Effect of Melatonin on the Biomass and Secondary Metabolite Production in Adventitious Root Culture of *Macrotyloma uniflorum*

By

Zenab Syed

Registration No: 02272111019

Department of Biotechnology

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad Pakistan

2023

DRSML QAU

Effect of Melatonin on the Biomass and Secondary Metabolite Production in Adventitious Root culture of *Macrotyloma uniflorum*

Researcher

Zenab Syed

Supervised by

PROF. Dr. BILAL HAIDER ABBASI

Thesis submitted to

The Department of Biotechnology

Quaid-i-Azam University, Islamabad

In the partial fulfillment of the requirements for the degree of

Master of Philosophy in Biotechnology

Department of Biotechnology

Faculty of Biological Sciences

Quaid-i-Azam University,

Islamabad, Pakistan

2023

DRSML QAU

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

DECLARATION OF ORIGINALITY

I hereby declare that the work "**Effect of Melatonin on the biomass and secondary metabolite production in adventitious root culture of *Macrotyloma uniflorum***" accomplished in this thesis is the result of my own research carried out in Plant cell culture Laboratory, Department of Biotechnology, Quaid-i-Azam University, Islamabad, Pakistan. This thesis has not been published previously nor does it contain any material from the published resources that can be considered as a violation of international copyright law. Furthermore, I also declare that I am aware of the term "copy right" and plagiarism. If, any copyright violation is found in this research work I will be responsible for the consequence of any such violation.

Signature: _________

Name: **Zenab Syed**

Date: ____________

DRSML QAU

Dedication

Every challenging work needs self-effort as well as guidance of elders especially those who are very close to our heart.

I dedicate my effort to my loving ***Father,***

my gorgeous ***Mother****, my amazing* ***Husband*** *and my*

supportive ***Siblings.***

Whose affection, love, encouragement and prayers made me able to achieve this honor.

Along the very hardworking and respected ***Supervisor.***

DRSML QAU

Table of contents

Acknowledgements ………………………………………………………………... **i**

List of Figures ………………………………………………………………... **ii**

List of Tables ……………………………………………………………… **iii**

Abbreviations ………………………………………………………………... **iv**

Abstract ……………………………………………………………………..... **v**

1. **Introduction** ……………………………………………………………… **1-2**
   1.2. Aims and objectives …………………………………………………….. 3-4
2. **Literature review** …………………………………………………………… **5**
   2.1. Botanical description of M. uniflorum ……………………………………….. 6
   2.2. Taxonomic Classification of *M. uniflorum* …………………………………... 7
   2.3. Common Names of *M. uniflorum* …………………………………………….. 8
   2.4. Geographical Distribution of *M. uniflorum* …………………………………... 9
   2.5. Cultivation and Ecology of *M. uniflorum* …………………………………….. 9
   2.6. Nutraceutical properties of M. uniflorum …………………………………… 10
   2.7. Traditional usage of M. uniflorum …………………………………………… 10
   2.8. Phytochemical Constituents of M. uniflorum ………………………………… 11
   2.9. Medicinal importance of *M. uniflorum* ……………………………………….. 13
   2.9.1 Hepatoprotective activity ………………………………………………..... 13
   2.9.2 Anti-microbial activity……………………………………………………….. 13
   2.9.3 Anti-HIV Activity…………………………………………………………… 14
   2.9.4 Anticarcinogenic activity…………………………………………………… 14
   2.9.5 Anthelmintic activity……………………………………………………….. 14
   2.9.6 Anti-inflammatory activity………………………………………………….. 15
   2.9.7 Anti-depressant activity……………………………………………………...16
   2.9.8 Anti-diabetic activity………………………………………………………16
   2.9.9 Anti-aging and skin photoprotective activity……………………………...16
   2.9.10 Anti-calcifying activity…………………………………………………17
   2.10 Importance of Plant tissue culture (PTC)…………………………………...17
   2.10.1 Application of Plant tissue culture techniques……………………………..18
   2.11 Micropropagation…………………………………………………………......18
   2.12 Callus culture………………………………………………………………...…18
   2.13 In vitro tissue culture of *M. uniflorum*……………………………………….19
   2.14 Elicitation…………………………………………………………………….19
   2.15 Melatonin as an elicitor……………………………………………………….20
3. **Materials and methods……………………………………………………21-27**
4. **Results and discussions…………………………………………………28-41**
5. **Conclusions………………………………………………………………41-42**
6. **References……………………………………………………………..43-50**

DRSML QAU

ACKNOWLEDGEMENTS

Millions of thanks to **Allah Almighty**, the Most Generous and the Most Merciful, who bestowed upon us the capacity to complete this study project as well as the health and ability to pursue knowledge and investigate some of the many aspects of his creation. Numerous mercies upon the **Holy Prophet Muhammad (S.A.W).**

I owe a great deal of gratitude and debt to my supervisor, **Prof. Dr. Bilal Haider Abbasi**, Professor of the Department of Biotechnology at Quaid-i-Azam University in Islamabad, for all his support, encouragement, and direction during this research project. Sincere gratitude to every one of the department's renowned teachers.

I would like to extend my heartfelt gratitude to my dear friends (**Hira Khan, Shazma Khan, Syed Ahsan Shahid, Amen Jehangir and Arslan Ali Toor**) for their time, constant support, and encouragement throughout my studies. Additionally, I would like to express my deep feelings of respect to my lab fellows (**Fazal Rehman, Nawal Abbasi, Sannia Batool, Aneeza Rafiq, Zain Asif, Razia Khurshid, Gouhar Zaman**) for their aspiring guidance and valuable suggestions during my laboratory experiments.

I owe my adored parents and siblings a great deal for this thesis. This thesis would not have been completed on time without their assistance, ongoing support, words of inspiration, advice, and guidance during every single day of my life.

May Allah bless every one of us with happiness and success for eternity! Ameen

DRSML QAU

List of Figures

S.no	FIGURES	PAGE NO
2.1	Various parts of *Macrotyloma uniflorum* (a) mature seeds (b) leaf shape (c) flower structure	**6,7**
2.2	Molecular structure of important phytochemicals of *M. uniflorum*	**12**
4.1	Callus morphology of Macrotyloma uniflorum **(A)** Control **(B)** Melatonin 0.2 mg/L **(C)** Melatonin 0.4 mg/L **(D)** Melatonin 0.6 mg/L **(E)** Melatonin 0.8 mg/L **(F)** Melatonin 1.0 mg/L **(G)** Melatonin 1.2 mg/L	**32**
4.2	Fresh callus biomass from root explant in response to different Melatonin concentrations	**33**
4.3	Dry callus biomass from root explant in response to different Melatonin concentrations	**34**
4.4	ORAC assay to estimate antioxidant activities in callus culture treated with various melatonin concentrations	**38**
4.5	CUPRAC assay to estimate antioxidant activities in callus culture treated with various melatonin concentrations.	**39**
4.6	FRAP assay to estimate antioxidant activities in callus culture treated with various melatonin concentrations.	**40**
4.7	CAA assay to estimate antioxidant activities in callus culture treated with various melatonin concentrations.	**41**
4.8	Antioxidant activities combined	**42**

DRSML QAU

List of Tables

S.NO	TABLES	PAGE NO
2.1	Taxonomic Classification of *M. uniflorum*	**8**
2.2	Phytochemical constituents of *M. uniflorum*	**12**
4.1	Effect of different melatonin concentrations on biosynthesis of polyphenolic metabolites in callus culture of *M. uniflorum*.	**36**

DRSML QAU

Abbreviations

FW	Fresh weight
DW	Dry weight
g/L	Gram per liter
µg/g DW	Microgram per gram dry weight
mg/L	Milligram per liter
g/L	Gram per liter
NAA	α-naphthalene acetic acid
MS	Murashige and Skoog
HPLC	High performance liquid chromatography
PTC	Plant tissue culture
ANOVA	Analysis of variance
TEAC	Trolox c equivalent capacity
ORAC	Oxygen radical absorbance capacity
CUPRAC	Cupric ion reducing antioxidant capacity
CAA	Cellular antioxidant activity
FRAP	Ferric ion reducing antioxidant power

DRSML QAU

Abstract

The underutilized pulse crop *Macrotyloma uniflorum*, also known as "Horse gram," has a high nutritional value and a variety of pharmacological effects. There have been no in vitro studies on the enhancement of the production of biomass and phytochemicals from adventitious root derived callus culture of *M. uniflorum*. Using adventitious roots as an explant cultured on MS media enriched with melatonin at varied concentrations, we have developed a workable strategy for production of biomass and pharmacologically important secondary metabolites in the study described here. Among all the various concentrations of melatonin the maximum biomass was obtained at the concentration of 0.6 mg/L of melatonin. The maximum biomass recorded was (FW = 370.25 ± 7.4 g/L, DW = 29.62 ± 0.88). While the maximum production of the phytochemicals were seen at a concentration of 1.0 mg/L of melatonin which means that there was a negative correlation between the production of the biomass and the enhancement of production of phytochemicals hence the study becomes a growth non associated study. The HPLC analysis confirmed the maximum accumulation of phytochemicals such as Gallic acid (9.24 ± 1.01 mg/g DW), Catechin (1.36 ± 0.01 mg/g DW), Caffeic acid (2.74 ± 0.07 mg/g DW), Epicatechin (2.45 ± 0.09 mg/g DW), Rutin (1.75 ± 0.05 mg/g DW), Myricetin (6.77 ± 1.03 mg/g DW), Daidzein (5.31 ± 1.07 mg/g DW), Genistein (4.11 ± 0.06 mg/g DW), Kaempferol (4.88 ± 0.11 mg/g DW). The antioxidant activities (in-vitro and in-vivo) showed highest levels at 1.0 mg/L of melatonin like in the case of production of phytochemicals. So, we can say that there is a positive correlation of the phytochemicals with the antioxidant potential. Therefore it can be said that these phytochemicals are contributing directly to the antioxidant activities of the callus. The maximum levels of antioxidant activities are ORAC = 567.52 ± 4.1 (µM TEAC), CUPRAC = 357.85 ± 0.62 (µM TEAC), FRAP = 431.12 ± 1.33 (µM TEAC) and CAA = 58.88%. These results show that biotic elicitor that is melatonin in this case has the potential to increase the biomass production of adventitious root culture of Macrotyloma uniflorum. And it is also useful in increasing the production of phytochemicals which are contributing to the antioxidant potential of the callus. Therefore, in light of the fact that melatonin effectively influences the development of medicinally significant phytochemicals, it can be inferred that M. uniflorum can play a key role in therapeutics due to the rise in the production of phytochemicals that directly impact antioxidant activities.

DRSML QAU

Chapter 1

Introduction

DRSML QAU

1. Introduction

Medicinal plants have been the foundation of traditional medicine for ages and are thought to be a source of physiologically active chemicals with therapeutic potential. More than 80% of the population, according to the World Health Organization depends on conventional herbal remedies for some of their main healthcare needs. (Oyebode, Kandala, Chilton, & Lilford, 2016).

In order to achieve universal health coverage, primary healthcare is a crucial aspect of healthcare. Traditional medicine is extremely important in nations with a lack of access to basic healthcare. It is widely utilized and has the potential to broaden both primary health care and universal health coverage. (Kim, Kim, Shin, Jang, & Ko, 2020).

Modern medications to treat many viral diseases have been mostly developed using natural substances obtained from plants. Plants' primary and secondary metabolites are the main source of commercially significant items including food additives, scents, pesticides, herbal cosmetics, colours, flavours, pesticides, pharmaceuticals, essential oils, natural rubber, gum, waxes, and tannins (Samarth, Samarth, & Matsumoto, 2017).

Plants in the family Leguminosae (also known as the Fabaceae) are considered legumes because they produce seeds in pods. Throughout the world, food legumes are regarded as the second-most important crop for human nutrition, right behind cereals, and are an integral part of a healthy diet. They are readily available, inexpensive, and a great source of high-quality proteins (nearly twice the amount found in cereals), carbohydrates, lipids, fiber, and micronutrients. Legumes offer fresh protein sources and other essential minerals that are helpful for overcoming micronutrient imbalances in the poor population. Plant legumes can be utilised to treat a variety of illnesses, like diabetes, cancer, heart disease, and ageing, because of their high phenolic content such as flavonoids, procyanidins, and phenolic acids (Dinore, Patil, Dobhal, & Farooqui, 2022).

An underappreciated nutraceutical crop among legumes *is Macrotyloma uniflorum*, often known as horse gramme or Dolichos biflorus. It is called horse gram owing to its widespread growth for horse feeding. It is a herbaceous perennial plant. The term Macrotyloma, particularly relates to knobby features on the pods, is derived from the Greek words macros (huge), tylos (ring), and loma (edge). (Blumenthal & Staples, 1993).

DRSML QAU

This growing herb is skinny, straight, and twining, bearing cylindrical tomentose stems. Trifoliate leaves possess lengthy stipules and a petiole. Roots have one deeply penetrating system and are branching with rounded, smooth nodules. Short, sessile, and cream whitish with a purple mark, the flowers are borne in auxiliary racemes. Typically, every pod contains six to seven reddish-brown seeds. Large areas of tropical and subtropical climates all over the world are used for its cultivation (Bhartiya, Aditya, & Kant, 2015).

Macrotyloma legumes have high nutraceutical properties that support a healthy diet for consumers. In addition to its dietary fibre, it provides a rich source of macronutrients (calcium, potassium, magnesium, phosphorus, zinc, and iron) and micronutrients (carbohydrate, protein, and fibre). A wide variety of potential phytopharmaceutical constituents, such as flavonoids, alkaloids, cardiac glycosides, hormones, aldehydes, tannins, starches, saponins, sterols, coumarins, and procyanidins, were detected in the seeds, roots, and leaves of *M. uniflorum*, during the phytochemical profiling. Such compounds make a substantial contribution to the plant's defense mechanism and even have antioxidant potential. (Gautam, Katoch, & Chahota, 2020)

According to academic experiments, *M. uniflorum* contains a phytochemical that seems to have hepatoprotective characteristics and antibacterial properties against a number of bacterial pathogens (Kaundal, Sharma, Kumar, Kumar, & Kumar, 2019). *M. uniflorum* seed extract exhibited anti-depressant and anti-diabetic efficacy in several preclinical studies. (Gupta, Badole, Bodhankar, & Sabharwal, 2011). It plays a significant role in both conventional and modern medicine. This plant's phytoconstituents are incorporated in the development of a variety of herbal therapeutic formulations, among them the pharmaceutical LI10903F entitled LOWAT, that has been proven to effectively regulate weight and inhibit lipogenesis and fat deposition (Kaundal et al., 2019) . Another study (L Rufus Auxilia, Sundari, & Daniel, 2013) demonstrated that Dolichin A and Dolichin B generated from horse gram seeds prevent the HIV virus.

The phenolics and flavonoids compounds present in medicinal plants are basically low molecular weight secondary metabolites. These are present in abundant amount as compared to primary metabolites. They work as a protective mechanism against various reactive oxygen species created under stressful conditions. Several biotechnological advancements, such as culture optimization, metabolic engineering, precursor feeding, and elicitation, have been applied in plant tissue culture

DRSML QAU

for the large-scale generation of highly desired secondary metabolites in in vitro cell culture. Elicitors are the key modulators that trigger the accumulation of phytoalexins (Rai, Saito, & Yamazaki, 2017).

In plant in vitro culture, biotic and abiotic elicitors are utilized to elicit different responses. According to (Patel & Krishnamurthy, 2013), biotic elicitors are substances with a biological origin/generated within living organisms, either from pathogens or by the plant itself, whose capabilities are linked to the receptors and operate by stimulating or suppressing a number of enzymes or signaling molecules. Whereas Abiotic elicitors are substances having nonbiological source that falls under the divisions of physical, chemical, and hormonal elements.

A significant indoleamine derivative is Melatonin (N-acetyl-5-methoxytryptamine). It is a phytohormone. The anti-inflammatory, antioxidant, and autophagic effects of melatonin are impressive. Plants' ability to respond to biotic and abiotic stress is affected by their level of melatonin. (Tan et al., 2000) found that the melatonin quantity in black and white mustard seeds was higher than that of the melatonin concentration in vertebrate blood. Because plants lack mobility compared to vertebrates and cannot relocate to evade harsh conditions, it is considered that plants synthesize melatonin at proportionally much higher quantities as a compensatory reaction. Numerous research studies have been published lately that put a spotlight on melatonin's contribution to cell division and root growth as its impact on plant physiology is examined.

The purpose of the current research was to evaluate how melatonin impacted the growth of *M. uniflorum* root-derived callus cultures in terms of biomass accumulation, phytoconstituents production, and antioxidant capacity. To the highest of our knowledge, there is no scientific literature that looks into the effects of melatonin on callus cultures made from adventitious roots originating from *M. uniflorum.*

1.1 Aims and objectives.

The following are the key aims and objectives of the current academic research:

- To develop a reliable methodology for *M. uniflorum* callus induction in vitro for the long-term development of bioactive metabolites.

DRSML QAU

- To investigate the effects of a biotic elicitor, in this case melatonin, on the morphology, growth, and accumulation of secondary metabolites in callus.
- To analyze and contrast the antioxidant potentials ORAC, CUPRAC, CAA and FRAP in callus cultures that were elicited and those that weren't.
- To investigate the relationship between phenolic compounds and the antioxidant capability of Macrotyloma uniflorum root-derived callus culture.

DRSML QAU

Chapter 2
Literature review

DRSML QAU

2. Literature review

2.1 Botanical description of *Macrotyloma uniflorum*

Macrotyloma uniflorum, a species of the Fabaceae family and popularly referred as horse grams, is an underappreciated legume. *Dolichos biflorus* (Auct) or *Dolichos uniflorus* (Lam) are two synonyms for it. It is a branching, twining, succulent, annual plant.30-90 cm-tall ascending herb having s mall trifoliate leaves with a length of 3.5 to 7.5 cm and a width of 2-4 cm are seen. The base of the leaflets is rounded, oblong, and fimbrillate. Erect, branching, cylindrical, and slightly hairy stems are present. It is covered with large amount of whitish hairs. (Mehra & Upadhyaya, 2013).

The taproot of this plant, which contains rounded and smooth nodules, produces a deep penetrating root system. Flowers are axillary inflorescences that are zygomorphic, bisexual, and yellowish cream with a purple mark. A slender, oblong fruit pod that contains 5 to 8 seeds, is thickly hairy, and is curled towards the tip. Seeds have quite a compressed oval or spherical form with a dark lustrous reddish-brown coloration (Bhartiya et al., 2015).

DRAFT ML QAU

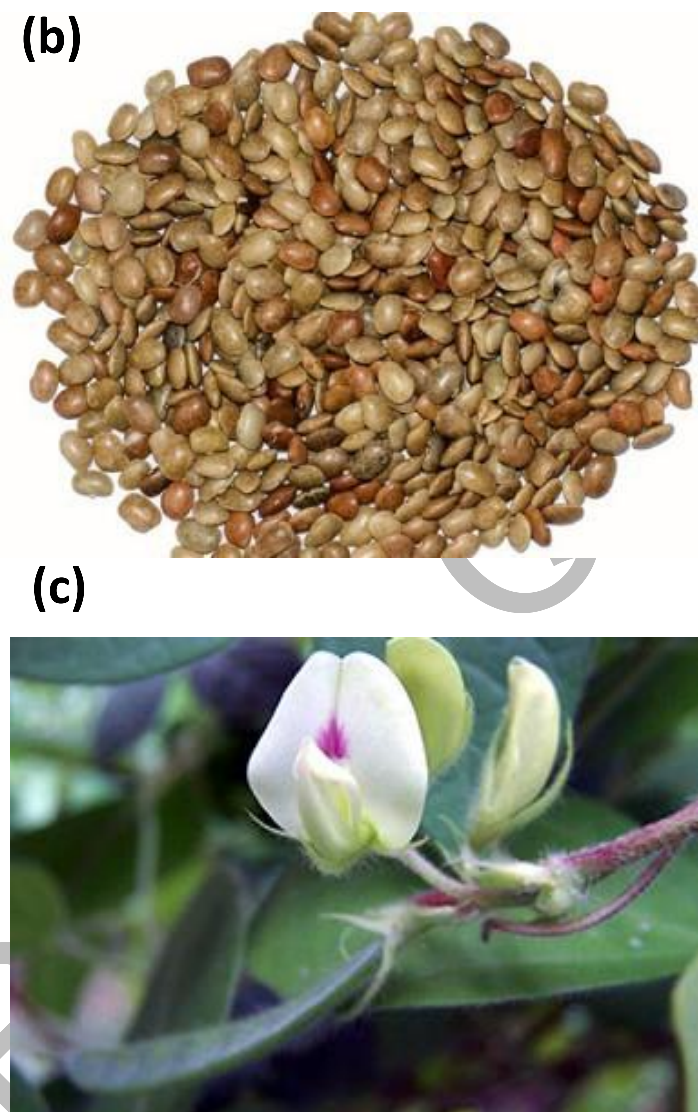


Figure 2.1 Various parts of Macrotyloma uniflorum.

(a) Leaf structure, (b) Mature seeds, (c) Flower shape.

Source: (a) https://www.indiamart.com/proddetail/dolichosbiflorus16093267462.html

(b) https://www.healthbenefitstimes.com/horse-gram/

(c) https://www.healthbenefitstimes.com/horse-gram/

DRAFT

2.2 Taxonomic Classification of *M. uniflorum*

Approximately 25 wild species make up the genus Macrotyloma, however only 9–15 of them are recognized in Asia. This plant is classified scientifically as follows (Ingle, Al-Khayri, Chakraborty, Narkhede, & Suprasanna, 2020).

Kingdom	Plantae
Class	Magnolyopsida (dicotyledons)
Subclass	Rosidae
Order	Fabales
Family	Fabaceae
Subfamily	Faboideae
Genus	Macrotyloma
Specie	Macrotyloma uniflorum

Table 2.1 Taxonomic Classification of *M. uniflorum*

DRSML QAU

2.3 Common Names of *M. uniflorum*

Here are some of the common names for this plant in many languages. This plant is recognized by a variety of alternative names around the world (Ingle et al., 2020).

➢ In Hindi and Urdu: Kulthi, Kulit

➢ In English: Horse gramme,

madaras gram, poor man pulse

➢ In Bengali: Kulthikalai

➢ In Tamil: Kollu, Kaanam

➢ In Telugu: Ulavalu or Guggillu

➢ In Kannada: Hurule,

➢ In Oriya: Kolatha

➢ In Gujrati: Kadthi Ni Dal

➢ In Sanskrit: Kulattha, Kulathika,

Sweta beej

➢ In Chinese: Bian Dou

➢ In Arabic: Habbul Kulth

2.4 Geographical Distribution of *M. uniflorum*

An herbaceous plant known as horse gram spreads throughout the subcontinent from South-East Asian to African nations. It is commonly grown in Australia, Burma, and other temperate and agricultural regions (Gupta et al., 2011). According to archaeobotanical research, horse gram is extensively dispersed around the planet, particularly in Asian and African nations like Nepal, China, India, and Pakistan (Patil & Kasturiba, 2019b).

2.5 Cultivation and Ecology of *M. uniflorum*

The annual legume *M. uniflorum* may thrive in more challenging environmental circumstances. Due to its tenacity and adaptation to changing environmental circumstances, it is native to tropical and subtropical regions of the world. It is cultivable in regions with yearly rainfall as low as 300–600 mm. Its cultivation requires a temperature of 20 to 30 °C and a pH of 5 to 7.5. This leguminous plant is an essential crop for famine and arid areas since it can endure drought stress and harsher weather conditions. It can thrive in soil with little organic matter and little nitrogen because of its flexibility. It can be found up to 1800 m above sea level (Patil & Kasturiba, 2019a).

DRSML QAU

2.6 Nutraceutical properties of M. uniflorum

The high nutraceutical properties of *M. uniflorum* are very helpful in preserving human health. For the first time, (Bharathi & Anand, 2016) used LC-MS to study the chemicals found in M. uniflorum and investigate their potential therapeutic and nutraceutical benefits for human health.

This legume has been documented as a treasure house of secondary metabolites like polyphenols, flavonoids, isoflavones, proteins, and its peptides, saponins, and lignans. These phytochemicals are responsible for exhibiting strong antioxidant, anti-inflammatory, anticarcinogenic, and free radical scavenging properties. It is a nutrient-dense legume as compared to other grown legumes that can be used as food supplements (Kouris-Blazos & Belski, 2016). It is a rich source of micro and macro nutrients containing high content of protein (18–29%), carbohydrate (57.2%), minerals (3.2%), dietary fiber (36.4%) and vitamins like thiamine (B1) (0.42 mg/100g), riboflavin (B2) (0.09 mg/100g), niacin (B3) (1.5 mg/100g) and vitamin C (1.0 mg/100 g) calcium (1.01 mg/g), magnesium (0.40 - 1.90 mg/g), iron (0.06 - 1.79 mg/g) and phosphorus (0.13 - 4.20 mg/g), zinc (0.02 - 0.07 mg/g), Manganese (0.09 - 8.21 mg/g) (Bhartiya et al., 2015).

2.7 Traditional usage of *M. uniflorum*

It is grown mainly to furnish feed and fodder for cattle and especially a horse. It makes excellent hay containing 16.2% protein and 1.8% fat. In some parts of India, it is cultivated as folklore medicine and eaten as a green leafy vegetable, because of the presence of relatively high mineral and anthocyanin content in leaves as compared to other common vegetables (Mandle, Salunke, Gaikwad, Dande, & Patil, 2012). *M. uniflorum* is a traditional pulse for people of the Indian Himalayan region, where it is consumes as whole, boiled, splits, dehulled, canned, grounded flour, and roasted (Vandarkuzhali & Narayanasamy, 2015). The whole horse gram seeds are used to make gravies and served with rice. It is a traditional dish in Uttarakhand and is used in various ethnic recipes like curry and papad. Horse gram seeds are infused with cow’s milk that is advantageous for the treatment of helminths disorders. People consume horse gram seed powder with curd to inhibit gastric ulcers. A decoction of the *M. uniflorum* root is used for treating leucorrhea and horse gram plant juice found to be curative against diarrhea (Kaundal & Kumar, 2020). During winter season horse gram are also used to keep the body warm and relieving cold and cough. Traditionally horse gram seeds paste is usually applied on skin for the treatment of skin

DRSML QAU

rashes and burn (Dulal, 2018). It can also be used for making fermented food products. A Processed horse gram flours are also used in making commercial products like soups and snacks and, confectionery, and several other bakery products (Thirukkumar & Sindumathi, 2014). The production and market chain of these underutilized legumes becomes dominating, as it performs an essential role in therapeutic food preparation and eliminating malnutrition in masses of developing countries (Gautam et al., 2020).

2.8 Phytochemical Constituents of *M. uniflorum*

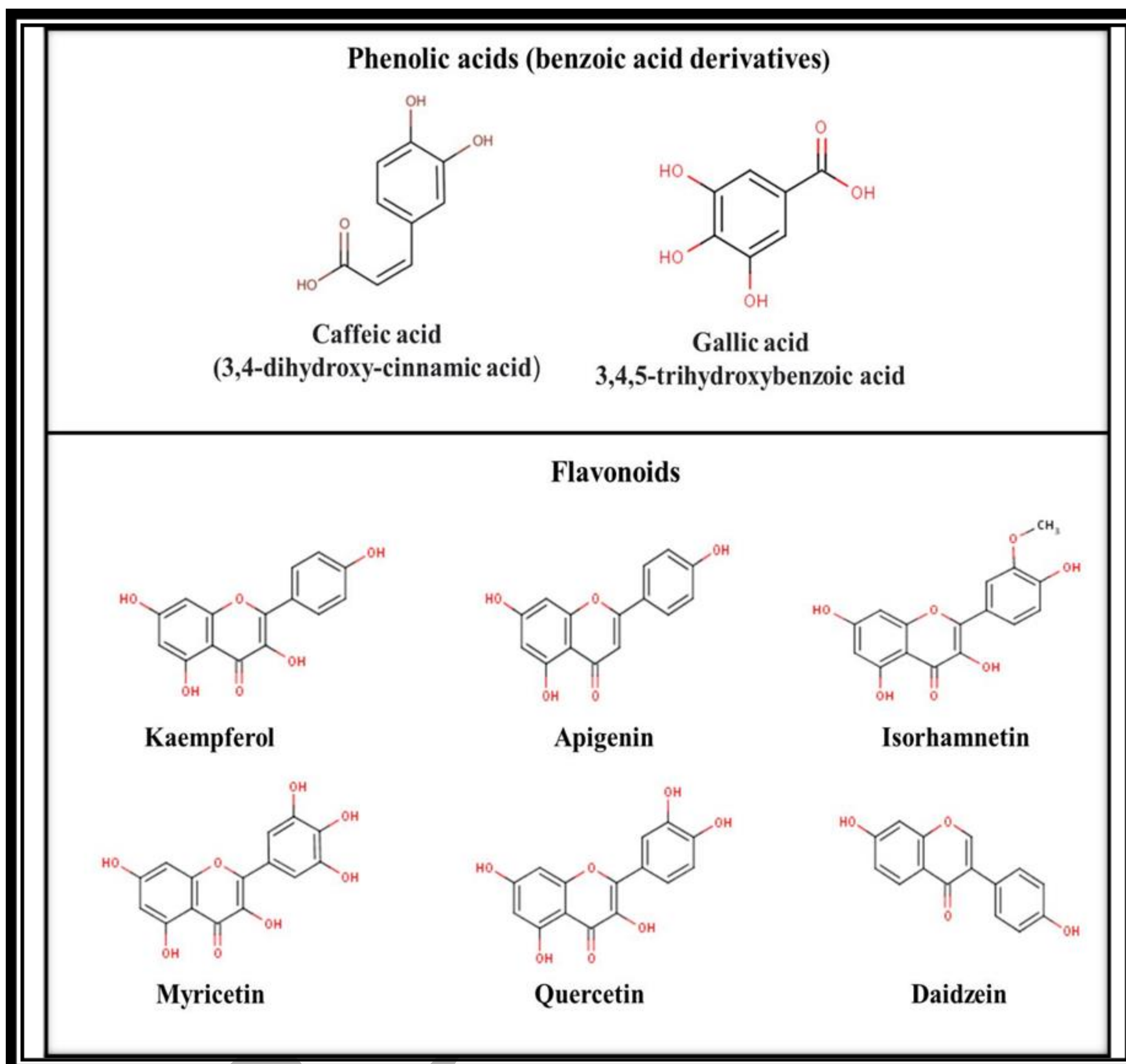
M. uniflorum upholds quantum of biologically active phytochemical compounds which comprises flavonoids (quercetin, kaempferol, and myricetin), alkaloids, cardiac glycosides, steroids, phenolic acids i.e., gallic acid and caffeic acid, tannins, carbohydrates, saponins, sterols, coumarins, procyanidins, syringic acid, phytic acid, vanillic acid, sinapic acid, p-coumaric acid, linoleic acid, ferulic acid and chlorogenic acid (Rlds & Erhss, 2017). Cyanidin, daidzein, malvidin, petunidin, delphinidin, and genistein (Dulal, 2018). (Goswami, 2017) performed Gas Chromatogram-Mass spectrometry (GC-MS) test of a seeds extract dissolved in methanol as solvent of *M. uniflorum*. The result showed the presence of forty various phytochemical and volatile composites including Acetoin, Acetic Acid, 2- Methyl-2-butenolide, Methyl Salicylate, 1-Hexanol, 2-Ethyl-hexan-1-ol, Ethyl Salicylate, Benzaldehyde, 2-Tridecanone, Linalool, 2,3-Butanediol, Butyric acid, Methionol, 2- Methylbutanoic Acid, Dimethyl adipate, Phenethyl acetate, Phenylacetaldehyde, Benzothiazole, β-Ionone, 2-Phenylethanol, Hexanoic Acid, Benzenemethanol, Geraniol, Guaiacol, Ethyl Benzoate, 1-Hexadecyne, Phenethyl acetate, Ethyl Salicylate, Octanoic Acid, γ-Nonalactone, 2-Pyrrolidinone, (E)-β-Damascenone, Phenol, p-Ethylguaiacol, Methyl Palmitate, 4-Vinylguaiacol, Pentadecanal, Ethyl tetradecanoate, 1-Tridecanol, Eugenol, Ethyl Palmitate, and Decanoic acid.. These phytochemicals exhibit significant therapeutic utility of this plant in developing novel drugs.

DRSML QAU

Category	Phytoconstituents
Flavonoids	Quercetin, Daidzein, myricetin, kaempferol, genistein
Phenolic acids (benzoic acid derivatives)	Syringic acid, Gallic acid, vanillic acid, protocatechuic acid, p-hydroxybenzoic acid,
Enzyme origin	Urease,α- and β glucosidase, b- Nacetylglucosaminidase, α-amylase,
Phenolic Carboxylic acids (cinnamic acid derivatives)	Sinapic acid, Caffeic acid, p-coumaric acid, chlorogenic acid, ferulic acid
Haemagglutinins, Tannins, Phytic acid	Agglutinin and lectins
Anthocyanins	Cyanidin, delphinidin, malvidin, petunidin

Table 2.2 Phytochemical constituents of *M. uniflorum* (Rlds et al., 2017).

DRSR CAU



DR

Figure 2.2 Molecular structure of important phytochemicals of *M. uniflorum*

(http://www.chemspider.com/Default.aspx)

2.9 Medicinal importance of *M. uniflorum*

The horse gram plant, popularly known as "nature's gentle drugs," has a diverse range of pharmacological properties. The following are a few of *M. uniflorum's* therapeutic characteristics:

2.9.1. Hepatoprotective activity

The methanolic seed extract of M. uniflorum has been shown to have in vivo hepatoprotective effects against hepatotoxicity induced by D-galactosamine and paracetamol in Wistar albino rats. The results of the study demonstrated that 400 mg/kg of a 95% methanolic extract of horse gramme seeds significantly reduced the induced hepatotoxic activity in Wistar albino rats (Parmar, Das, & Gohil, 2012).

2.9.2. Anti-microbial activity

In research, (Kaundal et al., 2019) reported that both fresh and dried sprouts of the legume *M. uniflorum* contained a number of phytochemicals with strong antibacterial properties against numerous human pathogenic bacterial strains, including adhesions of microbes, suppression of protein synthesis, lysis of cell membrane, and cleavage of proteolytic enzymes. The antibacterial action of the seed extract against Pseudomonas, E. coli, Bacillus subtilis, and Staphylococcus aureus is mostly due to tannin and phenolic compounds (Das, Gupta, Ansari, Pandey, & Rastogi, 2005)

Kawsar et al. (2008) investigated the impact of several extracts (dichloromethane, ethyl acetate, 1-butanol, and aqueous extracts) of horse gram seeds to screen antibacterial efficacy against 8 clinical bacterial and 6 clinical fungus strains. The outcome demonstrates that these extracts significantly affect the inhibition of microbial growth in gram-positive bacterial strains (Bacillus species and S. aureus), gram-negative bacterial strains (E. coli, S. typhi, Shigella dysenteriae, and Shigella sonnei), and several fungal strains. Ethyl acetate extract had the largest zone of inhibition of these extracts. *M. uniflorum* contains flavonoids that may be utilized to treat various skin conditions (Suriyavathana, Manikandan, Janeesha, Sandhya, & Ram, 2018).

2.9.3. Anti-HIV Activity

Horse gram have the potential to treat HIV conditions that are fatal. Dolichin A and Dolichin B, an isomeric pterocarpan (the second-largest category of iso-flavonoids) that display strong anti-HIV activity, have been found in the alcoholic extract of this plant. The two ligands (Dolichin A & Dolichin B) were successfully docked with proteases among the three HIV replicative enzymes, i.e., reverse transcriptase, integrase, and protease, according to the analysis of the in-silico

DRSML QAU

experiments' results. As a result of the rapid rate of HIV mutation, commercially available medications become resistant to HIV. Dolichin A and Dolichin, two lead chemicals produced from *M. uniflorum,* offer an alternate method of HIV treatment. This cutting-edge research will also assist in developing new medicines and alternative natural treatments for HIV that have fewer side effects (L. Rufus Auxilia & Sundari, 2013).

2.9.4. Anticarcinogenic activity

Several phenolics and bioactive compounds are present in horse gram that impart different biological activity such as antiapoptotic, anticarcinogenic, antiangiogenic, anti-inflammatory, and antiproliferative activity protecting against various cancer commonly occur in the human population. (Chakraborty & Abraham, 2016) observed the potent antitumor activity against human osteosarcoma cell line (MG 63) in methanolic and ethanolic extract of *M. uniflorum.* Genistein and daidzein present in horse gram sprouts are found to suppress proliferation of cells in (MCF-7) breast cancer cell lines (Sukanya & Gayathri, 2014). Moreover, *M. uniflorum* also is a rich source of phospholipids, phytosterol esters, and sterols, p-coumaric acid exhibiting antiulcer, protective, and healing properties against acute gastric ulceration (Aditya et al., 2019).

2.9.5. Anthelmintic activity

Alcoholic extracts from *M. uniflorum* seed showed stronger anthelmintic effects on Pheretima posthuma, an adult Indian earthworm. Considering its advantageous usage in dietary items for eliminating worms, this activity is caused by the presence of different alkaloids and phytosterols. Horse gramme seed extract in methanol, 50 mg/ml, paralyses and kills the worms more quickly than other extracts. When compared to the standard medication, albendazole citrate, it was discovered that this action had an equivalent impact (Sree, Soundarya, Ravikumar, Reddy, & Devi, 2014).

2.9.6. Anti-inflammatory activity

M. uniflorum is being used in Ayurvedic medicines for its anti-inflammatory purposes for centuries. (Giresha et al., 2022) assessed the in vitro anti-inflammatory activity of aqueous extract of *M. uniflorum*. Results showed that horse grain seeds extract can inhibit VRV-PLA2 (Viper arussellii snake venom PLA2) enzymes to a wider extent 87.56%. Another in vivo study was

DRSML QAU

conducted by (Mathew et al., 2014) in the carrageenin-induced paw edema model (female albino rat). Results revealed that 70% of methanolic extract of horse grains caused the significant dose-dependent inhibition of paw edema (73%) after 3 hours at the dose of 50mg/kg.

2.9.7. Anti-depressant activity

Depression was traditionally treated with horse gram in siddha, unani, and ayurveda medicine. In an ethanol extract of *Macrotyloma uniflorum*, (Zhu et al., 2018) phytochemically screen a variety of bioactive chemicals to determine their in vivo antidepressant effects in Wistar rats. The standardized ethanol extract of horse gram contains daidzein, genisteins, and isoflavones, according to analysis. (Zhu et al., 2018) also observed that the antidepressant properties of ethanolic extract at a dose (400 mg kgG1 /p.o.) reduced the mobility of rats. Numerous alkaloids and tyrosine kinase inhibitors are present, which contribute to its antidepressant properties.

2.9.8. Anti-diabetic activity

(Gupta et al., 2011) studied the -amylase inhibitor with antidiabetic potential that they extracted from horse gram seeds. The outcomes suggested that streptozotocin-nicotinamide-treated diabetic mice have antihyperglycemic activity in response to horse gram seed. The serum sugar level in diabetic mice is lowered, and mouse pancreatic amylase is inhibited. (3B)-stigmast-5-en-3-ol, linoleic acid present in horse gram also exhibit potent antidiabetic activity (Goswami, 2017).

2.9.9. Anti-aging and skin photoprotective activity

UV radiation is considered an important external factor causing photoaging of the skin. Free radicals are formed in the presence of UV causing degradation of unsaturated lipids in our skin. It results in structural changes in fibrillar proteins, elastin, and collagen. Dolichos biflorus have a protective effect against UV radiations (Miastkowska & Sikora, 2018). Antioxidants present in ethanolic, and aqueous extract of horse gram seed coat prevent skin cancers (Kaundal et al., 2019). Another study conducted by (Aditya et al., 2019) reported the potential usage of horse gram seed extract in treating skin disorders. (Moussou, Moser, Jeanmaire, Danoux, & Bardey, 2011) documented the potential effect of *M. uniflorum* callus extract in enhancing collagen production and its potential application in the cosmetic industry.

DRSML QAU

2.9.10. Anti-calcifying activity

Horse gram proved to be efficacious in curing kidney stones. One of its common names of *M. uniflorum* is “Gahot” (known by the people of Kumaon and Garhwal areas), which etymologically means to destroy kidney stones (Bhartiya et al., 2015). One study acclaimed that *M. uniflorum* exhibits litholytic and anticalcifying activity. Clinical studies also showed that horse gram seeds contain non-tannin, non-protein crystallization inhibitors that reduce the size of calcium oxalate crystals (Khare, Saraswat, & Khare, 2017). Phytic acid present in horse gram exhibit helps in destruction of kidney stones and can easily be removed from urine (Dulal, 2018). Another researcher Kaundal et al., (2020) reported that horse gram seeds extracts exhibit anti-uroliathiatic activity against, uric acid crystal as well as calcium phosphate crystals and calcium oxalate crystals.

2.10 Importance of Plant tissue culture (PTC)

Plant secondary metabolites can also be obtained from natural occurring plants that grow in the wild, however their industrial output is constrained by different ecological, geographical, and seasonal constraints. There are also a number of conventional cultivation techniques that are accessible, however they require a long time—possibly years—to grow plants to the point at which they began to produce the desired secondary metabolites. The PTC method offers a different platform to get beyond these constraints. This method involves aseptically cultivating plant cells, tissues, and organs in a synthetic nutrient medium with carefully monitored environmental factors (Chadipiralla, Gayathri, Rajani, & Reddy, 2020).

This process is mainly based on micropropagation which involves the rapid proliferation of cells from any small part of plant tissue (stem, leaf, axillary bud, root). Thereby extensively used for large-scale plant multiplication. This method is helpful for the speedy manufacture of bioactive chemicals that are significant from a commercial and medical perspective. Callus cultures are viewed as prospective bio factories because they offer a reliable production method and guarantee a consistent supply of goods including secondary metabolites without destroying the natural

DRSML QAU

habitat of the plants. It is devoid of a number of biotic and abiotic elements. (Rao & Ravishankar, 2002).

2.10.1. Application of Plant tissue culture techniques

- It is utilized to preserve the germplasm of significant medicinal plant species.
- It offers a useful method for crop development by raising crop output and quality.
- Expand genetic variations of therapeutic plants.
- It is used to create novel compounds that are not naturally found in local flora (Tariq Khan, Abbasi, Khan, & Azeem, 2017).

2.11 Micropropagation

Micropropagation represents an in-vitro rapid multiplication and clonal propagation of the plants from a tiny part of plant tissue. Under sterile environmental conditions, the plant has an ability to regenerate into a whole new plant. This biotechnological tool plays an crucial role for selection, multiplication, and germplasm conservation of the extinct and endangered medicinal plant species (Abbasi et al., 2016). This practice has been utilized for developing disease-free varieties of plants, germplasm conservation of endangered plants, genetic enhancements of traits and to produce high-quality secondary metabolites for drug designing (Khan et al., 2020)(I. Khan et al., 2020). In-vitro production of therapeutic plants has drawn a lot of interest during the last two decades. A variety of explants like rhizome, roots, leaves, shoot apex, bud scale, petals, cotyledon, and embryo axis have been used for raising medicinal plants. In-vitro protocol for micropropagation has been successfully established for *Mentha piperita, Ajuga bracteosa, Lallemantia Iberica, Lavandula angustifolia*, *Rosmarinus officinalis* and many other medicinal plant species (Tariq Khan et al., 2021).

2.12 Callus culture

The callus is defined as an undifferentiated mass of cell-induced from plant tissue under in vitro conditions. The main purpose of callus is to amplify the limited plant resources. Callus cultures protects natural plant's habitat and serve as potential source of phytochemicals that can directly be

DRSML QAU

isolated from callus culture. So there is no need to cut an entire plant. Phytohormones (auxin and cytokinin) either alone or in combination are also required for callus induction other than the nutrient medium. Callus exhibit totipotent potency enabling each cell to produce a whole new plant by direct regeneration or somatic embryogenesis. Secondary metabolites are produced in callus culture under stress conditions that can be enhanced by employing various biotic and abiotic factors (Fehér, 2019). Callus cultures of medicinal plants present economically viable means to produce new bioactive compounds that can be utilized in various industrial products including cosmetics and foods. It is potentially used in various commercial applications i.e., production of antibodies and recombinant proteins of therapeutic significance. They can be used to produce agriculture and horticulture plants by regeneration from callus (Benjamin, Ishaku, Peingurta, & Afolabi, 2019). In recent years pharmaceutical engineering has gained much more importance. In this process, various biological engineering techniques have been employed in callus culture to enhance the synthesis of useful secondary metabolites (Ogita, 2015).

2.13 In vitro tissue culture of *M. uniflorum*

By utilising various explants, including an epicotyl, hypocotyl, cotyledon, and a juvenile leaf of *M. uniflorum*(Mohamed & Jayabalan, 1996)effectively developed a reliable methodology for invitro callus induction. For callus induction, he discovered that MS medium supplemented with IAA/2,4-D (2.0 mg 1-along with 15% coconut milk and BAP (0.5 mg 1-1) was the most successful.

2.14 Elicitation

Plants produce secondary metabolites as a defense system to combat the infections caused by either internal or external stress. It is obvious that the secondary metabolite production in plantscan be enhanced by applying stress on plants.

Elicitors are compounds or bio-factors that stimulate various types of physiological alterations in plants, affecting their metabolic machinery which subsequently regulate biosynthesis of secondary metabolites in relatively larger amount. They are classified as biotic and abiotic elicitors depending on their nature (Patel & Krishnamurthy, 2013). Different techniques are applied on in vitro callus cultures of plants for high synthesis of useful phytochemicals. Among them, elicitation is one of the most efficient techniques in which secondary metabolites production is enhanced through

DRSML QAU

activating different metabolic pathways. Elicitation presents a promising avenue for the optimal biosynthesis of a secondary metabolite of the plant (Yang & Stöckigt, 2010).

The process of elicitation is directly correlated with enhanced biomass accumulation and bioactive compounds. The cell membrane-bound receptors identify the elicitors and activate the required genes by the signal transduction pathway by increasing the secondary metabolism (Saeed, Ali, Khan, Kayani, & Khan, 2017). All these stress-producing agents are called Elicitors. These elicitors are generally classified as biotic or abiotic elicitors (Baenas, García-Viguera, & Moreno, 2014). Chemical, hormonal or physical agents, the biotic elicitors might be the animal, plant, or microbial derivatives (Naik & Al-Khayri, 2016).

2.15 Melatonin as an elicitor

The pineal gland of animals produces the mammalian indoleamine neuro-hormone melatonin, which is recognised as a common and highly conserved chemical. It reduced stress brought on by a variety of chemical and environmental variables (Tan, Manchester, Esteban-Zubero, Zhou, & Reiter, 2015).

The existence of melatonin in different plant and animal species has been reported in more than 5700 studies. A number of medicinal plants have relatively high concentrations of melatonin, that is utilized to treat neurological problems. Melatonin is found both in the leaves and flowers of these plants (Murch, Campbell, & Saxena, 2001). The most important functions of MEL in plants are controlling circadian clock, development and growth and enhancing tolerance to a variety of environmental stresses (Kaur, Mukherjee, Baluska, & Bhatla, 2015).

DRSML QAU

Chapter 3
Material and Methods

DRSML QAU

3. Material and methods

All the experiments were performed under the supervision of Prof. Dr. Bilal Haider Abbasi at Plant Cell and Tissue Culture Laboratory, Department of Biotechnology, Quaid-i-Azam University Islamabad.

3.1. Chemicals and Equipment

The following chemicals were used in the current study: distilled water, sodium hydroxide, methanol, hydrochloric acid, and ethanol. Along with plant growth regulator (NAA) and melatonin as a biotic elicitor. The equipment utilized for this study include filter paper, a spatula, a blade, forceps, an autoclave (KP-30L, ALP Tokyo Japan), an electric balance (GF-300), a pH meter (Jenway 3305), a laminar flow transfer cabinet (ESCO), a burner, and glassware (Measuring cylinder, Erlenmeyer flask, glass beaker, petri dish).

3.2. Media preparation

The germination of seedlings of *M. uniflorum* took place in Murashige and Skoog basal medium (MS, 1962). Media preparation followed the protocol specified by (Abbasi et al., 2010). In order to do this, 30g of the sucrose and 4.4g of the MS medium were weighed in a weighing scale before being dissolved in distilled water in a flask to generate a total amount of 1 liter. The medium pH was kept at 5.65± 0.02 by use of (1.0 N) sodium hydroxide (NaOH) and (1.0 N) hydrochloric acid (HCL). The media were then solidified by adding 8g of agar, which was weighed before being added. After that, flasks were put inside the microwave for 5 minutes to get the media to boil and properly dissolve the agar. Each 100-ml Erlenmeyer flask was then filled with 30ml of the medium and firmly sealed using cotton and aluminum foil. To ensure that the media solidified adequately and to rule out any potential of contamination, these flasks were then sterilized by autoclaving each for 20 minutes at 121°C and 15-psi and were left overnight.

3.3. Surface sterilization

To get rid of the dust particles and other undesired elements that had formed on the glassware and utensils used in the research, they were all carefully washed with running water and detergent.

DRSML QAU

These were given time to dry before being properly wrapped in paper and sterilized in an autoclave for around twenty minutes at 121°C and 15 psi of pressure.

3.4. Explant collection, inoculation, and seed germination

M. uniflorum seeds were procured in perfect health and uniformity from the NARC's botanical garden in Islamabad. In a laminar flow (LFH) cabinet with a HEPA filter, inoculation was carried out in a sterile setting. Following this, an autoclaved flask holding 30ml of the medium was moved to LFH along with autoclave tools like a Petri dish, forceps, blade, distilled water, empty beaker, and ethanol. To lessen the risk of contamination, all of these devices were surface sterilized with 70% ethanol. For effective sterilization, the LFH door was shut and a UV lamp (GKL-511, 50 Hz, 19w) was turned on. After opening the LFH, turned off the UV light, and fans were turned on. Hands were sanitized with 70% ethanol prior to vaccination. Placed the flame of the spirit lamp next to the open Petri dishes with filter paper. Within the laminar flow hood, seeds were surface sterilized after being rinsed with water. They were first immersed in 0.1% HgCl2 (w/v) for one minute. It was then submerged in 70% EtOH for around 40 seconds. Finally, three rinses with sterile distilled water were performed to remove any remaining dust, and the surface was then dried on sterile whatman filter paper. There were four seeds inoculated per flask. Each experiment was carried out twice in duplicate. Following that, these flasks were placed in the growth chamber for 15-20 days in self-regulating environmental condition, such as temperature adjustment to 25°C and provision of a 16/8-hour photoperiod with light intensity of 40µmol $m^{-2}s^{-1}$ from the fluorescence light bulb (Philips Tornado Spiral).

3.5. Establishment of Callus culture

Macrotyloma uniflorum callus culture was established in the first experiment. For callus induction MS media (4.4 g/L) supplemented with sucrose (30g/L) and agar (8g/L) and 1M NAA was prepared (unpublished data). 10-day old plantlets grown (in-vitro) from seeds were chopped into small pieces, and 4-5 pieces were inoculated in each flask under LFH. Then, photoperiod (i.e., 16 hours light and 8 hours darkness) at intensity of 40 µmol $m^{-2}s^{-1}$ was applied to flasks containing inoculated explants. After 14–15 days, the callus began to grow. The callus had grown to its maximum size after 28 days.

DRSML QAU

3.6. Preparation of elicitor

Melatonin stock solution was prepared by dissolving 50mg melatonin with 50ml distilled water and was stirred continuously for an hour to achieve proper mixing.

3.7. Melatonin treatment on callus culture

Macrotyloma uniflorum callus culture was examined by shifting fresh callus of 0.5 g from earlier subcultured callus on MS media, which was optimized with hormone (1 NAA mg/L), and varied melatonin concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 µM). MS media deprived of melatonin were used as a control. The flasks with media and callus were kept in the culture room for four weeks. Following 28 days of inoculation, the callus was removed for further examination and activities.

3.8. ORAC

ORAC, which stands for Oxygen Radical Absorbance Capacity, is a tube analysis that evaluates the overall antioxidant activity of foods and other chemicals. By analyzing the suppression of peroxyl radical-induced oxidation, ORAC assay determines the antioxidants' power to break down radical chains. Under biological settings, peroxyl radicals are the most common free radicals involved in lipid oxidation in dietary and biological systems (Zhong & Shahidi, 2015). Method of ORAC assay by (Prior, Wu, & Schaich, 2005) was used. Briefly, 10 µL of the extracted material were combined with 190 mL of fluoresce that had been produced in 75 mM phosphate buffer (pH7.4). This mixture was then incubated for 20 min at 37 ± 1 °C with orbital shaking. The fluorescence intensity was then measured every five minutes for 2.5 hours at 37 °C using a fluorescence spectrophotometer (Bio-Rad) set with an excitation wavelength of 485 nm and an emission wavelength of 535 nm after the addition of 20 µL of 119.4 mM 2, 2′-azobis-amidinopropane (ABAP, Sigma-Aldrich). Assays were performed three times. To express capacity of antioxidant Trolox C equivalent antioxidant capacity (TEAC) was used.

3.9. CUPRAC

In the CUPRAC assay, antioxidants in the sample that have a leading thiol group are redox reduced with the CUPRAC reagent. The reagent self-reduces in this process to create a copper (I)-

DRSML QAU

neocuproine chelate complex, which results in a color observable at 450 nm (Özyürek et al., 2011). In a nutshell, 190 µL of the CUPRAC solution—which contains 10 mM Cu(II), 7.5 mM neocuproine, and 1 M acetate buffer pH 7—was combined with 10 µL of an extract. After 15 minutes of incubation at the room temperature (25 2 °C), the absorbance of reaction mixture was determined at 450 nm (BioTek ELX800; BioTek Instruments, Colmar, France). For this assay the method used by (Drouet et al., 2019) was applied.

3.10. Cellular antioxidant assay (CAA)

A cellular antioxidant experiment was performed to determine the amount of highly reactive oxygen species and nitrogen species in callus extract using the technique suggested by (Tungmunnithum et al., 2020). Six hours prior to the stimulation of stress brought on by RONS, all extracts were left to evaporate under nitrogen flow before being combined with (50 g/mL) DMSO solution and injected into the cells at the final concentration of 1 mg/mL. Following that, 1% (v/v) of dimethyl sulfoxide was supplied to the cell as the final concentration. A 0.1% of final volume of DMSO were added to the sample used as control. Horse gramme callus extracts were incubated with yeast cells for one night. Callus extracts were completely cleaned with PBS two times, followed by 10 minutes at 300C in the dark. The fluorescent signals were measured by use of a fluorimeter (BioRad, Marnes -la- Coquette, France) after repeated PBS washes ($\lambda em = 535$ nm, $\lambda ex = 505$ nm).

3.11. FRAP

The antioxidant FRAP assay is offered as technique for evaluating "antioxidant potential." It is an easy, automated test that measures the ferric reduction capacity of plasma. A colourful ferrous-tripyridyltriazine complex is produced when ferric to ferrous ion reduction occurs at a low pH. By contrasting the change in absorbance at 593 nm between test reaction mixtures and those containing ferrous ions in a known concentration, FRAP values are determined.

The FRAP potential of callus extract was examined using Benzie & Strain's methods (1996). In a nutshell, 190 mL of FRAP solution [containing 10 mM TPTZ (2,4,6-Tri (2-pyridyl)-s-triazine); 20 mM FeCl3 (ferric chloride hexahydrate); and 300 mM of acetate buffer of pH 3.6; ratio 1:1:10 (v/v/v)] was used to fully dissolve an aliquot of 10 mL of sample callus extracts. A microplate

DRSML QAU

reader (BioTek ELX800, BioTek Instruments, France) was used to measure absorbance at 630 nm following the incubation of the reaction mixture for 15 minutes at room temperature. The activity was carried out in triplicates, and antioxidant capabilities were demonstrated as (TEAC) Trolox-C-equivalent antioxidant capacity.

3.12. High performance liquid chromatography

3.12.1. Extraction method

In 20 ml of 80% (v/v) aqueous MeOH, quickly dissolve 0.5 g of the dry lyophilized powder using an Ultraturrax T25 mixer for 1 minute at 19,000 rpm. The glycosidic linkages were then hydrolyzed with the addition of 1 mol HCL to aid in the release of molecules (aglycones) that were there in a complex manner. These free chemicals are simple for an analyzer to find and measure. The ultrasound-mediated extraction was carried out at 50 °C for one hour with a fixed ultrasonic frequency of 45 kHz. The USC1200TH ultrasonic bath (Prolabo : inner dimension ; 300 multiply mm 240 mm multiply 200 mm) for ultra-sonication was used. It has a heating power of up to 400 W with adjustable frequencies and a 400 W electric power density (acoustic power of 1 W/cm^2). Additionally, it is fixed with temperature and frequency control monitoring equipment. After centrifugation, the supernatant was dried by allowing it to evaporate at 40°C, and it was then resuspended in 1 ml of citrate-phosphate buffer (pH 4.8) containing 5 units per milliliter of ß-glucosidase fromalmonds (Sigma) to allow for the release of aglycones over the course of four hours at that temperature. Before injecting the extract, the supernatant was centrifuged and filtered (0.45 µm). In order to produce nettle extracts with various quantities of caffeic acid, gallic acid, and other phenolic components, the following variables were chosen, extraction time, temperature, and ethanol concentration.

3.12.2. HPLC based quantification.

After ultra-sonification (UCS1200TH; 30KHZ frequency) was used to extract the lyophilized cell using 20ml water-methanol (80% v/v), each extract's centrifugation was done at 3,000 rpm for around fifteen minutes. The collected supernatant were then filtered with a syringe filter of 0.45 µm (Millipore, Molsheim, France) and were then injected for HPLC analysis. Important phenolic

DRSML QAU

and flavonoid components were separated and quantified using RP HPLC in a liquid chromatographic system fixed with an online degasser (Metachem Degasit), an autosampler (Prostar-230-pump), and a Prostar-335 Photodiode Array Detector (PAD) and then controlled by Galaxie 1.9.3.2 version software (Varian, Les Ulis, France) as the data processor. The separation was performed at 350C using a Purospher (Merck) RP-18 (250 x 4.0 mm) column with particle size of 5µm. Two solvents are needed for the mobile phase, consisting of Solvent A, 0.2% AcOH in HPLC grade water, and Solvent B, HPLC grade methanol. Throughout a run of an hour, the composition of mobile phase changes, with a nonlinear gradient 8% B for 36 minutes, 100% B for 30 to 35 minutes, 33% B for 28 minutes, and 30% B for 17 minutes, 8% B flowed for 0 minutes, 12% B for 11 minutes, and 12% B for 1 minutes. Detection was done fluorometrically at a 320nm wavelength. Compounds were identified by comparing their UV spectra and retention times to the UV spectra and retention times of genuine standards. Utilizing calibration 5-point curves with a minimum correlation coefficient of 0.9998, quantification was carried out. We purchased gallic acid, caffeic acid, epicatechin, ferulic acid, catechin, rutin, daidzein, genistein, myricetin, and kaempferol operating standards from Sigma-Aldrich (USA).

3.12.3. Statistical analysis

Every experiment listed above was carried out twice, in duplicates. Mean values of every experiment was calculated and standard error was determined using Microsoft excel program. All the graphs were created by using software Origin pro-2018 and Microsoft excel program.

DRSML QAU

Chapter 4
Results and Discussion

DRSMLQAU

4. Results and Discussion

4.1. Effect of melatonin on biomass accumulation

In the current study, we examined the effect of biotic elicitor that is melatonin in this case, in various doses, on callus generated from M. uniflorum plantlets that had been grown in-vitro. There are various factors that effect biomass accumulation which include elicitor concentration, culture type, age and contact time (Namdeo, 2007). The production of vital plant metabolites in culture media, callus induction, and callus growth are frequently significantly influenced by the presence of elicitors.

In different doses of melatonin treatment, a good callogenic response was seen at 0.6 mg/l (Figure 4.2) and the maximum biomass accumulated was (FW = 370.25 ± 7.4 g/L, DW = 29.62 ± 0.88). It demonstrates that in the current trials, 0.6 mg/L melatonin was quite successful at inducing calluses and increasing biomass. While at a concentration of 1.2 mg/L of melatonin, lowest biomass accumulation was observed (Figure 4.2) and the minimum biomass accumulated was (FW = 213.75 ± 8.8 g/L, DW = 18.25 ± 0.3). With an increase in melatonin content up to a certain point, the production of biomass steadily increased (figure 4.2). Following that concentration, biomass began to decline as melatonin levels rose. The discovered results are related to a prior work where a comparable tendency was seen in the biomass acumulation of Prunella vulgaris tissue culture that had been treated with melatonin (Fazal, Abbasi, Ahmad, & Ali, 2018b). Melatonin promotes the growth of elongated cotyledons, which are directly related to biomass accumulation in the *Lupinus albus* plant (Hernández-Ruiz & Arnao, 2008). *Brassica juncea's* roots grow more quickly when melatonin levels are lower; when levels are higher, however, the growth of the roots is inhibited. Exogenous melatonin has been demonstrated to increase plant root growth as a growth-stimulating chemical with an auxin-like effect. Through a variety of ways, melatonin modulates a wide range of biological and physiological functions. It carries out a variety of plant-related tasks, including acting as an anti-stress agent under biotic and abiotic stress, promoting plant growth, seed germination, biomass production, and so increasing plant yield (Qiao et al., 2019).

The highest concentration of melatonin had an inhibitory effect on callogenesis. Higher melatonin concentrations may have an inhibitory effect because they produce ROS, which limit cell

DRSML QAU

development and proliferation and cause apoptosis (Sarropoulou, Therios, & Dimassi-Theriou, 2012). Our research supports earlier studies that found increased melatonin doses had inhibitory effects on callogenesis (Fazal, Abbasi, Ahmad, & Ali, 2018a).

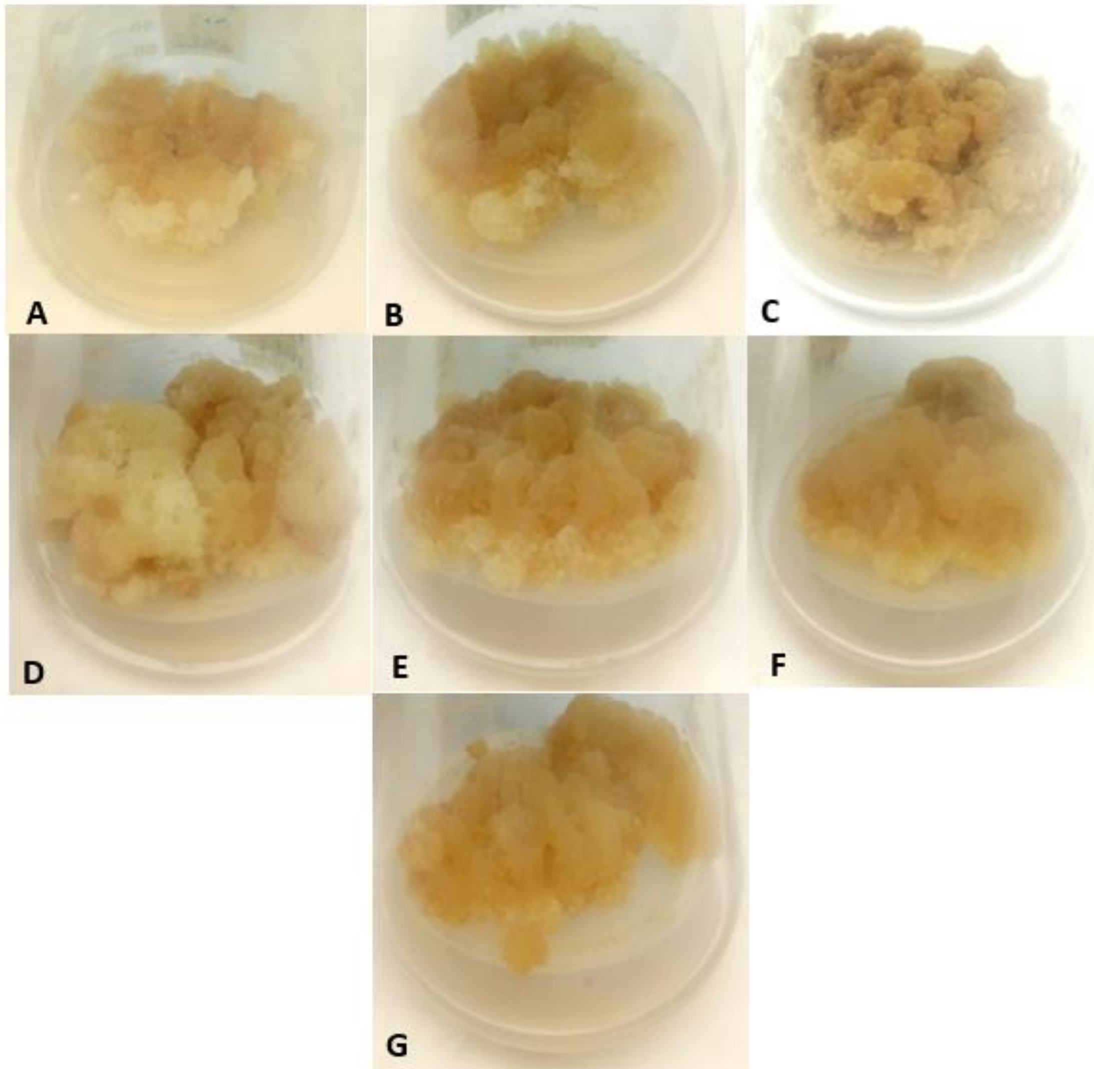


Figure 4.1 Callus morphology of Macrotyloma uniflorum

(A) Control **(B)** Melatonin 0.2 mg/L **(C)** Melatonin 0.4 mg/L **(D)** Melatonin 0.6 mg/L **(E)** Melatonin 0.8 mg/L **(F)** Melatonin 1.0 mg/L **(G)** Melatonin 1.2 mg/L

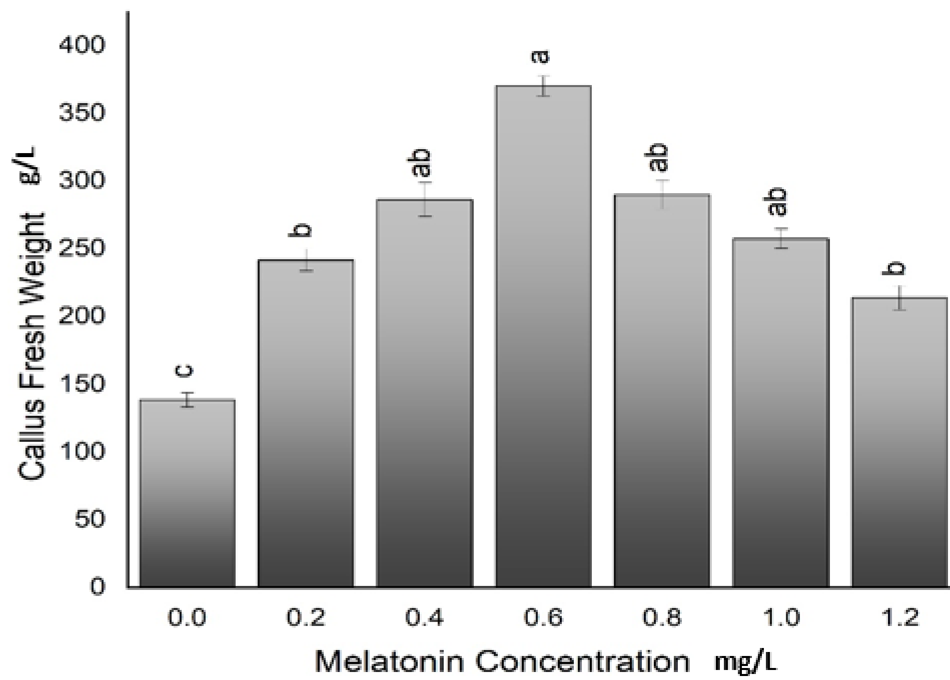


Figure 4.2 : Fresh callus biomass from root explant in response to different Melatonin concentrations

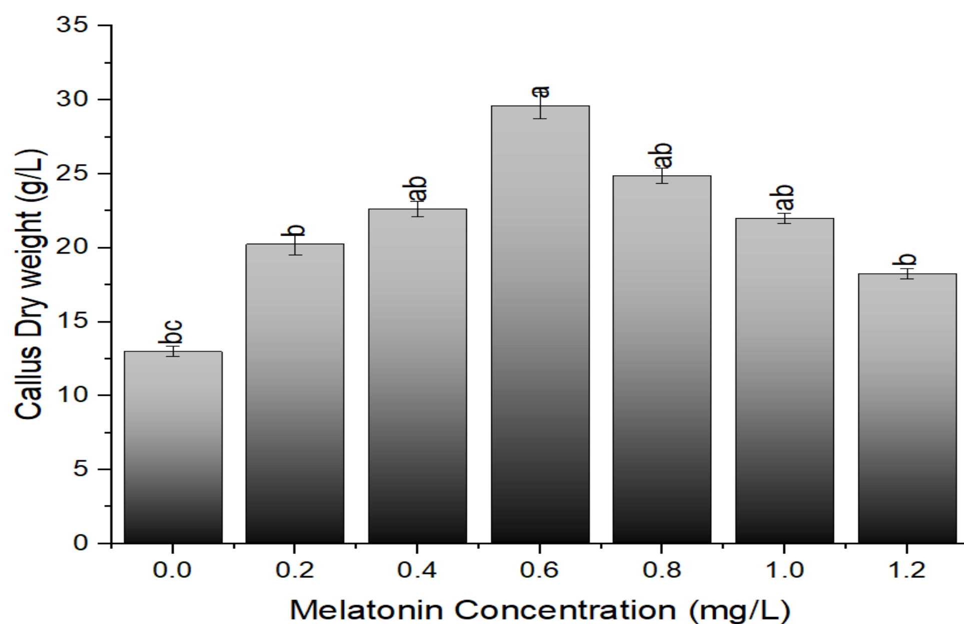


Figure 4.3: Dry callus biomass from root explant in response to different Melatonin concentrations

DR

4.2. HPLC based phytochemical analysis

HPLC is a useful method for quickly and accurately quantifying phenolic chemicals with high precision. In the current study, we used a reverse phase HPLC technique to quantify pharmacologically significant bioactive chemicals in *M. uniflorum* callus culture.The quantification of gallic acid, catechin, caffeic acid, epicatechin, ferulic acid,rutin, myricetin, daidzein, genistein and kaempferol was revealed by the RP-HPLC analytical results (Table 4.1).

Variable accumulation of highly valuable metabolites under the impact of different melatonin treatments was confirmed by the HPLC-based phytochemical study. The analysis revealed that the phytochemical production in the callus treated with melatonin was much more higher than that of the callus from control. The findings showed that, in comparison to in vitro intact plant cells, callus culture showed increased accumulation of secondary metabolites.

Eight phenolic acids were found in the ethanolic extract of M. uniflorum during RP-HPLC analysis by (Kawsar, Serajuddin, Huq, Nahar, & Ozeki, 2008). The most prevalent of the phenolic acids was specifically gallic acid (7.81 mg/100g of DW). In vitro callus culture had a substantially greater gallic acid concentration than seeds.

In this study, gallic acid showed highest accumulation among all of the other the phytochemicals. The highest concentration of gallic acid (9.25 mg / g DW) was shown at the concentration of 1.0 mg/L melatonin.

The second highest accumulating phytochemical was myricetin (6.78 mg/g DW) and was observed at 1.0 mg/L melatonin. The third highest accumulated phytochemical was Daidzein (5.31 mg/L melatonin) and it was also observed at 1.0 mg/L melatonin.

Furthermore, several other secondary metabolites were also detected during this HPLC analysis which are catechin, caffeic acid, epicatechin, ferulic acid,rutin, genistein and kaempferol. The highest concentrations of these compounds were also detected in callus treated with 1.0 mg/L melatonin. According to these investigations, in vitro callus culture produced under PGR treatments offers a more effective method for boosting the production of biologically active components.

In a separate study (Sreerama, Sashikala, & Pratape, 2010)carried out a quantitative phytochemical examination of horse gramme seeds. A number of flavonoids, including Kaempferol, Myricetin, and phenolic components, including gallic acid as well as caffeic acid, were found in the seeds, according to the HPLC examination.

The stem callus culture of *Fagonia indica* has also been shown to produce secondary metabolites (such as caffeic acid, gallic acid, kaempferol and myricetin) in vitro (Taimoor Khan, Ullah, Garros, Hano, & Abbasi, 2019). These metabolite concentrations are considerably lower than those seen in *M. uniflorum* callus culture, though.

DRSML QAU

Phytochemicals	Control	MEL 0.2	MEL 0.4	MEL 0.6	MEL 0.8	MEL 1.0	MEL 1.2
Gallic acid (mg/g DW)	0.80 ± 0.04	2.3 ± 0.01	3.50 ± 0.21	4.99 ± 0.17	5.15 ± 0.22	**9.24 ± 1.01**	3.33 ± 0.2
Catechin (mg/g DW)	0.18 ± 0.01	0.33 ± 0.05	0.63 ± 0.04	0.96 ± 0.03	1.00 ± 0.04	**1.36 ± 0.01**	0.18 ± 0.4
Caffeic acid (mg/g DW)	0.23 ± 0.03	0.65 ± 0.11	0.85 ± 0.03	1.25 ± 0.08	1.87 ± 0.09	**2.74 ± 0.07**	1.30 ± 0.03
Epicatechin (mg/g DW)	0.29 ± 0.06	0.45 ± 0.03	0.80 ± 0.06	1.37 ± 0.06	1.49 ± 0.07	**2.45 ± 0.09**	1.06 ± 0.09
Ferulic acid (mg/g DW)	0.25 ± 0.02	0.77 ± 0.10	0.96 ± 0.08	1.55 ± 0.09	1.73 ± 0,03	**2.81 ± 0.02**	1.23 ± 0.1
Rutin (mg/g DW)	0.16 ± 0.01	0.31 ± 0.06	0.66 ± 0.04	0.98 ± 0.05	**1.97 ± 0.08**	1.75 ± 0.05	0.78 ± 0.06
Myricetin (mg/g DW)	0.57 ± 0.07	1.01 ± 0.09	2.17 ± 0.15	3.50 ± 0.12	3.90 ± 0.17	**6.77 ± 1.03**	2.55 ± 0.04
Daidzein (mg/g DW)	0.53 ± 0.1	1.21 ± 0.07	2.62 ± 0.18	3.22 ± 0.11	3.59 ± 0.13	**5.31 ± 1.07**	2.56 ± 0.7
Genistein (mg/g DW)	0.55 ± 0.07	0.74 ± 0.04	1.73 ± 0.09	2.68 ± 0.09	2.98 ± 0.14	**4.11 ± 0.06**	2.12 ± 0.08
Kaempferol (mg/g DW)	0.36 ± 0.09	0.98 ± 0.08	1.83 ± 0.10	2.22 ± 0.07	2.47 ± 0.12	**4.88 ± 0.11**	1.76 ± 0.01

Table 4.1: Effect of different melatonin concentrations on biosynthesis of polyphenolic metabolites in callus culture of *M. uniflorum*.

DRSML QAU

4.3. Antioxidant activities

The anti-oxidant capacities of horse gram seeds have been the subject of numerous studies in the past (Goswami, 2017), however M. uniflorum callus made from its adventitious roots has not yet been investigated for its anti-oxidant potential. In light of this, various in vitro assays, including the (ORAC, CUPRAC, FRAP) assay and the in vivo CAA assay, were carried out to determine the antioxidant capacity of adventitious root derived callus produced under various melatonin treatments. Different phenolic and flavonoid components are linked to antioxidant properties in medicinal plants. To estimate the therapeutic potential of callus extract, it is required to assess its antioxidant capacity.

4.3.1. Antioxidant activities of callus treated with Melatonin

The following activities were performed on the callus obtained from different melatonin treatments.

4.3.2. ORAC

The objective of the current study was to investigate the impact of different melatonin concentrations on the total antioxidant capacity of callus derived from roots of *M.uniflorum*, utilising measuring technique (ORAC) based on several reaction mechanisms. The results obtained from this antioxidant screening are reported in Figure 4.4.

DRSML QAU

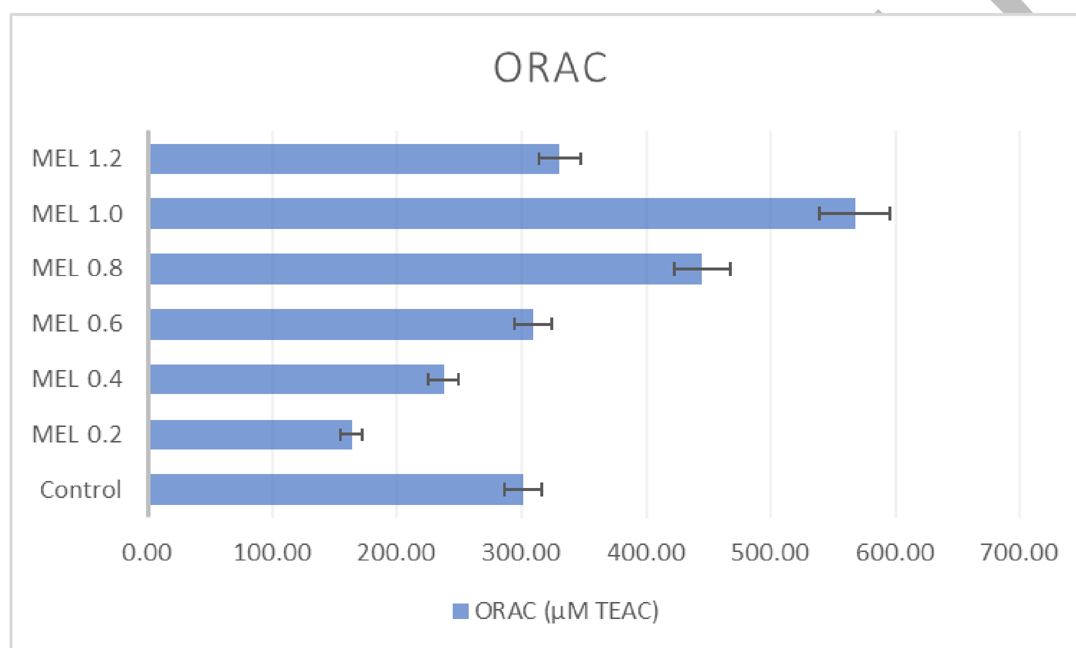


Figure 4.4: ORAC assay to estimate antioxidant activities in callus culture treated with various melatonin concentrations

The figure 4.4 reports that the highest ORAC activity (567.25 ± 23.02 µM TEAC) was observed at 1.0 mg/L of melaonin. While the ORAC activity for the control sample was (300.70 ± 13.2 µM TEAC). The lowest value of ORAC activity was observed at 0.2 mg/L melatonin which was (163.22 ± 8.4 µM TEAC).

4.3.3. CUPRAC

Total Antioxidant Capacity (TAC) assessments of both hydrophilic and hydrophobic samples are possible using CUPRAC. Figure 4.5 reports the findings of this antioxidant screening.

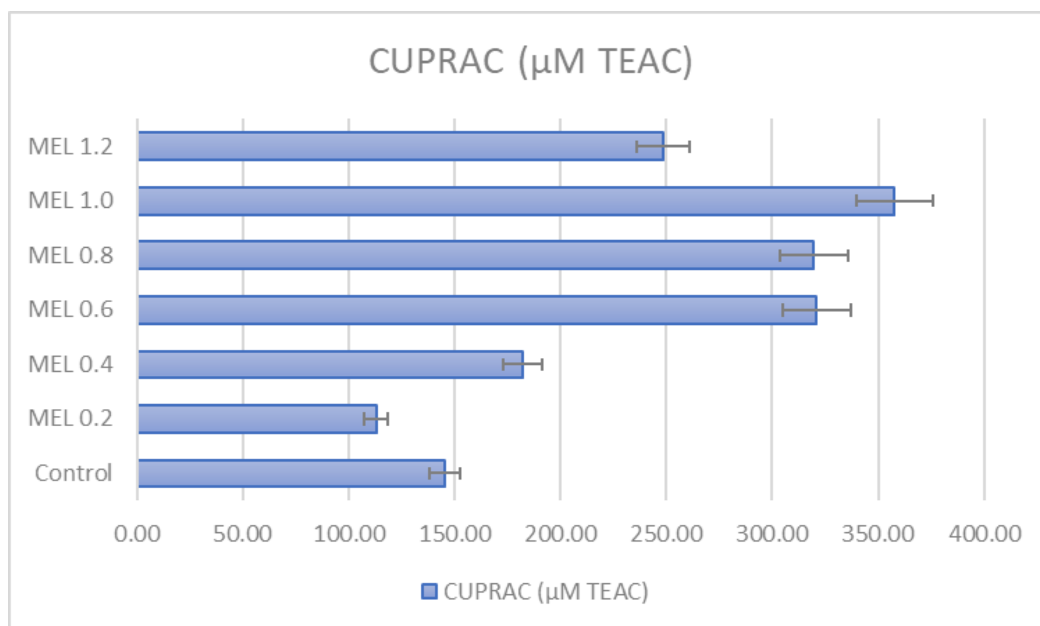


Figure 4.5: CUPRAC assay to estimate antioxidant activities in callus culture treated with various melatonin concentrations.

DRSML

The figure 4.5 represents the highest CUPRAC activity (357.85 ± 22.9 µM TEAC) and it was observed at 1.0 mg/L of melatonin. While the value of CUPRAC activity in the control samples was (145.21 ± 9.1 µM TEAC). The lowest value (112.90 ± 11.5 µM TEAC) of CUPRAC activity was recorded at 0.2 mg/L of melatonin.

4.3.4. FRAP

This assay is yet another essential instrument for determining a sample's antioxidant capacity because it mostly depends on the sample's capacity to convert Fe+3 to Fe+2 (an electron transfer mechanism). This test is done to find out how effective the phenolic chemicals in the sample are as antioxidants. The ability of callus produced in media enriched with various melatonin concentrations to reduce ferric iron was also evaluated.

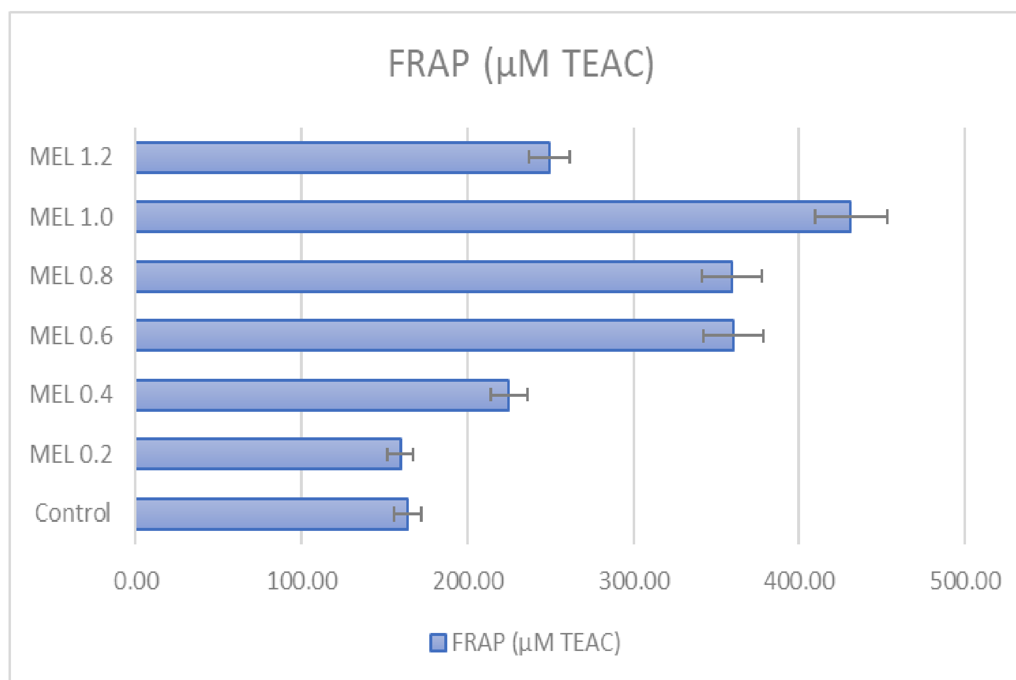


Figure 4.6: FRAP assay to estimate antioxidant activities in callus culture treated with various melatonin concentrations.

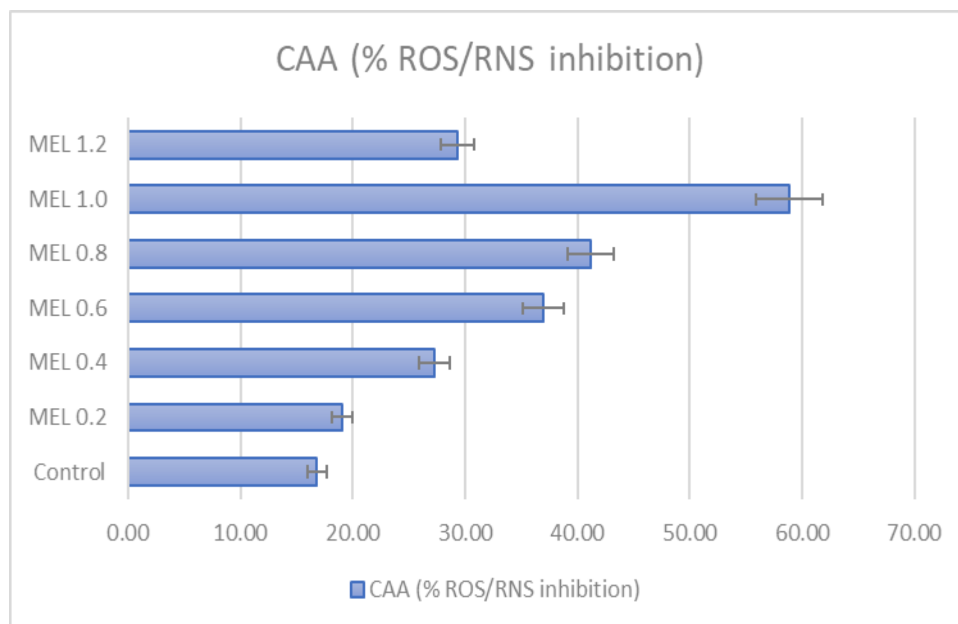
DRSML

Figure 4.6 reports: Among all the concentrations the highest FRAP activity (431.12 ± 26.1 µM TEAC) was observed at 1.0 mg/L of melatonin. These results are similar to a previous study done by (Nazir et al., 2020) where the FRAP activity was increased by the treatment of melatonin. Melatonin's antioxidant effect is primarily due to antioxidant enzymes that are triggered by it. This enzyme plays a critical function in defending plants from oxidative damage and boosts the effectiveness of the mitochondrial electron transport chain (Li et al., 2012). While the FRAP activity of the control sample was (163.95 ± 17.1 µM TEAC). The lowest value of the FRAP activity (159.62 ± 15.9 µM TEAC) was observed at 0.2 mg/L of melatonin.

4.3.5. CAA

This biologically representative approach is mostly used to evaluate the antioxidant capacity of phytochemicals, food extracts, and dietary supplements in cell culture. The level (percentage) of

inhibition of highly reactive oxygen species and nitrogen species (RONS) was also evaluated for extract from callus culture produced under various melatonin treatments, as shown in figure 4.7.



DRSML

Figure 4.7: CAA to estimate antioxidant activities in callus culture treated with various melatonin concentrations.

In callus extract cultured at 1.0 mg/L of melatonin, remarkable suppression of the ROS/RNS production (58.88%) was evaluated. At this concentration, a surprisingly substantial positive connection was found between the formation of secondary metabolites and cellular antioxidants. These results are supportive of previously performed study (Nazir et al., 2020) in which the suppression of the ROS/RNS production was enhanced by treatment of melatonin. The suppression of the ROS/RNS production in the control sample was 16.78%. The least suppression of the ROS/RNS production (19.03%) was observed at 0.2 mg/L of melatonin.

4.4 Antioxidant activities combined

Figure 4.8 shows the combined data for all of the antioxidant activities mentioned above.

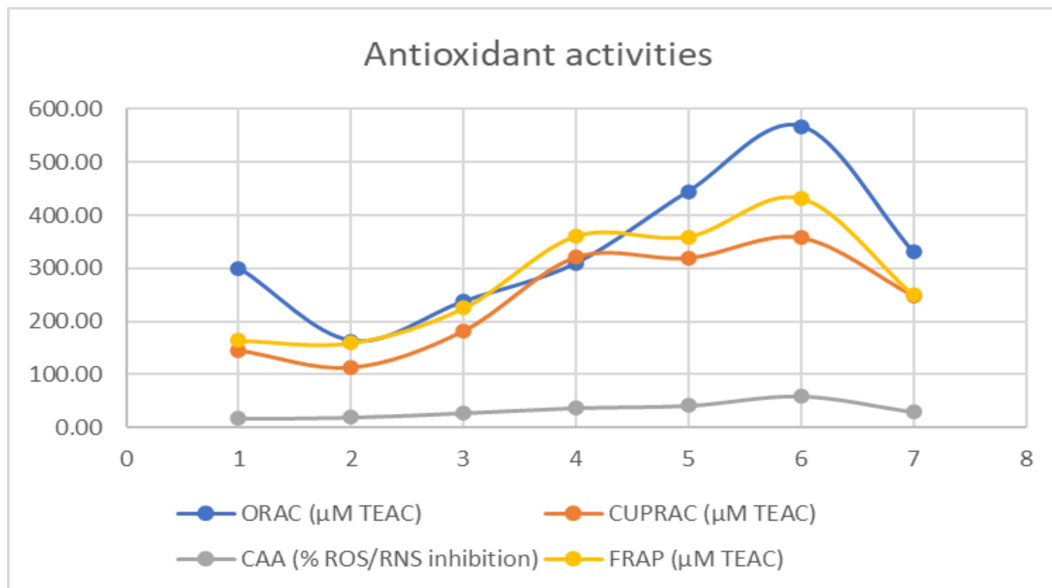


Figure 4.8: Antioxidant activities combined

DRSML

Conclusion

In the current work, the potential impact of various melatonin treatments as an elicitor on callus culture has been exploited for improved biomass accumulation and antioxidant activities. In comparison to other melatonin treatments, 0.6 mg/L of melatonin found to be highest efficient concentration for biomass accumulation. Additionally, phytochemical analysis using HPLC showed that caffeic acid, ferulic acid, rutin, myricetin, gallic acid, daidzein, genistein, epicatechin, and kaempferol had the maximum levels of production. The concentration of these secondary metabolites in calluses is greatest in callus treated with 1.0 mg/L of melatonin. Therefore, we can say that this is a growth non associated study. The production of phenolic compounds and antioxidant capacity also directly correlate, with both reaching their peak levels at 1.0 mg/L melatonin. Therefore, we can conclude that the antioxidant activities in the *Macrotyloma uniflorum* adventitious root culture are caused by these phenolic substances. It is evident that melatonin treatment of *Macrotyloma uniflorum* callus culture has the potential to further scale up the production of significant phytochemicals, even at industrial levels.

DRSML QAU

References

DRSML QAU

References

Abbasi, B. H., Ali, H., Yücesan, B., Saeed, S., Rehman, K., & Khan, M. A. (2016). Evaluation of biochemical markers during somatic embryogenesis in Silybum marianum L. *3 Biotech, 6*, 1-8.

Abbasi, B. H., Khan, M. A., Mahmood, T., Ahmad, M., Chaudhary, M. F., & Khan, M. A. (2010). Shoot regeneration and free-radical scavenging activity in Silybum marianum L. *Plant Cell, Tissue and Organ Culture (PCTOC), 101*(3), 371-376. doi: 10.1007/s11240-010-9692-x

Aditya, J., Bhartiya, A., Chahota, R. K., Joshi, D., Chandra, N., Kant, L., & Pattanayak, A. (2019). Ancient orphan legume horse gram: a potential food and forage crop of future. *Planta, 250*, 891-909.

Auxilia, L. R., & Sundari, T. M. (2013). Molecular Docking Studies of Dolichin A and B, Pterocarpans from Horsegram (Macrotyloma uniflorum) against HIV Replication Enzymes. *International Journal of Computer Applications, 75*, 19-23.

Auxilia, L. R., Sundari, T. M., & Daniel, R. R. (2013). Molecular Docking Studies of Dolichin A and B, Pterocarpans from Horsegram (Macrotyloma uniflorum) against HIV Replication Enzymes. *International Journal of Computer Applications, 75*(14).

Baenas, N., García-Viguera, C., & Moreno, D. A. (2014). Elicitation: a tool for enriching the bioactive composition of foods. *Molecules, 19*(9), 13541-13563.

Benjamin, E. D., Ishaku, G. A., Peingurta, F. A., & Afolabi, A. S. (2019). Callus culture for the production of therapeutic compounds. *American Journal of Plant Biology, 4*(4), 76-84.

Bharathi, V., & Anand, A. V. (2016). Chemical characterization from GC-MS studies of ethanolic extract of Macrotyloma uniflorum. *Research Journal of Pharmacy and Technology, 9*(3), 238-240.

Bhartiya, A., Aditya, J., & Kant, L. (2015). Nutritional and remedial potential of an underutilized food legume horsegram (Macrotyloma uniflorum): a review. *JAPS: Journal of Animal & Plant Sciences, 25*(4).

Blumenthal, M., & Staples, I. (1993). Origin, evaluation and use of Macrotyloma as forage-a review. *Tropical Grasslands (Australia)*.

Chadipiralla, K., Gayathri, P., Rajani, V., & Reddy, P. V. B. (2020). Plant tissue culture and crop improvement. *Sustainable Agriculture in the Era of Climate Change*, 391-412.

Chakraborty, P., & Abraham, J. (2016). Antimicrobial and cytotoxic effects of Macrotyloma uniflorum extract. *IJPPR, 8*(8), 1334-1340.

Das, I., Gupta, S., Ansari, S. A., Pandey, V., & Rastogi, R. (2005). In vitro inhibition and dissolution of calcium oxalate by edible plant Trianthema monogyna and pulse Macrotyloma uniflorum extracts. *Journal of crystal growth, 273*(3-4), 546-554.

Dinore, J. M., Patil, H. S., Dobhal, B. S., & Farooqui, M. (2022). Phytochemical analysis by GC-MS, LC-MS complementary approaches and antimicrobial activity investigation of Vigna unguiculata (L.) Walp. leaves. *Natural Product Research, 36*(21), 5631-5637.

Drouet, S., Leclerc, E. A., Garros, L., Tungmunnithum, D., Kabra, A., Abbasi, B. H., . . . Hano, C. (2019). A green ultrasound-assisted extraction optimization of the natural antioxidant and anti-aging flavonolignans from milk thistle Silybum marianum (L.) gaertn. fruits for cosmetic applications. *Antioxidants, 8*(8), 304.

Dulal, R. (2018). *Estimation of Protein and Secondary Metabolites of Macrotyloma uniflorum of Eastern Part of Nepal.* Central Departmental of Chemistry.

Fazal, H., Abbasi, B. H., Ahmad, N., & Ali, M. (2018a). Exogenous melatonin trigger biomass accumulation and production of stress enzymes during callogenesis in medicinally important Prunella vulgaris

DRSML QAU

L. (Selfheal). *Physiology and molecular biology of plants, 24*(6), 1307-1315. doi: 10.1007/s12298-018-0567-7

Fazal, H., Abbasi, B. H., Ahmad, N., & Ali, M. (2018b). Exogenous melatonin trigger biomass accumulation and production of stress enzymes during callogenesis in medicinally important Prunella vulgaris L.(Selfheal). *Physiology and molecular biology of plants, 24*, 1307-1315.

Fehér, A. (2019). Callus, dedifferentiation, totipotency, somatic embryogenesis: what these terms mean in the era of molecular plant biology? *Frontiers in Plant Science, 10*, 536.

Gautam, M., Katoch, S., & Chahota, R. K. (2020). Comprehensive nutritional profiling and activity directed identification of lead antioxidant, antilithiatic agent from Macrotyloma uniflorum (Lam.) Verdc. *Food Research International, 137*, 109600.

Giresha, A. S., Urs, D., Pundalik, S., Meti, R. S., Pramod, S. N., Supreetha, B. H., . . . Albogami, S. (2022). Sinapicacid Inhibits Group IIA Secretory Phospholipase A2 and Its Inflammatory Response in Mice. *Antioxidants, 11*(7), 1251.

Goswami, R. P. (2017). *Modulation of Bioactive Compounds in Underutilized Legume Horse Gram (Macrotyloma uniflorum) by Germination and Fermentation.*

Gupta, L. H., Badole, S. L., Bodhankar, S. L., & Sabharwal, S. G. (2011). Antidiabetic potential of α-amylase inhibitor from the seeds of Macrotyloma uniflorum in streptozotocin-nicotinamide-induced diabetic mice. *Pharmaceutical biology, 49*(2), 182-189.

Hernández-Ruiz, J., & Arnao, M. B. (2008). Melatonin stimulates the expansion of etiolated lupin cotyledons. *Plant Growth Regulation, 55*, 29-34.

Ingle, K. P., Al-Khayri, J. M., Chakraborty, P., Narkhede, G. W., & Suprasanna, P. (2020). Bioactive compounds of horse gram (macrotyloma uniflorum lam.[Verdc.]). *Bioactive Compounds in Underutilized Vegetables and Legumes*, 1-39.

Kaundal, S. P., & Kumar, R. (2020). Comparative Proximate Nutraceutical Study of Poor Man's Pulse, Horsegram [Macrotyloma uniflorum] with the Other Common Legume Crops: A Review. *European Journal of Nutrition & Food Safety*, 18-31.

Kaundal, S. P., Sharma, A., Kumar, R., Kumar, V., & Kumar, R. (2019). Exploration of medicinal importance of an underutilized legume crop, Macrotyloma uniflorum (Lam.) Verdc.(Horse gram): a review. *Int. J. Pharm. Sci. Res, 10*(7), 3178-3186.

Kaur, H., Mukherjee, S., Baluska, F., & Bhatla, S. C. (2015). Regulatory roles of serotonin and melatonin in abiotic stress tolerance in plants. *Plant Signaling & Behavior, 10*(11), e1049788.

Kawsar, S., Serajuddin, M., Huq, E., Nahar, N., & Ozeki, Y. (2008). Biological investigation of Macrotyloma uniflorum Linn. extracts against some pathogens. *J Biol Sci, 8*(6), 1051-1056.

Khan, I., Khan, M. A., Shehzad, M. A., Ali, A., Mohammad, S., Ali, H., . . . Ahmad, P. (2020). Micropropagation and production of health promoting lignans in Linum usitatissimum. *Plants, 9*(6), 728.

Khan, T., Abbasi, B. H., Khan, M. A., & Azeem, M. (2017). Production of biomass and useful compounds through elicitation in adventitious root cultures of Fagonia indica. *Industrial crops and products, 108*, 451-457.

Khan, T., Khan, M. A., Karam, K., Ullah, N., Mashwani, Z.-u.-R., & Nadhman, A. (2021). Plant in vitro culture technologies; a promise into factories of secondary metabolites against COVID-19. *Frontiers in Plant Science*, 356.

Khan, T., Ullah, M. A., Garros, L., Hano, C., & Abbasi, B. H. (2019). Synergistic effects of melatonin and distinct spectral lights for enhanced production of anti-cancerous compounds in callus cultures of Fagonia indica. *Journal of Photochemistry and Photobiology B: Biology, 190*, 163-171.

DRSML QAU

Khare, P., Saraswat, P., & Khare, N. (2017). Antiurolithiatic activity of ethanolic Dolichos biflorus leaf extract. *Eur J Biomed Pharm Sci, 4*(10), 758-760.

Kim, J. K., Kim, K. H., Shin, Y. C., Jang, B.-H., & Ko, S.-G. (2020). Utilization of traditional medicine in primary health care in low-and middle-income countries: a systematic review. *Health Policy and Planning, 35*(8), 1070-1083.

Kouris-Blazos, A., & Belski, R. (2016). Health benefits of legumes and pulses with a focus on Australian sweet lupins. *Asia Pacific journal of clinical nutrition, 25*(1), 1-17.

Li, C., Wang, P., Wei, Z., Liang, D., Liu, C., Yin, L., . . . Ma, F. (2012). The mitigation effects of exogenous melatonin on salinity-induced stress in Malus hupehensis. *Journal of Pineal Research, 53*(3), 298-306.

Mandle, V., Salunke, S., Gaikwad, S., Dande, K., & Patil, M. (2012). Study of nutritional value of some unique leafy vegetables grown in Latur district. *J Anim Sci Adv, 2*(3.1), 296-298.

Mehra, A., & Upadhyaya, M. (2013). Macrotyloma uniflorum Lam. A traditional crop of Kumaun Himalaya and ethnobotanical perspectives. *Int J Agric Food Sci, 3*(4), 148-150.

Miastkowska, M., & Sikora, E. (2018). Anti-aging properties of plant stem cell extracts. *Cosmetics, 5*(4), 55.

Mohamed, S. V., & Jayabalan, N. (1996). A protocol for Horsegram [Macrotyloma uniflorum (Lam.) Verdc.] callus induction. *Israel Journal of Plant Sciences, 44*(2-3), 143-145.

Moussou, P., Moser, P., Jeanmaire, C., Danoux, L., & Bardey, V. (2011). Dolichos Biflorus Extract For Use In Cosmetic Skin Treatment: Google Patents.

Murch, S. J., Campbell, S. S., & Saxena, P. K. (2001). The role of serotonin and melatonin in plant morphogenesis: regulation of auxin-induced root organogenesis in in vitro-cultured explants of St. John's wort (Hypericum perforatum L.). *In Vitro Cellular & Developmental Biology-Plant, 37*, 786-793.

Naik, P. M., & Al-Khayri, J. M. (2016). Abiotic and biotic elicitors-role in secondary metabolites production through in vitro culture of medicinal plants. *Abiotic and biotic stress in plants—recent advances and future perspectives. Rijeka: InTech*, 247-277.

Namdeo, A. (2007). Plant cell elicitation for production of secondary metabolites: a review. *Pharmacogn Rev, 1*(1), 69-79.

Nazir, M., Asad Ullah, M., Mumtaz, S., Siddiquah, A., Shah, M., Drouet, S., . . . Abbasi, B. H. (2020). Interactive effect of melatonin and UV-C on phenylpropanoid metabolite production and antioxidant potential in callus cultures of purple basil (Ocimum basilicum L. var purpurascens). *Molecules, 25*(5), 1072.

Ogita, S. (2015). Plant cell, tissue and organ culture: the most flexible foundations for plant metabolic engineering applications. *Natural Product Communications, 10*(5), 1934578X1501000527.

Oyebode, O., Kandala, N.-B., Chilton, P. J., & Lilford, R. J. (2016). Use of traditional medicine in middle-income countries: a WHO-SAGE study. *Health Policy and Planning, 31*(8), 984-991.

Özyürek, M., Güçlü, K., Tütem, E., Sözgen Başkan, K., Erçağ, E., Karademir Çelik, S., . . . Apak, R. (2011). A comprehensive review of CUPRAC methodology. *Analytical Methods, 3*, 2439-2453. doi: 10.1039/C1AY05320E

Parmar, H., Das, S., & Gohil, K. (2012). Hepatoprotective activity of Macrotyloma uniflorum seed extract on paracetamol and D-galactosamine induced liver toxicity in albino rats. *International Journal of Pharmacological Research, 2*(2), 86-91.

Patel, H., & Krishnamurthy, R. (2013). Elicitors in plant tissue culture. *Journal of Pharmacognosy and Phytochemistry, 2*(2), 60-65.

DRSML QAU

Patil, A. V., & Kasturiba, B. (2019a). A Study on Protein, in vitro Protein Digestibility, Antioxidant Activity and Total Polyphenol Content of Horsegram Varieties. *Int. J. Curr. Microbiol. App. Sci, 8*(2), 1936-1944.

Patil, A. V., & Kasturiba, B. (2019b). A study on sugar and mineral content of horsegram (Macrotyloma uniflorum (Lam) Verdc.) varieties.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of agricultural and food chemistry, 53*(10), 4290-4302.

Qiao, Y., Yin, L., Wang, B., Ke, Q., Deng, X., & Wang, S. (2019). Melatonin promotes plant growth by increasing nitrogen uptake and assimilation under nitrogen deficient condition in winter wheat. *Plant Physiology and Biochemistry, 139*, 342-349.

Rai, A., Saito, K., & Yamazaki, M. (2017). Integrated omics analysis of specialized metabolism in medicinal plants (Vol. 90, pp. 764-787): Wiley Online Library.

Rao, S. R., & Ravishankar, G. (2002). Plant cell cultures: chemical factories of secondary metabolites. *Biotechnology advances, 20*(2), 101-153.

Rlds, R., & Erhss, E. (2017). Medicinal and nutritional values of Macrotyloma uniflorum (Lam.) verdc (kulattha): A conceptual study. *Global Journal of Pharmacy & Pharmaceutical Sciences, 1*(2), 44-53.

Saeed, S., Ali, H., Khan, T., Kayani, W., & Khan, M. A. (2017). Impacts of methyl jasmonate and phenyl acetic acid on biomass accumulation and antioxidant potential in adventitious roots of Ajuga bracteosa Wall ex Benth., a high valued endangered medicinal plant. *Physiology and molecular biology of plants, 23*, 229-237.

Samarth, R. M., Samarth, M., & Matsumoto, Y. (2017). Medicinally important aromatic plants with radioprotective activity. *Future science OA, 3*(4), FSO247.

Sarropoulou, V. N., Therios, I. N., & Dimassi-Theriou, K. N. (2012). Melatonin promotes adventitious root regeneration in in vitro shoot tip explants of the commercial sweet cherry rootstocks CAB-6P (Prunus cerasus L.), Gisela 6 (P. cerasus× P. canescens), and MxM 60 (P. avium× P. mahaleb). *Journal of Pineal Research, 52*(1), 38-46.

Sree, V. K., Soundarya, M., Ravikumar, M., Reddy, T. R., & Devi, N. K. D. (2014). In vitro screening of Macrotyloma uniflorum extracts for antioxidant and anthelmintic activities. *Journal of Pharmacognosy and Phytochemistry, 3*(4), 06-10.

Sreerama, Y. N., Sashikala, V. B., & Pratape, V. M. (2010). Variability in the distribution of phenolic compounds in milled fractions of chickpea and horse gram: evaluation of their antioxidant properties. *Journal of agricultural and food chemistry, 58*(14), 8322-8330.

Sukanya, S., & Gayathri, G. (2014). Variability in the distribution of daidzein and genistein in legume sprouts and their anticancer activity with MCF-7 breast cancer cells. *Acad J Cancer Res, 7*(3), 173-178.

Suriyavathana, M., Manikandan, M., Janeesha, K., Sandhya, M., & Ram, K. (2018). Phytochemical screening and antimicrobial activity of aqueous seed extracts of Macrotyloma uniflorum. *World J Pharm Pharm Sci, 7*(9), 703-714.

Tan, D.-X., Manchester, L. C., Esteban-Zubero, E., Zhou, Z., & Reiter, R. J. (2015). Melatonin as a potent and inducible endogenous antioxidant: synthesis and metabolism. *Molecules, 20*(10), 18886-18906.

Tan, D.-X., Manchester, L. C., Reiter, R. J., Qi, W.-B., Karbownik, M., & Calvo, J. R. (2000). Significance of melatonin in antioxidative defense system: reactions and products. *Neurosignals, 9*(3-4), 137-159.

DRSML QAU

Thirukkumar, S., & Sindumathi, G. (2014). Studies on preparation of processed horse gram (Macrotyloma uniflorum) flour incorporated chappathi. *Int J Sci Res, 3*(3), 110-111.

Tungmunnithum, D., Abid, M., Elamrani, A., Drouet, S., Addi, M., & Hano, C. (2020). Almond Skin Extracts and Chlorogenic Acid Delay Chronological Aging and Enhanced Oxidative Stress Response in Yeast. *Life, 10*(6), 80.

Vandarkuzhali, P., & Narayanasamy, S. (2015). Horse Gram-A prospective functional food. *The Indian Journal of Nutrition and Dietetics*, 226-243.

Yang, L., & Stöckigt, J. (2010). Trends for diverse production strategies of plant medicinal alkaloids. *Natural product reports, 27*(10), 1469-1479.

Zhong, Y., & Shahidi, F. (2015). Methods for the assessment of antioxidant activity in foods *Handbook of antioxidants for food preservation* (pp. 287-333): Elsevier.

Zhu, Y., Li, X., Yang, W., Jia, H., Liu, C., Yash, P., & Souravh, B. (2018). Anti-depressant activity of standardized Macrotyloma uniflorum extract in experimental models of depression in rats. *International Journal of Pharmacology, 14*(6), 848-855.

DRSML QAU