

**REPUBLIC OF TURKEY  
ERCIYES UNIVERSITY  
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES  
DEPARTMENT OF AGRICULTURAL SCIENCE AND  
TECHNOLOGIES**

***IN VITRO (ROSMARINUS OFFICINALIS L.)* RESPONSE  
TO NANOPARTICLES AND THEIR EFFECTS ON  
SECONDARY METABOLITE AND GENETIC  
VARIATION**

**Prepared By  
Hussam Basim SALEH**

**Thesis Supervisor  
Assoc. Prof. Dr. Özhan ŞİMŞEK**

**M. Sc. Thesis**

**July 2023  
KAYSERİ**

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## COMPLIANCE WITH SCIENTIFIC ETHICS

I declare that all the information in this study was obtained following academic and ethical rules. I also state that I have fully cited and referenced all materials and results that are not inherent in this study, as these rules and behavior require.

Hussam Basim SALEH

Signature \_\_\_\_\_



## SUITABILITY FOR INSTRUCTION GUIDE

The MSc thesis entitled “*In vitro (Rosmarinus officinalis L.) Response to Nanoparticles and their effects on secondary metabolite and genetic variation*” has been prepared in accordance with the Erciyes University graduate school of natural and applied science thesis preparation and writing guide.

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**Hussam Basim SALEH**

2023, Kayseri

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AND GENETIC VARIATION***

**Hussam Basim SALE**

**Erciyes University, Graduate School of Natural and Applied Sciences  
Master's Thesis, July 2023  
Supervisor: Assoc. Prof. Dr. Özhan ŞİMŞEK**

**ABSTRACT**

The *Rosmarinus officinalis* is widely used as a medicinal herb or aromatic flavoring. Therefore, our study aimed to increase the accumulation of active secondary metabolites in plant tissue cultures by using nanomaterials and to evaluate their potential effect on the stability of the genetic material. The practical study was conducted in the plant biotechnology department at the Biotechnology Research Center/University of Al-Nahrain, Baghdad/Iraq. Callus cultures were created from the young rosemary leaves using MS medium containing different combinations of plant growth regulators (2,4-D, BA, TDZ) and then selecting the effective treatment for callus emergence and development for callus maintenance. The results showed that the most efficient treatment for callus formation contained 0.5 mg/l 2,4-D with 0.5 mg/l BA, giving a 100% germination rate and the highest significant rate for fresh and dry weights (936.4 and 69.5 mg, respectively), after one month of culture. When comparing the concentrations of secondary compounds between the leaves of the field plant and the callus cultures, HPLC analysis proved that most of the phenolic compounds in the leaves were higher than those in the callus cultures. The results stated above indicate that adding NPs (Ag and TiO<sub>2</sub>) into the MS culture medium at different concentrations (1, 2, 3, and 4 mg/l) had a positive impact on the callus fresh and dry weights and, additionally, enhanced the accumulation of certain beneficial plant phenolic compounds (such as caffeic acid, apigenin, ferulic acid, and rosmarinic acid), with varying quantities depending on the type and concentration of NPs employed. Notably, the treatment containing 4 mg/Ag-NPs exhibited superior performance in terms of augmenting the accumulation of phenolic compounds (such as luteolin, genkwanin, hesperidin, carnosic acid, and carnosol) in substantial quantities compared to the other treatments. Additionally, the RAPD-PCR technique showed that genetic variations in the callus exposed to nano-stress compared

to an untreated callus were determined and evaluated depending on the type and concentration of NPs used. This indicates the possibility of mutations occurring when exposed to different levels of NPs. Using nanomaterials as abiotic elicitation in *in vitro* cultures may be a promising technology for nano-biological applications used in developing the pharmaceutical industry sector.

**Keywords:** *Rosmarinus officinalis*, Nanoparticles, Elicitation, Callus induction.



# İN VİTRO (ROSMARINUS OFFICINALIS L.) NANOPARÇACIKLARA TEPKİ VE SEKONDER METABOLİT VE GENETİK DEĞİŞİKLİK ÜZERİNDEKİ ETKİLERİ

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## ÖZET

*Rosmarinus officinalis*, tıbbi bir bitki veya aromatik bir tatlandırıcı olarak geniş bir şekilde kullanılır. Bu nedenle, çalışmamız bitki dokusu kültürlerinde aktif ikincil metabolit birikimini artırmayı amaçlamış ve bunun genetik materyalin stabilitesi üzerindeki potansiyel etkisini değerlendirmeyi hedeflemiştir. Pratik çalışma, Biyoteknoloji Araştırma Merkezi/Al-Nahrain Üniversitesi, Bağdat/İrak'ta bulunan bitki biyoteknolojisi bölümünde gerçekleştirildi. Genç biberiye yapraklarından MS ortamı içinde bitki büyüme düzenleyicilerinin (2,4-D, BA, TDZ) farklı kombinasyonlarını içeren çağrıs kültürleri oluşturuldu ve çağrıs çıkışı ve gelişimi için etkili tedavi seçildi. Sonuçlar, çağrıs oluşumu için en verimli tedavinin 0,5 mg/l 2,4-D ile 0,5 mg/l BA içerdiğini gösterdi. Bu tedavi, %100 çimlenme oranı ve bir aylık kültürden sonra en yüksek anlamlı taze ve kuru ağırlık değerlerini (sırasıyla 936.4 ve 69,5 mg) verdi. Alan bitkisinin yaprakları ile çağrıs kültürleri arasındaki ikincil bileşik konsantrasyonlarını karşılaştırıldığında, HPLC analizi, yapraklardaki çoğu fenolik bileşiğin çağrıs kültürlerindeki daha yüksek olduğunu kanıtladı. Yukarıda belirtilen sonuçlar, MS kültür ortamına farklı konsantrasyonlarda (1, 2, 3 ve 4 mg/l) NPs (Ag ve TiO<sub>2</sub>) eklemenin çağrıs taze ve kuru ağırlıkları üzerinde olumlu bir etkisi olduğunu ve ayrıca bazı faydalı bitki fenolik bileşiklerinin (örneğin kafeik asit, apigenin, ferulik asit ve rosmarinik asit) birikimini artırdığını gösterdi. Bununla birlikte, NP türüne ve konsantrasyonuna bağlı olarak değişen miktarlarda. Özellikle, 4 mg/Ag-NPs içeren tedavi, diğer tedavilere göre önemli miktarda fenolik bileşik birikimini (örneğin luteolin, genkwanin, hesperidin, karnosik asit ve karnosol) artırma konusunda üstün performans sergiledi. Ayrıca, RAPD-PCR teknikleri, nano-strese maruz kalan çağrısularda tedavi edilmemiş çağrısularda göre genetik varyasyonların tespit edildiğini ve değerlendirildiğini gösterdi. Bu, farklı NP düzeylerine maruz kaldığında mutasyonların meydana gelebileceğini göstermektedir. Nanomalzemelerin in vitro kültürlerde abiyotik uyarım olarak kullanılması, farmasötik

endüstri sektörünün gelişiminde kullanılan nano-biyolojik uygulamalar için umut verici bir teknoloji olabilir.

**Anahtar Kelimeler:** Rosmarinus officinalis, Nanopartiküller, Elisitasyon, Kallus indüksiyonu.



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## LIST OF ABBREVIATIONS

<b>Abbreviations</b>	<b>Full name</b>
<b>BA</b>	Benzyladenine
<b>Con.</b>	Concentration
<b>2,4-D</b>	2,4-Dichlorophenoxy acetic acid
<b>DW</b>	Distilled water
<b>D.W.</b>	Dry weight
<b>F.W.</b>	Fresh weight
<b>HPLC</b>	High performance liquid chromatography
<b>LSD</b>	Least Significant Difference
<b>MS</b>	Murashige and Skoog medium
<b>PGRs</b>	Plant growth regulators
<b>RT</b>	Retention time
<b>TDZ</b>	Thidiazuron
<b>T.M.</b>	Total Mean
<b>UV</b>	Ultraviolet
<b>WHO</b>	World Health Organization
<b>CRD</b>	Completely Randomized Design
<b>DNA</b>	Deoxyribose Nucleic Acid
<b>pH</b>	Potential of Hydrogen
<b>RAPD</b>	Random Amplified Polymorphic DNA

## INTRODUCTION

Plants have been a valuable source of medicine for centuries, with traditional remedies like herbal treatments still being widely used today (Katzung et al., 2012).

The abundance and versatility of medicinal plants are attributed to their ability to produce organic compounds, called secondary metabolites or phytochemicals, which have medicinal properties and are commonly used as raw materials or co-factors in the pharmaceutical industry (Shanker & Shanker, 2016).

Although these compounds do not have a direct role in basic plant functions like photosynthesis, respiration, or carbohydrate, lipid, or protein synthesis, they provide defense against pests, pathogens, and other stresses (Taiz & Zeiger, 2002).

Rosemary, a member of the Lamiaceae family, is a popular medicinal plant that contains high levels of phenolic acids (Ziaková & Brandšteterová, 2003), and has been found to possess antibacterial, anti-inflammatory, and antioxidant properties (Stefanovits-bányai et al., 2003). Extracts from rosemary leaves have been researched for their potential as food additives and skin cancer inhibitors due to their strong antioxidant activity (Munné-Bosch & Alegre, 2001).

Plant cell suspension and callus cultures produce both primary and secondary metabolites that are found in the parent plant (Mehrabani et al., 2005).

Herbs have been used in the process of preparing medicines and treating a large number of diseases for several centuries, and the last decade witnessed a significant increase in the use of plants in treating human health problems, as the percentage of the world's population who will use herbal remedies and ancient methods reached The percentage of solutions to these problems ranges between 70 to 80%, as the presence of these plants and

their cheapness compared to modern drugs has increased their widespread use (Sharma et al., 2015)

Biotechnologies provided an opportunity to employ plant cells, tissues, and organs by cultivating them *in vitro* to obtain the required compounds. The plant tissue culture technology has a role in producing effective secondary metabolites on a commercial level, which is an alternative method to produce medicinal compounds from plants. It should be noted that amount of secondary metabolites is fluctuating in callus cultures; it may be more, few or none, than what is available *in vivo* (Isah, 2019).

Elicitation is a technique used to enhance the synthesis of secondary metabolites *in vitro*. Biotic and abiotic elicitors have been found to effectively promote the production of secondary metabolites in cell cultures by activating genes for *de novo* synthesis or driving physiological processes that result in increased accumulation of such products (Ramani & Chelliah, 2007; Ramawat & Goyal, 2004).

Nanoparticles (NPs) could act as abiotic stimulants for plants, especially in stressful environments, by encouraging the production of secondary metabolites like alkaloids, terpenoids, phenolic compounds, and flavonoids, and recently, NPs have been found to be a new way to get plants to make bioactive compounds (Selvakesavan et al., 2023)

It is still possible to increase the production of secondary compounds in plants under the technology of cultivating tissues and plant organs, and using the possibilities of any specific methods such as controlling the components of the nutrient medium for cultures and subjecting the crops to stress, as well as adding initiators to the nutrient medium for the cultivations, as stimulation or Irritation by exposing cultivated plants to stress factors is an important means of increasing the production of specific pharmaceutical compounds in different plants, including different medicinal plants, regardless of whether these stimuli are biological or non-living. In recent years, nanoparticles have been used. as catalysts for this purpose (Mastuti & Rosyidah, 2019)

Although the precise mechanism by which NPs enhance the synthesis of secondary metabolites in plants remains unclear, there is evidence to suggest that NPs can activate plant defense responses by initiating a signal transduction cascade and inducing an oxidative burst of ROS (Javed et al., 2021). As a result, it is conceivable that NPs promote

the production of secondary metabolites in plants by stimulating plant defense mechanisms and modulating secondary metabolism through multiple pathways.

**The aims of study:**

- 1- Induction of callus from *Rosmarinus officinalis L.* plant on culture medium containing various combinations of plant growth regulators (PGRs) and determining the best combination for inducing and maintaining callus tissue from leaf explants.
- 2- Quantitative and qualitative detection of important secondary metabolites using HPLC technique in the leaves of the mother plant and the callus resulting from the leaf explants.
- 3- Evaluation of the efficacy of different nanoparticles stimuli and their concentrations in the enhancement production of some medicinal important compounds in the callus culture of *Rosmarinus officinalis L.* plant.
- 4- Study of the genetic similarity or genetic variation between the callus culture after addition of nanoparticles.

## **CHAPTER 1**

### **LITERATURE REVIEW**

#### **1.1. Medicinal plants and herbs**

Medicinal plants contain a variety of secondary metabolites or products, which can have a significant physiological impact on mammals. These natural compounds are often used as medications or natural drugs, as they contain active principles that are effective in treating various diseases. Plant chemicals are increasingly being used for medicinal purposes (al-Sereiti et al., 1999).

The use of biotechnological technologies is vital for selecting, multiplying, and preserving medicinal plant genotypes. For the creation of high-quality plant-based medication, *in vitro* regeneration has huge potential. Various medicinal plants have been discovered to produce secondary metabolites *in vitro* using plant cell suspension cultures (Leena and Jaindra, 2003).

The micropropagation of various plant species, including those with therapeutic properties, has been achieved through *in vitro* cultivation. The success of this technique depends on several factors that influence the growth and development of medicinal plants (Withers & Alderson, 2013).

In 1978 WHO stated that medicinal plants are considered an excellent source for producing a variety of pharmaceuticals, as traditional medicines derived from these plants are used by approximately 80% of the population worldwide, including those living in industrialized nations (WHO, 1978).

## 1.2. *Rosmarinus officinalis* L.

For a considerable period, Rosemary has been extensively grown and is regarded as a highly significant medicinal plant (Stefanovits-bányai et al., 2003).

Rosemary is a globally grown plant with the Latin name "*rosmarinus*," which translates to "dew of the sea." It is a highly valued medicinal plant, and France, Spain, and Tunisia are the top producers of its essential oils. Typically, the plant is bushy, evergreen, and branches out, growing up to six feet tall, with stiff, leathery, and opposite leaves resembling pine needles. The plant flowers with light blue blooms from March to May. While there are variations with silver and gold-striped leaves, the green-leaved species is primarily used for medicinal purposes. For instance, the Corsican blue variation is a fragrant plant with porcelain-blue flowers and can grow up to 90 cm tall (Genders, 1977).

Miss Jessopp's Upright is a tall, hardy variety that can resist temperatures as low as  $-10^{\circ}\text{C}$ . It's ideal for hedging. (Brickell, 2019). Postractus is a ground cover that can be employed in a variety of situations. It is the least cold-hardy of the species, although it is quite wind tolerant. (Phillips & Rix, 1998)

Rosemary can be propagated through various methods, including seeds, cuttings, layering, and root division. It thrives in well-draining, arid soil and prefers sheltered areas like the base of a short wall with a south-facing exposure (Misra & Chaturvedi, 1984).

## 1.3. Rosemary classification

Kingdom: Plantae

Subkingdom: Tracheobionta – Vascular plants

Super division: Spermatophyta – Seed plants

Division: Magnoliophyta – Flowering plants

Class: Magnoliopsida – Dicotyledons

Subclass: Asteridae

Order: Lamiales

Family: Lamiaceae (Labiatae)

Genus: *Rosmarinus*

Species: *Rosmarinus officinalis* Linn. Rosemary (Stadhouders, 1990)



*Figure 1.1. (Rosmarinus officinalis L.) plant*

With around 220 genera and nearly 4000 species worldwide, the Lamiaceae (Labiatae) family is one of the largest and most distinctive flowering plant families. In the Mediterranean, genera such as Nepeta, Phlomis, Eremostachys, Salvia, and Rosmarinus contain a wide range of species (Jamzad et al., 2003).

#### **1.4. Popular uses of Rosemary**

Rosemary has a diverse range of uses, including being utilized as a culinary herb and for its therapeutic properties and fragrance in soap-making. Traditionally, it has been used to enhance memory, alleviate muscle discomfort and spasms, promote hair growth, and reinforce both the nervous and circulatory systems (Szapary, 2000).

Rosemary is associated with various beneficial effects, such as being an analgesic, antidepressant, anti-inflammatory, antispasmodic, astringent, carminative, diuretic, digestive, fungicidal, insecticide, relaxant, and reportedly acting as a heart tonic (Svoboda & Hampson, 1999).

Dried rosemary leaves, whether ground or whole, are frequently used to season a variety of dishes, including soups, stews, sausages, beef, fish, and poultry. Rosemary essential oil is a common ingredient in perfumes, cosmetics, creams, deodorants, hair tonics, and shampoos. Medicinally, Rosemary wine can be used externally as a stimulant and relaxant to alleviate muscle spasms, headaches, and nervousness. As a tonic, Rosemary is

beneficial for elderly individuals with poor circulation and is particularly effective in depressive states that are accompanied by overall debility and symptoms of cardiovascular weakness. The camphor molecule found in rosemary extract has a stimulating effect on the circulatory and neural systems, particularly in the vascular nerves, making it an ideal treatment for various types of chronic circulatory weakness, including hypotension (Al-Sereiti et al., 1999) (Table 1.1).

The antiseptic oil derived from rosemary is utilized in the treatment of various ailments, such as arthritis, colic, depression, memory loss, migraines, coughs, influenza, and diabetes (Offord et al., 1995)

Rosmarinic acid, ursolic acid, and apigenin are suggested to be responsible for the herb's anti-inflammatory properties (Aruoma et al., 1996).

Table 1.1. Therapeutic potential of *R. officinalis* plant (Al-Sereiti et al., 1999)

<b>Pharmacological action</b>	<b>Therapeutic potential</b>
Relaxation of bronchial smooth muscle	Bronchial asthma
Relaxant of smooth muscle	Antispasmodic
Reduction of leukotrienes	Bronchial asthma, Peptic ulcer, inflammatory diseases
Inhibition of lipid peroxidation	Hepatotoxicity, Atherosclerosis and <u>ischaemic</u> heart diseases, inflammatory diseases, <u>Asthenozoospermia</u>
Inhibition of the complement	Inflammatory diseases
Prevention of the carcinogen	Cancer (Protection)

## **1.5. Antioxidant activity of rosemary**

The phenolic diterpenes carnosol and carnosic acid account for 90% of the antioxidant activity of the chemicals found in rosemary leaves (Fadel & El-Massry, 2000)

### **1.5.1. Antimicrobial activity**

Pharmaceutical companies are interested in biologically active natural substances. The scientifically shown antibacterial action of various naturally occurring plant compounds has been the subject of numerous reports (Smith et al., 2002; Peng et al., 2005).

Rosemary ethanol extracts containing phytoconstituents offer a promising possibility for the development of modern chemotherapies against numerous microbes (Gachkar et al., 2007; Oyedemi et al., 2008).

Essential oils' antibacterial properties are crucial for both food preservation and the management of microbial-based diseases that affect both people and plants. The medicinal principle is thought to be an essential oil, although plant phenolics, particularly rosmarinic acid, are also important (Pattnaik et al., 1996)

### **1.5.2. Antibacterial activity**

Bacteria possess the genetic ability to obtain and propagate resistance to drugs, which can also be utilized as a therapeutic strategy (Cohen, 1992).

The growth of *Staphylococcus aureus* was inhibited by 12 compounds isolated from 35 plant species, and *Escherichia coli* was inhibited by 10 compounds. *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* were all inhibited in growth by substances derived from nine different plant species (Olano et al., 1996)

### **1.5.3. Antifungal activity**

Due to their antifungal properties, rosemary plant extracts are of great interest (Suhr & Nielsen, 2003); Savoia et al., 2004).

The antifungal effectiveness of rosemary extracts was evaluated, in vitro, against five distinct fungi: Phytophthora, Verticillium, Fusarium, Lycopersici, Phoma, and Botrytis. The results showed that all of the tested doses of rosemary extract exhibited antifungal activity against all of the examined fungi (Salamone et al., 2006).

The essential oils from rosemary were examined individually and in combination for their antifungal properties (Jang et al., 2008).

### **1.5.4. Rosemary secondary metabolites**

The Lamiaceae family has a wide variety of aromatic plants, with essential oils being produced by many of them (Chalchat et al., 1993).

Labiates are recognized for their essential oils, which are found in many family members. Various members of this family have yielded a large number of active essential oils. The presence of diterpenoids in the family's members is well-known. Rosemary is a popular houseplant that may be found all over the world.

The chemical analysis of rosemary aqueous extract revealed the presence of multiple substances, including rosmarinic acid, caffeic acid, chlorogenic acid, carnosic acid, rosmanol, carnosol, and various diterpenes, as reported by according to a study by (Al-Sereiti et al., 1999). Also, they reported that caffeic acid and its derivatives, specifically rosmarinic acid, are two vital constituents of rosemary.

Haas et al., (1993) stated that leaves of rosemary contain approximately 1-2.5% essential oil, consisting of various compounds such as cineol (30%), camphor (15-25%), borneol (16-20%), bornyl acetate (maximum of 7%), alpha-pinene (maximum of 25%), and others, which contribute to its complex flavor profile.

### **1.6. Essential oils (EOs)**

Essential oils are made from plant extracts that are stored in oil cells, glandular trichomes, and oil or resin ducts. These oils are predominantly composed of mono- and sesquiterpenes, as well as aromatic polypropanoids, which are produced via the mevalonic acid and shikimic acid pathways for terpenes and aromatic polypropanoids, respectively. Lavender, sage, peppermint, eucalyptus, geranium, jasmine, rose, lemon, orange, and rosemary are among the commonly utilized plants for extracting essential oils (Simon, 1990).

The essential oils of rosemary are made up of about sixty different components, with main compounds accounting for up to 89 percent of the total (Burt, 2004).

Essential oils are employed in a wide range of products and are also used in aromatherapy. These oils are rich in chemicals that assist plants in combating illnesses, and in keeping bacteria and parasites at bay. Numerous compounds have been identified within essential oils, with hundreds of them demonstrating antibacterial, antifungal, and antiparasitic properties (Pauli, 2006).

The essential oils extracted from the leaves and flowers of rosemary were examined, and it was discovered that the major constituents were camphor (32.33%) and  $\alpha$ -pinene (11.56%), as reported by (Offord et al., 1995). Furthermore, the quantity of essential oil present in rosemary leaves may range from 1.0 - 2.5 percent, depending on the plant's chemo type and growth stage during the time of harvest. This oil is almost colorless to pale yellow and has a stimulating taste, according to (Bauer et al., 2008). The primary components of rosemary oil, according to (Atti-Santos et al., 2005) are terpineol, camphene, borneol, bornyl acetate,  $\alpha$ -pinene, cineole, camphor, verbenone, and geraniol.

### **1.7. Diterpenes and triterpenes**

Carnosic acid, a diterpene found in rosemary in concentrations exceeding 4%, is the most prevalent antioxidant. According to (Ternes, 1992), carnosic acid decomposes into several less potent diterpenes, including carnosol, carnosic acid-methylester, epirosmanol, and 7-methyl-rosmanol. Diterpenes were found in the highest amounts in rosemary's leaf tissue, although not all tissues contained them. Diterpenes were also discovered in small amounts in rosemary's flowers, seeds, and stems, as well as in the herb itself at low concentrations. Diterpenes were not detected in the roots, according to (Munné-Bosch & Alegre, 2001).

### **1.8. Carnosic acid and Carnosol**

The two main phenolic diterpenes found in the leaves of *R. officinalis* were found to be carnosic acid and carnosol. The level of carnosol in dried rosemary leaves ranged from 0.2 to 0.4 percent, whereas carnosic acid concentrations ranged from 1.7 to 3.9 percent.

In vitro studies have demonstrated that carnosic acid can act as a precursor to phenolic diterpenes with  $\gamma$  and  $-\delta$  lactone structure. In the presence of oxygen, carnosic acid can break down into carnosol and rosmanol over several days (Abreu et al., 2008).

### **1.9. Phenolic compounds**

Phenolic compounds are a class of plant compounds that exhibit a wide range of functional activities, and they are responsible for many of the observed effects. These compounds are characterized by an aromatic ring with one or more hydroxyl substituents, including functional derivatives. Compared to other types of chemicals, phenolics have

relatively greater acidities due to the strong coupling between the aromatic ring and oxygen, while the link between oxygen and hydrogen is weaker (Fennema, 1996).

Some phenolic compounds, including eugenol, are considered essential oils and are often used as antibacterial agents. Rosemary has undergone extensive research on its antibacterial activities and contains 2-3% phenolic acids, including "caffeic acid, chlorogenic acid, labiatic acid, neochlorogenic acid, and rosmarinic acid in its leaves" (Genena et al., 2008)

### **1.10. Flavonoids and tannins**

Flavonoids, also known as bioflavonoids, are polyphenols that include flavonoids, iso flavonoids, and neo flavonoids. They exhibit anti-inflammatory, anti-allergic, and anti-cancer properties. One carbonyl group is present in flavones, which are known to be powerful antibacterial agents against a variety of pathogens. Their effectiveness is attributed to their capacity to bind to soluble extracellular proteins as well as bacterial cell walls. Diosmetin, Genkwanin, Eriocitrin, Hesperidin, and Hispidulin are some of the flavonoids found in rosemary, together with the two prevalent flavones Apiggenin and Lutein (Almela et al., 2006)

### **1.11. Rosmarinic acid**

Rosmarinic acid (RA) is a carboxylic acid and a naturally occurring polyphenol antioxidant found in many herbs of the Lamiaceae family. It is an ester of caffeic acid and 3,4-dihydroxy phenyllactic acid and has been isolated as a pure substance from the rosemary plant. RA has been shown to have numerous pharmacological effects including antiviral, antibacterial, anti-inflammatory, and antioxidant properties, which make it beneficial for human health. RA also has anti-allergic properties and inhibits the proliferation of murine cells and cyclooxygenase (Zelić et al., 2005).

The molecule of RA contains two phenolic rings, one derived from phenylalanine via caffeic acid and the other derived from tyrosine via dihydroxyphenyl-lactic acid. RA and caffeic acid are major tannin components that frequently co-occur in several members of the Lamiaceae family (Janicsak & Mathe, 1998).

### **1.12. *In vitro* production of secondary metabolites**

Plant cell and callus cultures are important tools in biotechnology for producing beneficial secondary metabolites, which are compounds traditionally derived from plants and used in medications (Robins, 1994). However, extracting and purifying these compounds can be difficult since they are produced in specialized cells at specific developmental stages (Dörnenburg & Knorr, 1995). Secondary metabolites include a diverse range of compounds, such as anthocyanins, carotenoids, flavones, coumarins, saponins, sesquiterpenes, steroidal alkaloids, sterols, tannins, and terpenoids (Srivastava & Srivastava, 2007).

Secondary metabolic pathways rely on substrates from primary metabolism, and the control of enzymes that use Co-A or its thioesters has a significant impact on the stimulation of secondary metabolism (Broeckling et al., 2005). By adjusting cultured conditions, some products can accumulate at higher levels in cultured cells than in intact plants. For example, rosmarinic acid from *Rosmarinus officinalis* L. and *Colleus blumei*, ginsenosides from *Panax ginseng*, and shikonin from *Lithospermum erythrorhizon* were found to accumulate at significantly higher quantities in cultured cells than in native plants (Balandrin & Klocke, 1988). *In vitro* systems such as callus cultures, cell suspension cultures, and root cultures have been used to enhance the accumulation of medicinal natural products (Rady et al., 2014).

### **1.13. Callus cultures**

Differential administration of growth regulators and management of parameters in the culture medium are used to induce callus development and subsequent differentiation. Cell division, cell growth, and tissue differentiation are triggered by endogenous growth factors or the addition of exogenous growth regulators to the nutritional medium (Leena and Jaindra, 2003).

Although calli obtained from both tissue culture and natural wounded callus cultures of dicotyledonous and monocotyledonous plants share some similarities in terms of their morphology, cellular structure, growth, and metabolism, there are also differences in these characteristics. Callus formation is generally better in juvenile tissue, which is the most physiologically active. The type, concentration, and ratio of auxin to cytokinin

required for callus formation also depend on the endogenous hormone content of the tissue (Torres, 1988). In the case of rosemary explants, it was found that the best medium for callus initiation was Murashige and Skoog (MS) media supplemented with 30 g/l sucrose and 1 mg/l NAA, IAA, and IBA (Yesil-Celiktas et al., 2007).

#### **1.13.1. Secondary metabolite production-related factors**

Various parameters that influence secondary metabolite synthesis in plant tissues were grown in cultures. Physio-chemical parameters are first modified to maximize growth and generation of secondary metabolites, followed by the selection of high-productive cells.

#### **1.13.2. Physical factors**

Various physical factors, including light, pH, and temperature, have been tested to determine their impact on the cultivation of plant cells (Murthy et al., 2014).

#### **1.13.3. Nutrient Factors**

Plant cells are typically grown in a nutrient-rich medium containing essential minerals, vitamins, and glucose sources. As totipotent cultures, plant cells possess the same capacity as whole plants to synthesize both primary and secondary metabolites. For instance, the synthesis of serpentine, an indole alkaloid, was investigated using different basal media, and it was found that the amount of serpentine produced is influenced by the composition of the basal medium used (Ahmad et al., 2014).

#### **1.13.4. Plant growth regulators**

A great number of studies have looked at how plant growth regulators (PGRs) affect the amounts of secondary metabolites in cultured cells. Plant cells require auxins and cytokinins, which are two types of growth regulators. Plant growth regulators appear to shift the cytoplasmic circumstances of product creation to greater or lower levels, rather than reacting with intermediate chemicals in the biosynthesis process (Ramawat & Goyal, 2004).

Plant growth and productivity are significantly influenced by auxins and cytokinins. Higher levels of auxins in the growth medium can lead to cell dedifferentiation, which in turn can reduce the production of secondary metabolites (Komamine, 1991).

Numerous studies have been conducted to examine the impact of plant growth regulators (PGRs) on the production of secondary metabolites in *Rosemary officinalis*. One such study found that treating Rosemary plants with MeJA can stimulate the biosynthesis of a variety of secondary metabolites (Li et al., 2022). Additionally, another study reported that the addition of 2,4-D and BA to MS medium can induce the formation of more secondary metabolites in callus cultures of Rosemary than in the leaves of the mother plant (Pérez-Mendoza et al., 2020).

#### **1.13.5. Precursors**

Precursors refer to molecules that are converted into secondary metabolites through minor structural modifications. These compounds can be added to plant cell cultures and incorporated into the production pathways of secondary metabolites (Asai & Nishioka, 1999). For example, the rosemary plant utilizes phenylalanine and tyrosine as precursors to produce rosmarinic acid. When these precursors are supplied to callus cultures derived from leaf tissues, the content of rosmarinic acid is increased, thereby improving the overall production of this valuable secondary metabolite (El-Naggar, 2006).

#### **1.13.6. Elicitation**

Elicitors are divided to microbial stress mediators (biotic elicitors) and stress agents (abiotic elicitors). Biotic elicitors included fungus, bacteria, polysaccharide like chitin and chitosan were used to enhance production of secondary metabolism (Ramawat & Goyal, 2004). moreover, Various abiotic agents such as UV rays, osmotic pressure, chemicals or heavy metals, and Nano particle acted as vigorous stress to increase formation of wide range of compounds in plant tissues (Radman et al., 2003) (Figure 1.2.)

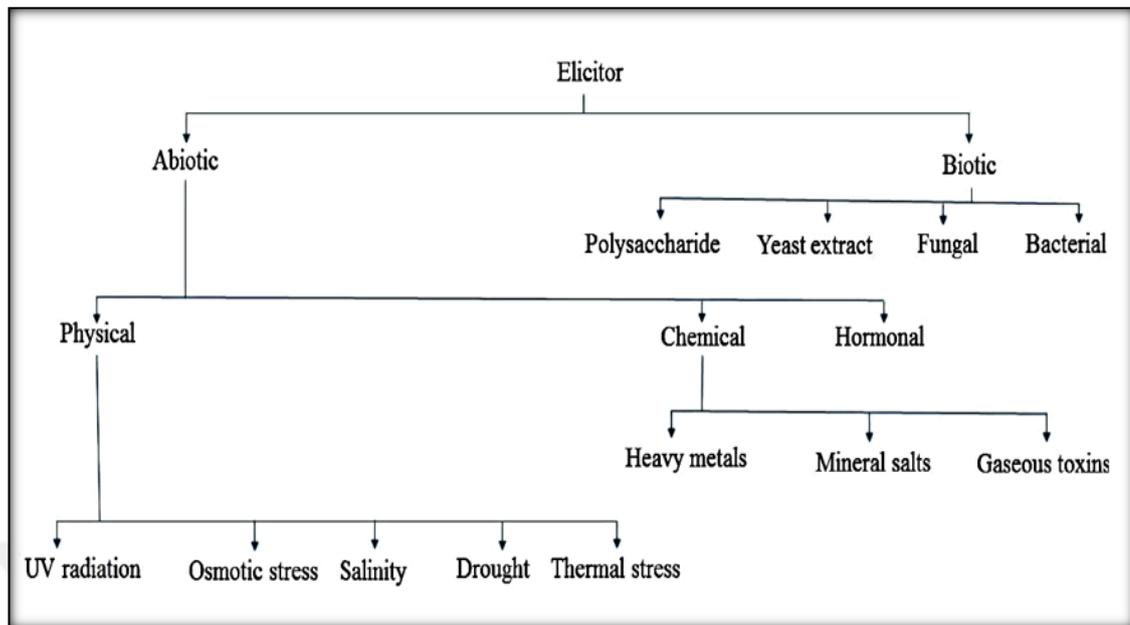


Figure 1.2. Classification of elicitors based on their "Nature" (Shanker & Shanker, 2016).

#### 1.14. Nano technology

Nanoparticles have the potential to positively impact the global economy, industry, and people. As the world continues to advance in agricultural production and the food industry, it becomes crucial to develop innovative approaches that enhance these areas. Nanotechnology can aid in diagnosing and treating diseases, as well as improving the ability of plants to absorb nutrients and resist infections from pests and pathogenic microorganisms ( Halder et al., 2019).

Several studies have been conducted to evaluate the potential beneficial or toxic effects of these NPs on plant growth, development and rate of photosynthesis and metabolism, especially on specialized plant metabolism in addition to their role in the plant defense and adaptation mechanism. Plants treated with them are also used as a source of biologically active compounds in Human industries as medicines to treat various diseases, food additives and cosmetics (Cardoso et al., 2018). The use of nanomaterials in a variety of sectors, including biological and medical systems, is the focus of nanoscience (I. Khan et al., 2019)

There are many reports indicating that the application of nanotechnology has a positive impact on plant tissue culture. Nanoparticles, or NPs, have also been extensively used to promote plant growth and increase crop yield, facilitate genetic modification of plants,

enhance the production of bioactive compounds, and provide protection against various environmental stressors. For instance, treating licorice seedlings with copper oxide (CuO) and zinc oxide (ZnO) resulted in increased levels of anthocyanins, flavonoids, glycyrrhizin, phenolic compounds, and tannins (Kim et al., 2017).

### **1.15. Nano applications in plant tissue culture technology**

One of the most active fields of research in modern materials science is the topic of nanotechnology. Based on certain qualities including size, distribution, and shape, nanoparticles display entirely new or better properties. Nanoparticles and nanomaterials are rapidly finding new uses. However, there is still a need for an affordable, commercially viable, and environment friendly synthesis route to produce silver nanoparticles. Nano crystalline silver particles have found tremendous applications in the fields of high sensitivity bio molecular detection and diagnostics, antimicrobials and therapeutics, catalysis, and micro-electronics (Jain et al., 2009)

Another scientific experiment targeted the compound copper Nano sulfate (CuSO<sub>4</sub>) by adding Nano copper sulfate to the MS medium for *Verbena bipinnatifida* plant and the results showed that the treatment 5mg/l was an increase in the height of branches that reached 52% Compared to the control treatment, as well as an increase in root length of 21% compared to the control treatment, and an increase in the fresh weight amounted to 39% compared to the control treatment, and in this experiment also, the phenolic compounds in the plant increased with an increase in concentration CuSO<sub>4</sub> nanoparticles (Genady et al., 2016).

Among the recorded research attempts to increase the secondary metabolite compounds of plants, the content of *vanilla planifolia* increased when adding silver nanoparticle to the MS medium of total phenols and antioxidant compounds as well as lipid peroxidase due to the increase in Reactive oxygen species (ROS) production, at concentrations 25 and 50 mg/l (Spinoso-Castillo et al., 2017).

The addition of zinc oxide was also tested in another study with different concentrations, and compensation of Humic acid for MS medium for the branches of *Lilium ledebourii* plant to estimate total phenols, anthocyanin pigment and flavonoids, the results showed that the highest amount of total phenols recorded in the NaO zinc oxide NPs is treatment

75mg/l and was better than the amount of Anthocyanin was produced in the 100 mg/l Humic acid treatment, while the highest amount of Flavonoids was achieved in the 25mg/l Nano Zinc Oxide treatment by interaction with 100mg/l Humic acid. (Chamani et al., 2015)

#### **1.16. Effect of TiO<sub>2</sub>-NPs on secondary metabolites**

The impact of TiO<sub>2</sub> nanoparticles (NP) on rosemary plant essential oil was assessed *Rosmarinus officinalis L.* Spraying seven doses of TiO<sub>2</sub> NPs—0 (control), 20, 40, 60, 100, 200, and 400 ppm—on the rosemary leaf was part of the experimental procedure. The outcomes showed that the quantity of rosemary essential oil was greatly impacted by the application of TiO<sub>2</sub>-NPs. With the application of TiO<sub>2</sub>-NPs, the peak area for the majority of the compounds rose. However, the peak area decreased at high TiO<sub>2</sub>-NP concentrations (greater than 200 ppm). Different settings allowed for the observation of the peak area of several substances, including myrecene, 2-butenal, 2-ethenyl-, 3-pinanone, isoborneol, and -terpineol. The groups that got 60 or 100 (Golami et al., 2018)

With another experiment to boost the production of secondary metabolites (phenolic and flavonoids compounds) in *Cicer arietinum L.* in vitro Secondary metabolites were quantified and quantified using high performance liquid chromatography HPLC in comparison to the mother plant. Titanium dioxide nanoparticles (TiO<sub>2</sub>) were used different concentrations to increase the production of secondary metabolites. The results showed that TiO<sub>2</sub> nanoparticle concentrations (4.5,6) mg/l caused highly significant production of most secondary metabolites from *Cicer arietinum L* callus embryo (Mohammed AL-oubaidi & Kasid, 2015).

These oxides are used in industry, Both ZnO and TiO<sub>2</sub> are frequently used in cosmetics due to their shared ability to filter UV and visible light Sun cream and glassware orders, too (Astruc, 2012).

#### **1.17. Effect of Ag-NPs on secondary metabolites**

Many studies reported that Nano silver has significant effect on production of various photochemical, in Onion Plant Ag NPs showed a significant improvement in the production of antioxidants in low concentrations. (Neal Stewart, 2015)

In a recent study, the dose-dependent effects of silver Ag-NPs nanoparticles were revealed and their effect on the production of terpenes in plants for *Thymus kotschyanus*, such as increasing the production of terpinyl acetate and increasing the production of essential oils from callus planting *Calendula officinalis*. -NPs on seed germination rate and phenol production of *Ricinus communis* and reaching the ideal concentration to increase all parameters of growth and production of phenolic substances. On the contrary, it was observed to stimulate the production of antioxidant phenolic compounds without severe toxic effects in hydroponics of *Bacopa monnieri* treated water hyssop. concentrations of Ag-NPs, while its addition led to an increase in the accumulation of anthocyanins without any negative effect on root growth, the opposite effect on rooting of *vanilla planifolia*, as these particles significantly enhanced the production of phenolic compounds (Chung et al., 2018)

Another study to find out the effect of Nano silver material by interfering with specific combinations growth regulators have investigate the combination of between 10mg/l Ag NPs and 2.5 mg/l BA and 0.1mg/l IAA added to MS medium in the *Tecomella undulata* plant gives the highest number of branches formed and the highest percentage of plant parts which produced new branches and the highest fresh weight of the plants and this is due to This combination affects the reduction of ethylene production and the researcher confirmed that the treatment of silver nanoparticles with 60 mg/l It had clearly reduced the growth of branches, meaning that the stimulating effect was reversed to Inhibitor at high concentration for Nano silver treatment (Aghdaei et al., 2012).

#### **1.18. Determination of secondary metabolites by High Performance Liquid Chromatography (HPLC)**

The analysis of phenolic acids compounds in rosemary leaves and callus extracts were carried out using an HPLC assay. The main benefit of HPLC over GC/MS is that it can analyze a considerably greater variety of components (Torre et al., 2001).

HPLC is a valuable tool for analyzing compounds that possess high polarity, large molecular weight, or are susceptible to thermal instability. The separation of the sample components in HPLC is accomplished by the interplay between the mobile phase, which is a solvent eluting through the column, and the stationary phase, which is composed of particles coated with specific chemicals. The extent of separation depends on how the

components interact with the stationary phase, which can be influenced by various physicochemical properties such as polarity or size (Peng et al., 2005).

The main phenolic antioxidants in rosemary, rosmarinic and carnosic acids, can be quickly and immediately determined using the HPLC method, which is reported (Troncoso et al., 2005).

Identification of phenolic and terpenic compounds in plant extracts that have been subjected to derivatization can be achieved by comparing their retention time and mass spectra with those of known derivatized substances. Alternatively, their spectral features can be compared with published data on phenolic and terpenic compounds (Razboršek et al., 2007).

### **1.19. DNA Markers and Genetic Variation**

The information that can be obtained about the extent of genetic variations between individuals contributes effectively to the expansion of knowledge of the genetic basis of sex and even the variety in the ways in which these plants are developed by the usual or modern methods. Its occurrence is considered the key to the survival of plant species, as with the loss of diversity, the plant becomes susceptible to infection with various pests, and these genetic variations can be obtained by crossing, chromosome duplication, or genetic mutations, whether they are spontaneous or induced, and include all inherited reactions in a living organism (Gepts, 2002). It should be noted that genes play a crucial role in the production of secondary metabolites, and plant cell cultures are capable of producing the same compounds as the whole plant (Lila, 2004).

Randomly Amplified Polymorphic DNA (RAPD) marker analysis is a molecular biology technique that uses short primers of random sequences ranging from 8 to 15 nucleotides in length. The amplification process results in the generation of complex patterns of Polymerase Chain Reaction (PCR) products, as the random sequence primers anneal to various regions of an organism's genome. Unlike traditional PCR analysis, RAPD does not require any prior knowledge of the DNA sequence of the target organism. The amplification of a DNA segment depends solely on the presence of complementary positions to the primer sequence. For instance, if the primers anneal too far apart or the 3' ends of the primers are not facing each other, no fragment will be produced. As a result,

any mutations that have occurred in the DNA template at the site that was previously complementary to the primer will prevent PCR product formation, leading to a distinct pattern of amplified DNA segments on the gel. The amplified fragments, typically ranging from 0.5-5 kb in size, are separated by agarose gel electrophoresis, and polymorphisms are identified by the presence or absence of bands of specific sizes (Bera & Saikia, 2002)

## **1.20. Applications**

RAPDs have been used for many purposes, ranging from studies at the individual level (e.g., genetic identity) to studies involving closely related species. RAPDs have also been applied in gene mapping studies to fill gaps not covered by other markers.

### **1.20.1. Limitations of RAPD**

The RAPD technique has certain limitations that need to be taken into account while analyzing results.

- Firstly, most RAPD markers are dominant, and it is not possible to determine whether a DNA segment is amplified from a heterozygous locus (1 copy) or a homozygous locus (2 copies). Co-dominant RAPD markers that can differentiate between the two are rare.
- Secondly, PCR is an enzymatic reaction, and the quality and quantity of the template DNA, concentration of PCR components, and PCR cycling conditions can greatly affect the outcome. Therefore, the RAPD technique is laboratory-dependent and requires carefully developed laboratory protocols to be reproducible.
- Finally, mismatches between the primer and the template DNA can lead to the absence or reduced amount of the PCR product, making the interpretation of RAPD results difficult (Polashock & Vorsa, 2002)

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1. Devices and materials

##### Devices

Table 2.1. List of devices used in the study.

<b>Devices</b>	<b>Company (Country)</b>
Analytical balance	Sartorius (Germany)
Autoclave	Tomy (Japan)
HPLC	Shimadzu (Japan)
Hi-glass bend sterilizer	HiMedia (India)
Hot plate stirrer	(Germany)
Laboratory oven	Fisher Scientific (USA)
Laminar air flow	Unitech (Korea)
Micropipette	Eppendorf (Germany)
pH-Meter	Hanna (USA)
Refrigerator	TEKA, (Spain)
Water distillatory	Stuart (UK)
Microwave	PenGuin (Germany)
Centrifuge	D-Lab (China)
Conventional PCR	AppliedBiosystem (USA)
Gel Electrophoresis	Cleaver Scientific (UK)
UV Transilluminator	UVP (UK)
Vortex	PHOENIX INSTRUMENT (Germany)
Quantus Florometer	Promega (USA)

## Chemicals

Table 2.2. List of the chemicals used in the study.

### Chemicals and biological materials

Products	Manufacturer (Country)
Agar-Agar	HiMedia (India)
Ethanol	HiMedia (India)
MS- medium	HiMedia (India)
Sucrose	Local market (Iraq)
Sodium hypochlorite (Beach)	Fas (Iraq)
DNA extraction kit	Add bio (korea)

### Plant growth regulators (PGRs)

Benzyladenine ( <b>BA</b> )	Sigma (USA)
Thiadiazuron ( <b>TDZ</b> )	Sigma (USA)
Dichlorophenoxyacetic acid ( <b>2,4-D</b> )	Sigma (USA)

### Elicitors

TiO <sub>2</sub> -NPs	Hongwunanometer (china)
Ag-NPs	Nanjing Nano Technology co,ltd (china)

### Standards used for HPLC Analysis

Caffeic acid	Sigma (USA)
Ferulic acid	Sigma (USA)
Rosmarinic acid	Sigma (USA)
Carnosol	Sigma (USA)
Carnosic acid	Sigma (USA)
Hesperidin	Sigma (USA)
Luteolin	Sigma (USA)
Apigenin	Sigma (USA)
Genkwanin	Sigma (USA)

## **2.2. Preparation of stock (PGRs) solutions**

The powders of plant growth regulators (**TDZ & BA**) were dissolved by few drops of suitable solvent of strong acid or base. While the powder of (2,4-D) was dissolved via few drops of 70% ethanol. Then sterilized distilled water was added to obtain final concentration 100 mg/l. All solutions were kept in dark bottles in the refrigerator.

## **2.3. Media preparation**

Murashige and Skoog (MS) medium components (Murashige and Skoog, 1962) were prepared by dissolving 4.9 g/l of medium in distilled water (DW), according to the manufacturer's instructions (Hi-media, India) which has all ingredients of macro and micronutrients with vitamins and Calcium Chloride. Different concentrations of PGRs with 3% Sucrose were added. The pH was adjusted to (5.7-5.8). Agar-Agar at 8 g/l was added to solidifying the medium. All prepared treatments were dispensed into clean glass tubes (2.5 x 8) cm for callus induction.

## **2.4. Media sterilization**

Steam sterilization by the autoclave (121°C, 15 psi pressure) was used for 20 min. All PGRs were sterilized by autoclave after being added to the culture media (Sharma et al, 2015)

## **2.5. Tools and glassware sterilization**

All tools were washed with detergent solution and rinsed in tap water. Washed glassware, and stainless-steel tools were dried and sterilized in laboratory oven at 150-200 °C for 2 hours. The plastic-ware (micropipette tips, etc.) were sterilized by autoclave at 121 °C for 30 min (Sharma et al, 2015)

## **2.6. Plant material and surface sterilization**

The mother plant was cultivated in the garden of Biotechnology Research Center, Al-Nahrain University. The fresh leaves were collected from mother plant and sterilized by washing 30 minutes in tap water, then immersed in 70% ethanol for 1 minutes, followed soaked in 2% sodium hypochlorite for 10 minutes with few drops of Tween 20, finally

washing 4-5 times with sterilized DW was applied to remove sterilization solution, all steps were done aseptic condition.

## 2.7. Callus induction

Homogeneous pieces of sterilized leaves were cut and planted on MS solidified medium enhanced with different concentrations of PGRs:

□ □ **Combination 1, (TDZ with 2,4-D):** different concentrations of **(2,4-D)** (0.0, 0.5, 1.0, and 1.5) mg/l combined with (0.0, 0.5, 1.0,) mg/l of **(TDZ)**

□ □ **Combination 2, (BA with 2,4-D):** different concentrations of **(2,4-D)** (0.0, 0.5, 1.0, and 1.5) mg/l combined with (0.0, 0.5, 1.0,) mg/l of **(BA)**

Each combination resulted in (12) treatments. Ten replicates of each treatment were kept at complete dark conditions at  $(25 \pm 2 \text{ }^\circ\text{C})$ , for 4 weeks.

## 2.8. Estimation of fresh and dry weight of callus

Fresh and dry weights of initiated callus were measured using analytical balance after 4 weeks of incubation in dark conditions. The fresh weight measurement was done under "laminar air flow cabinet" conditions. For the evaluation the dry weight, the initiated callus were dried in a laboratory oven at  $45 \text{ }^\circ\text{C}$  for 24 hours (Soni et al., 2018).

Figure 2.1 shows some of the work stages for the experiments of stimulating the emergence of callus and calculating its weights in the laboratory.

## 2.9. Maintenance of callus culture

The initiated callus from leaf explants were cultured on maintenance medium for one month to obtain an adequate amount of callus tissue. As a results The maintenance mediums was (0.5 mg/l BA with 0.5 mg/l 2,4-D).

## 2.10. Preparation stocks of Ag-NPs and TiO<sub>2</sub>-NPs

10mg/l each powder of Ag-NPs and Tio2 NPs were mixed with 100ml sterilized DW to prepare final stock concentration 100mg/l, the various concentrations (0, 1, 2, 3 and 4)

mg/l of each nanomaterial were directly added to the maintenance medium and sterilized by the autoclave.

### **2.11. Elicitation by nanoparticle**

After the maintenance stage, the callus tissues were cut into equal sections (about 200 mg) and planted on the same maintenance medium having different concentrations of NPs. All elicited treatments were kept at the dark condition. Fresh and dry weights of elicited callus were measured after 30 days of elicitation.

### **2.12. Quantification and qualification of some secondary metabolites in Rosemary by HPLC technique**

#### **2.12.1. Extraction method**

The elicited callus and leaves from the field plant were dried in a laboratory electrical oven at 45 °C for 24 h. The dried samples were grounded with a ceramic mortar and pestle to obtain a fine powder. One hundred (100 mg) of each sample was extracted with aid of reflux and sonication in 50 ml of 80% methanol [(80:20) methanol: water], for 45 min at room temperature with fixed frequency. The supernatant was transferred to a flask. The procedure was repeated three times and the pooled extract was concentrated by vacuum, and the volume adjusted to 100 ml with methanol. Aliquots were filtered through a 0.22µm Millipore filter before analysis (Dwivedi et al., 2017).

#### **2.12.2. Standards preparation for HPLC**

Ten mg of each standard were dissolved individually in 50 ml methanol to get 200 mg/l which was further diluted to obtain 25 mg/l as a final concentration.

#### **2.12.3. HPLC Analysis**

High-performance liquid chromatography (**HPLC**) instrument: Shimadzu-10 AV-LC as a HPLC system, with binary delivery pump model LC-10A shimadzu, and UV detector, at wavelength 280 nm.

- Column: zorbax C-18, 3 µm particle size, (50 x 2.0 mm) I.D.

- Flow rate: 1.2 ml/min

- Mobile phase: solvent A (deionized water acidified with 5% formic acid), solvent B (acetonitrile). Chromatographic separation was achieved with gradient program (linear gradient B% from 0 to 100% in 15 minutes).

20 µl of standard and sample were injected into HPLC and record the chromatogram.

The content of phenolic compounds in the samples was calculated according to formula below, by comparing the authentic standards peak area and their retention time (RT) to the peak area and (RT) of the examined samples.

(Mohammed, 2019)

$$\text{Concentration of sample } (\mu\text{g/ml}) = \frac{(\text{Area of sample}) \times \text{Conc. of standard} \times \text{dilution factor}}{(\text{Area of standard})}$$

#### **2.12.4. DNA Extraction**

The DNA extraction done by using (Addbio, Korea) extraction kit as manufacture instruction

1. the sample material (50~100 mg fresh weight) was grinded using a mortar and pestle.
2. The Proteinase K solution (20 mg/ml) 25 µl was added, then 200 µl of Lysis Solution and grinded sample was transfer to the 1.5 ml micro-centrifuge tube, mixed by vortexing, and incubated at 65°C for 30 min: the tube was inverted 2~3 times during the incubation for lysis.
3. The Precipitation Solution was added (100 µl) to the sample tube, mixed well by inverting and incubated for 15 sec. at 4°C.
4. The sample was centrifuged at 13,000 rpm for 5 min.
5. the clear lysate (supernatant) 200~300 µl was transferred of to a new 1.5 ml micro-centrifuge tube.
6. The Binding Solution was added 200 µl to the sample tube, and mixed well by pulse-vortexing for 15 sec.

7. The absolute ethanol was added 200  $\mu$ l and mixed well by pulse-vortexing for 15 sec.: After this step, briefly spin down to get the drops clinging under the lid.
8. The lysate was carefully transferred into the upper reservoir of the spin column with 2.0ml collection tube without wetting the rim.
9. The sample was centrifuged at 13,000 rpm for 1 min: the flow-through was poured off and the spin column was assembled with the 2.0 ml collection tube.
10. Washing 1 Solution was added 500  $\mu$ l of to the spin column with collection tube and centrifuged at 13,000 rpm for 1 min: the flow through was poured off and assembled the spin column with the 2.0 ml collection tube.
11. The washing 2 Solution was added (500  $\mu$ l) to the spin column with collection tube and centrifuged at 13,000 rpm for 1 min: Pour off the flow through and assemble the spin column with the 2.0 ml collection tube.
12. The spin column was dried by additional centrifugation at 13,000 rpm for 1 min to remove the residual ethanol in spin column.
13. The spin column was transferred to the new 1.5 ml micro-centrifuge tube.
14. The Elution Solution was added (75  $\mu$ l) to the spin column with micro-centrifuge tube, and let stand for at least 1 min.
15. The genomic DNA was eluted by centrifugation at 13,000 rpm for 1 min.

### **2.13. Genetic variation**

The standard RAPD technology use short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify total genomic DNA under low annealing temperatures by PCR technique. The amplification products are generally separated on agarose gels and stained with safe dye.

The RAPD-PCR reaction for investigating the interspecies diversity was carried out on rosemary DNA extracted from tissue culture and using 10 different primers as listed in Table 2.3, with the final volume of 25  $\mu$ L containing 12.5  $\mu$ L PCR master mix (Promega USA), 4  $\mu$ L DNA template, 1  $\mu$ L primer, and 7.5  $\mu$ L dH<sub>2</sub>O. The reaction condition

consisted of pre-heating at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 36 °C for 2 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min (Table 2.4).

Table 2.3. List of RAPD primers and their sequences used for random amplification of genomic DNA.

Primer	Sequence
OPS-19	GAGTCAGCAG
OPA-08	GTGACGTAGG
OPA-04	GGTCCCTGAC
OPA-06	AATCGGGCTG
OPA-09	GGGTAACGCC
OPD-02	GGACCCAACC
OPD-06	ACCTGAACGG
OPD-08	GTGTGCCCCA
OPD-10	GGTCTACACC
OPD-14	CTTCCCAAG

Table 2.4: Steps of program used for RAPD-PCR

Step	°C	Time	Cycle
Initial Denaturation	95	3 min	1
Denaturation	95	1	40
Annealing	36	2	
Extension	72	1	
Final Extension	72	10	1

### 2.13.1. Preparation of agarose gel

After PCR amplification, agarose gel electrophoresis was adopted to see the wild and mutant PCR products. One hundred ml of 1X Tris-acetate-EDTA (TAE) (Promega, USA) was taken in a beaker, then 1.5 gm (for 1.5%) agarose was added to the buffer, the solution was heated to boiling (using Microwave) until all the gel particles were dissolved,

*stargreen* (Bioneer) 140  $\mu$ l was added to the agarose, the agarose was stirred to get mixed and to avoid bubbles, the solution was allowed to cool down at 50-60 C°.

### **2.13.2. Casting of the horizontal agarose gel**

The agarose solution was placed into the gel tray after both the ends were coated, the agarose could solidify for 30 minutes at room temperature. The comb was carefully removed, and the gel was put in the gel tray. The tray was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 mm over the gel surface.

### **2.13.3. DNA loading**

The PCR products were loaded directly. For PCR products, 6  $\mu$ l was directly loaded to well. Electrical power was turned on at 3–5 volts/cm for 100 min. DNA moves from Cathode to plus Anode poles, the safe stained bands in gel were visualized using Gel imaging system. Figure 2.2, shows some steps of DNA extraction and PCR test.

### **2.13.4. Data Analysis**

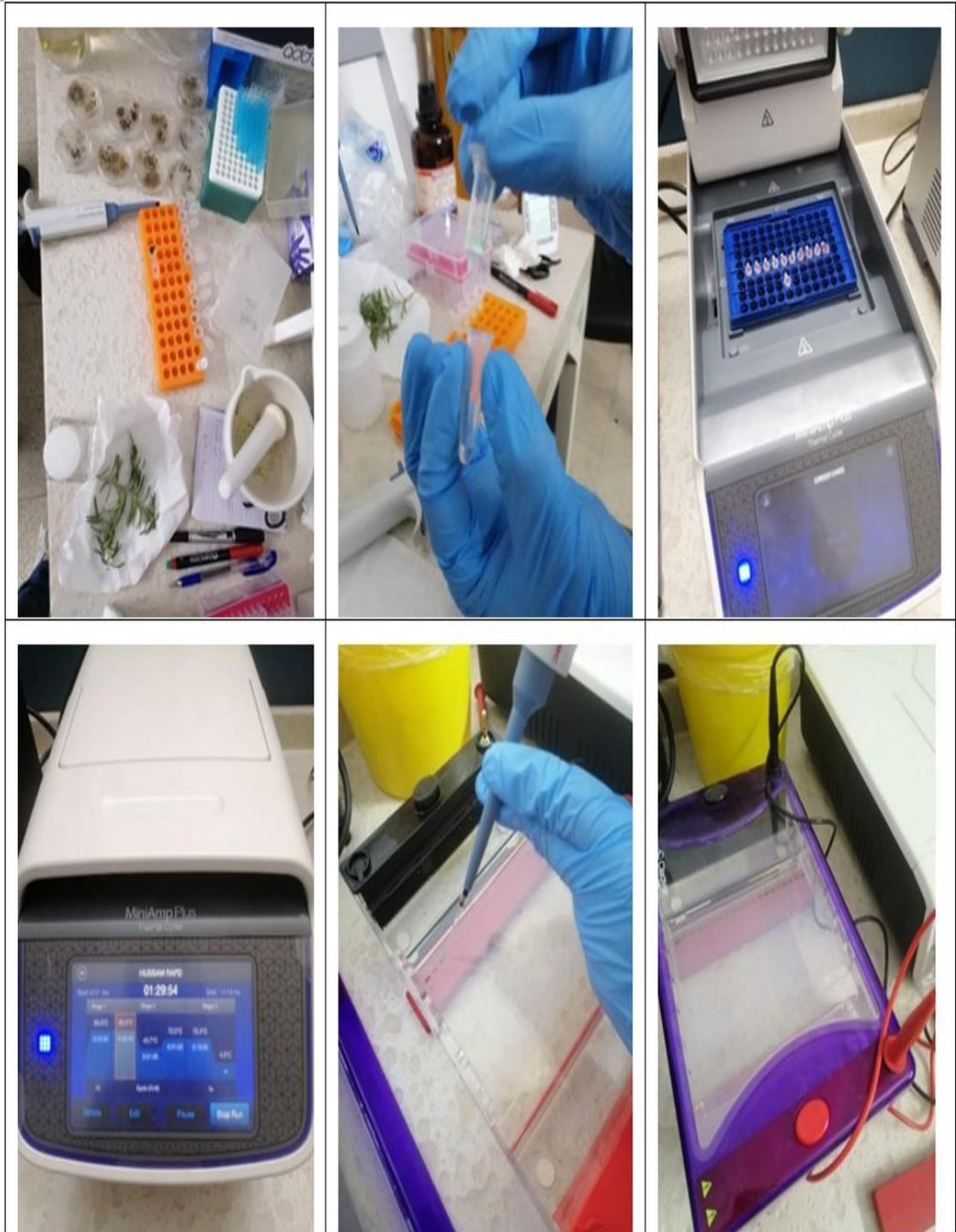
The RAPD bands were scored based on their presence (1) or absence (0) and each band was regarded as a locus.

### **2.13.5. Statistical analysis**

Each treatment of plant tissue culture and elicitation contained 10 replicates, while the quantification of SM via the HPLC device was carried out for three replicates. A complete randomized design (CRD) was applied as an experimental design. An ANOVA with the least significant differences (LSD) and Duncan tests were employed at a probability of 0.05 using SPSS version 23 software, and phylogenetic tree dendogram in PAST software Paleontological Statistics version 1.94b.



Figure 2.1. Images available depict the various stages involved in laboratory work for conducting tissue culture experiments.



*Figure 2.2.* Images available depict the various stages involved in laboratory work for PCR experiments.

## CHAPTER 3

### RESULTS

#### 3.1. Effects of different combination of PGRs on callus induction

##### 3.1.1. Effects of TDZ and 2,4-D on callus induction

In dark conditions, various concentrations of 2,4-D (0.0, 0.5, 1.0, and 1.5 mg/L) were added to the MS medium alone or in combination with 0.5 and 1.0 mg/L of TDZ. The development and generation of callus tissue were generally slow (started after four weeks) that required a considerable amount of time. Additionally, the texture of the callus tissue was predominantly watery, except for the treatment containing 1 mg/L TDZ, which resulted in the production of friable calluses as shown in (Table 3.1.)

Table 3.1. Morphology of callus culture induced by different concentrations of 2,4-D and TDZ and their combinations from Rosemary leaf explants

<b>Morphology of callus culture</b>				
<b>PGRs (mg/L)</b>	<b>(0.0) 2,4-D</b>	<b>(0.5) 2,4-D</b>	<b>(1.0) 2,4-D</b>	<b>(1.5) 2,4-D</b>
<b>0.0 TDZ</b>	No response	Brown & Watery	Brown & Watery	Brown & Watery
<b>0.5 TDZ</b>	Light green & Friable	Light green & Watery	Light green & Watery	Light green & Watery
<b>1.0 TDZ</b>	Light green & Friable	Light green & Watery	Light green & Watery	Brown & Watery

Moreover, Tables (3.2 and 3-3, and 3.4) illustrate the percentage of callogenesis, fresh and dry weights of calluses initiated from leaf explants. The treatments that had 0.5 mg/L 2,4-D resulted in the highest and most significant average values (73%, 469.0 and 33.4 mg, respectively) for induction rate, fresh, and dry weight. While the lowest average

values for fresh and dry callus weight (221.5 and 16.9 mg, respectively) were seen in treatments that contained 1.5 mg/L 2,4-D.

At the same time, TDZ at 1.0 mg/L resulted in the highest and significant average value (68%, 418.0 and 31.3 mg, respectively) for percentage, fresh and dry weight, while the lowest average values (60%, 267.9 and 20.1 mg, respectively) were seen in treatments with no TDZ added.

Furthermore, the treatment that fortified with 1.0 mg/L TDZ resulted in the highest mean (85%, 724.4 and 53.2 mg, respectively) for percentage, fresh and dry weight. Wears no callus formed during the control treatment (no PGRs).

Table 3.2. Effect of different concentrations of 2,4-D and TDZ and their combinations on percentage of callus induction from Rosemary leaf explants

Percentage of callus induction					
PGRs (mg/L)	(0.0) 2,4-D	(0.5) 2,4-D	(1.0) 2,4-D	(1.5) 2,4-D	Average
0.0 TDZ	0%	80%	80%	80%	60%
0.5 TDZ	60%	70%	65%	55%	63%
1.0 TDZ	85%	70%	60%	55%	<b>68%</b>
<b>Average</b>	48%	<b>73%</b>	68%	63%	

Table 3.3. Effect of different concentrations of 2,4-D and TDZ and their combinations on fresh weight (mg) of callus culture induced from Rosemary leaf explants

Fresh weight (mg)					
PGRs (mg/L)	(0.0) 2,4-D	(0.5) 2,4-D	(1.0) 2,4-D	(1.5) 2,4-D	Average
0.0 TDZ	0.0	422.2	332.8	316.6	267.9
0.5 TDZ	265.4	508.6	303.0	203.6	320.2
1.0 TDZ	<b>724.4</b>	476.4	326.8	144.4	<b>418.0</b>
<b>Average</b>	329.9	<b>469.0</b>	320.8	221.5	
<b>LSD (0.05)</b>	TDZ= 79.5    2,4-D= 91.8    TDZ*2,4-D = 102.7				

Table 3.4. Effect of different concentrations of 2,4-D and TDZ and their combinations on dry weight (mg) of callus culture induced from Rosemary leaf explants

Dry weight (mg)					
PGRs (mg/L)	(0.0) 2,4-D	(0.5) 2,4-D	(1.0) 2,4-D	(1.5) 2,4-D	Average
0.0 TDZ	0.0	33.2	24.4	22.8	20.1
0.5 TDZ	20.6	34.2	26.4	16.8	24.5
1.0 TDZ	<b>53.2</b>	32.8	28.0	11.2	<b>31.3</b>
<b>Average</b>	24.6	<b>33.4</b>	26.3	16.9	
<b>LSD (0.05)</b>	TDZ= 4.7    2,4-D= 5.4    TDZ*2,4-D = 6.8				

### 3.1.2. Effects of 2,4-D and BA on callus induction

The study evaluated the effects of different concentrations of 2,4-D alone or in combination with BA on callus tissue development in dark conditions. The results showed that the initiation of callus was started after three weeks (moderately slow). Morphology of callus tissue was predominantly yellow in color and friable in texture, except for the treatment containing 2,4-D alone, which produced brown and watery calluses (Table 3.5).

Table 3.5. Morphology of callus culture induced by different concentrations of 2,4-D and BA and their combinations from Rosemary leaf explants

<b>Morphology of callus culture</b>				
<b>PGRs (mg/L)</b>	<b>(0.0) 2,4-D</b>	<b>(0.5) 2,4-D</b>	<b>(1.0) 2,4-D</b>	<b>(1.5) 2,4-D</b>
<b>0.0 BA</b>	No response	Brown & Watery	Brown & Watery	Brown & Watery
<b>0.5 BA</b>	No response	Yellow & Friable	Yellow & Friable	Yellow & Friable
<b>1.0 BA</b>	No response	Yellow & Friable	Yellow & Friable	Yellow & Friable

**Tables (3-6, 3-7, and 3-8)** display the percentage of callogenesis and the fresh and dry weights of calluses derived from leaf explants. The treatments that included 0.5 mg/L 2,4-D resulted in the highest and most significant average values (95%, 733.2, and 54.9 mg, respectively) for callogenesis rate, fresh weight, and dry weight, whereas the lowest average values for all previous parameters (0.0) were observed in treatments that absence of 2,4-D.

In addition, the use of 0.5 mg/L BA resulted in the highest and most significant average values (75%, 574.2, and 41.2 mg, respectively) for callogenesis rate, fresh weight, and dry weight, while the lowest average values (64%, 350.1, and 25.7mg, respectively) were observed in treatments without BA.

It is worth noting that the interaction between auxin and cytokinin gave the highest values in treatments that contained 0.5 mg/L BA and 0.5 mg/L 2,4-D, which produced 100%, 936.4, and 69.5 mg, respectively, for callogenesis rate, fresh weight, and dry weight. So, it was selected as a maintenance medium for increasing the amount of callus tissue to use in elicitation tests. In contrast, no callus tissue formed in the control treatment without any plant growth regulators (PGRs).

Table 3.6. Effect of different concentrations of 2,4-D and BA and their combinations on percentage of callus induction from Rosemary leaf explants

Percentage of callus induction					
PGRs (mg/L)	(0.0) 2,4-D	(0.5) 2,4-D	(1.0) 2,4-D	(1.5) 2,4-D	Average
0.0 BA	0%	85%	83%	90%	64%
0.5 BA	0%	100%	100%	100%	75%
1.0 BA	0%	100%	93%	90%	70%
<b>Average</b>	0%	95%	92%	93%	

Table 3.7. Effect of different concentrations of 2,4-D and BA and their combinations on fresh weight (mg) of callus culture induced from Rosemary leaf explants

Fresh weight (mg)					
PGRs (mg/L)	(0.0) 2,4-D	(0.5) 2,4-D	(1.0) 2,4-D	(1.5) 2,4-D	Average
0.0 BA	0.0	525.1	421.8	453.4	350.1
0.5 BA	0.0	<b>936.4</b>	681.5	679.0	<b>574.2</b>
1.0 BA	0.0	738.0	493.9	290.3	380.5
<b>Average</b>	0.0	<b>733.2</b>	532.4	474.2	
<b>LSD (0.05)</b>	BA = 52.6	2,4-D= 60.7	BA *2,4-D = 85.9		

Table 3.8. Effect of different concentrations of 2,4-D and BA and their combinations on dry weight (mg) of callus culture induced from Rosemary leaf explants

Dry weight (mg)					
PGRs (mg/L)	(0.0) 2,4-D	(0.5) 2,4-D	(1.0) 2,4-D	(1.5) 2,4-D	Average
0.0 BA	0.0	41.3	30.3	31.1	25.7
0.5 BA	0.0	<b>69.5</b>	47.1	48.0	<b>41.2</b>
1.0 BA	0.0	53.9	33.6	20.9	27.1
<b>Average</b>	0.0	<b>54.9</b>	37.0	33.3	
<b>LSD (0.05)</b>	BA = 4.4	2,4-D= 5.1	BA *2,4-D = 7.2		

### 3.2. Elicitation of callus culture with different concentration of nanoparticles

#### 3.2.1. The impact of different concentrations of Ag-NPs and TiO<sub>2</sub>-NPs on the callus weight

The study examined the impact of varying levels of Ag-NPs and TiO<sub>2</sub>-NPs on callus growth. In general, the addition of NPs to the culture medium did not lead to phenotypic changes in the color and texture of callus tissue, but it was very similar to the control group (Figure 3.1).

Results presented in Figures (3.2 and 3.3) indicated that most of the treatments led to an increase in callus weight in comparison to the control group. The concentration of 3 mg/L

of Ag-NPs was found to be the most effective in terms of fresh and dry weight, with a mean of 542.5 and 32.1 mg, respectively. This concentration showed a significant difference from the control, where the mean fresh and dry weight were 388.3 and 25.1 mg, respectively. On the other hand, the optimal concentration for TiO<sub>2</sub>-NPs was 2 mg/L, which produced the highest mean of fresh and dry weight of 538.7 and 31.1 mg, respectively.

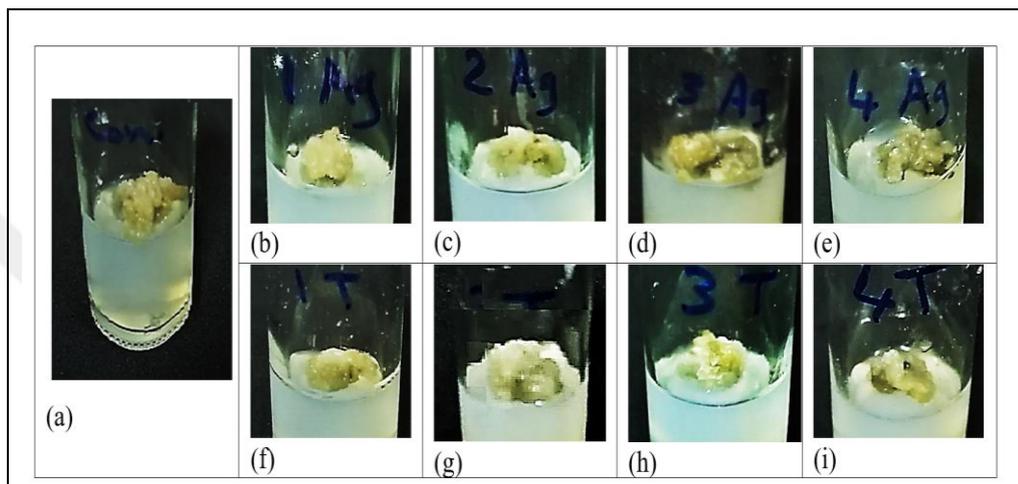


Figure 3.1. Morphology of calluses induced by various nanoparticle concentrations: (a) control; (b) 1 mg/L Ag-NPs; (c) 2 mg/L Ag-NPs; (d) 3 mg/L Ag-NPs; (e) 4 mg/L Ag-NPs; (f) 1 mg/L TiO<sub>2</sub>-NPs; (g) 2 mg/L TiO<sub>2</sub>-NPs; (h) 3 mg/L TiO<sub>2</sub>-NPs; (i) 4 mg/L TiO<sub>2</sub>-NPs.

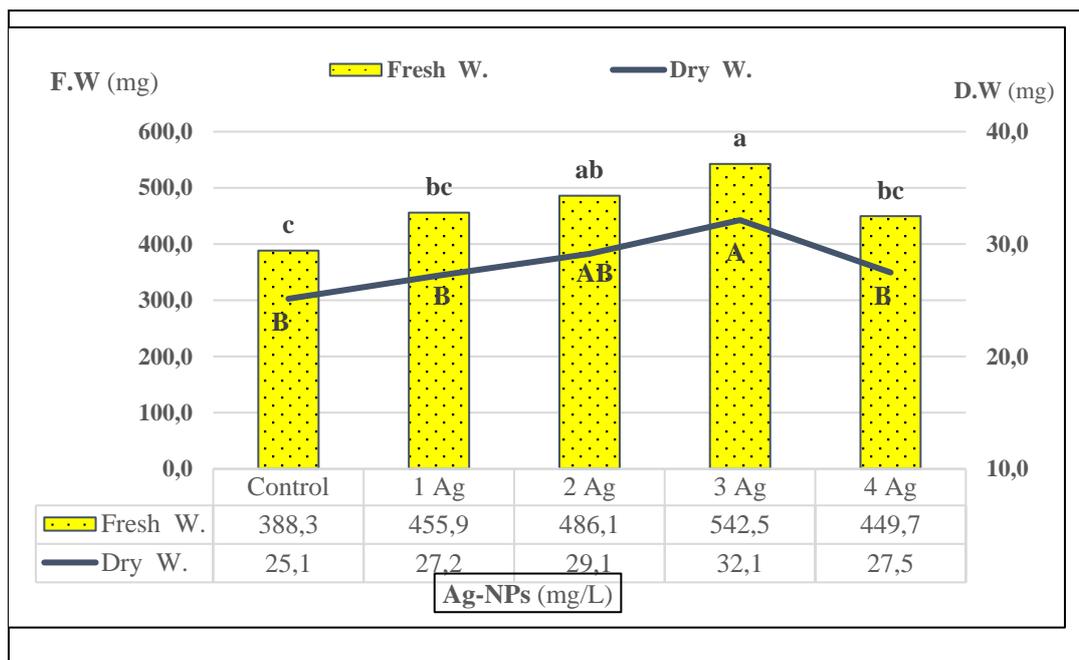


Figure 3.2. Effects of various Ag-NP concentrations on the callus fresh and dry weight in *R. officinalis* callus culture. Columns with the same letter are not significantly different, using Duncan's multiple range test at 5% level

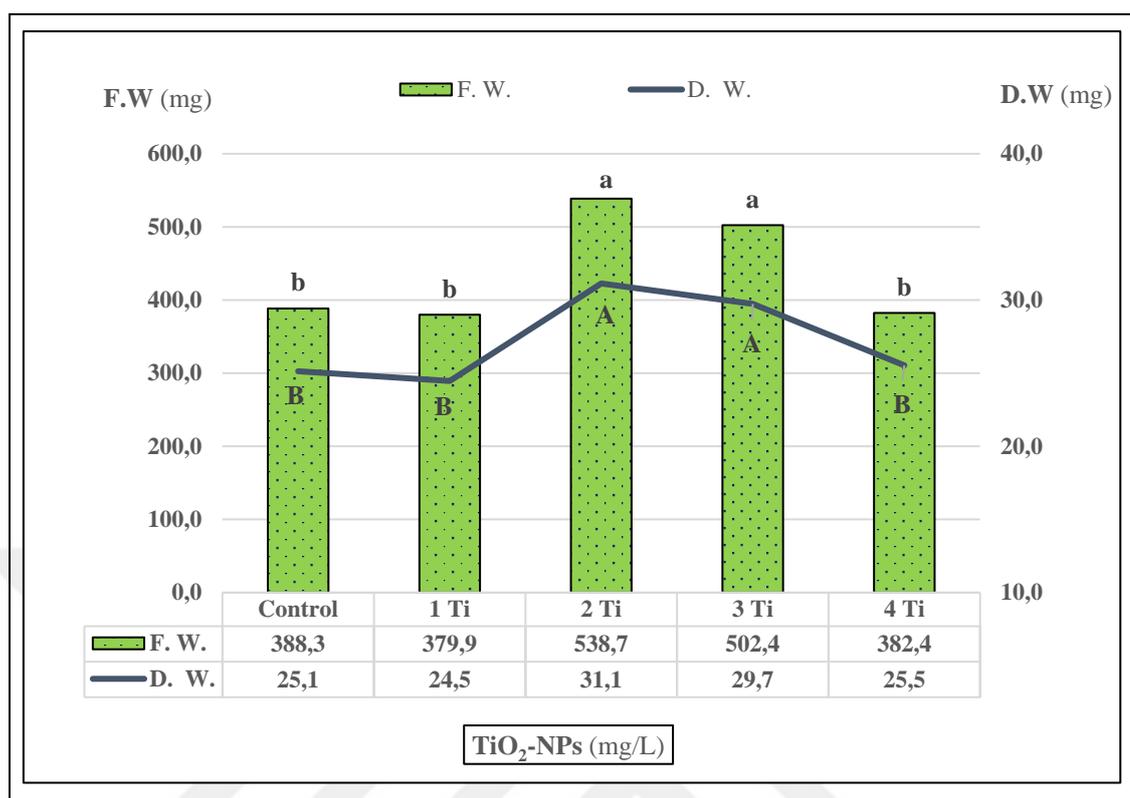


Figure 3.3. Impact of different concentrations of TiO<sub>2</sub>-NPs on callus fresh and dry weight in *R. officinalis* callus culture. Columns with the same letter are not significantly different, using Duncan's multiple range test at 5% level.

### 3.2.2. The impact of different concentrations of NPs on the content of certain phenolic compounds in *R. officinalis* using HPLC analysis

#### 3.2.2.1. Content of certain phenolic compounds in the callus culture and in leaves of *in vivo* plant

The graphic profile in Figure (3.4) and data in Table (3.9) illustrate the retention time R.T. (min) and area of nine phenolic compounds of *in vivo* plant (Leaves) and *in vitro* culture (Callus) compared to the reference standards used HPLC analysis. According to the comparison between these data, the concentrations of phenolic compounds were measured and corporate in (Figure 3.5).

Table 3.9. HPLC data report the R.T. (min) and area of nine phenolic compounds in leaves and callus of *R. officinalis* compared to the reference standards.

SN	Phenolic compounds	Reference standards		<i>In vivo</i> plant (Leaves)		<i>In vitro</i> culture (Callus)	
		R.T. (min)	Area	R.T. (min)	Area	R.T. (min)	Area
1	Caffeic acid	2.01	334965	2.04	136542	2.05	68119
2	Ferulic acid	3.20	274490	3.19	63449	3.02	57907
3	Rosmarinic acid	4.28	544628	4.28	952781	4.30	387625
4	Carnosol	5.11	390965	5.18	850106	5.14	569405
5	Carnosic acid	6.18	328460	6.21	387397	6.23	405880
6	Hesperidin	6.98	323910	6.93	100071	6.96	64097
7	Luteolin	8.17	333993	8.19	111033	8.21	51888
8	Apigenin	9.27	345297	9.28	46959	9.33	31230
9	Genkwanin	10.43	341746	10.45	236165	10.48	124263

Retention time (R.T.); Standards concentration were 25 µg/ml; Dilution factor was 20,

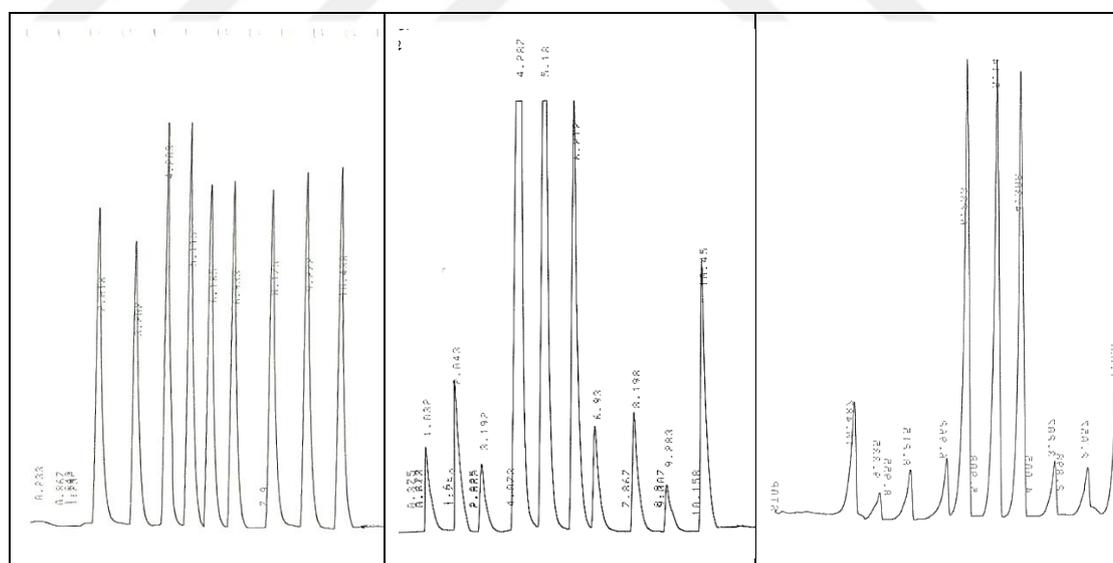
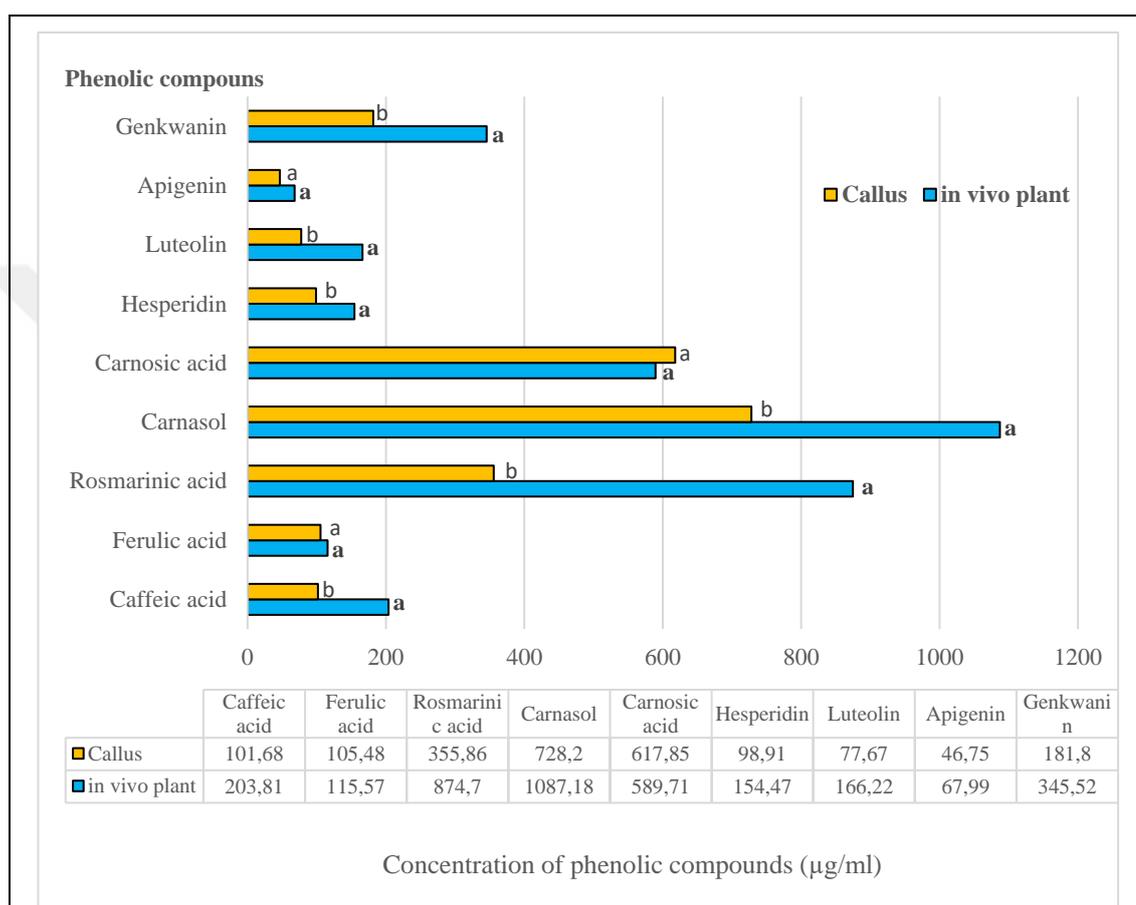


Figure 3.4. HPLC profile of separation nine phenolic compounds in leaves of *in vivo* plant and callus of *in vitro* culture: (A) standard compounds; (B) leaves sample; (C) callus sample.

The data in Figure (3.5) show that field leaves of *in vivo* plant had significantly highest means of most tested phenolic compounds, include: caffeic acid, ferulic acid, rosmarinic acid, carnosol, hesperidin, luteolin, apigenin, and genkwanin, that recorded (203.8, 115.5,

874.7, 1087.1, 154.4, 166.2, 67.9, and 345.5  $\mu\text{g/ml}$ , respectively) compared to *in vitro* callus culture.

Exceptionally, the content of carnosic acid compound (617.8  $\mu\text{g/ml}$ ) was higher in callus tissue compared to leaves of field plant (589.7  $\mu\text{g/ml}$ ).



**Figure 3.5.** Comparing the content of phenolic compounds ( $\mu\text{g/ml}$ ) in callus culture and leaves of *in vivo* plant (grown in field). Different letters in the same compound show significant differences, using Duncan's multiple range test at 5% level.

### 3.2.2.2. The impact of different concentrations of Ag-NPs on the accumulation of phenolic compounds

HPLC analysis was used in this study to explore the effect of Ag-NPs on the biosynthesis of phenolic components in rosemary callus cultures. Incorporating Ag-NPs into the callus culture medium resulted in noteworthy increases in the levels of the phenolic compounds analyzed, as displayed in Table (3.10). Among the tested concentrations, 4 mg/L of Ag-NPs exhibited the highest content of ferulic acid, carnosol, carnosic acid, hesperidin, luteolin, apigenin, and genkwainin, with significant differences observed in comparison

to the control treatment. Specifically, the quantities of these compounds reached 203.9, 1920.8, 2396.1, 1666.1, 856.6, 154.4, and 793.9  $\mu\text{g/ml}$ , respectively. Meanwhile, the maximum level of caffeic acid (310.9  $\mu\text{g/ml}$ ) was obtained at a concentration of 2 mg/L, whereas the highest amount of rosmarinic acid (1423.0  $\mu\text{g/ml}$ ) was achieved at a dose of 3 mg/L. It should be noted that the lowest content of phenolic compounds was observed in the control treatment.

Table 3.10. Content of phenolic compounds in the extract of *R. officinalis* callus cultures after 30 days of elicitation with Ag-NPs.

Phenolic compounds ( $\mu\text{g/ml}$ )	Ag-NPs (mg/L)				
	Control	1	2	3	4
<b>Caffeic acid</b>	101.7 c	108.9 c	<b>310.9 a</b>	300.7 a	232.7 b
<b>Ferulic acid</b>	105.4 c	159.3 b	133.3 bc	147.1 b	<b>203.9 a</b>
<b>Rosmarinic acid</b>	355.9 d	917.9 c	1057.7 b	<b>1423.0 a</b>	1357.3 a
<b>Carnosol</b>	728.2 c	1399.5 b	1535.6 b	1727.7 ab	<b>1920.8 a</b>
<b>Carnosic acid</b>	617.8 e	1218.1 d	1569.1 c	2031.7 b	<b>2396.1 a</b>
<b>Hesperidin</b>	98.9 e	435.0 d	508.2 c	934.0 b	<b>1666.1 a</b>
<b>Luteolin</b>	77.7 e	166.7 d	303.1 c	460.6 b	<b>856.6 a</b>
<b>Apigenin</b>	46.8 d	88.0 c	149.2 a	129.2 b	<b>154.4 a</b>
<b>Genkwanin</b>	181.8 e	358.9 d	550.8 c	746.2 b	<b>793.9 a</b>

Different letters within the same row show significant differences, using Duncan's multiple range test at 5% level.

### 3.2.2.3. The impact of different concentrations of TiO<sub>2</sub>-NPs on the accumulation of phenolic compounds

The biosynthesis of valuable secondary metabolites in elicitation calluses was significantly influenced by treatment with different doses of TiO<sub>2</sub>-NPs. Results from Table (3.11) indicated that the highest concentration of TiO<sub>2</sub>-NPs (4 mg/L) resulted in the highest content of ferulic acid, rosmarinic acid, carnosol, carnosic acid, hesperidin, luteolin, apigenin, and genkwanin, as compared to the control treatment (where the maximum content of these metabolites was 211.7, 974.7, 1565.4, 398.8, 178.2, and 538.6  $\mu\text{g/ml}$ , respectively). Caffeic acid content, on the other hand, was highest at 2 mg/L (443.2  $\mu\text{g/ml}$ ). The control treatment resulted in lower values of these metabolites.

Table 3.11. Content of phenolic compounds ( $\mu\text{g/ml}$ ) in extracts of *R. officinalis* callus cultures after 30 days of elicitation with  $\text{TiO}_2$ -NPs. Different letters within the same row show significant differences.

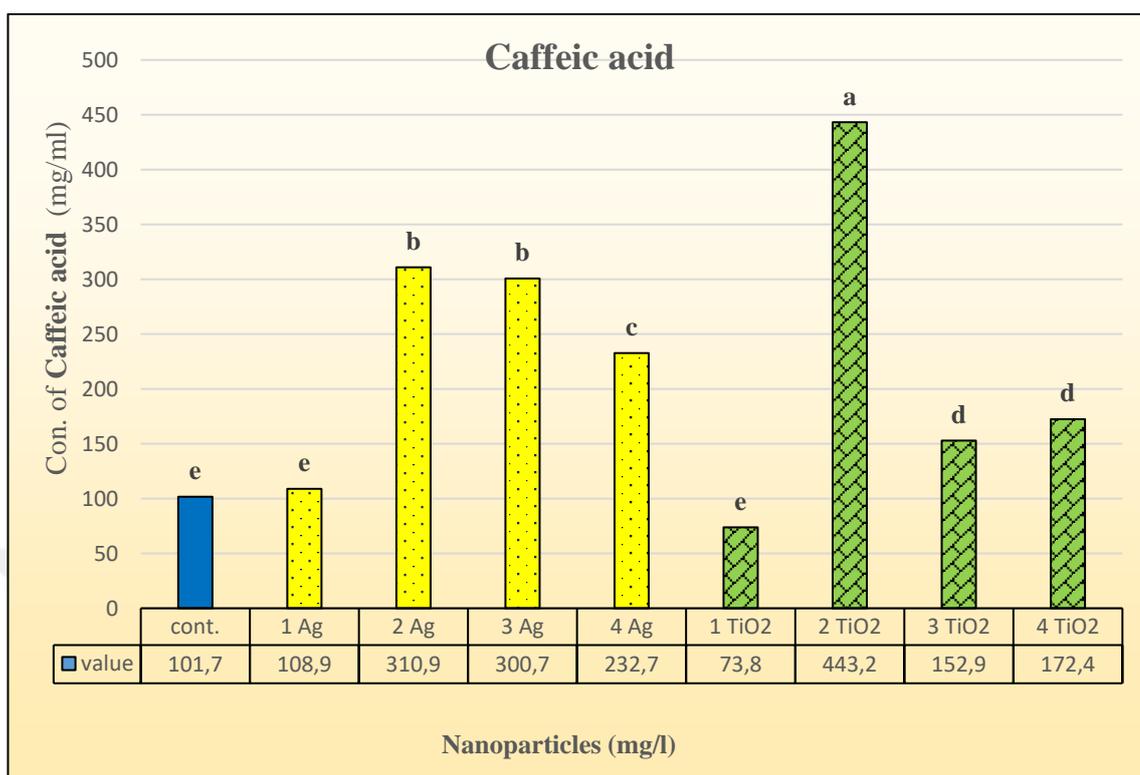
Phenolic compounds ( $\mu\text{g/ml}$ )	$\text{TiO}_2$ -NPs (mg/L)				
	Control	1	2	3	4
<b>Caffeic acid</b>	101.7 c	73.8 c	<b>443.2 a</b>	152.9 b	172.4 b
<b>Ferulic acid</b>	105.4 c	116.8 c	178.2 ab	161.0 b	<b>211.7 a</b>
<b>Rosmarinic acid</b>	355.9 e	535.5 d	634.5 c	792.3 b	<b>974.7 a</b>
<b>Carnosol</b>	728.2 d	1024.3 c	1096.1 c	1388.9 b	<b>1565.4 a</b>
<b>Carnosic acid</b>	617.8 d	646.2 d	954.7 c	1229.7 b	<b>1413.7 a</b>
<b>Hesperidin</b>	98.9 d	117.8 d	352.0 c	465.8 b	<b>656.5 a</b>
<b>Luteolin</b>	77.7 d	166.5 c	174.5 c	310.1 b	<b>398.8 a</b>
<b>Apigenin</b>	46.8 d	93.8 c	130.7 b	166.8 a	<b>178.2 a</b>
<b>Genkwanin</b>	181.8 d	292.7 c	255.9 c	384.2 b	<b>538.6 a</b>

Different letters within the same row show significant differences, using Duncan's multiple range test at 5% level.

#### 3.2.2.4. The comparison of the effects of different concentrations of Ag-NPs and $\text{TiO}_2$ -NPs on the accumulation of the examined phenolic compounds

##### 3.2.2.4.1. Effects of NPs on the accumulation of caffeic acid

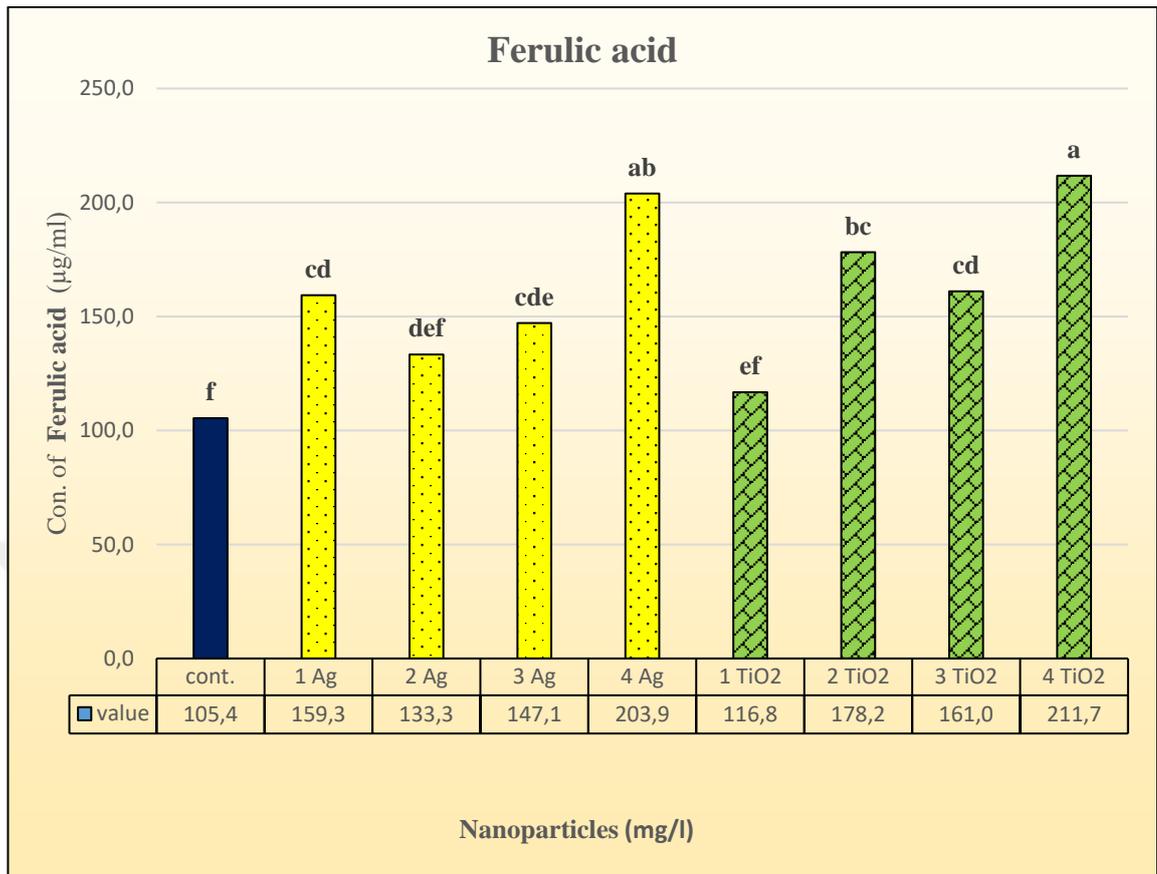
Different concentrations of nano-treatments led to a significant increase in the biosynthesis of caffeic acid. Among these, 2 mg/L  $\text{TiO}_2$ -NPs was found to be the most effective in triggering the accumulation of caffeic acid (443.2  $\mu\text{g/ml}$ ) in the callus culture, showing a significant increase compared to both the control and other treatments (as illustrated in (Figures 3.6)).



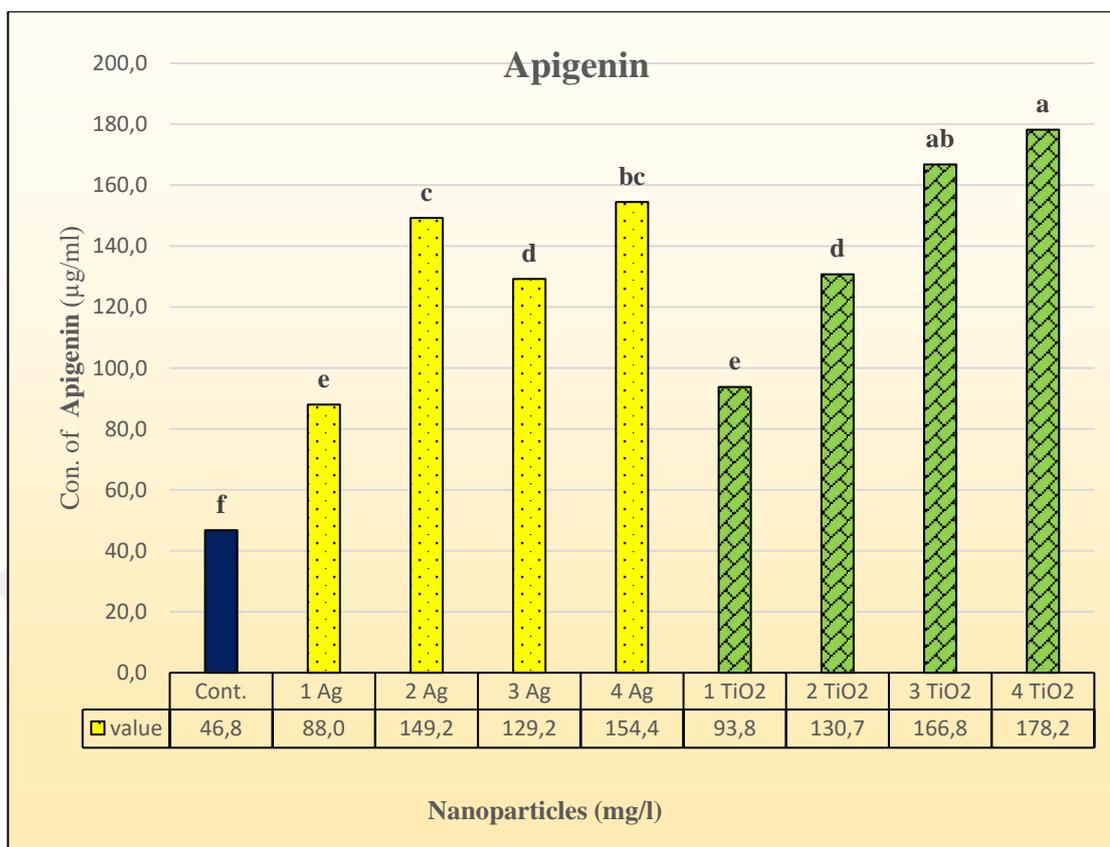
*Figure 3.6.* Effect of different concentrations of Ag-NPs and TiO<sub>2</sub>-NPs on the accumulation of **caffeic acid** compound in the *R. officinalis* callus culture. "Columns with the same letter are not significantly different, using Duncan's multiple range test at 5% level".

#### 3.2.2.4.2. Effects of NPs on the accumulation of ferulic acid and apigenin

Various concentrations of NPs were used to enhance the production of ferulic acid and apigenin compounds. The callus culture showed that the highest concentration of TiO<sub>2</sub>-NPs, which was 4 mg/L, had a big and positive effect on the biosynthesis of these compounds. The accumulation of ferulic acid and apigenin increased by 100.8% and 280.7%, respectively, compared to the control group, with the values (211.7 and 178.2 µg/ml, respectively) being significantly higher than other treatments. Figures (3.7 and 3.8) provide clear evidence of these results.



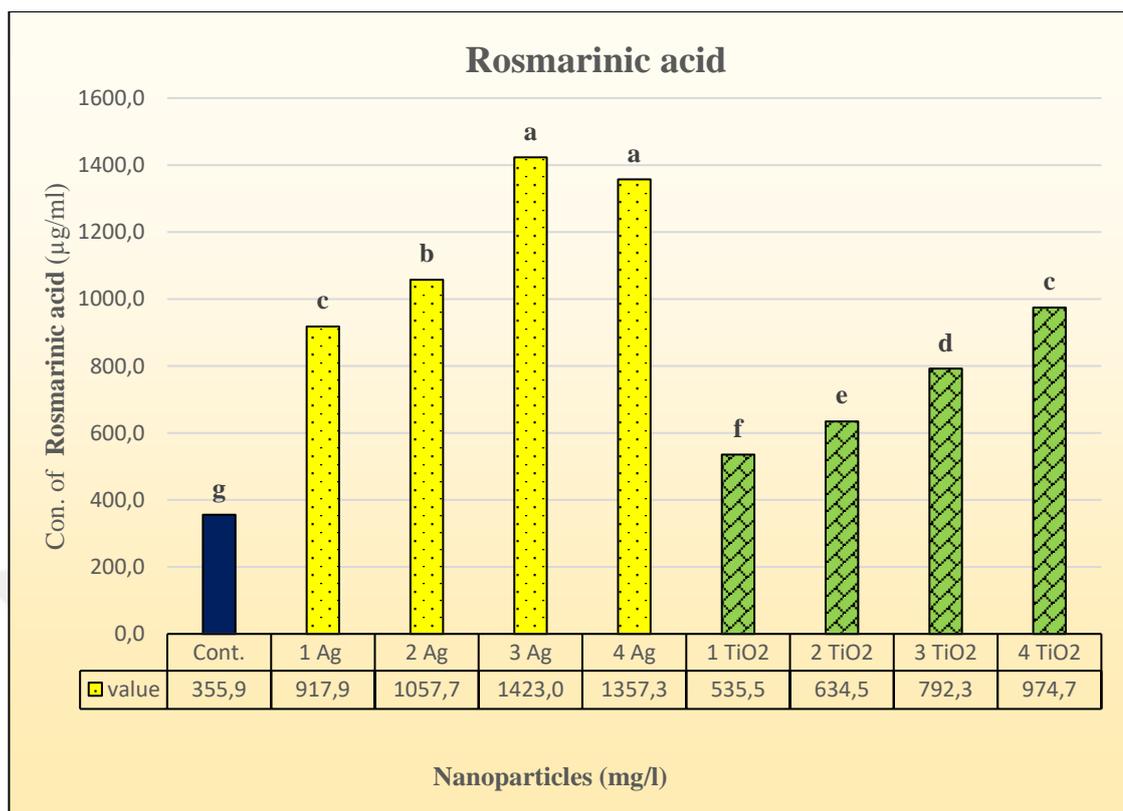
*Figure 3.7.* Effect of different concentrations of Ag-NPs and TiO<sub>2</sub>-NPs on the accumulation of **ferulic acid** in the *R. officinalis* callus culture. "Columns with the same letter are not significantly different, using Duncan's multiple range test at 5% level"



**Figure 3.8.** Effect of different concentrations of Ag-NPs and TiO<sub>2</sub>-NPs on the accumulation of **apigenin** compound in the *R. officinalis* callus culture. "Columns with the same letter are not significantly different, using Duncan's multiple range test at 5% level".

#### 3.2.2.4.3. Effects of NPs on the accumulation of rosmarinic acid

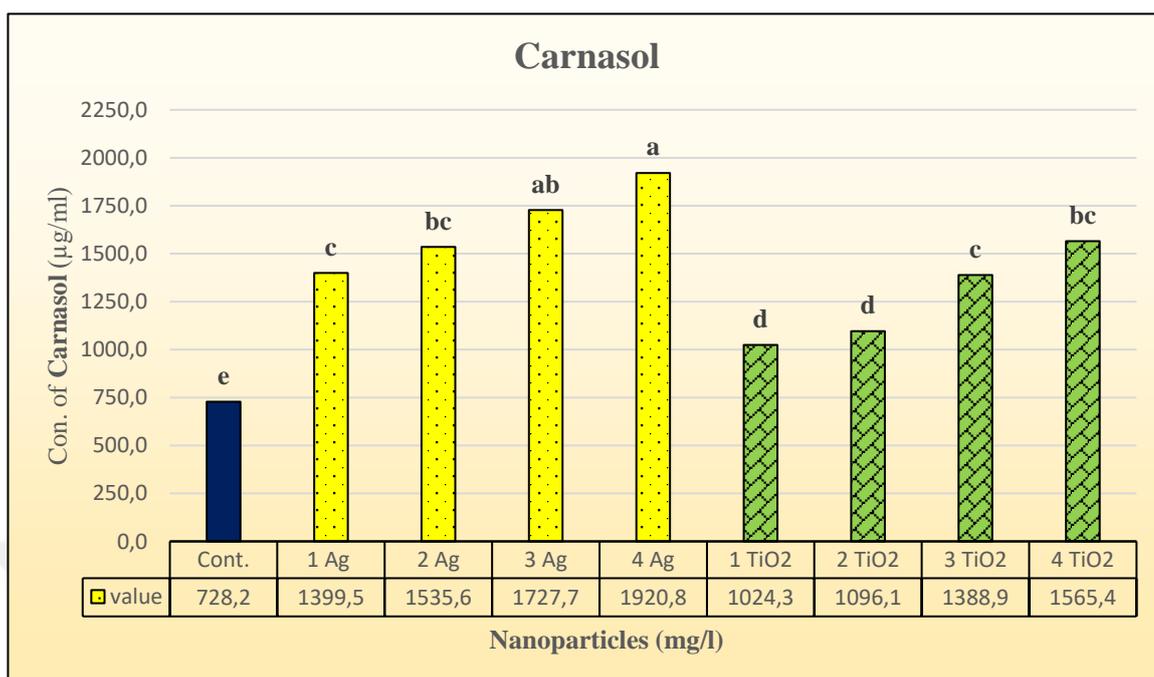
The application of various concentrations of NPs significantly improved the biosynthesis of rosmarinic acid. Among the different concentrations tested, 3 mg/L of Ag-NPs demonstrated a significant positive effect on the production of rosmarinic acid, resulting in a higher observed value of 1423.0 µg/ml compared to the control group and other treatments, as shown in (Figure 3.9).



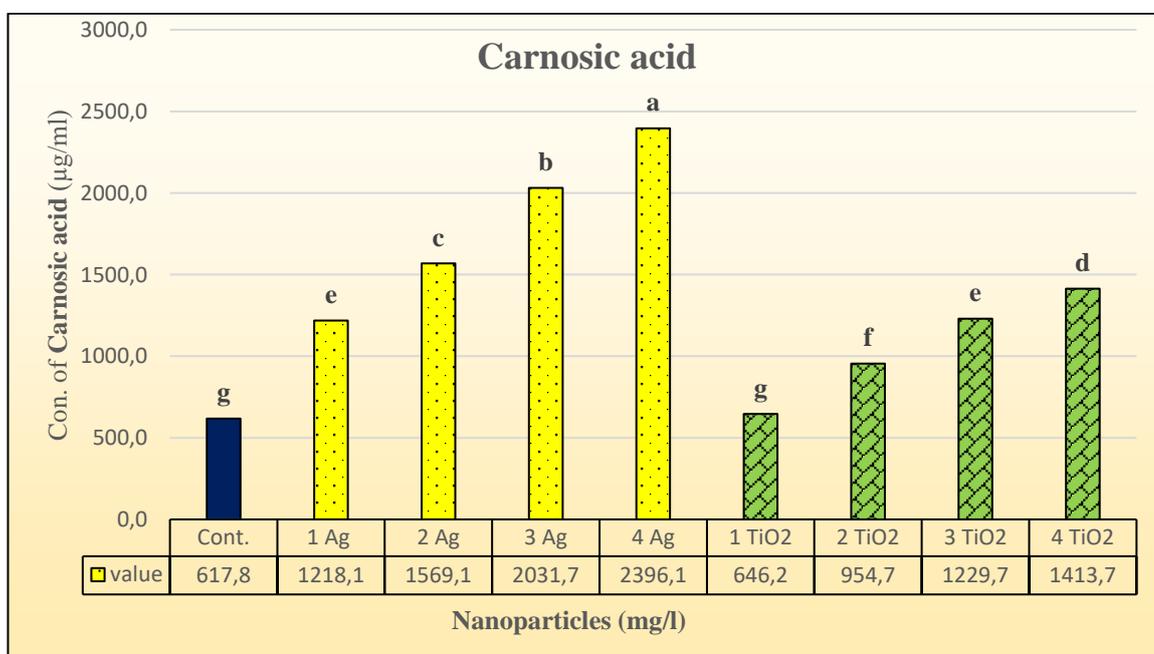
**Figure 3.9.** Effect of different concentrations of Ag-NPs and TiO<sub>2</sub>-NPs on the accumulation of **rosmarinic acid** in the *R. officinalis* callus culture. "Columns with the same letter are not significantly different, using Duncan's multiple range test at 5% level".

#### 3.2.2.4.4. Effects of NPs on the accumulation of carnosol, carnosic acid, hesperidin, luteolin, and genkwanin

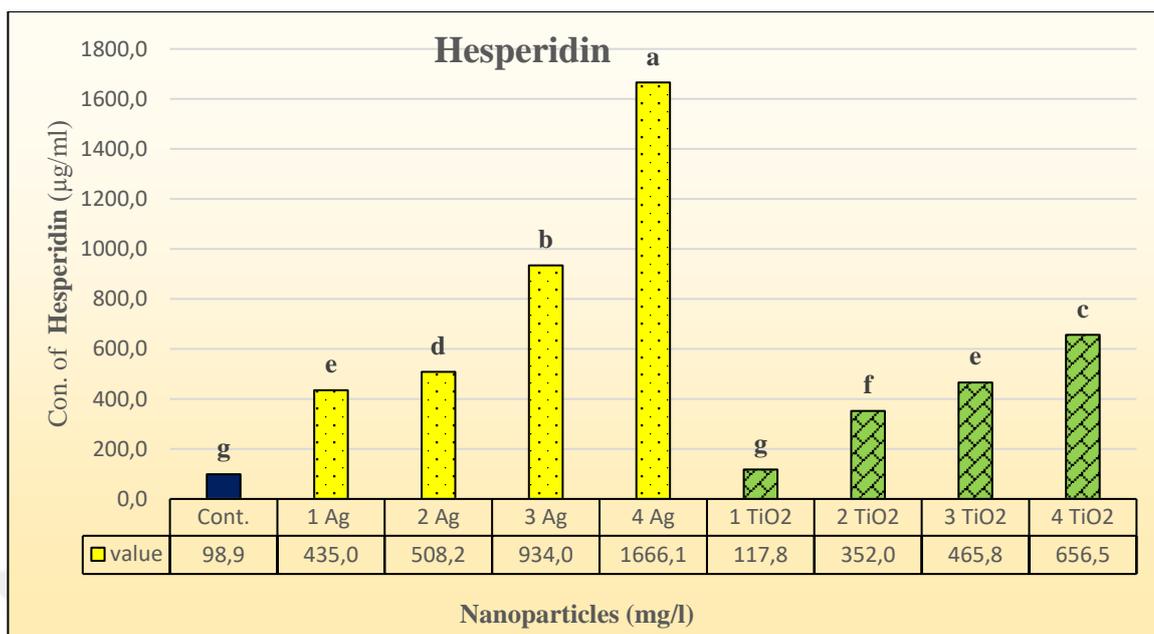
Figures (3.10, 3.11, 3.12, 3.13, and 3.14) demonstrate that all NP treatments led to a significant increase in the accumulation of the tested secondary metabolites compared to the control group. Ag-NPs at 4 mg/L had the highest impact on the biosynthesis of carnosol, carnosic acid, hesperidin, luteolin, and genkwanin, with carnosol reaching 1920 µg/ml and a 163.7% increase and carnosic acid reaching 2396.1 µg/ml with a 287.8% increase. Hesperidin increased to 1666.1 µg/ml with a 1584.6% increase, while luteolin increased to 856.6 µg/ml, indicating an increase of 1002.4%, and genkwanin showed an increase of 336.6% to reach 793.9 µg/ml.



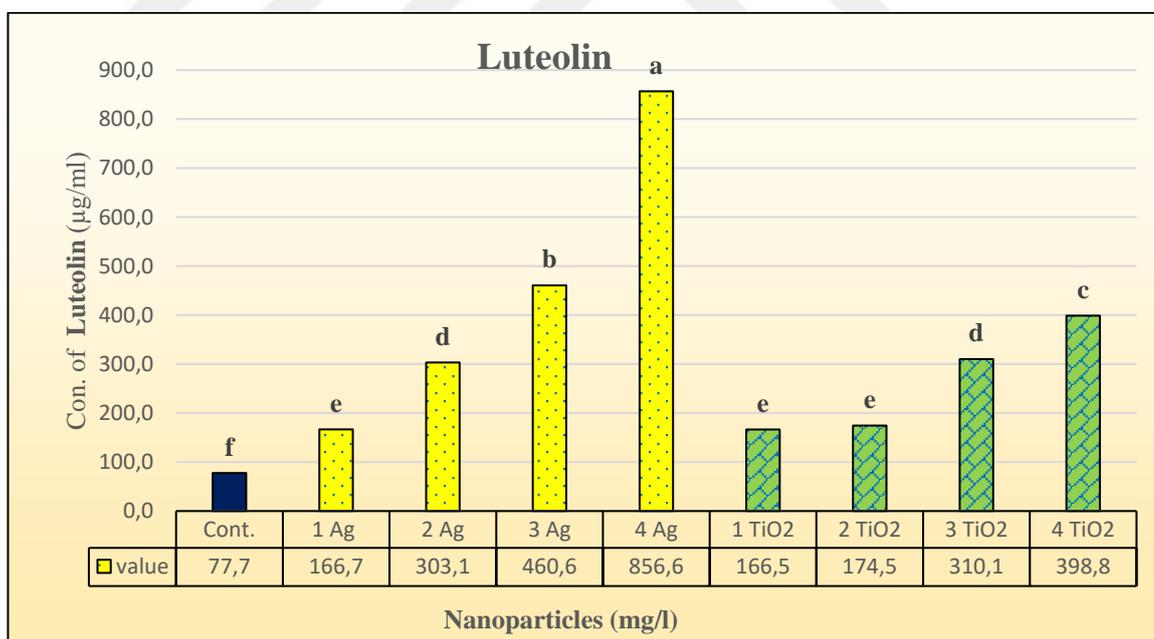
*Figure 3.10.* Effect of different concentrations of Ag-NPs and TiO<sub>2</sub>-NPs on the accumulation of **carnosol** compound in the *R. officinalis* callus culture. "Columns with the same letter are not significantly different, using Duncan's multiple range test at 5% level".



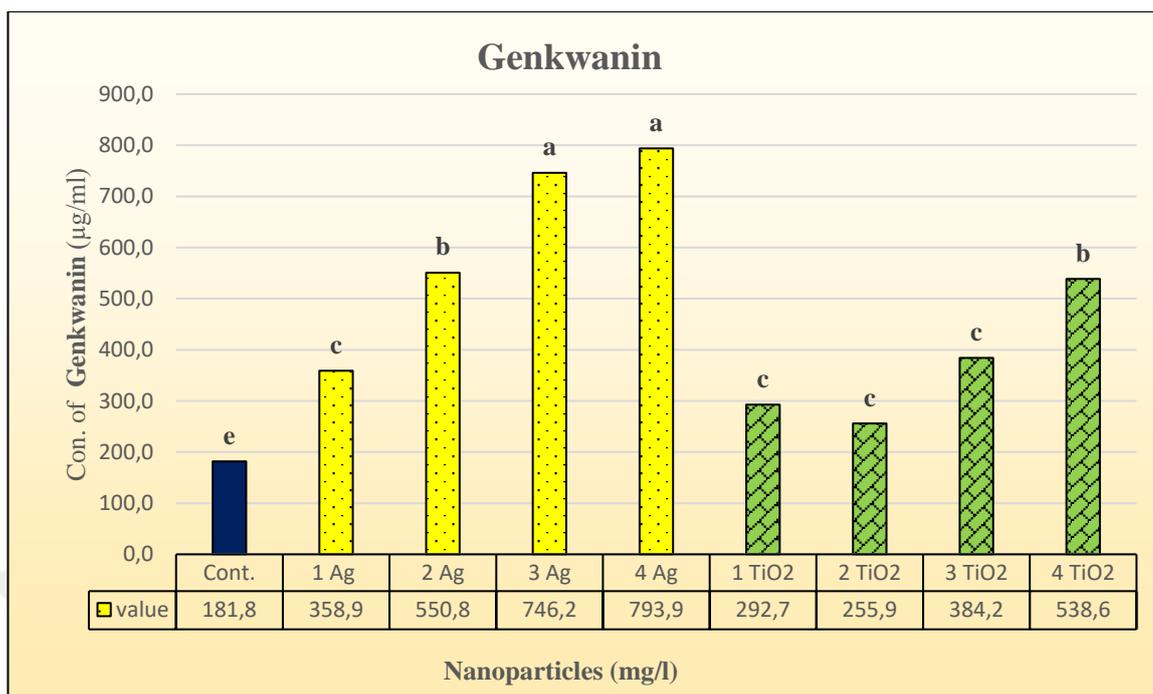
*Figure 3.11.* Effect of different concentrations of Ag-NPs and TiO<sub>2</sub>-NPs on the accumulation of **carnosic acid** compound in the *R. officinalis* callus culture. "Columns with the same letter are not significantly different, using Duncan's multiple range test at 5% level".



*Figure 3.12.* Effect of different concentrations of Ag-NPs and TiO<sub>2</sub>-NPs on the accumulation of **hesperidin** compound in the *R. officinalis* callus culture. "Columns with the same letter are not significantly different, using Duncan's multiple range test at 5% level".



*Figure 3.13.* Effect of different concentrations of Ag-NPs and TiO<sub>2</sub>-NPs on the accumulation of **luteolin** compound in the *R. officinalis* callus culture. "Columns with the same letter are not significantly different, using Duncan's multiple range test at 5% level".



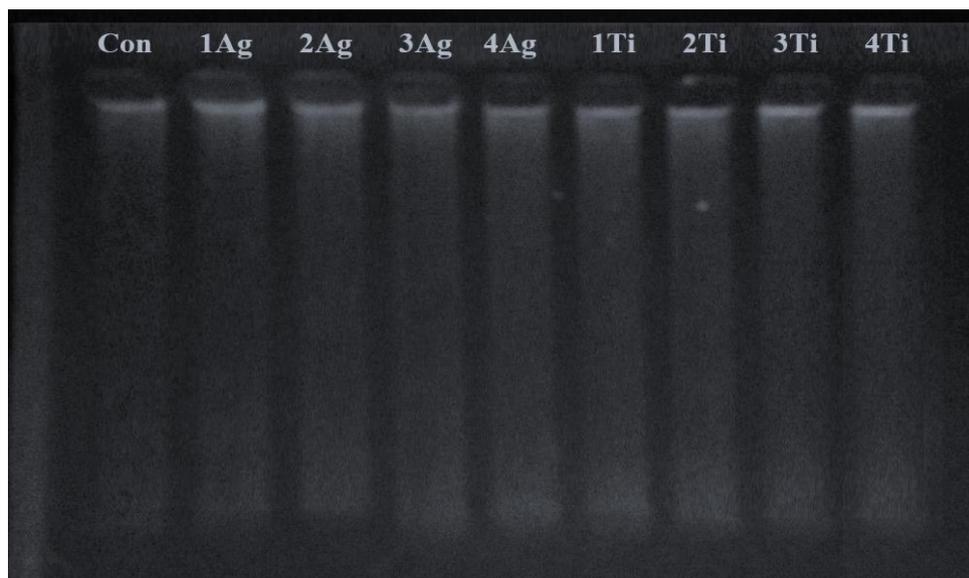
*Figure 3.14.* Effect of different concentrations of Ag-NPs and TiO<sub>2</sub>-NPs on the accumulation of **genkwainin** compound in the *R. officinalis* callus culture. "Columns with the same letter are not significantly different, using Duncan's multiple range test at 5% level".

### 3.3. The impact of different concentrations of Ag-NPs and TiO<sub>2</sub>-NPs on the genetic variation using RAPD PCR

#### 3.3.1. Extraction of DNA

The electrophoresis results demonstrated that the DNA extracted from both the control and NPs-treated calluses was of high quality and integrity. Briefly, genomic DNA concentrations ranged from 90-150 ng/µl and purification ranged from 1.67-1.85.

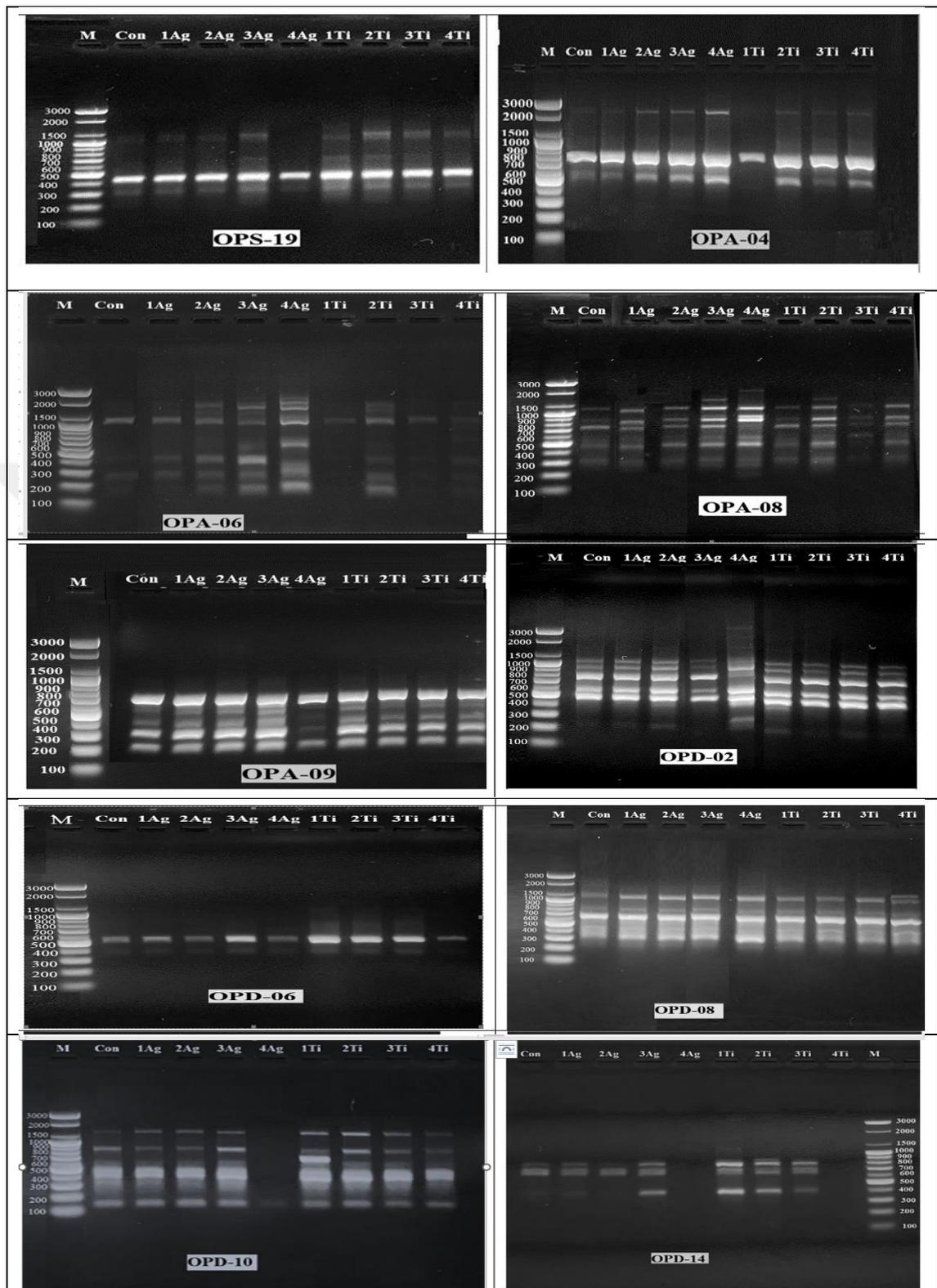
The DNA samples exhibited clear, sharp bands without any signs of degradation or smearing, indicating the absence of RNA, proteins, and other contaminants, as shown in Figure (3-15). The use of RedSafe stain allowed for the visualization and quantification of DNA fragments, which showed that the size and distribution of the fragments were consistent with intact genomic DNA. These results demonstrate the successful isolation and purification of genomic DNA from callus cultures of *R. officinalis*, which can be used for further genetic analysis and characterization.



*Figure 3. 15.* Electrophoresis separation clarifies the quality of DNA extracted from different samples of calluses (treated and non-treated with NPs), using 1% agarose gel, and RedSafe stain.

### **3.3.2. RAPD-PCR Amplification**

In this study, the RAPD-PCR technique was employed as a molecular technique that uses ten primers to determine DNA fingerprinting and identify the genetic variation in nine samples of *R. officinalis* calluses. The calluses were subjected to varying concentrations of Ag-NPs and TiO<sub>2</sub>-NPs, and their DNA was compared to the untreated control calli, as shown in (Figure 3.16).



**Figure 3.16.** PCR amplified products of nine samples of calluses using ten RAPD primers: OPS-19, OPA-4, OPA-6, OPA-8, OPA-9, OPD-2, OPD-6, OPD-8, OPD-10, and OPD-14. The samples are labeled as M= Molecular marker (100 bp), Con=control, 1Ag= 1 mg/L Ag-NPs, 2Ag= 2 mg/L Ag-NPs, 3Ag= 3 mg/L Ag-NPs, 4Ag= 4 mg/L Ag-NPs, 1Ti= 1 mg/L TiO<sub>2</sub>-NPs, 2Ti= 2 mg/L TiO<sub>2</sub>-NPs, 3Ti= 3 mg/L TiO<sub>2</sub>-NPs, and 4Ti= 4 mg/L TiO<sub>2</sub>-NPs.

### 3.3.3. Genetic Similarity

The data obtained from this study were analyzed to determine the genetic similarity (GS) and create a dendrogram. The genetic distance between different callus samples was estimated by comparing the shared amplification products of 10 primers. Using the agarose gels in Figure (3-16), the presence (1) or absence (0) of each DNA fragment for each primer was recorded, and a data matrix was constructed by evaluating RAPD profiles. The dendrogram was constructed using Jaccard's coefficient and Ward's methods, utilizing the PAST (PAleontological STatistics) computer software.

The highest genetic similarity (0.93939) was observed between 3Ag and 2Ti, while the lowest genetic similarity (0.57143) was observed between 4Ag and 1Ti (Table 3-11). These results supported by the constructed phylogenetic tree, showed different positions occupied by different nano treatments as they were analyzed separately in Figure (3-17).

Table 3.12. The genetic similarity between rosemary plant treatments stimulated with Ag-NPs and TiO<sub>2</sub>-NPs based on RAPD-PCR molecular indicators.

	Con	1Ag	2Ag	3Ag	4Ag	1Ti	2Ti	3Ti	4Ti
Con	1	0.89286	0.80645	0.8125	0.65714	0.81481	0.75758	0.89286	0.89286
1Ag		1	0.9	0.84375	0.68571	0.78571	0.84375	1	1
2Ag			1	0.9375	0.77143	0.76667	0.9375	0.9	0.9
3Ag				1	0.77778	0.71875	<b>0.93939</b>	0.84375	0.84375
4Ag					1	<b>0.57143</b>	0.72973	0.68571	0.68571
1Ti						1	0.71875	0.78571	0.78571
2Ti							1	0.84375	0.84375
3Ti								1	1
4Ti									<b>1</b>

When looking at the dendrogram in Figure (3-17), we can see that the different treatments of NPs in the callus culture were put into two main groups, A and B, and then each of those groups was split into two subgroups. Specifically, group A comprised the control, 1T, 3T, 4T, and 1Ag treatments, while group B was comprised of the 2T, 2Ag, 3Ag, and 4Ag treatments. The dendrogram further implies that, within group A, the Con treatment exhibited the greatest similarity to 1Ag, 4T, and 3T, with 1Ti following closely behind. On the other hand, within group B, 2T, and 3Ag were grouped together and showed the highest similarity to 2Ag, followed by 4Ag. As a result, it can be inferred that the 4Ag treatment was less similar to the 1T treatment.

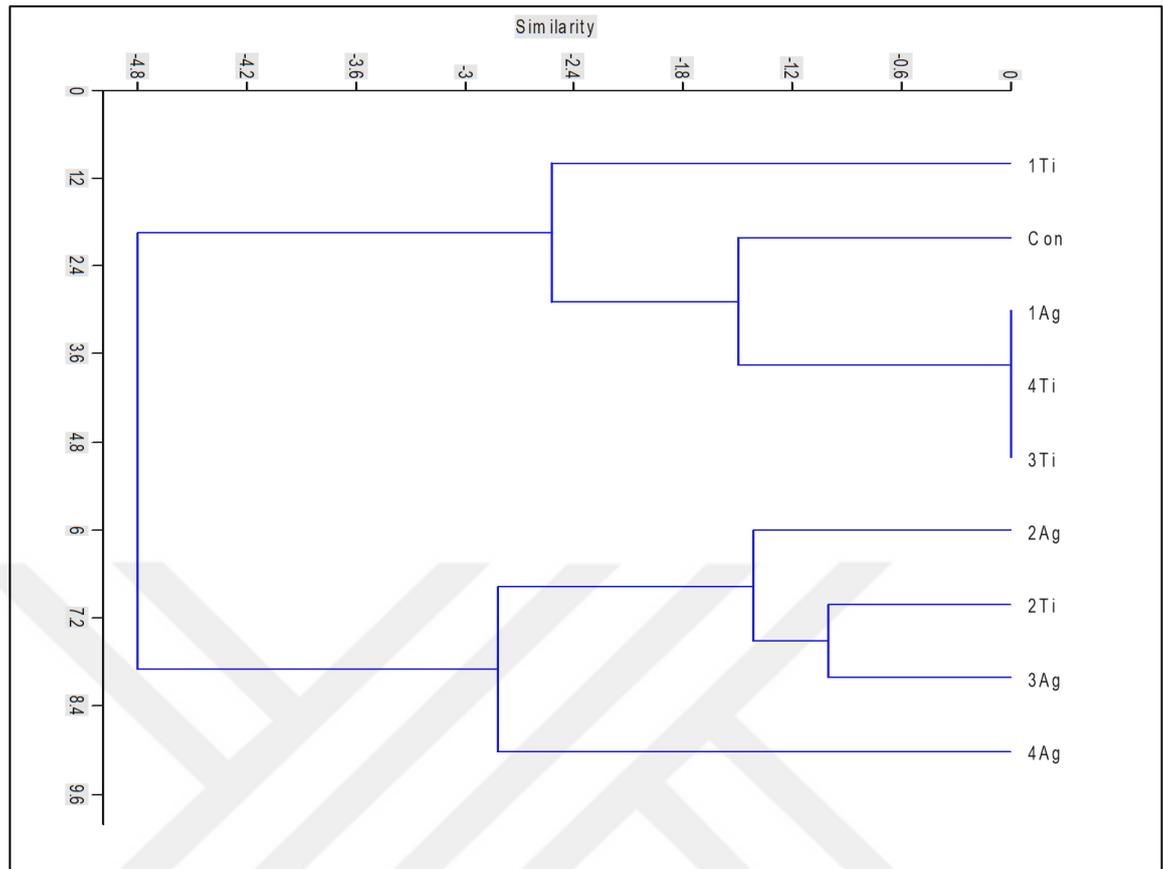


Figure 3.17. The tree diagram of the genetic relationship between stimulation coefficients with Ag-NPs and TiO<sub>2</sub>-NPs.

## CHAPTER 4

### DISCUSSION, CONCLUSION AND SUGGESTION

#### 4.1. Discussion

The study found that the specific kind and quantity of PGRs administered had an impact on the development and growth of calluses in *R. officinalis*. Various concentrations of auxin (2,4-D) resulted in a weaker callus with a brown, watery texture, whereas treatments containing only BA did not promote callus formation, but those containing TDZ did. It is noteworthy that the optimal combination of growth regulators consisted of equal amounts of 2,4-D and BA (0.5 mg/L each), which resulted in the highest fresh and dry weight of callus. Moreover, the absence of both auxin and cytokinin completely inhibited the induction of callus.

Currently, there is limited information available on the impact of plant growth regulators (PGRs) on callus formation in rosemary. However, a previous study examining the effect of a combination of BA, 2,4-D, and IAA added to MS medium on developing calluses found that the growth of *R. officinalis* was slow (Misra, 2002). Our study's results support another study's findings, which demonstrated that there was no callus formation in the treatment that lacked PGRs. The best combinations for stimulating callogenesis in *R. officinalis* were those containing auxin and cytokinin (El-Zefzafy et al., 2016).

2,4-D is a commonly utilized growth regulator for the stimulation of callus growth, as it induces cell division, promotes explant development, and facilitates callus formation and growth (Budisantoso et al., 2017). However, the concentration of 2,4-D applied and the duration of callus induction can impact the rate and texture of callus formation, and the optimal concentration of 2,4-D required for effective callus induction varies depending on the species of plant and type of tissue (Dalila et al., 2013).

The combination of 2,4-D with other plant growth regulators, such as BA, can also influence callus induction; for instance, the rate of callus induction may be greater in the

absence of BA, while the formation of compact and granular calluses may be increased with the addition of 1 mg/L of BA (Zang et al., 2016).

Boix et al. (2012) found that when 2,4-D and TDZ were used on *R. officinalis*, calluses formed. However, at concentrations of more than 0.5 mg/L, phenolic compounds could cause the calluses to turn brown because of oxidation. The development of oxidation and browning in calluses can pose a significant obstacle, and the buildup of phenolic compounds within the callus tissue is a primary contributor to this issue (Misra, 2002).

Thidiazuron (TDZ) is a synthetic plant growth regulator that has the same structure as cytokinins. It has been found to be a very effective and potent cytokinin agonist. In comparison to other cytokinins such as Kin and BA, TDZ has been shown to have greater potency. In plant tissue culture, TDZ has been used for a number of things, such as increasing the number of shoots, causing somatic embryogenesis, organogenesis, and callus formation. Nonetheless, the use of high concentrations of TDZ may lead to toxicity in plant cells and abnormal development of plant tissue cultures (Dewir et al., 2018).

The combination of auxin and cytokinin is crucial in callus induction because auxins alone can cause abnormal cell division, resulting in unorganized growth and somaclonal variation. In contrast, cytokinins alone can induce shoot formation. However, when auxin and cytokinin are combined, they act synergistically to promote cell division and differentiation, leading to the formation of organized callus tissues (Su et al., 2011).

Overall, the mechanisms of callus formation induced by auxin and cytokinin or their combinations involve the activation of genes related to cell division and differentiation, as well as the reprogramming of mature cells to a dedifferentiated state that can divide and differentiate into various cell types, including callus (Ikeuchi et al., 2013).

The variability in explant reactions can be explained by multiple factors, including the plant cells' ability to regenerate (Su et al., 2021), as well as their internal hormone levels, metabolism, and varying stages of maturity (Kaur et al., 2018); These factors collectively result in unique physiological responses that are affected by the levels of external PGRs levels (Cai et al., 2018).

As for the effect of adding NPs at different concentrations to the culture medium (maintenance medium) and using it as an abiotic stimulus in rosemary callus culture, the

results showed that both types of nanomaterials (Ag-NPs and TiO<sub>2</sub>-NPs) with all their concentrations used, showed a significant positive effect in stimulating the growth of callus mass and increase its fresh and dry weights, and also increased concentration of phenolic compounds in the callus tissue of Rosemary *in vitro* cultures.

Scientific studies have demonstrated that NPs have varying effects on callus formation. For instance, *Fagonia indica* exhibited an increase in callus weight when treated with 250 µg/mL of Iron-doped zinc oxide nanoparticles (Fe-ZnO-NPs) (A. U. Khan et al., 2021), while the use of 2 mg/L of zinc oxide nanoparticles (ZnO-NPs) or silicon dioxide nanoparticles (SiO<sub>2</sub> NPs) for 45 days increased the fresh weight of callus in *Salvadora persica* plants (Fouda et al., 2021). *In vitro* culture of *Punica granatum* showed significant increase in callus fresh and dry weight when treated with MgO-NPs at 10 mg/L (Al-Oubaidi and Al-Khafagi, 2018). Similarly, treatment with 60 mg/L of Ag-NPs resulted in a significant increase in the callus biomass of *Caralluma tuberculata* (Ali et al., 2019). Additionally, *Ocimum basilicum* culture showed the highest callus development rate and weight when treated with a medium containing 75 mg/L Al<sub>2</sub>O<sub>3</sub>-NPs (Dağlıoğlu et al., 2022).

On the other hand, it was observed that exposure to (ZnO-NPs) resulted in a decrease in the growth weights of callus cultures in *Brassica napus* (Kouhi & Lahouti, 2018). In general, the search results provided indicate that the impact of nanoparticles on callus weight relies on several factors, including the type of nanoparticle, concentration, and plant species. While certain NPs have been shown to increase callus weight, others have been found to decrease callus growth rate.

The distinctive characteristics of nanomaterials make them highly desirable for many plant culture systems due to their favorable physiological and phenotypic effects. Nanoparticles that are smaller than cell wall pore sizes can effortlessly enter the plasma membrane. By crossing the membrane using ion transport channels and/or transport proteins, nanoparticles may stick to membranes and interfere with organelles (Karimzadeh et al., 2019).

The potential benefits of Ag-NPs may be attributed to their ability to prevent ethylene signaling (Sadak, 2019). Similarly, the use of nano-anatase TiO<sub>2</sub> has been found to accelerate nitrogen metabolism in spinach (Yang et al., 2006). Additionally, Se-NPs have

been shown to alter protein phosphorylation in *Medicago interexta* plants (Selim et al., 2022).

Phenolic compounds have been identified as valuable active components present in the tissues of the rosemary plant. In this study, we quantitatively and qualitatively measured some of these compounds to compare the concentration differences between field-grown plants and calluses grown in the laboratory. Also, the investigation included studying of the effect of NPs on the accumulation of these compounds in callus cultures compared to the control group.

Our findings indicated that some compounds were present in higher amounts in field-grown plants, while others were found to have greater content in callus tissue. Moreover, the addition of nano concentrations to the culture medium increased the accumulation of phenolic substances in calluses exposed to nano stress, regardless of the concentration used.

Based on the search results, it has been found that the amounts of secondary metabolites in callus cultures and plants grown in the field can be different. Various types of secondary metabolites have different biosynthetic pathways depending on where the plant is in its growth cycle; additionally, PGRs are a key factor in cell growth, differentiation, and metabolite production (Fazili et al., 2022). The growth kinetics and accumulation of secondary metabolites in callus cultures are distinct from those observed in field-grown plants (Pan et al., 2020). The optimal concentration of the culture medium is a significant factor in controlling callus growth and metabolite synthesis (Rattan et al., 2021).

The impact of NPs on the synthesis of secondary compounds has been demonstrated to have a significant effect, consistent with prior research. For example, studies have shown that using Ag-NPs as plant stressors can increase the production of many secondary metabolites, such as carnolic acid and the total phenolic and flavonoid content in *R. officinalis* leaves (Soltanabad et al., 2019).

Ali et al. (2019) found that Ag-NPs caused an increase in the amount of phenylalanine ammonia lyase, total flavonoid, and total phenolic in the *Caralluma tuberculata* callus culture. Another study found that treating *Cucumis anguria* root cultures with 0.5-2 mg/L Ag-NPs increased the production of gallic acid, salicylic acid, caffeic acid, coumaric acids, ferulic acid, hesperidin, and other phenolic compounds (Chung et al., 2018).

TiO<sub>2</sub>-NPs were also found to increase the production of secondary metabolites in *Hypericum perforatum* callus cultures (Ebadollahi et al., 2019), and Hua et al. (2020), found that low doses of TiO<sub>2</sub>-NPs increased the storage of nutrients in *Coriandrum sativum*.

Recent research suggests that NPs may act as "elicitors" for secondary metabolite formation in plants by activating several cellular signal transduction pathways, such as ROS metabolism, Ca<sup>2+</sup> flux, and mitogen-activated protein kinases (Gohari et al., 2020). Vats (2018) say that when plants are stimulated, they make enzymes like SOD, CAT, POD, and APX that get rid of dangerous free radicals; this leads to the formation of different secondary metabolites, which give the plant a strong defense system and help the cells grow and develop normally.

The present study demonstrated that the application of NPs resulted in genetic variations in *R. officinalis* callus cultures at the DNA level, as evidenced by the use of ten primers in the RAPD-PCR technique.

According to the findings, the impact of NPs on genetic material was dose and type dependent, resulting in genetic modifications that resemble either DNA damage-induced mutations or rearrangements caused by NPs. This is in line with previous research that has shown that NPs can cause genetic diversity in plants in different ways. One of the mechanisms that has been studied the most is oxidative stress, in which NPs cause ROS to be made in plant cells, which damages DNA and can cause mutations by creating new variants in the DNA sequence of plants (Fouda et al., 2021).

Another mechanism by which NPs induce genomic variation is through the impairment of mitosis, alteration of DNA by inducing chromosomal anomalies, or by affecting DNA repair mechanisms (Karami Mehrian & De Lima, 2016). Furthermore, NPs can induce genetic diversity in plants through epigenetic modifications, where they alter DNA methylation, histone modification, and small RNA expression in plant cells. These changes can affect gene expression and phenotypic traits, leading to genetic diversity (Pogribna & Hammons, 2021).

## 4.2. Conclusion

The study highlights the significant impact of growth regulators and nanomaterials on the growth and accumulation of phenolic compounds in callus cultures of *R. officinalis*.

1. The specific type and quantity of growth regulators administered affect the development and growth of callus in *R. officinalis*.
2. The optimal combination of growth regulators consisted of equal amounts of 2,4-D and BA.
3. Both Ag-NPs and TiO<sub>2</sub>-NPs at various concentrations stimulated the growth of callus mass and increased the concentration of phenolic compounds in callus cultures.
4. The addition of NPs resulted in genetic variations in callus cultures, and the impact was dependent on the type and dose of NPs.
5. The findings provide useful insights for improving *in vitro* culture and phenolic compound production in *R. officinalis*.

Based on the findings, the following recommendations can be made:

1. The optimal combination of 2,4-D and BA can be used to enhance callus growth in *R. officinalis*.
2. The use of Ag-NPs and TiO<sub>2</sub>-NPs at appropriate concentrations can be a useful strategy to stimulate callus growth and increase phenolic compound production.
3. Careful consideration should be given to the type and dose of NPs used in callus cultures, as they can result in genetic variations.
4. The results of this study can be utilized to optimize *in vitro* culture methods and enhance phenolic compound production in *R. officinalis* for various applications.

## 4.3. Suggestions

Based on the findings of the study, the following suggestions can be made:

1. Further research should be conducted to investigate the molecular mechanisms underlying the genetic variations caused by NPs in callus cultures of *R. officinalis*.
2. The effect of other types of NPs on callus growth and phenolic compound production should be explored to determine their potential applications in the *in vitro* culture of *R. officinalis*.
3. The effect of different environmental conditions on callus growth and phenolic compound production should be studied to optimize the *in vitro* culture process.
4. The findings of this study can be extended to other plant species to investigate their potential applications in enhancing callus growth and phenolic compound production.

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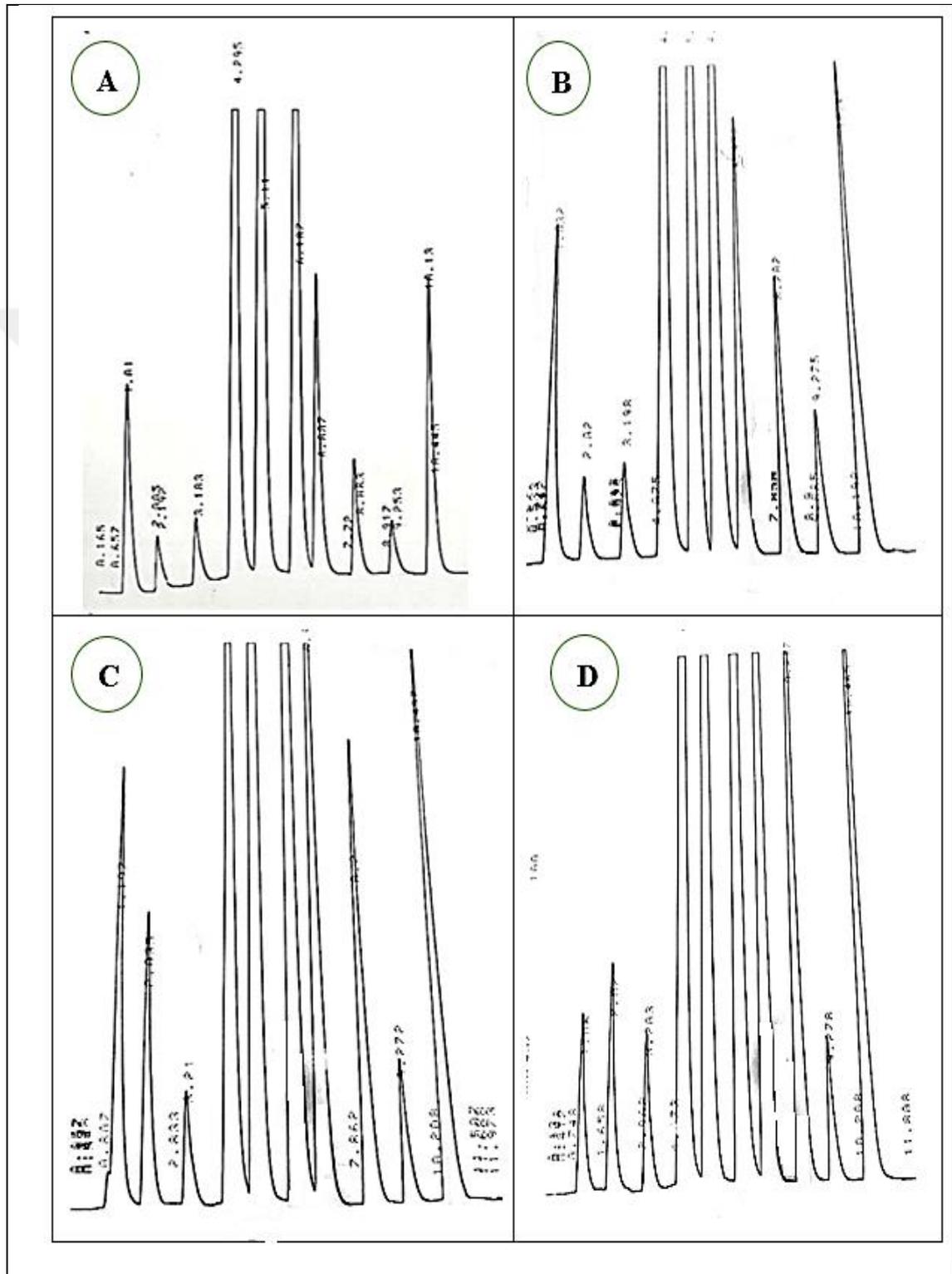
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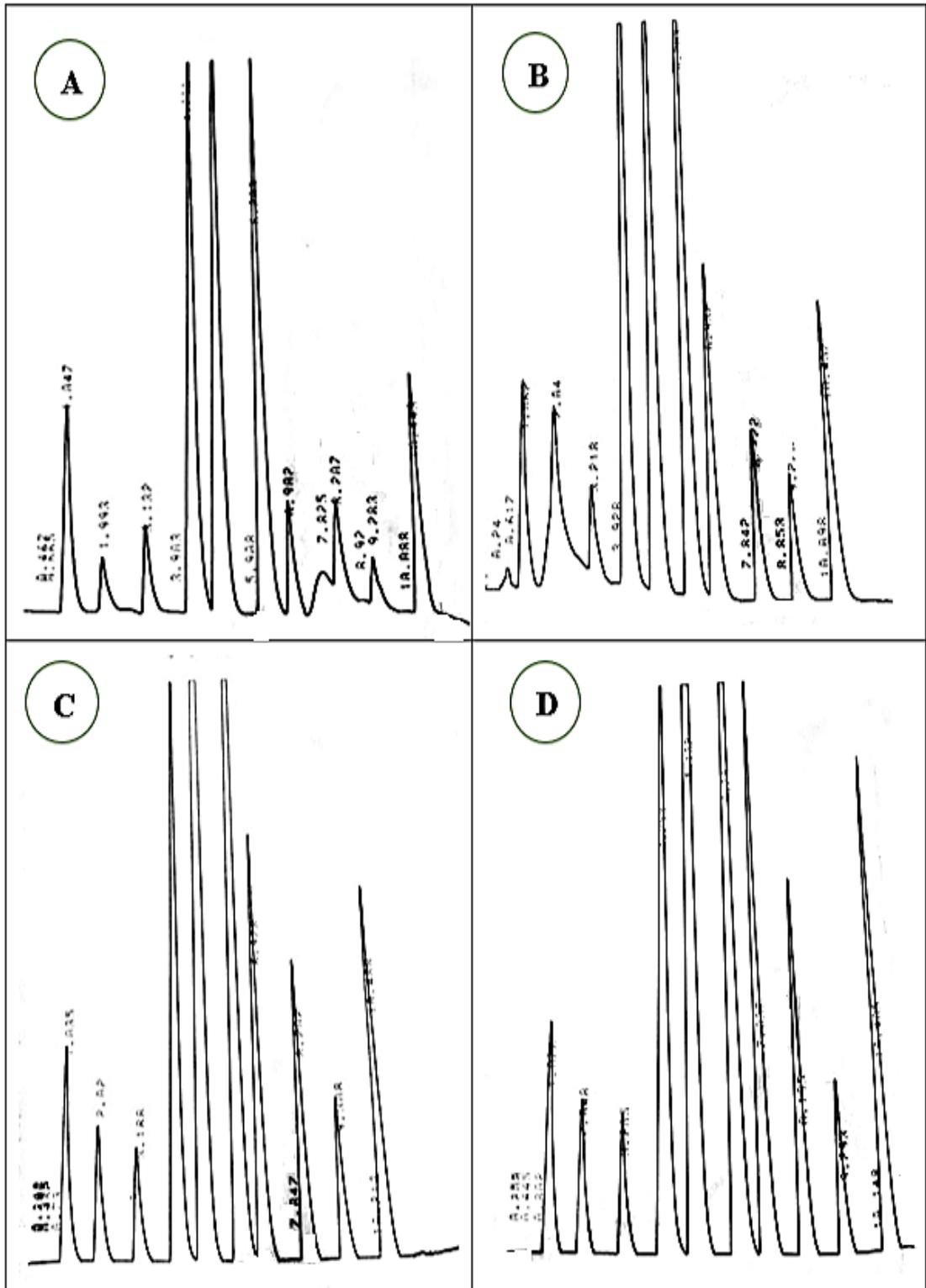
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## APPENDICES



**Appendix (1):** HPLC histogram of Ag-NPs treatments: (A) 1 mg/l, (B) 2 mg/l, (C) 3 mg/l, and (D) 4 mg/l.



**Appendix 2:** HPLC histogram of  $\text{TiO}_2\text{-NPs}$  treatments: (A) 1 mg/l, (B) 2 mg/l, (C) 3 mg/l, and (D) 4 mg/l.

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