

Araştırma Makalesi - Research Article

Green Synthesis, Optimization, and Characterization of Silver Nanoparticles from *Euphorbia rigida* Leaf Extract and Investigation of Their Antimicrobial Activities

Euphorbia rigida Yaprak Ekstraktından Gümüş Nanopartiküllerin Yeşil Sentezi, Optimizasyonu ve Karakterizasyonu ve Antimikrobiyal Aktivitelerinin Araştırılması

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ABSTRACT

Green synthesis is a biological method known for being environment-friendly, cheap and for retrieval of nanoparticles with ease, which is preferred more than chemical and physical methods. Various metallic nanoparticles with potent bioactivities have been synthesized using plant extracts. In this study, synthesis of silver nanoparticles (AgNPs) using the aqueous leaf extract of *Euphorbia rigida* was investigated. Their antibacterial activity was determined using microdilution methods. The optimization of the synthesis was performed via the adjustment of different parameters including pH, temperature, concentration of the plant extract, the different concentrations of AgNO₃, and synthesis time using UV-visible spectroscopy. Silver nanoparticles (Er-AgNPs) that were synthesized by *Euphorbia rigida* characterized by Dynamic Light Scattering (DLS) and Scanning Electron Microscope (SEM) analyses and their antimicrobial potential were tested on six different pathogens. The synthesis process resulted in spherical-shaped NPs with a size ranging between 50-75nm. The AgNPs demonstrated a strong antimicrobial activity against various pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecium*, *Staphylococcus epidermidis*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Listeria monocytogenes* and *Candida albicans*). The results showed that the synthesized Er-AgNPs can be used as a biological material for biomedical, pharmaceutical, biotechnology and industrial areas. In this study, the antimicrobial potential of silver nanoparticles which are prepared using *Euphorbia rigida* leaf extract is reported for the first time.

Keywords- Antimicrobial Activity, Dynamic Light Scattering, *Euphorbia Rigida*, Scanning Electron Microscope, Silver Nanoparticle

ÖZ

Yeşil sentez; çevre dostu ve maliyet açısından ucuz olması, kimyasal ve fiziksel yöntemlerden daha çok tercih edilen nanopartiküllerin kolay bir şekilde elde edilmesi ile bilinen biyolojik bir yöntemdir. Potansiyel biyolojik aktiviteye sahip çeşitli metalik nanopartiküller bitki özütleri kullanılarak sentezlenmektedir. Bu çalışmada, sütlegengil (*Euphorbia rigida*) yaprak sulu özütü kullanılarak, yeşil sentez yöntemi ile gümüş nanopartikülleri (AgNP) sentezlendi. Sentezlenen nanopartiküllerin antimikrobiyal aktiviteleri mikrodilüsyon yöntemi ile belirlendi. Yeşil sentezin optimizasyonu, sıcaklık, pH, sentez zamanı, AgNO₃ ve bitki özütünün farklı konsantrasyonları gibi farklı parametrelerin denenmesi ile gerçekleştirildi. *Euphorbia rigida* yaprak özütünden sentezlenen gümüş nanopartiküller (Er-AgNPs), Ultraviyole/görünür ışık absorpsiyon spektrofotometresi (Uv-

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vis), Dinamik ışık saçılımı (DLS), Taramalı Elektron Mikroskobu (SEM) analizleri ile karakterize edildi. Altı farklı patojene karşı antimikrobiyal aktiviteleri belirlendi. Bu çalışmada, *Euphorbia rigida* bitkisinin yaprak özütüne ait örneğin parçacık boyutunun histogramının 50 ile 75 nm olan küresel yapılı Ag nanopartiküller sentezlendi. Er-AgNPs'ler çeşitli patojenlere (*Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecium*, *Staphylococcus epidermidis*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Listeria monocytogenes* and *Candida albicans*) karşı güçlü bir antimikrobiyal aktivite gösterdi. Sonuç olarak, sentezlenen Er-AgNPs biyomedikal, farmasötik, biyoteknoloji ve endüstriyel alandaki uygulamalar için biyolojik bir materyal olarak kullanılabilir. Bu çalışmada, ilk kez *Euphorbia rigida* yaprak özütü kullanılarak hazırlanan gümüş nanopartiküllerin antimikrobiyal aktivitesi belirlendi.

Anahtar Kelimeler- Antimikrobiyal Aktivite, Dinamik Işık Saçılımı, Euphorbia Rigida, Taramalı Elektron Mikroskobu, Gümüş Nanopartikül

I. INTRODUCTION

A nanoparticle is a microscopic particle with size ranging from 10 to 1000 nm. Nanotechnology is a field of science that has a wide range of applications in biomedical sciences, pharmacology, food packaging materials, and personal care products, as well as its use as catalysts, drug carriers, and pollutants. In recent years, the field of nanotechnology has developed tremendously in terms of scientific research and applications [1]. The synthesis of nanoparticles was carried out by chemical, physical, and biological methods [2]. The synthesis of nanoparticles by biological method provides superiority to physical and chemical methods due to its low cost, simplicity of process, less chemical use, less energy requirement, and environmental friendliness [3]. Recently, many approaches by biological synthesis method have been developed to synthesize different nanoparticles. Fungi, bacteria, algae, yeast, and different green resources, such as plants are used for the synthesis of nanoparticles. Various methods, such as chemical reduction, ultrasonic radiation, laser ablation, evaporative cooling, sol-gel, microwave irradiation, photo reduction, thermal decomposition, and biological synthesis have been used to synthesize silver nanoparticles. Bacteria, fungi, actinomycetes, algae, and plant extracts are used for the synthesis of silver nanoparticles (AgNP) by biological method. Application areas of silver nanoparticles are agriculture, textile industry, cosmetics, food industry, as well as biosensor, catalyst in chemical reactions, optical receptor in nonlinear optics, antimicrobial agent, antiseptic for medical devices, and water disinfection. Silver nanoparticles have attracted more attention among metallic nanoparticles because of their various biological activities, including antioxidant, antibacterial, anticancer, antiinflammatory, antimicrobial and antifungal [4]. *Euphorbia* is the largest genus among *Euphorbiaceae* family, consisting of laticiferous plants. *Euphorbia* is represented by 106 species, with subspecies and variety total 116 taxa, 13 taxa of which are endemic and endemism ratio is near 9% in Turkey. Some *Euphorbia* species are used in the treatment of skin problems, cancer, wounds, migraines, and worms. Compounds isolated from the genus *Euphorbia* and its extracts have protective, cytotoxic, antimicrobial, antiinflammatory, anticancer, and antioxidant effects [5]. To our knowledge, there have been no reports of AgNPs synthesis using *Euphorbia rigidaleaf* aqueous extract (*E.rigida*) as a reducing agent. The present study aimed to use *E.rigida* for the synthesis of AgNPs and evaluate the biological activities of the prepared nanoparticles. The leaf extract was first analyzed for its content using different biochemical methods. The synthesis process was optimized depending on time, temperature, and concentrations. The prepared NPs were characterized by UV-Vis spectral analysis, dynamic light scattering (DLS), and scanning electron microscopy (SEM). Lastly, the AgNPs were tested for their antimicrobial effect over different pathogen strands.

II. MATERIALS AND METHODS

A. Preparation of *Euphorbia rigida* Leaf Aqueous Extract

Euphorbia rigida (Euphorbiaceae) was used as a source in this study. *Euphorbia rigida* (*E.rigida*) plant samples were obtained by the Department of Botany of the Department of Biology of the Ege University. The green leaves of the plant were collected, cleaned with distilled water. The leaves were then left for drying at room temperature. The dried leaves were pulverized by pasting in mortar (Retsch™ RM 200 Mortar). Then 10 gr *E.rigida* leaves mixed with 100mL distilled water then, boiled for 30 min at 90° C. The extract solution was left

to cool at room temperature and centrifuged at 10000 rpm for 20 min. The resulting *E.rigida* aqueous leaf extract filtered with Whatman No.1strainer paper and stored in refrigerator at +4 °C in dark glass bottle for use as stock solution.

B. Phytochemical Analysis of *Euphorbia rigida* Leaf Extract Content

Phytochemical screening analyses of *E.rigida* aqueous extract were made by standard biochemical methods [6].

C. Total Phenolic Compound Determination

The total phenolic compound content of plant extracts was determined by the spectrophotometric method according to the phenol reaction of Folin-Ciocalteu [7]. Briefly, 100 µL of *E.rigida* leaf extract was mixed with 1mL Folin- Ciocalteu reagent (10%) then, the mixture was incubated in the dark for 5 min at room temperature. After, sodium carbonate (Na₂CO₃, 7.5%, 800 µL) was added. The solutions were left for 60 min. and their absorbance was measured with a UV-Vis. spectrophotometer at 760 nm. Gallic acid (5-100 µg/mL) was used as a standard. The samples were repeated 3 times for each analysis and the total phenolic content was calculated as µg/mL gallic acid equivalent (µg/mL GAE) by using gallic acid calibration curve.

D. Total Flavonoid Compound Determination

Determination of total flavanoid content of plant extras was carried out by the spectrophotometric method [8]. The mixture contained 100 µL *E.rigida* leaf extract and 100 µL of 2% AlCl₃ solution dissolved in methanol. The samples were incubated for 10 min at room temperature and their absorbances were measured with a UV-Vis. spectrophotometer at 415 nm. Quercetin (50-100 µg/mL) was used as a standard. The samples were repeated 3 times for each analysis and total flavonoids were calculated using the quercetin standard curve.

E. Tannin Compound Determination

The method used for tannin determination is the same as the one described for phenolic content [7]. Instead of gallic acid as standard, tannic acid was employed for the preparation of the standard curve and after reading at 760 nm sample results were presented as tannic acid equivalent (TAE) µg/mL.

F. Silver Nanoparticles Green Synthesis

The optimization of the nanoparticles was performed by modifying various parameters. Different concentrations of silver nitrate (1.0, 2.0, 4.0, 6.0, 8.0, and 10 mM), *E.rigida* (0.04, 0.06, 0.08, 0.1 %), pH (3.0, 5.0, 7.0, 9.0, and 11), temperature (25, 37, 40, 50 and 80°C), and the reaction time (15, 30, 60, 90, 120, 180, and 240 min) were applied in order to determine the optimum parameters for the AgNPs synthesis using *E.rigida* leaf extract.

G. Characterization of The Synthesized Er-AgNPs

The silver nanoparticles obtained by using optimized parameters were characterized using appropriate spectral analysis methods. Absorption spectra of AgNPs solution was monitored by a UV-Vis spectrophotometer (Perkin Elmer Lambda 35 UV/VIS Spectrometer) from 300 to 800 nm. The diluted supernatant (1mL) of the Er-AgNPs sample was deposited in a quartz cuvette. The size and distribution of the Er- AgNPs was estimated using a Dynamic Light Scattering instrument (DLS, Malvaren Zetasizer Nano) in a polystyrene cuvette. The surface morphology of particles was analyzed using scanning electron microscopy (SEM).

H. Antimicrobial Assay

Antimicrobial activities of the Er-AgNPs were determined according to the microdilution method [9]. Antimicrobial activity of synthesized AgNP was tested against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 29998), *Enterococcus faecium* (DSM 13590), *Staphylococcus epidermidis* (ATCC 12228), *Salmonella enteritidis* (ATCC 12228), *Salmonella typhimurium* (ATCC 12228), *Listeria monocytogenes* (ATCC 25923) and *Candida albicans* (ATCC 10239) strains. The different pathogens were incubated in Mueller Hinton broth over night. Serial dilutions of the test sample with different concentrations were prepared. AgNPs (80 µL) from each dilution were transferred into 96-wells sterile microtitre plates and 20 µL of the microbial inocula were then added to obtain a final volume of 100 µL in each well. For each dilution series, equal volume (80 µL) of the test sample (AgNPs) and Mueller Hinton broth served as negative control while untreated cell suspensions were employed as positive controls. Gentamycin and flucytosine were respectively used as standard antibacterial and antifungal reagents. The plates were incubated at 37°C for 24 h. Microbial growth was assessed by eye inspection. MIC was defined as the lowest concentration of the test samples required to inhibit microbial growth after 24 h. The procedure was repeated three times. The results are expressed in µg/mL.

III. RESULTS AND DISCUSSIONS

A. Phytochemical Analysis of *Euphorbia rigida* Leaf Extract Content

The results of phytochemical analyses in the extracts of *E. rigida* leaves are given in Table 1. Phytochemical profiles proved the presence of flavonoid, anthocyanins, phenol, tannins, steroid and quinones. Phytochemicals including anthocyanins, phenols, flavanoids and steroid are thought to be effective in the stabilization and effective capping of silver nanoparticles. [6]. The flavonoid, phenolic, and tannin content of an aqueous extract of *E. rigida* leaves were found to be 0.086% /g leaf, 0.225%/g leaf, 0.182% / g leaf respectively. *E. rigida* leaves are a rich source of phytochemicals including anthocyanin, phenol, flavanoid compounds. Thus, they were believed to be a great tool for the green synthesis process of AgNPs. It has been suggested that, free hydroxyl and carboxylic groups of flavonoids and phenols of the plant are able to bind to the Ag⁺ surface and trigger the formation of AgNPs while the C-O, C-O-C and C-C groups may function as a stabilizer.

Table 1. Phytochemical profiles of *Euphorbia rigida* leaves

Phytochemical screening assay	<i>Euorphia rigida</i> extract
1 Tannins test (FeCl ₃ test)	+++
2 Alkoloids test (Mayer's test)	++
3 Flavonoids test (Lead acetate test)	+++
4 Anthocyanidines test (HCl/ammonia)	+
5 Carbohydrates test (molish's test)	+
6 Phenol test (FeCl ₃ test)	+++
7 Steroid test (Salkowski test)	++
8 Quinones test (conc.H ₂ SO ₄)	+
9 Coumarin test	+

“+” shows the presence of phytochemicals, “++” shows moderate concentration of phytochemicals, “+++” shows high concentration of phytochemicals

B. Preparation of Silver Nanoparticles

In this study, *E. rigida* leaf extract for the synthesis of silver nanoparticles was used. In the natural bioreductive extract environment of *E. rigida*, silver (I) cations have been found to be reduced to neutral silver (0) type and the colour was observed to become darker as it changed from light yellow to light brown (Figure 1). It is very well known that discoloration is caused by surface plasmon resonance of nanoparticles. The discoloration is mainly due to the stimulation of surface plasmon resonance in metal nanoparticles. The observed discoloration is an indicator of the formation of silver nanoparticles. Nanoparticles are known to originate from superficial plasmon vibrations. Our results were also confirmed by previous studies [10] that reported the formation of silver

nanoparticles using plant extracts. The change in bioreduction time can be caused by differences in the activity of enzymes present in leaf extract.

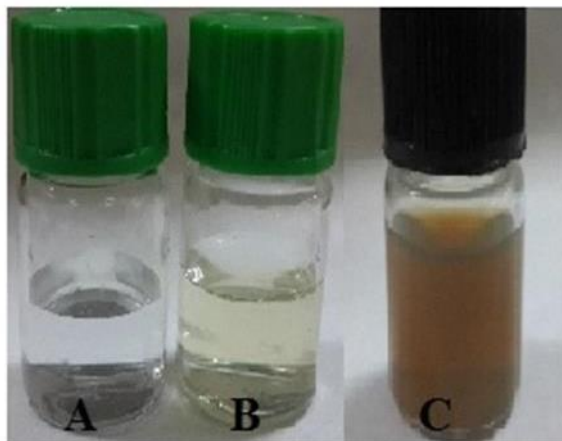


Figure 1. Biosynthesis of silver nanoparticles using *E. rigida* leaf extract; A) Silver nitrate solution B) *E.rigida* leaf extract and C) Er-AgNPs

C. Silver Nanoparticles Green Synthesis

The reduction of silver nitrate to silver nanoparticle using the plant leaf extract was showed by the colour change in the reaction solutions. Absorption was measured in the 300-800 nm wavelength range in the UV-visible spectrophotometer. Optimization of experimental conditions is necessary to obtain optimal conditions for the formation of silver nanoparticles. The optimization parameters involved in this study were pH, temperature, incubation period, silver nitrate concentration, and plant concentration.

D. The Effect of AgNO₃ Concentration on Silver Nanoparticle Synthesis

The effect of AgNO₃ concentration on the production of Ag nanoparticles was assessed by varying AgNO₃ concentration. Silver nanoparticles synthesized from 1 mM silver nitrate observed a Plasmon resonance band peak around 429 nm. At 1 mM concentration, a narrow band was observed with increased absorbance, while other concentrations observed a wide peak at 429 nm. The absorption was increased by increasing the concentration of silver ions from 1 mM to 5 mM (Figure 2). Due to the excess of functional groups in leaf extract, the nanoparticle synthesis and small size was begun quickly at 1 mM concentration. The optimization study showed that concentration has a significant effect on the synthesis of silver nanoparticles. This study concluded that the optimal concentration of AgNO₃ for nanoparticles synthesis is 1mM. In similar studies, the increased density was found to cause an increase in the concentration of nanoparticles. Higher concentration of silver nitrate results in a bigger size of the synthesized Er-AgNPs [11].

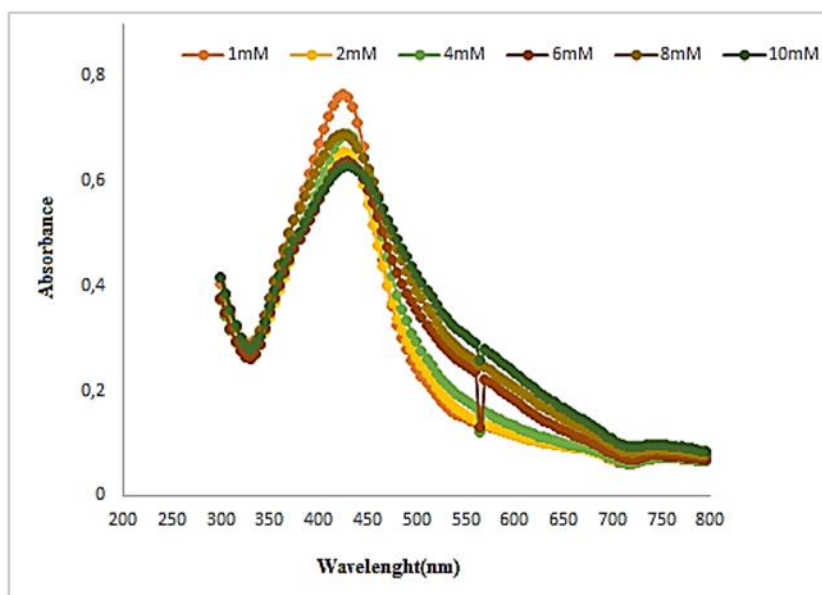


Figure 2. Absorption analysis of the Er-AgNPs synthesized at different concentrations of AgNO_3

E. The Effect of Temperature on Silver Nanoparticle Synthesis

Temperature is one of the important factors on the shape and size of silver nanoparticles. Varying the temperature (20, 30, 40, 50, and 80 °C) during the synthesis of AgNPs results in a rapid change in the solution color. This is an indication of the increase in the silver salt reduction (Figure 3). The synthesis of nanoparticles increases with increasing the reaction temperature [12]. The broadening peak (470 nm) was observed at low temperature, which indicates the formation of large-sized nanoparticles. The narrow peak (424 nm) was observed at high temperature (80°C), which means that the small-sized nanoparticles were formed. Finally, it was concluded that higher temperature was optimum for nanoparticles synthesis.

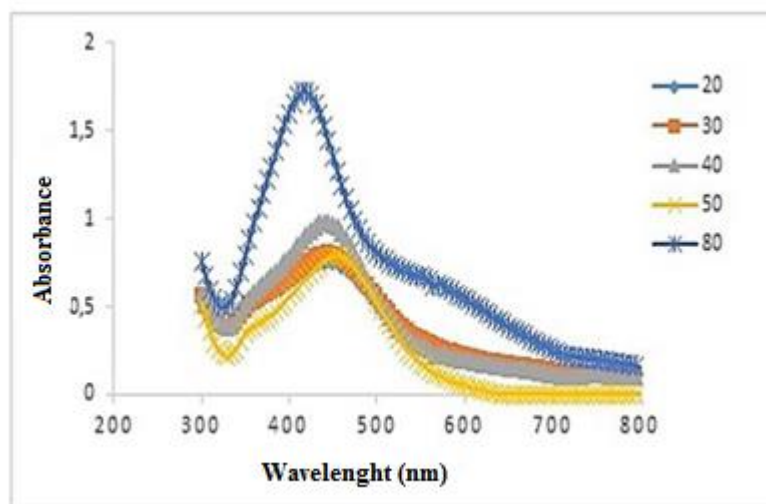


Figure 3. Absorption analysis of the Er-AgNPs synthesized at different temperatures

F. The Effect of Reaction Time on Silver Nanoparticle Synthesis

The effect of the reaction time on the formation of silver nanoparticles was investigated and the optimum conditions for the reaction were selected. The UV-Vis absorption of silver nanoparticles obtained from *E.rigida* leaf extract were taken at different time intervals (Figure 4). The absorption spectra of silver nanoparticles obtained from *E. rigida* leaf extract was measured and the best SPR peak was observed within 430 nm at 180 min. It was observed that UV-Vis absorption intensity incremented as the reaction time took longer. Reports from the literature indicate that the optimal time is reached when the color is stable and the SPR can be seen in a narrow shape [13].

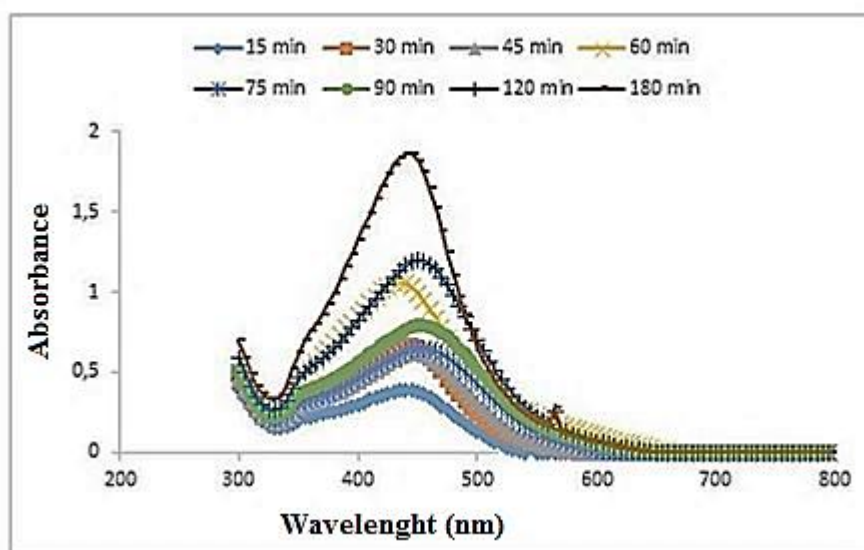


Figure 4. Absorption analysis of the Er-AgNPs synthesized at different contact time

G. The Effect of pH on The Silver Nanoparticle Synthesis

The influence of pH on the silver nanoparticles was investigated under different pH (Figure 5). The colour change of the solution from light yellow to brown showed the formation of Er-AgNPs. The pH is an important parameter that affects the stability, size and morphology of the nanoparticles. Because pH has the ability to change the charge of biomolecules, it can affect their caps and stabilizing ability. At alkaline pH (pH 9.0), maximum surface plasmon resonance band of the silver nanoparticles was observed at 420 nm. As the pH increases from 3 to 9, the absorption intensity also increases; however, at pH 11.0 the absorbance decreases again. The maximum formation of silver nanoparticles was observed at alkaline pH. Nahar et al. also reported that Ag-NPs show maximum stability at the pH 9.0 [14].

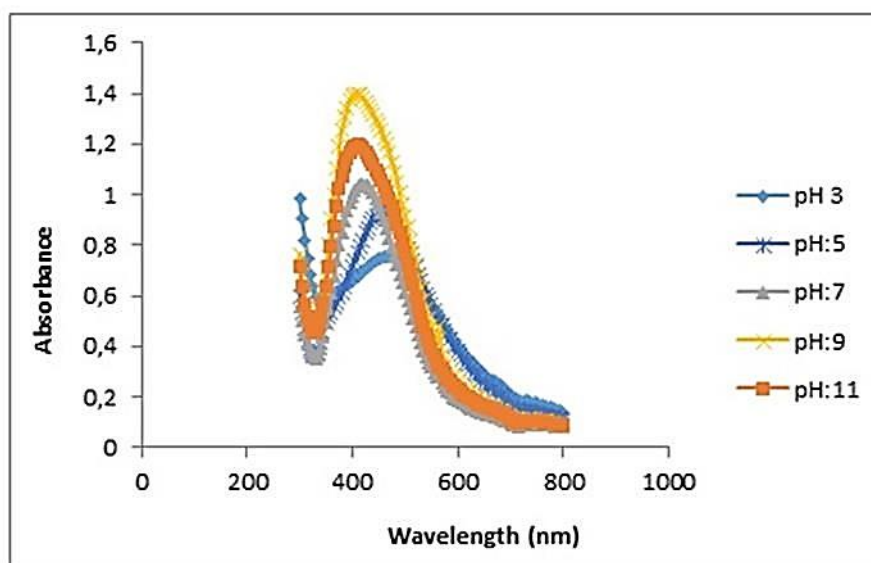


Figure 5. Absorption analysis of the Er-AgNPs synthesized at different pH

H. The Effect of Plant Extract Concentration on Silver Nanoparticle Synthesis

Biosynthesis of nanosilver particles at different concentrations of *E.rigida* plant extract ranging between 0.05% to 1% was characterized by UV-visible absorption spectroscopy. Results that are obtained indicate that AgNPs synthesized with the lowest concentration of *E.rigida* leaf extract showed an absorption band at 434 nm. The concentration (0.1%) of plant extract gradually shifts towards a lower absorption (434 nm) of the absorption band. However, the absorption band expands while the concentration of plant extract gradually increases. It reverts to significantly higher absorption (464 nm). It is thought that it may be due to the presence of large amounts of reductants in the reaction medium (electron-rich phytomolecules). This can cause rapid reduction of Ag ions. Rapid reduction of Ag ions facilitates further growth of nanoparticles. The absorption band of silver nanoparticles synthesized at the highest leaf extract concentration is 464 nm. In this study, it was decided that the optimum plant concentration for synthesizing small-sized nanoparticles was 0.1%. Unlike the characteristic peak, another peak was observed at 369 nm in the absorption spectrum of AgNPs synthesized from 0.25 – 1% extract concentration. This is known to be caused by phytomolecules of *E.rigida* attached to the AgNPs surface as stabilizing ligands [15].

I. Characterization of The Synthesized Er-AgNPs

In structural characterization studies of nanoparticles, particle size distributions in solution were studied using the DLS technique. The average particle size was determined to be 86 nm. It supports that nanoparticles are found in small clusters and do not form agglomerate stacks. In terms of particle size, compared to the DLS device, the SEM device gives values closer to reality. In this case, the DLS device gives information about the state of synthesis rather than the particle size. Simultaneously, the zeta potential values of nanoparticles were measured. AgNPs synthesized from euphorbia plant leaf extract were determined to have a zeta potential of 302.3 mV.

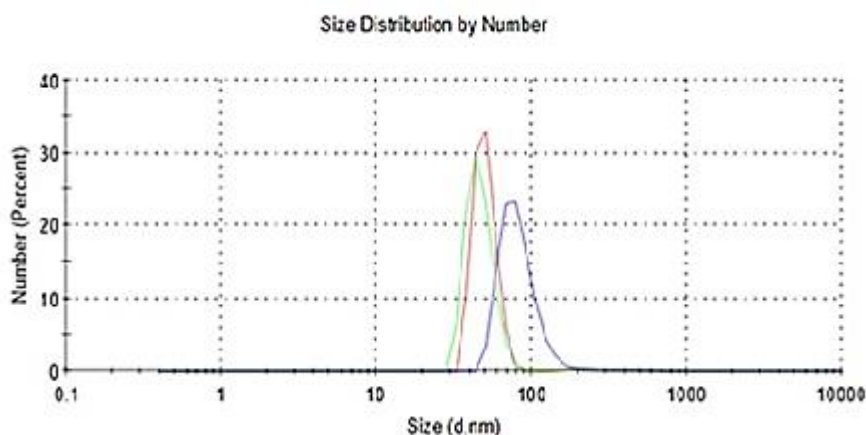


Figure 6. Zeta potential analysis of Er-AgNPs

The SEM results of synthesized Er-AgNPs showed in Figure 7. In the SEM images, the surface morphology of the silver nanoparticles was concluded to show a smooth, equal and spherical structure. It was thought that the reason for this circular shape could be the different quantity and nature of the capping agent found in the leaf extract used in AgNPs synthesis. The particle size histogram of *E. rigida* leaf extract ranged from 50 to 75 nm. Our results have been determined in accordance with previous studies [16]. This result indicates that *E. rigida* leaf extracts can act as a reducing agent in the production of silver nanoparticles. Since the size of the synthesized nanoparticles is less than 100 nm, the suitability of the particles for cell penetration can be mentioned.

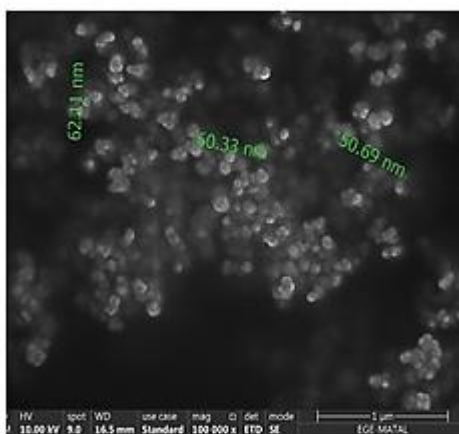


Figure 7. SEM analysis of Er-AgNPs

J. Antimicrobial Activities of the Green Synthesized Er-AgNPs

The antimicrobial effects of the prepared AgNPs were investigated against Gram-positive and Gram-negative bacteria, and a fungus. The determined MIC values for AgNPs were represented in (Table 2). The result showed that the highest antibacterial activity against *E. faecium* and *S. epidermidis* with MIC values were observed as 125 µg/mL. Given the results obtained in the current study and the previously reported results from green chemistry synthesized AgNPs [17,19], it is safe to say that silver NPs are a good option for antibacterial solutions with potential for health and biomedical related fields. The presence of plant biomolecules such as phenolic compounds, flavonoids and terpenoids in the extract, can increase the antimicrobial activity of green synthesized Er-AgNPs. The exact mechanics of antimicrobial activities of the prepared AgNPs are still not fully known [17].

Table 2. MIC values of ErAgNPs for different microorganisms

Microorganisms	MIC levels (µg/mL)		
	AgNPs	Gentamycin	Flucytosine
<i>Escherichia coli</i> (ATCC29998)	500	15.6	-
<i>Staphylococcus aureus</i> (ATCC 25923)	250	7.8	-
<i>Enterococcus faecium</i> (DSM 13590)	125	15.6	-
<i>Staphylococcus epidermidis</i> (ATCC 12228)	125	7.8	-
<i>Salmonella enteritidis</i> (ATCC 13076)	250	15.6	-
<i>Salmonella typhimurium</i> (ATCC 14028)	250	15.6	-
<i>Listeria monocytogenes</i> (ATCC 19111)	250	7.8	-
<i>Candida albicans</i> (ATCC 10239)	500	-	15.6

IV. CONCLUSIONS

Interest in the green synthesis method as a means of obtaining nanoparticles is increasing. The silver nanoparticles were produced by bio-reduction methods using aqueous leaves extract of *E. rigida*. The reduction of silver nitrate to silver nanoparticle was approved by UV-vis spectrophotometer, DLS and SEM techniques. Antimicrobial activity assessment of the AgNPs demonstrated a strong antimicrobial activity against important human pathogens (*E. coli*, *S. aureus*, *E. faecium*, *S. epidermidis*, *S. enteritidis*, *S. typhimurium*, *L. monocytogenes* and *C. Albicans*) With the increase in nanotechnological research, the synthesized Er-AgNPs will open a new field for the production of biomedical and industrial products and pharmaceutical industry to become more useful.

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