**T.R.**
**USKUDAR UNIVERSITY**
**INSTITUTE OF SCIENCE**

**DEPARTMENT OF MOLECULAR BIOLOGY**
**MASTER'S DEGREE PROGRAM OF MOLECULAR BIOLOGY**
**MASTER'S DEGREE THESIS**

# DETERMINATION OF THE SECONDARY METABOLITE CONTENT IN *Pimpinella anisum* L. (ANISE) CALLUS ELICITED BY GREEN SYNTHESIZED SILVER NANOPARTICLES

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**Thesis Advisor**
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# ÖZET

## YEŞİL SENTEZ YOLU İLE ELDE EDİLEN GÜMÜŞ NANOPARTİKÜLLERİN *Pimpinella anisum* L. (ANASON) KALLUS KÜLTÜRLERİNDE SEKONDER METABOLİT İÇERİĞİNE ETKİSİNİN BELİRLENMESİ

Nanopartikül-bitki etkileşimleri, özellikle farklı konsantrasyonlardaki nanopartikül uygulamalarının bitkiler üzerindeki büyüme, gelişme, fizyolojik ve moleküler etkileri günümüzde dikkat çeken bir araştırma konusudur. Bu çalışmada, *Salvia sclarea* yapraklarından yeşil sentez yoluyla elde edilen gümüş nanopartiküllerin (AgNp) farklı konsantrasyonlarda (0, 1.0, 5.0, 10 ve 20 mg/L) *P. anisum* kallus kültürlerinde kallus morfolojisi ve bitki sekonder metabolit (PSM) içeriği üzerindeki etkileri gözlemlenmiştir. Tüm ortamlarda kallus çapında zamana bağlı bir artış gözlenirken, en yüksek değerler kontrol grubunda gözlenmiştir. Kallus ağırlığı açısından uygulamalar arasında önemli bir fark bulunmamış ancak genel olarak 7. günden sonra tüm gruplarda azalma olduğu tespit edilmiştir. Maserasyonla elde edilen etanol hekzan ekstraktlarının GC-MS analizi, palmitik asit ve linolenik asit gibi yağ asitleri ile vanilil alkol ve 4-hidroksibenzoik asit gibi fenolik içeriklerinde önemli bir artış olduğunu göstermiştir. Elisitör olarak kullanılan sağlıklı bir yaklaşım olan yeşil sentez ile sentezlenen AgNP'lerin tıbbi ve ticari açıdan önemli PSM'lerin içeriğinde artışa neden olması potansiyel kullanımlarına ışık tutmaktadır.

**Anahtar kelimeler:** GC-MS analizi, Yeşil sentez, Maserasyon, Pimpinella anisum, Gümüş nanopartiküller

# ABSTRACT


## DETERMINATION OF THE SECONDARY METABOLITE CONTENT IN *Pimpinella anisum* L. (ANISE) CALLUS ELICITED BY GREEN SYNTHESIZED SILVER NANOPARTICLES

Nanoparticle-plant interactions are a research topic that attracts attention today, especially the growth, development, physiological, and molecular effects of nanoparticle applications at different concentrations on plants. In this study, the effects of silver nanoparticles (AgNp) obtained by green synthesis from *Salvia sclarea* leaves at different concentrations (0, 1.0, 5.0, 10, and 20 mg/L) on callus morphology and plant secondary metabolite (PSM) content in *P. anisum* callus cultures were observed. A time-dependent increase in callus diameter was observed in all the media, with the highest values observed in the control group. In terms of callus weight, there were no significant differences between the treatments, but in general, it was determined that there was a decrease in all groups after the 7th day. GC-MS analysis of ethanol hexane extracts by maceration showed a significant increase in fatty acids such as palmitic acid and linolenic acid content and phenolic contents such as vanillyl alcohol and 4-hydroxybenzoic acid. The fact that AgNPs synthesized by green synthesis, which is a healthy approach used as an elicitor, causes an increase in the content of medically important and commercially important PSMs, sheds light on their potential use.



**Keywords:** GC-MS analysis, Green synthesis, Maceration, *Pimpinella anisum*, Silver nanoparticles

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Hoping for a day when we live in a world of peace, justice, love, and freedom for all. A world where the pure innocence of individuals, regardless of their race, language, nationality, religion, or socioeconomic status, is preserved, cherished, and protected from the devastation of war and conflict.

**Ayşenur BOZKURT**

## DECLARATION

Herewith, I declare that I obtained all the information and documents in this study within the framework of academic rules, presented all visual, auditory, and written information and results by scientific ethics, did not falsify the data I used, referred to the sources I used by scientific norms, that my thesis was original except in the cases cited, produced by me and written under the Thesis Writing Guide of Uskudar University Institute of Science.

**12/06/2024**

**Ayşenur BOZKURT**

**Signature**

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## SYMBOLS / ABBREVIATIONS

| | |
|---|---|
| **µl** | Microliter |
| **2,4 D** | 2,4-Dichlorophenoxyacetic acid |
| **Ag** | Silver |
| **$AgNO_3$** | Silver Nitrate |
| **AgNP** | Silver Nanoparticle |
| **BAP** | 6-Benzylaminopurine |
| **CAT** | Catalase |
| **CSC** | Cell Suspension Culture |
| **g** | Gram |
| **MEP** | Methylerythritol-4-phosphate |
| **ml** | Milliliter |
| **mM AgNO3** | Millimolar |
| **MVA** | Mevalonate Pathway |
| **nm** | Nanometer |
| **NP** | Nanoparticle |
| ***P .anisum*** | *Pimpinella anisum* L. |
| **POD** | Peroxidase |
| **PPM** | Plant Primary Metabolite |
| **PSM** | Plant Secondary Metabolite |
| **PTC** | Plant Tissue Culture |
| **ROS** | Reactive Oxygen Species |
| ***S. sclarea*** | *Salvia sclarea* |
| **SM** | Secondary Metabolite |
| **SOD** | Superoxide dismutase |
| **SPR** | Surface plasmon resonance |
| **TMS** | Trimethylsilyl |
| **α** | Alpha |

# 1. INTRODUCTION

*Pimpinella anisum* L. (anise) fruits, also commercially called seeds, are very rich in trans-anethole content and it is what gives the aroma of the plant. Due to its content, it has been proven to help with gynecological diseases, gastrointestinal problems, and milk increase in women, and has become a natural alternative to the drugs used in diseases (Mahboubi & Mahboubi, 2021). In addition to these benefits, it has carminative and expectorant effects, and its antibacterial activities have been observed to have a lethal effect on parasites (Andallu & Rajeshwari, 2011). Anise essential oil is valuable in the perfume and pharmaceutical industries, and Türkiye is one of the important anise producers (N. Arslan et al., n.d.). *P. anisum* secondary metabolites (SMs) (phenolic compounds), which have prominent antioxidant and antimicrobial properties, are found in all parts of the plant but are most abundant in the seeds. *P. anisum* SMs (phenolic compounds), which have prominent antioxidant and antimicrobial properties, are found in all parts of the plant but are most abundant in the seeds. Naringin and p-coumaric are examples of phenolic compounds, anethole and limonene are examples of volatile compounds. SM contents vary according to geographical region, harvest time, and culture conditions. Anise is also rich in primary metabolites and contains important fatty acids such as palmitic, oleic, and linoleic (Spinozzi et al., 2023). The production of secondary metabolites, which have significant antioxidant and antimicrobial effects, can be achieved much more quickly and efficiently by using nanoparticles (NPs). The use of NPs as elicitors activates the plant defense mechanism through ROS activation within the plant cell and initiates the production of phytochemicals, including secondary metabolites (Chung et al., 2018). Although there are many chemical and physical synthesis methods of NPs, nanometals pollute the environment, and chemically synthesized NPs are costly in terms of production, require a lot of energy, and have the potential to pose a health hazard. In the green synthesis method, synthetic harmful chemicals are not used; plant extracts fulfill that task, and NP production thus has an environmentally friendly, sustainable alternative (Ying et al., 2022). Silver nanoparticles (AgNPs) are widely used as plant growth stimulants and secondary metabolite elicitors (Mahajan et al., 2022). In the studies conducted with AgNP in the literature, negative effects on plant development and root abscission were observed, while positive effects included an inhibitory effect on germination and shoot length, increased production of specific antioxidative enzymes, and increases in fresh biomass. It has been observed that AgNP can cause toxicity in plants

depending on concentration, and appropriate concentrations have been investigated and continue to be investigated in order to positively affect plant development and synthesize important compounds such as the desired PSMs (Fazal et al., 2016).

This study aimed to investigate the effects of AgNPs synthesized from *Salvia sclarea* (*S.sclarea*) leaves by the green synthesis method on *P. anisum* secondary metabolites and their impact on the development of *P. anisum* callus. Callus will be formed from anise seeds by plant tissue culture methods, and their diameters and weights will be measured at 7, 14, 28, and 35 days. The effect of AgNP on callus development will be analyzed. The callus will be lyophilized on the 35th day, and dried powdered *P.anisum* callus will be macerated with hexane and ethanol solutions. The hexane and ethanol extracts obtained by maceration will be derivatized. After derivatization, GC-MS analyses of the extracts will be performed, and changes in secondary metabolite content will be analyzed. This study, which will be carried out for the first time, will allow learning what kind of changes the AgNPs produced by green synthesis will cause in the chemical compounds and callus growth of aniseed plants in response to stress.

## 2. GENERAL INFORMATION

### 2.1 *Pimpinella anisum* L.

*Pimpinella anisum* L. (anise) belongs to the Umbelliferae (*Apiaceae*) family, which contains 434 genera and 3780 species. These are annual, biennial, or perennial herbaceous plants, most of which carry essential oils. The aromatic plants used in cosmetics and food industries' growing areas go from the northern temperate regions to the high altitudes of tropical regions (Giordano et al., 2022). The Apiaceae family's plants have characteristic flavors because of the oil and resin embedded in all plants, from roots to fruits. These plants are also rich in both secondary metabolites and phytochemicals (coumarins, triterpenoid saponins, flavonoids, terpenoids, steroids, and polyacetylenes) used in various industries and fields, including industrial products, pharmaceuticals, cosmetics, nutrition, drinks, and spices. Furthermore, they are widely utilized as conventional treatments for various illnesses, including respiratory, gastrointestinal, and reproductive conditions (Giordano et al., 2022; Spinozzi et al., 2021). *P. anisum*, part of the *Pimpinella* genus containing 180 species, is an annual herbaceous plant with white flowers that grows in Iran, Egypt, Türkiye, India, and other regions of the world that have warm environments.

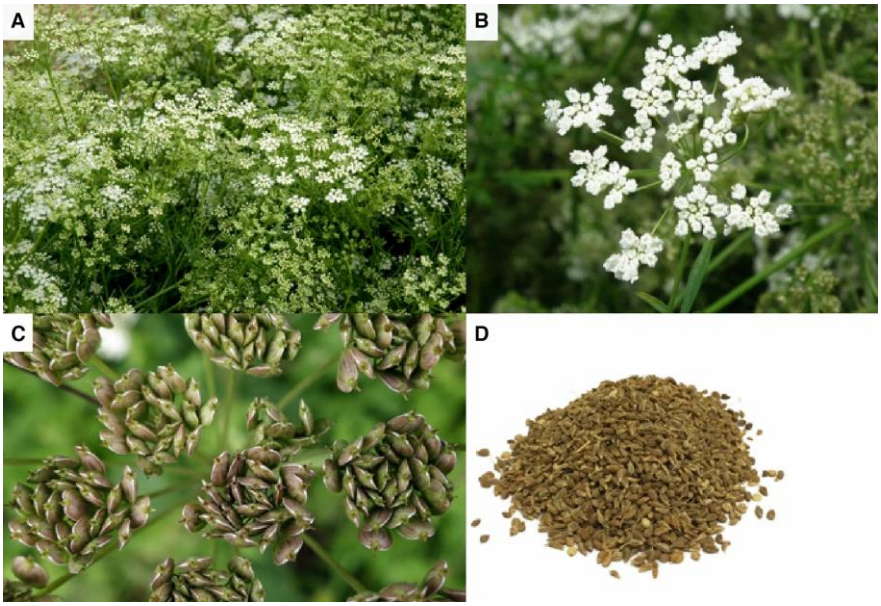


**Figure 1.** *Pimpinella anisum* (A), flower (B), and its' seeds (C and D) (Soussi et al., 2023)**.**

The Türkiye *Pimpinella* genus has 26 genera along with 8 endemic genera. The plant's height is around 30-50 cm. Its flowers are umbrella-shaped. Its fruits are 3-6 mm long and 1-3 mm wide. They are inverted pear-shaped, with short stems. They are gray-green or greenish-yellow in color and hairy on top (Betül Avcı et al., 2023; Gülçin et al., 2003; Shojaii & Abdollahi Fard, 2012).

A World Health Organization report states that there are 21.000 different plant species utilized worldwide for medicinal purposes. There are four thousand commonly used herbal remedies. However, only two thousand plants are commercialized as herbal drugs in Europe. 350 subspecies make up the total number of species that Türkiye imports and exports; 150 species are used in international trade. *P. anisum,* which has been used historically as a spice and medicine, is one of the cultivated aromatic and medicinal plants, and it holds a significant position in terms of usage and export share (Boztaş & Bayram, 2020). Anise has enjoyed widespread popularity across many regions due to its varied applications, which include flavoring, culinary, domestic, cosmetic, and medicinal uses. While the entire plant possesses a pleasant fragrance, it is the fruit, commonly known as aniseed, that has been highly esteemed since ancient times. Aniseed is among the oldest spices widely employed to flavor curries, bread, soups, sweets, dried figs, desserts, cream cheese, pickles, coleslaw, and egg dishes. It is also a preferred flavoring for alcoholic beverages in the Mediterranean region, including French Pastis, Pernod, Anisette, and Ricard, Greek Ouzo, Turkish Raki, Arabian Arak, South American Aguardiente, Russian Allasch, and Puerto Rican Tres Castillos. The essential oil derived from aniseed holds significant value in the perfume industry, dental care products as an antiseptic in toothpaste, mouthwashes, soaps, detergents, lotions, and skin creams, and in tobacco manufacturing, with a maximum use level of 0.25% oil in perfumes. Additionally, it is utilized to mask undesirable odors in drug and cosmetic products (Peter, 2001).

The flavoring ingredient of *P. anisum* is anethole ($C_{10}H_{12}O$), which is present in both the seeds and the leaves of the anise plant. It also shows inhibitory effects on chronic diseases. Similarly, *P. anisum* essential oil has a high concentration of terpenes ($C_5H_8$), flavonoids, and polyphenols that have been shown to have therapeutic effects against inflammatory, diabetic, and gastrointestinal disorders. Furthermore, certain leaf and seed extracts have shown strong inhibitory actions against viruses, fungi, and bacteria (Soussi et al., 2023). *P. anisum* has been reported to have a variety of therapeutic benefits on a

range of illnesses, including respiratory, gynecologic, neurological, and digestive issues. It has been observed that this plant's aqueous extract can postpone the start of seizures in mice that are brought on by picrotoxin. The anesthetic, hypothermic, muscle relaxant, and anticonvulsant properties of eugenol and estragole are well documented. Additionally, anethole has a relaxing effect on muscles (Gülçin et al., 2003).

Anise seeds have a volatile oil primarily composed of trans-anethole, constituting 1.5–6.0% of its mass. Additionally, these seeds contain lipids abundant in fatty acids like palmitic and oleic acids, comprising 8–11% of their mass. Furthermore, anise seeds consist of approximately 4% carbohydrates and 18% protein (Besharati-Seidani et al., 2005). The major compounds of Anise seeds are estragole, anethole, pseudoisoeugenol, coumarins, eugenol, scopoletin, estriol, methyl chavicol, umbelliferon, terpene hydrocarbons, anisaldehyde, polyacetylenes, and polyenes (Gülçin et al., 2003).

## 2.2 Nanoparticles

For centuries, nanostructures and nanoparticles (NPs) have been used for various purposes worldwide. Examples of this are that Mollusks use compact nanostructures of calcium carbonate to form a solid outer shell, and the sharp and strong damask steel sword used in the Middle East between 300 AD and 1700 AD contains carbon nanotubes and cementite nanowires. With the development of technology, nanostructures have been observed to exist with high-resolution transmission electron microscopy.

The starting point of today's research on NPs started with Sir Richard Feynman. Feynman's speech titled "There’s Plenty of Room at the Bottom" at the American Physics Society meeting at the California Institute of Technology on December 29, and his ideas in the article he published in 1960 inspired many people. In his article, Feynman attracted the attention of the Prediction Institute by talking about the endless possibilities that could arise if we could manipulate matter at the molecular level and the creation of small nano-scale "molecular machines" if we could carefully build complex molecules atom by atom, as a result of this in 1993 the Institute started the "Feynman Prize in Nanotechnology" to encourage young researchers to pursue this vision and still does. Thanks to these beginnings and the work of many scientists from the past to present, many historical innovations have occurred, and nanotechnology has reached its current state (Pande & Bhaskarwar, 2016). Nanotechnology has begun to attract attention and be used in many

areas with the advancement of technology. As a result, it has enabled outstanding progress in various scientific and technological disciplines, including chemistry, physics, biology, and electronics (Jelinek, 2015). Nanotechnology uses matter to create materials and structures at the molecular level with a size of 1 to 100 nanometers and in at least one direction called NPs (Pande & Bhaskarwar, 2016).

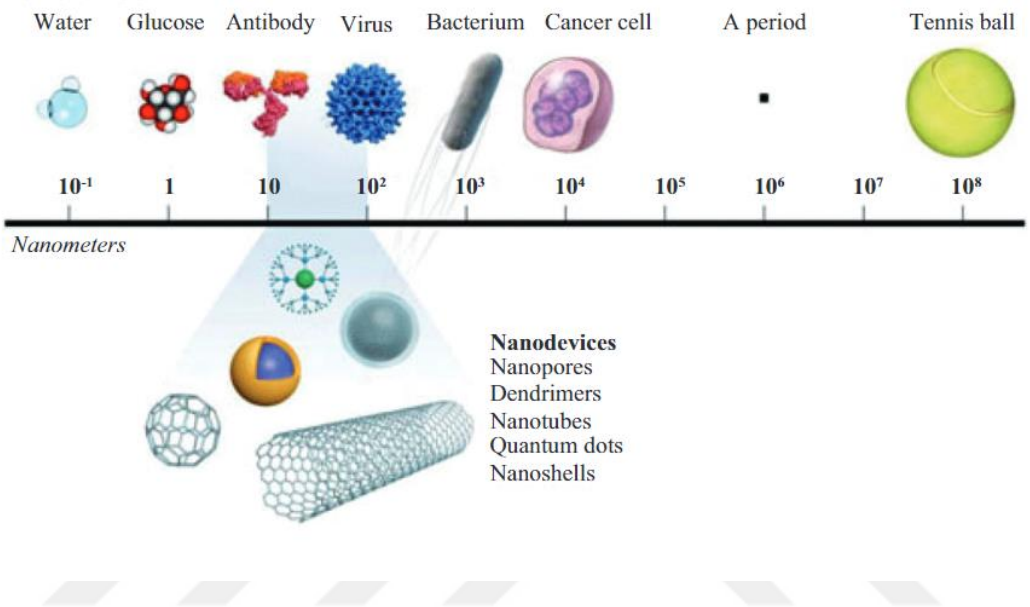


**Figure 2.** Examples of nanometer sizes (Pande & Bhaskarwar, 2016).

Nanotechnology, which has begun to be used in many areas with targeted drug delivery in the field of health and its use as a carrier for genes and drugs, has a significant potential for society, and new things, such as new nanomaterial production, continue to be discovered day by day. NPs used in biotechnology are used between 10 and 500 nm to communicate with biomolecules inside and on the surface of the cell and determine their various biochemical and physiochemical properties (Mody et al., 2010).

NPs already exist in nature; in addition, the production and use of nanomaterials have brought about some concerns about their environmental and toxicological effects in the atmosphere, ocean, and soil systems and their uptake and accumulation into plant cells, and studies have been conducted on this. As a result of the research, it has been observed that plants and phytoplankton create additional barriers by accumulating cell materials against nanomaterials of different thicknesses depending on environmental conditions, species, and their different tissue regions (Schwab et al., 2016). The

interaction of nanoparticles with biomolecules varies greatly due to various factors such as the shape of the nanoparticle, its size, and the production method. Today, nanoparticles are widely used in different fields, such as pharmaceuticals, energy, the car industry, the paint industry, and cosmetics. As a result of increasing needs in these areas, new nanoparticles are being developed, and there is potential that if high amounts of nanoparticles can not controlled, they can enter the air, soil, and water, reach plants, animals, and humans, and pose a danger to living health and the environment. In studies conducted with plants, the effect of nanoparticles is being tried to be fully understood. In various studies, depending on the type, size, zeta potential, and concentration of the nanoparticle, it causes fatal consequences in some plants, while positive results are obtained in others (Rastogi et al., 2017). For example, in the study, corn exposed to Cu-chitosan NPs showed a significant defense against Curvularia leaf spot (CLS) disease and played a growth-promoting role, and it also has the potential to be an alternative to the synthetic pesticides that are increasingly used and negatively affect the soil and the environment (Choudhary et al., 2017).

### 2.2.1 Types of Nanoparticles

Nanoparticles can have different structural forms, such as spheres, cylinders, tubes, or sheets, and can be further categorized into metal, silicate, organic, metal oxide ($MO_x$), polymer, and carbon nanoparticles (Pal et al., 2018). An ideal choice for drug delivery, organic nanoparticles, also called polymeric nanoparticles, are non-toxic and biodegradable. Examples include micelles and liposomes, hollow spheres, and nanocapsules and nanosphere, some of the most well-known shapes. Inorganic nanoparticles, which are widely used and attracted attention in biomedical sciences and engineering, are stable, biocompatible, non-toxic, and hydrophilic. Metal oxide nanoparticles ($MO_x$ NPs) have the potential to form metal oxides in different structures as a result of the reaction of metallic elements with oxygen. This oxidation is carried out in order to change the properties of the metal by turning it into metal oxide, thus increasing its reactivity and effectiveness. Magnetite, silicon dioxide, zinc oxide, iron oxide, and aluminum oxide are examples of metal NPs (Alhalili, 2023; Ijaz et al., 2020; Mody et al., 2010). Metallic nanoparticles such as iron oxide, silver nanoparticles, and gold nanoparticles have been widely used in diagnosis and treatment fields (Mody et al., 2010).

#### 2.2.1.1 Metallic Nanoparticles

Metal nanoparticles are small in size (1-100 nm) and have distinctive features such as surface area/volume ratio and pore size/structure. Moreover, although they can be synthesized from all metals, the most known and used types are iron, copper, zinc, gold, silver, aluminum, iron, cadmium, lead, and cobalt nanoparticles. Magnetic NPs (iron oxide), gold NPs, and silver NPs are among the types of NPs that are constantly used as therapeutic agents in diseases such as cancer (Ijaz et al., 2020; Mody et al., 2010). Although there are a wide variety of NPs, only some NPs, such as silver, copper, zinc oxide, copper oxide, titanium dioxide, and iron NPs from metal and metal oxide nanoparticles, are actively used for environmental safety. The effects of these nanoparticles are being tested in different plants to understand their toxicity and their effects on the growth of plant organs as a result of exposure (Rastogi et al., 2017).

Silver NPs (AgNPs), which are metallic nanoparticles synthesized by the green synthesis method, are preferred in different fields such as medicine, water treatment, and textiles due to their antibacterial properties (Mody et al., 2010; Pal et al., 2018). Studies on the use of nanoparticles in agricultural applications are being conducted, and different applications are being developed, showing that plants exposed to AgNp can have both positive and toxic effects, so NPs should be carefully analyzed for use in agricultural applications and tissue culture studies. In a study, it was observed that silver nanoparticles applied to barley plants at low concentrations increased the root elongation of the plant (Gruyer et al., 2014; Mahajan et al., 2022). 200 µl AgNP application was shown to increase the growth rate of chickpea, pigeon pea, and mungbean, as indicated by increases in root and shoot length and seed germination percentage; in addition, exposure to more than 200 µl AgNP caused toxicity and negatively affected plant growth (Singh et al., 2016). It has been proven that AgNps are also useful in the production of secondary metabolites by using it as an elicitor (Mahajan et al., 2022).

### 2.2.2 NP Synthesis

In the synthesis method of nanoparticles, which is divided into two approaches, top-down and bottom-up, the desired nanoparticle structures are obtained by breaking down the nanomaterials. In the top-down method, bulk material breaks into small particles by grinding and shredding, which is a physical method. In the bottom-up method, where chemicals and biological substances are used, it is necessary to bring

together small nanomaterials. The top-down method includes physical methods such as sonication, laser removal, and lithography, while the bottom-up method uses atoms and molecules by thermal decomposition method, Hydrothermal method, and Sol-gel methods (Alhalili, 2023; Pal et al., 2018). Nanoparticles produced by the physical approach are generally produced by laser ablation, annealing, metal sputtering, high-energy ball milling and evaporation, and condensation. Compared to the chemical approach, obtaining more homogeneous NPs and obtaining small-sized Np even at high concentrations shows that it is more convenient than the chemical approach. In chemical methods, reactions are carried out in solution, and silver ions are reduced, resulting in the formation of colloidal AgNPs that can be synthesized in large quantities. The reagents used during the synthesis are toxic and have the potential to harm the environment, but in the biological synthesis method of AgNp, the synthesis is carried out with one of the biological agents shown in Figure 3 and silver metal ion solution and does not require an external reducing agent (Pal et al., 2018).

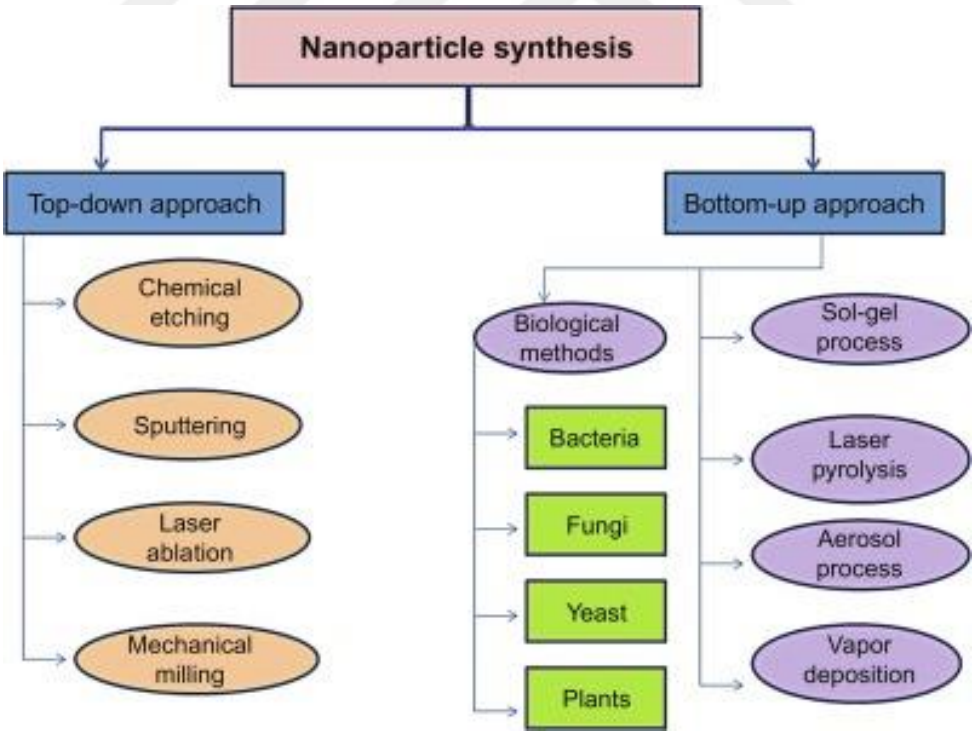


**Figure 3.** Methods used in nanoparticle synthesis approaches (Pal et al., 2018).

#### 2.2.2.1 Green Synthesis of AgNp

It has been observed that in the green synthesis method, a more environmentally friendly approach is achieved by using microorganisms, plants, and bio-based materials, and since no hazardous chemicals are used, unlike traditional methods, the formation of toxic waste is prevented. The green synthesis method has attracted great attention recently because it is sustainable and has many sources of bioactive compounds, especially the presence of various plants in nature. Another reason is that the plant reduces metal ions by facilitating the formation of nanoparticles. Leaves, roots, and seeds of the plant can be used in nanoparticle synthesis (Osman et al., 2024). Just as green synthesis is environmentally friendly, silver nanoparticles take the lead among nanoparticles. It has been proven to be useful in nanoremediation of environmental pollution by showing catalytic and electronic properties. Although AgNPs can be obtained by chemical and physical methods, green synthesis is preferred for sustainability and environmental protection reasons (Silva et al., 2024). "Living organisms such as fungi, bacteria, algae, and yeast are used in the green synthesis of silver nanoparticles (AgNP). However, plants stand out due to their ease of production, absence of disease-causing organisms, and environmental friendliness. Additionally, the plant's molecules, such as protein, saponin, terpene, and phenolic compounds, stabilize AgNP without requiring the addition of an external reducing agent, making production more cost-effective" (Pal et al., 2018; Roy & Das, 2015).

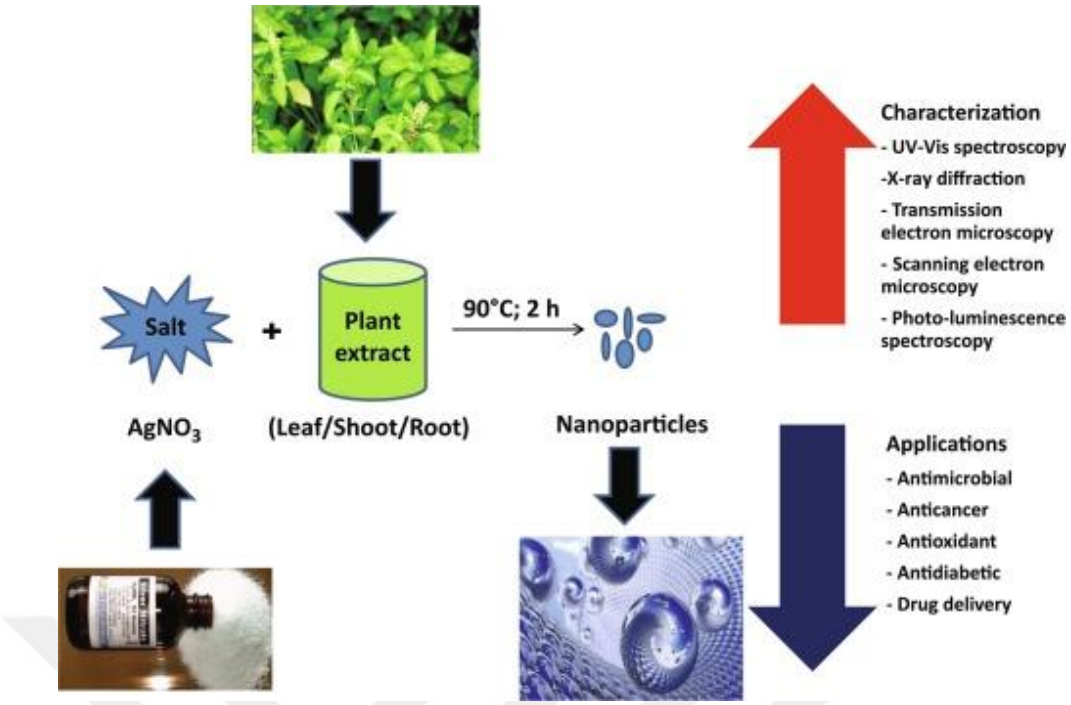


**Figure 4.** Green synthesis of AgNPs (Pal et al., 2018).

In a study, AgNps synthesized by the green synthesis method using *Chenopodium album* leaf extract destroyed *Candida albicans*, *Candida tropicalis*, and *Fusarium oxysporum* and prevented their growth (M. Kumar et al., 2024). In the study conducted by Anandalakshmi et al., AgNP synthesis was successfully achieved by the green synthesis method using the extract obtained from the leaves of *Pedalium murex*, and it was observed that these AgNPs showed high antibacterial properties against *E. coli and B. subtilis* (Anandalakshmi et al., 2016). In the study using *Glycyrrhiza glabra* leaf extract, it was observed that AgNPs with a well-dispersed spherical structure around 20 nm were successfully synthesized (Dinesh et al., 2012).

### 2.3 Secondary Metabolites

Phytochemicals produced in small amounts in plants as a defense system during biotic and abiotic stresses and make them adapt to the environment are called plant secondary metabolites (PSMs). Apart from being a defense mechanism, they participate in many biochemical pathways inside and outside the cell, which is why they play a key role in drug production. The amounts of plants used to obtain PSMs, which are found in small amounts in plants for uses such as medicine, food, and cosmetics, have put the plant species in danger of extinction. For this reason, PSM production by plant cell culture is

preferred. PSMs obtained through in vitro culture enabled the obtaining of higher quality and higher amounts of secondary metabolites and protected the plant population (Abdulhafiz et al., 2022; S. Kumar et al., 2022). PSMs, which play an important role in the growth process of plants, are formed as a result of going under some metabolic pathways of primary metabolites (PPMs) (proteins, carbohydrates, nucleic acids, and lipids) that play a role in the plant's ability to perform fundamental functions such as photosynthesis (Pott et al., 2019). When plants are exposed to physical, chemical, and biotic factors, they become stressed and begin to produce secondary metabolites as a defense. These factors can be abiotic stresses such as drought, heavy metals, temperature, pH changes in the soil, dryness, or biotic stresses such as diseases, insects, etc. PSMs are not only bioactive compounds synthesized as a defense mechanism against stress or to survive, but also, as a result of many studies, it has been shown that they are potential drugs and have many health benefits with their anti-inflammatory and antioxidant properties (Chaturvedi & Gupta, 2021; Joshi et al., 2024).

### 2.3.1 Classification of Secondary Metabolites and Their Uses

Although the biomolecules of the plant are divided into primary and secondary metabolites according to the activities they play, their distinction cannot be made precisely because some compounds are found in both primary and secondary metabolites (e.g., terpenoids) (Chaturvedi & Gupta, 2021). Secondary metabolites, divided into 3 main groups as phenolic compounds, terpenoids, and alkaloids, offer great promise in drug production with their antioxidant, anti-inflammatory, and anticancer properties (Anwar et al., 2019). Secondary metabolites perform their functions by concentrating in certain organs of plants, and their formation is specific to the cells and tissues of the plant. Terpenes constitute the largest group of secondary metabolites and produce toxins for protection from insects and mammals. They also play a key role in the photosynthesis and development of the plant. Nitrogen-containing secondary metabolites (e.g., alkaloids) protect to deter herbivores, are often toxic, and also protect against infections. Phenolic compounds form the basis of immunity that protects the plant from diseases. These compounds, derived from the shikimic acid pathway that is not found in animals, are involved in various plant defense mechanisms, such as protection against herbivorous insects and root parasites. Sulfur-containing secondary metabolites, which regulate sulfur

uptake in the plant, protect against microbial pathogens (e.g., parasites) in addition to their key role in plant growth (Pagare et al., 2015).

The synthesis of terpenes, which are insoluble, are the most abundant in plants, and attract attention in pharmaceutical production, is synthesized through the mevalonate (MVA) pathway in the cytoplasm and the methylerythritol-4-phosphate (MEP) pathway in plastids. Acetyl coenzyme-A (acetyl Co-A) brings together isoprenoids, forming the beginning of the synthesis of terpenes for both pathways. In the synthesis of these two pathways, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) intermediates are formed. While chlorophyll, gibberellin, and carotenoids are produced in the MEP pathway, brassinosteroids, triterpenes, polyphenols (including flavonoids), and sesquiterpenes, which are important for plant development, are produced in the MVA pathway. The MEP pathway, which produces phytohormones and photosynthetic pigments, is vital for plants (Anmol et al., 2024; Tian et al., 2022). Terpenes are divided into six groups according to their 5-carbon units: monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes, and polyterpenoids (Verma & Shukla, 2015). The pathways involved in the synthesis of phenolic acid are the shikimic acid pathway and malonic acid pathway, and many aromatic amino acid by-products, such as phenylalanine, are formed in the shikimic pathway (Anmol et al., 2024). Phenolics, which increase in response to abiotic and biotic stress, are divided into lignans, styrylpyrones, flavonoids, tannins, lignins, arylpyrones, coumarins and stibenes (Verma & Shukla, 2015). Saponins obtained from the MVA pathway are triterpenes and steroid glycosides and have soap-like properties used in the detergent industry (Mugford & Osbourn, 2013). Medically important nitrogen-containing Alkaloids are synthesized from the amino acids lysine, tryptophan, and tyrosine. They are divided into groups such as benzylisoquinoline alkaloids (BIAs), terpenoid indole alkaloids (TIAs), tropane, purine, etc (Verma & Shukla, 2015). The names of the pathways involved in the production of secondary metabolites are the Malonic-acid pathway, Shikimic-acid pathway, MVA pathway, and MEP pathway, the fundamental substance that enables their formation and turns into natural products such as aromatic amino acids, phenols, alkaloids, and terpenes at the end of synthesis is glucose produced by the plant through photosynthesis (Dhaniaputri et al., 2022).

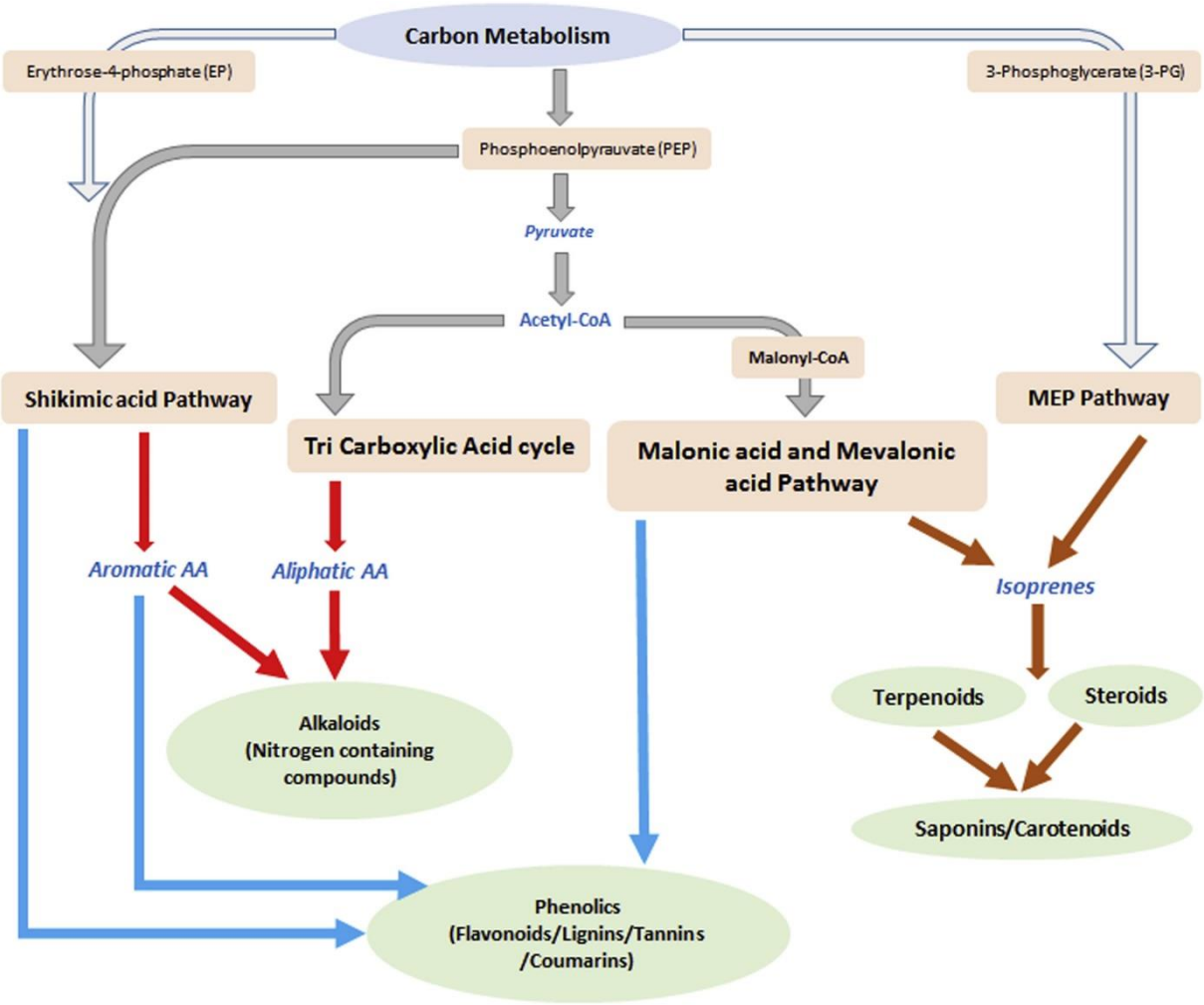


**Figure 5.** Carbon metabolism-related pathways that play a role in the synthesis of plant secondary metabolisms (Chaturvedi & Gupta, 2021).

### 2.3.2 Obtaining Secondary Metabolites by Maceration Method

Different extraction methods have been used to obtain PSMs stored in plants since prehistoric times, and essential oils used for therapeutic purposes have been obtained. Today, methods such as distillation, solvent extraction, and pressing are used to preserve the integrity of important components. Still, new techniques are also used to protect the environment, as shown in Figure 6 (Anmol et al., 2024).

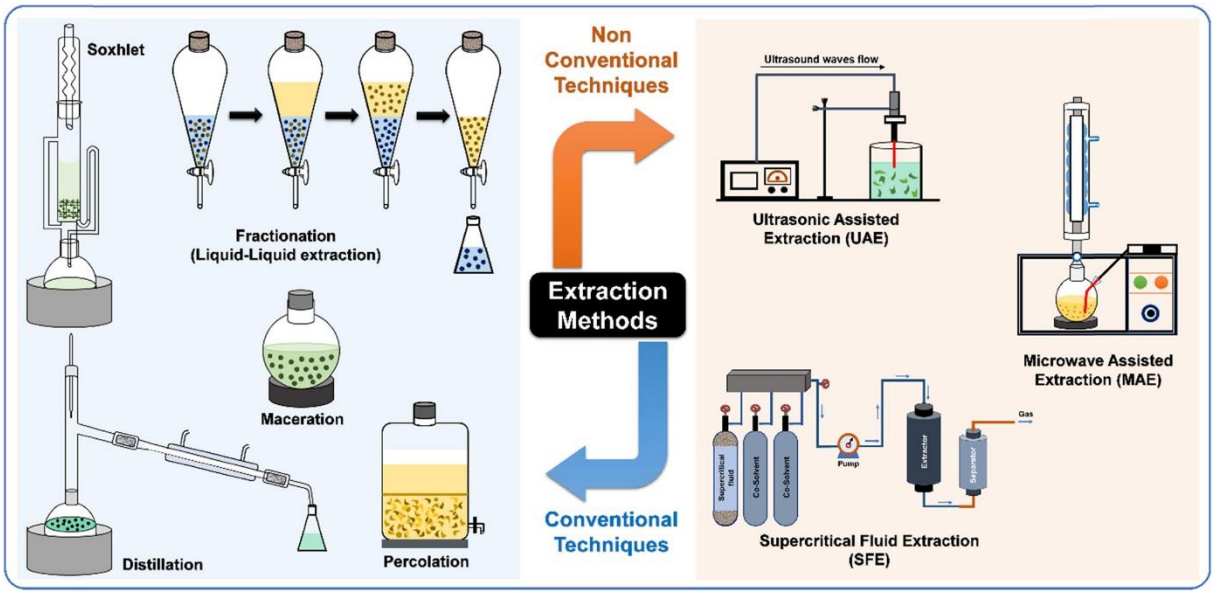


**Figure 6.** Extraction techniques (Anmol et al., 2024).

In the maceration method, the dried and powdered plant is mixed with solvent. The polarity of the solvent used to separate PMS is important, and the effect is tried to be increased by using factors such as shaking and temperature during the process. Then, by filtering the solvent and then drying it, PMSs are separated from the solvent, and crude extracts are obtained. Instead of using the plant directly, more amount of PMS is obtained by creating callus and cell suspension with explants obtained from plant organs such as leaves and roots with abiotic and biotic stress factors applied to plant tissue culture (PTC) (Anmol et al., 2024; Thirumurugan et al., 2018).

### 2.3.3 Effect of Elicitors on Secondary Metabolite Content

Plants undergo oxidative stress in the face of abiotic and biotic stresses and start to produce reactive oxygen species (ROS). To defend themselves from plant cells against this attack and minimize the damage, plant cells activate the antioxidant mechanism and produce endogenous enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) or increase the production of secondary metabolites by activating metabolic pathways. In the study conducted by Zhang et al., *Artemisia annua* L. hairy root culture was stimulated using silver-silica coated (Ag-$SiO_2$) core-shell nanoparticles, and it was observed that the artemisinin content increased by 3.9 times. This increase has been associated with oxidative stress (Marslin et al., 2017; B. Zhang et al., 2013). It has been concluded in the literature that increases in secondary metabolite content are induced

by the signals created by the plant against ROS. It is thought that the mitogen-activated protein kinase (MAPK) pathway, which is activated by the increased amount of cytoplasmic calcium ($Ca^{2+}$) due to the entry of AgNp from the cell wall, causes the reprogramming of secondary metabolites (Figure 7). ROS formed as a result of oxidative bursts are tried to be controlled by secondary metabolites. Excessive amounts of ROS can damage the nucleus and cell membrane, but at the same time, according to the research, it has been observed that small amounts of ROS contribute to the development of the plant (Ali et al., 2019; Marslin et al., 2017).

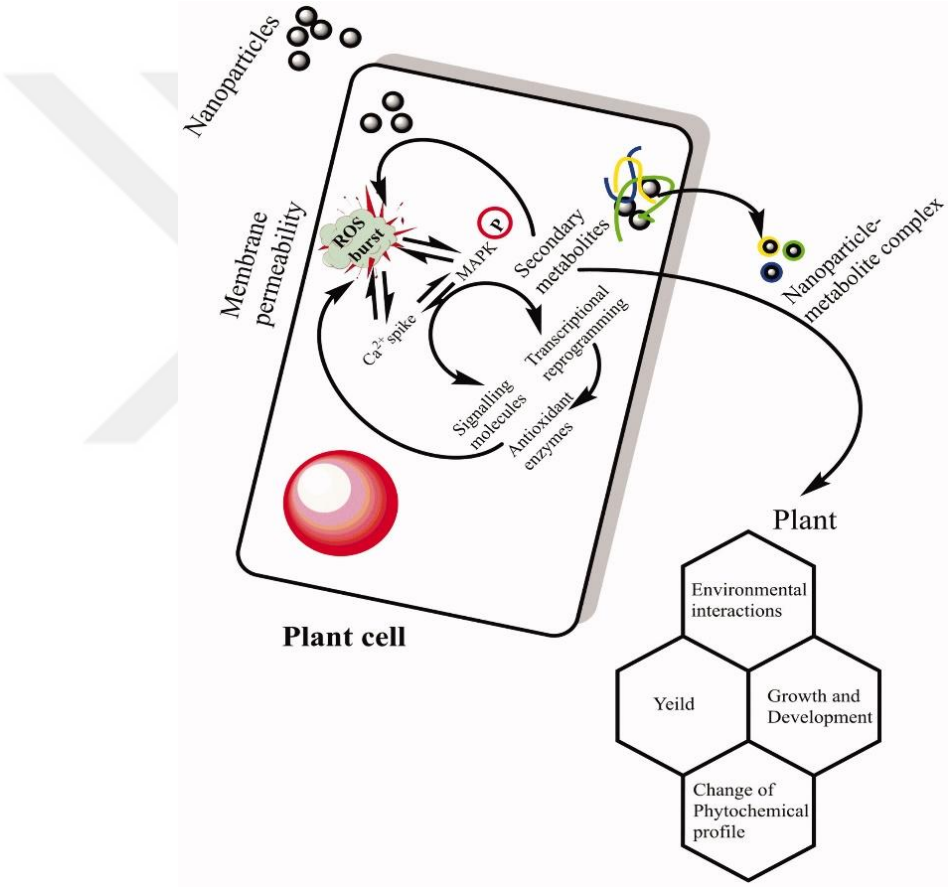


**Figure 7.** Antioxidant mechanism of the plant against AgNp (Ali et al., 2019).

In a study by Taghizadeh et al., an increase in total PSM contents was observed as a result of stimulation of the suspension culture of *Dracocephlum polychaetum* Bornm with static magnetic field (SMF) and iron oxide ($Fe_2O_3$) magnetic nanoparticles (MNP) (Taghizadeh et al., 2019). In a study showing the elector effects of AgNPs, it was concluded that AgNPs synthesized from *Alnus nitida* leaf extract and AgNPs added with urea increased

the metabolic content and biomass of wheat seeds by acting as elicitors at low concentrations (Khan et al., 2023).

# 3. MATERIALS AND METHODS

## 3.1 Materials

Tables 1 and 2 list the instruments/tools and chemical substances used for plant tissue culture, extraction, nanoparticle synthesis, and analysis in the study.

**Table 1.** Instruments and tools used in experiments

| **Instruments and Tools** | |
|---|---|
| pH meter | Ohaus ST5000-F |
| Magnetic Hotplate Stirrer | Scilogex MS-H-S |
| Biological Safety Cabinet | Hedlab X-BIO CLASS II |
| Plant Growth Chamber | Sanyo MLR-351 |
| Precision Balance | Radwag PS 4500.R2.M |
| Analytical Balance | Radwag AS 220.R2 |
| Centrifuge | Beckman Coulter Allegra X-22R |
| Autoclave | Hirayama HVE-50 |
| Ultrasonic Homogeniser | Bandelin/Sonorex HD 3100 |
| UV/Vis Spectrophotometer | Thermo Fisher Scientific GENESYS 180 |
| SEM (Scanning Electron Microscope) | Thermo Fisher Scientific Quattro S |
| Zetasizer Spectroscopy | Malvern Zetasizer-NanoS |
| Lyophilizer | Telstar CRYODOS -80 |
| Orbital Shaker | N-Biotek NB-T101M |
| Rotary Evaporator | Heidolph Hei-VAP Core |
| Ultrasonic Cleaner | Everest Ultrasonic CleanEx |
| Gravity Oven | Daihan Scientific WiseVen WON-105 |
| Gas Chromatography Mass Spectrometry (GC/MS) | Agilent Technologies 5975 Inert MSD |

**Table 2.** Chemicals used in experiments

| **Chemicals** | |
|---|---|
| Sucrose Crystallized | Duchefa, Cas 57-50-1 |
| Murashige & Skoog Medium | Duchefa |
| Plant Agar | Duchefa, Cas 9002-18-0 |
| 6-Benzylaminopurine (6-Bap) | Duchefa, Cas 1214-39-7 |
| 2,4-Dichlorophenoxyacetic Acid (2,4 D) | Duchefa, Cas 94-75-7 |
| N-Hexane | ISOLAB, Cas 110-54-3 |
| Ethanol Absolute | ISOLAB, Cas 64-17-5 |
| *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide | Sigma-Aldrich, Cas 24589-78-4 |
| Silver Nitrate ( $AgNO_3$) | Sigma-Aldrich, Cas 7761-88-8 |

## 3.2 Methods

### 3.2.2 Silver Nanoparticle Synthesis and Characterization

#### 3.2.2.1 Preparation of $AgNO_3$ Solution Stock

To prepare 100 ml of 1 molar silver nitrate ($AgNO_3$) solution, 16.99 g of $AgNO_3$ (169.9 g/l) was weighed on a precision scale and dissolved in 100 ml of distilled water. The prepared solutions were stored in dark glass bottles to prevent oxidation. The 10 mM $AgNO_3$ solution used in the synthesis was diluted to 1/100 from the stock solution.

#### 3.2.2.2 Preparation of Extract from *Salvia sclarea (S.sclarea)* Leaves

The extract of *S. sclarea* leaves was used for the synthesis of AgNPs using the green synthesis method. Leaves of the plants were collected and weighed to 10 g. After the leaves were washed with tap water, they were kept on drying paper for some time to remove excess water. The leaves, whose water was removed, were cut into small pieces in a beaker, 100 ml of distilled water was added (1 g: 10 ml), and the mixture was boiled in a magnetic stirrer for approximately 30 min (Figure 8). The mixture prepared in this

way was filtered through Whatman No.1 filter paper, transferred to falcon tubes, and centrifuged at 6000 rpm for 15 min. After centrifugation, the supernatant was collected.

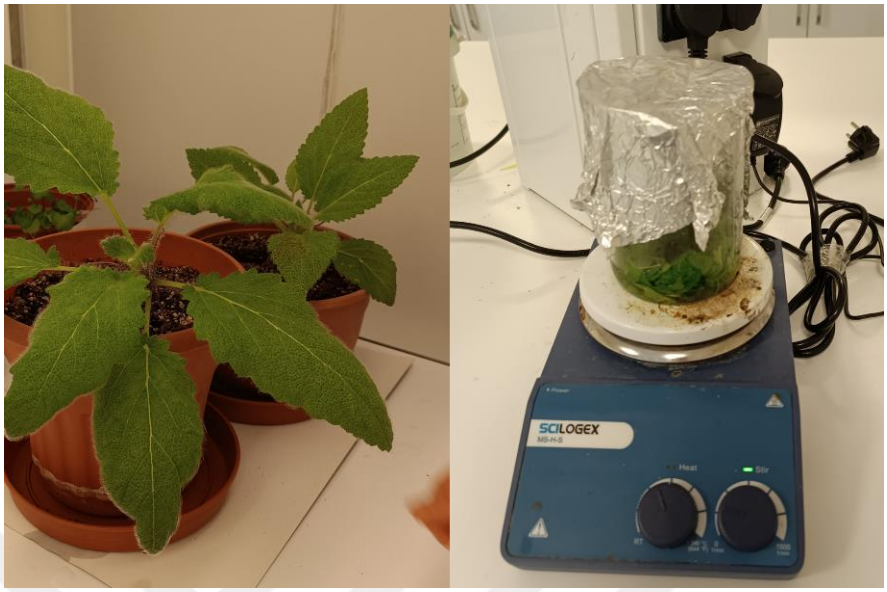


**Figure 8.** Preparation of extract from *S. sclarea* leaves.

### 3.2.2.3 Green Synthesis of Silver Nanoparticle Synthesis

For AgNP synthesis, the leaf extract and 10 mM $AgNO_3$ solution were mixed in a magnetic stirrer at a ratio of 1 ml $AgNO_3$: 9 ml plant extract. We understand that the reaction occurred by observing the color change (Figure 9). Afterward, the pH was adjusted to 9.3-9.4, the temperature was reduced from 55 °C to 35 °C, and the mixing process was continued for 45 min in a magnetic stirrer to bring the nanoparticles to the desired size. The mixture was then transferred to sterile centrifuge tubes and centrifuged at 10,000 rpm for 1 h. After centrifugation, the supernatant was removed, and the NP parts remaining at the bottom of the tubes were dried using a lyophilizer device.

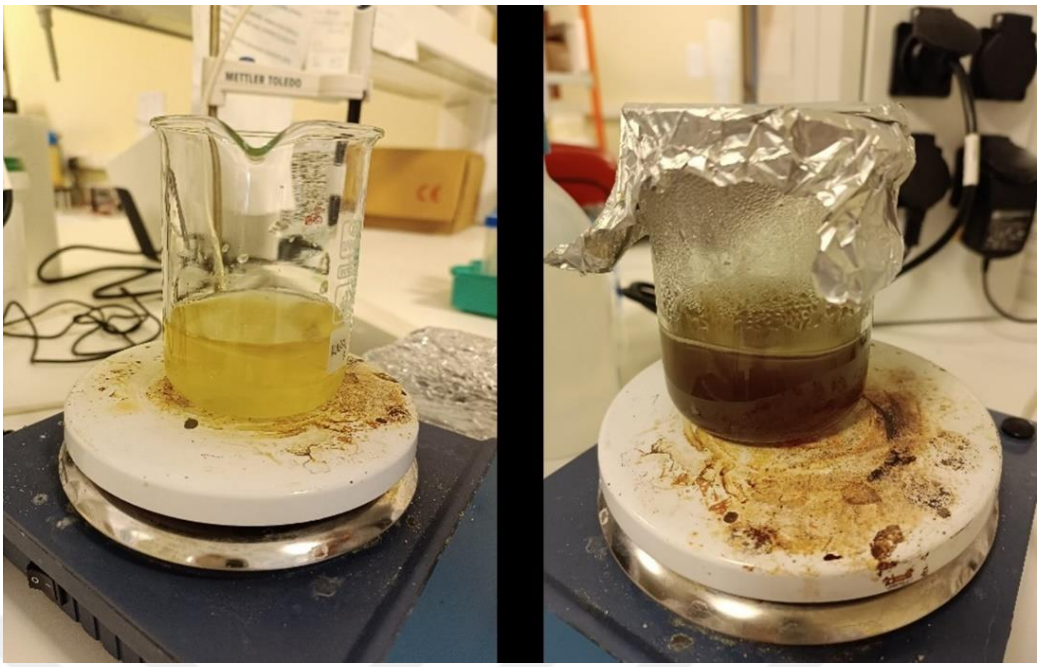


**Figure 9.** Synthesis of AgNPs.

#### 3.2.2.4 Silver Nanoparticle Characterization

Zeta Potential, Ultraviolet-visible spectroscopy (UV–Vis), and Scanning Electron Microscopy (SEM) were performed to evaluate the synthesized nanoparticles' structure, distribution, and size. Samples taken from *S.sclarea* plant extract after $AgNO_3$ addition at 30, 40, and 50 minutes were examined in UV-Vis spectroscopy in the 300-600 wavelength range to see whether the synthesis was successful or not. After UV-Vis analysis, the same solution was diluted 1:10 with water and sonicated, and Zeta Potential analysis was performed to evaluate their distribution, size, and stability. In the SEM analysis performed to examine the structure and size of AgNps, small samples taken from AgNps dried by freeze-dry method were mixed with 1-2 drops of distilled water, allowed to dry in the air, and then placed in the device and started to be examined.

### 3.2.3 Tissue Culture

#### 3.2.3.1 Sterilization of Working Environment, Tools, and Equipment

The experiments were performed in a biosafety cabinet. Ten minutes before work, cabin ventilation was turned on and cleaned with 70% alcohol. Culture media, tools, and equipment used during the experiments were sterilized by autoclaving at $121^0$C for 60

min. The forceps and scalpels used during the applications were kept sterile by intermittently passing them through alcohol and flame.

### 3.2.3.2 Preparation and Sterilization of Plant Culture Medium

Seed germination experiments of *P. anisum* grown in the Burdur province of Türkiye, MS (Murashige & Skoog, 1962) medium containing 3% sucrose and 0.7% agar was used as the main nutrient medium. A magnetic stirrer (5-10 minutes) was used to ensure complete dissolution. When dissolution occurred, the solutions whose pH was adjusted to 5.6-5.8 were autoclaved at $121^0$C for 60 minutes for sterilization. After autoclaving, the nutrient media were placed in the cabinet, poured into the plant culture box, and allowed to solidify.

### 3.2.3.4 Surface Sterilization and Germination of *P. anisum* Seeds

Surface sterilization of *P. anisum* seeds was achieved in the first stage by mixing the seeds in 20% bleach solution for 10 min using a magnetic stirrer. The seeds were then transferred into sterile distilled water and rinsed by mixing for 5 min using a magnetic stirrer, and this process was repeated three times. Since it was observed that contamination occurred in some seeds during the germination stage, the surface sterilization procedure was changed, the bleach solution was increased to 25%, the sterilization time was 15 min, and the washing time was 10 min. The sterilized seeds were transferred to MS medium (MS0), which did not contain plant growth regulators for germination.

### 3.2.3.5 Obtaining Aseptic Seedlings and Leaf Explant

Seeds transferred to MS0 medium and germinated were germinated at $24^0$C in a 16-hour light / 8-hour dark photoperiod, under 4000 lx fluorescence light, in an air-conditioning cabinet. The 5-6 weeks old aseptic seedlings obtained from germinated *P.* anisum seeds were used as explants. Leaf explants were planted in MS medium containing 2 mg/L 2,4-Dichlorophenoxyacetic acid (2,4 D), 30 g/L sucrose, and 7 g/L agar for callus formation (Figure 10).

**Figure 10.** Placement of *P. anisum* leaf explants on MS medium containing 2 mg/L 2,4-Dichlorophenoxyacetic acid (2,4 D), 30 g/L sucrose, and 7 g/L agar for callus formation.

### 3.2.4 Cell Suspension Culture

After the pH of the MS nutrient medium containing 2 mg/L 6-Benzylaminopurine (BAP), 2 mg/L 2.4 D, and 30 g/L sucrose was adjusted to 5.6-5.8, and it was autoclaved together with the conical flasks to be used in suspension culture at $121^0$C for 60 minutes to sterilize.

When callus formation was observed in the leaf explants planted in Petri dishes, the suspension culture was started by adding 1 g of callus to the flask to which 25 ml of medium was added. When callus formation was observed in the leaf explants planted in Petri dishes, suspension culture was started by adding 1 g of callus to the flask to which 25 ml of medium was added. The flasks were placed in an orbital shaker at 1200 rpm to run continuously. After 1 week, 25 ml of the new medium was added again, and at the end of the second week, when we obtained a blurry image (Figure 11), 1 ml of suspension cell culture taken from the conical flask was distributed into the solid MS medium containing 2 mg/L BAP, 2 mg/L 2,4 D, 30 g/L sucrose, and 7 g/L agar. After five weeks of culture, the developed calli were transferred to the same MS medium supplemented with 0 (control), 1.0, 5.0, 10, and 20 mg/L AgNp. (gram?)

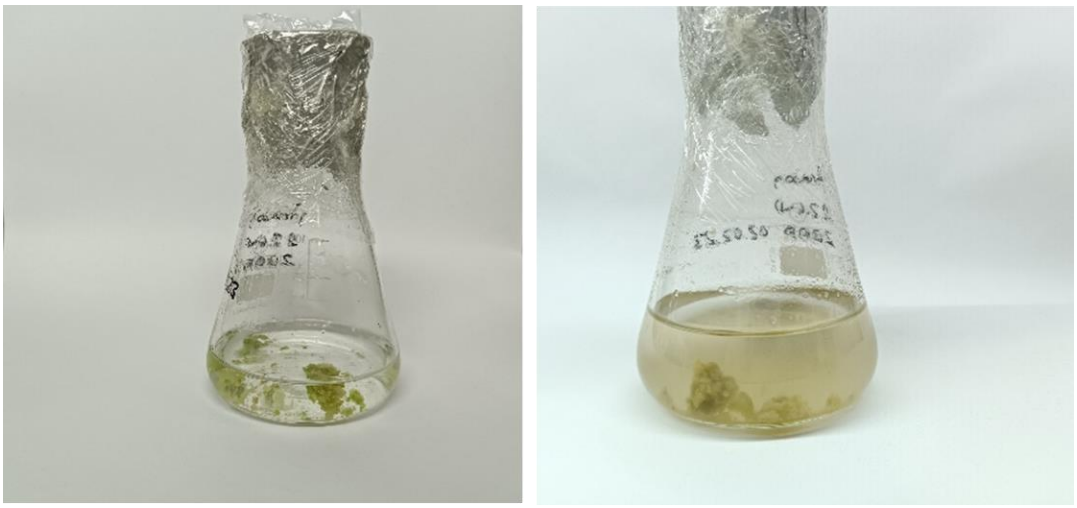

**Figure 11.** The first day and two weeks later of suspension culture established with *P. anisum* callus.

### 3.2.5 Determination of the Morphological Effect of Silver Nanoparticles on Anise Callus Diameter

The diameter of the calli was measured using a ruler 7, 14, 28, and 35 days after the initiation of the culture.

### 3.2.6 Analysis of Data

Tissue culture experiments were carried out in triplicate, and five explants were cultured in each repetition. The data's variance analysis was performed using the two-way analysis of variance (ANOVA), and the differences ($p \leq 0.05$) between each group were determined using Dunnet's Multiple Comparison test. Lastly, the data obtained were analyzed using GraphPad Prism-10 software.

### 3.2.7 Secondary Metabolite Content Analysis

#### 3.2.7.1 Maceration

The calli from the five different groups cultured for 5 weeks were removed from the water by lyophilization and completely dried. The freeze-dried samples were ground into powder and placed in conical flasks. The weights of dried calli are 2.02 g, 3.93 g,

4.06 g, 4.26 g, and 2.06 g, respectively, with control, 1.0 mg/L, 5.0 mg/L, 10 mg/L, and 20 mg/L AgNp. Hexane was added to cover the dried calli, and the mixture was kept in an ultrasonic bath for 1 h. While 50 ml of hexane was added to the control and 20 mg/L AgNp groups, 100 ml was added to the control, 1 mg/L, 5 mg/L, and 10 mg/L AgNp groups. After the ultrasonic bath, it was kept in hexane solvent for 1 night. After waiting for 1 night, the liquid was filtered with filter paper. Maceration was performed 3 times. The liquid fractions were then combined at the end of each filtration step (Figure 12). After filtration, crude hexane extracts were obtained by evaporating the hexane solvent in an evaporator at 40 °C. The amounts of hexane extracts obtained are 0.03 g, 0.04 g, 0.06 g, 0.03 g, and 0.05 g, respectively. The same procedures were carried out for ethanol extracts; 100 ml of ethanol solvent was added to each group, and crude ethanol extracts were obtained by evaporating in the evaporator at 45 °C. 0.42 g, 0.38 g, 0.38 g, 0.40 g, and 0.40 g of ethanol extract were obtained from the experimental groups, respectively.

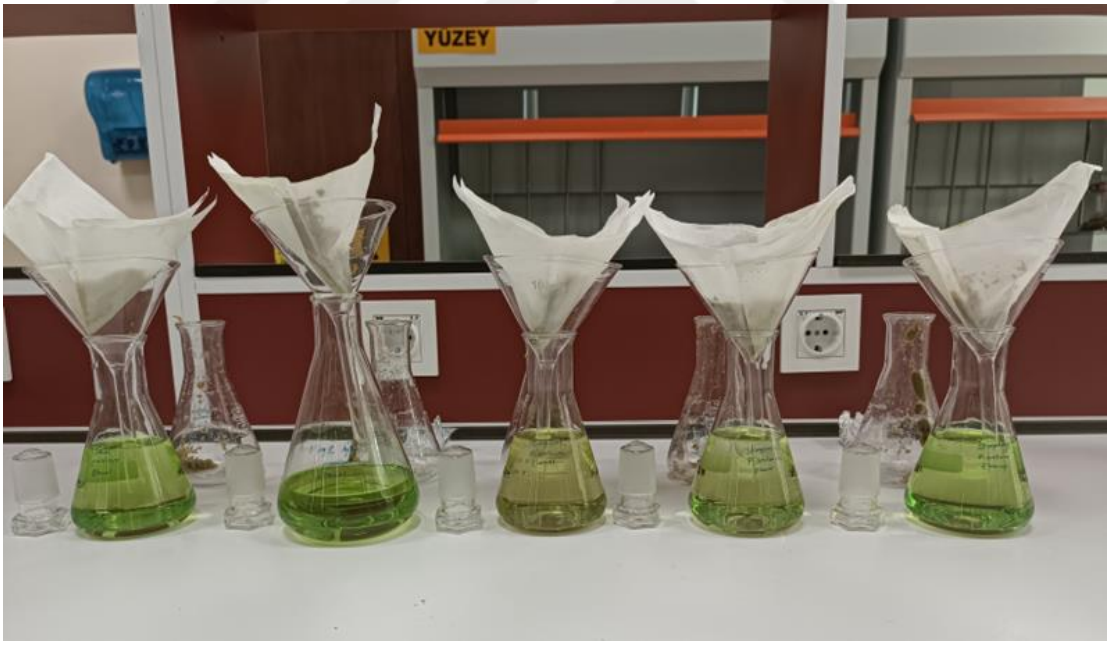


**Figure 12.** Filtration of ethanol extract on filter paper after maceration.

### 3.2.7.2 Trimethylsilyl (TMS) Derivatisation

The hexane and ethanol extracts obtained were subjected to derivatization before GC-MS analysis to determine the volatile and non-volatile components. The

derivatization processes used two different methods for the hexane and ethanol extracts. In the derivatization process of hexane extracts, 100 mg of hexane extract was weighed on a precision balance, and 50 µL of chloroform and 50 µL of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) were added. After vortexing for 20 s, the mixture was incubated at 65 Â °C for 60 min. After incubation, the solution was transferred to a gas chromatography (GC) vial and prepared for GC-MS analysis.

The derivatization process for the ethanol extracts consisted of several steps. For the derivatization process, 100 mg of the ethanol extract was weighed on a precision balance, and 4 mL of deionized water was added and suspended in a centrifuge tube. Then, 3 mL of ethyl acetate was added to the suspended centrifuge tube and shaken on a mechanical shaker for 15 min at room temperature. After shaking, the tube was centrifuged at 3600 × g for 5 min. The extraction process was performed in 2 repetitions. The supernatant collected as a result of the two extraction processes was transferred to a screw-cap glass tube and evaporated to dryness in a rotary evaporator at 40°C. Subsequently, 50 µL of chloroform and 50 µL of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) were added to the dry extract. After vortexing for 20 seconds, it was incubated at 65 °C for 60 minutes. After incubation, the solution was transferred to a GC vial and made ready for GC-MS analysis (Figure 13).

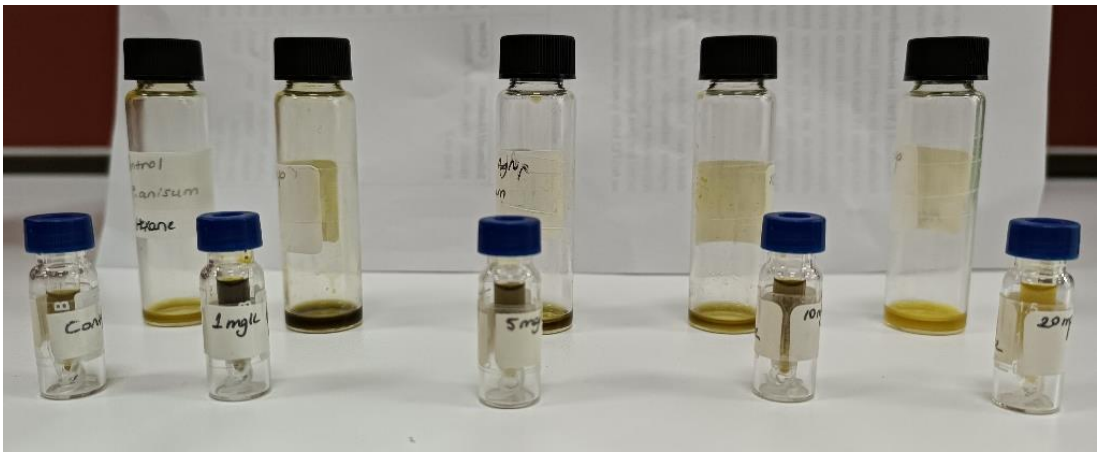


**Figure 13.** Hexane extracts were transferred to the GC vial at the end of the derivatization processes.

### 3.2.7.3 GC-MS Analysis

The GC-MS analysis of hexane and ethanol extracts obtained after derivatization was performed on an Agilent 5975 GC-MSD system with a helium mobile phase (1 mL/min) on an Innowax FSC column (60 m x 0.25 mm, 0.25 μm film thickness). The GC oven temperature was maintained at 60°C for 10 min, increased to 220°C at a rate of 4°C/min, maintained at this temperature for 10 min, and increased again to 240°C at a rate of 1°C/min. Injection was performed in splitless mode. The injection temperature was set to 250°C, the mass spectrometer was set to 70 eV ionization energy, and the mass spectrometer scanning range was set to m/z 35-450 atomic mass units. GC-MS analysis was performed in duplicate, and the data were averaged. Hexane

Secondary metabolite components were identified by comparing the relative lag times with the lag times of the original samples, or by comparing the relative lag times with a series of n-alkanes. In addition, identification was performed by comparing the mass spectrum profiles of the compounds using the commercial Wiley GC/MS Library and NIST17 mass spectrum libraries with the original compounds and known essential oil contents.

# 4. RESULTS

## 4.1 Silver Nanoparticle Synthesis and Characterization

The extract from *S.sclarea* leaves changed from light yellow to dark brown by adding $AgNO_3$ and increasing the pH to 9. This color change seen in the solution shows that *S.sclarea* components stabilize AgNp without adding an external agent, and the UV-VIS spectrometry shows that AgNp synthesis is carried out by reducing Ag+ to Ag0. The synthesis was also observed to be successful in the study by Arslan et al. (2024) (E. Arslan et al., 2024; Chung et al., 2018)Samples taken at 20, 30, 40, and 50 minutes of the reaction, which started with the color change in the solution, were analyzed in a UV-Vis spectrometer. A surface plasmon resonance (SPR) peak between 400 and 450 was observed at 30 minutes. It was concluded that AgNp synthesis occurred, and this minute was optimum for synthesis.

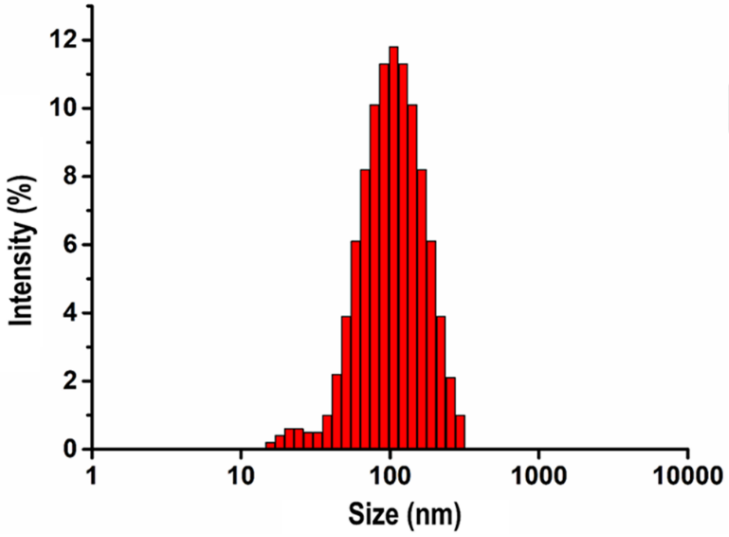


**Figure 14.** Particle size distribution of AgNPs (Ulusoy et al., 2024).

When the obtained AgNPs were examined using photon correlation spectroscopy, the particle sizes of AgNPs with -15.8 mV Zeta potential varied between 15 and 295 nm (mean approximately 88 nm) (Figures 14 and 15), and their structures were spherical in SEM analysis examined at 60,000x magnification under 2.00 kV high vacuum (Figure 16). The fact that the zeta potential is negatively charged indicates that AgNps have long-term stability and high dispersibility due to the repulsion of negative charges.

**System**

| | | | |
|---|---|---|---|
| **Temperature (°C):** | 25,0 | **Zeta Runs:** | 15 |
| **Count Rate (kcps):** | 274,7 | **Measurement Position (mm):** | 2,00 |
| **Cell Description:** | Clear disposable zeta cell | **Attenuator:** | 10 |

**Results**

| | | | Mean (mV) | Area (%) | St Dev (mV) |
|---|---|---|---|---|---|
| **Zeta Potential (mV):** | -15,8 | **Peak 1:** | -7,25 | 85,4 | 7,04 |
| **Zeta Deviation (mV):** | 61,3 | **Peak 2:** | 27,2 | 12,6 | 7,06 |
| **Conductivity (mS/cm):** | 0,0155 | **Peak 3:** | -77,2 | 1,2 | 3,93 |
| **Result quality :** | Good | | | | |

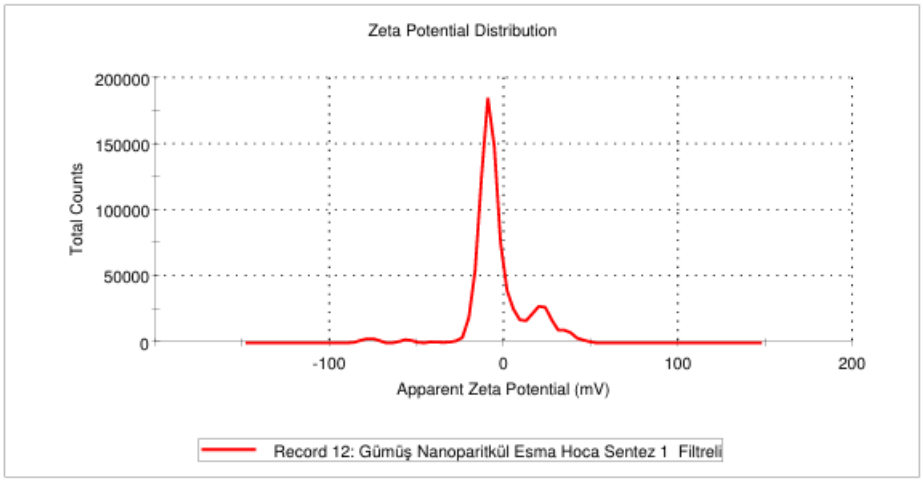


**Figure 15.** Zeta potential of AgNP.

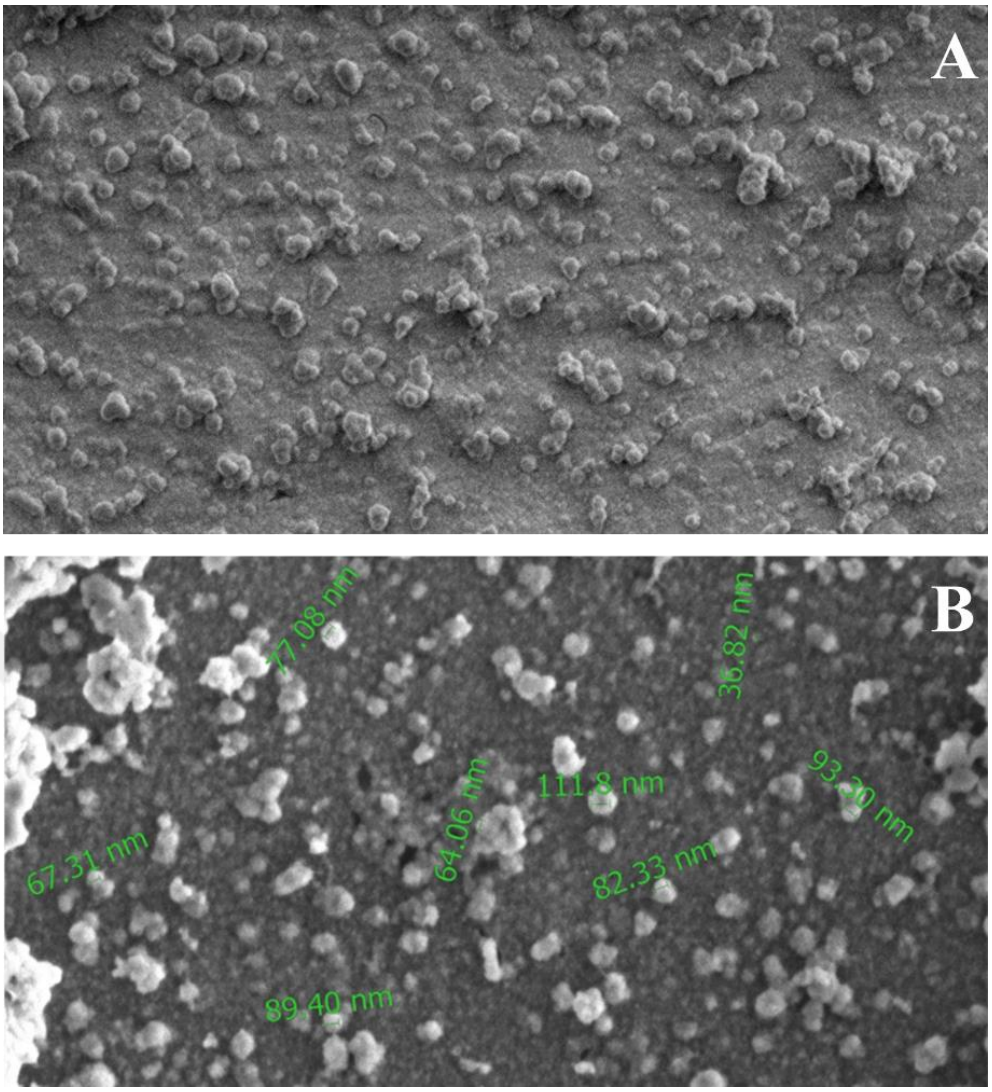


**Figure 16.** SEM images of the AgNPs.

### 4.2 Morphological Examination of *P. anisum* Callus Elicited by AgNPs

It was observed that the callus obtained from cell suspension culture (CSC) did not cause a pause in callus growth by observing the changes that occurred when the callus of CSC was exposed to different concentrations of AgNPs, as shown in Figure 17. Although the growth of callus obtained from CSC continued, the control group showed the best growth among the experimental groups, while the lowest callus growth was at 5 mg/L, as seen in Figures 17, 18, 19, 20, 21, and 22. In addition, at 5 mg/L and 10 mg/L, a decrease in the growth rate of CSC callus diameter was observed after the 14th day (within the measurements on the 28th and 35th days). It was determined that there were no significant differences between the treatments in terms of callus weight, there was a time-dependent decrease in callus weight in all treatments after the 7th day, and there was

a regression in weight decrease at 5 mg/L. The color change in the calli from green to yellow at 1 mg/L, 5 mg/L on day 35, 10 mg/L on day 14, and 20 mg/L on day 7 indicate that AgNPs cause toxicity in *P. anisum*.

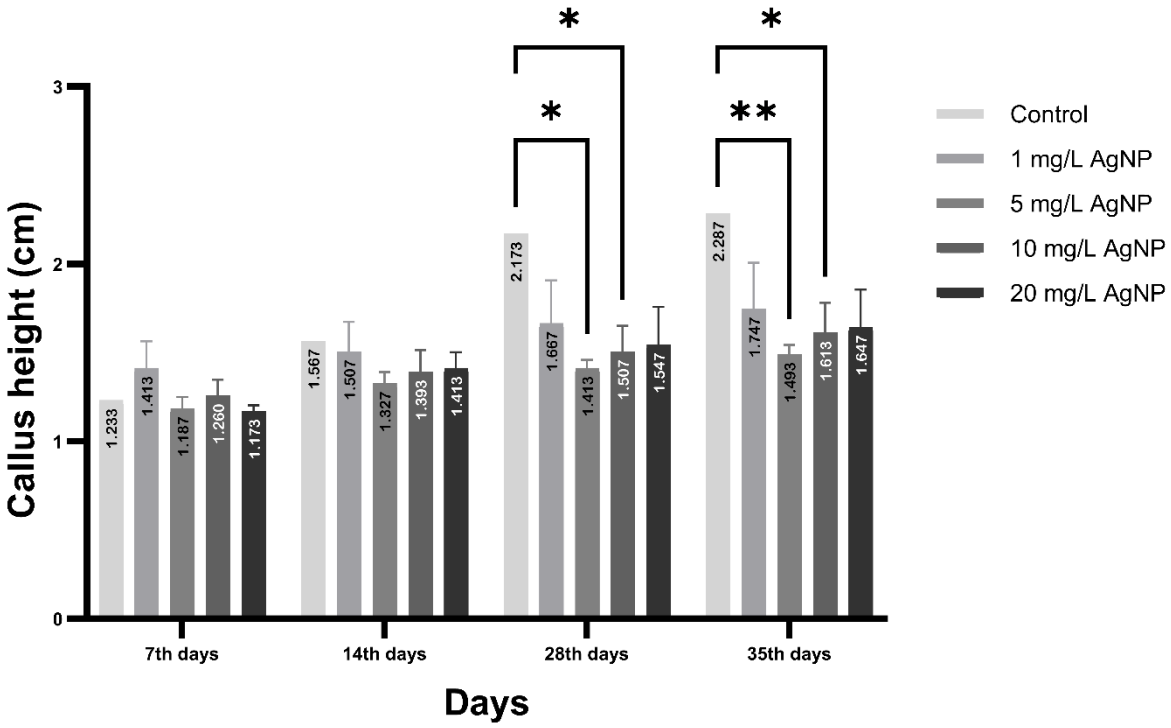


**Figure 17.** Mean diameter measurements of *P. anisum* callus.

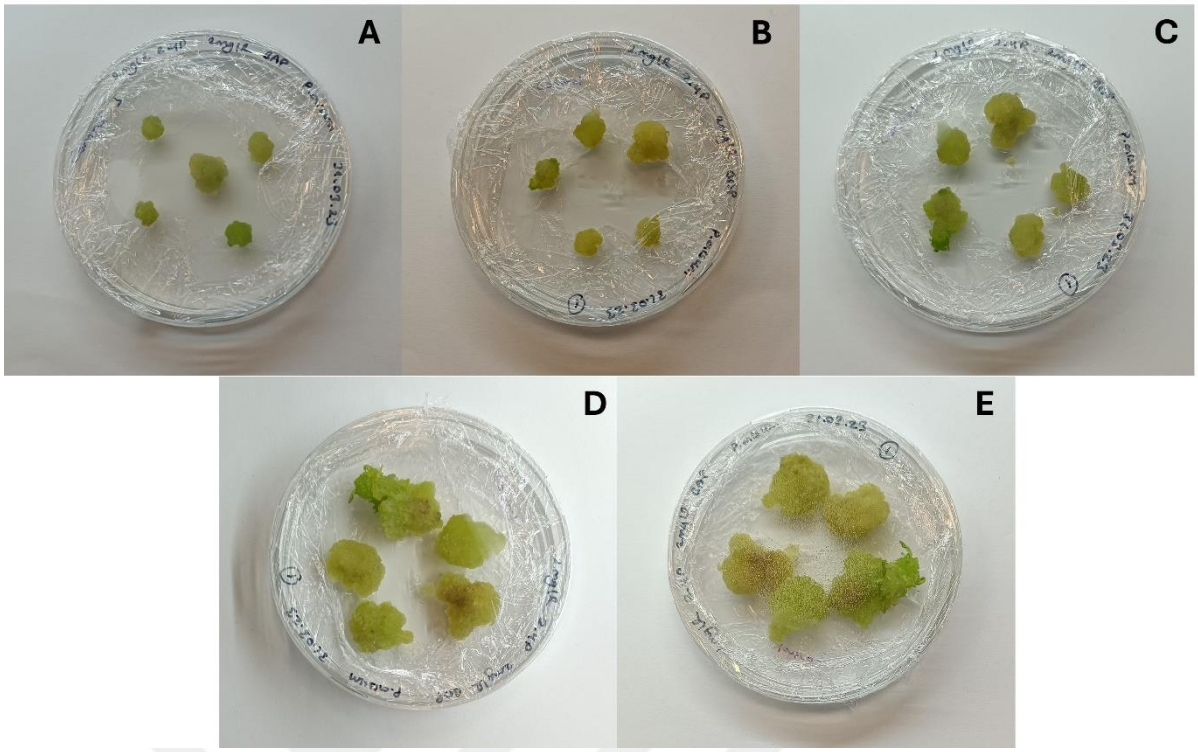


**Figure 18.** Images of the control group were taken at the initial stage (A), day 7 (B), day 14 (C), day 28 (D), and day 35 (E).

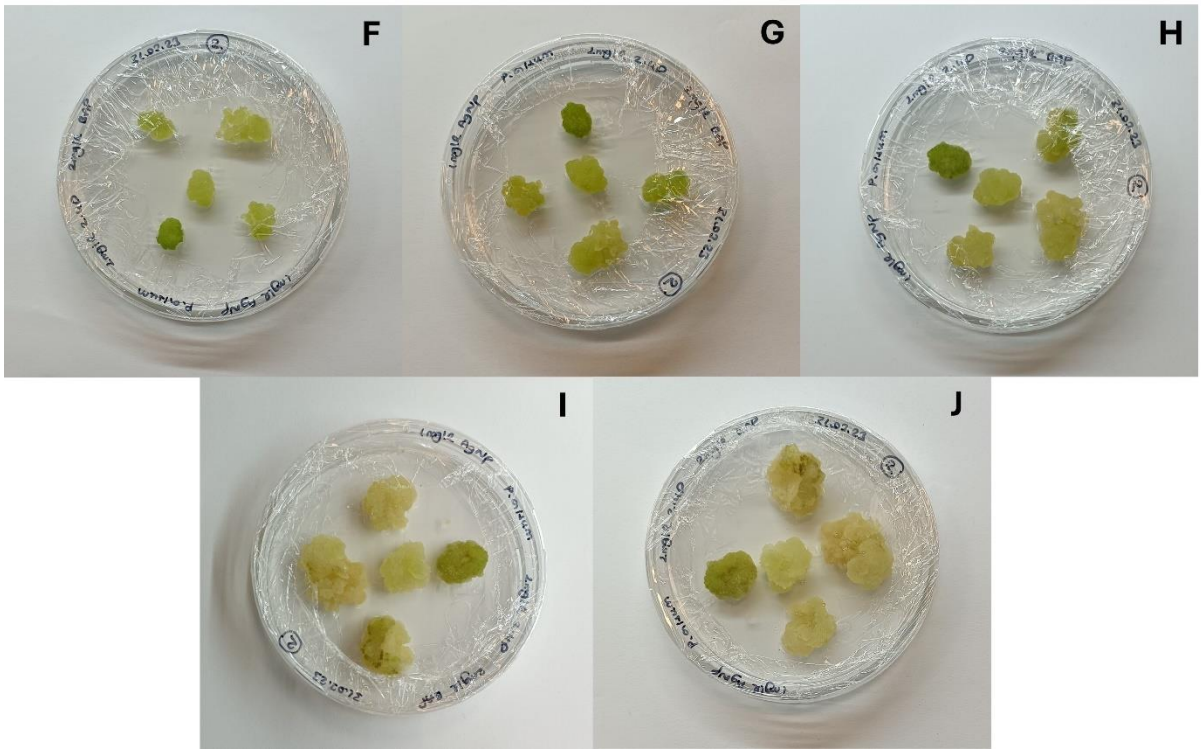


**Figure 19.** Images of the 1 mg/L AgNPs group were taken at the initial stage (F), day 7 (G), day 14 (H), day 28 (I), and day 35 (J).

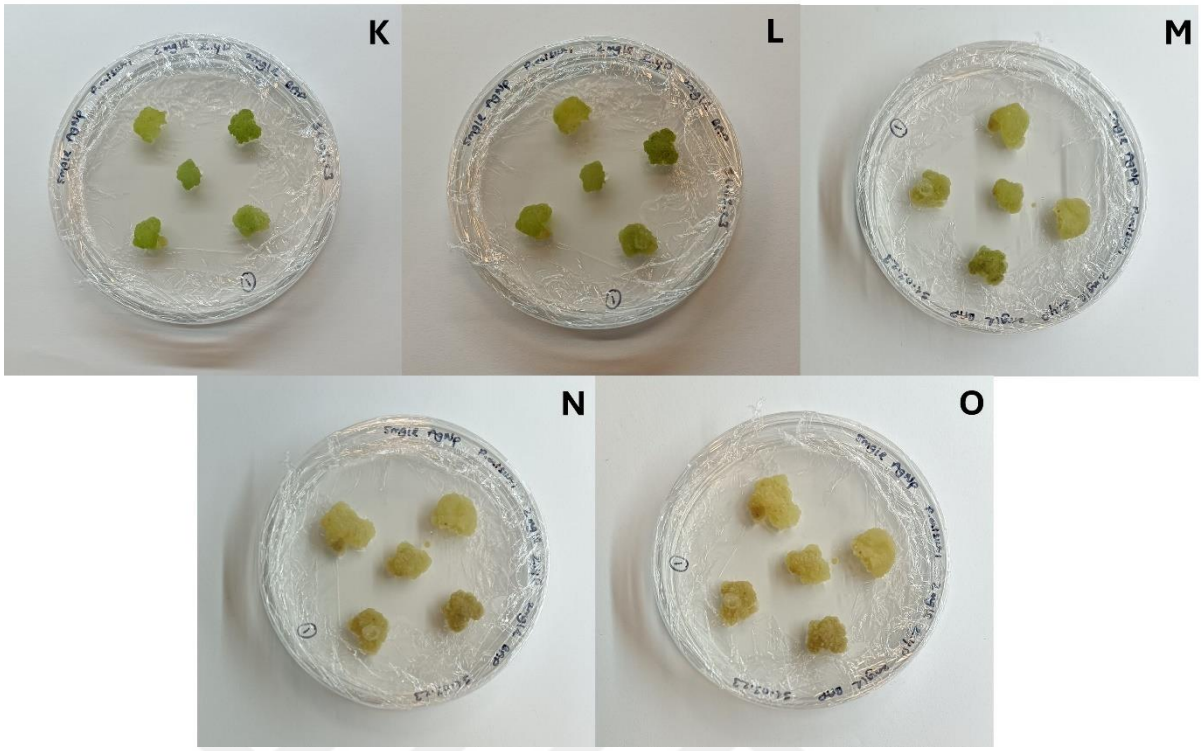


**Figure 20.** Images of the 5 mg/L AgNPs group were taken at the initial stage (K), day 7 (L), day 14 (M), day 28 (N), and day 35 (O).

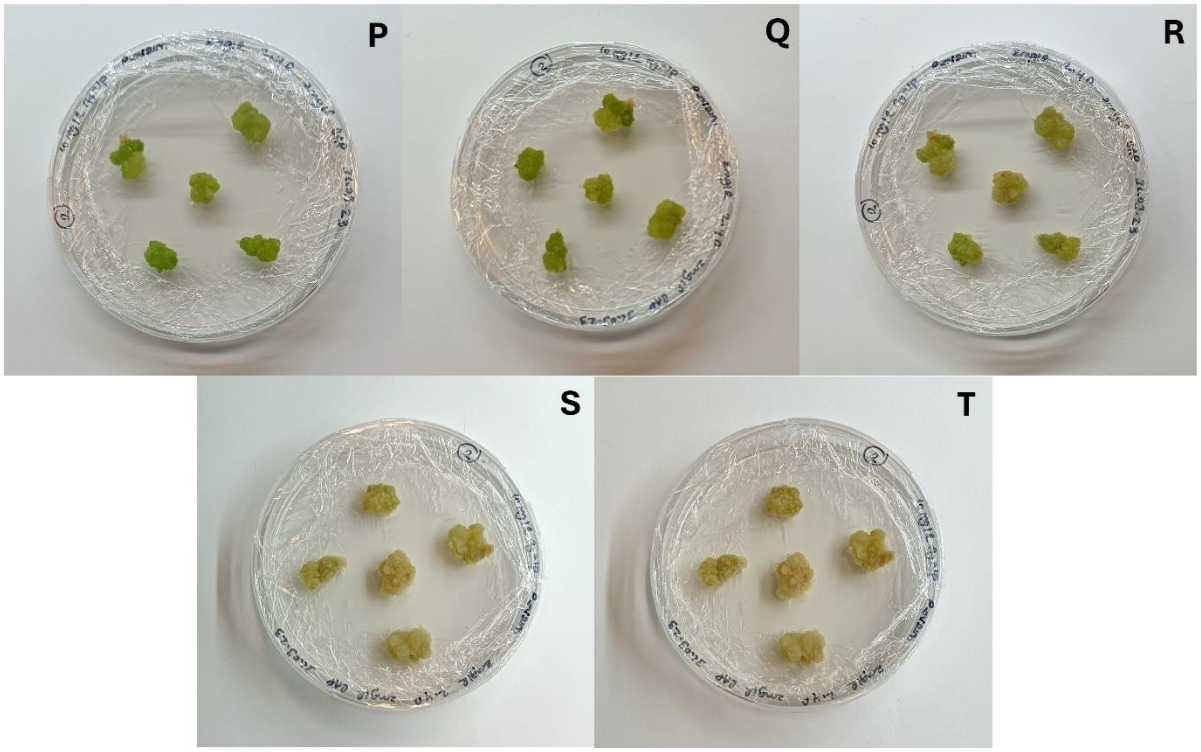


**Figure 21.** Images of the 10 mg/L AgNPs group were taken at the initial stage (P), day 7 (Q), day 14 (R), day 28 (S), and day 35 (T).

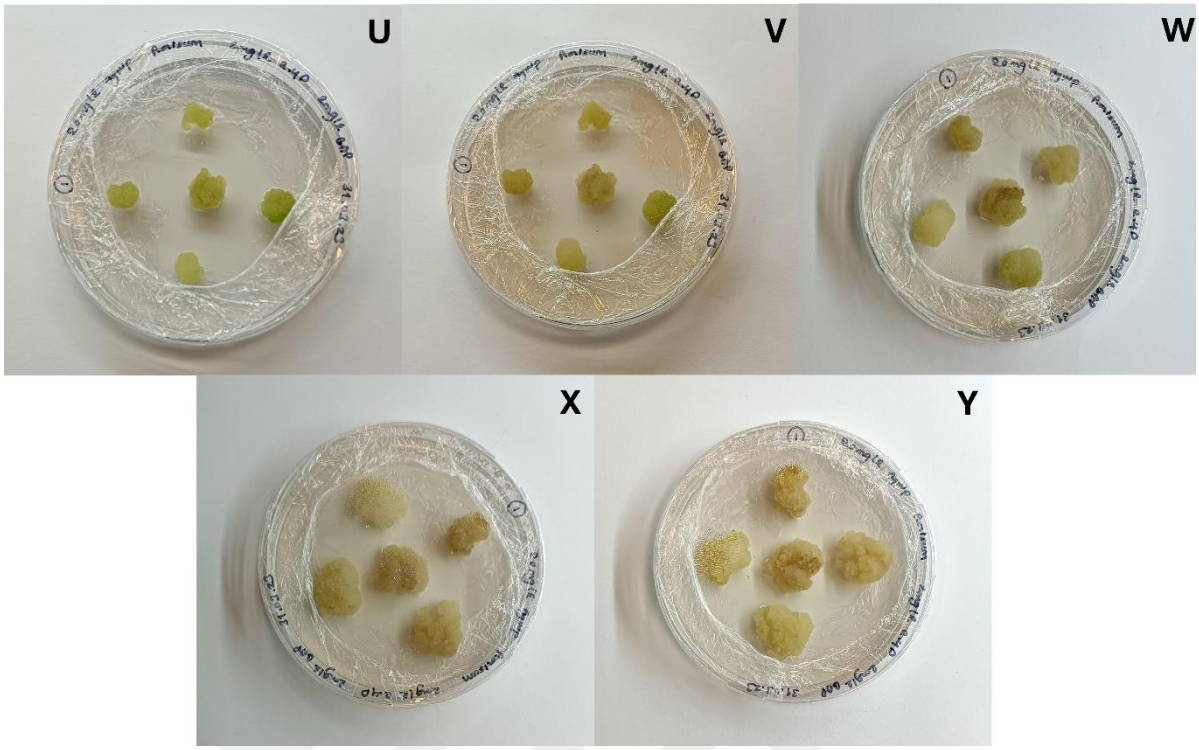


**Figure 22.** Images of the 20 mg/L AgNPs group were taken at the initial stage (U), day 7 (V), day 14 (W), day 28 (X), and day 35 (Y).

### 4.3 Analysis of Anise Secondary Metabolites

GC-MS analysis of hexane and ethanol extracts showed that fatty acid derivatives were the major group in the hexane extracts, whereas carbohydrate derivatives were the major group in the ethanol extracts. Palmitic acid TMS derivative, 9,12-octadecadienoic acid (Z, Z)-TMS derivative and α-Linolenic acid TMS derivative, which are fatty acid derivatives in hexane extract, were found in all experimental groups and were determined to be the major components (Table 1); While total fatty acid derivatives were 86.3% in the control group, it increased to 92.7-96.4 in the AgNP experimental groups. Palmitic acid, one of the defined photo components, has antioxidant properties (Gomathi et al., 2015).

Carbohydrate derivatives constitute the main group in ethanol extract with 69.2% (Table 2). D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 2) and β-D-Glucopyranose derivatives are the main components of this group. A sudden decrease was observed in this dominant group due to the increase in AgNP. This sudden decrease started at 5 mg/L and was also observed in the 10 mg/L and 20 mg/L groups, as well as a high

increase in fatty acid derivatives in these three experimental groups. Among the phenolic compounds, vanillyl alcohol and 4-Hydroxybenzoic were most abundant in the ethanol extract at 20 mg/L. Also, vanillyl alcohol was observed in all AgNP groups. The increase of fatty acids in hexane and ethanol extracts, which form a component of the phospholipid bilayer, may indicate that the plant is trying to remodel or synthesize its membrane to cope and adapt to the stress condition to which it is exposed (Zhang et al., 2018). Anethole, the main component of *P. anisum*, derives its strong aroma, has a strong antioxidant property, and was found in trace amounts in the 5 mg/L and 20 mg/L AgNP groups, although it was not found in the control group in the hexane extract analysis (Spinozzi et al., 2023).

**Table 3.** GC-MS analysis of hexane extracts of *P. anisum*

| RT | RRI | Compound | Control (%) | 1 mg/L AgNPs (%) | 5 mg/L AgNPs (%) | 10 mg/L AgNPs (%) | 20 mg/L AgNPs (%) |
|---|---|---|---|---|---|---|---|
| | | **Fatty Acid Derivatives** | | | | | |
| **28.568** | 1496 | Octadecanoic acid, TMS derivative | - | tr | 0.1 | 0.1 | 0.1 |
| **40.331** | 1890 | Dodecanoic acid, TMS derivative | - | - | - | 0.1 | 0.3 |
| **45.782** | 2108 | Myristic acid, TMS derivative | 0.4 | 0.4 | 0.1 | 0.5 | 0.6 |
| **48.687** | 2233 | Pentadecanoic acid, TMS derivative | 1.1 | 1.1 | 0.8 | 1.3 | 1.6 |
| **53.200** | 2422 | Palmitic asit, TMS derivative | 30.2 | 28.6 | 23.4 | 39.9 | 48.8 |
| **54.182** | 2457 | Heptadecanoic acid, TMS derivative | 0.4 | 1.6 | 4.7 | 1.1 | 1.8 |
| **59.078** | 2606 | Oleic acid, (Z)-, TMS derivative | 1.8 | - | 1.9 | 5.5 | - |
| **59.304** | 2612 | Stearic acid, TMS derivative | - | - | - | - | 8.9 |
| **61.103** | 2655 | Cis- 9,12-Octadecadienoic acid (Z, Z)- TMS derivative | 35.2 | 41.4 | 32.9 | 33.0 | 19.1 |
| **62.654** | 2692 | α- Linolenic acid, TMS derivative | 16.3 | 22.2 | 31.8 | 12.4 | 6.2 |
| **66.249** | 2770 | 11-Eicosenoic acid (E)-, TMS derivative | 0.9 | - | - | - | - |
| **68.995** | 2826 | Arachidic acid, TMS derivative | - | - | - | 1.0 | 2.5 |
| **83.051** | 3088 | Behenic acid, TMS derivative | - | 0.3 | 0.7 | 1.3 | 2.8 |
| | | ***n*-alkane derivative** | | | | | |
| **28.856** | 1504 | Pentadecan | - | tr | tr | - | 0.1 |
| | | **Phenolic content** | | | | | |
| **29.369** | 1520 | Vanillyl alcohol, 2TMS derivative | 2.2 | 1.8 | 0.7 | 2.7 | 3.8 |
| **39.565** | 1862 | t-Anethole | - | tr | 0.1 | - | 0.1 |
| | | **Carbohydrate derivatives** | | | | | |
| **38.022** | 1805 | D-(-)- Tagatofuranose, pentakis (trimethylsilyl) ether (isomer 1) | 1.0 | - | - | - | - |
| **38.485** | 1822 | D-(-)- Tagatofuranose, pentakis (trimethylsilyl) ether (isomer 2) | 2.9 | - | - | - | - |
| | | **TOTAL** | **92.4** | **97.4** | **97.2** | **98.9** | **96.7** |

RT: Retention time; RRI: Relative retention time

**Table 4.** GC-MS analysis of ethanol extracts of P. anisum

| RT | RRI | Compound | Control (%) | 1 mg/L AgNPs (%) | 5 mg/L AgNPs (%) | 10 mg/L AgNPs (%) | 20 mg/L AgNPs (%) |
|---|---|---|---|---|---|---|---|
| | | **Fatty Acid Derivatives** | | | | | |
| **28.636** | 1497 | Octadecanoic acid, TMS derivative | - | - | 0.1 | 0.1 | 0.1 |
| **33.664** | 1655 | Butanedioic acid, 2TMS derivative | - | - | 1.0 | 1.6 | 1.5 |
| **34.133** | 1670 | 2-Butenedioic acid, (E)-, 2TMS derivative | - | - | 0.2 | - | - |
| **37.634** | 1791 | Pentanedioic Acid, 2TMS derivative | - | - | tr | - | - |
| **45.906** | 2113 | Myristic acid, TMS derivative | - | - | 0.2 | 0.4 | 0.4 |
| **48.646** | 2231 | Pentadecanoic acid, TMS derivative | - | - | 1.0 | 0.8 | 0.7 |
| **49.910** | 2288 | Hexadecanoic acid, ethyl ester | 1.0 | - | - | - | - |
| **51.306** | 2347 | Heptadecanoic acid, ethyl ester | - | 2.3 | - | - | 1.3 |
| **52.702** | 2405 | Palmitic acid, TMS derivative | - | - | 40.1 | 40.4 | 38.8 |
| **54.354** | 2464 | Heptadecanoic acid, TMS derivative | - | - | 1.8 | 0.8 | 1.0 |
| **58.410** | 2588 | 9-Octadecanoic acid, (Z)-, TMS derivative | - | - | - | 5.0 | 5.4 |
| **58.914** | 2602 | Stearic acid, TMS derivative | - | - | 4.3 | - | - |
| **59.594** | 2619 | Tridecanoic acid | - | 41.9 | - | - | - |
| **61.627** | 2667 | 9,12-Octadecadienoic acid (Z, Z)-TMS derivative | - | - | 32.8 | 17.8 | 4.6 |
| **62.706** | 2693 | α- Linolenic acid, TMS derivative | - | - | 6.1 | 5.5 | 1.1 |
| **66.679** | 2779 | 11-Eicosenoic acid | 15.6 | - | - | - | - |
| **83.018** | 3087 | Linoelaidic acid | 7.6 | - | - | - | - |
| | | ***n*-alkane derivative** | | | | | |
| **29.011** | 1509 | Pentadecan | - | - | 0.1 | 0.3 | 0.8 |
| **33.079** | 1635 | Hexadecane | 0.7 | - | - | - | - |
| **35.418** | 1713 | Heptadecane | - | - | 0.1 | 0.3 | 0.8 |
| | | **Phenolic content** | | | | | |
| **29.369** | 1520 | Vanillyl alcohol, 2TMS derivative | - | 2.2 | 1.4 | 6.8 | 10.9 |
| **40.490** | 1896 | Salicylic acid, 2TMS derivative | - | - | 0.6 | - | - |
| **44.510** | 2055 | 4-Hydroxybenzoic acid, 2TMS derivative | - | - | 1.3 | 1.3 | 3.7 |
| **40.276** | 1888 | Thiamazole, 2TMS derivative | - | - | 0.2 | - | - |
| | | **Carbohydrate derivatives** | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **36.768** | 1760 | β-D-Ksilopiranoz, 4TMS derivative | 3.0 | 2.5 | - | - | - |
| **36.985** | 1768 | β-Galaktofuranoz, TMS derivative | 9.5 | - | - | - | - |
| **37.665** | 1792 | D-(-)- Tagatofuranose, pentakis (trimethylsilyl) ether (isomer 1) | - | 6.1 | 0.9 | 6.7 | 4.4 |
| **38.05** | 1806 | D-(-)- Tagatofuranose, pentakis (trimethylsilyl) ether (isomer 2) | 26.2 | 24.9 | 0.4 | 1.1 | 11.6 |
| **39.644** | 1865 | β-D-Mannopyranose, 5TMS derivative | 11.1 | 5.9 | - | - | - |
| **42.997** | 1994 | β-D-Glukopiranoz, 5TMS derivative | 19.4 | 8.9 | - | - | - |
| | | **TOTAL** | **94.1** | **94.7** | **92.6** | **88.9** | **87.1** |

RT: Retention time; RRI: Relative retention time

# 5. DISCUSSION

In previous studies, it was concluded that the molecules in the plant stabilized AgNp by reducing $Ag^{+}$ to $Ag^{0,}$ and the synthesis was successful. In AgNP synthesis with aqueous extract of *Barringtonia acutangula* (L.) leaves and phytochemicals such as flavonoids, steroids, and terpenoids were found to act as reducing agents on AgNPs, and the color change from yellow to brown was observed to occur by stimulation of the surface plasmon vibration of AgNPs (Porrawatkul et al., 2017).

In our AgNP synthesis process, after $AgNO^{3}$ was added to the *S. sclarea* leaf extract, a color change in the solution was observed from light yellow to dark brown, and an SPR peak between 400 and 450 nm was observed in the UV-VIS spectrometer analysis at 30 min. The compounds in *S. sclarea* leaf extract acted as reducing agents and converted silver ions into AgNPs. In this direction, we can say that *S. sclarea* leaf extract is successful in AgNP synthesis using an environmentally friendly, sustainable green synthesis method.

In the study by Jiang et al., an increase in intracellular silver content accompanied by root abscission and a decrease in biomass was observed with increasing concentration in *Spirodela polyrhiza* plants exposed to AgNP (Jiang et al., 2012). In a study investigating the elicitor effect of AgNPs obtained from *Syzygium aromaticum* flowers on *Rubia tinctorum* root culture, an increase in total radical scavenging activity was observed, and it was concluded that AgNPs play an increasing role in biomass depending on their concentration (Nartop et al., 2023). In another study, it was concluded that AgNP phytotoxicity was related to the concentration of NPs in AgNP-treated *Physalis peruviana* cultured in vitro. Low concentrations increased plant growth and biomass, while high concentrations decreased the development of plant organs and caused changes in the antioxidant mechanism and plant structure (Timoteo et al., 2019).

When our callus diameters of CSCs exposed to AgNP were examined, the best yield was obtained in the control group, while the lowest callus growth was observed at 5 mg/L and 10 mg/L AgNPs, with a sudden decrease in callus diameter on days 28th and 35th days. No significant differences were observed in callus weight between the groups; however, in general, a time-dependent decrease in callus weight was observed in all groups after day 7. The color change from green to yellow at 1 mg/L, 5 mg/L on day 35,

10 mg/L on day 14, and 20 mg/L on day 7 indicated that AgNPs caused an increase in toxicity in *P. anisum* at high concentrations.

In line with the results we obtained in our experiments and comparisons with previous studies, it was concluded that AgNPs accumulated in *P. anisum* calluses cause toxicity and enter various organelles, causing membrane damage through ROS production, and the plant tries to reshape or synthesize its membrane in the face of this stress. It was concluded that AgNPs accumulated in *P. anisum* calluses caused toxicity and entered various organelles, causing membrane damage through ROS production, and the plant tried to reshape or synthesize its membrane in the face of this stress (Jiang et al., 2012; Yan & Chen, 2019; Zhang et al., 2018).

The PSM content in *P. anisum* varies depending on the geographical region and cultural conditions in which it is grown. It is rich in PSMs and fatty acids from primary metabolites; palmitic acid, a saturated fatty acid, and linoleic and oleic acids, which are unsaturated fatty acids, are abundant examples. Phenolic acids and flavonoids are very important in plants because of their antioxidant and antimicrobial properties and are concentrated in the fruit (seed) part of *P. anisum* (Cahoon & Li-Beisson, 2020; Spinozzi et al., 2023). Changes in ROS as a result of the plant encountering stress cause peroxidation of polyunsaturated fatty acids. It has been concluded that the decrease in biomass after exposure to AgNp may have a negative effect on plant development by causing damage to DNA, lipids, and proteins through excessive ROS production as a result of oxidative stress. When excessive ROS is produced, plant cells defend themselves using antioxidative enzymes and PSMs (e.g., phenolics and flavonoids) (Iori et al., 2023; Lala, 2021). Studies have shown that the amounts of total phenolic compounds and total flavonoids in AgNPs synthesized using *Chenopodium murale* leaf extract are higher than in *C. murale* extract (Abdel-Aziz et al., 2014). Our GC-MS analyses observed an increase in the amounts of linoleic acid and palmitic acid, which are well-known and have economic value (Tables 1 and 2).

In a study analyzing *Oryza sativa* L. (rice) exposed to titanium dioxide ($TiO_2$) NPs, rice appeared to suppress oxidative stress by increasing the amount of non-enzymatic antioxidants such as unsaturated fatty acids, amino acids, and secondary metabolites. As a result of the increase in non-enzymatic antioxidants, the content of secondary metabolites, amino acids, and fatty acids also increased. Nano-$TiO_2$ induced

ROS production, and since linolenic acid is abundant in plant membrane lipids, an increase in the amount of linolenic acid in NP-exposed rice leaves was observed, which was associated with membrane lipid peroxidation (Wu et al., 2017).

In the analysis of our hexane extracts of P. anisum, palmitic acid TMS, 9,12-octadecadienoic acid (Z, Z)-TMS, and α-linolenic acid TMS derivatives formed the main group. While total fatty acid derivatives were 86.3% in the control group, they increased to 92.7-96.4 in the AgNP experimental groups. The main group in the ethanol extract of P. anisum was carbohydrate derivatives (69.2 %). D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 2), and β-d-glucopyranose were the most abundant derivatives. Due to the increase in AgNP, a sudden decrease was observed in this dominant group, starting at 5 mg/L, 10 mg/L, and 20 mg/L, while a high increase in fatty acid derivatives was observed in the same groups.

In a previous study, the effects of AgNPs, copper nanoparticles (CuNPs), and gold nanoparticles (AuNPs) on *Artemisia absinthium* were investigated, and better results were obtained with AgNPs in terms of total phenolic and flavonoid content produced by plants as a defense against stress (Hussain et al., 2017). In a study conducted by Chung et al., an increase in phenolic and flavonoid content was observed in the phytochemical analysis of biosynthesized AgNPs applied to bitter gourd CSC after 28 days, and a remarkable increase in hydroxybenzoic acid content was observed compared to the control group (Chung et al., 2018a). In another study, an increase in phenolic compounds, such as hydroxybenzoic and flavonols, was observed in the elicitation of hairy root cultures of Chinese cabbage with CuO (copper oxide) NPs (Chung et al., 2018b). In our GC-MS analysis of *P. anisum* ethanol extract, vanillyl alcohol and 4-hydroxybenzoic derivatives from phenolic compounds were detected, and the highest concentration was 20 mg/L.

# 6. CONCLUSIONS AND RECOMMENDATIONS

This study showed that nanoparticles synthesized from *S. sclarea* leaves obtained by the green synthesis method were successfully synthesized and increased the fatty acid content of *P. anisum* CSCs. In light of the investigations carried out in the initial studies, it was concluded that this increase was due to the disruption of the plant cell membrane caused by toxicity. Phenolic compounds were found to contain vanillic alcohol and 4-hydroxybenzoic acids, whereas trace amounts of t-anethol were found at 20 mg/L. It is thought that this has the potential to create preliminary information in studies to be carried out in the production of medically important PMSs. In the hexane extract of fatty acids, palmitic acid increased significantly after 10 mg/L, and linolenic acid increased significantly after 1 mg/L. The increase in palmitic acid and other fatty acids, which have antioxidant properties, against AgNp stress suggests that PSMs can be obtained in an effortless and environmentally friendly way by tissue culture and that investigating the effect of other medicinal plants on the content of plants with medicinal value can yield good results in many areas, such as agriculture, health, and cosmetics industries.

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