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Green bio-inspired synthesis, characterization and activity of silver nanoparticle forms of *Centaurea virgata* Lam. and the isolated flavonoid eupatorin

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Abstract: A green synthesis method of silver nanoparticles (AgNPs) using *Centaurea virgata* Lam. extract and the isolated compound eupatorin was investigated in this study. Ultraviolet-visible (UV-Vis) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM)/energy-dispersive X-ray (EDX) spectroscopy, thermal gravimetric analysis, X-ray diffraction analysis and zeta potential were used for characterization of AgNPs. The UV-Vis spectrum exhibited a characteristic absorption band at 420 nm for monodisperse nanoparticles. FTIR measurements also proved the formation. X-ray diffraction patterns showed peaks at (110) and (112), which are characteristic for hexagonal crystals and also showed peaks at (111), (200) and (240), which are characteristic for orthorhombic crystals. The TEM images of AgNPs show that the morphology of AgNPs was predominantly spherical. Obtained AgNPs were highly stable according to the zeta potential values. The nitric oxide scavenging activity, which is also related to anticancer activity, of AgNPs was evaluated. It can be concluded that *C. virgata* Lam. extract and eupatorin can be used as a reducing agent for potential antioxidant AgNP formation.

Keywords: AgNPs; *Centaurea virgata* Lam.; eupatorin; isolation; nitric oxide scavenging activity.

1 Introduction

Nanotechnology is one of the most important areas in modern science technologies in recent years. It takes science in the frame of 1–100 nm particle size [1]. The word

nanotechnology was first used by Nario Taniguchi in 1974. The word “nanotechnology” comes from the Greek origin word “nano” which means dwarf [2].

Nanomaterials have a wide application area such as physics, chemistry, electronic, optic and biomedical [3]. The most important advantage of nanoparticle utilization is to increase or develop the special properties which appear in the nanoscale dimensions of the material. Methods such as using chemical and physical processes can be listed as follows; microwave dielectric, heating reduction, ultrasonic irradiation, radiolysis, solvothermal synthesis and electrochemical synthesis [3–6]. In synthetic methods silver nanoparticles (AgNPs) are obtained in liquid state. Therefore, they are stable only in the form of metal salts at low concentrations [5]. Besides, heavy organic solvents and toxic reducing agents are used in most synthetic methods such as hydrazine N,N dimethylformamide and sodium borohydride. These chemicals are highly reactive and pose potential environmental toxicity and biological hazards [7]. Increased interest in green synthesis is due to increased environmental awareness [3, 7]. Raveendran and his friends were the first ones which realized nanoparticle formation by green synthesis. β -D-Glucose was used as reducing agent and starch as capping agent to prepare silver nanoparticles [8]. After this a significant increase in the use of green synthesis was observed. Microorganisms, enzymes, plants and plant extract can be used as reducing agents in green synthesis. In plant extracts as our research secondary metabolites like flavonoids, phenolic acids, terpenes from plants helps reducing the metal ions for nanoparticle formation [9].

Centaurea is a well-known genus from the Asteraceae family and has a lot of species in Turkey. *Centaurea virgata* Lam. is a plant which grows in different regions of Turkey such as Elazığ, Diyarbakır and Konya. As a result of ethnobotanical studies, it has been shown that this plant was used for ulcers, diabetes and allergy in folk medicine. Chemical studies demonstrate that *Centaurea* genus mostly includes flavonoids, phenolic acids, sesquiterpene lactones, triterpenes and lignans [10]. According to the secondary metabolite content of *Centaurea* genus, the plant *C. virgata* was thought to be used as a reducing agent [9]. After nanoparticle

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formation, the usage of the plants would be increased and developed. In this research we demonstrate the formation of silver nanoparticles using *C. virgata* plant extract and eupatorin, a flavonoid compound which isolated from the extract. Nitric oxide activities of each sample before and after formation was evaluated.

2 Materials and methods

2.1 Plant material

Centaurea virgata Lam. was collected from Elazig, Turkey (2011). The plant was identified by Doc. Dr. Ugur Cakilcioglu from Munzur University. The voucher specimen was deposited in Ege University Faculty of Pharmacy, Pharmacognosy Department with the code 1461.

2.2 Preparation of plant extract

The aerial parts of the plant (1.5 kg) were first extracted with methanol. After evaporation, the residue was dissolved in methanol (Sigma-Aldrich, St. Louis, MO, USA) (MeOH):water (H₂O) (50:50). This solution was initially extracted with 5 × 500 ml n-hexane and then 5 × 500 ml chloroform (Sigma-Aldrich, St. Louis, MO, USA) (CHCl₃). The organic phase was evaporated until dryness.

2.3 Isolation of eupatorin from the chloroform extract

The antioxidant activities of n-hexane, chloroform and methanol:water extracts were investigated. The chloroform extract (CV) was found to be the most active one according to a nitric oxide assay. Therefore, it was used to isolate the main compound eupatorin. Open column chromatography was performed by using the solvents shown in Table 1. As a result, 12 main fractions were obtained. Fraction **CVI₁₀₋₁₁** from this chromatography was further fractionated by VLC (vacuum liquid chromatography) using solvent systems I, II, III, IV and V (Table 1). C₁ (subfraction) was further fractionated by preparative thin layer chromatography using solvent system V. Eupatorin (2.8 mg) was obtained by this purification. Another main fraction **CVI₇** was crystallized by using acetone and 258.6 mg eupatorin was crystallized.

Table 1: Solvent systems.

No.	Solvent systems	Ratio
I	Cyclohexane:chloroform:methanol	(5:9:1)
II	Cyclohexane:chloroform:methanol	(8:9:1.5)
III	Cyclohexane:chloroform:methanol	(4:10:1.5)
IV	Cyclohexane:chloroform:methanol	(4:9:2)
V	Chloroform:methanol	(98:2)

2.4 Quantification of eupatorin by liquid chromatography-mass spectrometry

During the isolation process, eupatorin was found to be the major compound of the chloroform extract. The eupatorin content was examined by liquid chromatography-mass spectrometry (LC-MS) by a Shimadzu Liquid Chromatographic System. Eupatorin was selected for reduction of AgNPs.

2.5 Synthesis of AgNPs

Some 10 g of chloroform extract was dissolved in 10 ml of methanol. Each 5 ml of the extract solution was mixed with 100 ml AgNO₃ (Sigma-Aldrich, St. Louis, MO, USA) solution. After 60 min incubation at 55°C (LAB-LINE MaxQ6000), centrifugation by a Beckman Coulter Optima L100 XP branded centrifuge for 30 min at 4750 rpm and 24°C was performed. The supernatant part was gently poured and the residue was lyophilized. For lyophilization, a Labconco instrument was used. The same procedures were administrated also for the isolated eupatorin.

2.6 Characterization of AgNPs

2.6.1 Ultraviolet-visible spectroscopy: UV characterization is a preliminary study for nanoparticle synthesis. Synthesized AgNPs absorption peaks were examined to prove that functional groups were connected to silver ions [11]. Ultraviolet-visible (UV-Vis) spectra were recorded as a function of the reaction time on a Thermo Evolution Array UV-Vis spectrophotometer (Evolution Array; Thermo Scientific, Waltham, MA, USA) in the range of 200 nm–800 nm at room temperature. Quartz cuvettes were used for the experiment. The reaction mixture was checked every 30 min between 0 and 150 min.

2.6.2 Fourier transform infrared spectroscopy: The Fourier transform infrared (FTIR) characterization method was used to determine the stability and the responsible biomolecules to be attached to the silver ions [12]. Potassium bromide pellets were made for each experiment. A Perkin-Elmer, Spectrum 100 spectrometer was used for the analyses in the range of 4000–280 cm⁻¹ (Perkin Elmer Inc., Wellesley, MA, USA). According to these analyses, we can also determine the bonds responsible for the nanoparticle formation.

2.6.3 Transmission electron microscopy analysis: Transmission electron microscopy (TEM) analysis was performed to take a microscope image and determine the particle size [13]. The AgNPs images were taken by a Libra 120 transmission electron microscope (Carl Zeiss, Oberkochen, Germany). The microscope takes images by using electronic radiation and detects the electrons which are transported through the sample.

2.6.4 Scanning electron microscopy/energy-dispersive X-ray spectroscopy: Scanning electron microscopy (SEM) analysis gives more detailed information about the physical characteristics rather than TEM analysis [14]. This characterization method shows the size and shape of the nanoparticles. An SEM 250 FEG Quanta instrument was used for the determination. An energy-dispersive X-ray (EDX)

study was used to learn about the elemental composition. For the analysis, an SEM 250 FEG Quanta instrument was used with an EDX detector (Oxford Azteck).

2.6.5 Thermal gravimetric analysis: The thermal gravimetric analysis method was used for characterization with a digit temperature rise to determine the thermal stability of the AgNPs [15]. A Perkin-Elmer Diamond TG/DTA (Thermogravimetric and Differential Thermal Analysis) was used for the experiments.

2.6.6 X-ray diffraction analysis: The nanoparticles were analyzed for the formation of AgNPs at room temperature by examining their diffraction Rigaku Ultima IV X-ray diffractometer (Rigaku Corp., Tokyo, Japan) was used as a device. The device was operated at a voltage of 45 kV. Copper potassium alpha radiation (40 mA) was also used.

2.6.7 Particle size distribution and zeta potential: Both particle size distribution and zeta potential are critical physical parameters for the information of homogeneity, stability and functionality [16]. High zeta potential (+, -) shows the stability. Particle size distribution and polydispersity index were examined at room temperature. A polydispersity index higher than 0.5 shows that the formation is not homogenous. The angle was 137° . Zeta potential was also evaluated at room temperature using the angle 17° , with 78.5 dielectric constant under an electrical field of 15 v/cm. The instrument was the same for both experiments: a Nano-ZS Zetasizer (Zetasizer-Nano-ZS, Malvern, UK). Samples were diluted with distilled water.

2.6.8 Determination of total phenolic content: Determination of total phenolic content of *C. virgata* extracts was performed using the Folin-Ciocalteu method [17]. Some 0.1 ml of each extract was mixed with 2.8 ml distilled water. Amounts of 2 ml 2% sodium carbonate and 0.1 ml 0.1 N Folin-Ciocalteu reagent were added to the mixture. The absorbance of the mixture was measured at 750 nm after incubation for 30 min at room temperature. Distilled water was used as blank solution. A UNIQCAM 8625 UV/Vis spectrometer was used for the analysis. The standard curve was determined by gallic acid.

2.7 Determination of total flavonoid content

Total flavonoid content of *C. virgata* extracts was determined using the aluminum chloride colorimetric method [18]. Some 0.5 ml of each extract was mixed with 1.5 ml ethanol, 0.1 ml 10% aluminum chloride solution and 2.8 ml distilled water. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm by the UNIQCAM 8625 UV/Vis spectrometer. Quercetin was used as control.

2.8 Nitric oxide radical scavenging assay

Nitric oxide radical scavenging activities of the CV, eupatorin and the nanoparticles of *C. virgata* extract (AgNP_{scv}) and eupatorin (AgNP_{se}) were determined. A nitric oxide radical scavenging assay was done according to the Griees reaction. Griees reagent is a mixture of equal amounts of 0.1% N-(1-naftyl)etylenediamine dihydrochloride (NED) and 1% sulfanilic acid. The nitrite standard curve was prepared by

diluting 0.1 M nitrite standard [19]. NED and sulfanilic acid solution were incubated at room temperature for 30 min. Extracts were mixed by Griees reagent and distilled water and incubated for 30 min. After the incubation, the absorbance was measured at 548 nm by the UNIQCAM 8625 UV/Vis spectrometer.

3 Results and discussion

Eupatorin was isolated from the chloroform (CHCl₃) phase of the methanol (MeOH) extract of *C. virgata* by means of multistep chromatography (open column chromatography), vacuum liquid chromatography (VLC), preparative thin layer chromatography and crystallization. The concentration of eupatorin in the chloroform extract was determined by LC-MS (Table 2), and it was found that it contains 2.96% eupatorin.

UV-Vis spectroscopy was performed before and after formation of AgNPs. During the formation of nanoparticles, the aqueous solution was examined for its UV spectrum every 30 min for 150 min. The color change from yellow to brownish after incubation shows that AgNPs induce surface plasmon resonance (SPR). The SPR was due to the presence of free electrons present on AgNPs.

The spectrum (Figure 1) proves the formation of a new peak at about 420 nm, which is characteristic for monodisperse AgNPs [20]. Additionally having SPR range between 390–420 nm shows that particles are in small range around 25–50 nm. The AgNPs are expected in spherical shape because of the SPR around 410–450 nm. Longer wavelength shows smaller size of AgNPs [21]. For eupatorin and the AgNP_{se} color change was observed, however, the peak around 420 nm could not be seen because of more polydisperse formation (Figure 2) [22]. Because the characteristic peaks for all AgNPs were between 200 nm and 400 nm and the intensity was changed, the investigations were continued.

The FTIR spectrum of the extract gives absorbance bands as 3418 cm⁻¹, 2931 cm⁻¹, 1715 cm⁻¹, 1656 cm⁻¹, 1365 cm⁻¹, 1077 cm⁻¹ and 818 cm⁻¹. After the formation of AgNPs from CV, the absorbance band 3418 cm⁻¹, which is responsible for

Table 2: Quantification of eupatorin.

	Eupatorin (μg)
CHCl ₃ extract	405.98
	405.06
	405.80
Average	405.61
STD	0.4876
%	2.97

Figure 1: Ultraviolet-visible (UV-Vis) spectrum of CV and AgNP_{SCV}.

Figure 2: Ultraviolet-visible (UV-Vis) spectrum of eupatorin (EUP) and AgNP_{SE}.

the O-H stretching, was shifted to 3434 cm^{-1} [21]. Also, the intensity of absorbance bands characteristic for a lactone ring, 1715 cm^{-1} , 1656 cm^{-1} and 1608 cm^{-1} , decreased and shifted to 1763 cm^{-1} [23]. This shows that nanoparticle formation has broken the ring. A band at 2395 cm^{-1} occurred because of the reaction of the extract with AgNO_3 [11]. This band is responsible for O-C-O (Figure 3). For eupatorin, there were characteristic bands for flavonoids such as C=O at 1651 cm^{-1} , O-H at 3419 cm^{-1} , benzopyrane ring at 1046 cm^{-1} and between 800 cm^{-1} and 500 cm^{-1} , benzene substitutions.

The FTIR spectrum of AgNP_{SE} showed lower intensity for all bands and O-H and C=O bands were shifted. All these alterations prove AgNP formation, especially based on O-H stretching and C=O stretching (Figure 4) [24].

TEM scanning is very important to learn about morphology and particle size. Figure 5 demonstrates that AgNP_{SCV} is spherical and about 40–50 nm. As we see (Figure 6), AgNP_{SE} images show that nanoparticles are also spherical. Particle size distribution was shown in two different ranges: 45–55 nm and 25–30 nm.

Figure 3: Fourier transform infrared (FTIR) spectra of CV and AgNP_{SCV}.

Figure 4: Fourier transform infrared (FTIR) spectra of eupatorin (EUP) and AgNP_{SE}.

An SEM scanning microscope is capable of physical characterization, crystallography and particle size determination [14, 25].

According to Figure 7, AgNP_{SCV} crystals dispersed equally, which proves homogeneity. Particle size was determined at about 40–45 nm. More polydisperse nanoparticles of AgNP_{SE} shows that nanoparticle sizes are in two different ranges: 25–30 nm and about 50 nm (Figure 8). Based on the investigations of EDX analyses, a sharp silver peak for both nanoparticles was observed (Figures 9 and 10), which proves the silver crystals [26].

Thermal gravimetric analyses were performed to prove thermal stability. The first weight loss of AgNP_{SCV} started at 200–250°C. This first step shows that the water molecules

bind softly to biomolecules loss. Up to 500°C, the 53.597% loss was found because of the bioorganic compound desorption. The first weight loss for AgNP_{SE} began at 193.12°C because of water loss. After 500°C, the total weight loss was 75%, which shows AgNP_{CV} is more thermostable than AgNP_{SE} [15].

The X-ray diffraction pattern of AgNP_{SCV} gave fingerprint diffraction peaks for AgNPs (Figure 11). The crystalline planes of nanosilver are (111), (200), (220), (240) and (400). There are two forms of crystals: orthorhombic and cubic [21, 27]. The (111), (200) and (240) crystalline planes are characteristic for orthorhombic crystal forms.

In Figure 12, hexagonal and monoclinic crystals of AgNP_{SE} showed distinct diffraction peaks. Crystalline

Figure 5: Transmission electron microscopy (TEM) image of AgNP_{SCV}.

Figure 7: Scanning electron microscopy (SEM) image of AgNP_{SCV}.

Figure 6: Transmission electron microscopy (TEM) image of AgNP_{SE}.

Figure 8: Scanning electron microscopy (SEM) image of AgNP_{SE}.

planes of silver nanoparticles are (110), (220), (222). (110) and (112) planes proves the hexagonal form of the crystals [27].

The repellency of the nanoparticles to each other is verified by the negative zeta potential. This increases the stability of the formulation. The repulsion protects them from forming an association and therefore protects them from agglomeration [28]. According to Figures 13 and 14, zeta potential results prove highly stable nanoparticles both for AgNP_{SCV} and AgNP_{SE} in the order of -25.6 and -22.6 [21, 29]. Particle size distributions of AgNP_{SCV} and AgNP_{SE}

Figure 9: Energy-dispersive X-ray (EDX) analysis of AgNP_{SCV}.

Figure 10: Energy-dispersive X-ray (EDX) analysis of AgNP_{SE}.

Figure 11: X-ray diffraction (XRD) pattern of silver nanoparticles obtained from AgNP_{SCV}.

Figure 12: X-ray diffraction (XRD) pattern of silver nanoparticles obtained from AgNP_{SE}.

show, respectively, 38.65 nm and 38.0 nm (Figures 15 and 16) [30].

Total phenolic and flavonoid contents of the extracts are shown in Table 3. Moreover, nitric oxide scavenging activity was determined for all extracts and the nanoparticles. The results are shown in Table 4. AgNP_{SCV} and AgNP_{SE} were more active than CV extract and eupatorin. After

Figure 13: Zeta potential of AgNP_{SCV}.

Figure 14: Zeta potential of AgNP_{SE}.

Figure 15: Particle size distribution graphic of AgNP_{SCV}.

Figure 16: Particle size distribution graphic of AgNP_{SE}.

Table 3: CV total flavonoid and phenolic content.

Sample	% Total flavonoid content	% Total phenolic content
CV	3.86 ± 0.01	4.86 ± 0.003

Table 4: Nitric oxide scavenging activity of CV, AgNP_{SCV}, eupatorin (EUP), AgNP_{SE}.

Samples	% Scavenging activity
CV	3.44 ± 0.001
AgNP _{SCV}	7.48 ± 0.002
EUP	1.83 ± 0.001
AgNP _{SE}	6.85 ± 0.012

nanoparticle formation, eupatorin and CV became more active than prior to. This demonstrates that after nanoparticle formation, activity potential changes and this is very important for medical uses.

4 Conclusion

To our knowledge, this is the first study of *C. virgata* and eupatorin nanoparticle formation and comparison of their activity. Bioreduction of the silver ions was demonstrated. CV extract and eupatorin were used as reducing agents. Characterization proved the stable active nanoparticle formation. Activity investigations of the extract, eupatorin, AgNP_{CV} and AgNP_E showed that AgNPs of the starting materials became more active. Our results suggest that AgNPs are more active. The ecofriendly formation, less toxicity and greater activity of AgNP_{SCV} and AgNP_{SE} makes them promising for use in different medicinal areas.

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