control, where the same volume of extraction solvent was added.

3.8 HPLC

HPLC conditions adapted from Hosaka *et al.* (2012), with some modification in the separation conditions to improve anthocyanins separation. Shimadzu HPLC system with Inertsil ODS-4 (I.D. 6.0 x 150 mm, GL Science Inc.). The flow rate was fixed at 1.0 ml/min with a detection wavelength of 350 and 530 nm and a column temperature of 40°C. The solvent system was composed of a mobile phase A

(MeCN/HOAc/H_2O/H_3PO_4 = 6:8:83:3) and B (MeCN/HOAc/ H_2O/H_3PO_4 = 10:8:79:3). The test samples were eluted with a linear gradient of 0 to 100% of B in A for 10 to 30 min at a flow rate of 0.2 ml/min and then with 100% of B for 30 min. The standard solution for each sample was prepped by dissolving 100 µL of extract in 1 mL of methanol, resulting in a methanol concentration of 50 g/ml. The polyphenol content was determined by associating the intensity of UV absorption spectra with the retention time of the sample to a reference standard and calculating the intensity of the absorbance.

3.9 Statistical analysis

All of the experimental treatments had three duplicates and were carried out in the same setting. Standard deviation and mean values were calculated using in-built functions of Microsoft Excel 2019 software. Origin pro-2018 software was used to create all the graphs and images that were edited to enhance visibility on Canva software (basic version).

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Influence of UV-C and melatonin on biomass accumulation

The impact of UV-C radiation and exogenous melatonin on the growth of *Alcea rosea* callus cultures was evaluated through the measurement of their fresh and dry weight in response to defined concentrations of UV-C and melatonin elicitor (Figure 4.1). The optimum biomass accumulation (fresh weight (FW): 359.13 g/L, dry weight (DW): 12.99 g/L) was achieved at UV-C (30 mins exposure) with the lowest biomass being accumulated at UV-C (70 mins exposure) (FW: 166.83 g/L, DW: 6.97 g/L), whereas the accumulated biomass at control was FW: 207.97 g/L and DW: 8.43 g/L (Figure 4.1). Longer radiation exposure times ensued significant reduction in the biomass accretion which could possibly be the result of irreversible changes induced by UV-C stress leading to apoptosis (Gai *et al.*, 2016; Nawkar *et al.*, 2013; Xu *et al.*, 2016). The results were similar to previous studies reporting optimum biomass accumulation of *Fagonia indica* callus cultures at UV-C exposure of 30 mins (Abbasi *et al.*, 2021). Stimulatory effects on callus growth of medicinal plants under the influence of UV-C radiations have been reported previously (Xu *et al.*, 2015; Xu *et al.*, 2016).

Additionally, the impact of exogenous melatonin on callus growth and development was studied at varying melatonin concentrations. The optimum biomass accumulation was achieved at 3 mg/L melatonin concentration (FW: 226.43 g/L, DW: 10.20 g/L), while the control accumulated biomass (fresh weight (FW: 207.97 g/L, DW: 8.43 g/L). Whereas the least amount of biomass aggregation (FW: 152.15 g/L, DW: 5.90 g/L) was at 25 mg/L melatonin concentration (Figure 4.1). A trend of significant reduction of biomass growth was observed at increased concentrations of melatonin, which was in agreement with previously conducted studies (Nazir *et al.*, 2020).

Inhibitory response of callus at higher melatonin concentrations can be possibly due to reactive oxygen species which interfere with the proliferation of callus resulting in cell death (Abbasi *et al.*, 2011; Fazal *et al.*, 2016). The results are in relation to the studies conducted in past, reporting significant reduction of biomass at higher concentrations of melatonin (Fazal *et al.*, 2018; Sarropoulou *et al.*, 2012).

4.2 Quantification of polyphenols in *Alcea rosea* callus cultures by HPLC

The study quantified 9 polyphenols (anthocyanins) produced by *Alcea rosea* callus cultures being subjected to elicitor stress by UV-C and melatonin. Optimum production of anthocyanins was by the callus under melatonin (25 mg/L) stress (15.92 µg/g DW) followed by callus under melatonin (15 mg/L) stress (15.32 µg/g DW) , upon comparison with the control (7.47 µg/g DW), the anthocyanins produced were found to be 2.13 and 2.05 folds higher respectively. The least number of anthocyanins accumulated was in callus supplemented with melatonin (1 mg/L) i.e., 6.78 µg/g DW. UV-C exposure of about 40 mins produced maximum anthocyanins (13.67 µg/g DW) compared to control and other UV-C exposure times (Table 4.1).

In the present work, HPLC quantification of compounds revealed that malvidin 3-O-malonylglucoside was the optimal anthocyanin (3.89 µg/g DW) produced and similar higher concentrations of malvidin 3-O-malonylglucoside (which constituted almost 22% - 26% of the total anthocyanins) were observed in calluses where melatonin was used as an elicitor. Malvidin and its glycosides have potential anti-inflammatory, antioxidant, and anti-cancer activities (W. Huang *et al.*, 2013).

As for UV-C stress derived callus the optimum production (13.67 µg/g DW) of total anthocyanins was observed at 40 minutes exposure time with the minimum (7.45 µg/g DW) accumulation at UV-C 30 minutes exposure time. The trend was also followed in this experiment with malvidin 3-O-malonylglucosidase being the most accumulated anthocyanins at each exposure time.

Other than malvidin and its glucosides, delphinidin, cyanidin and petunidin derivates were also detected. The structures of the observed anthocyanins along with the chromatogram are shown in figure 4.3. The dark color of *Alcea rosea* nigra flowers is attributed to the presence of anthocyanins and are also reported to have significant pharmacological importance (Hosaka *et al.*, 2012; W.-Y. Huang *et al.*, 2014; W. Huang *et al.*, 2013).

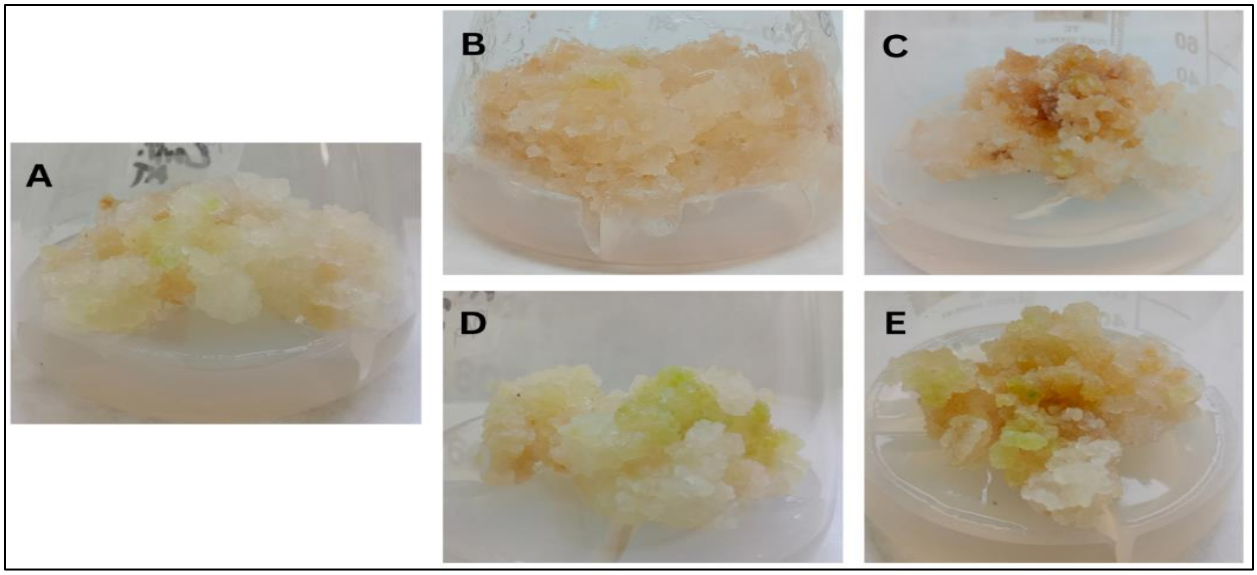

Figure 4-1 Callus morphology of *Alcea rosea* callus cultures

The letters represent different concentrations of UV-C exposure and Melatonin. **(A)** Control **(B)** UV-C exposure at 30 mins **(C)** UV-C exposure at 70 mins **(D)** Melatonin (3 mg/L) **(E)** Melatonin (25 mg/L)

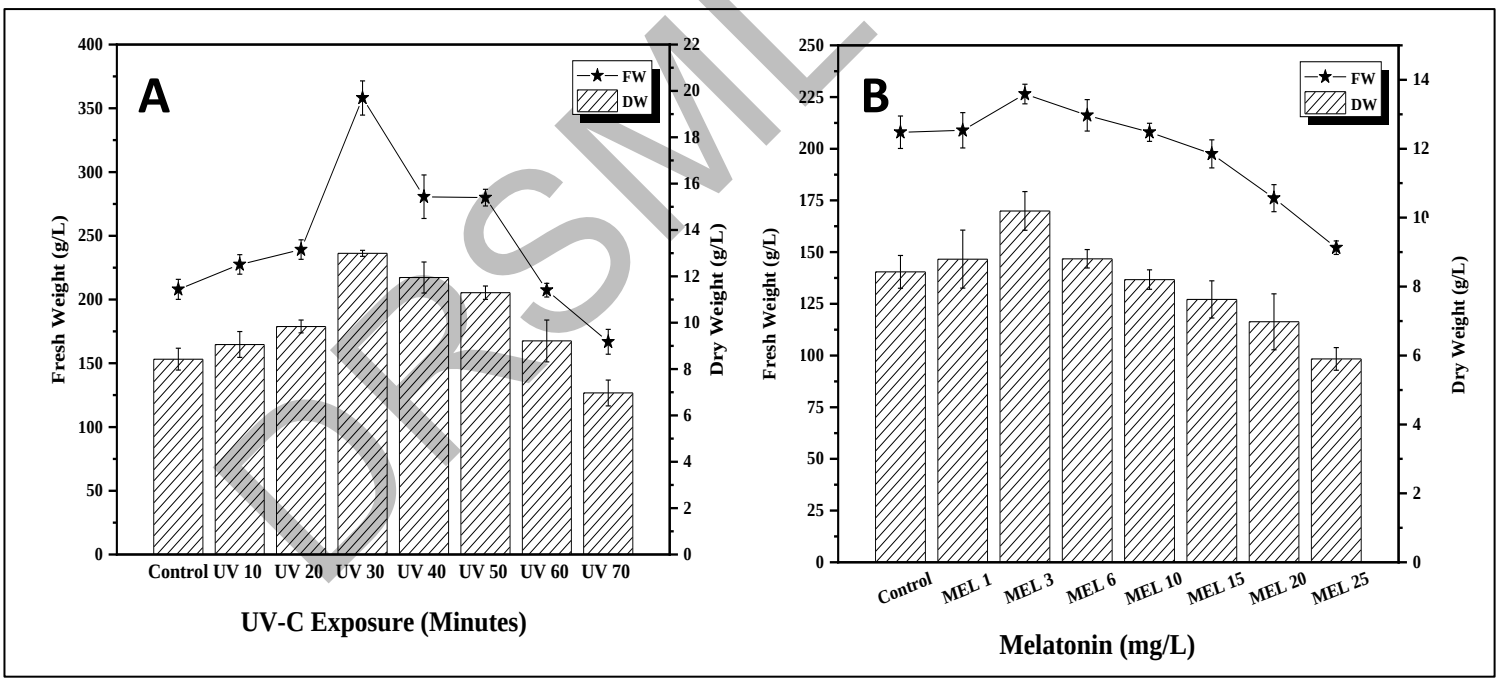

Figure 4-2 Biomass accumulation of *Alcea rosea* callus cultures

(A) FW and DW under UV-C exposure, **(B)** FW and DW under exogenous melatonin stress. The values are mean of triplicates with the standard deviation applied.

4.3 Evaluation antioxidant and anti-aging potential of *Alcea rosea* callus extracts

The antioxidant potential of callus cultures of *Alcea rosea* under different elicitors (UV-C and Melatonin) was analyzed by utilizing both the in vitro and in vivo antioxidant assays. Invitro assays were based upon the hydrogen atom transfer (ORAC) and electron transfer (CUPRAC) mechanism of antioxidant action. The in vivo cellular antioxidant activity (CAA) of the callus extracts was evaluated to determine their aptitude to inhibit reactive oxygen species (ROS) and reactive nitrogen species (RNS). The obtained results of these assays are shown in Figure 4.4, and it was observed that the hollyhock callus extracts exhibited significant antioxidant action. Extract from callus (exposed to UV-C 30 mins exposure) displayed highest antioxidant activity for ORAC and CUPRAC assays with values of 960.57 and 556.23 µM TEAC, respectively. Control showed maximum CAA activity of 78.18 % (ROS/RNS Inhibition) which was followed closely by UV-C (30 mins exposure) with a value of 78.08 % (ROS/RNS Inhibition). In contrast, the lowest antioxidant activity was observed in callus exposed to melatonin (1 mg/L) with µM TEAC values of 332.78 and 200.85 for ORAC and CUPRAC respectively. Meanwhile, the lowest CAA activity of 47.93% (ROS/RNS Inhibition) was observed at UV-C (20 mins exposure). HAT-based assay (ORAC) showed higher antioxidant potential than ET-based assay (CUPRAC) in all the callus extracts indicating the involvement of at least one anthocyanin's involvement in HAT-based antioxidant mechanism.

The next step of investigation into the anti-aging potential of hollyhock involved the assessing the anti-aging potential of callus extracts. At an established concentration of 50 µg/mL, the in-vitro inhibitory capacities of elastase, tyrosinase, and AGEs (advanced glycation end products) were investigated.

Elastase is an enzyme that participates in the degradation of extracellular matrix components in the dermis. This degradation process results in alterations to the skin, such as the formation of deep wrinkles, decrease in skin tone and elasticity (Boran, 2018; Coricovac *et al.*, 2015; Liyanaarachchi *et al.*, 2018). Age-related tyrosinase dysfunctions become more noticeable with ageing and can therefore cause cutaneous melanoma on top of pigmentation disorders like freckles or melisma (Briganti *et al.*, 2003). Age-related illnesses and oxidative stress have been linked, and this could cause an accumulation of AGEs (Finkel *et al.*, 2000; Gkogkolou *et al.*, 2012). Hence, compounds having the potential to inhibit these enzymatic processes or activities develop interest from cosmetic industry.

The results are represented in table 4.2. The values are expressed as percentages of relative activities. The maximum amount of Tyrosinase inhibition activity (53.18%) was observed in callus

exposed to melatonin (3 mg/L) with the control (NAA+TDZ) showing 31.88% inhibition, whereas the least amount of inhibition activity was observed in callus extract exposed to UV-C exposure (50 mins) with a meager value of 12.31% inhibition. Moreover, the Elastase inhibition activity showed 46.34% inhibitory activity at control with the maximum amount of activity reaching 73.07% value in callus extract exposed to melatonin (25 mg/L). Similarly, the minimum inhibitory activity was also observed in callus exposed to melatonin (1 mg/L) with a value of 13.87%. Two types of AGEs (vesperlysine-like AGEs and pentosidine-like AGEs) inhibitory activities were also evaluated. In comparison, pentosidine-like AGEs showed greater inhibitory activity in both, calli exposed to UV-C and calli exposed to melatonin, with the maximum inhibitory percentage of 70.2 was reported at melatonin (3 mg/L) which was a significant activity when compared with the minimum percentage inhibitory activity of 18.70 observed in callus under UV stress (20 mins). For vesperlysine-like AGEs, minimum activity (14.93%) was observed in callus exposed to UV stress (50 mins). In contrast, the maximum (36.8%) inhibitory percentage was exhibited melatonin (25 mg/L) concentration.

Tyrosine inhibitory activity and Elastase inhibitory activity of 53.18% and 73.07% was achieved respectively, which signifies the possible potential use of *Alcea rosea* callus extracts in the cosmetic industry for their role in reducing skin pigmentation and retaining skin elasticity in old age. Significant AGEs inhibitory activity also shed some light onto the role of *Alcea rosea* callus extracts in their possible use as elevating some effects of ageing or their possible role in prevention of disease cause by AGEs.

Table 4-1 Quantification of anthocyanins in callus cultures of *Alcea rosea* through HPLC

Elicitors		Phytochemicals (µg/g DW)									
		Delphinidin 3-O-glucoside	Delphinidin 3-O-rutinoside	Cyanidin 3-O-glucoside	Cyanidin 3-O-rutinoside	Petunidin 3-O-glucoside	Petunidin 3-O-rhamnosylglucoside	Malvidin 3-O-glucoside	Malvidin 3-O-rhamnosylglucoside	Malvidin 3-O-malonylglucoside	Total Anthocyanins
UV-C (Minutes)	Control	0.95 ± 0.05	0.90 ± 0.03	1.25 ± 0.04	0.42 ± 0.02	0.83 ± 0.05	0.95 ± 0.06	0.18 ± 0.001	0.26 ± 0.01	1.72 ± 0.09	7.47 ± 0.15
	UV 10	1.36 ± 0.06	1.72 ± 0.09	1.54 ± 0.08	0.76 ± 0.04	1.12 ± 0.03	1.38 ± 0.08	0.25 ± 0.005	0.35 ± 0.03	3.00 ± 0.2	11.49 ± 0.15
	UV 20	1.28 ± 0.04	1.25 ± 0.04	1.64 ± 0.04	0.53 ± 0.02	1.19 ± 0.01	1.50 ± 0.1	0.23 ± 0.001	0.33 ± 0.01	2.31 ± 0.16	10.26 ± 0.11
	UV 30	0.90 ± 0.07	0.87 ± 0.05	1.45 ± 0.06	0.38 ± 0.001	0.78 ± 0.04	0.87 ± 0.02	0.32 ± 0.03	0.22 ± 0.005	1.67 ± 0.06	7.45 ± 0.17
	UV 40	1.76 ± 0.06	1.61 ± 0.08	2.27 ± 0.1	0.74 ± 0.03	1.75 ± 0.1	1.69 ± 0.07	0.26 ± 0.005	0.33 ± 0.03	3.26 ± 0.2	13.67 ± 0.2
	UV 50	1.39 ± 0.05	1.35 ± 0.067	1.88 ± 0.09	0.65 ± 0.04	1.22 ± 0.06	1.39 ± 0.03	0.27 ± 0.01	0.34 ± 0.02	2.73 ± 0.1	11.22 ± 0.10
	UV 60	1.09 ± 0.03	0.97 ± 0.05	1.36 ± 0.05	0.42 ± 0.01	0.99 ± 0.02	1.28 ± 0.01	0.98 ± 0.06	0.23 ± 0.005	1.78 ± 0.08	9.08 ± 0.13
	UV 70	1.36 ± 0.05	1.23 ± 0.03	1.66 ± 0.04	0.57 ± 0.02	1.17 ± 0.09	1.31 ± 0.06	0.25 ± 0.003	0.33 ± 0.01	2.51 ± 0.2	10.40 ± 0.2
Melatonin (mg/L)	Mel 1	0.88 ± 0.04	0.76 ± 0.06	1.24 ± 0.06	0.35 ± 0.01	0.78 ± 0.04	0.81 ± 0.03	0.15 ± 0.001	0.26 ± 0.03	1.55 ± 0.05	6.78 ± 0.09
	Mel 3	1.04 ± 0.03	0.97 ± 0.04	1.29 ± 0.9	0.41 ± 0.02	0.90 ± 0.02	1.29 ± 0.04	0.19 ± 0.005	0.27 ± 0.004	1.99 ± 0.05	8.35 ± 0.11
	Mel 6	1.96 ± 0.08	1.78 ± 0.09	2.39 ± 0.13	0.83 ± 0.02	1.68 ± 0.07	1.84 ± 0.05	0.36 ± 0.02	0.48 ± 0.05	3.63 ± 0.14	14.95 ± 0.16
	Mel 10	1.08 ± 0.06	0.93 ± 0.06	1.38 ± 0.1	0.48 ± 0.01	0.93 ± 0.05	1.05 ± 0.03	0.30 ± 0.009	0.27 ± 0.005	2.52 ± 0.1	8.94 ± 0.08
	Mel 15	1.92 ± 0.09	1.80 ± 0.07	2.48 ± 0.16	0.83 ± 0.06	1.79 ± 0.06	1.96 ± 0.05	0.34 ± 0.01	0.49 ± 0.03	3.70 ± 0.15	15.32 ± 0.4
	Mel 20	1.35 ± 0.05	1.14 ± 0.06	1.67 ± 0.09	0.56 ± 0.01	1.34 ± 0.05	1.26 ± 0.04	0.22 ± 0.003	0.36 ± 0.003	2.50 ± 0.06	10.40 ± 0.25
	Mel 25	2.07 ± 0.1	1.88 ± 0.08	2.57 ± 0.15	0.85 ± 0.02	1.79 ± 0.08	1.99 ± 0.06	0.39 ± 0.0	0.51 ±0.02	3.89 ± 0.09	15.92 ± 0.16

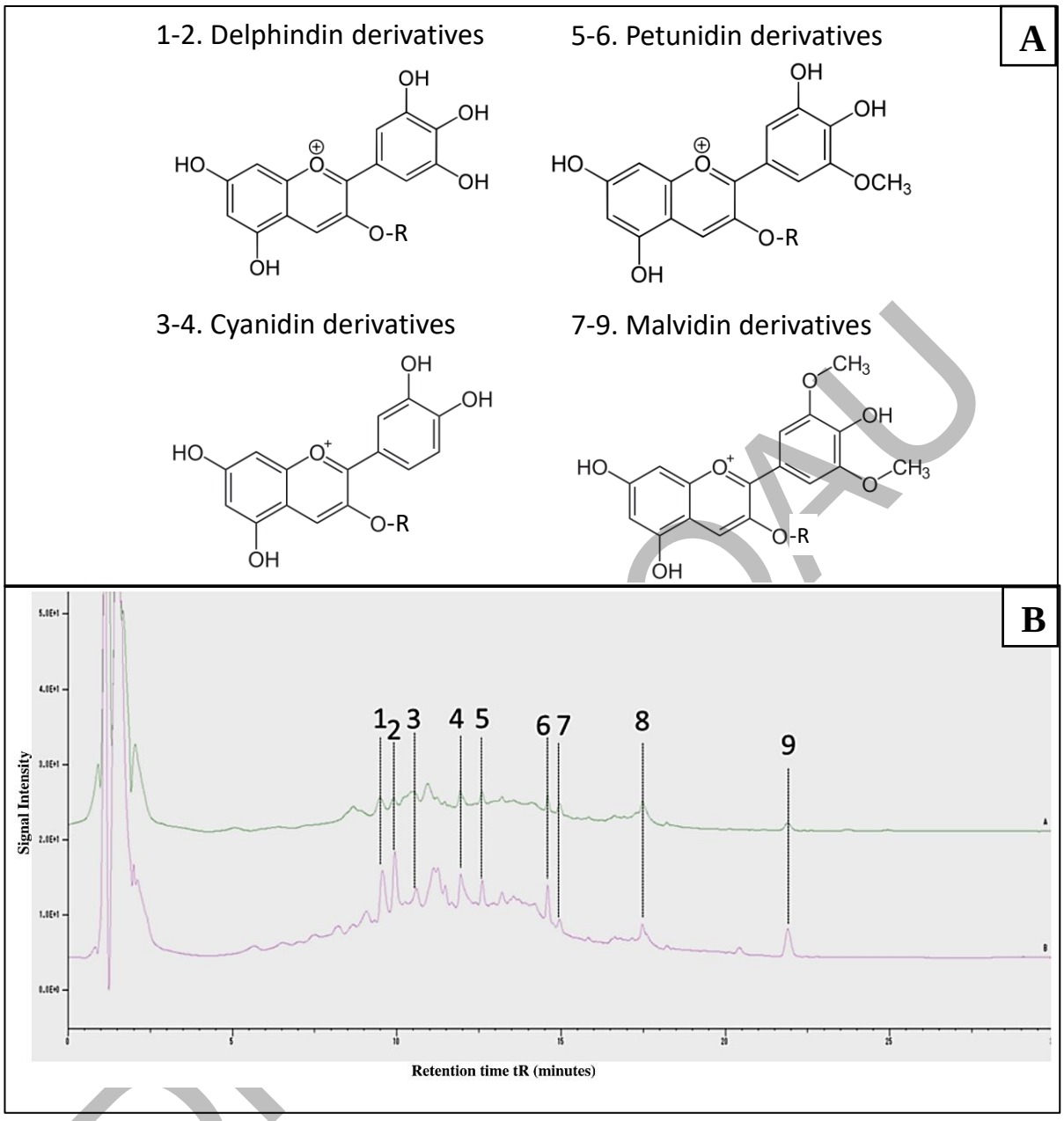

Figure 4-3 Chemical Structure and Chromatogram of *Alcea rosea*

Figure **(A)** chemical structural illustrations of anthocyanins accumulated in callus extracts of *Alcea rosea*, Delphinidin derivatives (**1-2**), Cyanidin derivatives (**3-4**), Petunidin derivatives (**5-6**) and Malvidin derivatives (**7-9**); **(B)** Typical HPLC chromatogram for *rich (pink)* and *poor (green)* anthocyanins accumulation in callus extracts of *Alcea rosea* (recorded at 530 nm).

Table 4-2 Anti-aging activities of *Alcea rosea* callus cultures

Activities of callus extracts of *Alcea rosea* represented as percentage activities, in which DMSO was utilized as control, under the influence of UV-C exposure and exogenous melatonin.

Elicitor		Anti-Aging Activities			
		Tyrosinase (% inhibition)	**Elastase (% inhibition)**	**Vesperlysine-like AGEs (%inhibition)**	**pentosidine-like AGEs (%inhibition)**
UV-C (Minutes)	**Control**	31.88 ± 1.59	46.34 ± 2.3	33.95 ± 1.5	29.01 ± 1.1
	UV 10	22.97 ± 1.2	34.73 ± 1.9	23.01 ± 1.0	20.55 ± 1.4
	UV 20	17.08 ± 0.8	23.23 ± 1.7	19.35 ± 1.2	18.70 ± 0.8
	UV 30	45.81 ± 1.9	30.71 ± 1.0	33.25 ± 1.5	57.03 ± 3.1
	UV 40	26.55 ± 1.1	37.49 ± 1.4	23.27 ± 1.0	30.06 ± 1.4
	UV 50	12.31 ± 0.5	20.92 ± 1.5	14.93 ± 0.4	20.82 ± 1.5
	UV 60	23.46 ± 1.0	29.14 ± 1.0	23.26 ± 1.0	31.11 ± 1.1
	UV 70	19.33 ± 0.6	32.91 ± 1.3	25.35 ± 1.1	22.64 ± 1.3
Melatonin (mg/L)	**Mel 1**	13.28 ± 0.4	13.87 ± 0.8	16.9 ± 0.2	21.8 ± 0.9
	Mel 3	53.18 ± 2.3	37.50 ± 1.5	36.5 ± 1.9	70.2 ± 2.5
	Mel 6	19.43 ± 0.7	43.46 ± 2.0	23.2 ± 1.2	21.8 ± 0.2
	Mel 10	37.51 ± 1.8	32.11 ± 1.8	24.8 ± 1.5	42.0 ± 1.6
	Mel 15	23.08 ± 1.1	43.17 ± 2.1	22.8 ± 1.0	25.2 ± 1.5
	Mel 20	49.57 ± 2.3	41.00 ± 1.4	31.7 ± 1.8	57.1 ± 2.1
	Mel 25	44.65 ± 2.0	73.70 ± 3.8	36.8 ± 1.7	35.4 ± 1.1

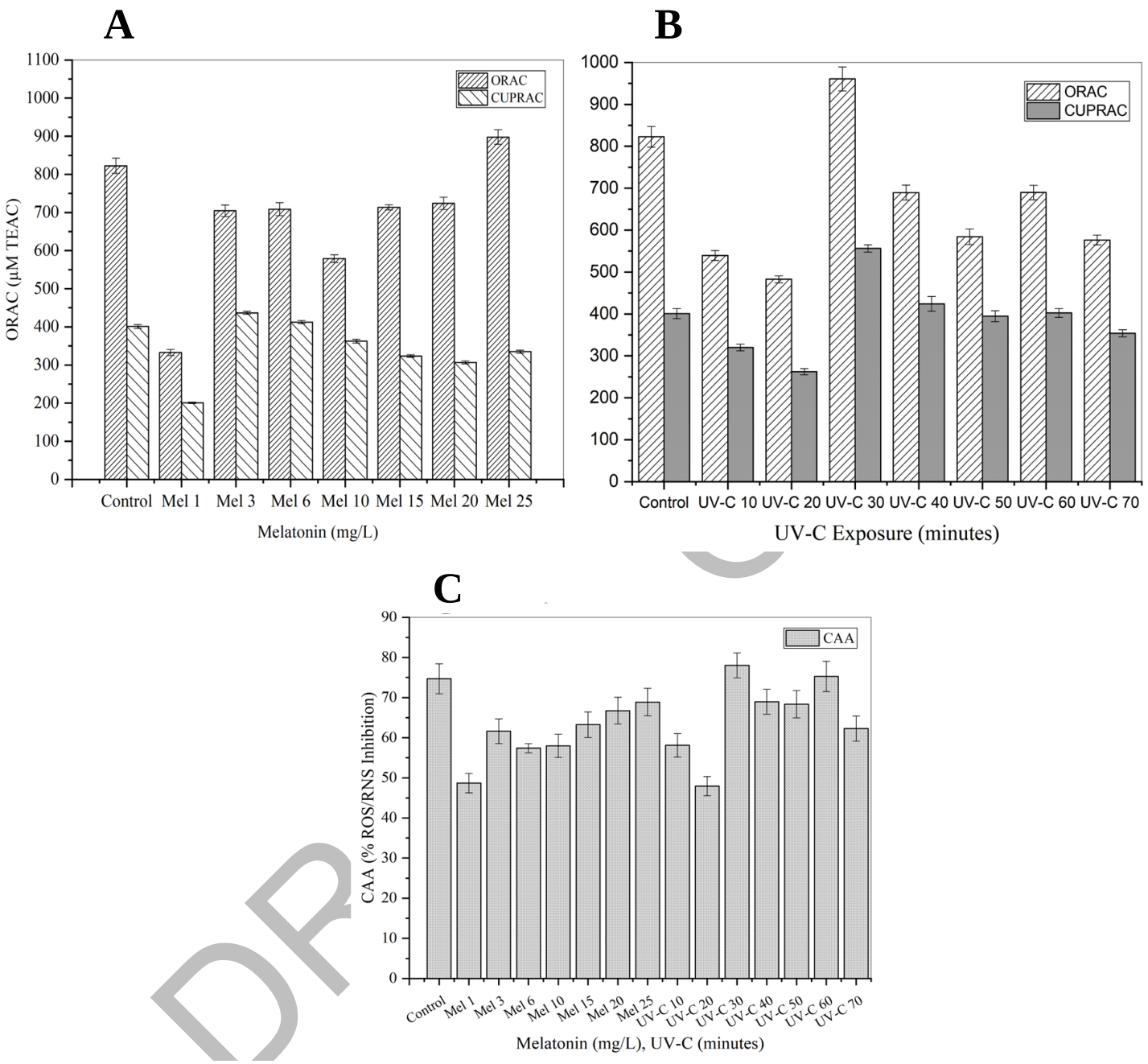

Figure 4-4 Antioxidant activities of *Alcea rosea* callus extract

Antioxidant activities of callus cultures of *Alcea rosea* under UV-C and melatonin stress. The values are means of triplicates. TEAC (Trolox Equivalent Antioxidant Capacity µM). **(A)** and **(B)** represents the antioxidant activity ORAC and CUPRAC; **(C)** is representative of CAA antioxidant assay.

4.4 Conclusions

This study evaluated the effects of UV-C and melatonin as an elicitor for *Alcea rosea* callus cultures on the biomass accretion and accumulation of bioactive compounds. Amidst the various concentrations of elicitors (melatonin and UV-C) the optimum values of FW and DW were obtained in callus cultures under UV-C (30 mins) stress, 359.16 g/L and 12.99 g/L respectively). HAT-based assay (ORAC) showed higher antioxidant potential than ET-based assay (CUPRAC) in all the callus extracts, which was indicative of the involvement of at least one phytochemical in the HAT-based antioxidant mechanism. A marked increase in antioxidant activities was observed signifying that the callus extracts of *Alcea rosea* can serve as an agent for alleviating antioxidant stress response effectively. CAA (% ROS/RNS inhibition) assay showed maximum activity at control (78.18%) which was succeeded by callus under UV-C exposure (30 mins) with an inhibition percentile of 78.08. Prominent anti-aging activity assays of tyrosinase, elastase and AGEs are representative of the possible potential use of *Alcea rosea* callus cultures in the commercial cosmetic industry for a range of applications involving hyperpigmentation, maintaining skin elasticity, and preventing effects of old age by inhibiting age-related reactive oxygen/nitrogen species. However, further study into the genes involved in the expression and production of anthocyanins, detected in current study, responsible for antioxidant and anti-aging activities is required to better incorporate *Alcea rosea* into the cosmetic industry.

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