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ORIGINAL ARTICLE

Phytochemical prospecting, green synthesis of Silver nanoparticles from *Euphorbia helioscopia* and its antibacterial activity

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Abstract

Euphorbia helioscopia, a traditional medicinal herb, possesses various pharmaceutical applications for human diseases. In the present study, the ethanol and hexane extracts of E. *helioscopia* leaves were subjected to phytochemical screening to identify the presence of secondary metabolites. The concentrations of alkaloids, phenols, and flavonoids were determined quantitatively to evaluate the medicinal properties of the plant extracts. The ethanolic extract showed a higher yield of various secondary metabolites, specifically, phenol showed a high degree of precipitation. Silver nanoparticles (AgNPs) were then green synthesized from the leaf extract and characterized by UV–Visible spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), and Scanning Electron Microscope (SEM). The presence of elemental silver is confirmed by the sharp peaks in the UV-Visible at 442 nm. The FTIR spectrum showed the presence of various functional groups from the plant extract that fabricated and activated the AgNPs. The synthesized NPs were found to be spherical with slight aggregation in the SEM micrograph. The crude plant extracts and AgNPs were compared for antibacterial activity at various concentrations. AgNPs exhibited higher inhibitory activity against selected Gram-positive and Gram-negative pathogens than the crude extracts. The enhanced activity of AgNPs may be attributed to the phenolic content of the plant extracts. Hence, the present study confirms that E. *helioscopia* leaves have potential antimicrobial activity and also act as an efficient source for AgNPs with remarkable pharmacological properties that can be further evaluated to develop them as a promising drug candidate.

Keywords: *AgNPs; Antibacterial Activity; Euphorbia helioscopia; Phytochemicals; Quantification.*

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INTRODUCTION

One of the major public health concerns is the spread of infectious disease caused by the emergence of contagious microbial agents that have developed resistance to antibiotics due to mutations. The toxicity, low efficiency, and high cost of existing drugs lead to a search for novel alternatives from natural sources at low cost with the least side effects [1]. 80% of the world's population depends on traditional medicines from plants because they are the paragon for therapeutically potential drugs that are easily available, safe, less expensive, and efficient [2]. However, such plants should be investigated to better understand their properties and safety [3].

Euphorbiaceae is a plant family with more than 2000 species distributed worldwide, and 14 species were identified in the Yercaud Hills of Salem district, Tamilnadu [4]. *Euphorbia*

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helioscopia, a genus of flowering plant belonging to this family, is a herbaceous annual plant that grows in temperate regions of the Northern Hemisphere. The plant is traditionally used in the treatment of cholera, malaria, skin eruptions, bacillary dysentery, osteomyelitis, and glactagogue [5]. The leaves of *E. helioscopia* showed laxative, antibacterial, antiviral, cytotoxicity, antifungal, antihelminthic, antioxidant, antiallergic, antitumor and vasodepressor activities [6-8].

Nanotechnology is the study of nano-sized particles with varied chemical compositions, morphology, organized dispersity, and uniqueness. A diverse group of metallic nanoparticles such as gold, silver, lead, and platinum has been synthesized and exhibited wide applications in optical sensing, bio-labelling, spectrally selective coatings for the absorption of solar energy, food additives, textile industry, water treatment, and data storage [9-11]. Among the metallic nanoparticles (NPs), silver nanoparticles (AgNPs) fascinated more importance in biology and medical research. Recent studies revealed that AgNPs could release silver ions (Ag^+) that interact with the cell membrane and cell wall components of the bacteria and alter their penetrability and cellular respiration, increase dephosphorylation of phosphotyrosines that interfere with cell signal transduction, and kill the cells. AgNPs have catalytic effects at low concentrations, surface-enhanced Raman scattering effect, and other unique physiochemical properties. Studies also showed that the AgNPs have broad-spectrum antimicrobial, wound healing, anti-cancer, anti-platelet, anti-inflammatory,and anti-plasmodial activities [12-16].

Top-down and bottom-up physicochemical approaches are commonly used in the synthesis of NPs. Physical methods such as condensation, evaporation, and laser ablation and chemical methods that involve the reduction of metal ions in chemically reducing substances and radiation, favour the successful formation of small metal clusters or aggregates [17-19]. Yet, these methods are highly economical, toxic, and pose various biological threats to ecosystems. Hence, an alternative, eco-friendly, and sustainable approach is highly required for the optimized synthesis of NPs. The use of microorganisms (bacteria, fungi, and actinomycetes) and plant biomass or its extracts could be a green alternative to produce NPs sustainably [20]. Comparatively, the presence of bioactive metabolites like flavonoids, alkaloids, saponins, tannins, etc., in the plant extracts reduces the metal ions efficiently into NPs that are safe, easily accessible, cost-effective, and non-toxic. Optimized fabrication of copper oxide nanoparticles (CuONPs), cadmium oxide nanoparticles (CdONPs), cerium oxide nanoparticles (CeO_2 NPs), and selenium nanoparticles (SeNPs) using plant extracts were obtained for various applications [21-23]. However, the mechanism of NP synthesis using phytochemicals as reducing agents is a potential area that requires wide research exploration.

Based on the above literature studies and the promising interest in green synthesis technology, a study was carried out to produce AgNPs from *E. helioscopia*. The plant *E. helioscopia* is least studied for its potency in nano research. In the present work, the phytochemical constituents from leaf extracts of *E. helioscopia* were analyzed qualitatively and quantitatively. AgNPs were green synthesized from the leaf extracts and characterized. The antibacterial activity of the plant extracts and AgNPs is evaluated and compared against selected Gram-positive and Gram-negative bacteria. The study correlates the plant's antimicrobial properties to its efficiency in the synthesis of AgNPs, a greener alternative for a variety of medical applications.

EXPERIMENTAL

Collection of Plant Materials

The fresh leaf samples of *E. helioscopia* were collected randomly from the Yercaud Hills, Tamil Nadu. Sample materials were washed under running tap water and air dried under ventilation at room temperature. The chemicals used for the study were of analytical grade and obtained from Laba Chemie Limited, India, Merck India Limited. Silver nitrate ($AgNO_3$) is obtained from Sigma-Aldrich chemicals.

Preparation of Extracts

The plant samples were homogenized using mortar and pestle and ground into powder form. About 50 g of powdered crude sample was subjected to sequential extraction with 250 mL of ethanol and hexane separately in increasing order of polarity using the soxhlet extraction method by packing it uniformly into a thimble. The process of extraction was carried out continuously for 24 hours or untill the solvent in the siphon tube of the

extractor became colorless. The solvent was then evaporated by heating on hot plate at 30-40ºC. The dried extracts were stored in refrigerator at 4ºC for future use. Various phytoconstituents are analyzed and identified from the concentrated extracts.

Phytochemical Screening

Preliminary phytochemical analysis was carried out in both the concentrated extracts of *E. helioscopia* as per standard methods described by Brain and Turner method [24] and Evans method [25].

Detection of alkaloids (Mayer s test)

500 µL of crude extracts were dissolved individually in 400 µL of dilute hydrochloric acid and filtered. The filtrate was then treated with 400 µL of Mayer's reagent. The formation of a yellow cream precipitate in the extract shows the presence of alkaloids.

Detection of Flavonoids

500 µL of crude plant extracts were treated with 400 µL of sulfuric acid. The formation of an orange color indicates the presence of flavonoids in the extract.

Detection of Steroids (Liebermann- Burchard test)

2 mL of acetic anhydride and 400 µL of sulfuric acid were added to 500 µL of the plant extracts. The color change from violet to blue or green in the samples indicates the presence of steroids.

Detection of Terpenoids (Salkowski's test)

To the 500 µL of the plant crude extract, 400 µL of chloroform and 3ml of concentrated sulfuric acid were added carefully to form a layer. A reddish brown coloration of the inner face indicates the presence of terpenoids.

Detection of Anthroquinones (Borntrager's test)

About 500 µL of the extracts was boiled with 10% hydrochloric acid for few minutes in a water bath. It was filtered and allowed to cool. Equal volumes of chloroform were added to the filtrate. A few drops of 10% ammonia were added to the mixture and heated. The formation of a pink color indicates the presence anthraquinones.

Detection of Phenols (Ferric Chloride test)

The extracts were treated with a few drops of 5% ferric chloride solution. The formation of a bluish black color indicates the presence of phenol.

Detection of Saponins (Froth test)

About 0.2 g of the extracts were shaken with 5ml of distilled water. The presence of saponins is indicated by the formation of froth (the appearance of creamy, stable, persistent small bubbles).

Detection of Tannins (Ferric chloride test)

A small quantity of extracts were mixed with water and heated on water bath. The mixture was filtered, and 0.1% ferric chloride was added to the filtrate. A dark green color formation indicates the presence of tannins.

Detection of Carbohydrates (Fehling's test)

0.2 g filtrate is boiled in a water bath with 0.2 mL each of Fehling solutions A and B. A red precipitate indicates the presence of sugar.

Detection of Oils and Resins (Spot test)

The plant extract solution was applied to filter paper. The transparent appearance of the filter paper indicates the presence of oils and resins.

Quantitative Phytochemical Analysis
Estimation of Alkaloids

The alkaloid content of *E. helioscopia* was estimated by Harborne method [26]. 5ml of ethanol extract were added to 2 mL of 5% sulphuric acid and kept at 28 °C for 4 hours. 1 mL of ammonia solution was then added, and finally 2.5 mL of chloroform was added. Alkaloid precipitated was filtered, washed, and dried at 80° C in the oven. The content of alkaloid was then calculated as follows.

Total Alkaloids Percentage = Weight of alkaloid X 100 / Weight of Sample Taken

Estimation of Flavonoids

The total flavonoids in the plant extract were estimated by Zhishen *et al*. [27] method. 5ml of plant extract were diluted with 1 mL distilled water, followed by addition of 1 mL of 5% sodium nitrate solution. The mixture was incubated for 10 minutes and 0.15 mL of aluminium chloride solution was added. Then 0.5 mL of 4% sodium hydroxide is added and made up the volume to 2.5 mL by distilled water. The mixture was incubated again for 15 minutes at room temperature. The absorbance is measured at 510 nm in a

spectrophotometer.

Determination of Total phenols

The total phenolic component in *E. helioscopia* extract was estimated by Siddhuraju and Decker, [28]. 5 ml of ethanol extract were diluted with 10ml of distilled water in a 50 ml flask. The mixture is incubated for 10 minutes at room temperature. Then 2 ml of 0.1 N ammonium hydroxide was added and allowed to react for 20 minutes. 2.5 ml of amyl alcohol was then added and incubated for 5 hours in room temperature. The absorbance was read at 760nm.

Synthesis of Silver Nanoparticles

The ethanolic extract of *E. helioscopia* was used for the synthesis of AgNPs (pH 7.5). To 1 mL of plant extract, 9 mL of 1 mM $AgNO_3$ was added and incubated at room temperature for a period of 24 hours at 25°C in the dark. The leaf extracts and $AgNO_3$ solution were used as controls throughout the experiment. The color change to brown-yellow solution indicates the reduction of $AgNO_3$ to AgNPs. The final solution was centrifuged at 18,000 rpm for 25 minutes. The collected pellets were stored at -4°C.

Characterization of Silver Nanoparticles
UV-Visible Analysis

The reduction of pure Ag^+ ions was monitored by measuring the UV-Visible spectrum of the reaction medium after diluting a small aliquot of the sample into distilled water. The color change in the reaction mixture was recorded through visual observation, and the spectra's were taken at different time intervals using a UV-Visible spectrophotometer (Perklin- Elmer, Lamda 35,Germany) up to 24 hours between 350 nm to 500 nm.

FTIR analysis

The chemical composition of the synthesized AgNPs was studied using Fourier transform infrared spectrum analysis (FTIR) (Perkin-Elmer LS-55- Luminescence Spectrometer). The solutions were dried at 75°C and the dried powders were characterized in the range 4000–400 cm^{-1} with a resolution of 4cm^{-1} using the KBr pellet method.

SEM Analysis

The morphological features of synthesized AgNPs were studied by Scanning Electron Microscope (SEM) (JSM-6480 LV). After 24 hours of the addition of $AgNO_3$, the SEM slides were prepared by making a smear of the solutions on the slides. A thin layer of platinum was coated to make the samples conductive. Then the samples were characterized in the SEM at an accelerating voltage of 20 KV.

Screening of Antibacterial Activity

All the bacterial cultures were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Two Gram-positive pathogenic bacterial strains such as *Bacillus subtilis (B. subtilis)* (MTCC1133), *Staphylococcus aureus (S. aureus*) (MTCC2940), and three Gram-negative bacterial strains such as *Escherichia coli (E. coli)* (MTCC40), *Klebsiella pneumoniae (K. pneumoniae)* (MTCC2405) and *Pseudomonans aeruginosa (P. aeruginosa)* (MTCC424) were used to determine the antimicrobial activity of *E. helioscopia* leaf extract and AgNPs synthesized from the plant extract. The young bacterial broth cultures were prepared before the screening procedure.

Preparation of inoculums

Stock cultures were maintained at 4°C on slopes of nutrient agar. Active cultures of experiment were prepared by transferring a loop full of cells from the stock cultures to test tube of Mueller-Hinton Agar (MHA) for bacteria that were incubated without agitation for 24 hours at 37°C and 25°C respectively. The cultures were diluted with fresh Muller-Hinton broth to achieve optical densities corresponding to 2.0 X 10^6 colony forming units (CFU/ml) for bacteria.

Antibacterial susceptibility test

The disc diffusion method [29] was used to screen the antimicrobial activity. *In-vitro* antimicrobial activity was screened by using Mueller Hinton Agar (MHA) obtained from Hi-media (Mumbai). The MHA plates were prepared by pouring 15 ml of molten media into sterile petriplates. The plates were allowed to solidify for 5 minutes, and 0.1% inoculums suspension was swabbed uniformly, and the inoculums were allowed to dry for 5 minutes. Ethanolic extracts (C) and AgNPs (S) of the plants are prepared at 30 µg/mL (C30, S30) and 60 µg/mL (C60, S60) concentrations and loaded on the sterile disc of 6 mm diameter. Chloramphenicol, a standard

Table 1. Comparative analysis of phytochemical constituents in ethanol and Hexane extract of *E. helioscopia.*

S.No	Phytochemical constituents	Chemical tests	Observations	Extracts	
				Ethanol	Hexane
1.	Alkaloids	Mayer's test	Cream color Reddish brown solution/ precipitate	++	-
2.	Flavonoids	Lead acetate test H_2SO_4 test	Yellow orange Reddish brown / Orange color precipitate	++	-
3.	Steroids	Liebermann-Burchard test	Violet to blue or Green color formation	+	-
4.	Terpenoids	Salkowski test	Reddish brown precipitate	-	-
5.	Arthroquinone	Borntrager's test	Pink color	-	-
6.	Phenols	Ferric chloride test Lead acetate test	Deep blue to Black color formation White precipitate	++	+
7.	Saponin	Froth test	Stable persistant	+	-
8.	Tannin	Ferric chloride test	Brownish green / Blue black	+	+
9.	Carbohydrates	Fehling's test	Yellow / brownish / blue / green color	+	+
10.	Oils & Resins	Spot test	Filter paper method	-	+

Note: ++ - Very Abundant
+ - Abundant
- - Not Detected

antibiotic of concentration 1 mg/mL was used as positive control. The extracts were allowed to diffuse for 5 minutes, and the discs were placed on the surface of the medium. The plates were incubated at 37°C for 24 hours. Formation of clear zone indicates bacterial growth inhibition and the diameter were measured in mm.

RESULTS AND DISCUSSION

Phytochemical Profiling

E.helioscopia extracts prepared through soxhelt extraction using ethanol and hexane as solvents were compared and found that ethanolic extract were higher with yield of 21.28%. The study also showed the presence of alkaloids, flavonoids, steriods, phenols, saponin, tannin and carbohydrates in ethanolic extract and phenol, tannin, carbohydrates, oils and resins in hexane extract (Table 1). The ethanolic extracts contained more secondary metabolites than hexane extracts, specifically phenol showed high degree of precipitation in ethanol extract. Terpenoids and Arthroquinone was absent in both extracts. Saleem *et al.* [30] showed that the methanol is the best solvent for extraction of phytochemicals from *E. helioscopia* but in the present study, ethanolic extracts showed better results of phytochemicals. The quantitative phytochemical estimation specifies that the plant extracts contain a significant amount of alkaloid, flavonoids and phenolic content.

Quantitative estimation of Phytochemicals

The Gravimetric analysis of total alkaloid contents in ethanolic extract of *E. helioscopia* was found to be 27.3%. Alkaloids have one heterocyclic nitrogen atom and also found with organic acids in the form of salts that contributes therapeutic property in the plant materials. Purest form of alkaloids has antibacterial, analgesic, sedative and antispasmodic property and it is used as basic medicinal agents [31]. It is present in high proportion than other phytochemicals.

The total flavonoid content from the plant extract was estimated to be 7.5%. Flavonoids have antioxidant, anticancer and antiinflammatory properties. Arun *et al.* [32] showed that *E. helioscopia* contains high content of flavonoids in methanolic extracts while in the present study flavonoids are found in medium concentrations than other compounds in ethanolic extracts.

The total phenolic content in the ethanolic extracts was found to be 37.86% that was higher than alkaloids. The phenolic compounds may contribute to the free radical scavenging property, antibacterial activity and anticancer property of the plants [33, 34].

Synthesis of Silver Nanoparticles

Generally, Ethanolic and crude extract of plant samples was used to synthesize the AgNPs by bio-reduction method. Synthesize of AgNPs is identified by the color changes from faint light to colloidal brown in the solution [35]. The AgNPs synthesized from the ethanolic extract of *E. helioscopia* were characterized by UV-Visible spectrophotometer, FTIR and SEM.

UV-Visible Analysis

The formation of AgNPs using ethanolic plant

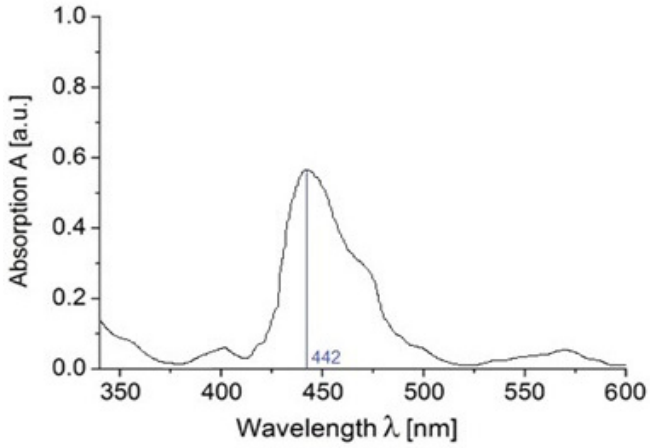

Fig. 1. UV analysis of AgNPs from *E. helioscopia.*

extract was confirmed by measuring the UV-visible spectrum at wavelengths ranging from 350 to 650 nm. The sample showed sharp peaks and proper baseline at 442 nm with the absorption of 0.59. The broadening of peak indicates that the particles are polydispersed and the UV-Visible spectrum obtained is shown in Fig.1. The colour change in the solution confirms the formation of AgNPs. They are formed by the reduction of Ag^{+} to Ag^{0}. The size, shape, and the interactions of plant metabolites with AgNPs lead to the excitation of surface plasmon resonance in aqueous solution that can be measured by spectroscopic methods. Usually the absorption peak of AgNPs lies in the range of 400-500 nm. Nirmala & Sridevi [36] reported Pa-AgNPs from endophytic bacteria that exhibited a absorption at 410 nm. Similar results were also reported in many green synthesized AgNPs [37-40] that confirms the characteristics of AgNPs.

FTIR analysis

FTIR measurement was carried out to identify possible biomolecules of *E. helioscopia* extract responsible for the formation and stabilization of nanoparticles. The calculated spectra clearly reflect the well-known dependence of NPs optical properties, viz. the resonance wavelength, the extinction cross-section, and the ratio of scattering to absorption, on the nanoparticle dimensions. The FTIR spectrum of AgNPs is shown in (Fig. 2) shows absorption peaks at 3398.46 cm^{-1} is primary amines (weak to medium), 3190.87 cm^{-1} is intermolecular hydrogen bonded OH (Strong), 2184.20 cm^{-1} for aromatic methane (Week), 1649.21 cm^{-1} is primary amines (Medium to Strong), 1384.96 cm^{-1} for tertiary butyl (Medium), 1034.28 cm^{-1} is cycloalkanes (Strong), 754.13 cm^{-1} for ortho disubstituted (Strong) and 698.10 cm^{-1} is aromatic methane (Week). The results obtained coincide with the earlier findings of similar AgNPs FTIR prediction [36-39] that revealed the presence of hydroxyl, alkene and amine groups in the bacterial extract acting as reducing, capping and stabilizing agents of Ag^{+}.

SEM Analysis

SEM image was recorded and is shown in Fig. 3. It is evident that the AgNPs synthesized were spherical in shape and it is in the diameter range of 5 nm – 6.3 nm. Similarly, Nasrollahzadeh *et al*. [39] green synthesized AgNPs from *E. helioscopia* Linn leaf extract for the synthesis of propargylamines.

Major secondary metabolites present in the plant leaves were determined by the phytochemical screening among which, the phenolic compounds were available in major composition that attributes to the anti-inflammatory, anticancer and antioxidant properties of the plant. These phytochemicals donates electrons for the reduction of Ag^{+} to nanosilver Ag^{0} in the plant extracts. They also influence the shape, size, and surface agglomeration of the NPs formed at standard conditions of neutral pH, room temperature and optimal concentrations. They also act as capping and stabilizing agents of NPs that enhances the antimicrobial ability of the AgNPs [12, 14, 21-22].

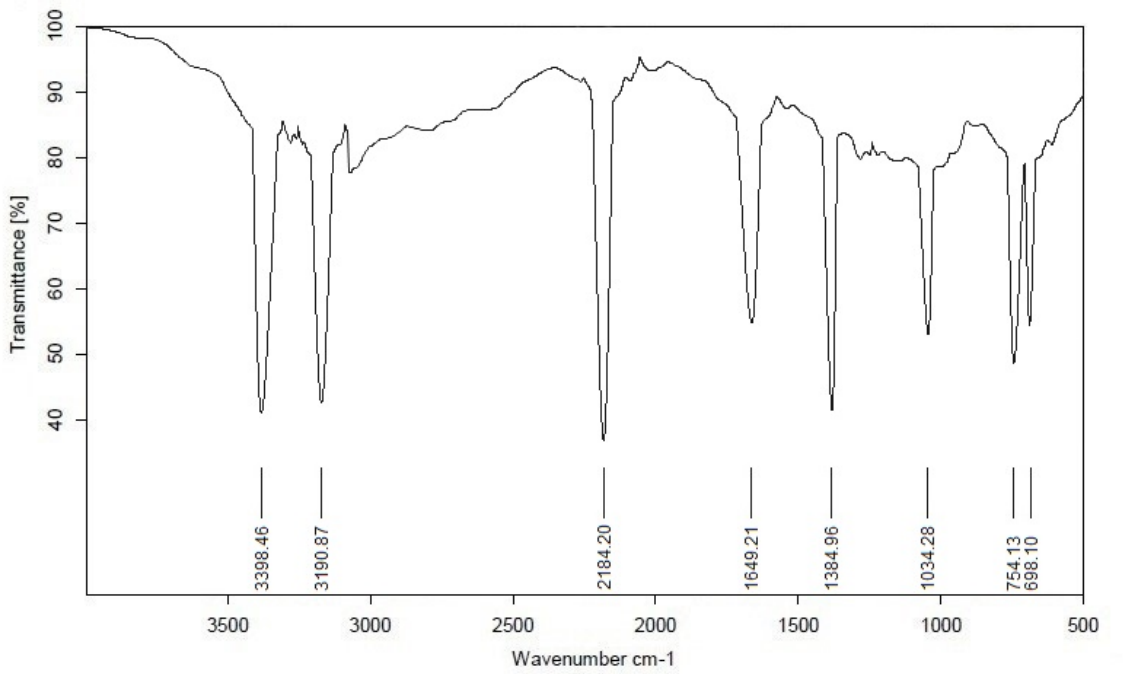

Fig. 2. FTIR analysis of AgNPs from *E. helioscopia.*

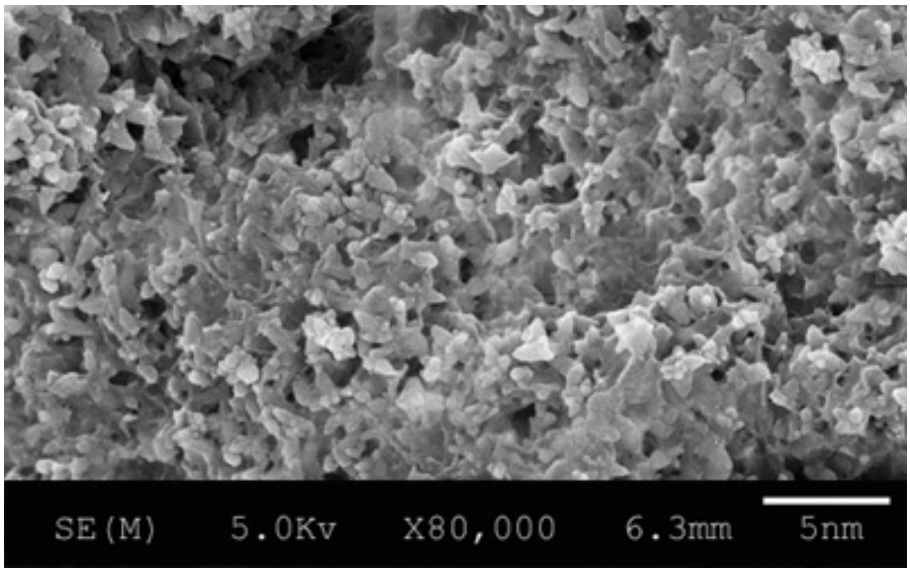

Fig. 3. SEM analysis of AgNPs from *E. helioscopia.*

Antibacterial susceptibility test

Screening of antimicrobial properties of ethanolic extracts (C) and AgNPs (S) of the *E. helioscopia* plant samples showed antimicrobial activities against Gram positive *B. subtilis, S. aureus,* and Gram negative *E. coli, K. pneumoniae, P. aeruginosa* (Table 2, Fig. 4a-4e, Fig. 5). The intensity of inhibition widely varied with microbial strains. This variability of inhibition may be due to the resistance capacity linked to the bacterial groups or to the nature of the compounds present in the plant extracts. The AgNPs showed higher activity than ethanolic extracts by forming higher inhibition zone. Among the bacterial strains *K.*

Table 2. Diameter of inhibition zone of control (C), Plant extracts (C30, C60) and AgNPs (S30, S60) of *E. helioscopia.*

S.No	Organisms	C (mm)	C30 (mm)	C60 (mm)	S30 (mm)	S60 (mm)
1	*B. subtilis*	16	7	9	15	23
2	*S. aureus*	20	7	14	19	24
3	*E. coli*	18	8	13	9	21
4	*K. pneumoniae*	18	7	12	11	26
5	*P. aeruginosa*	17	7	13	19	22

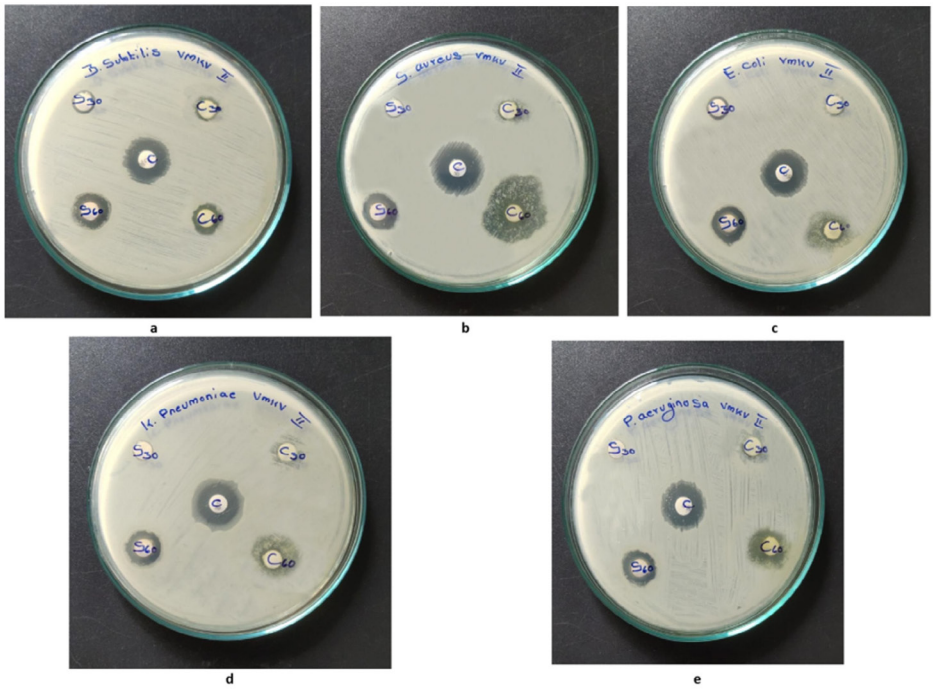

Fig. 4. Antibacterial activity of control (C), AgNPs (S30, S60) and Plant extract (C30, C60) of *E. helioscopia* against a. *B. subtilis,* b. *S. aureus,* c. *E. coli,* d. *K. pneumonia,* e. *P. aeruginosa.*

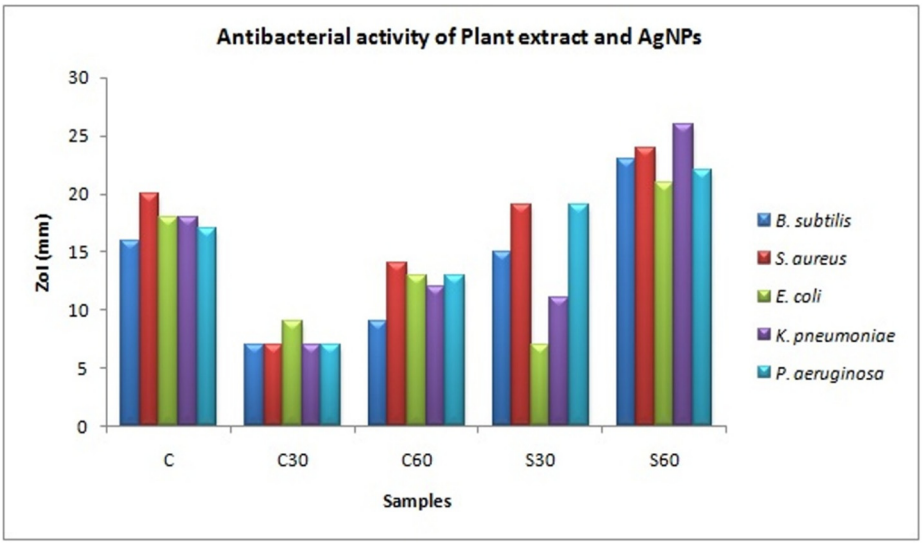

Fig. 5. Antibacterial activity of control (Chloramphenicol), Plant extract (C30, C60) and AgNPs (S30, S60) of *E. helioscopia.*

pneumoniae was more sensitive to S60 extracts. Kirbag *et al*. [40] also showed that the latex of *E. virgate* showed maximum activity against *K. pneumoniae* that was concurrence with the present study. The AgNPs at concentration 60µg/ml showed more inhibition than at 30µg/ml concentration. *S. aureus* also showed significant inhibition zone compared to other bacterial strains in S30 and S60 plant extracts. Elumalai *et al*. [41] also showed that the AgNPs green synthesized from leaf extract of *E. hirta* exhibited antimicrobial property. The result was also in concurrence with the earlier reports of AgNPs on antibacterial activity [41-43].

Apart from physicochemical methods, biological techniques emerge as an ecofriendly and cost-effective method for AgNPs synthesis. Though the plant extracts of *E. helioscopia* are found to have antibacterial activity, the AgNPs biosynthesized from the plant showed more activity. The nanoscale range of particle size, concentrations and ability to penetrate and damage the cell wall of microorganisms increased its efficiency as antibacterial agent than the crude plant extracts.

CONCLUSION

Medicinal plants are widely screened for their phytochemical constituents, which are emerging as novel drug candidates. The current study provides pertinent information on the phytochemical composition of ethanol and hexane extracts of *E. helioscopia*, with the ethanolic extract signifying higher activity of secondary metabolites. AgNPs were further synthesized from the plant extract, and their optimal size and structural formation were elucidated by spectral studies. The functional groups of the plant metabolites capping the AgNPs were also identified. The antibacterial screening of the plant extracts and the AgNPs against the bacterial pathogens showed significant inhibition with broad spectrum activity. Concentration at 60µg/ml of AgNPs showed highest antibacterial activity against the selected pathogenic organism than other concentrations and its crude extracts. The phenolic content in the plant extracts, nanoscale size and shape of the AgNPs may contribute to this enhanced activity. Thus, *E. helioscopia* will be a potential source of bioactive compounds that aid in the green synthesis of AgNPs with wide pharmacological applications.

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CONFLICTS OF INTEREST

The authors do not have any conflicts of interest.

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