Effect of Lupeol on Growth, Phytochemistry, and Antioxidant Potential of *Brassica Nigra* Shoot Tip Culture

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Declaration

I hereby declare that the work presented in this thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously published or presented for any other degree or certificate.

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Dedication

I dedicate my work to my parents who have always loved me unconditionally and whose good example has taught me to work hard for the things that I aspire to achieve.

Humna Sajjad

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First and Foremost Alhamdulillah to ALLAH Almighty for He is the most merciful and compassionate, WHO gave me knowledge, strength and courage all the way to complete my MPhil project. I can never thank HIM enough for the blessings HE bestowed upon me, most profoundly for creating me among the Ummat e Muhammad *(sallallahu alayhi wa aalihi wa sallam).*

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Humna Sajjad

List of Abbreviations

BAP	6- Benzylamino purine
DW	Dry weight
FC	Folin ciocalteu
FW	Fresh weight
GAE	Gallic acid equaivalent
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

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Abstract

Brassica nigra, also known as Black Mustard, is an important medicinal plant with a wide range of pharmacological properties. Lupeol belongs to the triterpenoid family. It is a secondary metabolite that occurs naturally and is known to play an essential role in the complex systems of plant development and defense. Present study aims to investigate the effects of different concentrations of lupeol (1 µM, 5 µM, and 10 µM) on the growth characteristics, phytochemical content, as well as enzymatic and non-enzymatic antioxidant responses in *Brassica nigra* shoot tip culture. Among all tested samples optimal concentration for growth enhancement was observed at 10 µM lupeol. Both root and shoot length showed significant increases, with 109% and 77% increments. Fresh weight of roots increased up to 243% while dry weight enhanced up to 132% at 10 µM lupeol. Likewise, fresh weight (120%) and dry weight (103%) of shoots increased at 10 µM lupeol. Maximum total phenolic content was observed to increase at 10 µM lupeol in roots (87%) and shoots (98%). Similarly, flavonoid content showed 24% increase in roots and a 100% increase in shoots at 10 µM lupeol. Superoxide dismutase activity in roots and shoots of plants was found higher at 10 µM lupeol 160%, and 145%, respectively. Similarly, increase in the antioxidant enzyme (POD) activity by 246% in roots and 722% in shoots, respectively. DPPH assay revealed FRSA of 59% and 53% at 10 µM lupeol concentration of roots and shoots, respectively. Maximum ABTS scavenging activity of roots (47.8%) and shoots (47.3%) was observed at 10 µM lupeol. Significant Metal chelating activities of lupeol treated roots and shoots confirm their function as an efficient metal chelator agent at 10 µM lupeol by 52% and 49.4%, respectively. The total reducing potential of *B. nigra* roots (123%) and shoots (396%), total antioxidant potential of *B. nigra* roots (300%) and shoots (258%) were all gradually increased at 10 µM lupeol. This study shows the significance of lupeol as a promising elicitor and as a protective agent against oxidative damage in plants.

CHAPTER 1

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Botanical Description of *Brassica nigra*

Brassica nigra, also known as "black mustard," is a winter annual herb, belonging to the Brassicaceae family. This herb attains a stature of approximately 1 m upon maturity. Its leaves display petiolate attributes, arranged alternately, and possess a dense covering of dark green trichomes. The bottom leaves are large, characterized by a rough texture and irregularly sinuate-dentate margins. These leaves exhibit a pinnate arrangement, featuring a large terminal lobe accompanied by smaller lower lobes. Upper leaves, in contrast, are smoother and have moderate lobing (Rakow, 2004). The flowers of this plant appear as small, luminous yellow cruciform blossoms with four petals, encompass tetradynamous stamens and a bicarpellate pistil. This florid manifestation predominantly occurs during summer in May and June. The onset of flowering transpires approximately 45 days post-seeding. The botanical yields fruit spanning the period from June through October. The seeds are tiny and have minor pits. The seeds appear differently in comparison to those of brown mustard seeds. They measure around 2 mm or smaller and tend to be slightly oblong rather than spherical, their color shifting from dark reddish-brown to black, and typically covered in white pellicles. Additionally, they possess a much stronger smell compared to the white variety (Podsędek, 2007; Cartea *et al.,* 2011).

1.2 Taxonomic Classification

The Brassicaceae family encompasses around 350 genera and close to 3,500 species. Among the noteworthy genera affiliated with this family are Brassica, Crambe, Thlaspi, Camelina, and Sinapis. The most significant genus within this family is Brassica, encompassing crops such as., *Brassica rapa, Brassica napus L, and, Brassica oleracea L*. This genus is further delineated into various subcategories, including oilseed, forage, vegetable plants, and condiment (Jawaher *et al.,* 2022). The taxonomic placement of the *Brassica nigra* plant within the hierarchy is shown in Table 1.1.

Table 1.1 Classification of *Brassica nigra* plant

Kingdom	Plantae
Clade	Angiosperms
Clade	*Eudicots*
Clade	*Rosids*
Order	*Brassicales*
Family	*Brassicaceae*
Genus	*Brassica*
Species	*Brassica nigra*
Binomial name	*Brassica nigra*

1.3 Vernacular Names

Brassica nigra is acknowledged by various vernacular names across different regions of the world. The ensuing list features some of the prevailing names for this plant in a multitude of languages:

- Senafich (Amharic)
- Moutarde noir (French)
- Senape nera (Italian)
- Zwarte mosterd (Dutch)
- Mostarda preta (Portuguese)
- Mostaza negra (Spanish).
- Abba (Singalese)
- Biji sawi hitam (Malaysian, Indonesian)
- Rai (Hindi) (Thomas *et al.*, 2012)

1.4 Geographical Distribution of *Brassica nigra*

B. nigra is believed to have its origins predominantly in the Asia Minor-Iran vicinity; however, its contemporary distribution encompasses the wild habitats of the Mediterranean, extending across central Europe, the Middle East, and the highlands of Ethiopia. Analogous to other mustards, black mustard finds utility both as a culinary green vegetable and as a source of a spice that has etched its presence in both ancient and contemporary food. Its application as a spice is traceable back to the historical civilizations of Egypt, Babylonia, India, Greece, and China. The cultivation of black mustard as a field crop gained momentum post the medieval era. Furthermore, this botanical entity provides a notably beneficial edible oil (Kaur *et al.,* 2022).

1.5 Production and Cultivation

Mustard represents a cool-season agricultural product, well suited to a short growing season. Its cultivation is predominantly established in arid regions, as such conditions foster superior seed quality, (Rosengarten, 1969). The plant tends well-drained and well-aerated soils that evade waterlogging and exhibit resistance to drought. Insufficient aeration of soil perpetuates hindered growth. Optimal performance occurs when mustard is cultivated in soil approximating a near-neutral pH, yet it is also amenable to alkaline and moderately saline soil compositions (Pruthi, 2001).

The mustard seeds, characterized by their diminutive size, necessitate placement in a damp, compact, and shallow seedbed to facilitate prompt germination and emergence. Ample moisture provision augments an extended flowering phase, the duration of which positively correlates with enhanced yield outcomes. Storing the seeds for prolonged periods mandates maintaining a moisture content below 10%. In the process of drying mustard, meticulous attention must be paid to preventing the seed temperature from exceeding 52 °C, as this can potentially damage intrinsic enzymes. Such damage could subsequently hinder the hydrolysis of glucosinolates into isothiocyanates, the key agents contributing to the hot characteristic of the mustard. (Thomas *et al.*, 2012).

1.6 Phytochemical Constituents of *Brassica nigra*

Phytochemicals, chemical compounds derived from plants, play a crucial role in human health by providing defense mechanisms against various diseases. These compounds exhibit anti-oxidative properties, effectively neutralizing free radicals, which are byproducts of biochemical processes. They safeguard essential biomolecules and offer protection against a spectrum of physiological conditions, including neurological and cardiac disorders (Uttara *et al.,* 2009). Numerous Brassica species have undergone inspection to uncover their bioactive phytochemical constituents and antioxidant potential. Notably, the chemical composition of the crop demonstrates considerable variability contingent on mustard variety, cultivation region, and growth conditions. Brassica plants stand out as substantial repositories of medicinally significant phytochemical compounds (Danlami *et al.,* 2006).

Remarkably, *brassica nigra* boasts an energetic profile, containing an average of 23-30% fixed oil, 29-36% protein, and 12-18% carbohydrate content. Among the roster of bioactive phytochemical components prevalent in numerous Brassica species are carotenoids (zeaxanthin, lutein, β-carotene), polyphenols, phenolic acids, alkaloids, flavonoids, tannins, anthocyanins, saponins, phytosterols chlorophyll, glucosinolates, terpenoids, phytosteroids, glycosides, vitamin E, Vitamin C, aliphatic and aromatic amines (Shahawany *et al.,* 2016). These compounds underpin the manifold biological activities exhibited by *B. nigra* plants against various diseases, rendering them efficacious in human therapeutics. The edible components of these plants boast an array of beneficial attributes, including antimicrobial, antimalarial, antidiabetic, antiulcer, anti-hyperlipidemic, anti-aging, antioxidant, anti-proliferative, neuroprotective, and anti-genotoxic (Unal *et al.,* 2014).

Notably, leaves, seeds, and callus of these plants contain an assortment of compounds such as phenolics (gallic acid, catechin, epicatechin, myricetin, quercetin, and rutin), flavonoids, sinigrin, cyanogenic and cardiac glycosides, glutathione reducing sugars, tannins, phlobatannins, saponins, alkaloids, and volatile oils. These components collectively contribute to antioxidant and antiradical activities, as evidenced by assays measuring DPPH, and ABTS radical scavenging capacity. Black mustard (*B. nigra*) is characterized by the dominant presence of 2-propenyl (allyl) glucosinate (sinigrin), which upon hydrolysis produces allyl isothiocyanate, commonly referred to as volatile oil. In addition, minor volatile constituents released through enzymatic hydrolysis

encompass methyl, phenyl, butyl, sec-butyl, 3-butenyl, 4-pentenyl, isopropyl, 3-methylthopropyl, benzyl, and β-phenylethyl groups (Nawaz *et al.,* 2018). Notably, gas chromatography-mass spectrometry (GC-MS) analysis of essential oil from *B. nigra* seeds revealed prominent constituents, including 9,12-octadecadienoyl chloride, di-(9-octadecenoyl)-glycerol, 1-(hydroxymethyl)-1,2-ethanediyl ester, and hexadecanoic acid (Jawaher *et al.,* 2022).

The conventional method for screening phytochemical content in *B. nigra* extracts involves thin-layer chromatography (TLC), which facilitates the separation and identification of anthraquinone glycosides, saponins, volatile alkaloids, flavonoids, and oils. Phytochemical exploration of explants yields twenty TLC spots, comprising 5 anthraquinone glycosides, 4 tannins, 3 saponins, 3 alkaloids, 3 flavonoids, and 2 volatile oils (Obi *et al.,* 2009).

1.7 Pharmacological Properties of *Brassica nigra*

As documented in the current body of literature, *Brassica nigra* is renowned for its abundance in a multitude of biologically active constituents, with links to a diverse range of potential health advantages such as antioxidative, cytotoxic activity, antimicrobial, and antidiabetic properties.

1.7.1 Antioxidant Profiling

Brassica species contain antioxidants such as ascorbic acid, alpha-tocopherol, phenolic acids (sinapic acid, gallic acid, caffeic acid, and ferulic acids), carotenoids (lutein, beta-carotene), and flavonoids (quercetin, rutin, and kaempferol). These compounds are known to act as antioxidants, neutralizing free radicals and protecting the immune system (Salehi *et al.,* 2021). Furthermore, because they participate in the breakdown of peroxidase, neutralization and adsorption of ROS, as well as the scavenging of singlet and triplet oxygen, phenolic are more effective at protecting the body against chronic diseases such as cancer and atherosclerosis, as well as ageing and inflammation, and neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Verma *et al.,* 2019).

Additionally, there is a notable link between the compositional profile of Brassica species and their antioxidant ability, with a particular emphasis on polyphenols, especially flavonoids. This emphasis is founded on the superior antioxidant efficacy exhibited by phenolic compounds,

surpassing that of vitamins and carotenoids (Mullen *et al.,* 2007). Notably, *B. nigra* (black mustard) seed extract showed strong DPPH inhibition, reaching 94.24% inhibition. A comprehensive in vivo assessment involving female albino rats exposed to cadmium chloride, the ability of the aqueous extract of *B. nigra* seeds to reduce oxidative stress induced on by cadmium chloride was elucidated by CdCl2. Furthermore, this extract demonstrated effectiveness in counteracting the toxic impact on hematological parameters and lung tissue (Shen *et al.,* 2010).

1.7.2 Cytotoxic Activity

Cancer drugs carry toxicity that impacts not only cancerous cells but also normal cells. To mitigate cancer risk in various cancer types, such as lung, colon, breast, and gastric cancer, natural compounds must be used (Kwak *et al.,* 2016). The substantial antiproliferative and preventive influence exerted by Brassica vegetable seeds on tumor cells, particularly in colon and lung cancer scenarios, can be attributed to their abundant reservoirs of bioactive constituents such as phenolics, flavonoids, and glucosinolates. Notably, beyond their antioxidant prowess, phenolics and flavonoids exhibit discernible antitumor properties (Tang *et al.,* 2010).

The ethanolic extract of *B. nigra* seeds displayed cytotoxic effectiveness by reducing the viability and clonogenic proliferation of H-1299 and A-549 cells, triggering cellular apoptosis in a concentration and time dependent manner, and increasing caspase-3 activity (Fang *et al.,* 2021). In a different setting, an isothiocyanate-rich hydro-alcoholic extract of *B. nigra* seeds showed an antiproliferative effect on liver tissue in mice subjected to phenobarbital at a dose of 800 mg/kg. This effect was underscored by the amelioration of histopathological alterations, including moderate diffuse proliferation and eosinophilic cytoplasm (Ghanbari-Movahed *et al.,* 2021).

1.7.3 Antimicrobial Activity

Aside from their agricultural and nutritional significance, an array of investigations has indicated that Brassica seeds possess a high level of antimicrobial and fungicide potential against a variety of important pathogens (Bellostas *et al.,* 2007). Brassica seeds are a primary source of protein, glucosinolate, essential oil, and polyphenol. The bioactive agents isolated from B. nigrin seeds, gluconasterthalin (phenethyl glucosinolate) and sinigrin, glucotropeoline (benzyl glucosinolate) have been identified as effective against a variety of fungi, including *Botrytis cinerea, Fusarium oxysporum, Aphanomyces euteiches var. pisi, Gaeumannomyces graminis var. tritici, Pseudocercosporella herpotricoides, Verticillium,* and *Rhizoctonia solani*, (Wang *et al.,*

2019). The collective body of research data offers compelling indications that Brassica seeds, along with their derivative compounds such as functional peptides, sulforaphane, and GLS, hold significant promise as natural antimicrobial assets within the realms of both the food and pharmaceutical industries (Khaliq *et al.,* 2022).

1.7.4 Antidiabetic Activity

The global prevalence of diabetes stands as a significant contributor to both mortality and morbidity. Multiple research investigations have indicated the potential for hypoglycemia to be induced by Brassica seeds (Grover *et al.,* 2002). In-depth exploration, encompassing in vitro and animal studies, has been undertaken to assess the impact of Brassica seed extracts on diabetes. The findings from these endeavors revealed notable outcomes. Specifically, when administering oil derived from Brassica seeds to diabetic Strain-Zebrafish (STZ) rats at dosages of 500mg/kg and 1000mg/kg body weight respectively, a discernible reduction in blood glucose levels was observed. The levels dropped from 335mg/dL to 280mg/dL and from 330mg/dL to 265mg/dL, at 4 hours respectively, in comparison to the diabetic control group (Grover *et al.,* 2003).

Additionally, the test groups had a marked increase in Body Weight, Liver Glycogen Content, Plasma Insulin Levels, and a decrease in Glycosylated Haemoglobin (GHA), Malondialdehydehydrogen (MDA) decreased and reduced glutathione substances (GSH) increased. The results of the study showed remarkable antihyperglycaemic effects of the B. Nigrum seed oil at both doses (Thirumalai *et al.,* 2011).

1.8 Industrial Importance

Brassica nigra holds considerable significance within both the culinary and healthcare sectors. In the culinary domain, the seeds of this plant are incorporated as condiments and spices. There are forty species of Brassica, of which three are most commonly used as spices. Notably, among these, *B. nigra* stands out for its distinctive pungent taste. However, harvesting *B. nigra* seeds proves relatively challenging in comparison to the other two types, namely B. juncea and B. alba. Historical records highlight the ancient utilization of *Brassica nigra* seeds as a spice in regions such as India, Greece, and the Middle East. The seeds of black mustard are skillfully ground to create a finely powdered mustard meal, which can be effectively stored in its dried form. This meal serves as the basis for renowned condiments like English mustard and French mustard

when combined with vinegar. These condiments, known for their piquant flavors, are commonly served on tables.

The inclusion of white mustard (*Sinapis alba L.*) mitigates its intensity, resulting in the creation of milder Dutch or German mustard. In regions like North America and Europe, other herbs are also complemented to further enhance the traditional mild flavor achieved with starchy ingredients such as honey and sugar. This amalgamation of components leads to a diverse range of mustard-enhanced recipes, spanning meat dishes, sauces for seafood, meat, snacks, and salads, as well as, and as a cream stabilizer in mayonnaise. Additionally, *Brassica nigra* aids in the production of honey characterized by its light hue and delicate flavor profile (Vaughan and Hemingway, 1959).

1.9 Agriculture Importance

Brassica nigra serves a dual purpose as both a cover crop and a green manure crop within agricultural contexts. Its fast growth rate and dense foliage make it an excellent choice for weed control and erosion control. During the winter months, *Brassica nigra* can achieve an impressive soil coverage of up to 80%. *Brassica nigra* plays a crucial role in enriching the soil. It adds organic matter to the soil, enhancing its structure, and also produces valuable chemical compounds that facilitate nutrient availability for future crops (Haramoto *et al.,* 2004). Moreover, *Brassica nigra* is notably recognized for its biofumigation capability. As its plant remnants decompose in the soil, they release volatile chemicals known as isothiocyanates. These compounds exhibit potent antifungal, antibacterial, and nematocidal properties, allowing them to naturally control soil-borne pests and diseases, thereby reducing reliance on synthetic chemical interventions (Jat *et al.,* 2019). The seeds of *Brassica nigra* represent a substantial reservoir of essential nutrients, including proteins, dietary fibers, minerals, and vitamins. Additionally, its leaves are consumed as part of salads. Incorporating *Brassica nigra* into one's diet contributes to a well-rounded nutritional intake and supports overall health (Vicas *et al.,* 2019).

1.10 Importance of Plant Tissue Culture (PTC)

Plant secondary compounds can be derived from wild plants found in nature, but their commercial extraction faces limitations due to environmental, geographical, and seasonal factors. Traditional cultivation methods can be used, yet they entail extended periods, sometimes spanning years, to cultivate plants that yield the desired secondary compounds. The Plant Tissue Culture

(PTC) approach emerges as a viable solution to overcome these challenges. The PTC method involves cultivating plant cells, tissues, and organs in a sterile artificial medium under controlled environmental conditions (Chadipiralla *et al.,* 2020). This technique is primarily rooted in micropropagation, which involves rapidly multiplying cells from tiny plant tissue fragments such as stems, leaves, axillary buds, and roots. As a result, it becomes an effective tool for large-scale plant reproduction. Notably, this technology offers the advantage of swiftly generating commercially and medicinally valuable bioactive compounds within a concise timeframe (Rao & Ravishankar, 2002).

1.10.1 Applications of PTC

- Tissue culture has introduced a method for propagating and genetically enhancing commercially important plants.
- Tissue culture aids in investigating the metabolism, growth, physiology, reproduction, and nutritional needs of plants within controlled settings.
- It offers a potent approach for enhancing crop quality and yield.
- Mutagens are introduced into single-cell liquid cultures to initiate mutations.
- Tissue culture enables the development of embryos that might not typically survive within seeds, resulting in new generations; it's also applicable for interspecific hybridization.
- It allows the creation of numerous plants amidst the absence of seeds or crucial pollinators.
- Somatic embryogenesis facilitates the large-scale production of artificial seeds.
- Plantlets can be swiftly generated using minimal plant tissue.
- Tissue culture plays a role in synthesizing new bioactive compounds not naturally present in the wild species.
- Germplasm conservation of valuable medicinal plant species is also achieved through tissue culture.
- By fostering genetic diversity, tissue culture contributes to the advancement of medicinal plant varieties (Khan *et al.,* 2017).

1.11 Micropropagation

Micropropagation involves the rapid in vitro multiplication and clonal propagation of plants using even the tiniest sections of plant tissue. In a controlled and sterile environment, these plant fragments can regenerate into completely new plants (Abbasi *et al.,* 2016). This biotechnological approach holds immense significance in safeguarding and conserving extinct and endangered medicinal plant species through selection, propagation, and germplasm preservation. This technique has also found applications in the development of disease-free plant varieties, genetic enhancement, and the production of high-quality secondary metabolites for pharmaceutical purposes (Khan *et al.,* 2020).

The last couple of decades have witnessed a growing interest in the in vitro cultivation of medicinal plants. Various plant parts like roots, rhizomes, shoot apex, leaves, petals, bud scales, embryo axes, and cotyledons have been utilized as explants to cultivate medicinal plants. Notably, a range of medicinal plant species such as *Lavandula angustifolia, Ajuga bracteosa, Mentha piperita, Rosmarinus officinalis,* and *Lallemantia Iberica,* among others, have effectively been subjected to in vitro micropropagation techniques (Khan *et al.,* 2021).

1.11.1 Shoot tip-Culture

Shoot tip culture is the fastest and the most reliable method for generating plantlets via micropropagation, all while evading somaclonal variation – the genetic and epigenetic shifts encountered in clonal propagation. Explants or shoot tips encompass the shoot apical meristem, unexpandable leaves at varying growth stages, and several 1 cm-long leaf primordia. The process involves introducing these explants into media enriched with cytokinin, a plant hormone. Cytokinin's presence leads to the inhibition of apical dominance and stimulates the formation of a highly branching shoot system. Subsequently, careful manipulation of these developing shootlets takes place within the rooting medium to foster the growth of plantlets. (Bhatia *et al.,* 2015)

1.12 Stages Involved in Shoot-Tip Culture Micropropagation

The complexity of the in vitro cloning or micropropagation process can be broken down into four distinct stages. These stages were first identified in 1978 by Murashige, with stages I to III encompassing in vitro conditions and stage IV occurring in a greenhouse setting (Gosal et al., 2010). The introduction of stage 0 into the overall micropropagation system was made in 1981 by the team of Debergh and Mane (Torres, 1989).

1.12.1 Stage 0: Stock/Elite Plants Selection

When it comes to micropropagation, the initial step is to choose seeds or plants that have the right traits for large-scale propagation. After selecting seeds/high-quality plants, they are kept in a controlled environment characterized by low humidity, regulated irrigation, and the absence of systemic microbial infections. This process typically spans around 2 to 3 months (Cassells & Doyle, 2006).

1.12.2 Stage I: Aseptic Culture Establishment

During this phase, the selected explants from Stage 0 are prepped for inoculation. They undergo treatment with an appropriate sterilizing agent and are then placed onto a well-defined culture medium. The shoot tips go through surface sterilization, involving chemicals like 5% sodium hypochlorite, 0.1% mercuric chloride, or 70% alcohol. The choice (in combination or alone) and duration of treatment depend on the extent of surface contamination (Husain & Anis, 2009). Once sterilized, the explants are introduced to the MS medium. This medium is enriched with growth regulators, vitamins, and sucrose. For micropropagation, a cytokine-containing (1-3 mg/ L 6-Benzyl Amino Purine) culture medium is used with a lower concentration of Auxin (Naphthenic Acetic Acid) (Saini & Jaiwal, 2002).

1.12.3 Stage II: Multiplication of the Explants

The process of explant multiplication involves the time-consuming growth of shoots from explants. Occasionally, only one shoot emerges from the apical shoots, which is then utilized to obtain numerous nodal explants. Subsequently, nodal explants are introduced to media enriched with cytokines to stimulate multiple shoot growth. Additionally, multiple shoots can also be generated by somatic embryogenesis, or direct organogenesis, on explants. In micropropagation, a single explant yields about five to six shoots within 4 to 5 weeks. In an ideal scenario with full plant survival, a single explant could potentially result in 510–612 plants over a year (Tilkat et al., 2009).

1.12.4 Stage III: Germination of Somatic Embryo or Rooting of Regenerated Shoots

Rooting in the fresh medium is initiated by the emergence of multiple shoots during stage II. Each separated shoot is introduced to a rooting medium containing auxins. Alternatively, under humid conditions, some shoots are rooted directly in the soil. These newly rooted plants are susceptible to moisture. For somatic embryos, germination precedes the formation of plantlets.

Following a hardening process, the plantlets are gradually transitioned to soil. The hardening medium, like pearlite, peat, or vermiculite, retains ample moisture and is kept in high humidity conditions before transfer to the soil (Hussain et al., 2012).

1.12.5 Stage IV: Hardening

During the hardening phase, the stage III plantlets transition from controlled in vitro conditions to the external soil environment. This process instills resistance to stress, moisture, and diseases, shifting the plants from their initial heterotrophic state in vitro to an autotrophic state. Protecting the plantlets from direct sunlight and gradually reducing relative humidity are crucial steps. Plantlets develop strong root systems and cuticular wax on aerial parts during this acclimatization. Subsequently, the plantlets become ready for transplantation into open fields (Marana et al., 2009).

1.13 Tissue Culture Studies of *Brassica nigra*

Various species within the Brassicaceae family have been subjects of study in plant tissue culture, offering an alternative approach for breeding economically significant plants. Plant tissue culture enables the propagation of rare and valuable plants (Collin, 2001). This technique can induce qualitative and quantitative changes that can be useful for the production of plant secondary metabolites by altering nutrient and hormonal conditions. Several plant parts, such as shoots, hypocotyls, petioles, leaf discs, and peduncles, have been utilized for in vitro regeneration. In an example, Jain et al. (1988) demonstrated that changes in the enzymes of the glycolysis pathway occur in *B. nigra* petiole suspension cultures after a week of phosphate deficiency. The same thing was seen with cotyledonic explants where callus and shoot formation served as growth indicators. Furthermore, *B. nigra* seed cultures, cultivated with varying nanoparticle concentrations, exhibited increased levels of oxidative enzymes. The ensuing discussion focuses on the chemical synthesis of metal oxide nanoparticles, their characterizations, and a lab study of their toxic effects on important medicinal plants (Jain *et al.,* 1988).

1.14 Elicitation

Plants create secondary metabolites (SM) as a defense mechanism against both internal and external stress-induced diseases. Subjecting plants to stress can enhance secondary metabolite production. Elicitors, which can be chemicals or bio-factors, trigger various physiological changes in plants. This alteration affects their metabolic processes and leads to a substantial

increase in secondary metabolite production (Patel et al., 2013).

In vitro callus cultures, multiple strategies are employed on plants to boost the production of valuable phytocompounds. Among these methods, elicitation stands out as a successful approach for optimizing the production of a plant secondary metabolite. It also relates closely to augmented biomass growth and the generation of bioactive compounds (Yang & Stöckigt, 2010).

Elicitors, upon interacting with cell membrane-bound receptors, trigger signal transduction pathways that activate relevant genes. All these stress-inducing agents fall under the umbrella term "elicitors" (Isah, 2019). They are classified as either abiotic or biotic (Baenas et al., 2014). Abiotic elicitors encompass light, metals, salinity, osmotic changes, temperature variations, drought, and hormonal signals. On the other hand, biotic elicitors encompass plant cell wall components (like pectin, and cellulose) and microorganisms (such as extracts from fungi, yeast, and bacteria) (Naik & Al-Khayri, 2016).

1.15 Lupeol

Lupeol is a naturally occurring triterpene compound, which is believed to be essential for the intricate mechanisms of plant defense and growth. This bioactive substance, marked by its distinctive chemical makeup and exceptional traits, serves as an elicitor, effectively boosting the production of secondary metabolites. Additionally, its noteworthy therapeutic and pharmacological effects have garnered huge interest of scientists.

1.15.1 Chemical Properties

- **Chemical formula:** C30H50O
- **Molecular weight:** 426.72 g/mol
- **Other Names:** Fagarasterol, Clerodol
- **Melting point:** 215-216 °C (Saleem *et al.,* 2009).
- **Structural composition:** In lupane, a hydroxyl group substitutes the hydrogen at the 3beta position, transforming it into a pentacyclic triterpenoid. The structure comprises a five-membered ring (with an envelope conformation) and four six-membered rings (with chair conformations). These rings are interconnected in a transorientation (Corrêa *et al.,* 2009).
- **Physical appearance:** a white crystalline solid at room temperature (Corrêa *et al.,* 2009).

- **Solubility:** It dissolves in organic solvents such as chloroform, ether, and ethanol, yet its hydrophobic properties prevent solubility in water.
- **Stability:** According to Corrêa et al. (2009), the structure remains relatively stable under standard conditions.

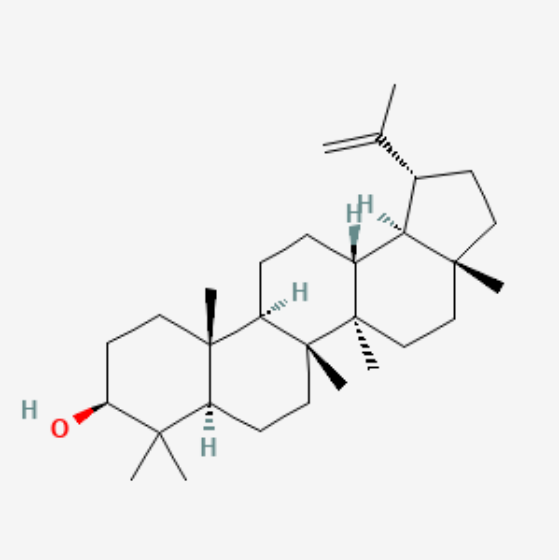

Figure 1.1 Structure of Lupeol (Pubchem)

1.15.2 Agricultural Importance of Lupeol

Triterpenoids assume a crucial role in plant defense mechanisms. Notably, lupeol- a triterpenoid, has demonstrated its ability to counteract oxidative stress in model animals. Its effectiveness against free radicals and reactive oxygen species is recognized. Lupeol alleviated the stress response and gradually decreased the number of antioxidative molecules (enzymatic and nonenzymatic). By manipulating salt concentrations during the study, improvements were noted in the biochemical and antioxidative responses within both parts of Brassica plants.

The study emphasizes the importance of Lupeol in agriculture. It serves to alleviate stress, shield plants from oxidative harm, and maintain cellular redox equilibrium amidst stressors. Lupeol's presence was also found to stimulate increased root growth, aiding in counteracting the toxic impact of salt. With the need to elevate food crop productivity by 70% by 2050 to sustain the growing population, lupeol can be employed as an elicitor to address these imperative requirements (Zia et al., 2022).

1.15.3 Medicinal Importance of Lupeol

The core structure of a pentacyclic triterpene compound contributes significant stability and rigidity to the molecule. The presence of lupeol across different plant species emphasizes its widespread occurrence (Zhang et al., 2022). Comprehensive scientific research has dug into its pharmacological characteristics, revealing its potential in areas such as anti-inflammatory, antiproliferative, and antihyperglycemic activities. As a precursor molecule, Lupeol facilitates the synthesis of Betulinic Acid, a triterpenoid compound that has demonstrated potent anticancer attributes. This remarkable natural product is particularly abundant in the bark tissue of birch trees (Kumari & Kakkar, 2012).

1.15.4 Ecological Importance of Lupeol

Lupeol plays a critical role in the complex formation of nodules in the leguminous plant species, Lotus japonicus. It is primarily responsible for regulating the expression of the enzyme ENOD40, which is widely known to be involved in the formation and growth of nodules. According to Delis et al., (2011), the OSC3 gene is the exclusive driver of lupeol production within L. japonicus roots and nodules. By regulating the expression of the ENOD40 gene, Lupeol has a major influence on the molecular mechanisms that lead to the formation of nodules and thus contributes to the successful functioning of the plant and nitrogen-fixing bacteria. Additionally, Lupeol is a component of the cuticular wax surface of castor beans (Ricinus communis), where it is suggested to play a potential role in shielding the plant against dehydration (Guhling et al., 2006).

1.16 Aims and objective

Objectives of the study are:

- To investigate the effect of Lupeol on growth and proliferation of shoot-tip culture of *Brassica nigra*
- To determine the phytochemical analysis of *B. nigra* shoot-tip culture.
- To determine the antioxidant enzyme analysis of *B. nigra* shoot-tip culture.
- To determine the non-enzymatic analysis of *B. nigra* shoot-tip culture.

CHAPTER 2

2 MATERIAL 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

Seeds were germinated utilizing Murashige and Skoog basal medium (Murashige & Skoog, 1962). In an electronic balance, sucrose (30 g) and MS medium (4.4 g) were weighed and dissolved in distilled water in an Erlenmeyer flask to bring the total amount up to 1 liter. The pH of the medium was then adjusted at 5.65 ± 0.02 by using hydrochloric acid (1.0 N) and sodium hydroxide (1.0 N). The medium was then solidified by adding 8 g agar to it. The flasks were then microwaved for five min, or until the medium began to boil and the agar was properly dissolved. After that, medium (30 mL) was transferred into each Erlenmeyer flask (100 mL), and the flasks were firmly sealed with aluminum foil and cotton. Finally, these flasks were autoclaved for 20 min at temperature (121°C) and pressure (15-psi) and then left overnight to ensure that the medium solidified correctly and that there was no possibility of contamination (Abbasi *et al.,* 2010)

2.3 Surface Sterilization

To remove dust and other contaminants that had accumulated on the research tools and glassware, they were thoroughly cleaned with detergent and running tap water. These were allowed to dry before being wrapped carefully in paper and sterilized utilizing an autoclave at 121°C for around 20 minutes at 15-psi pressure.

2.4 Explant Collection, Inoculation, and Seed Germination

In a sterile environment, inoculation was performed in a laminar flow hood (LFH) cabinet. The LFH walls and floors were carefully swabbed with ethanol (70%) before usage. After that, autoclaved flasks having medium (30 mL) were moved to LFH, together with autoclaved tools

such as a forceps, Petri dish, distilled water, blade, ethanol, and an empty beaker. To limit the risk of contamination, each of these tools was surface sterilized with ethanol (70%). For efficient sterilization, the LFH door was closed and the UV light was switched on for 20 minutes. Hands were sterilized with 70% ethanol before inoculation. The spirit lamp was flamed and placed near the opened Petri plate with a filter paper. After rinsing the *Brassica nigra* seeds with distilled water, they were surface sterilized in the LFH. These seedlings were soaked in detergent TWEEN-20 for two minutes followed by rinsing with tap water. Subsequently, they were subjected to a 70% ethanol treatment lasting for 5 to 10 minutes. Afterward, the seeds underwent a surface sterilization process lasting for 5 minutes using a 0.1% solution of mercuric chloride (HgCl2). Following the surface sterilization, the seeds underwent three rounds of washing with sterile water to eliminate any residual traces of mercuric chloride. Each flask accommodated six seeds for inoculation. For the explant, shoot tips were extracted from seedlings that had germinated in vitro.

2.5 Shoot tip Culture Establishment

In vitro germinated explant seedlings had their shoot tips excised and ranged in size from 0.5 to 1.0 cm. Explants were placed without surface sterilization on an MS basal medium supplemented with varying concentrations of plant growth regulator lupeol, BAP, and NAA. Each of the three lupeol concentrations (1µM, 5µM, 10µM) was tested with four replicates. 4 Replicates for of Benzylaminopurine (BAP) and 4 Replicates for 1µM concentration of BAP Hormone (NAA) as positive controls (Table 1). Six explants were kept in each flask, which had three flasks per treatment. Three flasks were first kept in a 25 °C growing chamber in the dark. Then, using white LEDs (380–780 nm, PPFD 15 mol/m2/s), they were switched to an 8–16 dark/light cycle once the seedlings had grown.

2.6 Fresh and Dry Weight of Roots and Shoots

After 19 days, the plant's shoots and roots were trimmed and separated. Using an electronic measuring balance, their fresh weight (FW) was determined. Subsequently, the plants were positioned on Petri plates and placed in a 37°C incubator for 3 days to facilitate low-temperature drying. After drying, the roots and shoots were once again weighed using the measuring balance to obtain the dry weight (DW).

2.7 Phytochemical Analysis

The phytochemical analysis of test samples of roots and shoots of *brassica nigra* were carried out by measuring the total phenolic and total flavonoid contents.

2.7.1 Total Phenolic Contents

TPC of test samples were identified using the method described previously by Astill *et al.,* 2001.

For TPC, 3 stock solutions were prepared.

1. **Folin-Ciocalteu reagent:** Folin-Ciocalteu reagent stock solution in distilled water was produced 10 times diluted solution.
2. **Sodium carbonate solution:** Sodium carbonate was produced as a 6% stock solution in distilled water.
3. **Gallic acid:** 4 mg of Gallic acid was dissolved in 1 ml of DMSO to create a stock solution.
4. **Test samples** prepared in 1ml DMSO.

Procedure

Gallic acid and DMSO were carefully added to each well of a 96-well plate for the first 20 µL of the test samples, standard and blank (i.e., sample/stock solution). Following that, 90 µL of freshly made FC (Folin- Ciocalteu) reagent that had been 10 times diluted was added to the test samples, standard, and blank. The plate was then incubated for 5 min and 90 µL of Na2CO3 solution was mixed to the reaction mixture after incubation. The plate was then incubated for 1 hr and absorbance was measured at 650 nm by using microplate reader.

Analysis

For the standard, a calibration curve was drawn at various concentrations and the total phenolic content of the test samples were evaluated in accordance to the value obtained by that curve (R2 value). The resultant TPC was calculated as µg Gallic acid equivalent per mg extract (µg GAE/mg extract).

2.7.2 Total Flavonoid Contents (TFC)

Total flavonoid contents of the test samples were determined using the method previously described by Almajano *et al.,* 2008.

For TFC three stock solutions were prepared.

1. **Aluminium chloride:** Stock solution of 10% (10g/100ml) aluminium chloride was prepared in distilled water.
2. **Potassium acetate:** 1 M (98.15 g/L) potassium acetate solution was prepared in distilled water.
3. **Quercetin:** 4 mg/ml Quercetin was prepared in DMSO.
4. **Test samples** prepared in 1ml DMSO.

Procedure

Firstly, 10 µL of aluminium chloride solution (10%) was added to the 96-well microplate along with 20 µL of the blank, standard, and test sample. Following the addition of 10 µL of potassium acetate (1 M) solutions, 160 µL of distilled water was added to bring the total amount to 200 µL. The plate was then incubated for 30 min, and the samples' optical densities were determined at 415 nm using a microplate reader (Bioteck, USA).

Analysis:

The calibration curve of the standard was drawn using Quercetin under varying final concentrations. The equation obtained using calibration curve was used to evaluate the total flavonoid content of the test samples (R2 value), the assay was performed in triplicate. While the values were obtained in µg Quercetin equivalent per mg plant extract (µg QE/mg extract).

2.8 Antioxidant Enzymes Analysis

For the determination of different enzymes activities, fresh roots were taken, washed with distilled water and then grinded in phosphate buffer (pH 7.0), were transferred to Eppendorf tubes and centrifuged at 10000 rpm for 10 minutes. The supernatant was collected and was used for a number of different enzymatic assays that includes;

2.8.1 Super-oxide Dismutase (SOD)

SOD activity was measured using the Beauchamp and Frodovich, 1971, technique.

Procedure

First 500ml of 0.05M phosphate buffer (pH 7.8) was prepared. Then 0.0279g of Na2EDTA, 0.0014g of methionine and 0.049g of NBT were dissolved in 100ml of phosphate buffer (pH 7.8) and 20ml from step 2 was collected and volume was raised up to 100ml of phosphate buffer (pH 7.8). After that fresh riboflavin solution was prepared by adding 1.13mg of riboflavin in 100ml of phosphate buffer (pH 7.8), 40ml of step 4 was collected and volume was raised up to 100ml by phosphate buffer (pH 7.8). Finally, blank was created by mixing 0.25 millilitres of step 5 with 1 millilitre of phosphate buffer (pH 7.8).

Analysis

To determine the level of enzymatic activity, the subsequent calculation was employed:

A = ELC

Letters A, C, E, and L are used to represent the sample absorbance, the enzyme concentration (measured in nM/min/mg FW), the extinction coefficient (measured in 6.39 mM^{-1} cm^{-1}), the length of the wall (0.25 cm), and the enzyme concentration (E).

2.8.2 Per-oxidase Activity (POD)

Per-oxidase activity was performed by using the method of vetter *et al*., 1958 that was modified by gorin and hidema, with some modifications.

Procedure

50µl of sample was mixed with 0.675ml of 100Mm MES buffer (pH 5.5), 100µl of 0.1% Phenylenediamine and 0.3µl of 0.05% hydrogen-per-oxide (H_2O_2). Absorbance was recorded at 485nm for 3 minutes.

Analysis

To determine the level of enzymatic activity, the subsequent calculation was employed:

A = ELC

Letters A, C, E, and L are used to represent the sample absorbance, the enzyme concentration (measured in nM/min/mg FW), the extinction coefficient (measured in 6.39 mM-1 cm-1), the length of the wall (0.25 cm), and the enzyme concentration (E).

2.9 Non-Enzymatic Analysis

In a pestle and mortar, the dried roots and shoots were transferred before being placed in eppendorf tubes. The weight of crushed roots and shoots were measured once again and 1ml of DMSO was added to each tube. These test samples were than subjected to different biological assays including;

2.9.1 DPPH Free Radical Scavenging Assay

The antioxidant activity of the test sample against (DPPH) was measured using a slightly modified technique reported by Clarke et al. (2013).

For DPPH, stock solutions were prepared as;

9.6 mg of DPPH was dissolved in 100 ml of analytical methanol to create a stock solution, which was then sonicated for 30 minutes. The stock solution of test sample was prepared by dissolving 4mg/ml in DMSO with final concentration of 200 µg/ml. The solution of Ascorbic acid, 1mg/1ml was used as standard and DMSO was used as blank.

Procedure:

In this assay first of all 10 µL of test sample, standard and blank was transferred in the 96 microplate with pipette and then 190 µL of DPPH was transferred and mixed. The reaction mixture was incubated in dark for 1 hr at 37ºC. The optical density of the samples were measured at 515 nm using microplate reader.

Analysis:

The formula (1 - A / B)*100 was used to determine the percent inhibition of the test sample. A is the represents the DPPH solution's O.D. with sample. B represents the O.D. of the negative control, which contains the reagent but not the sample.

2.9.2 ABTS Radical Scavenging Activity

The test samples' 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid potential was assessed in accordance with the steps outlined by Wang et al. (2012).

Procedure:

2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was mixed in a 1:1 ratio with 2.45 mM of potassium persulfate to create the ABTS reagent. The reagent was treated to a period of darkness lasting 12-18 hours at ambient temperature, after which it was subsequently diluted with methanol in a ratio of 1:2. The absorbance was measured, then diluted until it attained a value of 0.7 0.02 at a wavelength of 540 nm. In a 96-well microtiter plate, 10 µl of samples were reacted with 100 µl of ABTS reagent for the assay. After 10 minutes of incubation at room temperature in the dark

Analysis

The absorbance of the plate was measured at 540 nm.

2.9.3 Metal Chelating Potential (MCP)

The test samples' metal chelating capacity was determined in accordance with the steps outlined by Wang et al. (2012).

Procedure:

The experiment was carried out in a 96-well plate. 100 µl of each sample were placed into each well, and then 50 µl of a 2 mM $FeCl_2$ solution was added. The plate was incubated in the dark for 10 minutes. Following that, 20 µl of 5 mM ferrozine was added to each well and incubated for 5-10 minutes.

Analysis

The absorbance was checked against EDTA as a positive control at 562 nm. Metal chelating efficiency was determined by MC ability%

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

2.9.4 Total Antioxidant Capacity (TAC)

Brassica nigra shoot tips' total antioxidant activity was determined using the method reported by Clarke et al. (2013) with a few modifications.

For TAC stock solutions were prepared as;

Stock solution of TAC reagent was prepared by mixing 1.63ml of concentrated sulphuric acid, 0.1918g of NaH_2PO_4 and 0.247g of ammonium molybdate in 50ml of distilled water. First of all add sulphuric acid in distilled water and then add other salts. 4mg/ml stock solution of test sample was used for this procedure while 1 mg/ml solution of Ascorbic acid was used as standard and DMSO was used as blank.

Procedure

In the total antioxidant assay, 900 µL of TAC reagent was added to Eppendorf tubes after 100 µL of stock solution or test samples were first added. The tubes were then incubated at 95ºC for 90 min. After incubation the reaction mixtures were cooled at room temperature and then 200 µL of uniformly mixed solution were transferred to 96 well plate.

Analysis

The absorbance was measured at 630 nm by using micro plate reader.

2.9.5 Total Reducing Power Assay (TRP)

Total reduction potential of the test samples were evaluated according to the procedure described by Jafri *et al.*, (2017).

Stock solution of 0.2 M Phosphate buffer (pH 6.6), potassium ferricyanide, trichloroacetic acid and ferric chloride was prepared for TRP assay.

1. **0.2 M Phosphate buffer stock solution;** 1.42 g of $Na_2HPO_4.2H_2O$ and 1 g of NaH_2PO_4 were dissolved in 50 ml distilled water (PH 6.6).
2. **1% Potassium ferricyanide:** 1 g/100 ml distilled water.
3. **10% Trichloroacetic acid:** 10 g/100 ml distilled water.
4. **1% Ferric chloride:** 0.1 g/100 ml distilled water.
5. **Test sample:** 4 mg/ml Stock solution of the test samples was used for this procedure
6. **Ascorbic acid:** 1 mg/ml solution was used as standard and DMSO was used as blank.

Procedure:

For total reducing power assay 100 µL of the test samples was transferred to Eppendorf tubes and then mixed with 200 µL of phosphate buffer (0.2 M, pH 6.6) and 250 µL of 1% potassium ferricyanide solution. The reaction mixtures were then incubated for 20 min at 50ºC. After

incubation, the reaction mixture was acidified with 200 µL of 10% trichloroacetic acid. The resulting mixtures were centrifuged at 3000 rpm for 10 min. 150 µL of supernatant layer of each centrifuged mixture was transferred within microplate and mixed with 50 µL of 0.1% ferric chloride solution.

Analysis

The optical density was measured at 630 nm using microplate reader. The results were expressed as µg Ascorbic acid equivalent per mg extract (µg AAE/mg extract).

2.10 Statistical Analysis

The layout of the experiment was entirely random. For each concentration, four flasks were prepared. The biochemical and antioxidant assays were conducted in triplicate. The statistical method of analysis of variance (ANOVA) was employed to examine the differences between means, and the least significant difference (LSD) test was utilized for post hoc analysis. Furthermore the results were statistically analysed using LSD at 0.05 percent probability.

CHAPTER 3

3 RESULTS

3.1 Morphological Characteristics

3.1.1 Root Length

Lupeol treated explants significantly increased the root length of *Brassica nigra* shoot-tip culture in concentration dependent manner as compared to control. Maximum root length was exhibited by L10 µM followed by L5 µM and L1 µM with the increase of 109.8%, 86.9%, and 60.1%, respectively. However, there were no signs of root development in the presence of BAP. In addition, when compared to lupeol-treated explants, the application of NAA resulted in a significant reduction in root length of 60.13%. This shows that the presence of lupeol acts as a growth stimulant, causing a significant increase in the root length of lupeol treated *B. nigra* explants as shown in Table 3.1.

3.1.2 Shoot Length

When compared to the control, lupeol-treated plants significantly increased the shoot length of *Brassica nigra* shoot-tip culture explants in a concentration-dependent way. Maximum shoot length was exhibited by L10 µM followed by L5 µM and L1 µM with the increase of 77. 1%, 53%, and 34%, respectively. However, BAP inhibited shooting and the resultant shoot length was (3.0 cm) which is 66.6% short as compared to control (8.9 cm). Moreover, shoot length was observed to decrease in NAA (10%) as in comparison to lupeol treated samples which showed that presence of lupeol overall act as an growth stimulator and caused an increase in the shoot length of *B. nigra* treated explants.

3.1.3 Number of Leaves

Results showed that lupeol treated plant shoots have significant increase in number of leaves in concentration dependent manner as compared to control (8 number of leaves). Maximum number of leaves were observed by L10 µM followed by L5 µM and L1 µM with the increase of 22, 18, and 14 number of leaves, respectively. However, number of leaves were observed to decrease in BAP (10 number of leaves) and NAA (11 number of leaves) in comparison to lupeol

treated samples which showed thar presence of lupeol acts as a growth stimulant, causing a significant increase in the number of leaves of lupeol treated *B. nigra* explants (Figure 3.2)

Table 3.1 Length of roots and shoots and number of leaves of *Brassica nigra* in presence of BAP, NAA, and lupeol. Values represent means± standard errors from triplicates.

Concentrations (µm)	Root length (cm)	Shoot length (cm)	Number of leaves
Negative Control	5.1±0.25 [e]	8.9±0.44 [e]	8±0.44 [f]
10µM BAP	Callus on the base	2.9±0.14 [f]	10±0.5 [e]
1µM NAA	7.2±0.36 [d]	9.8±0.49 [d]	11±0.55 [d]
1µM Lupeol	8.2±0.40 [c]	11.9±0.59 [c]	14±0.7 [c]
5µM Lupeol	9.5±0.47 [b]	13.5±0.67 [b]	18±0.9 [b]
10µM Lupeol	10.7±0.53[a]	15.7±0.788 [a]	22±1.1 [a]

3.1.4 Fresh and Dry Weight of *B. nigra* Root

A concentration dependent increase was observed in FW and DW of *B. nigra* roots. Results showed that lupeol treated explant have significant increase in their FW and DW of roots as compared to control. Highest value of FW were observed by L10 µM followed by L5 µM and L1 µM with the increase of 120%, 82.9%, and 42.8%, respectively. However, when compared to the control, the application of BAP resulted in a significant 42% reduction in fresh weight. Compared to lupeol-treated explants, the application of NAA resulted in a significant reduction in FW of 26%.

DW of roots showed an increase in lupeol-treated plants in comparison to control. Highest value of DW were observed by L10 µM followed by L5 µM and L1 µM with the increase of 103.1%, 63.3%, and 30.2%, respectively. However, when compared to the control, the application of BAP resulted in a significant 46% reduction in dry weight. Compared to lupeol-treated explants, the application of NAA resulted in a significant reduction in DW (11%). This highlights lupeol's growth-stimulating potential, which caused a significant increase in the fresh and dry weight of the roots of the lupeol treated *B. nigra* explants.

3.1.5 Fresh and Dry Weight of *B. nigra* Shoot

A concentration dependent increase was observed in FW and DW of *B. nigra* shoots. Results showed that lupeol treated explant have significant increase in their FW and DW of shoots as compared to control. Highest value of FW were observed by L10 µM followed by L5 µM and L1 µM with the increase of 243.4%, 206%, and 48.9%, respectively. Compared to lupeol-treated explants, the application of NAA resulted in a significant reduction in FW of 30.7%.

DW of shoots showed an increase in lupeol-treated plants in comparison to control. Highest value of DW were observed by L10 µM followed by L5 µM and L1 µM with the increase of 132.7%, 81%, and 34%, respectively. Compared to lupeol-treated explants, the application of NAA resulted in a significant reduction in DW (8.6%). This highlights lupeol's growth-stimulating potential, which caused a significant increase in the fresh and dry weight of the shoot of the lupeol treated *B. nigra* explants.

Table 2.2 Fresh weight and dry weight of roots and shoots of *Brassica nigra* plants under BAP, NAA, and lupeol. Values represent means ± standard errors from triplicates.

Concentrations (µm)	Roots FW(g)	Roots DW(g)	Shoots FW(g)	Shoots DW(g)
Negative control	0.098 ± 0.049^{c}	0.058 ± 0.0029^{c}	6.5 ± 0.32^{d}	0.38 ± 0.019^{e}
10µM BAP	-	-	3.7 ± 0.18^{e}	0.20 ± 0.010^{f}
1µM NAA	1.28 ± 0.06^{bc}	0.063 ± 0.00315^{bc}	8.2 ± 0.41^{cd}	0.432 ± 0.02^{e}
1µM lupeol	1.46 ± 0.07^{b}	0.078 ± 0.0039^{b}	9.7 ± 0.48^{c}	0.504 ± 0.025^{c}
5µM lupeol	3.01 ± 0.15^{ab}	0.105 ± 0.045^{ab}	11.9 ± 0.59^{b}	0.632 ± 0.03^{b}
10 µM lupeol	3.38 ± 0.16^{a}	0.135 ± 0.006^{a}	14.4 ± 0.72^{a}	0.786 ± 0.04^{a}

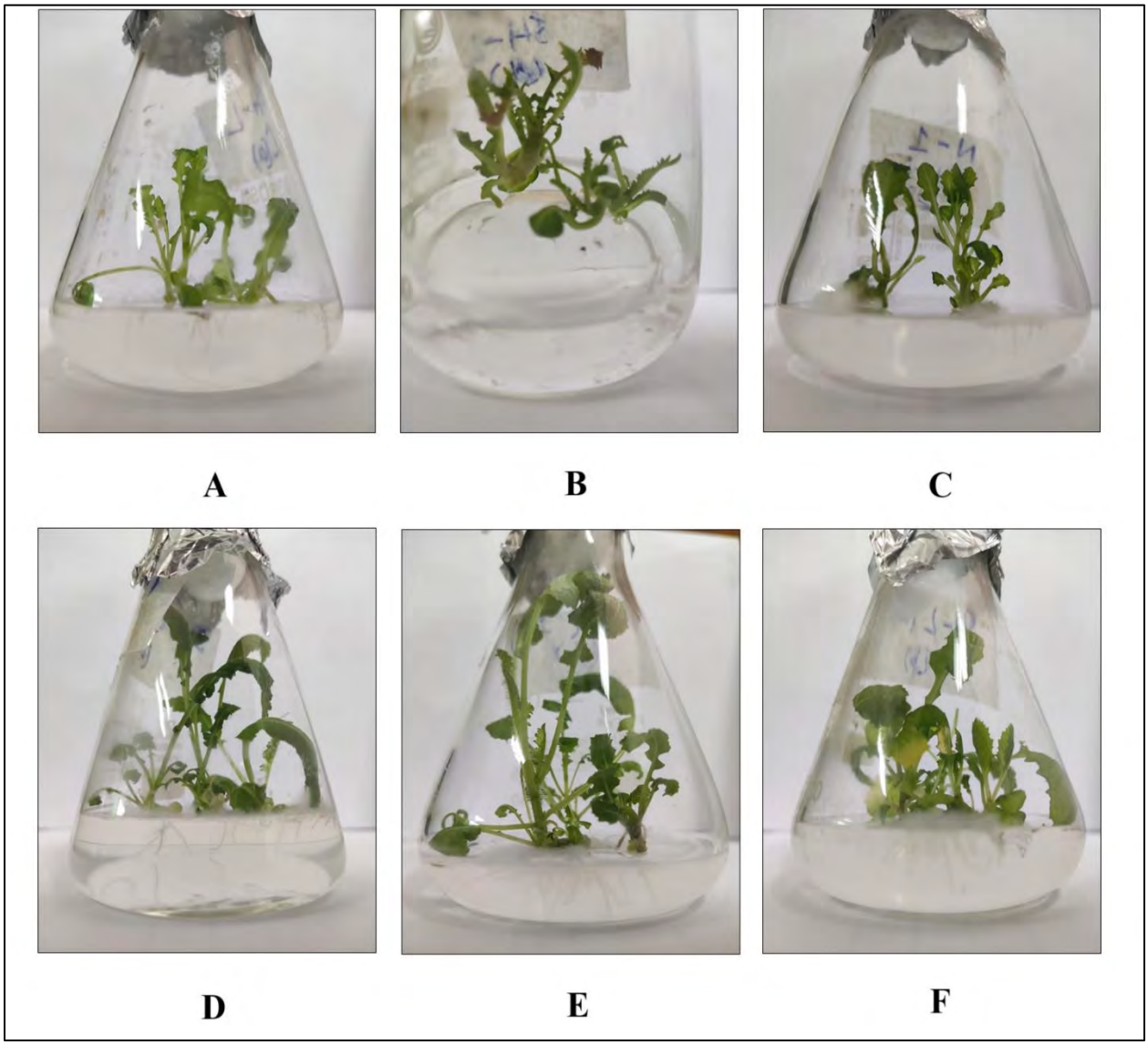

Figure 3.1 Growth of Brassica nigra in the presence of BAP, NAA, and Lupeol at the 2nd week (A) Negative control, (B) 10 µM BAP, (C) 1 µM NAA, (D) 1 µM Lupeol, (E) 5 µM Lupeol, and (F) 10 µM Lupeol.

Figure 3.2. Impact of BAP, NAA, and lupeol on root length, shoot length, and number of leaves of Brassica nigra. (After 6 weeks – at the time of harvesting) (A) Negative control, (B) 10 µM BAP, (C) 1 µM NAA, (D) 1 µM Lupeol, (E) 5 µM Lupeol, and (F) 10 µM Lupeol.

3.2 Phytochemical Screening

3.2.1 Total Phenolic Content

Roots

Total phenolic content was found significantly increased in *B. nigra* roots due to treatment of lupeol as compared to the negative and positive controls. In Lupeol treated *B. nigr*a roots, concentration dependent total phenolic content was observed (Figure 3.3). At lupeol 10 µM (L10), maximum phenolic count in root was noted with 31.04 µgGAE/mg D.W followed by lupeol 5 µM (L5) and 1 µM (L1) with the TPC of 25.23 µgGAE/mg and 22.20 µgGAE/mg, respectively. Comparatively, lupeol treated *B.nigra* roots exhibit the higher count of phenolic content than positive controls NAA (19.31 µgGAE/mg at 1 µM). Lupeol treatment to *B.nigra* roots at 1 µM, 5 µM , and 10 µM remarkably enhanced the total phenolic content by 33%, 52%, and 87%, respectively, compared to control (16.67 µgGAE/mg DW). In lupeol treated samples, minimum TPC was noted in L1 µM exposed plant roots (22.20 µgGAE/mg).

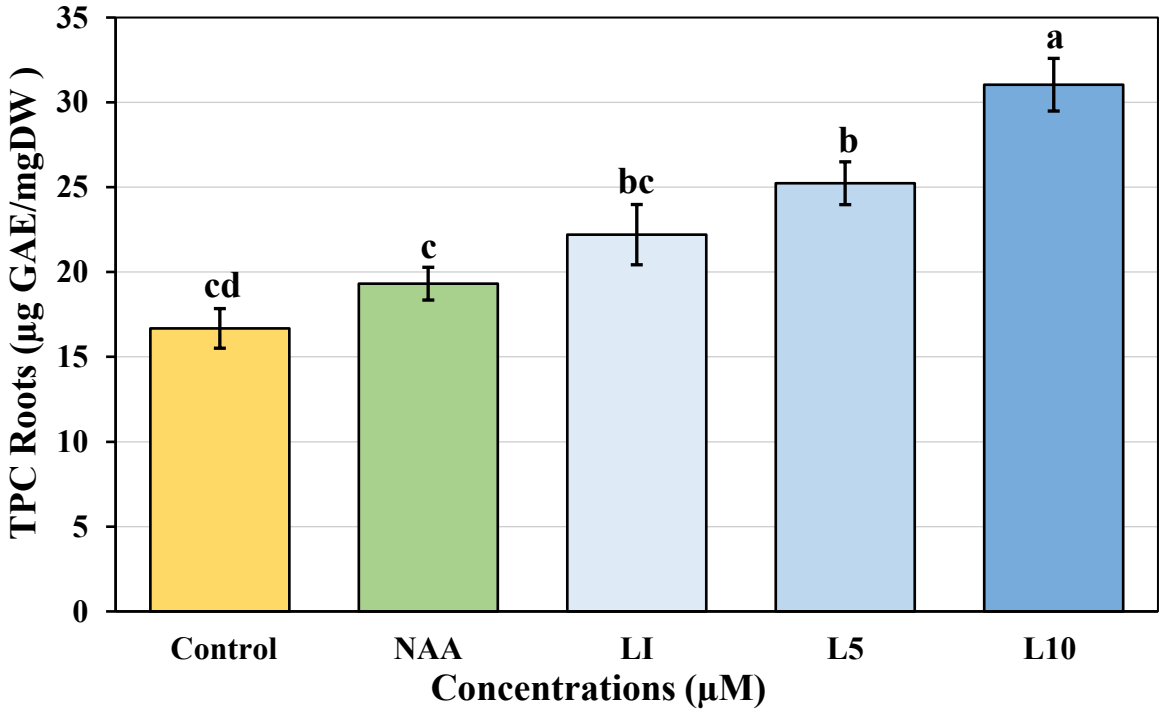

Figure 3.3. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM) and NAA (1 µM) on Total Phenolic content of *B.nigra* roots. *Data is the mean of (± standard errors) three triplicates.

Shoots

Total phenolic content was observed to be increased in *B. nigra* shoots treated with varied concentrations of lupeol (1 µM, 5 µM, and 10 µM) (Figure 3.4). The plants exposed to the lupeol concentration at L1µM resulted in a total phenolic content of shoots with 27.05 µgQE/mg DW, while at L5 µM it was 29.8 µgGAE/mg D.W, and at L10 µM it further increased to 37.44 µgGAE/mg DW. Lupeol treatment of *B.nigra* shoots at L1 µM, L5 µM, and L10 µM significantly increased the total phenolic count by 43%, 57%, and 98%, respectively compared to control (18.8 gGAE/mg DW). Similarly, *B. nigra* shoots treated with lupeol have higher phenolic content than the positive controls BAP (25.17 µgGAE/mg DW at 10 µM), and NAA (24.89 µgGAE/mg DW at 1µM). Overall, shoots exposed to 10 µM lupeol exhibit the highest phenolic contents.

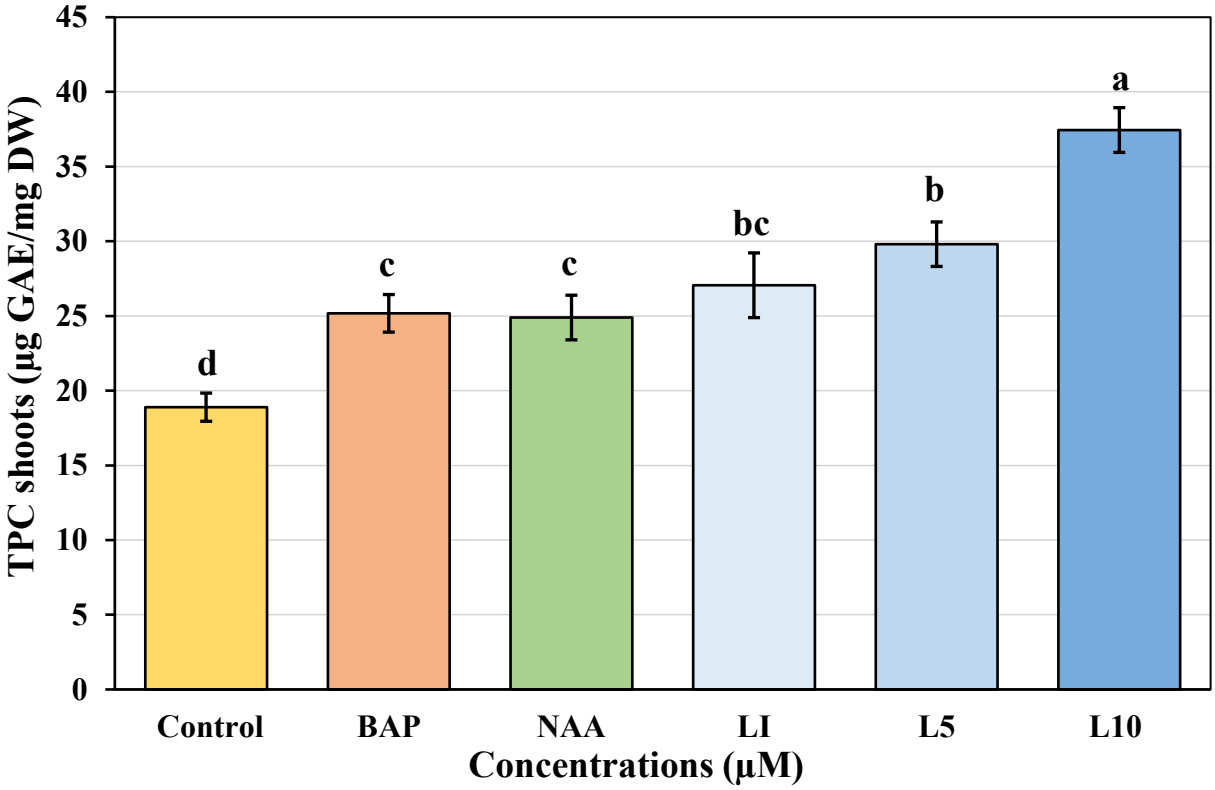

Figure 3.4. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM), BAP (10 µM), and NAA (1 µM) on total phenolic content of *B.nigra* shoots. *Data is the mean of (± standard errors) three triplicates.

3.2.2 Total Flavonoid Content

Roots

Total flavonoid content was remarkably increased in roots of *B. nigra* plants treated with lupeol concentrations (Figure 3.5), which was 15.47 µgQE/mg DW (at L1 µM), 17.44 µgQE/mg DW (at L5 µM) and 20.83 (at L10 µM) as compared to control (9.05 µgQE/mg DW). A concentration dependent increase in TFC was observed as the maximum flavonoid content was quantified by roots exposed to L10 µM (130%) and minimum content at L1 µM exposed roots (70%). Comparatively, lupeol treated roots also showed significant increase in TFC against positive controls BAP and NAA where BAP (10 µM) had the TPC values of 8.91 µgQE/mg DW and NAA (1 µM) had 12.53 µgQE/mg DW, respectively.

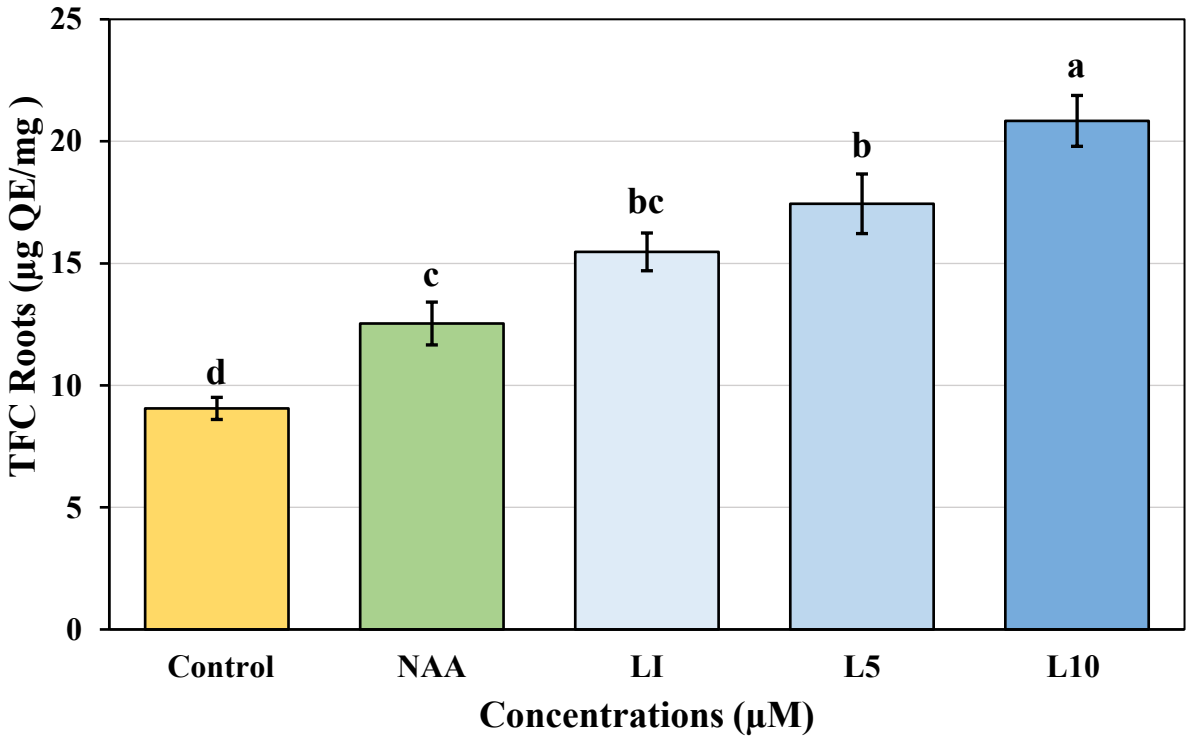

Figure 3.5. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM) and NAA (1 µM) on total flavonoid content of *B.nigra* roots. *Data is the mean of (± standard errors) three triplicates.

Shoots

Total flavonoids content was significantly increased in lupeol treated shoots of *B. nigra* plants with 16.56 µgQE/mg DW (L1 µM), 18.28 µgQE/mg DW (L5 µM), and 24.61 µgQE/mg DW (L10 µM). Flavonoids contents of shoots were improved by 85%, 37%, and 24% respective to the treated concentrations of lupeol i.e. L 10 µM, L5 µM, and L1 µM when compared to the control (13.29 µgQE/mg) (Figure 3.6). Furthermore, NAA at 1 µM also exhibit considerable flavonoid contents (15.43 µgQE/mg DW) but 10 µM BAP had least count of flavonoids (12.91 µgQE/mg DW) which was also less than control (13.29 µgQE/mg DW). Among lupeol treated samples, L10 µM had maximum flavonoids content followed by L5 µM and L1 µM.

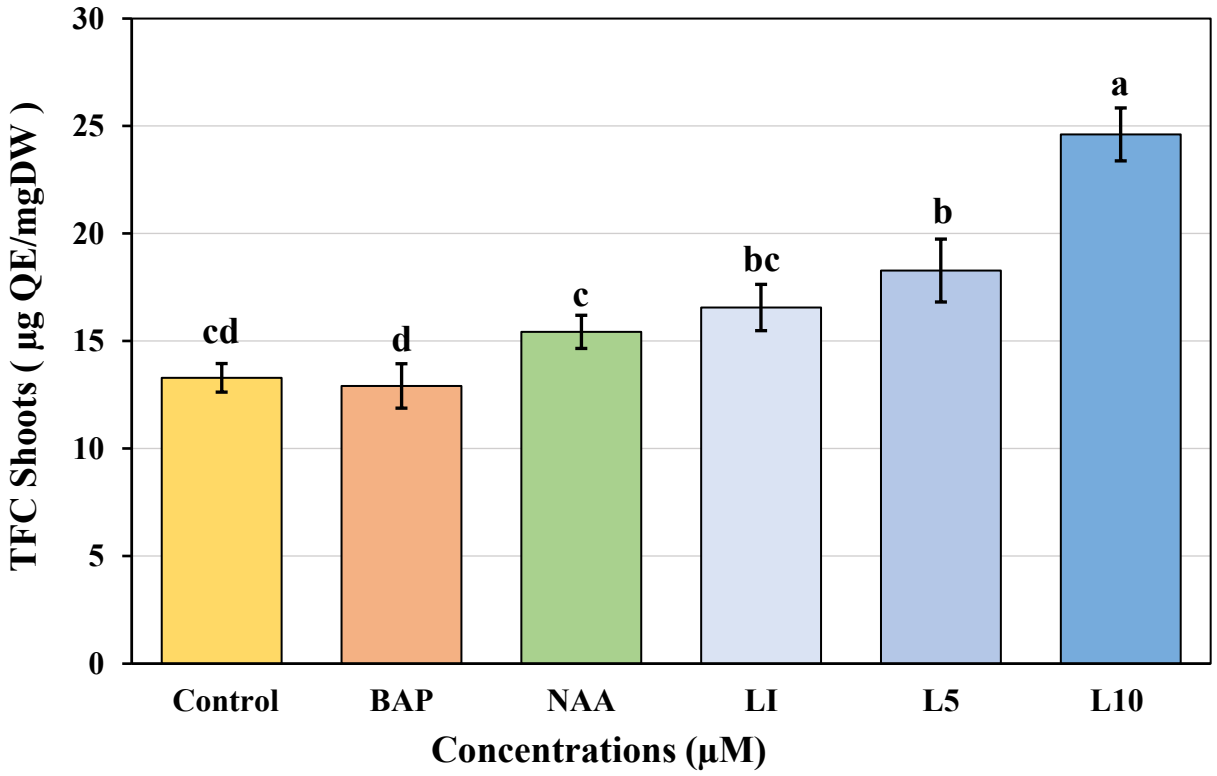

Figure 3.6. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM), BAP (10 µM), and NAA (1 µM) on total flavonoid content of *B.nigra* shoots. *Data is the mean of (± standard errors) three triplicates.

3.3 Antioxidant Enzymes Analysis

3.3.1 Superoxide Dismutase (SOD) Activities

Roots

Superoxide dismutase activity in roots of *B. nigra* plant increased gradually by increasing the concentration of lupeol as compared to control. Minimum enzyme activity was observed at L1 µM which was 0.055 nM/min/mgFW, however, at L5 µM and L10 µM, maximum enzyme activity was recorded with 0.071 nM/min/mgFW and 0.078 nM/min/mgFW. NAA (10 µM) also exhibit the same enzyme activity (0.053 nM/min/mgFW) like that of L1 µM (0.055 nM/min/mgFW) of lupeol treated concentration in roots. If we compare it to control, there was decrease in the enzyme activity of plants with the value of 0.036 nM/min/mgFW as illustrated in figure 3.7. Lupeol treatment to *B.nigra* roots at 1 µM, 5 µM, and 10 µM remarkably enhanced the SOD activity by 85%, 139%, and 160%, respectively, compared to control.

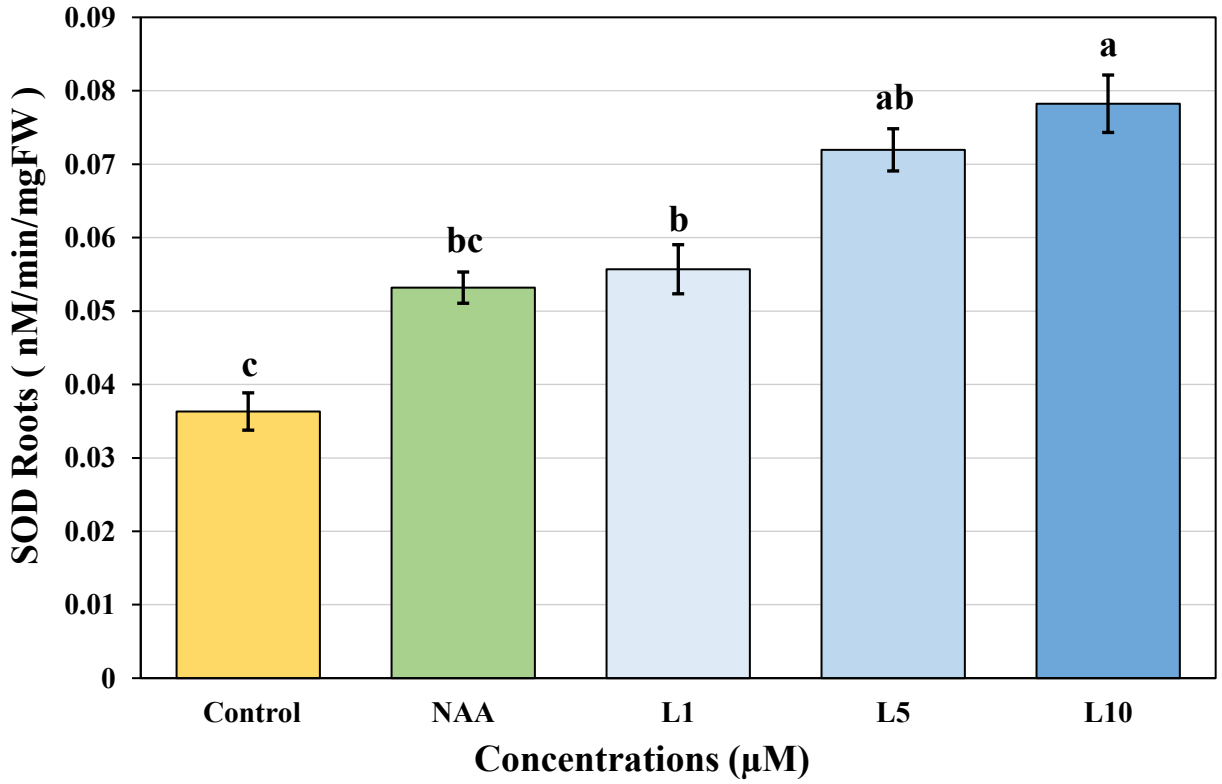

Figure 3.7. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM) and NAA (1 µM) on Superoxide dismutase activities of *B.nigra* roots. *Data is the mean of (± standard errors) three triplicates.

Shoots

The lupeol treated plant shoots resulted in a significant increase superoxidase (SOD) activity. The minimum SOD activity was observed at a concentration of L1 µM, quantifying 0.078 nM/min/mgFW. The maximum SOD activity was observed at a concentration of L10 µM, quantifying 0.098 nM/min/mgFW as compared to positive control 1 µM NAA (0.06 nM/min/mgFW) and 10 µM BAP (0.055 nM/min/mgFW). Lupeol significantly affect the SOD activity in plant shoots at various concentrations of 1 µM, 5 µM, and 10 µM by 95%, 115%, and 145%, when compared to the negative control (0.046 nM/min/mgFW) as presented in figure 3.8.

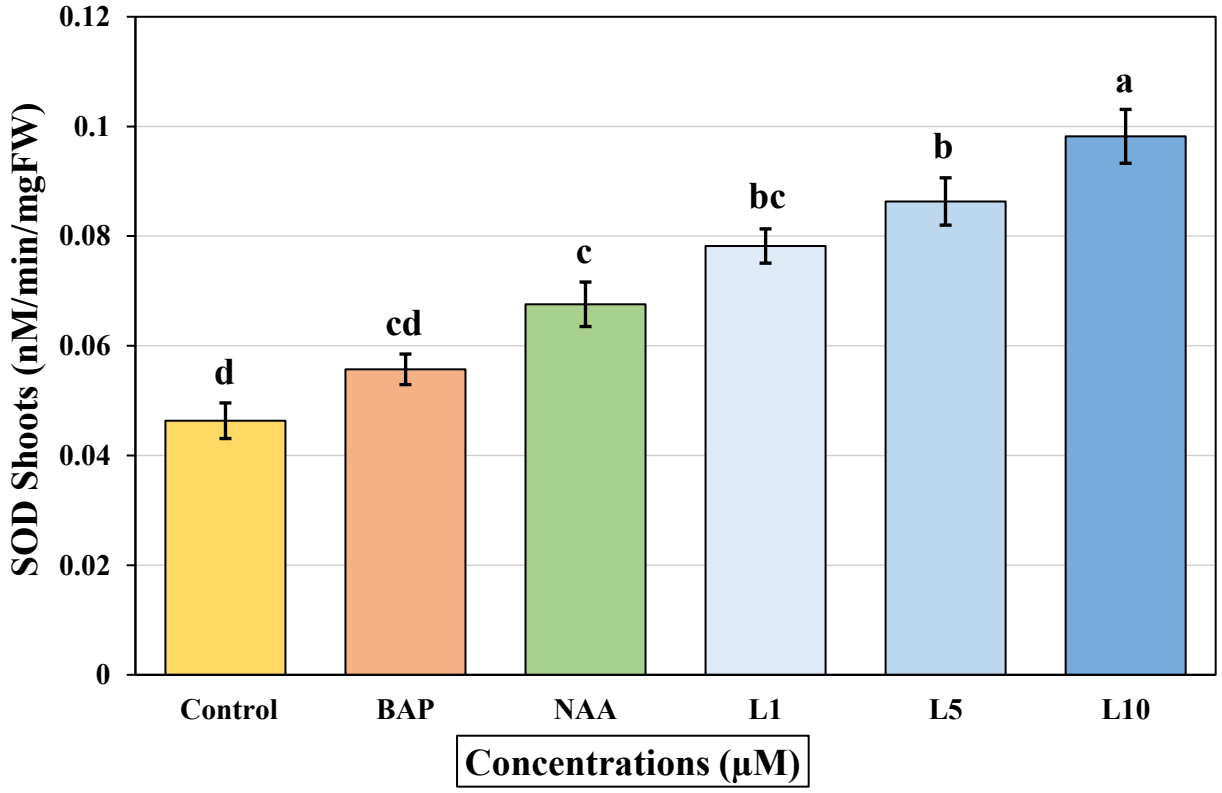

Figure 3.8. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM), BAP (10 µM), and NAA (1 µM) on superoxide dismutase activities of *B.nigra* shoots. *Data is the mean of (± standard errors) three triplicates.

3.3.2 Peroxidase (POD) activities

Roots

Treatment of plant roots with varying concentrations of lupeol remarkably increased the POD activity in concentration dependent manner (Figure 3.9). In lupeol treated roots, minimum POD activity was observed at L1 µM which was 1.15 nM/min/mgFW and maximum POD activity was noted at 10 µM which was 1.69 nM/min/mgFW as compared to positive controls NAA (0.98 nM/min/mgFW at 1 µM). However, L5 µM showed the modest enzyme activity of 1.41 nM/min/mgFW. Lupeol treatment of *B.nigra* roots at L1 µM, L5 µM, and L10 µM significantly increased the antioxidant enzyme (POD) activity by 136%, 188%, and 246%, respectively compared to control (0.49 nM/min/mgFW).

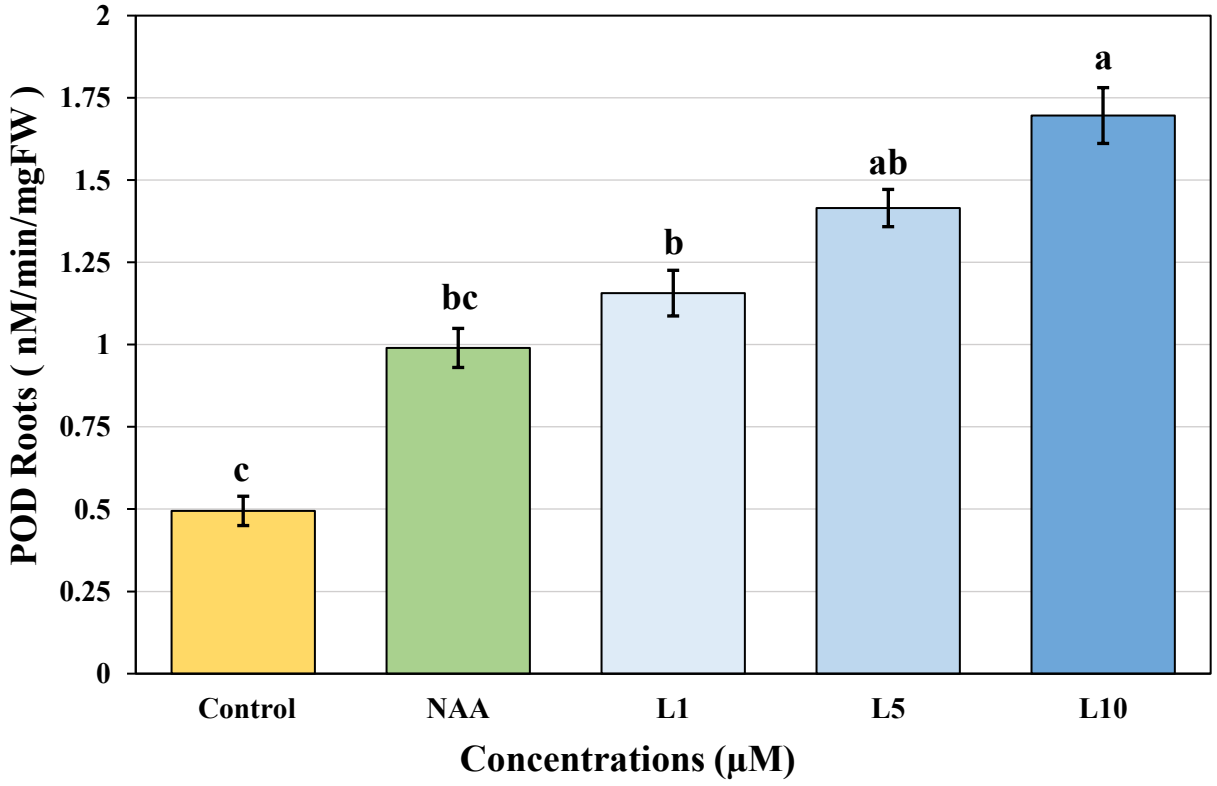

Figure 3.9. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM) and NAA (1 µM) on Peroxidase activities of *B.nigra* roots. *Data is the mean of (± standard errors) three triplicates.

Shoots

A noteworthy increase in the antioxidant enzyme (POD) activity of *B. nigra* shoots was noted due to treatment of lupeol as compared to the negative and positive controls. In Lupeol treated *B. nigra* shoots, concentration dependent POD activity was observed (Figure 3.10). At lupeol 5 µM and 10 µM in shoots, maximum POD quantification was noted with 2.17 nM/min/mgFW followed by L1 µM with the 2.14 nM/min/mgFW. Comparatively, lupeol treated *B.nigra* shoots exhibit the higher activities of POD than positive controls NAA (1.49 nM/min/mgFW at 1 µM) and BAP (0.78 nM/min/mgFW at 10 µM). Lupeol-derived elicitation in *B. nigra* shoots result in significant POD activity at all experimental lupeol concentrations of 1 µM (712%), 5 µM (722%), and 10 µM (722%) as compared to control (0.26 nM/min/mgFW).

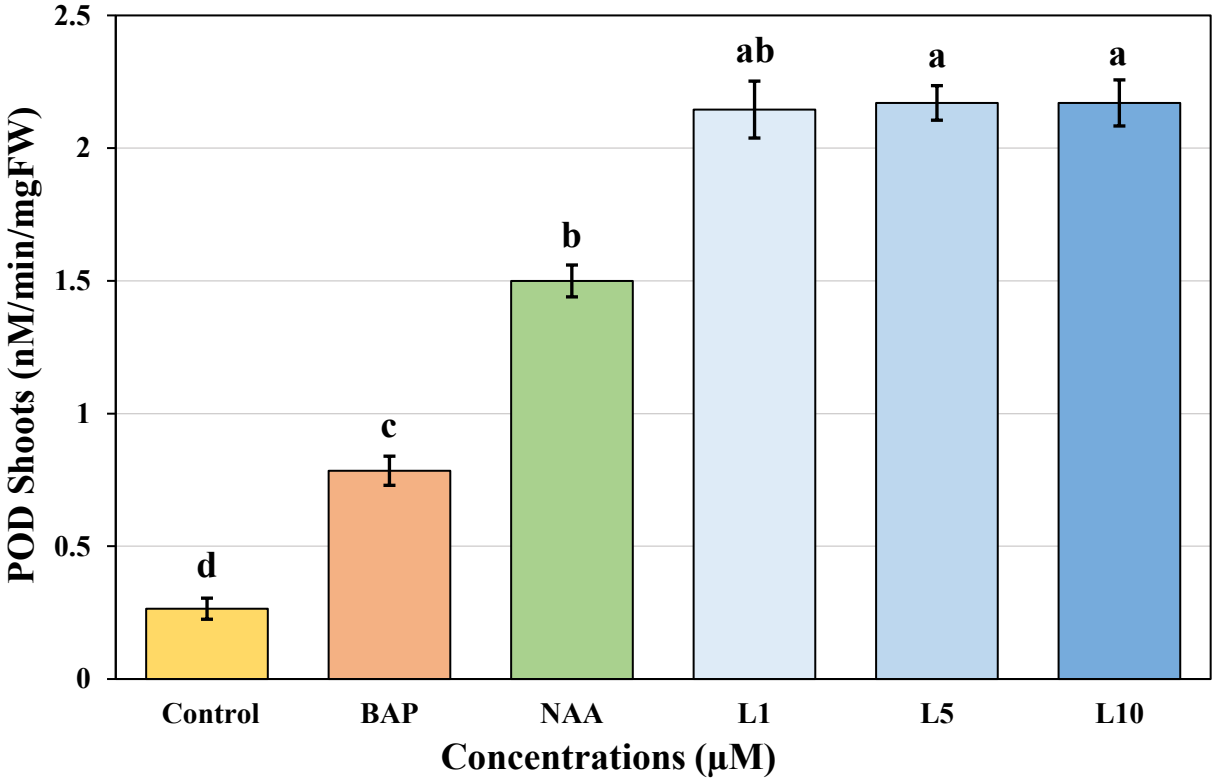

Figure 3.10. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM), BAP (10 µM), and NAA (1 µM) on Peroxidase activities of *B.nigra* shoots. *Data is the mean of (± standard errors) three triplicates.

3.4 Non-Enzymatic Analysis

3.4.1 Free radical scavenging activity (FRSA)

Roots

Lupeol-derived elicitation in *B. nigra* roots result in increased free radical scavenging activity at all experimental lupeol concentrations as compared to controls (Figure 3.11). At L1 µM, FRSA observed was 30% which further increased up to 34% and 59% at L5 µM and L10 µM. Hence, Concentration dependent scavenging activity was observed in lupeol treated plant roots as the L10 µM concentration showed the highest scavenging activity while L1 µM exhibit the lowest scavenging of free radicals. In comparison to the FRSA of lupeol treated roots, significant decrease in scavenging activity was observed in control by 16% and NAA by 17%.

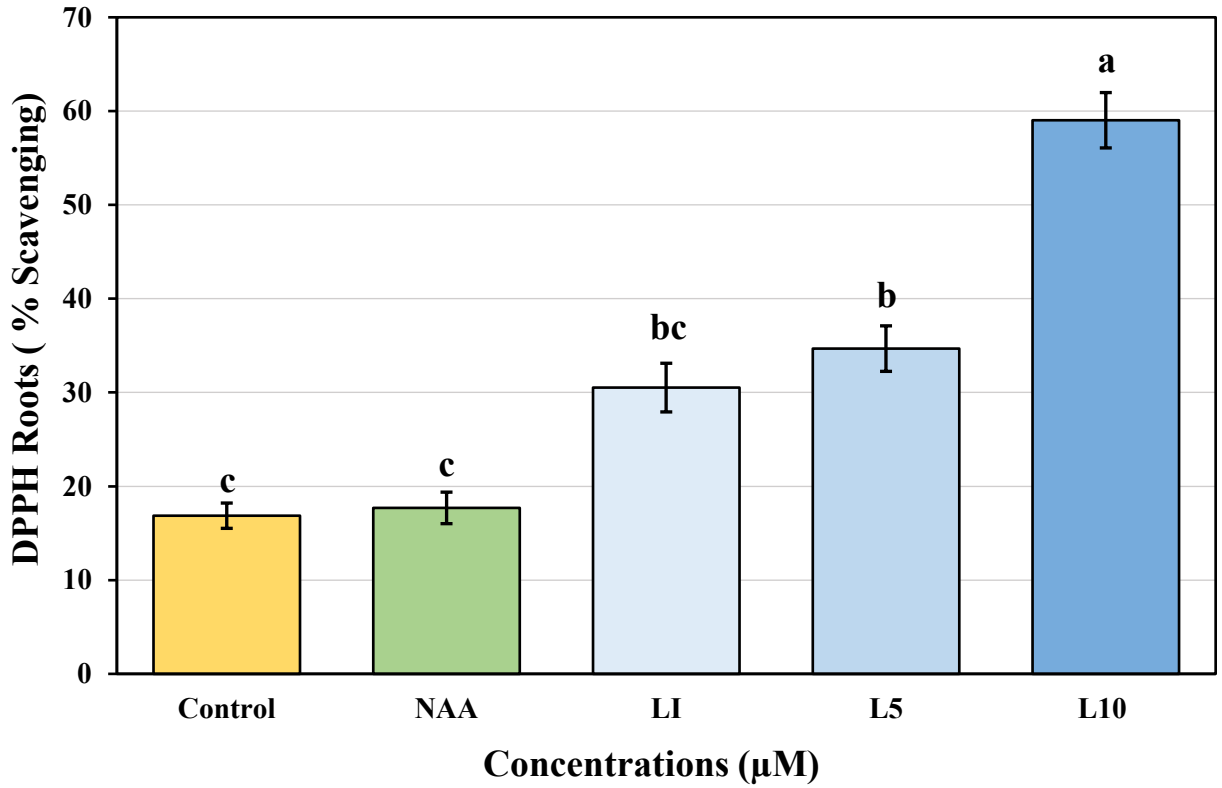

Figure 3.11. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM) and NAA (1 µM) on free radical scavenging activity of *B.nigra* roots. *Data is the mean of (± standard errors) three triplicates.

Shoots

By the treatment of lupeol, the free radical scavenging activity in *B. nigra* plant shoots was increased relative to the control, with a minimal activity of 34% at L1 µM and a maximum activity of 53% at L10 µM, respectively (Figure 3.12). Whereas L5 µM exhibit the modest scavenging activity with 38%. A decreasing trend was observed in scavenging activity of BAP (26%) and NAA (23%) as compared to lupeol treated plant roots. The application of lupeol resulted in a significant increase in scavenging activity compared to the negative control (17.6%).

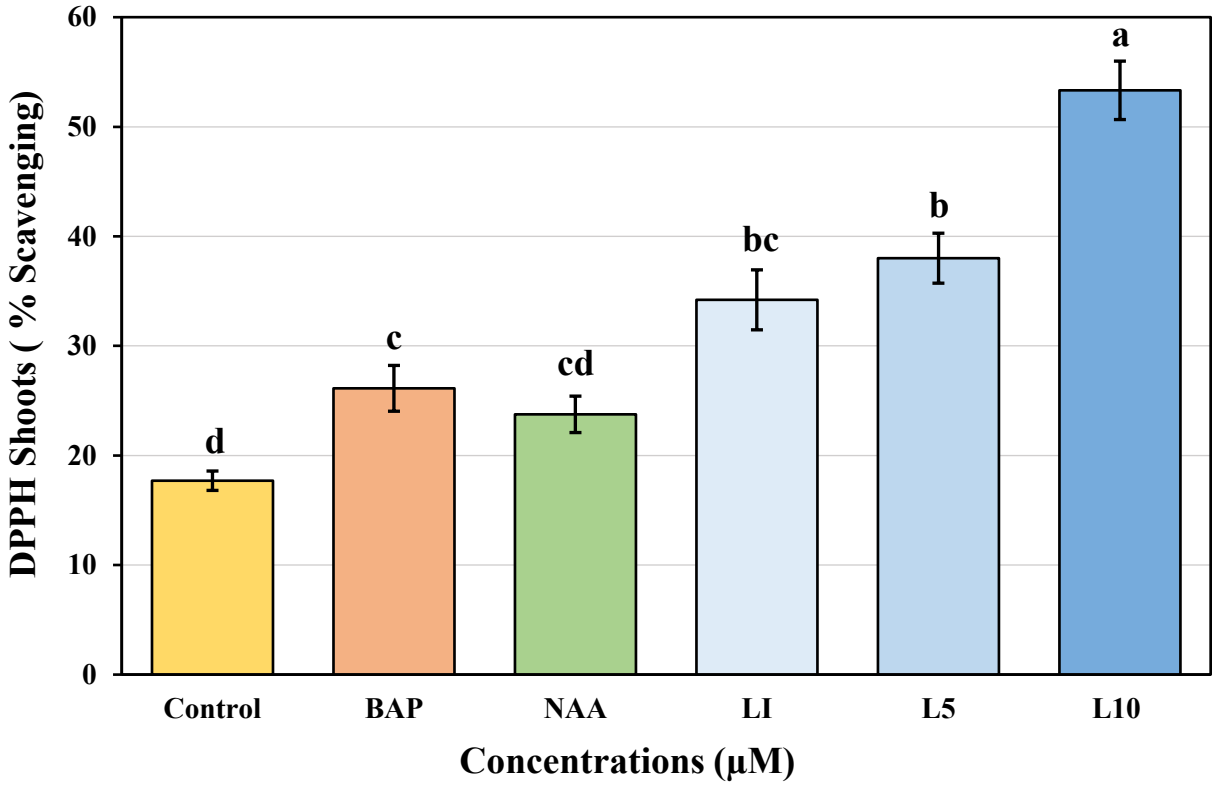

Figure 3.12. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM), BAP (10 µM), and NAA (1 µM) on free radical scavenging activity of *B.nigra* shoots. *Data is the mean of (± standard errors) three triplicates.

3.4.2 ABTS scavenging activity

Roots

The 2, 2'-azino-bis-ethylbenzthiazoline-6-sulfonic acid (ABTS+) radical cation was used to study the radical scavenging activity. Noteworthy scavenging potential in ABTS investigation was observed by the roots of *B. nigra* plants due to the treated lupeol concentrations compared to the control (Figure 3.13). ABTS scavenging activity increased as the concentrations of lupeol increased, which was 26% at L1 µM, 30.4% at L5 µM, and 47.8% at 10 µM ABTS activity, in contrast to positive control 1µM NAA (14.6%). The negative control sample (5.85%) showed a notable decrease of 5.85% in ABTS activity as compared to lupeol treated samples and NAA. However, a notable increase in ABTS activity was noticed at higher concentration of 10 µM of lupeol.

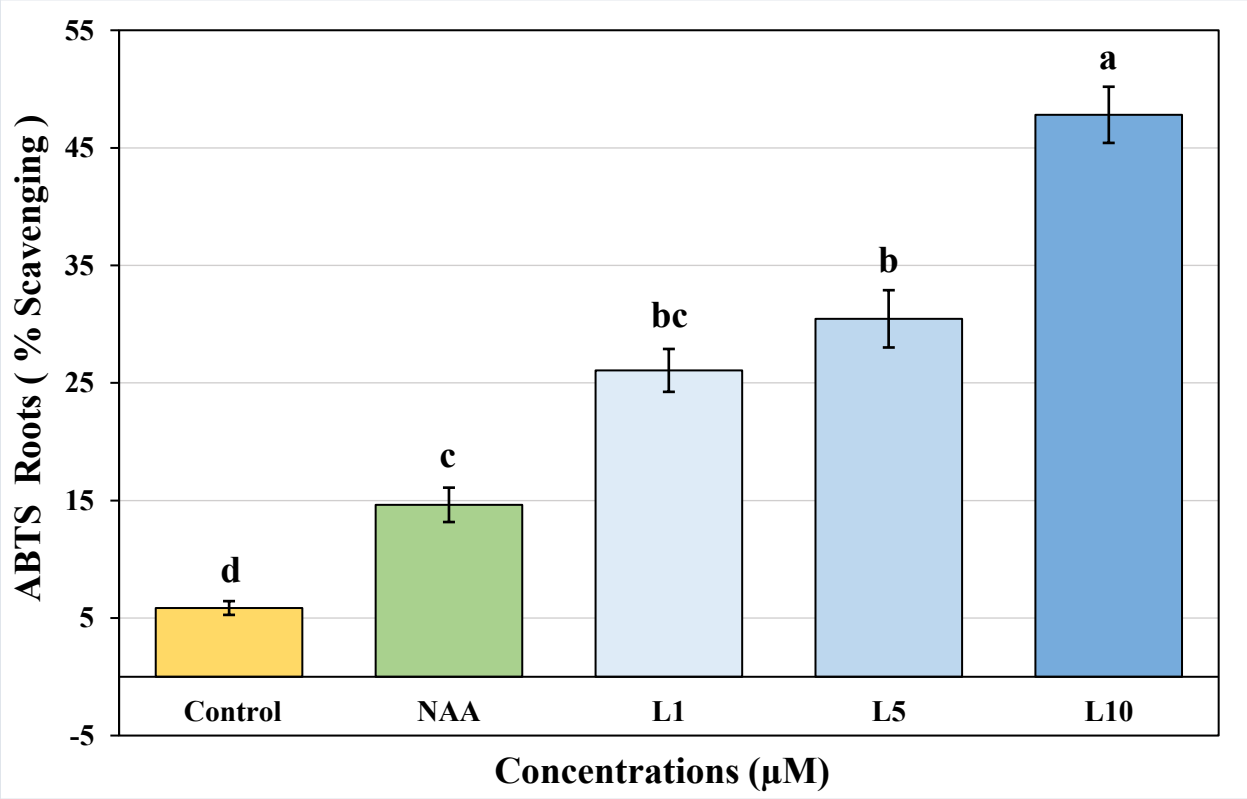

Figure 3.13. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM) and NAA (1 µM) on ABTS scavenging of *B.nigra* roots. *Data is the mean of (± standard errors) three triplicates.

Shoots

ABTS scavenging activity in shoots of lupeol-treated *B. nigra* plants increased with the increase of lupeol concentration from 1 µM to 5 µM and 10 µM (Figure 3.14). Maximum ABTS scavenging was observed at L10 µM with the 47.34% activity while minimum activity was noticed in L1 µM and L5 µM with the 36% and 34.7% scavenging respectively. In comparison to the ABTS scavenging of lupeol treated shoots, significant decrease in scavenging activity was observed in control by 8.3%, BAP by 15.6%, and NAA by 18%.

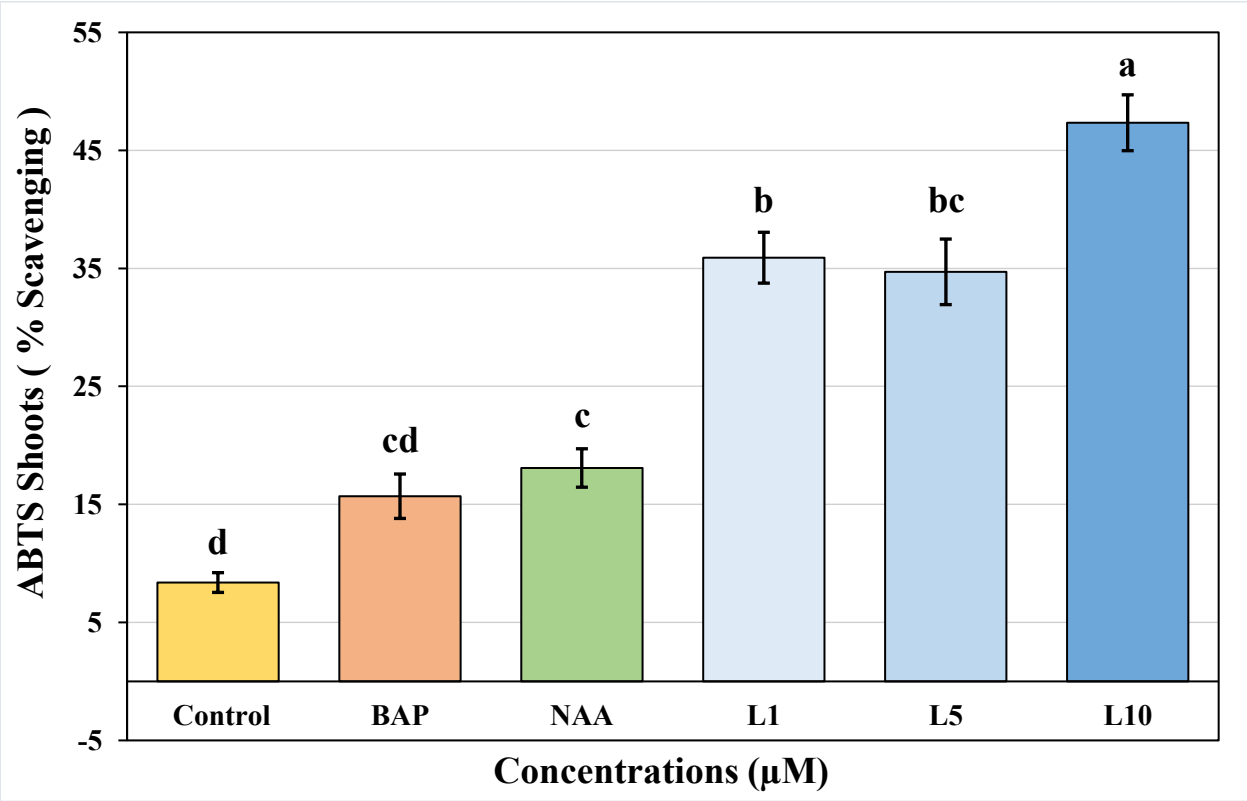

Figure 3.14. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM), BAP (10 µM), and NAA (1 µM) on ABTS scavenging of *B.nigra* shoots.

*Data is the mean of (± standard errors) three triplicates.

3.4.3 Metal chelating Potential (MCP)

Roots

Lupeol treated plant roots ability to chelate transition metals can also be used to estimate antioxidant activity. In MC activity, increasing trend was observed in lupeol treated shoots of *B. nigra* plants from 1µM to 5µM, and 10 µM of lupeol, resulted in significant activities, and computed as 39%, 41%, and 52%, respectively. The significant MC activities of lupeol treated roots confirm their function as an efficient metal chelator agent in comparison to NAA and control which had comparatively decreased chelation potential as 16% by NAA and 15.6% by control (Figure 3.15). The higher lupeol concentration L10 µM has a marked effect on the metal chelation potential of plant roots (52%).

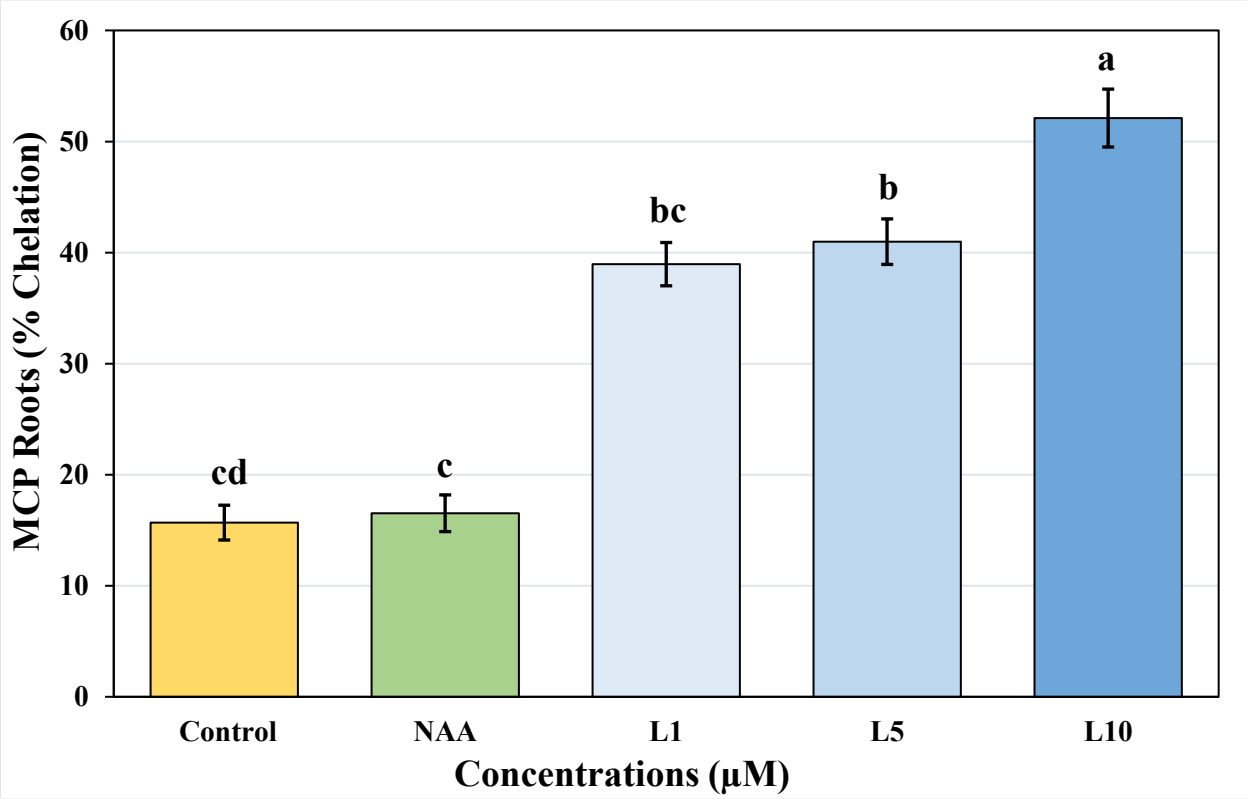

Figure 3.15. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM) and NAA (1 µM) on metal chelating potential of *B.nigra* roots.*Data is the mean of (± standard errors) three triplicates.

Shoots

Results showed that lupeol treated plant shoots have significant metal chelation capacity in concentration dependent manner as compared to control (12.31%) (Figure 3.16). Maximum metal chelation capacity was depicted by L10 µM followed by L5 µM and L1 µM with the 49.4%, 40%, and 37%, respectively. However, MC activity was observed to decrease in BAP (17%) and NAA (20%) in comparison to lupeol treated samples which showed that presence of lupeol overall affect the antioxidant capcity of *B. nigra* treated roots in terms of their metal chelation potential.

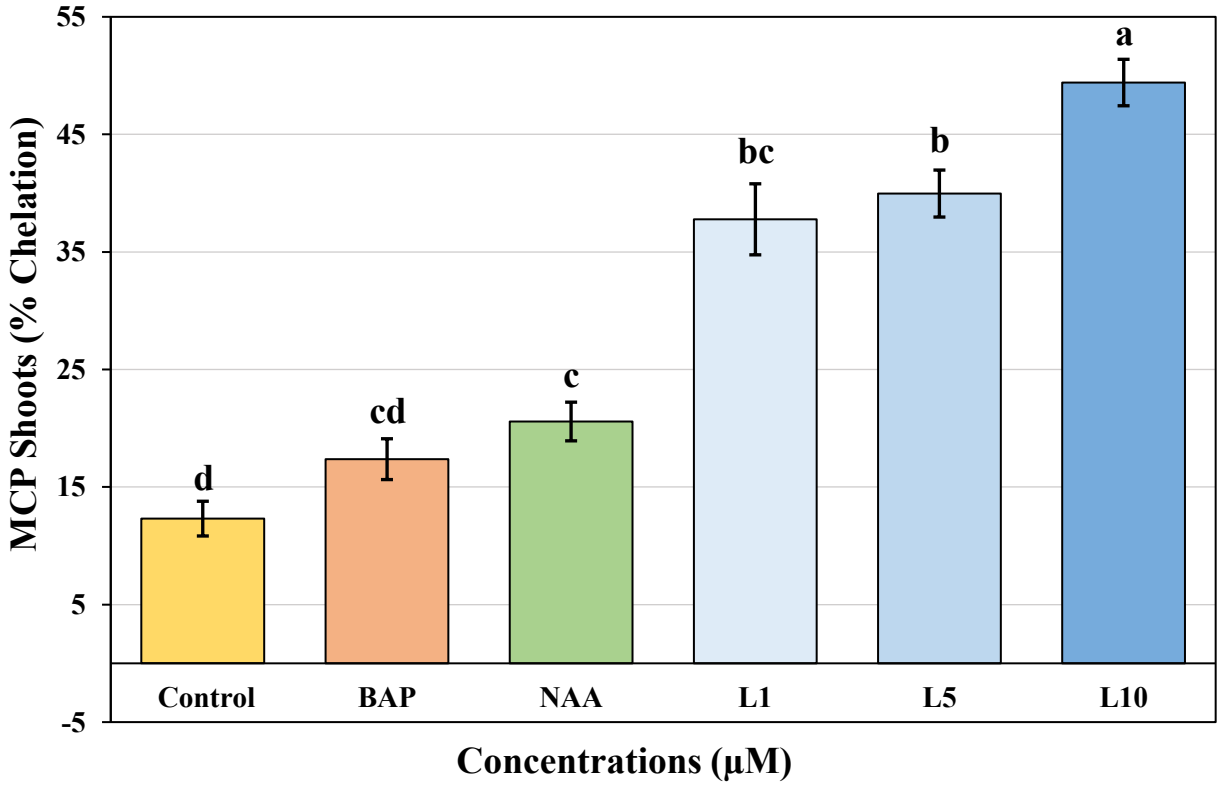

Figure 3.16. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM), BAP (10 µM), and NAA (1 µM) on Metal chelating potential of *B.nigra* shoots.

*Data is the mean of (± standard errors) three triplicates.

3.4.4 Total antioxidant capacity (TAC)

Roots

Total antioxidant capacity assay results show that there was a significant increase of antioxidants in lupeol treated plant roots as compared to control group. In lupeol treated roots, at L1 µM, 44.02 µgAAE/mg DW of TAC was observed which further increased up to 59.11 µgAAE/mg DW and 85.14 µgAAE/mg DW at 5 µM and 10 µM. Positive control NAA showed a decrease in total antioxidant capacity by 25.23 µgAAE/mg DW while control showed further decrease in TAC with 21.27 µgAAE/mg DW. Overall, plant roots exposed to all the experimental concentrations of lueopl (1 µM, 5 µM, and 10 µM) comparatively showed higher antioxidant count than 1 µM NAA and control (Figure 3.17).

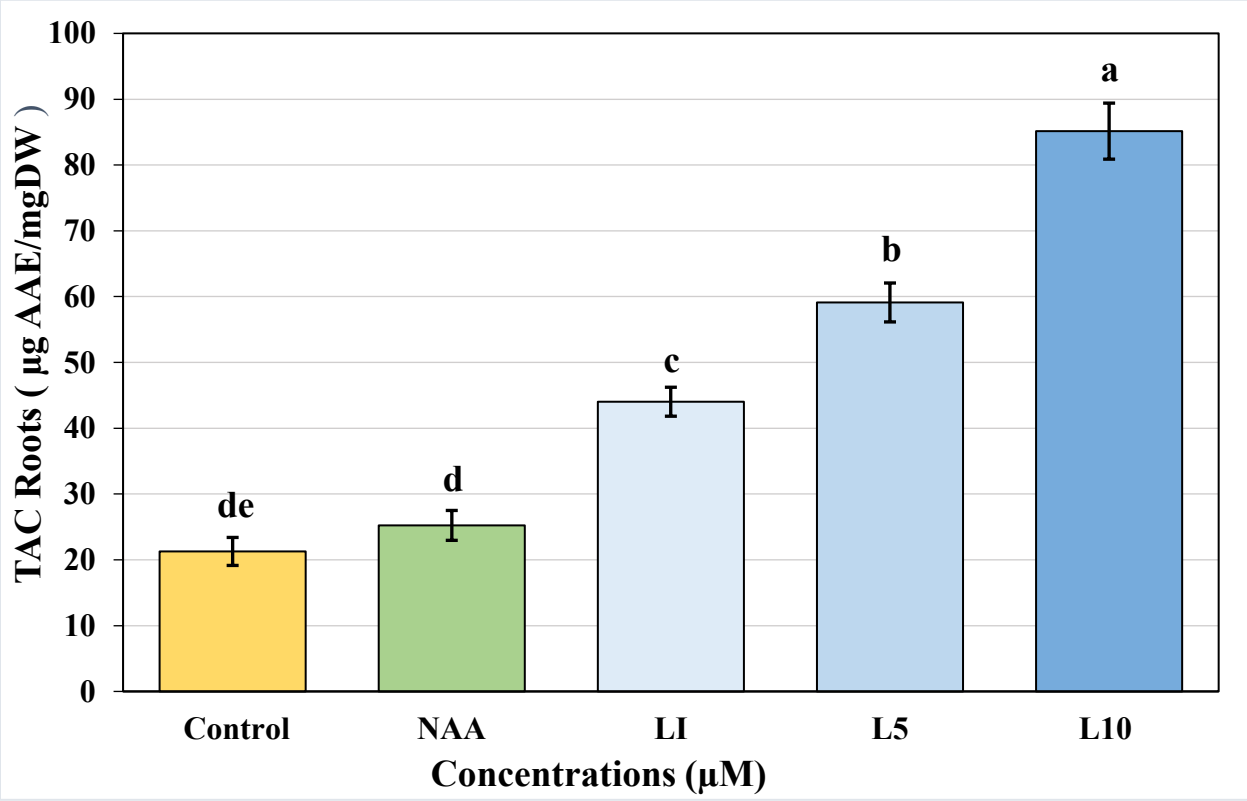

Figure 3.17. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM) and NAA (1 µM) on total antioxidant capacity scavenging of *B.nigra* roots.

*Data is the mean of (± standard errors) three triplicates.

Shoots

In the lupeol treated plant shoots, a notable increase in the overall antioxidant capacity was noted in comparison to the control (23.59 µgAAE/mg DW) as illustrated in Figure 3.18. At the concentration of L1 µM, the total antioxidant capacity (TAC) was measured in shoots was 57.9 µgAAE/mg DW, while at L5 µM, it increased to 62.2 µgAAE/mg DW. Furthermore, a significant increase in TAC was observed at L10 µM with the antioxidant count of 84.28 µgAAE/mg DW. Considerable TAC values were also quantified in positive controls 10 µM BAP (37.47 µgAAE/mg DW) and 1 µM NAA (30.23 µgAAE/mg DW). However, overall controls showed less antioxidant potential than lupeol treated plant shoots.

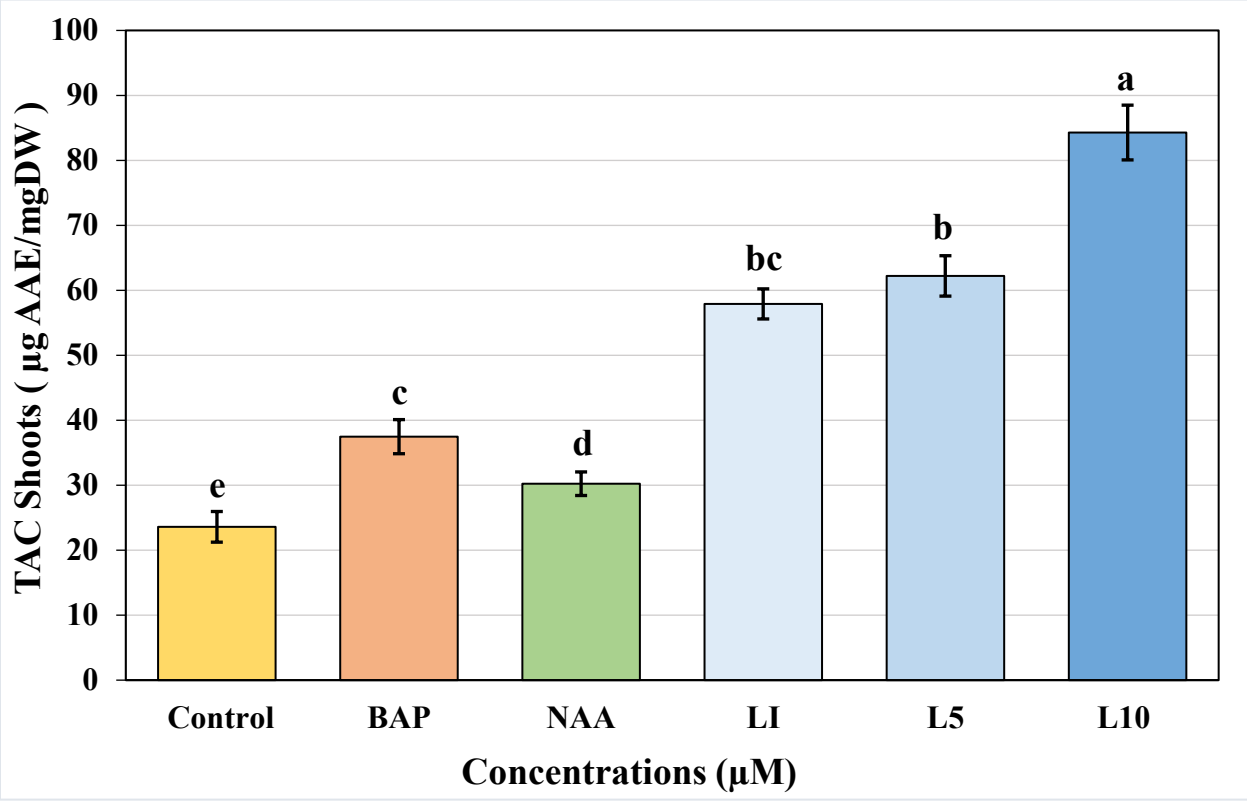

Figure 3.18. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM), BAP (10 µM), and NAA (1 µM) on total antioxidant capacity of *B.nigra* shoots.

*Data is the mean of (± standard errors) three triplicates.

3.4.5 Total Reducing Power Assay

Roots

Lupeol-stimulated elicitation in *B. nigra* roots result in increased total reducing power at all experimental lupeol concentrations as compared to controls (Figure 3.19). At L1 µM, TRP observed was 46.23 µgAAE/mg DW which further increased up to 53.22 µgAAE/mg DW and 74.13 µgAAE/mg DW at L5 µM and L10 µM. Hence, Concentration dependent reducing activity was observed in lupeol treated plant roots as the L10 µM concentration exhibit the highest number of reductones while L1 µM exhibit the lowest count of reductones. In comparison to the TRP of lupeol treated roots, significant decrease in reducing activity was observed in control by 33.14 µgAAE/mg DW and NAA by 39.64 µgAAE/mg DW.

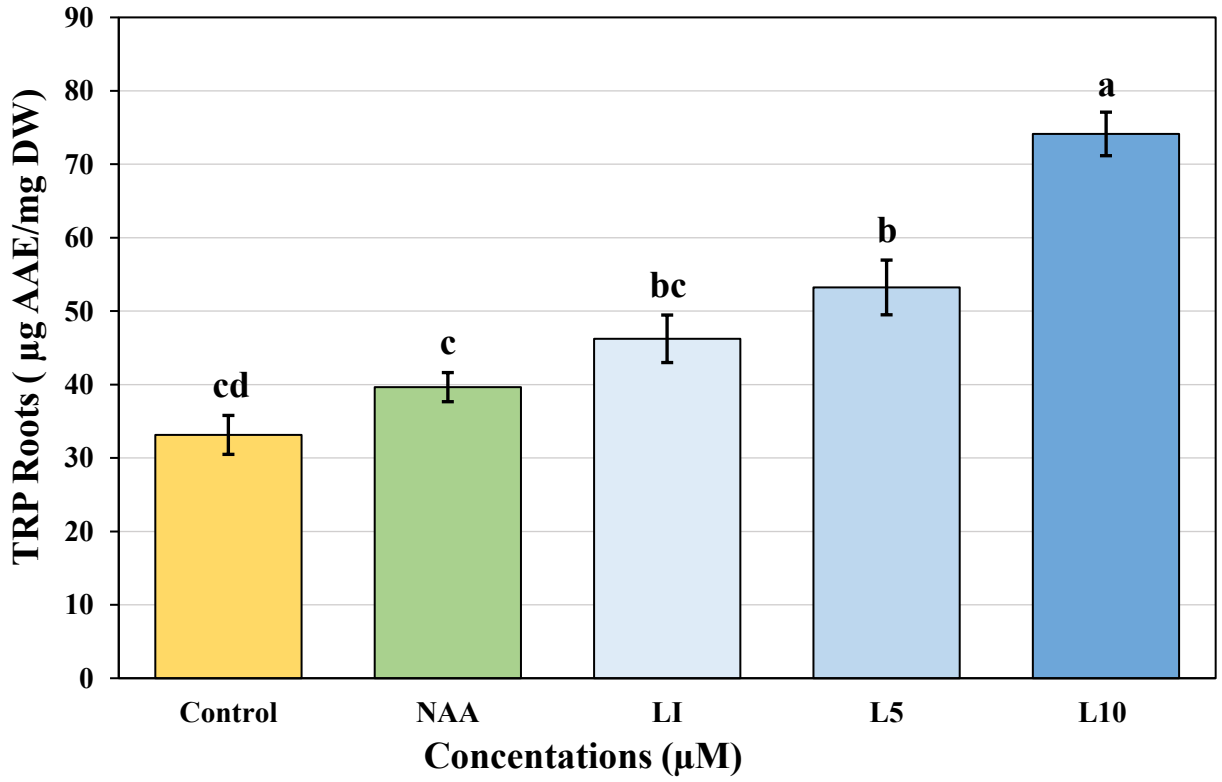

Figure 3.19. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM) and NAA (1 µM) on total reducing power of *B.nigra* roots.

*Data is the mean of (± standard errors) three triplicates.

Shoots

A notable enhancement in the total reducing power was observed in the shoots of *B. nigra* plants treated with varied concentrations of lupeol (L1 µM, L5 µM, and L10 µM), in comparison to the control (19.64 µgAAE/mg DW) (Figure 3.20). Maximum reducing power in shoots was observed at lupeol concentration of L10 µM, resulting in 97.58 µgAAE/mg DW of reducing capacity. The minimum reducing power was observed at a concentration of L1 µM with 61.29 µgAAE/mg DW as compared to the L10 µM, while the L5 µM also showed the considerable reduction capacity with 70 µgAAE/mg DW. Consequently, an increase in TRP was observed as a result of the lupeol treatment to plant shoots, in comparison to the positive control BAP (26.72 µgAAE/mg DW) and NAA (22.44 µgAAE/mg D.W) with noticeable decreased TRP.

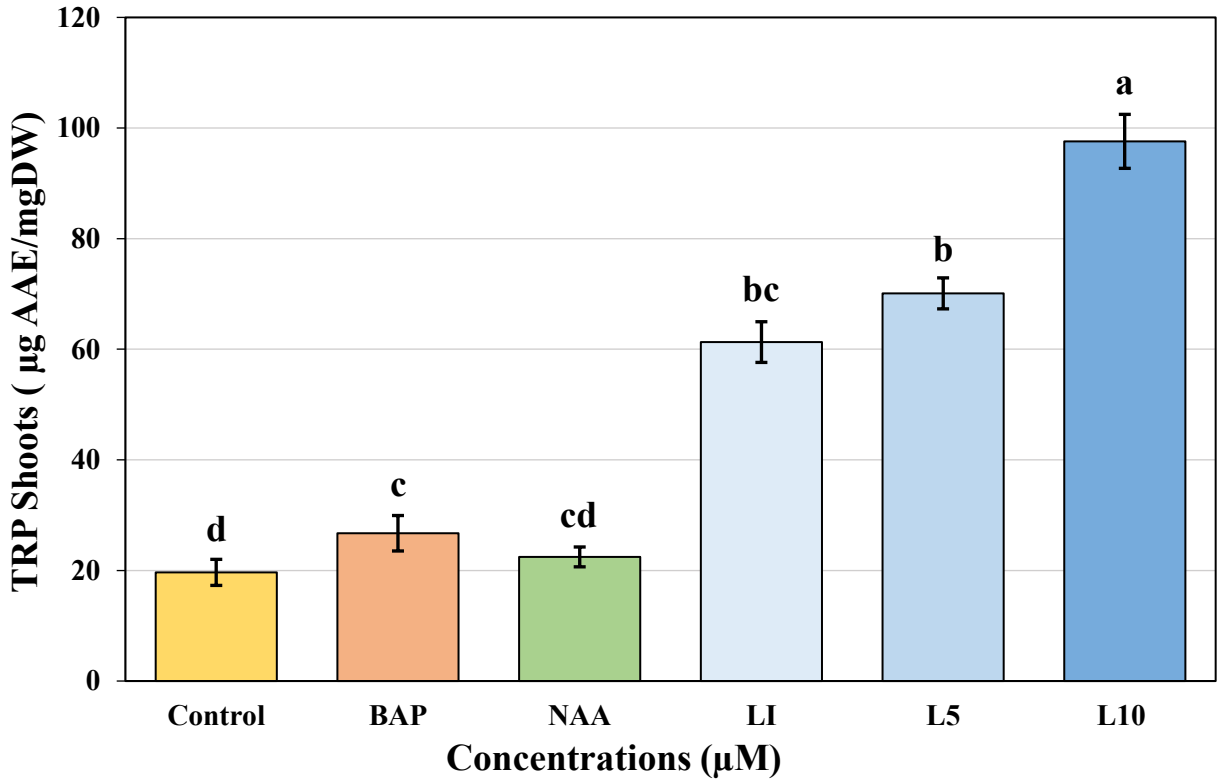

Figure 3.20. Effect of lupeol concentrations (1 µM, 5 µM, and 10 µM), BAP (10 µM), and NAA (1 µM) on total reducing power of *B.nigra* shoots.

*Data is the mean of (± standard errors) three triplicates.

CHAPTER 4

4 DISCUSSION

Plant growth regulators and secondary metabolites play crucial roles in plant development and stress response. Plants possess diverse categories of secondary metabolites, which are classified into three major groups; terpenes or terpenoids, phenolics and N-containing compounds. Thousands of the secondary metabolites are included in each of these groups. The previous studies focused on the enhanced concentration of secondary metabolites in plants by various abiotic and biotic elicitors which include salt, heavy metals or some chemicals as abiotic elicitors and phytohormones like jasmonic acid, abscisic acid, ethephon, and salicylic acid as biotic elicitors (Kumar and Srivastava 2016). But the main aim of our study is to use the triterpenoid lupeol that function as an elicitor to enhance the secondary metabolite production in *B. nigra* plantsIsoprenoids or terpenes (derived terpenoids) are the biggest class of secondary metabolites and play a significant role in plant development, growth, interactions with the environment, tolerance to stress, and resistance to predators (Bustamante *et al.,* 2020). Numerous triterpenoids are also reportedly implicated in the defence systems of plants, according to Cardenas *et al.* (2019). A pentacyclic triterpenoid called lupeol is present in many plants, fruits, and vegetables. Lupeol has been linked to oxidative stress in animals. (Sharma *et al.,* 2020). Recently Zia et al (2021) studied the significant effects of lupeol on mitigation of oxidative stress within salt stressed plants. Lupeol is a naturally occurring secondary metabolite and can have role in plant defense and it also acts as precursor for other triterpenoid metabolites which are usually involved in plant development and defense (Szakiel *et al.,* 2012). This study aims to investigate the effects of different concentrations of lupeol (1 µM, 5 µM, and 10 µM) on the growth characteristics, phenolic and flavonoid contents, as well as enzymatic and non-enzymatic antioxidant responses in *Brassica nigra*.

The study found that lupeol acts as a growth stimulant, promoting root and shoot length, and biomass accumulation in *B. nigra*. A concentration-dependent increase in fresh weight and dry weight was seen in *B. nigra* plants exposed to a range of lupeol concentrations as compared to control groups with significant differences. Lupeol had an impact on the accumulation of biomass. Biomass accumulation is associated with primary metabolism mainly Nitrogen and Carbon

metabolism that contributes in cellular growth and cell components (Goncalves, Antes *et al*., 2009). The optimal concentration for growth enhancement was observed at 10 µM lupeol. Both root and shoot length showed significant increases, with 109% and 77% increments, respectively, at this concentration as compared to control. Similarly, fresh and dry weights of roots and shoots were substantially enhanced at 10 µM of lupeol, Fresh weight of roots increased up to 243% while dry weight enhanced up to 132% at L10 µM. Likewise, fresh weight and dry weight of shoots increased up to 120% and 103%, respectively. This positive effect of lupeol on growth aligns with previous research. One possible explanation for this phenomenon is that triterpenoids are known to have significant involvement in several physiological and developmental mechanisms within the plant. The potential mechanisms underpinning this phenomenon may be attributed to the interaction between lupeol and plant growth regulators, including auxins and cytokinins, which are recognized for their ability to regulate several physiological processes in plants.

Phenolic biomolecules are synthesized through the shikimate/phenylpropanoid pathway and function as potent free radical scavengers in living cells. Flavonoids are polyphenolic compounds that are ubiquitous in nature (Rice-Evans, 1999). Both the phenolics and flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health such as antiviral, anti-allergic, antiplatelet, anti- inflammatory, antitumor and antioxidant activities. Flavonoids also protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite (Al-Snafi, 2020)

Basically, elicitors induce stress responses in plant that stimulate the synthesis of secondary metabolites due to oxidative stress (Naik, Al-Khayri *et al.* 2016). Lupeol as an elicitor like other elicitors such as Jasmonic acid (JA) and Salicylic acid (SA) are related to signaling molecules (Giri, Zaheer *et al.* 2016). These molecules can initiate signal transduction pathways that lead to the stimulation of various transcriptional genes which cause production of endogenous molecules involve in defense mechanism (Zhai, Jia *et al.,* 2017, Singh and Dwivedi 2018). This study evaluates the effect of different concentrations of lupeol on *B. nigra* shoot tip culture and their morphological and phytochemical parameters like phenolic and flavonoid contents. The result indicated that the addition of lupeol together with culture media had more beneficial effects on plant growth and defense responses as compared to control. Plant phenolic compounds are directly related to their free radical scavenging activity and may contributed directly in antioxidative action

(Bidchol, Wilfred *et al.,* 2011). The total phenolics contents (TPC) and total flavonoid contents (TFC) were analyzed in lupeol treated root and shoot tip culture of *B. nigra* as shown in Figure 3.3 and 3.4. The result demonstrate that phenolic contents of both shoots and roots of lupeol treated *B. nigra* exhibited a significant increase in concentration dependent manner. At 10 µM of lupeol, *B. nigra* shoots and roots exhibiting a remarkable 98% and 87% increase in phenolic contents. Similarly, the flavonoid content in both shoots and roots demonstrated an upregulation, with shoots displaying a striking 100% enhancement and roots exhibiting a more moderate 24% increase. The reason for the increased response is that higher experimental concentration of lupeol L10 favour the enhancement of phenolic and flavonoids contents in plants. The enhanced production of phenolics and flavonoids compounds is related to the physiological state and defensive response of plant or tissues (Uchendu, Paliyath *et al.,* 2011). In this study the phenolics contents increased at high concentration of lupeol whereas, 10 µM BAP and 1 µM NAA did not act as elicitor for the production of phenolic contents followed by same trend in flavonoid content. A positive correlation was found in biomass accumulation and total phenolics and flavonoid contents in *B. nigra* shoot tip and root cultures.

Plants have a variety of detoxifying mechanisms to protect them from the accumulation of ROS, including antioxidant enzymes like POD and SOD and non-enzymatic antioxidants like TPC and TFC (Sharma *et al.,* 2013). The superoxide dismutase (SOD) and peroxidase (POD) are iso-enzyme that differentiated by many physical and chemical factors and amino acid sequence with same catalyze reaction (Chong, Abdullah *et al.,* 2005). The in-vitro antioxidant enzymatic activities (SOD and POD) were also analyzed for the study of effect of lupeol on the shoot tip culture of *B. nigra* as shown in Figure 3.7, 3.8, 3.9, and 3.10. In recent study, MS-media fortified with varying concentrations of lupeol was found with enhanced SOD and POD activities. Superoxide dismutase activity in roots of plants was found higher under different lupeol concentrations of L1 µM (160%), L5 µM (139%), and L10 µM (85%) as that of control (0.046 nM/min/g FW) followed by similar trend in plant shoots. Treatment of plants with lupeol elevated the enzyme activity in a concentration-dependent manner. Earlier studies suggest the association of antioxidant enzyme activation to stress-induced. This mechanism is to combat ROS-generated oxidative damage made to plants (Husen *et al.,* 2018). The results are consistent with the findings of Zia et al., (2021) where the application of lupeol on plants resulted in higher antioxidant enzyme activities in salt-stressed *B. nigra* plants. The enhancement in SOD and POD may related to the

systemic signaling in plants to get through with biotic and abiotic stress (Wasternack and Parthier 1997).

The production of ROS and other free radicals cause shift in metabolic pathways is due to environmental stress that is responsible for damaging membranes, plant cells, DNA, lipids and proteins directly or indirectly (Sergiev, Todorova *et al.,* 2019). Due to oxidative stress, plant synthesized various metabolic compounds mainly, Phenolics and flavonoids that performed scavenging activity on these free radicals (Gill, Tuteja *et al.,* 2010, Mohamed, Akladious *et al.,* 2017, Rehman, Ullah *et al.,* 2017). In this study, the antioxidant potential of *B.nigra* shoot tip and root culture was studied by using antioxidant assays which shows a positive correlation between antioxidant efficiency and accumulation of secondary metabolites on different concentrations of lupeol. This may be due to oxidative stress caused by different lupeol concentrations. To investigate antioxidant ability of plant shoots and root, performing more than one scavenging test as DPPH, ABTS, and metal chelating assays. Generally, secondary metabolites are considered as an antidot for various disorders that cause antioxidant potential in the plant tissue to enhanced (Ullah, Tungmunnithum et al., 2019). The free radical scavenging activity (DPPH and ABTS) and metal chelating activity of shoot tip and roots induced on lupeol treated media (for all concentrations) was higher than the control. It suggest that the addition of lupeol in culture medium may contributed in higher antioxidant activity (Zia *et al.,* 2020). In our study, higher DPPH, ABTS, and MC activities were noted at L10 as compared to control. The role of DPPH free radical scavenging activity is to prevent different diseases caused by oxidative stress. The obtained results showed that all lupeol concentrations of *B. nigra shoots and roots* exhibit enhanced radical scavenging and metal chelating activities due to their hydrogen transfer ability. The efficacy of lupeol in enhancing the antioxidant activities was observed when compared to the control groups. There is a direct correlation of total polyphenolic contents (TPC and TFC) and antioxidant activities. This is because the phenolics and flavonoid are highly responsible for antioxidant potential of plants (Abraham, Bhatt *et al.,* 2011, Fazal, Abbasi *et al.,* 2016). A related study on *T. pallida* show highly correlation between total polyphenolic contents and free radical scavenging activity (Huang, Ou *et al.,* 2005). Free radical scavengers possess the ability to directly neutralize peroxide radicals, thereby halting the progression of the chain reaction. Plant balanced their production by phytochemicals that act as antioxidant for scavenging free radical (Pinto, Sousa *et al.,* 2005, Chen, Chan et al., 2009).

The findings of the present investigation revealed notable disparities in the antioxidant activity and overall reducing capacity of plants when exposed to varying concentrations of lupeol. Lupeol significantly affect the TRP of *B. nigra* shoots and roots with a notable increase of 396% and 123% respectively, at a concentration of L10 µM. The shoots of *B. nigra* showed significant reduction potential at all lupeol concentrations whereas roots at L10 µM only exhibit the considerable reduction potential as compared to controls. A gradual increase in the antioxidant activity was observed in lupeol treated *B. nigra* shoots (146-258%) and roots (106-300%) by increasing the concentration of lupeol and maximum activity was observed at L10 µM. BAP and NAA did not show enhanced antioxidant activities as compared to lupeol treated roots and shoots. Compounds having high reducing power act as primary and secondary antioxidants and reduce the oxidized intermediates of lipid peroxidation processes (Kalita, Tapan *et al.,* 2013). Hydrogen atoms are donated by reductant molecules, and free radical chains are broken up (Abdel-Hameed *et al.,* 2009). Efficient electron donors include phenols and reductants (Shon *et al*., 2003). Free radicals are produced in plants under stress conditions. In response to stress, antioxidants are produced to combat against their damaging effects. Phytochemicals react with free radicals and protects the plant against oxidative damage of tissues and biomolecules (Anandjiwala, Bagul et al., 2008).Lupeol demonstrated a dose-dependent relationship with antioxidant activity. Total reducing power (TRP) and total antioxidant capacity (TAC) levels increased with higher concentrations of lupeol, suggesting its potential in enhancing the antioxidant defenses of *B. nigra*. Hence, this study shows the significance of lupeol as a promising growth regulator and as a protective agent against oxidative damage in plants.

4.1 Conclusion and Future Perspective

In the field of plant biotechnology, tissue culture is one of the most popular regenerative techniques. Due to high humidity in the culture flask, low light intensity, and hetero- or mixotrophic form of nutrition, in vitro plants are particularly fragile. Because of this, they are unable to produce secondary metabolites in an efficient manner, which inhibits plant growth and development as well as membrane fluidity, lipid peroxidation, water usage efficiency, and net photosynthetic rate. These factors lead to oxidative stress in plants. The plant activates a variety of enzymatic and non-enzymatic defence systems in response to the oxidative stress brought on by various stimuli. One such procedure is the synthesis of secondary metabolites. A pentacyclic triterpenoid called lupeol is present in a wide range of plants, including fruits and vegetables. It is a naturally occurring secondary metabolite that is known to have a significant role in plant growth, interactions with the environment, ability to withstand environmental stress, and predator defense. When plants are under stress, lupeol acts as an elicitor. Lupeol-treated plants reduced the stress in plants and improved biomass and plant length in the *Brassica nigra* shoot tip culture. The current study on lupeol as an elicitor strongly suggests that it has a useful role in the agricultural industry to alleviate stress by protecting the plant from oxidative damage. Additionally, it is suggested that it will enhance the yield of food crops to meet the demands of billions of around the people.

Chapter 5

5 References

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