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Research article

Antibacterial efficacy of green synthesized silver nanoparticles from rosemary, pennyroyal, and eucalyptus extracts against E. coli and S. aureus bacteria

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ABSTRACT

Bacteria, including those causing hospital-acquired infections, have become a significant concern for human health due to their resistance to common antibiotics. Silver nanoparticles possess highly antimicrobial properties and can be applied in various medical and healthcare contexts. The purpose of this research is to produce silver nanoparticles through a bio-based (green synthesis) method using extracts from the leaves of rosemary, pennyroyal, and eucalyptus plants and to investigate their antibacterial activity. Extracts from the leaves of rosemary, pennyroyal, and eucalyptus plants were prepared and added to a silver nitrate solution in the process of synthesizing silver nanoparticles. The production of silver nanoparticles in the solution was investigated by recording the color changes during the experiment and measuring the absorption levels across different wavelengths using a spectrophotometer. The antimicrobial effects exhibited by the silver nanoparticle solution were investigated and confirmed targeting both Staphylococcus aureus and Escherichia coli (E. coli) strains using the agar well diffusion method. Nanoparticles with diameters approximately ranging from 18 to 80 nanometers were successfully synthesized, exhibiting a varied assortment of spherical geometries and a notable purity level of 88% silver. Furthermore, nanoparticles synthesized from rosemary plant extract exhibited superior antibacterial properties compared to those from other plant extracts.

KEYWORDS

Silver nanoparticles
Escherichia coli
Staphylococcus aureus
Green synthesis

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1. Introduction

Over the last several years, with the increase in environmental issues, there has been considerable emphasis on green chemistry, and the endeavor to synthesize nanomaterials using eco-friendly approaches has significantly increased [1, 2]. Green chemistry is a research or innovative approach that involves practical applications through the design, development, and production of efficient products that can mitigate hazardous components for health. New green technology projects are prioritized to minimize the potential hazards of nanotechnology applications for both humans and the environment [3]. Nanoparticles produced by chemical methods, which are widely used today have raised significant concerns associated with the utilization of harmful and noxious chemicals, as well as the resulting environmental damages. Nano-biotechnology has emerged as an exceptionally promising scientific and nanotechnological domain in the modern age [4]. Nanoparticles (NPs) exert a profound influence on various facets of human life owing to their distinctive properties,

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including size, shape, and morphology. Metallic nanoparticles, such as silver, titanium, gold, and platinum, stand out for their diverse applications in scientific fields like pharmaceuticals, nanomedicine, and biomedical engineering [5].

Bacteria are among the factors that cause hospital-acquired infections and contribute to healthcare-related issues [6]. The excessive utilization of antibiotics has led to an increase in bacterial resistance, posing challenges in treatment and becoming a serious global health threat to hospitalized individuals. Finding alternatives to antibiotics will be an effective solution to prevent the aggravation of this problem.

Nanoparticles can be categorized into several distinct types. Nanoparticles can be distinguished by their morphology, physical characteristics, size, and chemical properties. Nanoparticles encompass a variety of classifications, including carbon-based, ceramic, metal, polymeric, semiconductor, and lipid-based nanoparticles, each characterized by distinct properties and applications [7]. Furthermore, nanoparticle varieties are commonly dichotomized into two main classifications: organic nanoparticles, derived from carbon-based materials, and inorganic nanoparticles, typically composed of metal or mineral elements. In nanoparticle classification, the first cluster encompasses dendrimers, compact polymeric nanoparticles, micelles, liposomes, and hybrid nanoparticles. In contrast, the second cluster comprises fullerenes, quantum dots, as well as metal, metal oxide, and silica nanoparticles [8–10].

Among metallic nanoparticles, silver nanoparticles are among the most commonly used particles within the domain of nanotechnology, and their applications in the nanoworld are continuously expanding each day. Silver nanoparticles find extensive applications in electronic, optical, pharmaceutical, healthcare, and catalytic fields due to their distinct physical and chemical characteristics. The broad utilization of silver nanoparticles can be attributed to their effective antibacterial properties. Silver nanoparticles are considered toxic to pathogenic agents but almost harmless to foods and tissues, and therefore to humans. Silver particles in larger dimensions behave as a metal with low reactivity. However, when reduced to smaller dimensions in the nanometer range, their antimicrobial properties increase by more than 99%. This enhancement in antimicrobial efficacy makes them suitable for applications in improving surgical procedures and combating infections. Researchers have increasingly focused on using plants as sustainable and abundant sources for synthesizing biocompatible nanoparticles. This approach offers several benefits, including biocompatibility, non-toxicity, cost-effectiveness, and the production of highly pure nanoparticles [11]. Various secondary metabolites such as enzymes, proteins, essential oils, carbohydrates, lipids, phenols, tannins, acids, vitamins, pigments, sulfur, resins, and terpenes are essential contributors to the synthesis of metallic nanoparticles within plants [12].

So far, the successful use of pine, mulberry, and magnolia extracts for the synthesis of silver nanoparticles, yielding particles ranging in size between 15 and 500 nanometers, has been reported [13]. Gardea-Torresdey and colleagues researched on synthesizing gold and silver nanoparticles, employing alfalfa plant extract for the process [14]. The production of nanoparticles through eco-friendly green biochemistry techniques is a safe and non-toxic process that embraces various microorganisms like bacteria, fungi, and plants to ensure environmental friendliness. Both unicellular and multicellular organisms have been used for the production of nanomaterials, including mineral nanoparticles [15].

Rosmarinus officinalis L., a species classified under the Lamiaceae family, is extensively recognized for its widespread use due to its aromatic attributes. Rosemary exhibits a range of biological activities, including anti-cancer, antimicrobial, anti-inflammatory, and antioxidant properties. Furthermore, it has proven beneficial in improving memory and mitigating anxiety and stress [16, 17]. Eucalyptus camaldulensis Dehnh., a constituent of the Myrtaceae family, is of interest for its therapeutic potential. The decoction derived from its leaves is traditionally employed to alleviate symptoms associated with respiratory and urinary tract bacterial infections, asthma, and sore throat [18, 19]. Mentha pulegium L., commonly referred to as pennyroyal, is distinguished within the Mentha genus by its unique olfactory profile. The herb's aerial parts are traditionally employed in folk medicine for treating various digestive tract ailments, including dyspepsia, flatulence, and intestinal colic, highlighting its significance in herbal therapeutics [20, 21].

Rosemary, pennyroyal, and eucalyptus plants have wide distribution regions around the world. Silver nanoparticle production using these plants can be an easy and cost-effective solution, while also providing a useful application for these plants. Compared to traditional methodologies, green synthesis method is more cost-effective, simpler to implement, and more environmentally sustainable, thereby qualifying as a "green" approach. The objective of this research is to investigate the potential of rosemary, pennyroyal, and eucalyptus plant extracts in synthesizing silver nanoparticles. Furthermore, the research seeks to assess the antimicrobial effectiveness of the synthesized silver nanoparticles on pathogens responsible for hospital-acquired infections. This investigation will profoundly advance the exploration of natural biomass and its applications within the realms of nanotechnology and nanoscience.

2. Materials and methods

In the process of preparing the plant extract, both the leaves and flowering stems of rosemary, pennyroyal, and eucalyptus plants were collected. After undergoing three washes with distilled water, they were dried and weighed using a balance. A quantity of 20 grams of these dried plants was placed in a beaker with 100 milliliters of distilled water and boiled for 60 minutes. Following cooling, the mixture underwent filtration using Whatman 40 filter paper and was subsequently stored at a temperature of 3 °C until required for further use.

Silver nitrate solutions with concentrations of 0.1 M, 0.01 M, and 0.001 M were prepared, each in a volume of 100 milliliters. Subsequently, the solutions underwent agitation on a magnetic stirrer, followed by the addition of 3 milliliters of plant extract to each. The prepared mixtures were incubated in darkness to promote the generation of silver nanoparticles. The transition of the solution's color from yellow to dark brown indicated the successful synthesis of silver nanoparticles. Photonix Ar 2015 UV-Vis array spectrophotometer was utilized to measure the absorbance of the produced silver nanoparticles at different wavelengths.

The colloidal solution containing the synthesized silver nanoparticles was purified by centrifugation at 12,000 rpm for 20 minutes. Subsequently, the supernatant was decanted and the precipitate underwent three washes with distilled water. After each washing step, the sample was subjected to centrifugation. The dried precipitate obtained after purification was utilized for the characterization of the

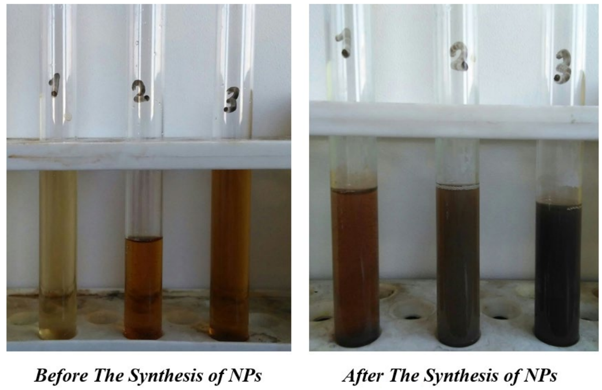

Fig. 1. Formation of silver nanoparticles in the plant extracts of 1- rosemary, 2- pennyroyal, and 3- eucalyptus after 80 minutes.

formed silver nanoparticles, including particle morphology determination and EDS analysis. For this purpose, the MIRA3 FEG-SEM Tescan Scanning Electron Microscope available at the Central Laboratory of the University of Tabriz was used.

The antibacterial property evaluation was performed using 5 mm discs soaked in pure plant extracts of rosemary, pennyroyal, and eucalyptus, as well as in silver nitrate solution, silver nanoparticles, and one disc as the control. Staphylococcus aureus, a gram-positive bacterium, was cultured on Baird-Parker agar, and Escherichia coli, a gram-negative bacterium, was cultured on Meuller-Hinton agar. After incubating the bacterial cultures for 15 minutes to allow the bacteria to adhere to the agar surface, the aforementioned discs were placed on the plates with a suitable distance between them. The plates were then incubated at 37 °C for 24 hours. Following this timeframe, the diameter of the inhibition zone surrounding each disc was assessed using a glass ruler.

3. Results and discussion

Once the prepared plant extracts were introduced into the silver nitrate solution, a gradual shift in the solution's color ensued. Following an 80-minute duration, the solution transitioned to a dark brown hue, signifying the emergence of silver nanoparticles within the solution.

The investigation of the absorption spectrum in the range of 200 to 900 nanometers reveals an elevation in the absorption rate at higher concentrations, with the highest absorption observed at approximately 420 nanometers, as illustrated in Fig. 2. The detection of a peak within this range confirms the synthesis of silver nanoparticles. This peak, observed between 400 and 450 nanometers, is indicative of silver nanoparticle formation and is associated with their surface plasmon resonance (SPR). The SPR phenomenon arises from the excitation of free electrons within the nanoparticles, leading to a resonant oscillation that corresponds to the observed peak. The absorbance data clearly shows that higher concentrations correlate with increased absorbance, reinforcing the successful synthesis of silver nanoparticles.

This finding is consistent with literature reports and highlights the reliability of SPR as a diagnostic tool for nanoparticle synthesis. The distinct absorption peak serves as robust confirmation of silver nanoparticle formation, providing a reliable method for monitoring the synthesis process.

To investigate the diameter and morphology of the nanoparticles, SEM microscope images were obtained and are presented in Fig. 3. Based on the SEM images, it was demonstrated that the nanoparticles are spherical and have an approximate diameter ranging from 18 to 80 nanometers. Although their shape closely resembles silver

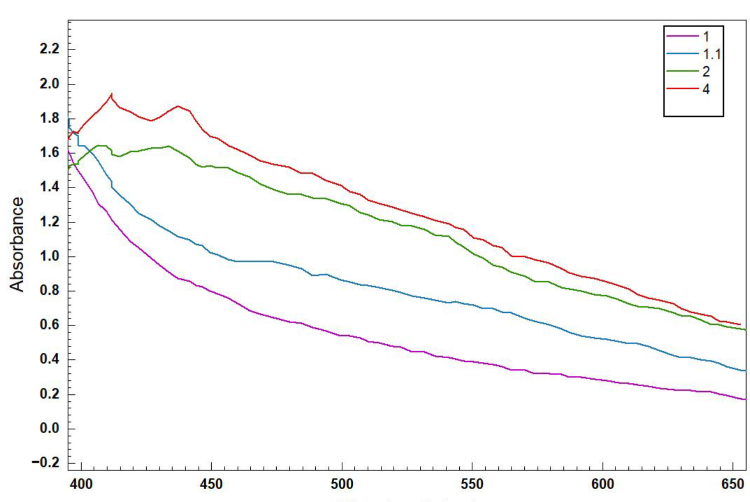

Fig. 2. Changes in absorption rate with the concentration of silver nitrate solution: 1) control sample, 2) 0.001 M, 3) 0.01 M, and 4) 0.1 M, which demonstrate the concentrations of $AgNO_3$.

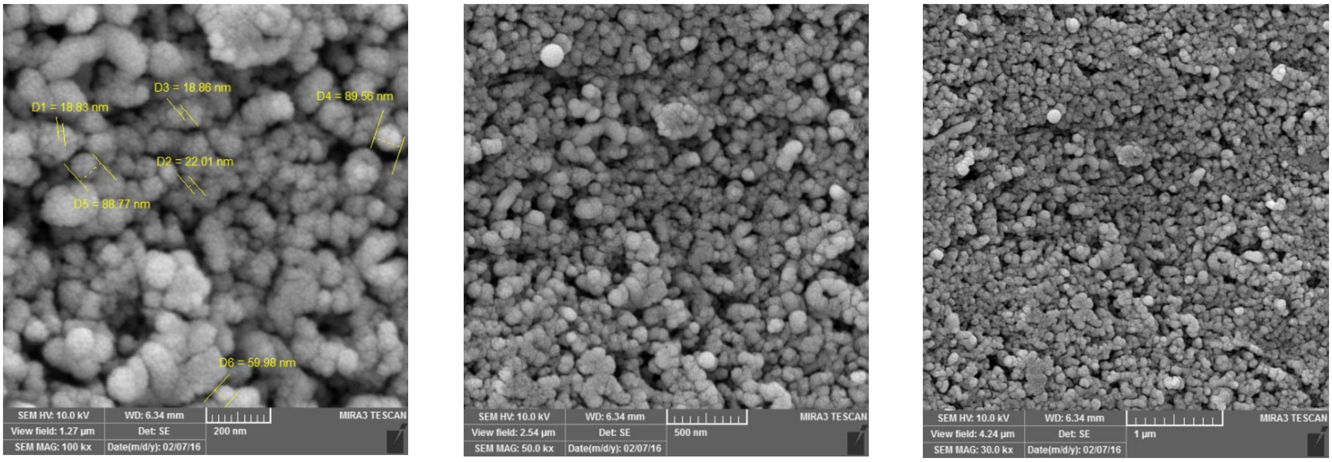

Fig. 3. SEM images of synthesized silver nanoparticle samples.

nanoparticles synthesized by other plants, the size of the nanoparticles obtained in this study is smaller than the sizes reported by many other researchers [22], who mostly reported sizes between 71 to 110 nanometers.

The elemental composition of the synthesized silver nanoparticles is depicted in Fig. 4, obtained through Energy Dispersive X-ray Spectroscopy (EDS) analysis. The EDS spectrum prominently features peaks corresponding to silver (Ag), confirming that silver is the predominant element present in the nanoparticles. The most significant peaks, located around the 3 keV region, are characteristic of silver, indicating its successful incorporation into the nanoparticles. Additionally, the spectrum reveals minor peaks for carbon (C), chlorine (Cl), and potassium (K), suggesting the presence of these elements in trace amounts. These minor peaks are likely attributable to residual impurities from the synthesis process or potential environmental exposure during sample preparation.

The EDS analysis indicates that the synthesized silver nanoparticles exhibit a high purity level of approximately 88%. This high degree of purity underscores the effectiveness of the synthesis method employed, as evidenced by the minimal intensity of impurity peaks relative to the silver peaks. Therefore, the EDS analysis confirms the successful production of high-purity silver nanoparticles, validating the synthesis protocol used in this research study.

The analysis of the particle size distribution for the nanoparticle sample was conducted using a size distribution curve. A histogram representing the frequency distribution of particle diameters was plotted alongside a normal distribution curve to fit the data. The particle sizes, measured in nanometers (nm), were statistically analyzed to derive the mean (μ) and standard deviation (σ) of the particle diameters. The size distribution histogram in Fig. 5 shows a significant number of particles with diameters concentrated at the lower end of the size spectrum, peaking sharply around the smallest size intervals. The frequency of particles diminishes as the diameter increases, forming a positively skewed distribution. The overlaid normal distribution curve, characterized by a mean (μ) of approximately 40.79 nm and a standard deviation (σ) of about 55.94 nm, provides a statistical representation of the size distribution. This model indicates that the majority of the particles are smaller, with sizes tapering off as they approach larger

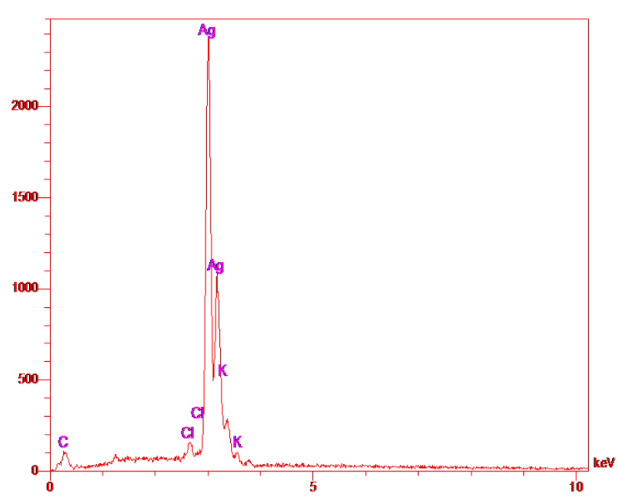

Fig. 4. EDS characterization of synthesized silver nanoparticle specimen.

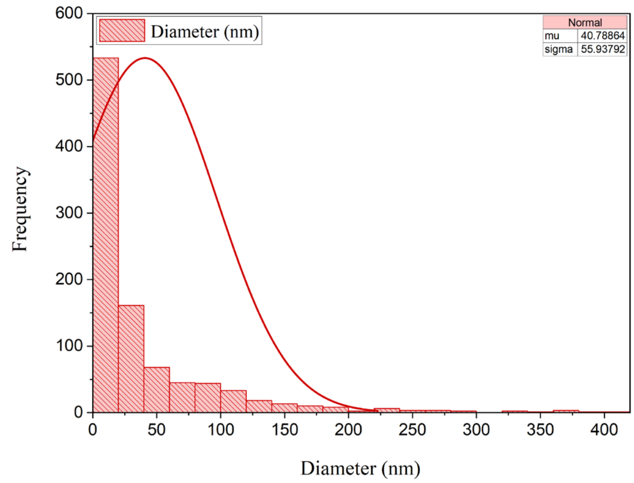

Fig. 5. Size distribution curve of synthesized silver nanoparticles.

diameters. The analysis reveals that the estimated average particle diameter falls predominantly within the range of 18–80 nm, encompassing the central tendency and variability of the particle sizes in the sample. The particle size distribution is critical in determining the suitability of nanoparticles for various applications.

Smaller particles, as indicated by the peak in the histogram, typically exhibit higher surface area-to-volume ratios, which can enhance reactivity and interaction with other substances. The positively skewed distribution suggests a prevalence of smaller particles with a few larger outliers, a common trait in many nanoparticle synthesis processes. The normal distribution fit, while not perfectly representative of the skewed nature of the data, provides a useful approximation for understanding the general trend and variability within the sample. The derived mean and standard deviation offer insights into the central tendency and spread of the particle sizes, respectively. Overall, the particle size distribution analysis indicates that the nanoparticles in the sample predominantly have diameters between 18 and 80 nm, with a mean size of approximately 40.79 nm and a standard deviation of 55.94 nm. This distribution suggests a higher concentration of smaller particles, which is typical for many nanoparticle synthesis methods. Understanding this distribution is essential for predicting the behavior and potential applications of these nanoparticles in various fields.

The suppressive impact on the proliferation of Escherichia coli and Staphylococcus aureus bacterial strains in the culture medium was investigated using 5 mm discs prepared from pure plant extracts of rosemary, pennyroyal, and eucalyptus, as well as silver nitrate solution, silver nanoparticles, and a control disc. The results are presented in Table 1 and Fig. 6. The diameter of the inhibition zone for bacteria on the Escherichia coli plate was larger than that on the Staphylococcus aureus plate, indicating a greater inhibitory effect of silver nanoparticles against Escherichia coli bacteria. Furthermore, no zone of inhibition was observed around the control disc.

The antimicrobial performance of biologically synthesized nanoparticles is related to their size. Antibacterial properties are observed in the range of 9 to 130 nanometers. The synthesized particles in this study had sizes ranging from 18 to 80 nanometers, which contributed to their antimicrobial properties. In general, the antibacterial properties depend on the nanoparticles' surface area. Reduced particle sizes result in increased contact surface areas, enhancing their antibacterial effectiveness, which is consistent with literature reports [23].

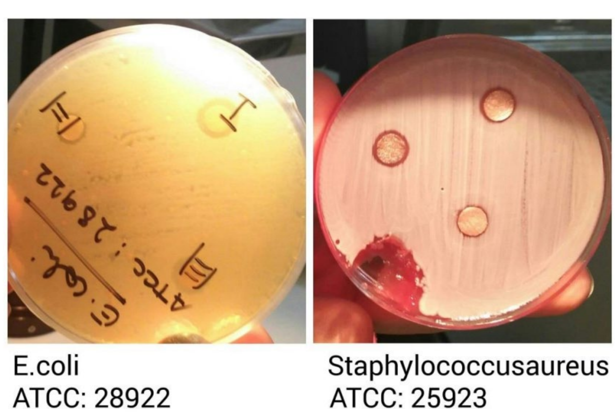

Fig. 6. Images of the inhibition zone in the Escherichia coli and Staphylococcus aureus bacterial plates.

Table 1. Mean diameter of inhibition zones for the studied bacteria against the prepared discs in millimeters.

Bacteria	Rosemary extract	Pennyroyal extract	Eucalyptus extract	Silver nitrate	Silver NPs (rosemary)	Silver NPs (pennyroyal)	Silver NPs (eucalyptus)
Staphylococcus aureus	0	0	0	0	8	7	6
E. coli	0	0	0	0	10	6	9

A study conducted by Guzman et al. [24] in 2012 explored the antimicrobial properties of silver nanoparticles against both gram-positive and gram-negative bacteria. The research revealed that the effectiveness of silver nanoparticles is concentration-dependent, with a more pronounced impact observed on gram-negative bacteria compared to gram-positive bacteria. According to their findings, the dissimilar reactions of gram-negative and gram-positive bacteria strains to silver nanoparticles stem from distinctions in their cell wall structures. Gram-negative bacteria feature a less robust, thinner cell wall, while their outer surface is characterized by a layer of negatively charged lipopolysaccharides. The negative charge existing on the surface of bacterial cells facilitates the interaction between the cells and silver nanoparticles, which carry a minor positive charge, aiding in their attachment to the bacterial cell. Initially, this interaction initiates a breach in the bacterial cell wall. Subsequently, as nanoparticles penetrate the bacterial cell, they disrupt its growth, ultimately resulting in bacterial death. The outcomes of this study are in concordance with contemporary research findings.

Silver nanoparticles, like other biomaterials, are capable of causing toxic effects in living organisms, and the toxicity of these particles depends on their characteristics and route of entry. The toxicity induced by silver nanoparticles stems from oxidative stress, which triggers the sequestration of nanoparticles within the cellular cytoplasm and nucleus, consequently fostering the production of free radicals. Silver nanoparticles are initially separate particles that, owing to their extensive surface area, have the highest level of toxicity, which decreases over time as the particles agglomerate. The toxicity of silver nanoparticles is dual; while they affect bacteria at concentrations of 20–25 ppm, they are harmless and even biocompatible for human cells [25–28].

Nanomaterial synthesis stands out as one of the most vibrant domains within current nanoscience research. Silver nanoparticles possess characteristics that render them effective in inhibiting bacterial growth and exerting antibacterial effects. Silver ions can form bonds with electron-donating entities found in biomolecules, such as glucose, oxygen, and nitrogen. Silver nanoparticles disrupt the structure of the bacterial outer membrane, causing the gradual release of molecules such as lipopolysaccharides and purines from the cytoplasmic region. Once silver nanoparticles penetrate the bacterial cell, they deactivate essential enzymes and trigger bacterial death by generating hydrogen peroxide. Numerous methods exist for synthesizing nanoparticles; however, both physical and chemical approaches can be superseded by environmentally friendly “green” methods. These green techniques involve the use of substances with reduced environmental impact, thereby mitigating pollution.

Research indicates that silver ions can markedly augment the production of oxygen species, notably superoxide anion radicals, thereby instigating oxidative stress within cellular environments and across molecular surfaces, organelles, and cellular structures as a whole. Secondary metabolites, encompassing phenolic compounds and flavonoids, within plants, contribute to their antimicrobial and antioxidant attributes, thereby serving as protective agents against oxidative cellular damage. Certain plant species possess the capacity to bioreduce Ag^{+} ions to Ag^{0}, thereby facilitating the synthesis of nanoparticles with inherent antioxidant properties. Consequently, the biosynthesis of nanoparticles utilizing microorganism and plant-based approaches has emerged as a focal point of interest within the scientific community, representing an environmentally sustainable and biocompatible approach.

4. Conclusions

The conducted experiment has yielded promising results, demonstrating the successful fabrication of silver nanoparticles using extracts derived from rosemary, pennyroyal, and eucalyptus. This approach has proven to be not only rapid but also cost-effective, highlighting its potential as a robust biological method. Nanoparticles with diameter sizes ranging from 18 to 80 nanometers were successfully synthesized, displaying a diverse array of spherical geometries with a high purity level of 88% silver. The UV-Vis results of the synthesized silver nanoparticles indicate that the increasing concentration of $AgNO_3$ has a direct effect on producing finer silver nanoparticles with enhanced antibacterial efficiency. The morphology of the nanoparticles closely resembles that of silver nanoparticles synthesized using alternative botanical sources. However, the size distribution observed in this study is notably smaller than the ranges commonly reported in the literature, which typically fall between 71 and 110 nanometers. A comparison of the efficiency of botanical extracts on the antibacterial effectiveness of nanoparticles demonstrates that silver nanoparticles resulting from the reduction of silver nitrate with rosemary extract exhibit superior bacterial killing activity. Given the demonstrated antimicrobial activity of these nanoparticles, their application is suggested in various fields for preventing contamination and the spread of infectious agents.

CRediT authorship contribution statement

Shanli Salahi: Conceptualization, Methodology, Validation, Writing – review & editing.
Tayyebeh Ghaffari: Conceptualization, Methodology, Validation, Writing – original draft, Project Administration.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Declaration of competing interest

The authors declare no competing interests.

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