



BIOSYNTHESIS OF SILVER NANOPARTICLES FROM THE NESTS OF THE PAPER WASP, *ROPALIDIA MARGINATA* AND THE MUD WASP, *SCELIPHRON CAEMENTARIUM* AND THEIR ANTIMICROBIAL ACTIVITIES

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ABSTRACT

Nanotechnology forms one of the emerging subjects of research with regard to the modern biological and material sciences. In the present study we have reported, bio synthesis of silver nanoparticles (AgNps) from the nests of the paper wasp, *Ropalidiamarginata* (PE) and mud wasp, *Sceliphroncaementarium* (ME) and confirmed their antimicrobial activities. The AgNps were characterized by UV-Visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), Scanning electron microscope (SEM), Energy dispersive electron spectroscopy (EDAX) and X-ray diffraction (XRD). The AgNps were evaluated for antimicrobial properties both antibacterial and antifungal. The board peaks were obtained at 389 nm for PE and 400 nm for ME with UV-Vis surface plasmon resonance studies confirmed that the synthesized nanoparticles are AgNps. The FTIR data showed prominent peaks for both which indicated the presence of phenolics compounds and proteins in the synthesis of AgNps. Scanning electron microscopic (SEM) studies revealed that the nanoparticles were spherical in shape with size ranging from 0.1 to 0.5 µm for PE and 0.2 to 0.5 µm for ME. EDAX analysis showed 75.48 weight percentage of Ag present in PE and 72.57 % in ME indicated the purity of sample. The AgNps of PE showed potent antibacterial activity against *S. pyogenes*, *S. aureus*, *E.coli*, *K. pneumoniae*, *B. subtilis*, *S.paratyphi* and antifungal activity against *C. albicans*, *T. viride* and *A. fumigatus*. The PE AgNps produced zone of inhibition of 13 mm against *K. pneumoniae* and 8 mm against *T.viride* at 30µg/ml. The AgNps of ME showed potent antibacterial activity against *S. pyogenes*, *S.aureus*, *E.coli*, *K.pneumoniae*, *Bacillus subtilis*, *S. paratyphi* and antifungal activity against *Candida albicans*, *T. viride*, *Afumigatus*. The PE AgNps produced zone of inhibition of 12 mm against *S. aureus* and 9 mm against *T. viride* at 30 µg/ml. Pencillin standard drug (10µg) was used as positive control for both bacteria and fungus.

KEYWORDS: Silver nanoparticles, *Ropalidiamarginata*, *Sceliphroncaementarium*, nest extracts, antibacterial activity, UV-Vis, FTIR, SEM, XRD.



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INTRODUCTION

In the recent years nanotechnology has gained a great impetus and represents the most interesting and fascinating research areas of modern sciences. The nano particles have unique physical and biological properties and these factors contribute for the extensive study and application of AgNPs in the various biological, biomedical, environmental, food and agriculture, catalytic, electrical and electrochemical applications¹. Several authors have successfully synthesized silver nanoparticles (AgNPs) using metals like iron, copper, calcium, gold, palladium, zinc and silver. The various methods by which the AgNPs can be synthesized are namely chemical methods²⁻⁵, physical methods⁶⁻⁸ and biological methods⁹⁻¹¹. Chemical methodology followed for the synthesis includes chemical reduction, electrochemical, irradiation assisted chemical and pyrolysis¹². Metal precursor, reducing agents and stabilizing or a capping agent is the essential requirements for the synthesis of AgNPs in solution. Borohydride, sodium citrate, alcohol, Ascorbic acid and hydrazine compounds are the most commonly used reducing agents. Compared to chemical methods the physical methods generally have a fast processing time as they do not require lethal and highly reactive chemicals and includes arc-discharge, physical vapor condensation, energy ball milling method¹³ and direct current magnetron sputtering. The AgNPs synthesized with the help of physical method tend to have a narrow size distribution which provides an advantage over chemical methods. Consumption of high energy is the only demerits about the physical methodology. An alternative approach for the biological synthesis of AgNPs is possible with the herbal extracts and/or microorganisms which seems to have several advantages over the chemical and physical methods as these routes are simple, cost-effective, eco-friendly and can be easily scaled up for higher yields or production. Synthesis of AgNPs from the metabolites from arthropods is yet another promising field of

nanotechnology and has been widening among the researchers¹⁴. The nests of *Polistes* have been reported to be rich in cellulose and 20 prevalent amino acids have been isolated from these nests such as valine, proline, alanine, glycine and serine.¹⁵⁻¹⁶ The nests of *S.caementarium* also have been found to contain more amounts of proteins, amines, magnesium, calcium, iron, sulphur, phosphorus, potassium phenol, chlorine, and aluminum in varying quantities¹⁷. Even though arthropods synthesize a wide variety of metabolites like cobwebs, nests, cocoons, and silk, these biomaterials have not been effectively utilized for the green synthesis of AgNPs and there are not many literature reports are available in this aspect¹⁸⁻¹⁹. Hence an attempt was made to synthesize AgNPs using the nests of *R.marginata* and *S.caementarium* and further examine for the antibacterial and antifungal properties. This report is the first of kind towards the use of the nests of the paper wasp and mud wasp for the biosynthesis of AgNPs. *Ropalidiamarginata* belongs to the genus *Ropalidia* and subfamily Polistinae, family Vespidae and order Hymenoptera. The genus *Ropalidia* has around 136 species, mainly limited to the Old World²⁰⁻²¹. They build paper nests, hence the name paper wasps and live in colonies. These wasps exhibit primitive eusociality, consisting of a single reproductive queen who is the foundress of the colony²². There exists a morphological similarity among the non-reproductive female workers and the queen. Physical dominance is expressed by the queen to control workers; instead she uses pheromones in order to suppress other female workers from overtaking queenship²³. Usually the size of the colony is moderate and it may contain hundreds of brood. The nests constructed by these wasps is not covered by envelope is called open type, having more than one pedicels²⁴⁻²⁵. They feed on the plant fibers, masticate the cellulose part of it and mix with saliva to build the papery nests and are mostly found in closed spaces with small openings in natural and man-made structures (Figure1).



Figure 1
Nest building by *Ropalidiamarginata*

Sceliphroncaementarium is a cosmopolitan wasp belonging to the genus *Sceliphron*, subfamily Sceliphrinae, family Sphecidae, of order Hymenoptera. Around 9716 species of Sphecidae falling under 318 genera has been described worldwide²⁶. These wasps

exhibit a great morphological and biological diversity. The adult wasps feed on nectar and honeydew of flowers, spiders and other smaller insects that belonging to several orders. The females construct mud nests and stock it with paralyzed prey for the developing wasp

larva²⁷. Sphecid wasps have also been studied by several authors in the recent years and around 17 species from the four genus of Subfamily Sphecinae have been reported from various parts of India²⁸⁻³². The nests are built by the female *Sceliphron* in the dry and sheltered sites preferably nearer to the human habitations using the mud collected from the from water puddles. A single is constructed initially and are

provisioned with spiders, upon which eggs are laid (Figure 2). The nest is sealed by the female before she starts her work on the construction of another cell. An aggregate cell eventually consists of more than one cell and is covered by mud as several layers. Sometimes when the cells are not fully stocked by the spiders, they are sealed on the temporary basis at night.



Figure 2
Nest building by *Sceliphroncaementarium*

MATERIALS AND METHODS

Preparation of Paper and Mud Wasp nest extract

Nests of paper wasp and mud wasp were collected from Coimbatore, Tamil Nadu, India. The nests were taken to the laboratory and washed with distilled water so as to remove dust and other extraneous materials. The washed nests were air-dried at room temperature (30 ± 2 °C) and kept in air-tight container until further use. The method was modified and previously reported²⁵. About 0.1 g of each of the nest were weighed and hydrolyzed with 10 ml of 0.1-M NaOH at 90 °C for 1 h. The hydrolyzed nests were allowed to cool,

centrifuged at 4000 rpm for 30 min, and used without further purification.

Biosynthesis and separation of Silver nanoparticles

The silver nanoparticles were synthesized using a constant volume (1ml) of the sample extracts under various experimental conditions. Aqueous solution of 1mM AgNO₃ was prepared and used for the synthesis of silver nanoparticles. 5 ml of the aqueous extract of paper wasp nest (PE) and mud wasp nest (ME) were mixed separately with 95 ml of AgNO₃ for the synthesis of silver nanoparticles. The formation of silver nanoparticles was confirmed by the color change from brown to ash color (Figure 3 and Figure 4).

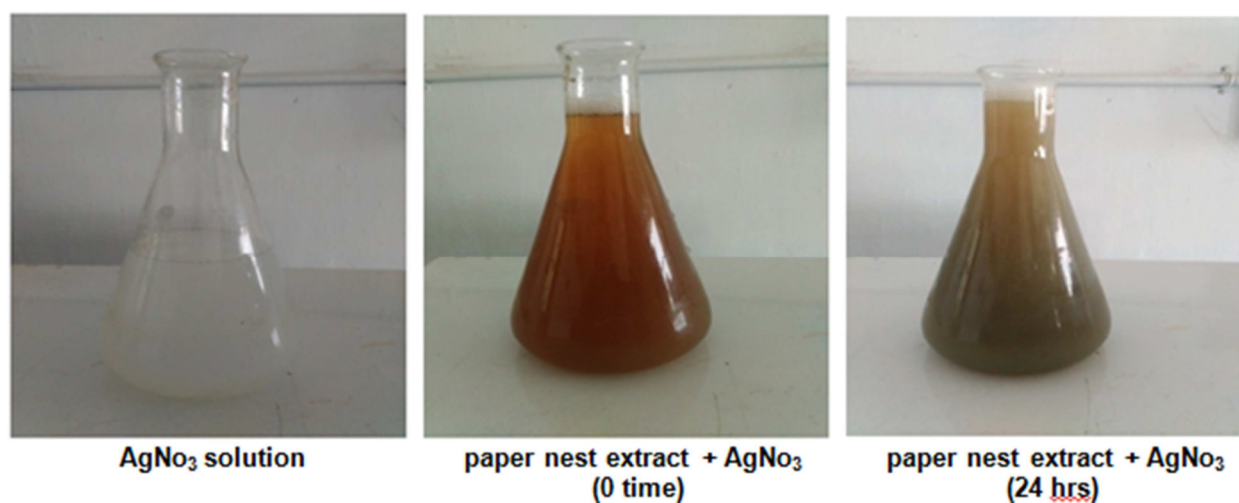


Figure 3
Biosynthesis of AgNps using paper wasp nests

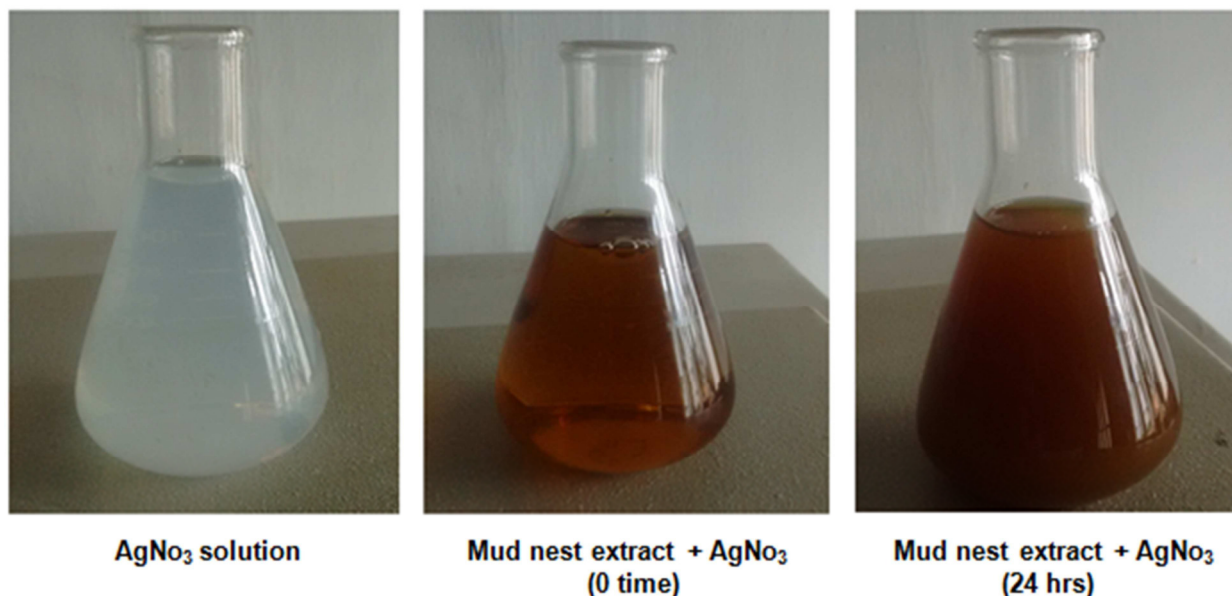


Figure 4
Biosynthesis of AgNps using mud wasp nests

The appearance of ash color after 3 hours indicated the formation of silver nanoparticles. The synthesized silver nanoparticles were separated by centrifugation (Spectrofuge 7M) at 13,000 rpm for 15 minutes. The process was repeated by dispersion of pellets in water to obtain colored supernatant solutions. The samples were then stored at -4°C for further use.

Characterization of silver nanoparticles

The Ultraviolet-visible (UV-vis.) absorption spectroscopy is a valuable technique for the characterization of nanoparticles like the noble metals because they are intensely colored and show absorptions due to surface plasmon oscillations. The reduction of pure Ag^+ ions was monitored through visual observation of the color change and by measuring the UV-Vis spectrum of the reaction medium at 5–10–12 hours. UV-Vis spectral analysis was done by using UV-Vis spectrophotometer UV-2450 (Shimadzu). Fourier transform infrared (FTIR) spectroscopy analysis was carried out using (Nicolet b6700 FTIR, Thermo Scientific) spectrometer on the powder sample of AgNps. The AgNps solution was centrifuged at 10,000 rpm for 20 min. The solid residue obtained were dried at room temperature and used for FTIR measurements by KBr pellets technique. The structures of the AgNps were analyzed by using XRD. The pure AgNps were obtained by repeated centrifugation at 5000 rpm for 30 minutes. The redispersion of pellet of AgNps was done by using 10 ml of deionized water. The purified AgNps were dried by freezing and then collected. The crystalline nature of nanoparticles was examined using an X-ray diffractometer (XRD) from X-pert pro equipped with $\text{Cu K}\alpha$ radiation source using Ni as filter operated at voltage of 40kV and a current of 30 mA. The particles were allowed to settle for 3–5 min on the carbon coated copper grid, the excess liquid flicked off with a wick of blotting paper and the grids were then air dried before SEM viewing. Micrograph was obtained using a Hitachi S-4500 (JEOL, USA) operating at 200 kV. The elemental characterization of metal nanoparticles was conducted using energy dispersive X-ray (EDX) analysis. The samples were dried and coated on to

carbon film and performed on JEOL –MODEL 6390 SEM instrument equipped with Thermo EDX attachments.

Antimicrobial activities of synthesized AgNPs

The extracts of the synthesized AgNPs nests of the paper wasp, *R. marginata* and the mud wasp, *S.caementarium* were analyzed for their antimicrobial activity against the gram positive bacterial strains like *Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacillus subtilis* and gram negative bacterial strains like *E. coli*, *Klebsiella pneumonia*, *Salmonella paratyphi*. Antifungal studies were also carried out on selective four fungal like *Candida albicans*, *Aspergillus fumigates* and *Trichodermaviride*. The bacterial and the fungal strains were procured from Department of Microbiology, Hindusthan College of Arts and Science, Coimbatore. Standard protocol was followed with regard to the well diffusion method³⁴. For antibacterial evaluation, each bacterial culture (100 μl) was grown overnight (18 h) in peptone water and seeded over Mueller–Hinton Standard Agar plates (Hi Media Laboratories Private Limited, Mumbai, India). Thereafter, the plates were bored using cork borer (5 mm) to create wells, to which 10 μl (10 cells /ml) of graded concentrations (10–30 μg /ml) of AgNPs prepared by dispersion in sterile distilled water were added. The plates were incubated at 37°C for 24 h, for the examination of zones of inhibition, which were measured. Pencillin (10 μg) was used as standard. The antifungal activities of the biosynthesized AgNPs were evaluated using the standard methods³⁵ by incorporating graded concentrations of AgNPs into potato dextrose agar plates, which were then inoculated with agar plug of 5 mm of 10 days old cultures of the above mentioned fungal strains. In the control experiments, fungal plugs were inoculated on PDA plates without the incorporation of AgNPs. All the plates were incubated at $28 \pm 2^{\circ}\text{C}$ for 72 h. Pencillin (10 μg) was used as the standard drug. The diameters of fungal growths in all the plates were measured.

STATISTICAL ANALYSIS

The data obtained were analyzed using SPSS software (version 12). Students (paired) 't' test was used for analysis of comparison. The data were presented as mean \pm standard deviation (SD). Probability value (p) of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The nest extract of (PE and ME) with pH 10.0 catalyzed bio reduction of silver ions to produce metallic AgNPs within 5 min. The AgNPs was brownish in color which stabilized after 10 min of reaction. The formation of colloidal silver nanoparticles were indicated by the color

change ranging from light yellow, yellow brown to dark brown³⁶⁻³⁸. The composition of bioreductant molecules present in the nests of the wasps are held responsible for the color changes which influences the surface plasmon resonance. The nests of *R.marginata* and *S.caementarium* are rich in proteins, amino acids that readily serve as bioreductant molecules for the green synthesis of AgNPs. Therefore this work has further shown that metabolites of paper nest wasp and mud nest wasp could use for the synthesis of nanoparticles. Colloidal AgNPs were monitored by using UV-Vis spectroscopy from 190-750nm scan range. D The peaks were obtained at 389nm for PE and 400nm for ME respectively (Figure 5 a, Figure 5b).

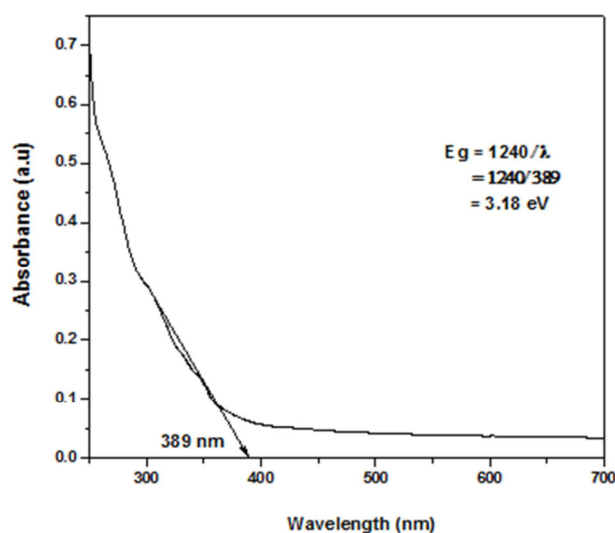


Figure 5a

UV-Vis spectrum of biosynthesized AgNPs using PE.

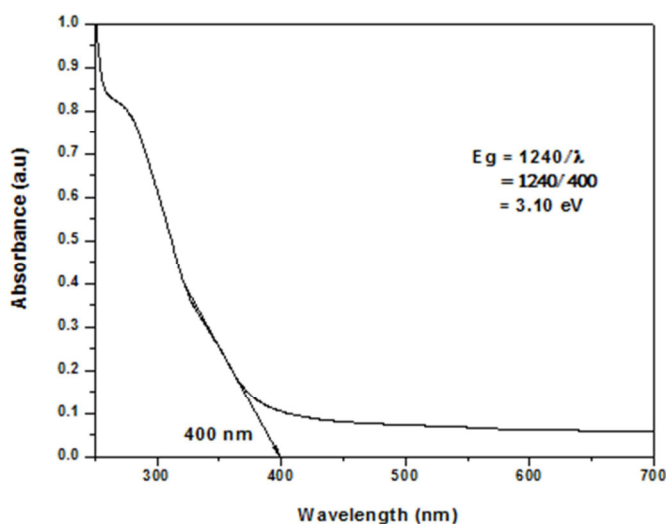


Figure 5b

UV-Vis spectrum of biosynthesized AgNPs using ME

The observed maximum absorbance falls within the range previously reported³⁹ here, the nanoparticles absorb light at different wavelength and get excited due to change density at the interface between conductor and insulator of UV-Vis to give a respective peak. This mechanism is known as surface plasmon resonance.

FTIR spectrums of synthesized AgNPs (PE and ME) were carried out to know the possible biomolecules responsible for the capping and stabilization of nanoparticles. For this, the sample was analyzed in the scan range from 4000 to 500cm⁻¹ of near IR spectra by FTIR (Figure 6a, Figure 6b).

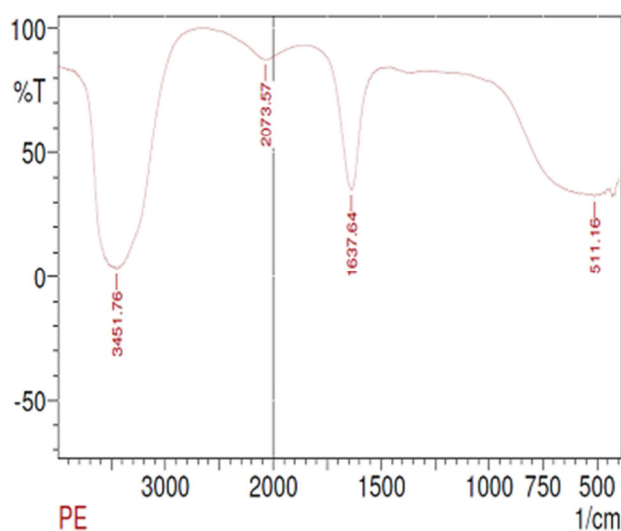


Figure 6a

FTIR spectrum of biosynthesized AgNPs using PE.

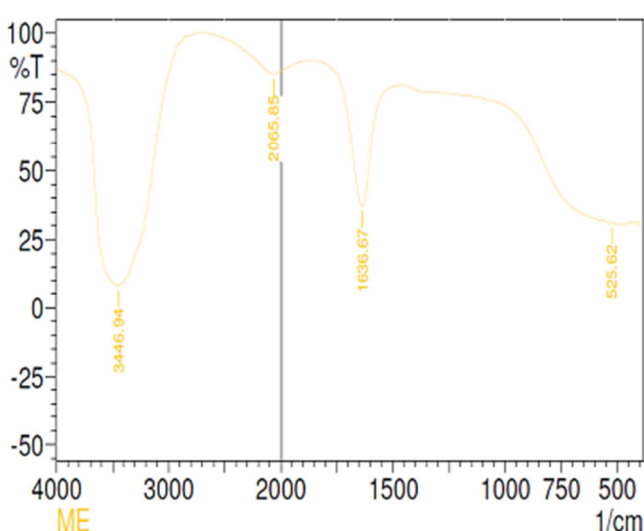


Figure 6b

FTIR spectrum of biosynthesized AgNPs using ME

The board peaks obtained for PE at 3451.76, 2073.57, 1627.64 and 511.76 cm^{-1} and ME at 3446.94, 2065.85, 1636.67 and 525.62 cm^{-1} . The major peak at 3451.76 and 1627.64 cm^{-1} for PE and 3446.94 and 1636.67 cm^{-1} for ME correspond to N-H amines or O-H stretch of carboxylic acid, C=C stretch of alkenes or C=O stretch of amides respectively⁴⁰⁻⁴¹ indicating the involvement of phenols and protein present in the biosynthesis of AgNPs. This suggest that the hydroxyl group of phenols and amide groups of proteins forming a layer of the

nanoparticles, act as capping agents to prevent agglomeration and provide stability to the reaction medium. X-ray diffraction analysis was carried out to confirm the nature of nanoparticles. The XRD pattern shows three peaks of X-axis like 32.22°, 38.10° and 46.21° corresponding to 261, 176 and 153 Bragg reflections of Y-axis for PE and X-axis like 37.90°, 31.99° and 45.98° corresponding to 320, 163 and 90 Bragg reflections of Y-axis for ME respectively.(Figure 7a, Figure 7b).

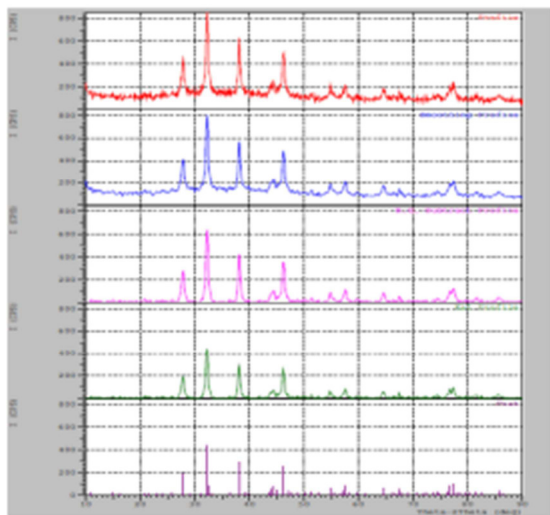


Figure 7a
X-Ray diffraction spectrum of synthesized AgNPs using PE.

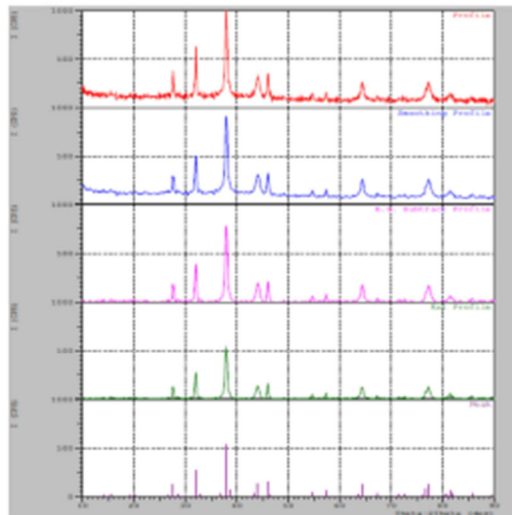


Figure 7b
X-Ray diffraction spectrum of synthesized AgNPs using ME.

A higher modification study was carried out with SEM analysis to know the size and shape of the nanoparticles along with crystalline nature. 10mm resolution studies of synthesized AgNPs showed 0.1- 0.5 μm for PE and 0.2-0.5 μm for ME (Figure 8a, Figure 8b) which are well dispersed without any form of aggregation.

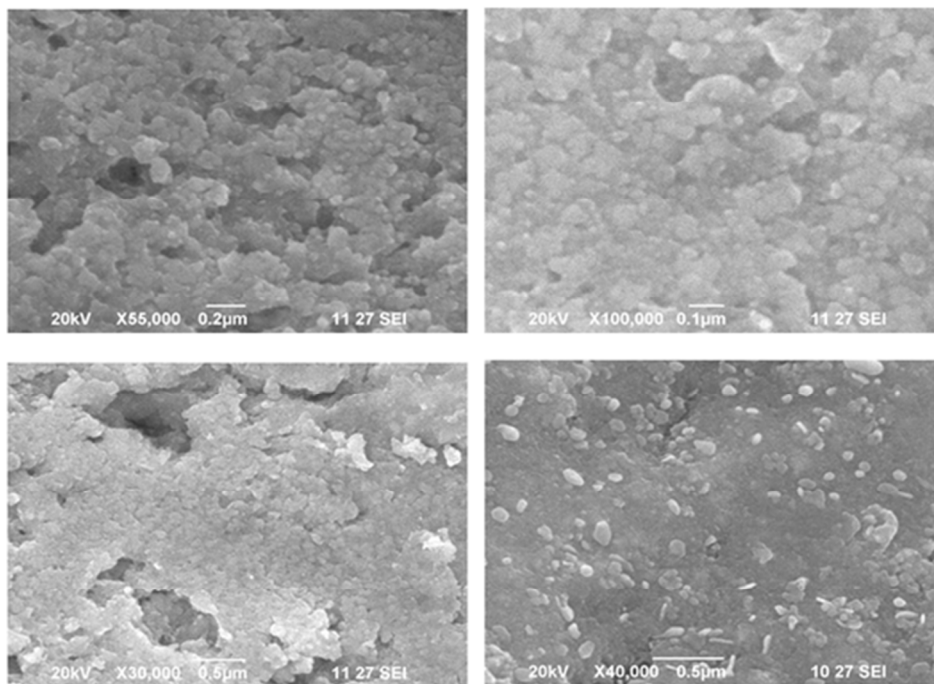


Figure 8a
Biosynthesized AgNPs with SEM analysis show size range from 0.1- 0.5 μm (PE)

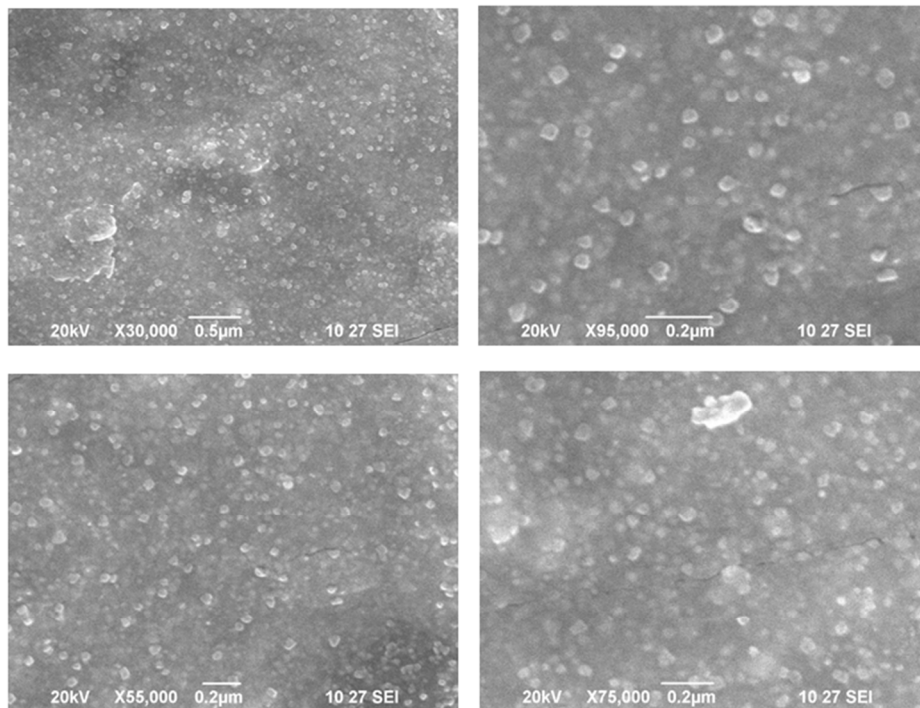


Figure 8b
Biosynthesized AgNPs with SEM analysis show size range from 0.1- 0.5µm (ME)

The EDAX analysis of synthesized sample showed 75.48% weight percentage of Ag metal along with 11.82% of chlorine and 12.71% of oxygen for PE and 72.57% silver with 5.42% of chlorine and 22.01% of oxygen for ME (Figure 4a,b) indicated both the samples (PE and ME) having high purity of silver nanoparticles (Table 1) (Figure 9a,b).

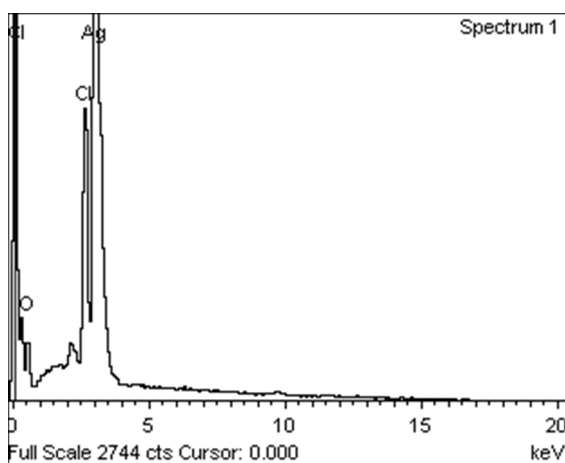


Figure 9a
EDAX analysis of AgNPs using PE showed weight percentage of Ag metal.

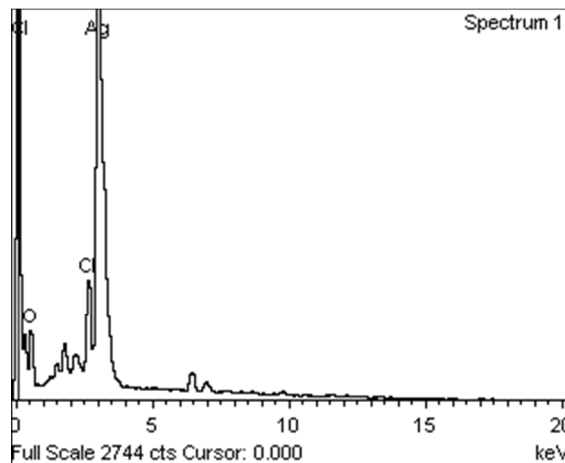


Figure 9b
EDAX analysis of synthesized AgNPs using ME showed weight percentage of Ag metal

Table 1
EDAX analysis of synthesized AgNPs showing the weight percentage of Ag metal (PE & ME)

S.No	Element (PE)	Weight (%)	Element (ME)	Weight (%)
1	O	12.71	O	22.01
2	Cl	11.82	Cl	5.42
3	Ag	75.48	Ag	72.57

**PE- AgNPs synthesized from paper wasp nests*
**ME-AgNPs synthesized from the mud wasp nests*

Antimicrobial Activities of AgNps

Antimicrobial activity of bio synthesized AgNps were analyzed on gram negative and gram positive bacterial strains growing on nutrient agar medium and fungal strains growing on potato dextrose agar medium. The inhibition of zone of each extract (PE and ME) was compared with standard drug Pencillin for both the activities. The (PE) AgNps showed highest inhibitory activity on *Klebsiella pneumonia* followed by *Bacillus subtilis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella paratyphi* and for fungal strains *Trichodermaviride* followed by *Candida albicans* and *Aspergillus fumigatus*. The mud paper wasp extract showed highest inhibitory activity on *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella paratyphi*, *Klebsiella pneumonia*, *Bacillus subtilis* and *Escherichia coli* whereas fungi *Trichodermaviride*, *Aspergillus fumigatus* and *Candida albicans*. Based on these studies 0.1 -0.5 μm size and shape of the nanoparticles showed higher inhibitory

antimicrobial activity. AgNps with larger surface area provide better contact with microorganisms³³. Thus these particles are capable to penetrate the cell membrane or attach to the bacterial and fungus surface based on their size. Bacterial growth and proliferation are adversely inhibited by the adhesion of ultra-small sized AgNps onto the cell wall of bacteria, resulting in changes in the cell wall which in turn is unable to protect the interior of the cell. Through the penetration of silver nanoparticles into the bacterial cell, it leads to DNA damage, or even cell death, by altering its normal functioning of bacterial DNA. The interaction of Ag⁺ ions with the proteins containing sulfur present in the bacterial cell wall irreversibly causes the disruption of the bacterial cell wall. Therefore, the nanoparticles synthesized from the paper and the mud wasp nests were found to have potent antibacterial and antifungal activities. The antimicrobial activity reports are shown in Figure 10, Table 2-5.

Table 2
Antibacterial activity of paper wasp AgNPs (PE)

S. No	Pathogenic bacteria	AgNPs (PE)			Standard (Penicillin)
		Zone of inhibition (mm)			
		10 μl	20 μl	30 μl	
1	<i>Streptococcus pyogenes</i>	5.0 \pm 0.50	8.0 \pm 0.50	9.0 \pm 0.27	3.0 \pm 0.34
2	<i>Staphylococcus aureus</i>	4.0 \pm 0.15	5.0 \pm 0.40	9.0 \pm 0.26	4.0 \pm 0.41
3	<i>Escherichia coli</i>	5.0 \pm 0.28	6.0 \pm 0.26	8.0 \pm 0.25	3.0 \pm 0.21
4	<i>Klebsiella pneumoniae</i>	5.0 \pm 0.58	10.0 \pm 0.25	13.0 \pm 0.58	3.0 \pm 0.58
5	<i>Bacillus subtilis</i>	3.0 \pm 0.55	10.0 \pm 0.23	12.0 \pm 0.50	4.0 \pm 0.45
6	<i>Salmonella paratyphi</i>	3.0 \pm 0.20	4.0 \pm 0.25	4.0 \pm 0.30	4.0 \pm 0.50

The experiment was conducted in triplicates (n=3), \pm SD

Table 3
Antifungal activity of paper wasp AgNPs (PE)

S. No	Pathogenic fungus	AgNPs (PE)			Standard (Penicillin)
		Zone of inhibition (mm)			
		10 μl	20 μl	30 μl	
1	<i>Candida albicans</i>	3.0 \pm 0.25	5.0 \pm 0.55	7.0 \pm 0.30	4.0 \pm 0.32
2	<i>Trichodermaviride</i>	5.0 \pm 0.26	6.0 \pm 0.54	8.0 \pm 0.35	4.0 \pm 0.35
3	<i>Aspergillus fumigatus</i>	3.0 \pm 0.52	5.0 \pm 0.51	6.0 \pm 0.33	4.0 \pm 0.38

The experiment was conducted in triplicates (n=3) \pm SD

Table 4
Antibacterial activity of mud wasp AgNPs

S.No	Pathogenic bacteria	Mud wasp AgNPs			Standard (Penicillin)
		Zone of inhibition (mm)			
		10 μl	20 μl	30 μl	
1.	<i>Streptococcus pyogenes</i>	3.0 \pm 0.52	8.0 \pm 0.50	10.0 \pm 0.25	3.0 \pm 0.41
2.	<i>Staphylococcus aureus</i>	6.0 \pm 0.57	7.0 \pm 0.32	12.0 \pm 0.22	3.0 \pm 0.55
3.	<i>Escherichia coli</i>	5.0 \pm 0.59	6.0 \pm 0.80	7.0 \pm 0.33	3.0 \pm 0.48
4.	<i>Klebsiella pneumoniae</i>	6.0 \pm 0.85	8.0 \pm 0.22	10.0 \pm 0.35	3.0 \pm 0.21
5.	<i>Bacillus subtilis</i>	2.0 \pm 0.22	4.0 \pm 0.25	10.0 \pm 0.39	4.0 \pm 0.25
6.	<i>Salmonella paratyphi</i>	3.0 \pm 0.35	8.0 \pm 0.25	10.0 \pm 0.24	4.0 \pm 0.38

The experiment was conducted in triplicates (n=3) \pm SD

Table 5
Antifungal activity of mud wasp AgNPs

S. No	Pathogenic fungus	Mud wasp AgNPs Zone of inhibition (mm)			Standard (Penicillin)
		10 μ l	20 μ l	30 μ l	
1.	<i>Candida albicans</i>	3.0 \pm 0.21	5.0 \pm 0.45	6.0 \pm 0.27	4.0 \pm 0.27
2.	<i>Trichodermaviride</i>	3.0 \pm 0.26	6.0 \pm 0.41	9.0 \pm 0.35	4.0 \pm 0.25
3.	<i>Aspergillusfumigatus</i>	4.0 \pm 0.27	5.0 \pm 0.58	7.0 \pm 0.50	4.0 \pm 0.32

The experiment was conducted in triplicates (n=3) \pm SD

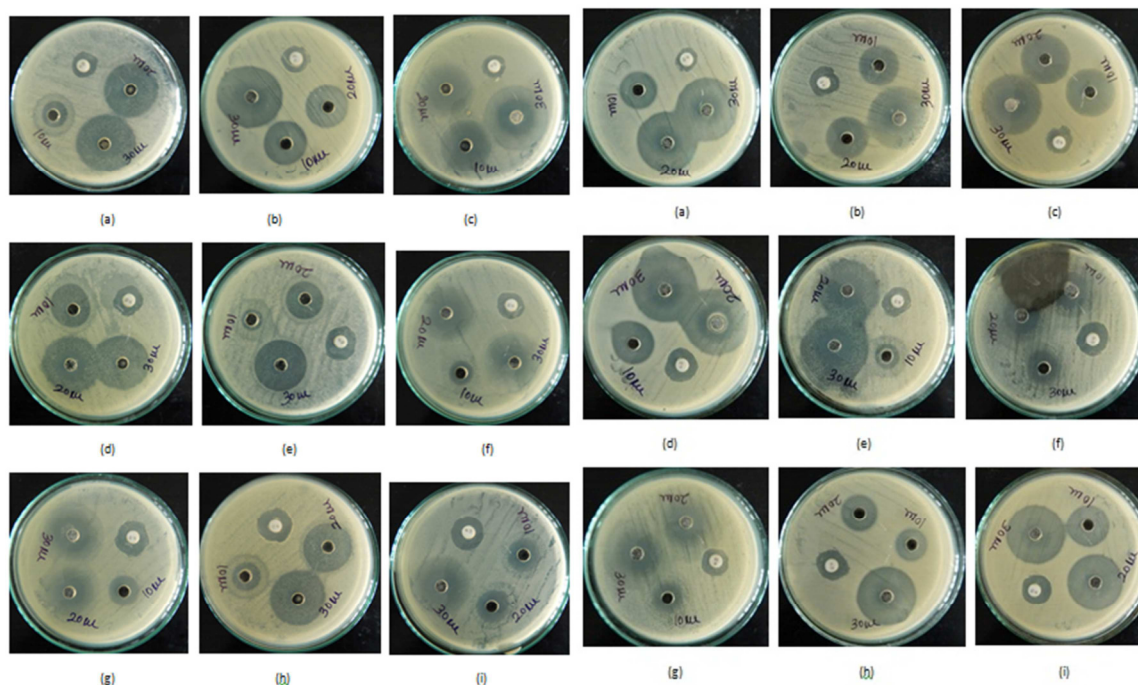


Figure 10

Antimicrobial activity of biosynthesized silver nanoparticles from mud and paper extract against (a) *S. pyogenes*, (b) *S. aureus*, (c) *E. coli*, (d) *K. pneumoniae*, (e) *B. subtilis*, (f) *S. paratyphi*, (g) *C. albicans*, (h) *T. viride*, (i) *A. fumigatus*.

CONCLUSION

The biosynthesis of silver nanoparticles using the nests of the paper wasp, *R. marginata*(PE) and mud wasp, *S. caementarium*(ME) provides with an eco-friendly and an effective alternate for the chemical synthesis of nanoparticles. The size of these silver nanoparticles varied from 0.1-0.5 μ m for PE and 0.2-0.5 μ m for ME and the characterization was using UV-vis spectrophotometer, FTIR, SEM, EDAX and XRD techniques. Remarkable antimicrobial activities were exhibited against disease causing pathogens by the biosynthesized silver nanoparticles from the paper and mud wasp nests (PE and ME). And since these silver nanoparticles have the potentiality as effective antimicrobial agents, the biomaterials of arthropods

have a huge prospect of being applied in nanobiotechnology and towards commercialization.

AUTHOR CONTRIBUTION STATEMENT

Mrs. Susheela P conceptualized and gathered the data with regard to this work. Dr. Rosaline Mary and Dr. Radha. R analyzed these data and necessary inputs were given towards the designing of the manuscript. All authors discussed the methodology and results and contributed to the final manuscript.

CONFLICT OF INTEREST

Conflict of interest declared none.

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