Organogenic Response of *Brassica nigra* Stem Explant by Lupeol and Their Phytochemical Analysis

BY

Muhammad Mustajab Khan

Registration No: 02272113007

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Department of Biotechnology

Faculty of Biological Sciences

Quaid-i-Azam, University

Islamabad, Pakistan

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Organogenic Response of *Brassica nigra* Stem Explant by Lupeol and Their Phytochemical Analysis

By
Muhammad Mustajab Khan
Supervisor
Dr. Muhammad Zia

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DECLARATION

I hereby confirm that the work and research presented in this thesis are primarily my own efforts, except where I have clearly acknowledged the contribution of others. This thesis is an original composition and has not been previously submitted for any other degree or academic qualification.

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Muhammad Mustajab Khan

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I, Muhammad Mustajab Khan, son of Muhammad Mashiyat Ullah, with the registration ID 02272113007, am pursuing an MPhil in Biotechnology at the Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan. I hereby confirm that my thesis, titled "Organogenic Response of *Brassica nigra* Stem Explant by Lupeol and Their Phytochemical Analysis," is a genuine work of research carried out under the guidance of Dr. Muhammad Zia. This work has not been submitted or published anywhere else.

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DEDICATION

This work is wholeheartedly dedicated to my beloved parents. Their steadfast support, endless encouragement, invaluable advice, and loving prayers have been the guiding lights on my journey, shaping me into who I am today.

Muhammad Mustajab Khan

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LIST OF ABBREVIATIONS

DMSO	Di-methyl-sulfoxide
DPPH	2,2-diphenyl -1-picrylhydrazile
DW	Dry weight
FC	Folin ciocalteu
FW	Fresh weight
GAE	Gallic acid equaivalent
BAP	Benzylaminopurine
LSD	Least significance difference
NAA	Naphthalene acetic acid
PGRs	Plant growth regulators
POD	Peroxidase
ROS	Reactive oxygen species
SD	Standard deviation
SE	Somatic embryogenesis
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TFC	Total flavonoid content
TPC	Total phenolic content
TRP	Total reducing power

ABSTRACT

The primary objective of this study was to investigate the impact of Lupeol, a triterpenoid molecule, on *Brassica nigra*. The therapeutic potential of this plant is significant, and Lupeol is recognized for its contributions to plant defense and growth. This study examined the effect of different concentrations of Lupeol (1 µM, 5 µM, and 10 µM) on the biochemical and growth parameters of *Brassica nigra*. Developmental factors such as root and shoot length, as well as fresh and dry weight, exhibited a remarkable increase compared to control. The study indicates significant increase in root (296%) and shoot (65%) lengths at 10 µM Lupeol. Fresh weight of both roots and shoots showed 149% and 67% increase, respectively while the dry weight increased up to 6% and 116%, respectively at 10 µM Lupeol. Enzymatic activities of Superoxide Dismutase (SOD) and Peroxidase (POD) exhibited maximum activity at a concentration of 5 µM Lupeol, which aligns with the observed trends in other biochemical indicators. A significant proportion of the biochemical indicators, including Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant activities such as DPPH, ABTS, Metal Chelating (MC), TAC, and TRP exhibited highest levels at a Lupeol concentration of 5 µM. Total phenolic content (TPC) in shoots reached its maximum level at this particular dose, measuring 37.68 µgGAE/mg DW. At 5 µM Lupeol, TFC in shoots was 21.76 gQE/mg DW, ABTS activity was 45%, MC activity was 54.97%, Total Antioxidant Capacity (TAC) was 84.28 gAAE/mg DW, and Total Reducing Power (TRP) was 95.20 µgAAE/mg DW. The scavenging activity of 2,2-diphenyl-1- picrylhydrazyl (DPPH) was most pronounced in shoots at the aforementioned concentration, exhibiting a scavenging rate of 60%. These results indicate that the 5 µM Lupeol treatment has a complex range of bioactive effects on plant shoots, alongside its impact on growth parameters like fresh and dry weight. In conclusion, Lupeol appears to be a potent bioactive molecule that promotes growth and protects Brassica nigra from oxidative damage.

CHAPTER 1

1 INTRODUCTION AND REVIEW OF LITERATURE

1.1 *Brassica nigra*

Black mustard, also referred to as *Brassica nigra*, is a plant species that has considerable impact on both the medical and agricultural industries. Due to its many valuable qualities and applications, this member of the Brassicaceae family is grown for generations. They are primarily farmed for their condiment potential. Due to its cruciferous flowers, these plants are easily differentiated from other flowering plants (Augustine et al., 2013). The *B. nigra* plant can grow up to 2 m tall and can induce flowering without vernalization. The flowers have a yellow color, and the seeds can be found in shades ranging from dark to brown having 1 to 2 mm in diameter (Rakow, 2004). Evidence of historic cultivation of Brassica species in the Middle East, India, China, Rome, and Greece, where *B. nigra* seed was used as a spice and a medicine, has shown the origin of these species (Warwick et al., 2006).

1.2 Medicinal Importance

1.2.1 Traditional Medicinal Uses

Different civilizations have a long history of using *B. nigra* as a traditional medicine. The plant's medicinal benefits have been used in relation to its seeds, leaves, and stems. *B. nigra* has been used in traditional medicine to treat symptoms like respiratory difficulties, rheumatic disorders, digestive issues, and skin infections.

1.2.2 Anti-inflammatory and Analgesic Properties

According to studies, *B. nigra* has strong analgesic and anti-inflammatory effects. The plant is a possible option for the development of natural anti-inflammatory medications since extracts from several portions of the plant have shown inhibitory effects on inflammatory mediators (Ayati et al., 2021).

1.2.3 Antimicrobial Activity

Extracts from the *B. nigra* plant have shown notable antibacterial efficacy against various diseases, including bacteria, fungi, and viruses (Feeny & Rosenberry, 1982). Because of its antibacterial properties, *B. nigra* is a useful plant for creating organic antimicrobial agents or for use as a component of topical treatments for skin infections and wound healing.

1.2.4 Antioxidant and Anticancer Effects

In *B. nigra*, bioactive substances such as glucosinolates and phenolic compounds are present, which adds to the plant's strong antioxidant properties. These anti-oxidants aid in scavenging dangerous free radicals and guard against illnesses brought on by oxidative stress. Additionally, studies indicate that *B. nigra* inhibits the development of tumors and causes the death of cancer cells (Foster & Duke, 2000; Ahmed et al., 2020).

1.3 Agricultural Importance

1.3.1 Cover Crop and Green Manure

Brassica nigra is frequently used as a cover crop or green manure crop. It is a great option for weed suppression and erosion management due to its quick growth and heavy foliage. As a winter cover crop, brassicas may provide more than 80% of soil coverage (Haramoto & Gallandt, 2004). *B. nigra* contributes organic matter to the soil, enhances soil structure, and produces useful chemicals that encourage nutrient availability for subsequent crops.

1.3.2 Biofumigation

The ability of *Brassica nigra* to biofumigate is widely established. Its plant leftovers emit volatile chemicals known as isothiocyanates as they break down in the soil. Strong antifungal, antibacterial, and nematocidal properties of these chemicals allow them to naturally manage soil-borne pests and diseases while decreasing the need for synthetic chemical treatments (Larkin & Lynch, 2018).

1.3.3 Nutritional Value

The seeds of *B. nigra* are an abundant source of essential nutrients, such as proteins, dietary fibers, minerals, and vitamins (Danlami, 2016). Its leaves are eaten as a salad. *Brassica nigra* contributes to a balanced nutritional intake and promotes overall health when included in diets.

1.4 Plant Growth Regulators

All plant species possess inherent hormones that facilitate their ability to adapt and flourish within their respective environments. These hormones exert regulatory control over several aspects of an organism's physiology, including growth, development, morphology, and physiological function. Hormones, acting as signaling molecules, traverse the internal pathways of plants, selectively reaching target cells to initiate physiological reactions. Various plant organs, including buds, leaves, and roots, are responsible for the production of these substances (Rademacher, 2015). Hormones exert effect on several aspects of cellular processes in plants, including cell division, cell growth, cell structure, and function, as well as the plant's

response to external stressors (Ferguson & Grafton-Cardwell, 2014). The effects and responses of plant hormones can vary significantly based on factors such as the specific cells or tissues they target, the developmental stage of the plant, the relative concentration of hormones in relation to other hormones, the availability and absorption of nutrients and water, as well as the prevailing weather and climatic circumstances (Ferguson & Grafton-Cardwell, 2014).

The investigation of hormones to enhance crop growth and productivity has been ongoing since the 1930s (Rademacher, 2015). These hormones regulate and influence several physiological processes in plants (Ferguson & Grafton-Cardwell, 2014). It is very difficult to isolate, identify, and extract enough of these hormones for lab testing since they are only found in very small concentrations in plant tissue (Rademacher, 2015). Synthetic hormones also effectively regulate several biological processes, including the growth and differentiation of roots, stems, leaves, flowers, and fruits (Flasiński & Hąc-Wydro, 2014). The aforementioned finding resulted in the development of synthetic hormone compounds, commonly referred to as plant growth regulators.

The utilization of PGRs has been demonstrated to augment the process of germination, as well as the subsequent growth and development of plants, through their ability to modulate the hormonal activity inside the plant (Hopkins & H¨uner, 2009). The response of plants to PGRs is influenced by several aspects, including plant type, stimulant, dosage, timing, growth stage, and application site (Mitchell, 1942).

PGRs are categorized into five primary types, namely auxins, gibberellins, cytokinins, ethylene, and abscisic acid. The regulation of phytohormonal activity has been demonstrated by recently identified PGRs, including brassinosteroids and jasmonates (Rademacher, 2015). These hormones work together to control every aspect of plant life.

1.5 Lupeol

1.5.1 Introduction

Lupeol, a naturally occurring triterpene compound, has been extensively studied and identified in a diverse range of plant species, including but not limited to succulent fruits like mangoes and various medicinal plants such as the renowned Aloe Vera. This biologically active compound, with its distinct chemical structure and unique properties, has garnered significant attention from the scientific community due to its potential therapeutic applications and pharmacological activities. Lupeol belongs to the triterpenoid family, which encompasses a vast array of naturally occurring compounds widely distributed in the plant kingdom.

1.5.2 Chemical properties

of Lupeol include:

Chemical formula: C30H50O

Molecular weight: 426.72 g/mol

Other Names: Fagarasterol, Clerodol

Structure: Lupane in which a hydroxyl group replaces the hydrogen at the 3beta position, making it a pentacyclic triterpenoid. It has one five-membered ring (in an envelope conformation) and four six-membered rings (in chair conformations). These rings are bonded together in a trans orientation (Corrêa et al., 2009).

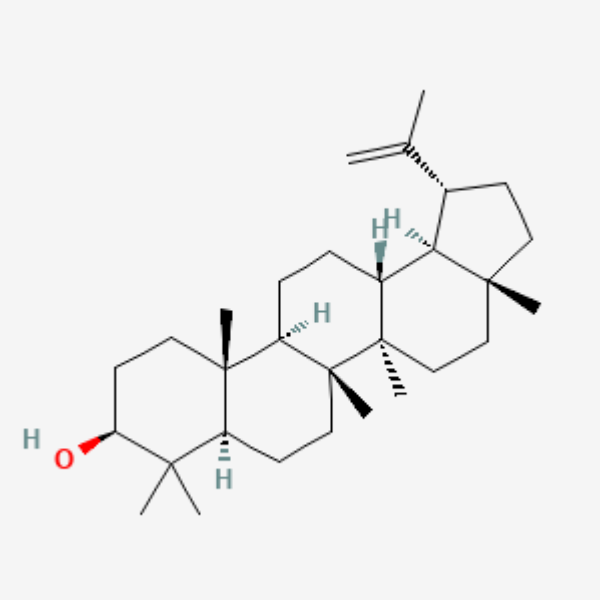

Figure 1.1 *Structure of Lupeol*

Melting point: 215-216 °C (Saleem, 2009).

Physical appearance: a white crystalline solid at room temperature (Corrêa et al., 2009).

Solubility: It is soluble in organic solvents including chloroform, ether, and ethanol, but not in water due to its hydrophobic nature.

Stability: The structure is fairly stable under standard conditions according to Corrêa et al. (2009).

Its presence in numerous botanical sources defines its ecological significance and evolutionary importance. The structural backbone of a pentacyclic triterpene skeleton is characterized by the fusion of five rings, which imparts remarkable stability and rigidity to the molecule. The occurrence of Lupeol in various plant species, including fruits and medicinal plants, highlights its ubiquitous nature and ecological relevance. Extensive scientific investigations have been conducted to explore the multifaceted properties of this compound, with a particular focus on its potential therapeutic efficacy. These investigations have delved into various aspects of its pharmacological profile, shedding light on its anti-inflammatory, antiproliferative, and antihyperglycemic activities. By meticulously examining its mechanisms of action, researchers have sought to unravel the intricate molecular pathways through which this compound exerts its beneficial effects. Through these rigorous scientific endeavors, a growing body of evidence has emerged, underscoring the promising potential of this compound as a valuable therapeutic agent in the realm of medicine.

1.5.3 Natural sources of Lupeol in plants

Lupeol is present in medicinal plants such as American ginseng, Shea butter plant, Tamarindus indica, Allanblackia monticola, Himatanthus succuba, Celastrus paniculatus, Zanthoxylum riedelianum, Leptadenia hastata, Crataeva nurvala, Bombax ceiba and Sebastiania adenophora used by native people in North America, Latin America, Japan, China, Africa, and Caribbean islands and it is also found in olive, fig, mango, strawberry, red grapes, Japanese Pear, white cabbage, pepper, cucumber, tomato, carrot, Cajanus cajan, Capsicum annuum, Coccinia grandis, Cucumis sativa (Saleem, 2009). Table 1.1 represents the list of plants that have been reported to contain significant quantities of Lupeol.

Table 1.1: List of plants containing Lupeol

S.no	Plants	Scientific name	Content	Reference
Medicinal plants				
1	Elm plant	Ulmus spp.	880 µg/g bark	(Nguemfo et al., 2009)
2	Bitterroot	Apocynum cannabinum		(Imam et al., 2007)
3	Aloe leaves	Aloe vera L.	280 µg/g dry leaf	(Nguemfo et al., 2009)
Vegetables				

4	Tea	Camellia sinensis	-	(Imam et al., 2007)
5	Cucumber	Cucumis sativus	-	(Imam et al., 2007)
6	Carrot	Daucus carota	-	(Imam et al., 2007)
7	Capsicum	Capsicum annum	-	(Imam et al., 2007)
8	Common pea	Pisum sativum	-	(Imam et al., 2007)
9	Tomato	Lycopersicon esculentum	-	(Imam et al., 2007)
Fruits				
10	Grapes	Vitis vinifera		(Imam et al., 2007)
11	Mango fruit	Mangifera indica L.	180 µg/g	(Nguemfo et al., 2009)
12	Japanese pear	Pyrus pyrifolia	175 µg/g twig bar	(Nguemfo et al., 2009)
13	Olive fruit	Olea europa L.	3 µg/g of fruit	(Nguemfo et al., 2009)
14	Date palm	Phoenix dactylifera	-	(Imam et al., 2007)
15	Ginseng oil	-	15.2 mg/100g	(Nguemfo et al., 2009)
16	Common fig	Ficus carica	-	(Imam et al., 2007)
17	Guava	Psidium guajava	-	(Imam et al., 2007)
18	Mango sps (peel like banana	Mangifera pajang Kosterm	> 300 (µg/ml)	(Ahmad et al., 2015)
19	White mulberry	Morus alba	-	(Imam et al., 2007)

1.5.4 Role of Lupeol in Plants

Lupeol, a naturally occurring triterpenoid compound, has been observed to manifest in various plant species in its unmodified form, predominantly within resins or waxes, as described by Szakiel et al. (2012). Although the mechanism for Lupeol biosynthesis is widely recognized, it is still not completely understood how it is regulated (Li et al., 2021). Additionally, it assumes a pivotal role as a precursor for the biosynthesis of other specialized triterpenoid metabolites, which are known to play crucial roles in the complex mechanisms of plant defense and development.

It has been found to play a crucial role in the intricate process of nodule formation in *Lotus japonicus*, a legume plant species. This pivotal involvement is primarily attributed to its ability

to regulate the expression of the ENOD40 gene, which is known to be intricately associated with the establishment and development of nodules in leguminous plants. Delis et al. (2011) also found that the OSC3 gene is the only factor responsible for the production of Lupeol in *L. japonicus* roots and nodules. Through its regulatory influence on ENOD40 gene expression, Lupeol exerts a profound impact on the molecular mechanisms underlying nodule formation, thereby contributing to the successful symbiotic relationship between the plant and nitrogen-fixing bacteria (Delis et al., 2011).

Lupeol is also a component of the castor bean plant's (Ricinus communis) cuticular wax surface, where it has been postulated that it may serve a physiological function in safeguarding the plant against the detrimental effects of dehydration (Ortwin Guhling et al., 2006).

Furthermore, it is worth noting that Lupeol serves as the precursor molecule for the synthesis of betulinic acid, a triterpenoid compound that exhibits remarkable anticancer properties (Jin et al., 2019) (André Moreira Cunha et al., 2021). This intriguing natural product is known to accumulate specifically in the bark tissues of birch trees (Cárdenas et al., 2019). The potent anticancer activity of betulinic acid has been extensively investigated and has shown promising results in various preclinical studies (Jiang et al., 2021) (Oliveira-Costa et al., 2022).

1.5.5 Medicinal Importance of Lupeol

Lupeol, a naturally occurring triterpenoid compound, can be found abundantly in a diverse range of consumable fruits, vegetables, and various medicinal plants. Over the past ten years, an abundance of scientific investigations has been undertaken to explore the pharmacological properties of Lupeol among which some are shown in Table 1.2. These studies have established that Lupeol exhibits a wide array of pharmacological effects, including but not limited to its remarkable anticancer, antioxidant, anti-inflammatory, and antimicrobial activities.

Table 1.2: Medicinal properties of Lupeol

Effect	Model/Cell lines	Activity	Reference
Neuroprotection	Mice	Neuroinflammation was prevented by inhibiting activated glial cells and inflammatory mediators. Reduced Aβ and beta-secretase-1 (BACE-1) expression and	(Ahmad et al., 2020)

		improved memory and cognitive function.	
Anti-inflammatory	Male Swiss albino mice	Significant pro-inflammatory cytokine suppression was observed.	(Ahmad et al., 2010)
Anti-oxidative	Rabbit nucleus pulposus cells (NPCs)	Increased the anti-oxidative stress in the mitochondria, which prevented high-glucose-induced apoptosis in NPCs.	(Guo et al., 2018)
Anti-oxidative	Male Swiss albino mice	Suppressed BPO-induced oxidative stress, ODC activity, and increased thymidine incorporation in murine skin.	(Saleem et al., 2001)
Apoptosis	Human non-small cell lung cancer (NSCLC)	Reduced EGFR and STAT3 activity and induced apoptosis in NSCLC cells.	(Tae Rin Min et al., 2019)
Anti-inflammatory	Human neutrophils	Suppressed the production of superoxide by blocking tyrosyl phosphorylation of a 45.0-kDa protein in neutrophils.	(Yamashita et al., 2002)
Anti-inflammatory	Rat hepatocytes	Restored levels of antioxidant enzyme, lowered lipid peroxidation, inhibited ROS production, and prevented mitochondrial depolarization.	(Kumari & Kakkar, 2012)
Anti-inflammatory	Human skin in-vitro	Downregulated NF-kappaB expression in keratinocytes and upregulated matrix metalloproteinase (MMP)-2, indicating anti-inflammatory activity.	(Pereira Beserra et al., 2018)

Apoptosis	Human, HCC cell lines SMMC7721 and HepG2.	HCC cell lines SMMC7721 and HepG2 were induced to undergo apoptosis.	(He et al., 2011)
Apoptosis	Human colorectal cancer (CRC) cells	Enhanced apoptosis in CRC cells that have Wnt/β-catenin signaling that is always on.	(Tarapore et al., 2013)
Tumor Repression	Human OS cells	Repressed the tumor by regulating the expression of an oncogene miR-212-3p, which targeted HMGA2	(Zhong et al., 2020)
Apoptosis	Human, in-vitro 2D cell line models (HEp-2, UPCI:SCC-131)	Increased the expression of p53 which in turn induced the expression of Bax and turned on the apoptosis pathway.	(Bhattacharyya et al., 2016)
Cell Cycle Arrest	Human cervical carcinoma (HeLa) cells	Induced S-phase cell cycle arrest, decreased S-phase Cyclins and CDKs, and elevated the expression of cyclin-dependent kinase inhibitors, p21 at transcription and translation.	(Prasad et al., 2018)
Anti-inflammatory	Neonatal rat cardiomyocytes (NRCMs)	Inhibited nuclear translocation of NF-B p65 and decreased inflammatory cytokines.	(Li et al., 2022)
Anti-proliferative	Human, MDA-MB-231 breast cancer cells	Reduced TNBC proliferation via the PI3K/mTOR pathway-induced autophagy and inhibited TNBC metastasis via EMT by autophagy-mediated Twist1 downregulation.	(Zhang et al., 2022)

1.6 Organogenesis

1.6.1 Definition

Organogenesis refers to the initiation and development of an organ. Inducing organogenesis is a crucial aspect of plant tissue culture for regenerating plants from the culture. During the process of organogenesis, the emergence of new plants occurs through the development of axillary or adventitious buds in a controlled laboratory environment. These buds undergo elongation to form shoots and subsequently give rise to adventitious roots. The process of organogenesis can frequently be accomplished using tissues derived from seedlings, and occasionally from fully developed trees (Meilan, 2004).

There are two types of organogenesis:

- Indirect Organogrnesis
- Direct Organogenesis

1.6.2 Indirect Organogenesis

Indirect organogenesis refers to organ development that occurs via the callus rather than the embryo. Although clonal fidelity cannot be guaranteed through the induction of plants, this method may be well suited for mass production and the selection of somaclonal variants with desirable traits. Transgenic plants can be cultivated through the induction of plants during the callus phase. This is achieved by either transforming the callus, then regenerating the plant from it, or by first transforming an explant and then cultivating a callus and shoots from it (Bhatia & Bera, 2015).

1.6.3 Direct Organogenesis

Direct organogenesis refers to the process by which buds or shoots are formed directly from tissue, skipping the callus stage. Improved multiplication rates (high number of plants per explant) have been achieved through the use of direct organogenesis in plant propagation, leading to lower operational costs. More transgenic plants have been generated using this method. And because it guarantees the production of uniform planting material without genetic variation, it has been used for clonal propagation (the genetic multiplication of a cultivar without sexual reproduction) (Gallego, n.d.).

1.7 Role of Lupeol in Plant Tissue Culture

As of the present moment, it is worth noting that there exists a significant lack of comprehensive and substantial data about the exogenous administration of Lupeol in the

context of plant tissue culture. The existence of this research gap serves to highlight the imperative requirement for conducting comprehensive studies that aim to provide a thorough understanding of the potential effects and implications of this particular compound about the intricate processes of in vitro plant growth and development.

1.8 Aims and Objectives

- To comprehensively analyze the impact of Lupeol on the development of *Brassica nigra* stems explant.
- To evaluate the existing research gaps about the utilization of Lupeol in plant tissue culture and subsequently provide potential avenues for further investigation.
- To broaden our knowledge of the response of *Brassica nigra* to Lupeol and to examine its potential advantages in terms of plant development and crop improvement.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Chemicals and Equipment

Chemicals employed in this study include methanol, plant growth regulators, hydrochloric acid, ethanol, sodium hydroxide, mercuric chloride, and distilled water. The equipment comprises forceps, blades, spatula, filter paper, pH meter, autoclave, electrical balance, spirit lamp, laminar flow transfer cabinet, and glassware (glass beaker, petri dish, measuring cylinder, Erlenmeyer flask).

2.2 Media preparation

All of the tests were conducted with standard MS (Murashige and Skoog, 1962) media. The seeds were germinated in a growth regulator-free environment using the MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose for in-vitro germination. Using 0.1 M NaOH and 0.1 M HCl, the media pH was brought up to 5.8. Gelrite (0.7%) was added as a solidifying agent and dissolved via heating. The media was dispensed at a rate of 30 mL per 100 mL conical flask. The medium was autoclaved at 121°C and 15 pounds per square inch for 20 mins.

2.3 Seed sterilization and preparation of explants

After being washed twice with running water and treated with detergent tween-20 for 2 mins, the seeds of *B. nigra* were subjected to a 5-10 minute soak in 70% ethanol. The seeds were surface sterilized with mercuric chloride ($HgCl_2$) at a concentration of 0.1% for 5 mins following washing. After surface sterilization, three rounds of washing in sterile water were carried out to eliminate any remaining $HgCl_2$. Six seeds were planted in each flask. The evenly spaced points from seedlings' stems that had germinated in vitro were cut off and used as explants.

2.4 Inoculation and culture condition

Stems of in vitro germinated seedlings were cut off at a length of 0.5 to 1.0 cm to be used as explants. The explants were inoculated onto MS basal medium that had been supplemented with varying concentrations of plant growth regulators (Lupeol, BAP, and NAA) without undergoing any surface sterilization. There were three different Lupeol concentrations used, with each having four replicates: 1 µM, 5 µM, and 10 µM. The NAA hormone, at 1 µM, was

used in four replicates as a positive control. Also serving as positive controls were four replicates of a single concentration of 10 µM of the BAP hormone (the table includes NAA, BAP, and Lupeol concentrations). Six explants were inserted into each flask for each treatment. The flasks were kept at 8/16 dark/light cycle using white LEDs (380-780 nm, PPFD 15 mol/m2/s).

2.5 Morphological Characteristics

The root and shoot lengths were measured using the standard method after the plants had been harvested after 29 days. As well, the number of leaves was calculated.

2.6 Fresh and dry weight of roots and shoots

After 19 days, the plants' shoots and roots were cut off and separated, and their fresh weight was determined using an electronic measuring balance. Then, for the next three days, the plants were dried slowly at 37 ℃ in an incubator using Petri dishes. As soon as the roots and shoots were dry, they were weighed on the measuring balance.

2.7 Determination of antioxidant enzyme activities

To separate the enzyme activities of the shoots and roots, they were harvested fresh, washed in distilled water, crushed in phosphate buffer (pH 7.0), placed in individual Eppendorf tubes, and centrifuged for 10 mins at 10000 rpm. The collected supernatant was then put to use in several different enzymatic assays, including;

2.7.1 Super-oxide Dismutase (SOD)

Superoxide dismutase (SOD) activity was measured using the method described by Ullah et al. (2019). The following chemical components were required,

Ingredients: 1 mM EDTA, 130 mM methionine, 0.75 mM NBT, and 0.02 mM riboflavin.

Preparing the reaction mixture of 200 µL required mixing 20 µL EDTA, 20 µL methionine, 20 µL NBT, 78 µL phosphate buffer, 2 µL riboflavin, and 60 µL enzyme extract in a 96-well plate. Except for the control well, all the wells contained the same combination of chemicals. There was no enzyme extract used in the control group. Following 7 mins of incubation under fluorescent light, the mixture's absorbance was measured using a microplate reader set to 560 nm, and the following formula was applied to express the enzyme's activity:

$A = ELC$

The absorbance of sample (A), enzyme concentration (nM/min/mg FW) (C), extinction coefficient (6.39 mM-1 cm-1) (E), and wall thickness (L) are given in this equation.

2.7.2 Per-oxidase activity (POD)

The POD activity of extracts from fresh *B. nigra* shoots and roots were measured using Lagrimini's (1992) technique.

100 mM guaiacol, dH2O, 27.5 mM hydrogen peroxide, and a 50 mM KH_2PO_4 buffer were used. In a 96-well plate, a reaction mixture of 200 µL was prepared by combining 40 µL of KH_2PO_4 buffer with 20 µL of guaiacol, 20 µL of each sample, 100 µL of dH_2O, and 20 µL of H_2O_2. Except for the control well, all the wells contained the same combination of chemicals. There was no enzyme extract used in the control group. Absorbance at 470 nm was measured with a microplate reader, and the following formula was used to express enzymatic activity:

A = ELC

Here, A: sample absorbance, C: enzyme concentration (nM/min/mg FW), E: extinction coefficient (6.39 mM-1 cm-1), and L: length of the wall (0.25 cm).

2.8 Biological and biochemical screening of roots and shoots

The dried roots and shoots were ground up with a pestle and mortar and then placed in Eppendorf tubes. One milliliter of DMSO was added to each test tube after reweighing the crushed roots and shoots. Afterward, these samples were put through several biological assays, including:

2.8.1 Phytochemical screening

For the phytochemical analysis, the total phenolic and total flavonoid contents of test samples of roots and shoots of *B. nigra* were determined.

2.8.1.1 Determination of total phenolic contents (TPC)

To determine the TPC of test samples, we used the procedure described by Astill et al. (2001). 20 µL of the sample, blank, and standard (sample, DMSO, and Gallic acid), respectively, were added to each well of the 96-well plate. Then, 90 µL of freshly diluted FC (Folin- Ciocalteu) reagent was added to the test samples, standard, and blank. After 5 mins, 90 µL of Na_2CO_3 solution was added to the plate and the reaction was allowed to proceed. After incubating for an hour, the absorbance at 650 nm was measured using a micro-plate reader.

The total phenolic content of the samples was calculated using the value obtained from the calibration curve (R2 value), which was plotted at different concentrations for the standard. The TPC was calculated as µg Gallic acid equivalent per milligram of extract (g GAE/mg extract), and the assay was performed three times for reliability.

2.8.1.2 Determination of total flavonoid contents (TFC)

Total flavonoid content was calculated using the protocol described by Almajano et al. (2008). First, 20 µL of each the test sample, standard, and blank were poured into the 96-well microplate, and then 10 µL of the aluminum chloride solution (10%) was added. After adding 10 µL of a 1 M potassium acetate solution, the volume was brought up to 200 µL by adding 160 µL of distilled water. Optical density was measured at 415 nm after 30 mins of incubation using a microplate reader (Bioteck, USA).

Quercetin was used to generate the standard calibration curve at a range of final concentrations. The total flavonoid content was estimated by using an equation obtained from a calibration curve (R2 value), and the assay was performed in triplicate. The results were expressed as µg of Quercetin equivalent per milligram of plant extract (mg QE/mg extract).

2.8.2 Antioxidant assays

The antioxidant capacities of *B. nigra* roots and shoots were evaluated using many different assays, such as;

2.8.2.1 DPPH free radical scavenging assay

Antioxidant activity against DPPH was evaluated using a method similar to that described by Clarke et al. (2013). After pipetting 10 µL of the test sample, standard, and blank into each well of the 96 microplates and mixing, 190 µL of DPPH was added and allowed to sit. For one hour, the reaction mixture was kept at 37°C in the dark. The optical density of the samples was measured at 515 nm using a microplate reader.

The percentage of inhibition in the test sample was calculated using the following formula:

Percent inhibition of the test sample = (1 – A / B) *100

Here

A= O.D of DPPH solution with the sample.

B= O.D of negative control (containing the reagent except for the sample).

2.8.2.2 Determination of total antioxidant capacity (TAC)

The total antioxidant activity of *B. nigra* shoots and roots were determined using a technique based on that described by Clarke et al. (2013) with minor modifications.

The total antioxidant capacity assay began with the addition of 100 µL of stock solution/test samples to Eppendorf tubes, followed by the addition and thorough mixing of 900 µL of TAC reagent. The test tubes were then incubated at 95°C for 90 mins. When the reaction mixtures had finished incubating, they were cooled to room temperature, and then 200 µL of the uniformly mixed solution was dispensed into a 96-well plate. The absorbance at a wavelength of 630 nm was measured using a microplate reader.

2.8.2.3 Total Reducing Power Assay (TRP)

The total reduction potential of the test samples was calculated using the method described by Jafri et al. (2017).

Total reducing power was measured by adding 100 µL of the test material to 200 µL of phosphate buffer (0.2 M, pH 6.6) and 250 µL of 1% potassium ferricyanide solution in Eppendorf tubes. Then, for 20 mins, the reaction mixtures were kept at 50°C. After incubating the reaction mixture with 200 µL of 10% trichloroacetic acid, the pH was brought down. The resulting blends were centrifuged at 3000 rpm for 10 mins. The supernatant (150 µL) from each centrifuged mixture was combined with 50 µL of 0.1% ferric chloride solution in a microplate.

A microplate reader was used to measure the optical density at 630 nm.

The data was presented as the amount of Ascorbic acid (AAE) in micrograms per milligram of extract (µg AAE/mg extract).

2.8.3 Determination of metal chelating activity

Using the method described by Zia et al. (2021), the reaction was performed on a 96-well plate. Each well had 100 µL of sample and 50 µL of 2 mM FeCl2 poured into it. The plate was left to incubate in the dark for 10 mins. Next, 20 µL of 5 mM ferrozine was added to each well, and the plates were re-incubated for 5-10 mins. The Absorbance was measured at 562 nm against EDTA as a positive control. Efficiency in chelating metals was determined by

MC ability%

[(Absorbance of control – absorbance of sample)/ absorbance of control)] *100

2.9 Statistical analysis

The experimental design was completely arbitrary. A total of four flasks were set up for each concentration. Mean and standard deviation (SD) are presented for the results. Triplicate samples were used for the biochemical and antioxidant tests. Further analysis of means at $p<0.05$ was performed using analysis of variance (ANOVA) and least significant difference (LSD).

CHAPTER 3

3 RESULTS

3.1 Morphological Characteristics

3.1.1 Root Length

When compared to the control, the root length increased by 71% at 1µM of Lupeol, 2.99 times at 5µM, and a massive 3.97 times at 10µM of Lupeol. The positive control treated with NAA at a concentration of 1µM exhibited noticeable effects on the growth of roots, although to a lesser extent when compared to the observed effect of Lupeol. Comparing the application of NAA to the negative control, the length of the roots increased significantly by 18%.

3.1.2 Shoot Length

In comparison to the control group, the shoot length exhibited a significant increase of 37% at a concentration of 1µM of Lupeol. Moreover, at concentrations of 5µM and 10µM of Lupeol, the root length experienced a substantial increase of 51% and 65%, respectively. A concentration of 1µM of NAA as a positive control demonstrated discernible impacts on shoot growth, although to a lesser degree than the observed effect of Lupeol. When comparing the application of NAA to the negative control, it was observed that the length of the roots exhibited a significant increase of 21%. Another positive control with 10µM of BAP inhibited shooting and the resultant shoot length was 1.76 cm which is 82% short compared to the negative control (10.43 cm).

Table 1.1 **Length of roots and shoots and number of leaves of *B. nigra* and presence of BAP, NAA, and Lupeol.** Values represent means± standard errors from triplicates. The alphabets after each value indicate significant difference in values at $p<0.05$.

Concentration (µM)	Root Length (cm)	Shoot Length (cm)	No. of Leaves
Negative Control	5.26±0.50	10.43±0.60	8
BAP-10	Callus on the base	1.76±0.25	4
NAA-1	6.23±0.90	12.7±0.75	16
L-1	9.03±0.61	14.3±0.45	19
L-5	15.76±1.40	15.76±0.55	22
L-10	20.86±1.79	17.26±0.47	27

Figure 3.1 *Impact of Lupeol NAA and BAP on root length, shoot length, and number of leaves of Brassica nigra stem culture. (a) Negative Control, (b) BAP-10*µM*, (c) NAA-1*µM*, (d) L-1*µM*, (e) L-5*µM*, (f) L-10*µM

3.1.3 Fresh weight

A notable augmentation in both fresh weight (FW) and dry weight (DW) of Brassica roots was seen when comparing control plants to those cultivated in varying concentrations of Lupeol. Comparing Lupeol concentrations of 1µM, 5µM, and 10µM, it was found that the FW of Brassica roots increased by as much as 70%, 115%, and 148%, respectively, compared to the control. The observed increase was similarly significant in comparison to the positive control plants treated with 1µM of NAA.

The shoot fresh weight (FW) of Brassica was shown to be significantly enhanced with increasing concentrations of Lupeol. Specifically, a 26% increase was reported at a concentration of 1µM, a 30% increase at 5µM, and a notable 67% increase at 10µM, when compared to the control group. In comparison, positive control plant shoots that were grown with a concentration of 1µM NAA and 10µM BAP exhibited a respective increase in fresh weight (FW) of 17% and 13%. However, in contrast to the Lupeol-treated plants, the positive control plants demonstrated a decrease in fresh weight.

3.1.4 Dry weight

Adding Lupeol at concentrations of 1µM, 5µM, and 10µM, respectively, increased 89%, 165%, and 200% in the dry weight of Brassica roots, compared to the control. This increase was significant when compared to positive control plants treated with 1µM of NAA.

The shoot dry weight of Brassica showed an increase with higher concentrations of Lupeol. A 70% increase was observed at 1µM, a 96% increase at 5µM, and a 116% increase at 10µM compared to the control. Positive control plants grown on 1µM NAA and 10µM BAP also showed a 50% and 37% increase in dry weight of shoots, respectively. However, the positive control plants displayed lower dry weight than those treated with Lupeol.

Table 3.2 Fresh weight and dry weight of roots and shoots of *B. nigra* plants under NAA, BAP, and Lupeol. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.

Concentration (µM)	Roots FW (mg)	Roots DW (mg)	Shoots FW (mg)	Shoots DW (mg)
Negative Control	1.038±0.05	6.7±0.33	1.657±0.08	1.26±0.06
BAP-10	-	-	1.872±0.09	1.73±0.08
NAA-1	1.274±0.08	9.8±0.49	1.903±0.08	1.89±0.09
L-1	1.765±0.11	1.27±0..6	2.098±0.11	2.15±0.10
L-5	2.239±0.11	1.78±0.08	2.158±0.10	2.48±0.12
L-10	2.584±0.12	2.02±0.10	2.776±0.13	2.73±0.13

3.2 Phytochemical screening

3.2.1 Total Phenolic Content

Roots

Total phenolic contents were found to increase in shoots of *B. nigra* grown under Lupeol, (19.06, 23.35, and 21.72 µgGAE/mg DW). Minimum content was observed at 1 µM which was 19.06 and maximum at 5 µM which was 23.35 µgGAE/mg DW. Compared to the positive control (17.18 µgGAE/mg DW) NAA-1µM, phenolic content was higher at all the concentrations of Lupeol. While negative control showed the least phenolic content compared to all others.

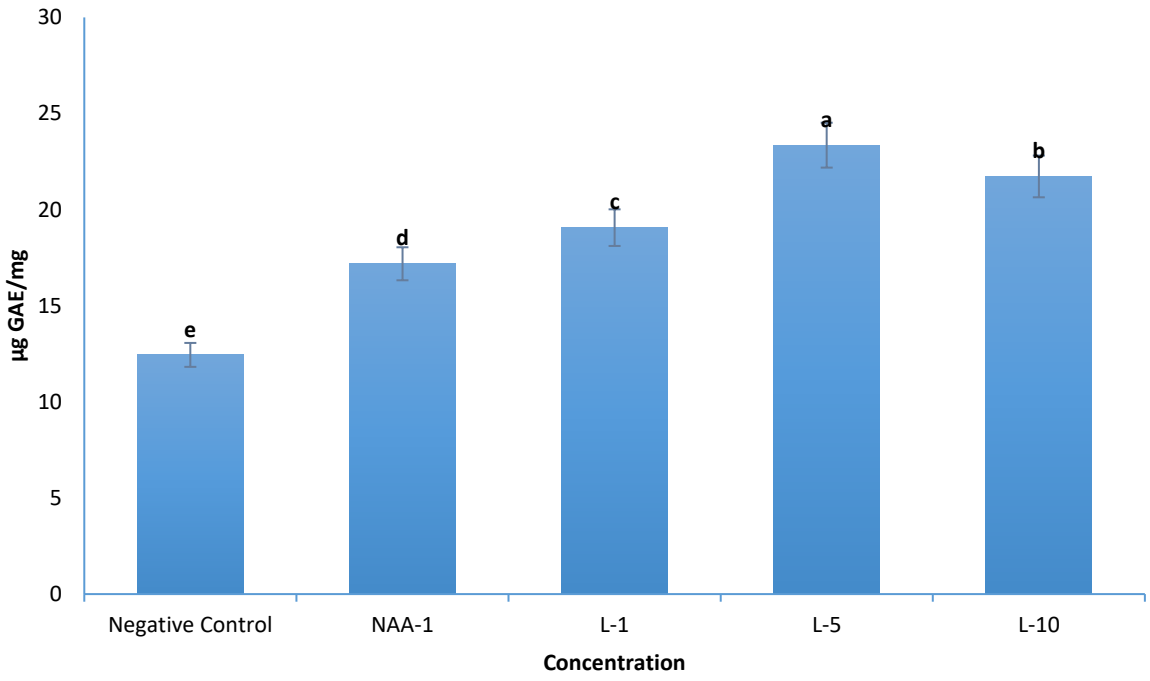

Figure 3.2 *Impact of Lupeol and NAA on total phenolic content of B. nigra roots. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at p<0.05.*

Shoots

Due to the presence of Lupeol, the total phenolic content in the shoots of *B. nigra* was found to be increased in comparison to the control, with a minimum of 32.97 and a maximum of 37.68 µgGAE/mg DW. at 1 and 5 µM, respectively. Compared to the positive controls 1 µM NAA (26.94 µgGAE/mg) and 10 µM BAP (29.72 µgGAE/mg), phenolic content was higher at all the concentrations of Lupeol. While negative control showed the least phenolic content compared to all others. Comparing Lupeol to a negative control revealed an increase in TPC content. However, at 10µM of Lupeol, the TPC is 35.94 µgGAE/mg DW. which is 4.6% less in comparison to 5 µM.

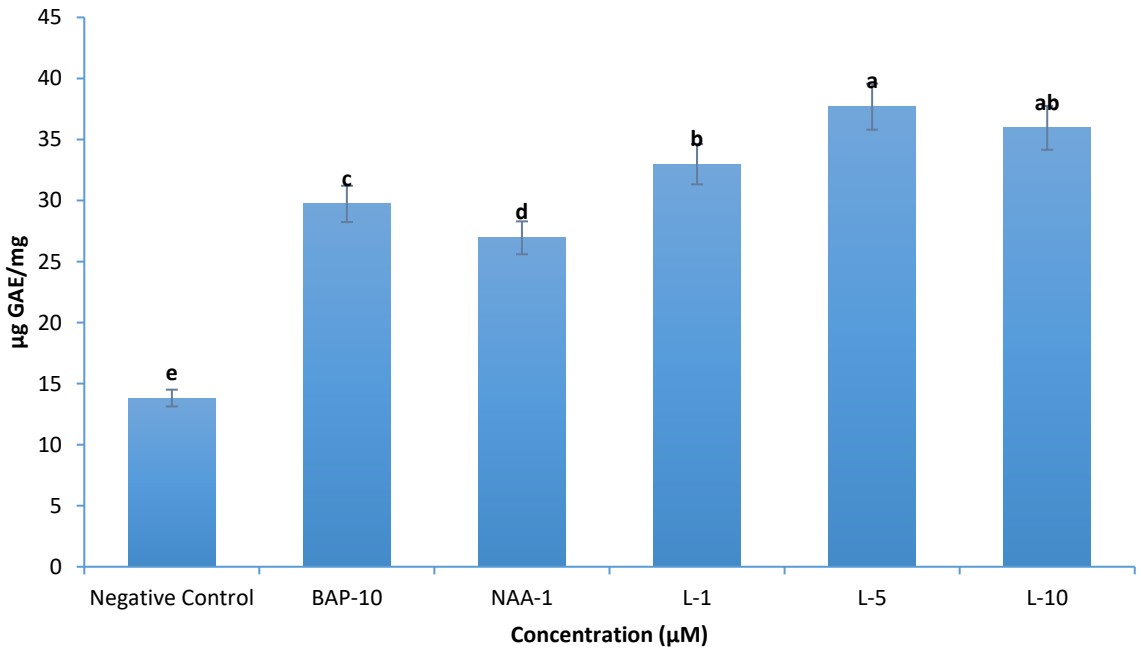

Figure 3.3 *Impact of Lupeol, NAA, and BAP on total phenolic content of B. nigra shoots. Values represent means ± standard errors from triplicates. The alphabets on each value represent the significant difference among values at p<0.05.*

3.2.2 Total flavonoid content

Roots

The roots exhibited an increase in total flavonoid content when cultivated in the presence of Lupeol. The concentration of Lupeol at 1µM resulted in a total flavonoid content of 12.28 µgQE/mg DW, while at 5 µM it was 16.6 µgQE/mg DW, and at 10 µM it reached 15.97 µgQE/mg DW. The positive control NAA-1µM yielded a total flavonoid content of 10.60 µgQE/mg DW. The application of Lupeol resulted in a significant increase in the Total flavonoid content when compared to the negative control.

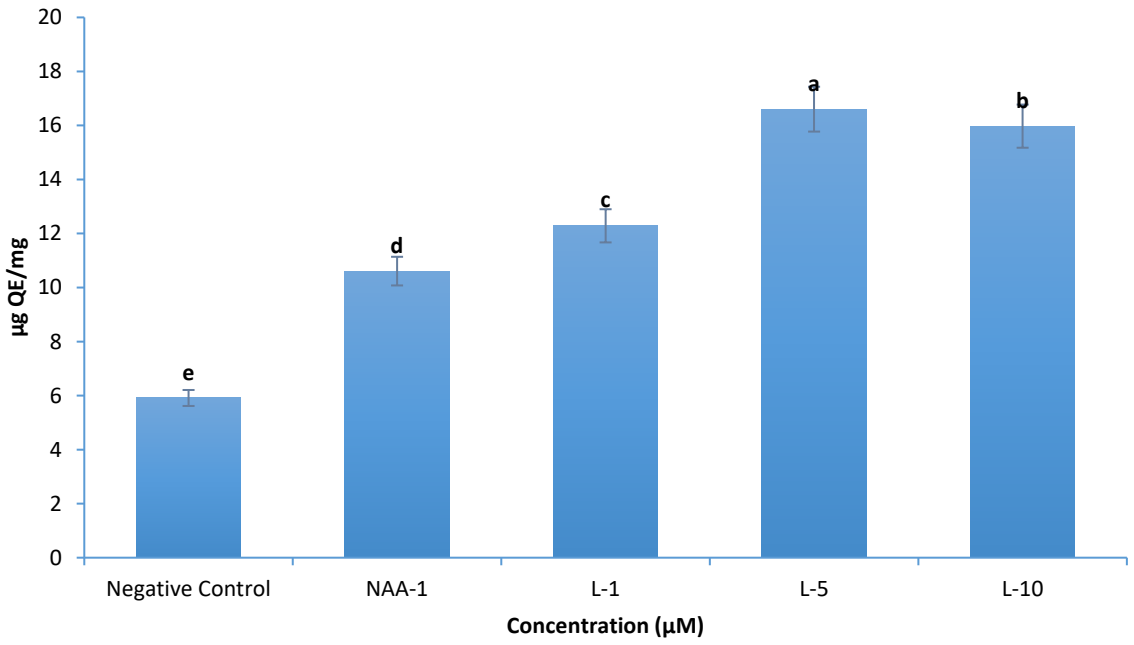

Figure 3.4 *Lupeol and NAA impact on total flavonoid content of Brassica nigra roots. Lupeol and NAA impact on total flavonoid content of Brassica nigra roots. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

Shoots

The total flavonoid content in *Brassica nigra* shoots was found to be higher than that of the control, with minimum values of 15.13 gQE/mg DW and maximum values of 21.76 gQE/mg DW at 1 µM and 5 µM, respectively. Compared to the positive control 10 µM BAP (10.18 µgQE/mg), Lupeol significantly increased the Total flavonoid content at all the concentrations. TFC in 5 µM Lupeol-treated plants was 22% more than those treated with 10 µM of Lupeol.

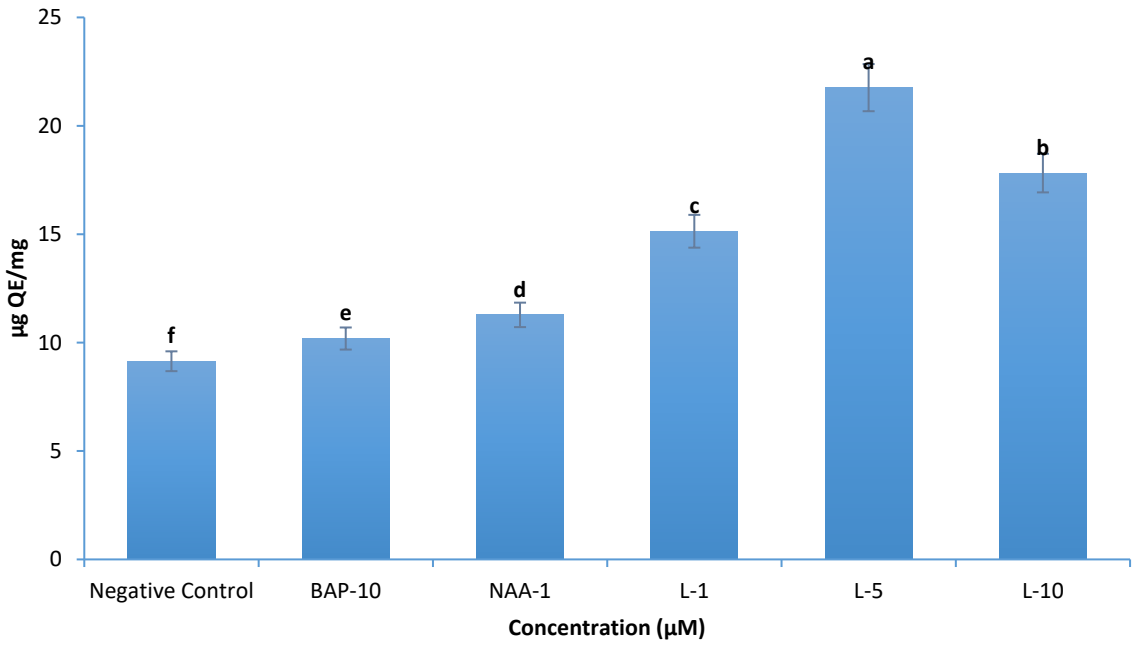

Figure 3.5 *Lupeol, NAA, and BAP impact on total flavonoid content of B. nigra shoots. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

Antioxidant assays

3.2.3 DPPH

Roots

In the presence of Lupeol, the DPPH scavenging activity in *B. nigra* plant roots was increased relative to the control, with a minimal activity of 33.49% at 1 µM and a maximum activity of 40.61% at 5 µM. In comparison, the positive control NAA-1µM yielded DPPH-scavenging activity of 25.29%. The application of Lupeol resulted in a significant increase in the DPPH-scavenging activity when compared to the negative control (17.33%).

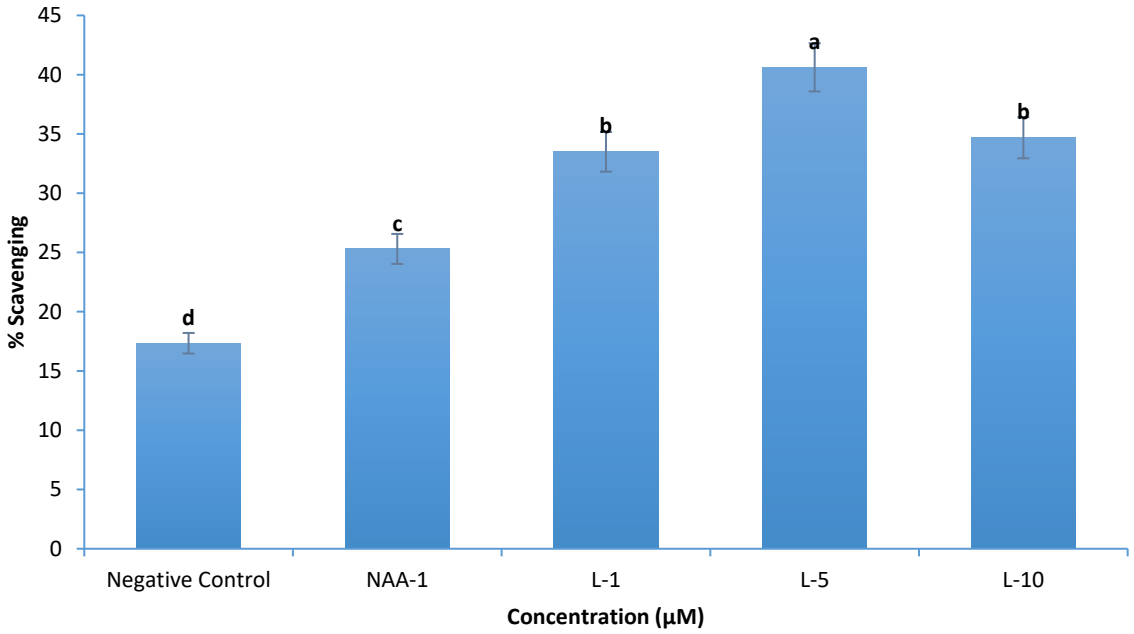

Figure 3.6 *DPPH free radical scavenging activity of Brassica nigra roots under the impact of Lupeol and NAA. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

Shoots

In the shoots of *B. nigra* plants grown in the presence of Lupeol, DPPH scavenging activity increased at all concentrations. Compared to positive and negative controls, these elevations in DPPH scavenging activity were huge. 39% activity at 1 µM, 60% at 5 µM, and 47% at 10 µM of Lupeol treated plants. In contrast, negative control has the least DPPH scavenging activity of 17% while BAP-10 µM and NAA-1 µM have 31% and 26% activity respectively.

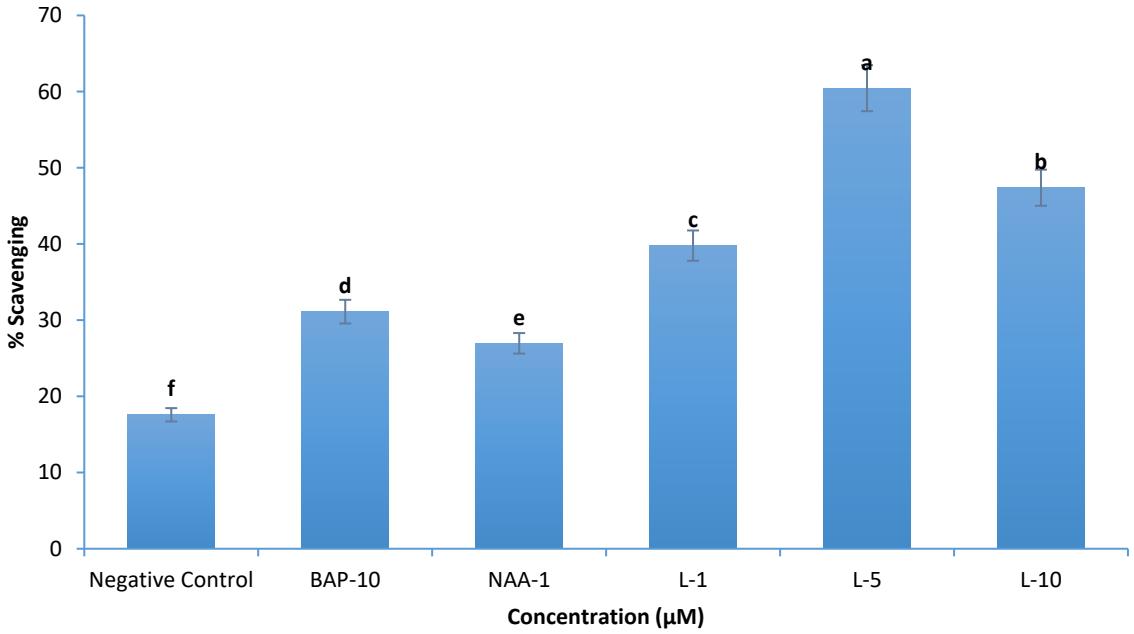

Figure 3.7 *DPPH free radical scavenging activity of B. nigra shoots under Lupeol, NAA, and BAP. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at p<0.05.*

3.2.4 ABTS

Roots

Significant variation in ABTS activity was observed in the roots of *B. nigra* plants under the influence of Lupeol as compared to the control. In comparison to positive control 1µM NAA (23%) and negative control (13.56%), ABTS activity increased at different concentrations of Lupeol, which was 25% at 1 µM, 29.92% at 5 µM, while at 10 µM ABTS activity was 27.39%.

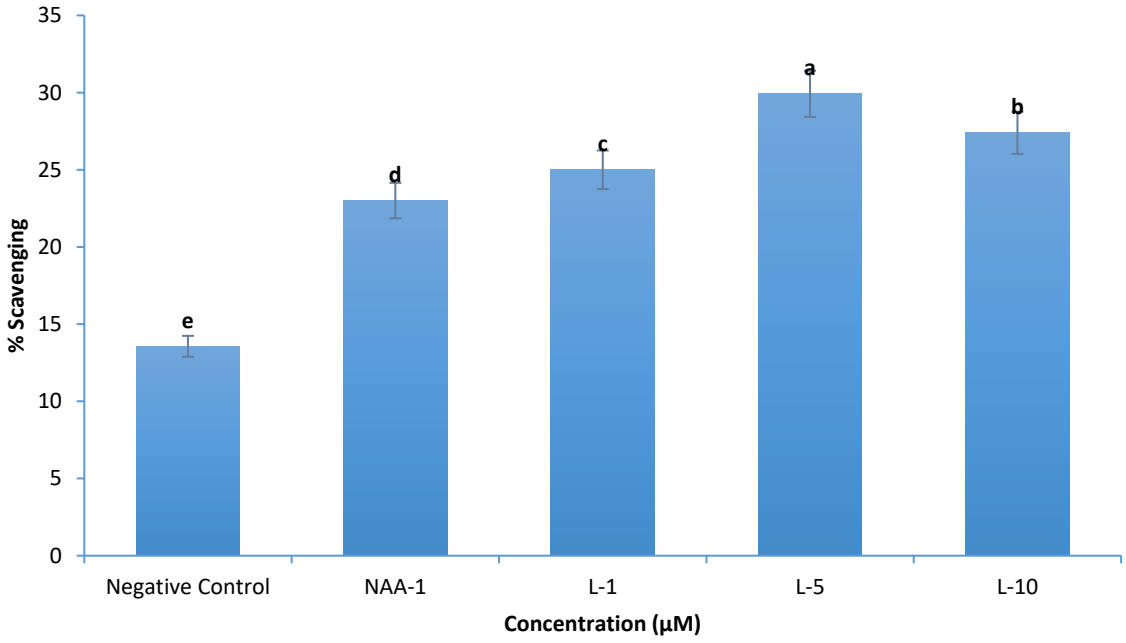

Figure 3.8 *ABTS free radical scavenging activity of B. nigra roots under Lupeol and NAA. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at p<0.05.*

Shoots

ABTS activity in shoots of Lupeol-treated *B. nigra* plants decreased at 1 µM as compared to positive control NAA-1 µM (25.26%) and BAP-10 µM (26.32%), which was 22.34%. However, at higher concentrations of Lupeol ABTS activity was increased significantly, which was 45.07% at 5 µM and 32.71% at 10 µM. Negative control had the least ABTS activity (9.7%).

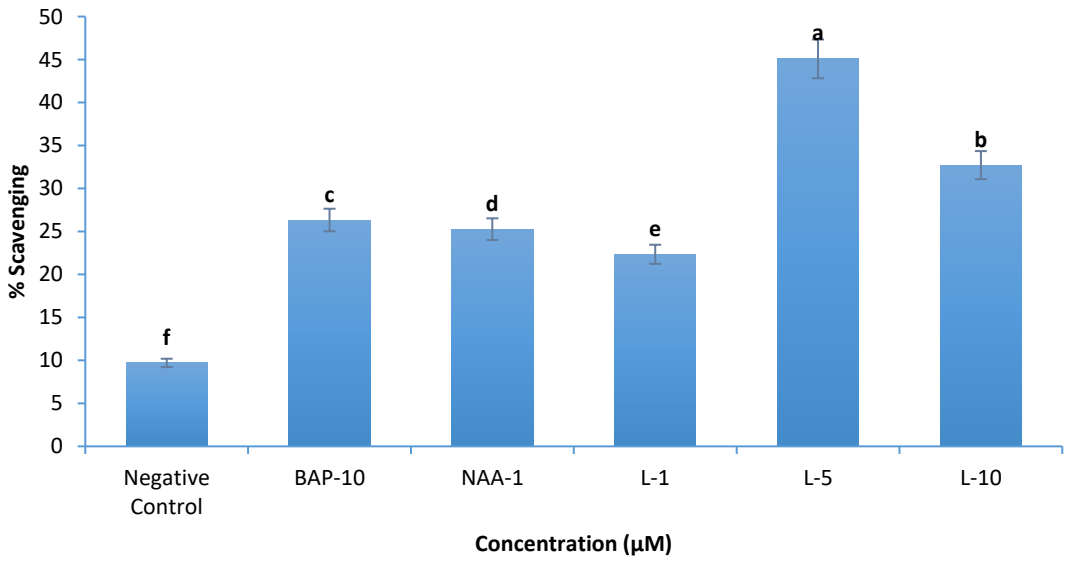

Figure 3.9 *ABTS free radical scavenging activity of B. nigra shoots under the impact of Lupeol, NAA, and BAP. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

3.2.5 Metal chelating

Roots

An increase in metal chelating activity was observed in the roots of *B. nigra* plants grown under the influence of Lupeol as compared to the negative control (11.29%) and positive control NAA-1 µM (15.51%), which was 31.02% at 1 µM, 44.51% at 5 µM and at 10 µM it was 37.77%.

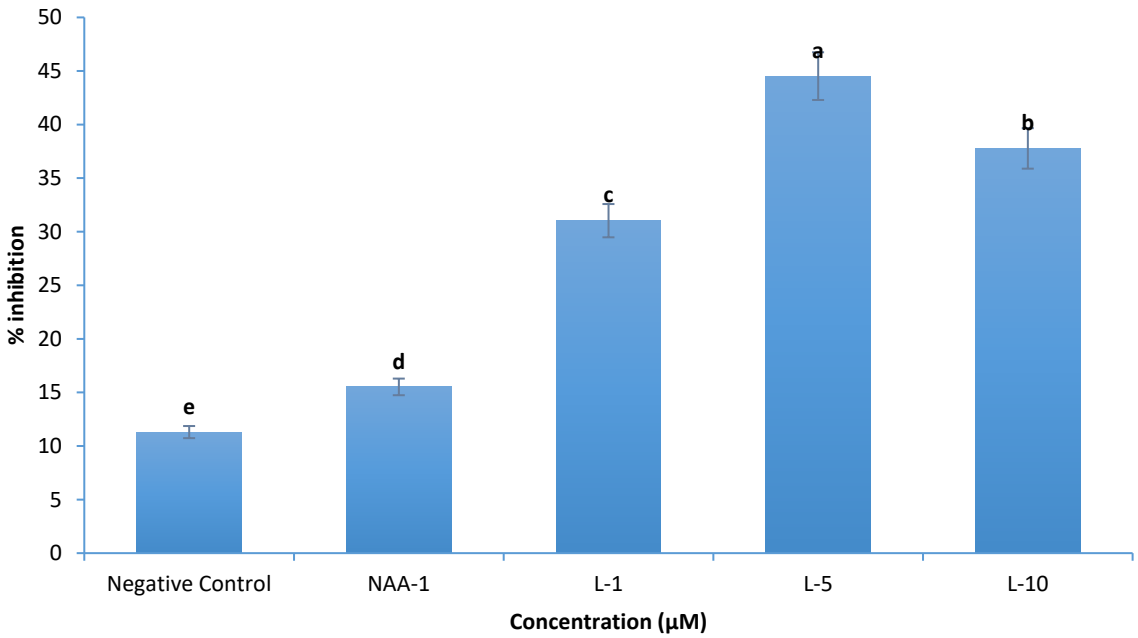

Figure 3.10 *Impact of Lupeol and NAA on the metal chelating activity of B. nigra roots. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

Shoots

In shoots of *B. nigra* plants MC activity increased at 1µM, 5µM, and 10 µM of Lupeol in comparison to positive controls NAA-1µM (19.89%) and BAP-10µM (27.15%), which was 35.58%, 54.97%, and 43.33% respectively. MC activity also increased in comparison to negative control (16.52%).

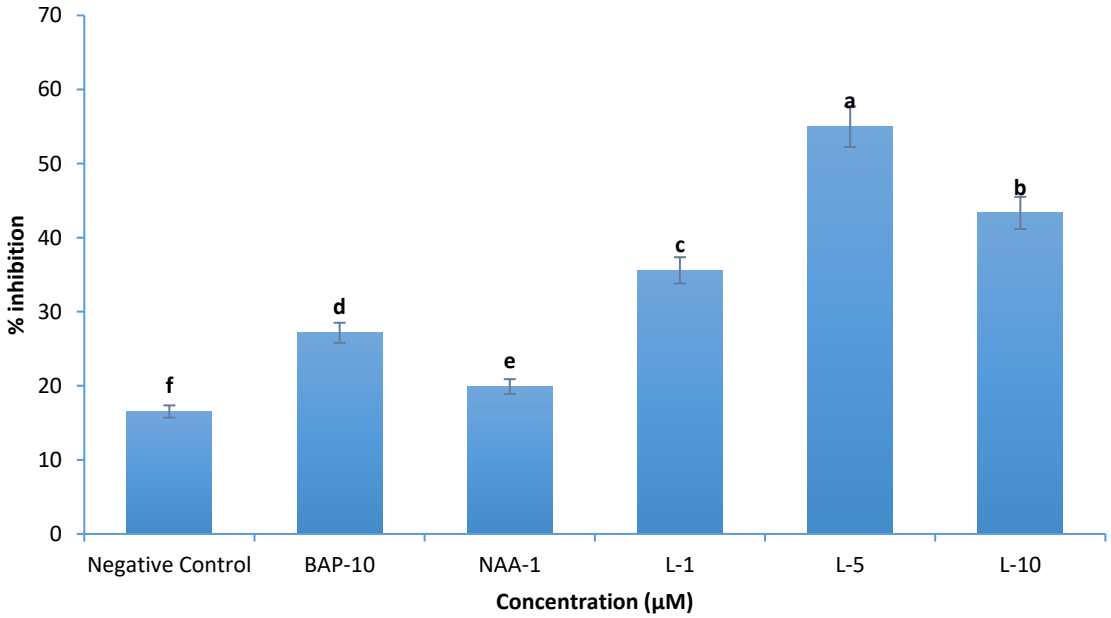

Figure 3.11 *Impact of Lupeol, NAA, and BAP on the metal chelating activity of B. nigra shoots. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

3.2.6 Total antioxidant capacity (TAC)

Roots

An elevation in the overall antioxidant capacity was noted in the roots of plants cultivated in the presence of Lupeol, in comparison to the positive control NAA (30.06 µgAAE/mg DW). Specifically, at a concentration of 1 µM, the total antioxidant capacity (TAC) was measured at 49.97 µgAAE/mg DW, while at 10 µM, it increased to 60.23 µgAAE/mg DW. Furthermore, a TAC of 60.23 µgAAE/mg DW was observed at a concentration of 5 µM. In contrast, the negative control had the lowest TAC (24.8 µgAAE/mg DW).

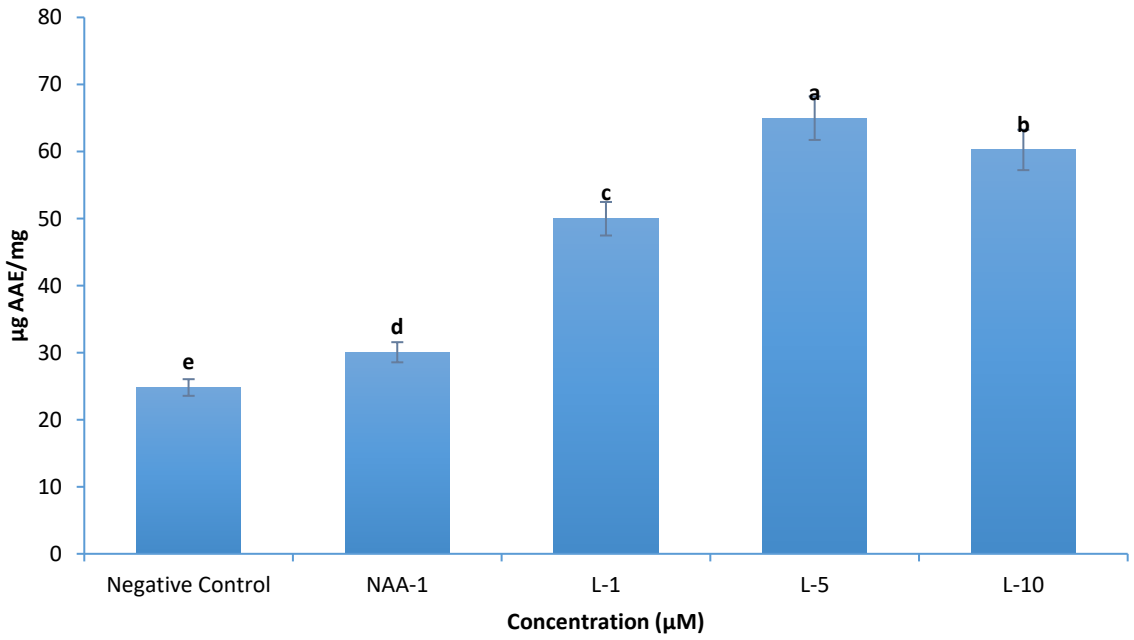

Figure 3.12 *Total antioxidant capacity of B. nigra roots against Lupeol and NAA. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

Shoots

TAC decreased at all the concentrations 1 µM, 5 µM, and 10 µM, to 66.78 gAAE/mg DW, 84.28 gAAE/mg DW, and 75.83 gAAE/mg DW, respectively, in the shoots of *B. nigra* plants cultivated in the presence of Lupeol. These values surpass the antioxidant capacities of the positive controls NAA-1 µM and BAP-10 µM, which measured 33.42 µgAAE/mg DW and 36 µgAAE/mg DW, respectively. In comparison to the negative control, which exhibited a total antioxidant capacity of 25.83 µgAAE/mg DW, the presence of Lupeol resulted in a significant increase in total antioxidant capacity.

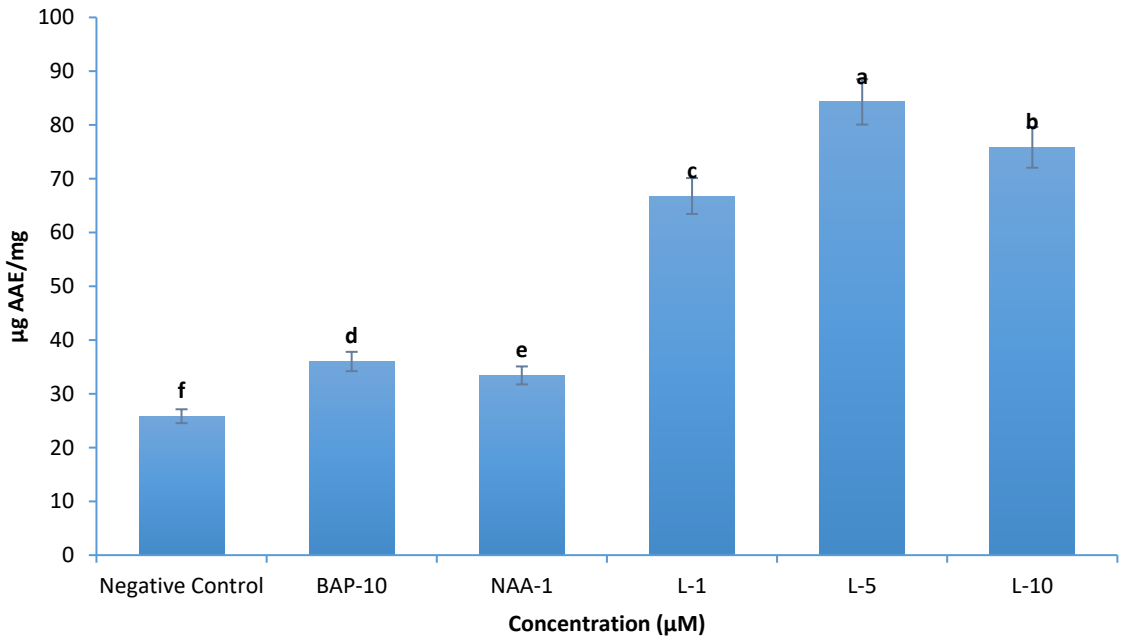

Figure 3.13 *Total antioxidant capacity of B. nigra shoots against Lupeol, NAA, and, BAP. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

3.2.7 Total reducing power (TRP)

Roots

A notable enhancement in the total reducing power was observed in the roots of Brassica plants cultivated under the influence of Lupeol, in comparison to the positive control NAA-1 µM (35.53 µgAAE/mg DW). The minimum reducing power was observed at a concentration of 1 µM, measuring 43.18 µgAAE/mg DW, while the maximum reducing power was observed at a concentration of 5 µM, measuring 78.74 µgAAE/mg DW. An overall increase in TRP was observed as a result of the application of Lupeol, in comparison to the negative control (31.08 µgAAE/mg DW).

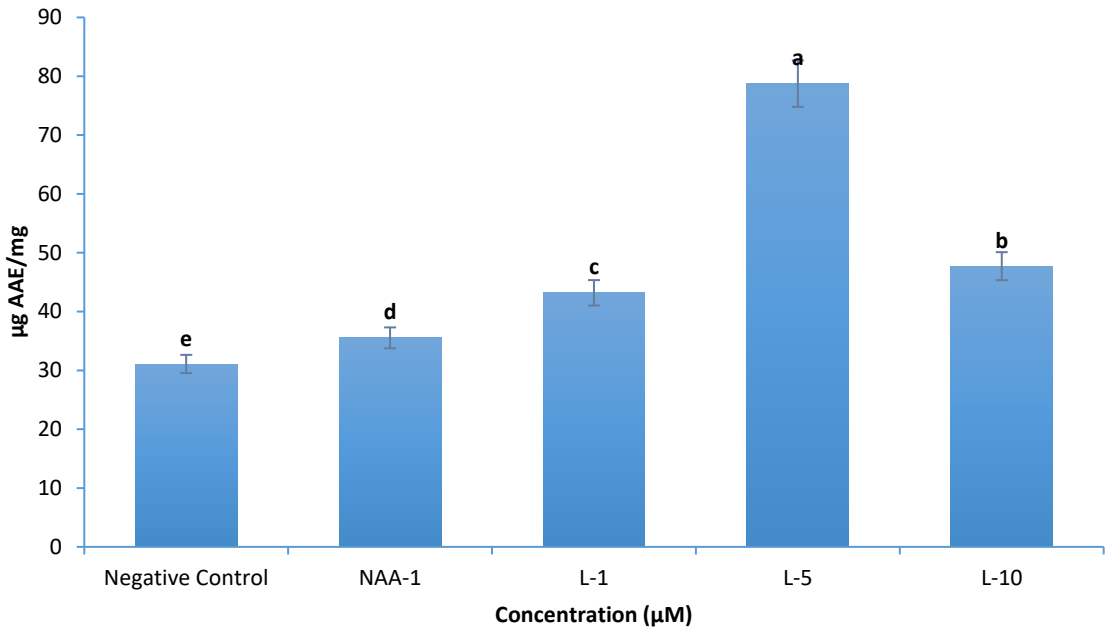

Figure 3.14 *Total reducing power of B. nigra roots against Lupeol and NAA. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at p<0.05.*

Shoots

The shoots of *B. nigra* plants exhibited a tremendous increase in TRP levels at all concentrations of Lupeol as compared to all other controls. Compared to positive controls NAA-1(40.46 µgAAE/mg DW) and BAP-10 (45.98 µgAAE/mg DW) increase in TRP levels was observed at concentrations of 1 µM (84.91 µgAAE/mg DW) and 5 µM (95.20 µgAAE/mg DW) and 10 µM (67.87 µgAAE/mg DW). The application of Lupeol resulted in a noticeable increase in TRP levels when compared to the negative control (35.86 µgAAE/mg DW).

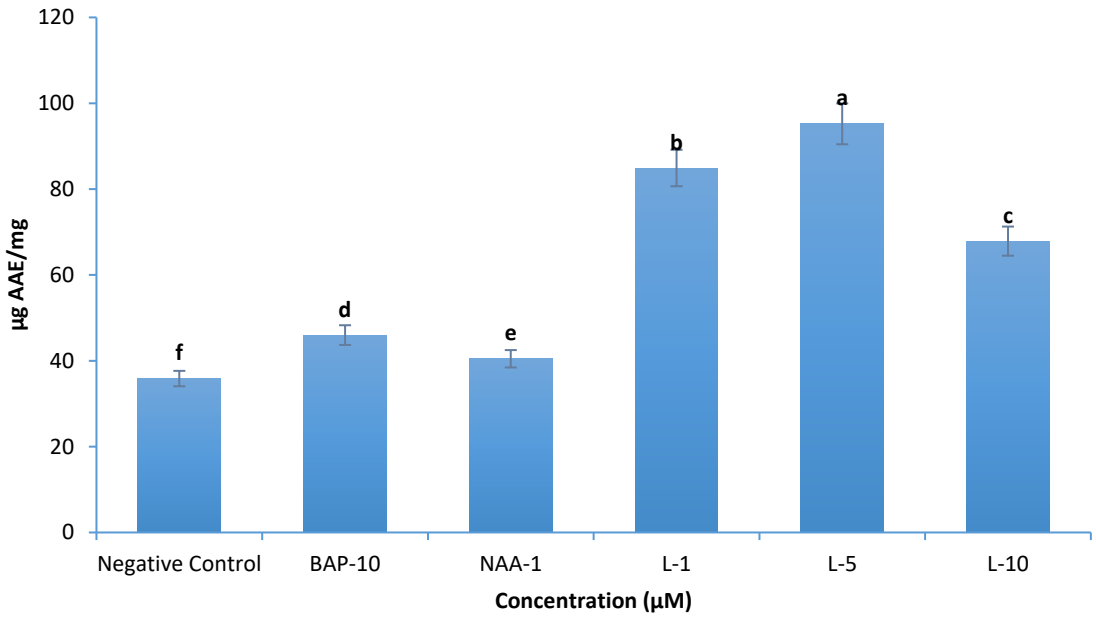

Figure 3.15 *Total reducing power of B. nigra shoots against NAA, BAP, and Lupeol. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

3.3 Determination of antioxidant enzyme activities

3.3.1 Superoxide dismutase (SOD)

Roots

An elevation in the activity of Superoxide dismutase was noted when Lupeol was present, in comparison to the positive control NAA (0.045 nM/min/mg), in the roots of the *B. nigra* plant. The lowest level of activity was recorded at a concentration of 1 µM, yielding an activity rate of 0.062 nM/min/mg FW. Conversely, the highest level of activity was observed at a concentration of 5 µM, resulting in an activity rate of 0.083 nM/min/mg FW. An increase in the activity of superoxide dismutase (SOD) was observed in plants cultivated under varying concentrations of Lupeol (0.062, 0.083, and 0.073 nM/min/mg FW), in comparison to the negative control (0.043 nM/min/mg FW), as depicted in Figure 3.16.

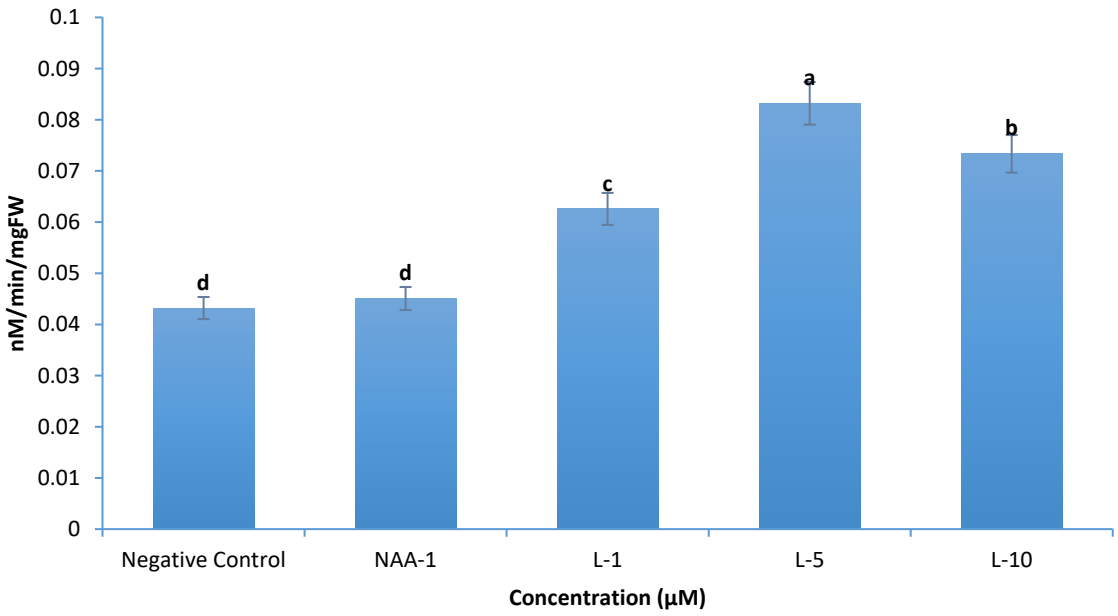

Figure 2.16 *Activity of superoxide dismutase of B. nigra roots against NAA and Lupeol. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

Shoots

Superoxide dismutase activity was increased at all the concentrations of Lupeol as compared to positive control 10µM BAP (0.049 nM/min/mg FW) and positive control 1 µM NAA (0.06 nM/min/mg FW) in shoots of *B. nigra*. However, the highest superoxide dismutase activity was observed at a 5 µM concentration of Lupeol which was 0.086 nM/min/mg FW. While in comparison to the negative control (0.041 nM/min/mg FW), superoxide dismutase activity increased in all concentrations, 1 µM (0.073 nM/min/mg FW), 5µM (0.086 nM/min/mg FW) and 10 µM (0.075 nM/min/mg FW) of Lupeol.

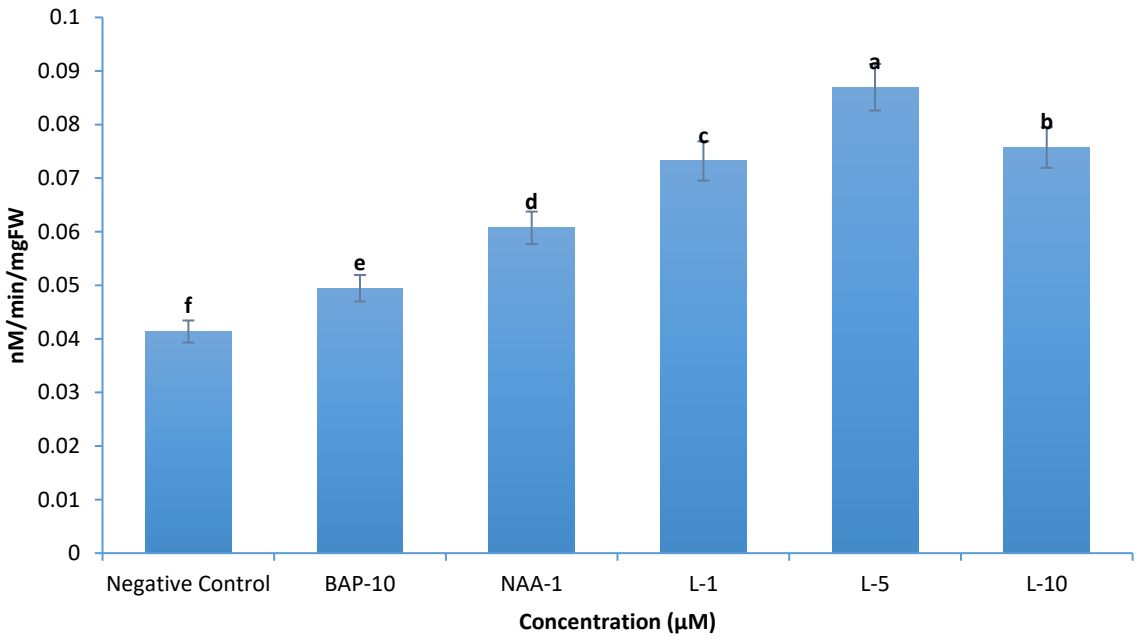

Figure 3.17 *Activity of Superoxide dismutase of B. nigra shoots against NAA, BAP, and Lupeol. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

3.3.2 Peroxidase (POD)

Roots

The presence of Lupeol increased peroxidase (POD) activity. The minimum POD activity was observed at a concentration of 1 µM, measuring 1.55 nM/min/mg FW. Conversely, the maximum POD activity was observed at a concentration of 5 µM, measuring 2.1 nM/min/mg FW as compared to negative control (1.00 nM/min/mg FW). The peroxidase (POD) activity exhibited an increase at only 10 µM concentration of Lupeol (2.1 nM/min/mg FW) when compared to the positive control NAA-1 µM (1.86 nM/min/mg FW).

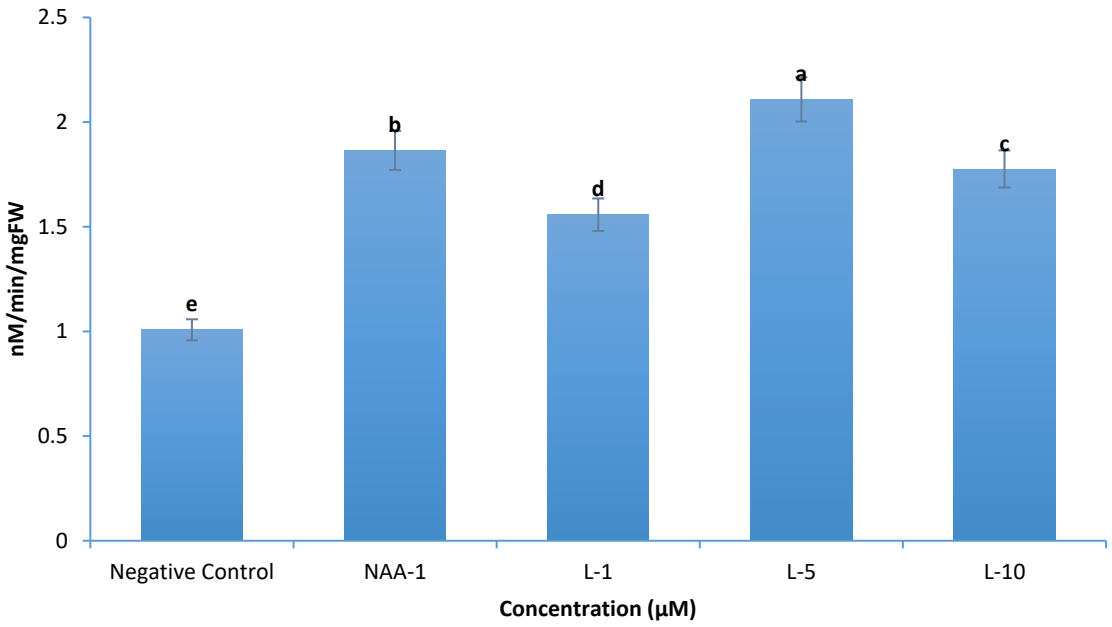

Figure 3.18 *Activity of peroxidase in B. nigra roots against NAA and Lupeol. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

Shoots

POD activity was found to increase in the presence of Lupeol where minimum POD activity was observed at 1 µM which was 1.68 nM/min/mg FW and maximum POD activity was observed at 5 µM which was 2.29 nM/min/mg FW as compared to positive controls NAA-1 µM (1.63 nM/min/mg FW) and BAP-10 µM (1.59 nM/min/mg FW). POD activity was higher at all the concentrations of Lupeol (1.68, 2.29, and 1.81 nM/min/mg FW) in comparison to the negative control (1.42 nM/min/mg FW).

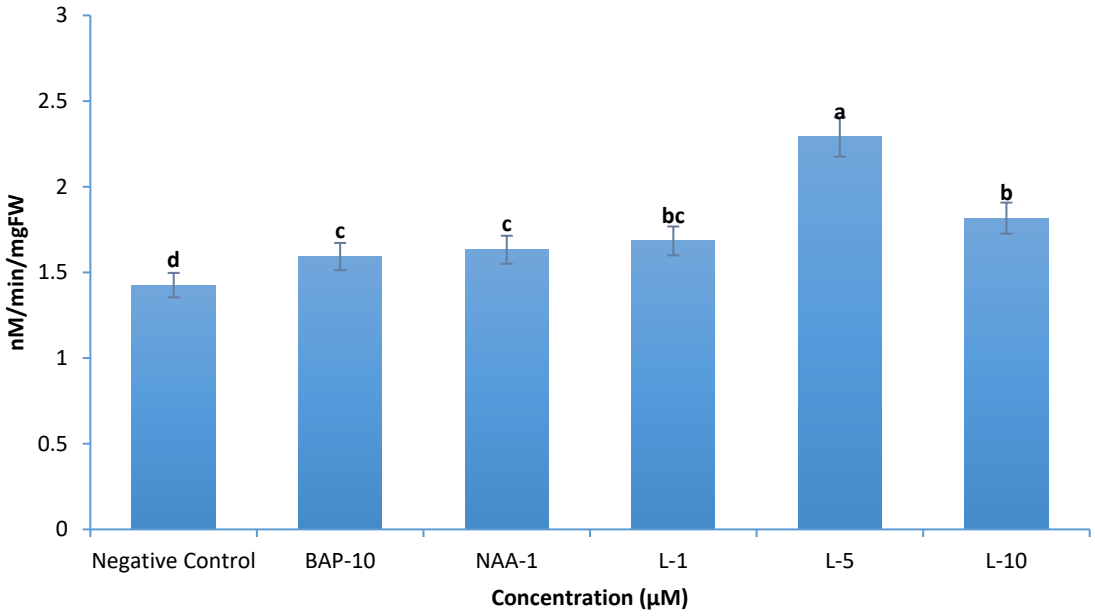

Figure 3.19 *Activity of peroxidase of B. nigra shoots against NAA, BAP, and Lupeol. Values represent means ± standard errors from triplicates. The alphabet after each value indicates a significant difference in values at $p<0.05$.*

CHAPTER 4

4 Discussion

4.1 Discussion

The primary findings of this study revolve around the effects of various concentrations of Lupeol on the various characteristics of *B. nigra*. At a concentration of 1µM of Lupeol, there was a 71% increase in root length compared to the control. This increase was even more pronounced at 5µM and 10µM of Lupeol, with root lengths increasing by 2.99 times and 3.97 times, respectively. In terms of shoot length, there was a 37% increase at 1µM of Lupeol, and further increases of 51% and 65% at 5µM and 10µM of Lupeol, respectively. While NAA at a concentration of 1µM did lead to increased root and shoot lengths, the effects were less pronounced than those observed with Lupeol. A similar trend was observed in fresh and dry weights of root and shoot. The process of biomass accumulation is closely linked to primary metabolism, particularly nitrogen and carbon metabolism, which play a significant role in cellular growth and the formation of cell components (Goncalves, Antes, *et al*., 2009). The results are also consistent with a previous study conducted by Zia et al. (2022), which showed a positive impact of Lupeol on growth indices in the presence of salt-induced stress. A plausible hypothesis for this phenomenon posits that triterpenoids are recognized for their substantial participation in several physiological and developmental processes across the plant domain. The underlying mechanisms that may explain these phenomena might be linked to the interaction between Lupeol and plant growth regulators, such as auxins and cytokinins, which are known for their capacity to regulate many physiological processes in plants.

The significance of these findings lies in the potential applications of Lupeol in agriculture and plant biotechnology. Given the pronounced effects on root and shoot growth, Lupeol could be explored as a potential growth stimulant for crops, leading to increased yields and productivity. Phenolic molecules are produced via the shikimate/phenylpropanoid pathway and serve as highly effective scavengers of free radicals within living cells. Flavonoids, which are polyphenolic chemicals, are widely present in the natural environment (Rice-Evans, 1999). Total Phenolic Content (TPC) in *B. nigra* shoots increased at all concentrations of Lupeol, peaking at 5 µM with 23.35 µgGAE/mg DW. This was higher than both positive and negative controls. The phenolics and flavonoids have gained significant attention in recent times due to their potential advantageous impacts on human health, including antiviral, anti-allergic, antiplatelet, anti-inflammatory, anticancer, and antioxidant properties. Flavonoids have the

ability to safeguard cells from the detrimental impacts of reactive oxygen species, including singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals, and peroxynitrite (Al-Snafi, 2020). Total Flavonoid Content (TFC) in roots and shoots also increased across Lupeol concentrations, peaking at 5 µM with 16.6 µgQE/mg DW in roots and 21.76 gQE/mg DW in shoots, surpassing control levels. Phenolics, a class of non-enzymatic antioxidants, have garnered considerable attention due to their capacity to protect plants against the buildup of reactive oxygen species (ROS) (Sharma et al., 2012). The findings of this study indicate that the application of Lupeol to plants resulted in a greater accumulation of phenolic and flavonoid compounds, as compared to control groups. According to Zia et al. (2022), it can be inferred that the application of Lupeol stimulates the synthesis of secondary metabolites in plants as a reaction to abiotic stress.

The DPPH free radical scavenging assay was one of the main assays utilized. This test holds significant importance in the field of antioxidant research as it offers valuable insights into the capacity of a molecule or extracts to counteract free radicals, which are frequently associated with cellular harm and the aging process (Lobo et al., 2010). In DPPH antioxidant assays, both roots and shoots showed increased scavenging activity under the influence of Lupeol. Root activity peaked at 5 µM with 40.61% scavenging, while shoot activity was highest at the same concentration with 60% scavenging, both markedly higher than control values. In ABTS activity assays, roots showed an increase of 25-29% across different Lupeol concentrations, while shoots initially decreased but later rose significantly. In Metal Chelating (MC) tests, root activity increased to 44.51% at 5 µM, and shoots showed a peak of 54.97% at the same concentration.

To prevent the buildup of ROS, plants have several detoxifying mechanisms in place, including non-enzymatic antioxidants like TPC and TFC as well as antioxidant enzymes like POD and SOD (Sharma et al., 2013). Total Antioxidant Capacity (TAC) in roots peaked at 60.23 µgAAE/mg DW at 5 and 10 µM, while in shoots, TAC reached 84.28 gAAE/mg DW at 5 µM. For Total Reducing Power (TRP), root activity was highest at 78.74 µgAAE/mg DW at 5 µM, and shoots peaked at 95.20 µgAAE/mg DW at the same concentration. Both BAP and NAA did not exhibit enhanced antioxidant activities in comparison to the roots and shoots treated with Lupeol. Compounds with significant reducing capacity function as both primary and secondary antioxidants, effectively mitigating the formation of oxidized intermediates during lipid peroxidation processes (Kalita, Tapan et al., 2013). The iso-enzymes superoxide dismutase (SOD) and peroxidase (POD) differ from one another by a variety of physical and chemical properties as well as amino acid sequences while catalyzing the same reaction

(Chong, Abdullah et al., 2005). As indicated in Figures 3.16 - 3.19, the in-vitro antioxidant enzyme activities (SOD and POD) were also examined for the assessment of the impact of Lupeol on the stem culture of *B. nigra*. Superoxide Dismutase (SOD) activity in roots and shoots was maximized at 5 µM, recording 0.083 and 0.086 nM/min/mg FW, respectively. Peroxidase (POD) activity in both roots and shoots was highest at 5 µM, measuring 2.1 nM/min/mg FW in roots and 2.29 nM/min/mg FW in shoots. The outcomes align with the research conducted by Zia et al. (2021), which showed that the utilization of Lupeol on plants led to increased activities of antioxidant enzymes in *B. nigra* plants subjected to salt-induced stress. The increase in SOD and POD activities may be associated with the systemic signaling mechanisms in plants that enable them to cope with both biotic and abiotic stress (Wasternack and Parthier, 1997).

Overall, most assays showed peak activity at 5 µM of Lupeol, indicating a generally positive but non-linear effect on various biochemical markers.

4.2 Conclusion

This research shows that Lupeol improves the development and physiology of *Brassica nigra*. The potential of Lupeol as a growth stimulant in agriculture stems from its noticeable effects on root and shoot growth and its influence on phenolic and flavonoid components. The consistent peak activity at a concentration of 5 µM in several assays indicates that the effects of Lupeol are both statistically significant and effective. This finding implies that the use of larger doses of synthetic chemicals, which may have adverse effects, might potentially be minimized. In light of the pressing demand for sustainable agricultural methods that optimize crop output while reducing environmental impact, the findings of this study regarding the potential of Lupeol offer promising prospects for further investigation in enhancing agricultural productivity and resilience.

CHAPTER 5

5 References

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