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Antibacterial Activity of Synthesized Silver Nanoparticle from Polyherbal Extract against Human Pathogens

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Abstract

Polyherbal formulations are known for various medicinal properties. The aim of this study was to biosynthesis of Silver nanoparticle (AgNps) using polyherbal extract and to evaluate their effect on human bacterial pathogens. Polyherbal extract silver nanoparticles were synthesized and the presence of AgNPs was confirmed by UV-Visible (UV-VIS) spectrophotometer and Scanning Electron Microscopic (SEM) analysis. The effect of polyherbal AgNps were evaluated against human bacterial pathogens by agar well diffusion method. The UV-VIS spectrophotometer, SEM analysis and the agar well diffusion method confirmed the potential antibacterial activity of biosynthesized polyherbal AgNPs against common bacterial pathogens such as *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Propionibacteriun acnes, Streptococcus oralis, Enterobacter feacalis, Proteus vulgaris, Aeromonas hydrophila, Corynebacterium diphtheriae* and *Actinomycetes Sp*. Therefore, we suggest that polyherbal AgNPs can be used for the treatment of bacterial diseases.

Keywords: Polyherbal formulation, Silver nanoparticles, Bacterial pathogens, Agar well diffusion, Antibacterial activity

Introduction

Nanotechnology is the application of science to a molecular level regulation of matter [1]. Due to their extremely small size and high surface-to-volume ratio, nanoparticles are of great interest, resulting in chemical and physical variations in their properties [2-4]. There are also many uses for nanoparticles in various fields such as medical imaging, nanocomposites, sensors, drug delivery and tumor hyperthermia [5-8]. Metal nanoparticles in medicine and pharmacy have many significant uses [9]. Green synthesis ascends as an incipient approach that has more advantages over the way, NPs are synthesized in physical and chemical ways [10,11].

The most popular nanoparticles used for biomedical applications and in the emerging interdisciplinary nanobiotechnology field are gold and silver [9]. Silver (Ag) NPs emerge as potential antimicrobial agents by showing robust antiviral, antibacterial, antifungal and antiinflammatory activities that highlighted their importance [10]. Plant-assisted NP synthesis is more effective in achieving a higher yield than the microbial synthesis. Plants have many metabolites and biochemicals (e.g. polyphenols) that can act in the synthesis of biogenic NPs as both a stabilizing and reducing agent. NPs plant mediated synthesis is environmentally sustainable (evitating the use of hazardous chemicals) and economical [12].

AgNPs are used as nano-carriers for drugs and antibiotics that help to improve antibiotic activity against resistant microbes [13]. Antibacterial activity of AgNPs depends on its size and shape. Pal *et al.* [14] found that if the size of AgNPs decreases, their surface area increases, resulting in an increased binding affinity to molecules. AgNPs are converted to silver ions as it reaches the cell and begin to interact with cellular biomolecules that cause cell damage. It hinders replication of DNA by binding to DNA [15]. It interferes with the cycle of cell division by linking membrane proteins and cellular proteins that help to separate the cells [16]. The mechanism of action of nano-particles of metal and metal oxide against bacteria is still not very clear and a number of studies are going across worldwide [17]. Studies have shown that AgNPs have induced the formation of pits and gaps on the membrane surface of bacteria by releasing ions which further interact with disulphide or sulfhydryl groups of enzymes causing metabolic pathway disruption that ultimately leads to death of bacterial cells [18].

Morinda citrifolia is one of the most significant traditional medicinal plant in Polynesia [19]. Medicinal characteristics such as anticancer, antitumor, antidiabetic, anti-aging, antimicrobials, etc. have been fully investigated scientifically abroad as a result of several commercial noni products currently available [20].

Caesalpinia bonduc L. is a medicinal herb of the Caesalpiniaceae family, widely distributed throughout the world. It has been considered an important remedy in Indian traditional plant medicine for the treatment of several diseases. The plant exhibits antiproliferative, antipsoriatic, antitumor, larvicidal, contractile muscle, hepatoprotective, anticonvulsant, antifilarial activity, etc. Phytochemical analysis of revealed the presence of alkaloids, flavonoids, glycosides, saponins, tannins, and triterpenoids [21].

Nigella sativa (Family Ranunculaceae) is a medicinal plant which is commonly used around the world. The medicinal effects were investigated and a wide variety of its pharmacological activities were evaluated including antidiabetic, anticancer, immunomodulator, analgesic, antimicrobial, anti-inflammatory, spasmolytic, bronchodilator, hepato-protective, gastro-protective, antioxidant, etc. The plant's therapeutic properties are due to the presence of thymoquinone, which is a significant bioactive component of the essential oil [22].

Based on the above investigation of the medicinal plants, this study has a main objective to propose an efficient biosynthesis of silver nanoparticles with proven antibacterial activities. Synthesis of silver nanoparticles using medicinal plants are environment friendly and offers an excellent alternative to the development of antibiotics. Most of the previous research work are done by using single plant and not the synergistic effect of the different medicinal plants for an antibacterial activity. The present study aims to evaluate the synergistic effect of antibacterial activity of the green synthesized silver nanoparticles using polyherbal formulation of selected medicinal plants against the human pathogens.

Materials and methods

Chemicals and reagents

The nutrient agar medium was purchased from Himedia (India). Silver nitrate was purchased from Fisher Scientific (Pittsburgh). The bacterial species *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Propionibacterium acnes, Streptococcus oralis, Enterobacter faecalis, Proteus vulgaris, Aeromonas hydrophila, Corynebacterium diphtheriae and Actinomycetes sps* were purchased form MTCC, Chandighar, India. Whatman filter paper was purchased from Millipore, USA.

Collection and authentication of plant materials

The plant materials *C. bonduc, M. citrifolia* and *N. sativa* were collected from Palakarai, (La: 10.8109°N, Lon: 78.6959°E), Tiruchirappalli, Tamil Nadu, India. The collected plants were authenticated by Dr.S. John Britto, The Director, Raphinat Herbarium and Centre for Molecular Systematic, Tiruchirappalli, Tamil Nadu. India. The plant materials were shown in **Figure 1**.

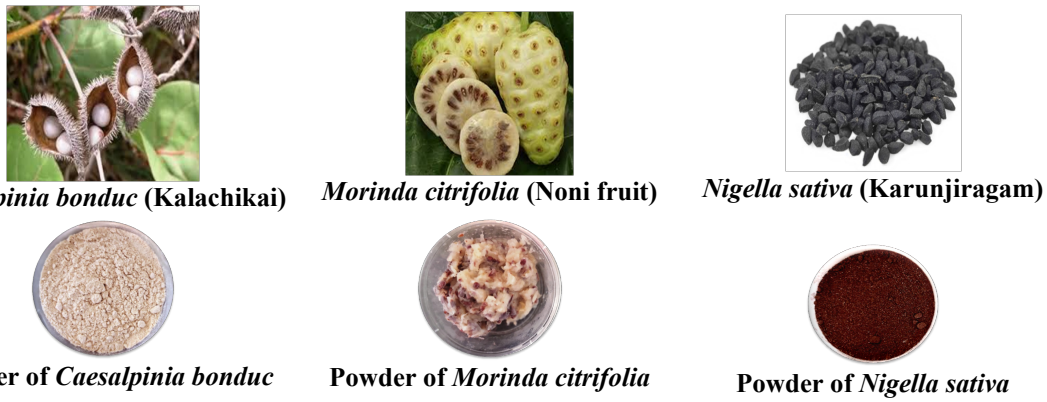


Figure 1 Collection of traditional medicinal plants for extraction.

Preparation of polyherbal formulation

The *C. bonduc, N. sativa* seeds were washed thoroughly and shade dried. *M. citrifolia* was cut into fine pieces and ground well. The polyherbal formulation was prepared at 5:3:2 ratio of *M. citrifolia* (5): *C. bonduc* (3): and *N. sativa* (2) and heated at 60 °C with 100 mL of double distilled water for 60 min. Then the extract was primarily filtered with tissue paper and then secondary filtration with the Whatman filter paper. The filtered extract was used for the synthesis of silver nanoparticles.

Bio-synthesis of AgNPs

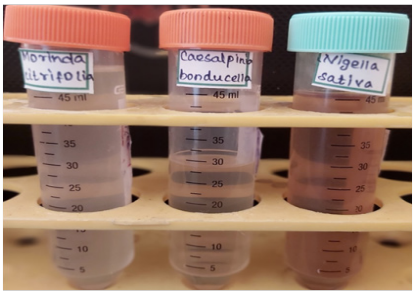
The filtered extract of polyherbal formulation was used for the synthesis of AgNPs. To synthesize AgNps from polyherbal formulation, different ratio of 0.01N Silver nitrate ($AgNO_3$) and polyherbal formulation extract was taken (5:5, 6:4, 7:3, 6:4, 8:2, 9:1). The mixture was incubated at room temperature for 24 h. The best ratio was taken for bulk synthesis of silver nanoparticles. Synthesized nanoparticles shown in **Figure 2**.

Characterization of AgNPs

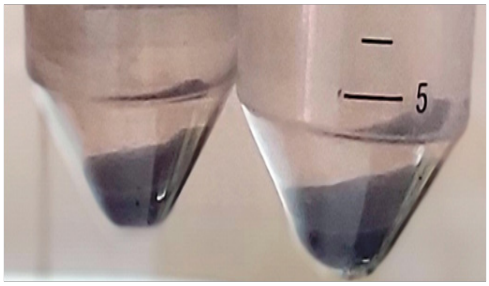
The green synthesized silver nanoparticles along with $AgNO_3$ solution were analysed using UV-Visible spectrophotometry and their size and shape was analysed through the SEM (Scanning Electron Microscope) analysis.

UV-Vis spectrophotometry analysis

The bio reduction of AgNPs nanoparticles was monitored periodically by UV-Visible spectroscopy. The samples used for analysis were diluted with 2 mL deionized water and subsequently measured by the UV-Visible spectrum at regular different time intervals. A UV-Visible spectrograph of silver nanoparticles was recorded as a function of time by using a quartz cuvette with water as reference and were recorded at a scanning speed of 200 to 1,100 nm.



Aqueous extract



Biosynthesized AgNps

Figure 2 Polyherbal plant extract of the selected plants and green synthesis of silver nanoparticles.

SEM analysis of biosynthesized AgNPs

The biosynthesized silver nanoparticles were characterized using high resolution SEM (Scanning Electron Microscope) analysis. The samples were prepared by simple drop coating of the suspension of silver solutions onto an electric clean glass and allowing the solvent (water) to evaporate. The samples were left to dry completely at room temperature.

Nutrient broth

Nutrient broth was prepared by dissolving 2.8 g of nutrient medium (HiMedia) in 100 mL distilled water and boiled to dissolve the medium completely. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Nutrient agar medium

The medium was prepared by dissolving 2.8 g of the Nutrient Agar Medium (HiMedia) in 100 mL of distilled water and autoclaved at 15 lbs pressure at 121 °C for 15 min, and poured onto 100 mm petriplates (25 - 30 mL/plate) while still molten.

Agar well diffusion method

Petri plates containing 20 mL nutrient agar medium were seeded with 24 h culture of selected bacterial strains. Wells were cut and different concentration of samples (1,000, 500, 200 and 100 µg/mL) was added. The plates were then incubated at 37 °C for 24 h. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Gentamicin antibiotic was used as a positive control. The values were calculated using Graph Pad Prism 6.0 software (USA).

Statistical analysis

The difference in estimated parameters between the groups was analyzed using one-way ANOVA with Bonferroni's test. Data expressed as Mean ± Standard Deviation. All parameters were analyzed at 95 % confidence intervals and p-value < 0.05 was considered to be statistically significant. Statistical analysis of the data was performed using Graphpad Prism version 6.00 for Windows, GraphPad Software, San Diego California USA.

Results and discussion

Biosynthesis and characterization of polyherbal AgNPs

Green synthesis provides advancement over physical and chemical methods as it is environmentally friendly and cost-effective, easily scaled up for large-scale synthesis and no use of high pressure, temperature, energy and toxic chemicals is required in this method [23]. Several NPs that were developed biologically demonstrated excellent inhibition against many pathogenic microorganisms. Some of the nano-materials surprisingly destroyed the numerous microbial species that developed high drug resistance [24]. The metal nanoparticles formed by plants have been shown to be more stable compared with those formed by other species. Plants can reduce metal ions more rapidly than fungi or bacteria. Additionally, plant extracts are in order to use an easy and safe green method for scale-up and industrial production of well-dispersed metal nanoparticles [9]. In the present study, AgNPs were synthesized from the polyherbal extract. Total AgNps reduction was observed at a ratio of 5:5. Reduction of silver nanoparticles occurred immediately after $AgNO_3$ solution was added to the polyherbal extract, it is due to the presence in the leaf extract of active molecules [22].

UV-Visible spectrophotometric analysis

The UV-Visible spectrophotometric analysis confirmed the presence of silver nanoparticles. **Figure 3(a)** displays a UV-Vis spectra of AgNP's by the peak representation at 400 - 450 nm.

SEM analysis

The SEM is commonly used as an example of the direct interactions between NPs and biological materials [25]. **Figure 3(b)** shows a SEM images associated to the AgNP's synthesized by polyherbal extract. AgNP's with round shaped morphologies and well dispersed can be appreciated. This fact indicates that the green synthesis process through of polyherbal extract, offer a high bioreduction efficiency.

XRD analysis

In order to support the results obtained, **Figure 3(c)**. shows a XRD pattern corresponding to thte AgNP's. This result confirmed the formation of silver nanoparticles.

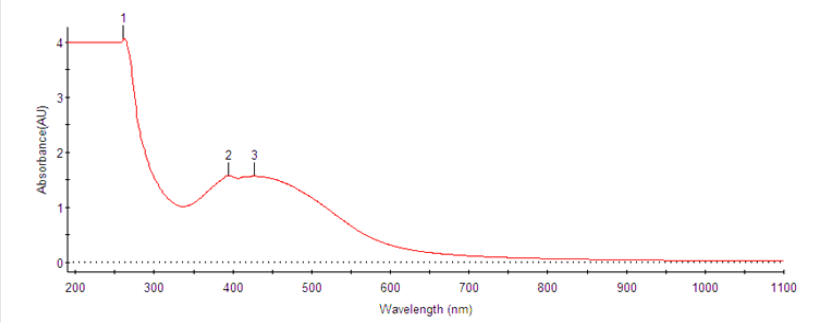


Figure 3(a) UV spectrophotometer analysis of polyherbal AgNps

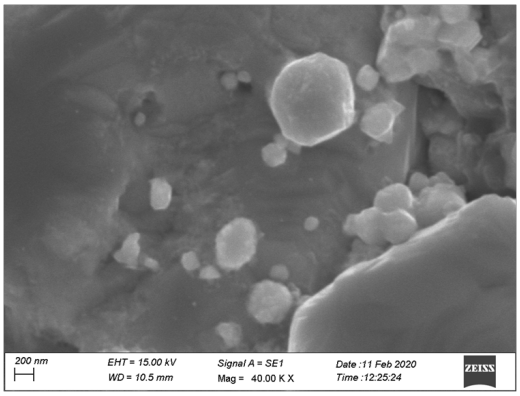


Figure 3(b) SEM image of polyherbal AgNps

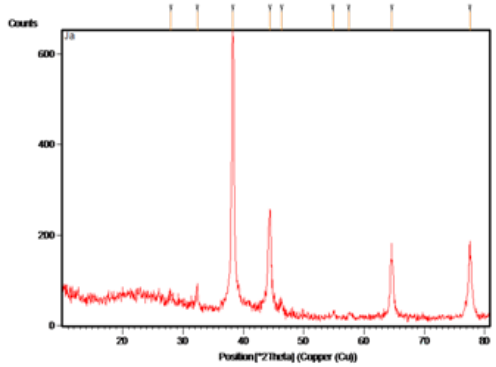


Figure 3(c) XRD pattern of polyherbal AgNps

Figure 3 (a) - (c) Characterization of polyherbal AgNPs by UV-Vis spectrophotmeter, SEM and XRD (X-ray diffraction) analysis.

Antibacterial activity of polyherbal AgNPs

The antibacterial properties of polyherbal AgNPs evaluated against selected 12 different bacterial species was highly inhibited. The maximum antibacterial activity of polyherbal AgNPs was observed in *S. pyogenes* and *P. auerogino*sa (15 mm). The zone of inhibition values was shown in **Table 1** and the tested species by agar well diffusion method is shown in **Figure 4**. All the AgNP's polyherbal extract solutions show inhibitory effect.

The synthesized silver nanoparticle of polyherbal extract was tested at different concentrations and compared it with the standard antibiotic. **Figures 4(a)** and **4(b)** shows the zone of inhibition of the synthesized silver nanoparticle of the polyherbal extract against, *S. aureus* and *S. pyogenes*. It was observed as 15 ± 1.5, 14 ± 1.0, 12.5 ± 1.0, 12 ± 0.5 mm at the concentration of 1,000, 500, 200 and 100 µg/mL, respectively. It was found to be low when compared to standard antibiotic (21 ± 2.0 mm) against *S. pyogenes*.

The nanoparticles synthesized from leaf extracts of *Acalypha indica* (Euphorbiaceae) had excellent antimicrobial activity against pathogens, *E. coli and V. cholera* (Minimum Concentration Inhibitor (MIC) = 10 mg mL^{-1}) [26]. Biosynthesized silver nanoparticles from *Ocimum sanctum* (tulsi) leaf extract have shown significant antimicrobial activity against microorganisms of both gram-negative (*E. coli*) and gram-positive bacteria (*S. aureus*) [27]. Biosynthesis of silver nanoparticles was investigated with the *Cacumen platycladi* extract. Reducing sugars and flavonoids in the extract were mainly responsible for the bioreduction of the silver ions, and their reductive capability promoted at 90 °C, leading to the formation of silver nanoparticles (18.4 ± 4.6 nm) with narrow size distribution. The nanoparticles were experiencing strong antibacterial activity against *E. coli* and *S. aureus* [28].

The nanoparticles produced had substantial antimicrobial activity against pathogenic microorganisms like *S. aureus, Candida tropicalis, K. Pneumoniae* and *C. Krusei* [29]. The present study also revealed that, the zone of inhibition (**Figure 4(a)**) of the synthesized silver nanoparticle of the polyherbal extract was observed as 12 ± 0.5, 10 ± 1.5, 9 ± 0.5, 8 ± 1.5 mm at the concentration of 1,000, 500, 200 and 100 µg/mL, respectively. It was found to be low when compared to standard antibiotic (18 ±

1.5 mm) against *S. aureus*. **Figure 4(c)** shows the zone of inhibition of the synthesized silver nanoparticle of the polyherbal extract was observed as 11 ± 2.5, 9 ± 0.5, 8 ± 0.5, 7 ± 1.5 mm at the concentration of 1,000, 500, 200 and 100 µg/mL, respectively was found to be low when compared to standard antibiotic (18 ± 2.5 mm) against *E. coli*.

The antimicrobial activity of copper oxide nanoparticles against many bacterial species such as *Klebsiella pneumoniae, P. aeruginosa, Shigella* and *Salmonella paratyphi* was determined by Mahapatra *et al*. [30]. Nanoparticles were assumed to cross the bacterial cell membrane to damage the crucial bacterial enzymes which further induce cell death. **Figure 4(d)** shows the zone of inhibition of the synthesized silver nanoparticle of the polyherbal extract was observed as low (11 ± 0.5, 9 ± 1.5, 8 ± 0.5 mm) in the concentration of 500, 200 and 100 µg/mL of green synthesized nanoparticles, respectively and high (15 ± 1.5 mm) at the concentration of 1,000 µg/mL of the AgNPS when compared to standard antibiotic (12 ± 0.5 mm) against *P. aeruginosa*.

The spherical silver nanoparticles (40 - 50 nm) were made using the *Euphorbia hirta* leaf extract [31]. These nanoparticles possessed antibacterial ability and successful properties against *Bacillus cereus* and *S. aureus*. In the present study, **Figure 4(e)** shows the zone of inhibition of the synthesized silver nanoparticle of the polyherbal extract. It was found to be low in the concentration of 500, 200 and 100 µg/mL of green synthesized nanoparticles and same zone of inhibition is observed at the concentration of 1,000 µg/mL of the AgNPs when compared to standard antibiotic (12 ± 0.5 mm) against *B. cereus*.

The zone of inhibition of the synthesized silver nanoparticle of the polyherbal extract was observed as high in all the concentration (13 ± 0.5, 11 ± 1.5, 10 ± 1.0, 9 ± 0.5 mm) of 1,000, 500, 200 and 100 µg/mL, respectively when compared to standard antibiotic (9 ± 1.5 mm) against *P. acnes* (**Figure 4(f)**) and *S. faecalis* (**Figure 4(h)**).

AgNPs could release ions and therefore produce free radicals, disturb the equilibrium of electrons between groups of electron donors and inactivate the organisms' enzymes and DNA. In addition, they caused holes in the bacterial membrane to disrupt the balance of cellular ions. Therefore, they can be used to inhibit pathogen development [32]. The zone of inhibition of the synthesized silver nanoparticle of the polyherbal extract was observed as 14 ± 1.5, 12 ± 0.5, 11 ± 1.5, 10 ± 0.5 mm at the concentration of 1,000, 500, 200 and 100 µg/mL, respectively. Thus the polyherbal extract possessed antibacterial activity but it was found to be low at all concentrations of the synthesized silver nanoparticles when compared to standard antibiotic (19 ± 0.5 mm) against *P. vulgaris,* and also the same result was observed in *C. diphtheria, Actinomycetes Sp, S. oralis,* and *A. hydrophila* (**Figures 4(i) - 4(l)**).

The highest concentration (1,000 µg/mL) of the synthesized silver nanoparticle of the polyherbal extract showed highest zone of inhibition when compared to other concentration (500, 200 and 100 µg/mL) of the AgNPs of the polyherbal extract. Exact mechanism of AgNPs against bacteria is still unlcear. Some researchers have proposed that AgNPs' activity on bacteria may be due to its ability to penetrate into the cell [16], free radical formation [33,34], silver ion inactivation of proteins in the cell [35] and development of reactive oxygen species (ROS) [36]. AgNP's have the capacity to hold fast to the bacterial cell membrane and penetrate into the cytoplasm, and produce structural changes in the cell. The result of the present study showed that highest concentration of the AgNP's shows the highest antibacterial activity. In this sense, the cell membrane of the bacterial strains evaluated in the study exhibits a major resistance to the AgNP's in low concentration.

Table 1 Zone of inhibition of polyherbal AgNps against the human bacterial pathogens.

Name of the test sample	Name of the test organism	Zone of inhibition (mm) Mean ± SD				
		1,000 µg/mL	500 µg/mL	200 µg/mL	100 µg/mL	AB
Polyherbal AgNps	*Staphylococcus aureus*	12.5 ± 0.50	10.0 ± 1.50	9.0 ± 0.50	8.0 ± 1.50	18.0 ± 1.50
	Streptococcus pyogenes	15.0 ± 1.50	14.0 ± 1.00	12.5 ± 1.00	12.0 ± 0.50	21.0 ± 2.00
	Escherichia coli	11.0 ± 2.50	9.0 ± 0.50	8.0 ± 0.50	7.0 ± 1.50	18.0 ± 2.50
	Pseudomonas aeruginosa	15.0 ± 1.50	11.0 ± 0.50	9.0 ± 1.50	8.0 ± 0.50	12.0 ± 0.50
	Bacillus cereus	12.0 ± 0.50	11.0 ± 1.00	9.0 ± 0.50	8.0 ± 0.50	12.0 ± 0.50
	Propionibacteriun acnes	13.0 ± 0.50	11.0 ± 1.50	10.0 ± 1.00	9.0 ± 0.50	9.0 ± 1.50
	Streptococcus oralis	9.5 ± 1.00	7.5 ± 1.50	6.0 ± 1.00	5.0 ± 1.50	10.0 ± 1.00
	Streptococcus feacalis	10.0 ± 0.50	10.0 ± 0.50	9.0 ± 0.50	8.0 ± 0.50	5.0 ± 0.50
	Proteus vulgaris	14.0 ± 1.50	12.0 ± 0.50	11.0 ± 1.50	10.0 ± 0.50	19.0 ± 0.50
	Aeromonas hydrophila	12.0 ± 0.50	10.0 ± 2.50	11.0 ± 0.50	9.0 ± 0.50	18.0 ± 1.50
	Corynebacterium diphtheria	11.0 ± 2.50	9.0 ± 0.50	10.0 ± 1.50	9.0 ± 0.50	16.0 ± 2.50
	Actinomycetes sps	11.0 ± 0.50	10.0 ± 1.50	9.0 ± 0.50	9.0 ± 0.50	15.0 ± 1.00

Values are expressed in Mean ± Standard Deviation.

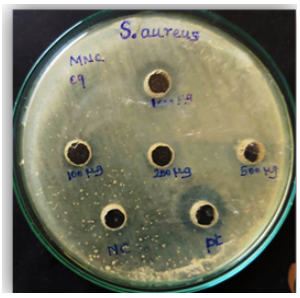


Figure 4(a) *S. aureus*

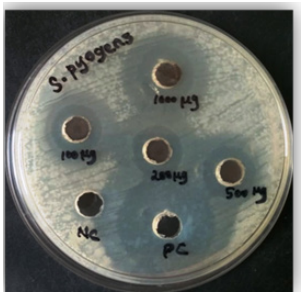


Figure 4(b) *S.pyogenes*

Figure 4(c) *E. Coli*

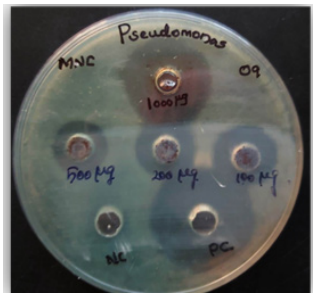


Figure 4(d) *P. aeruginosa*

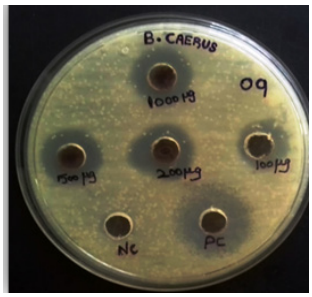


Figure 4(e) ***B. cereus***

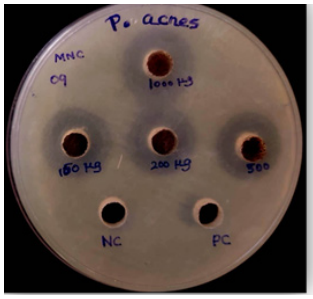


Figure 4(f) ***P. acnes***

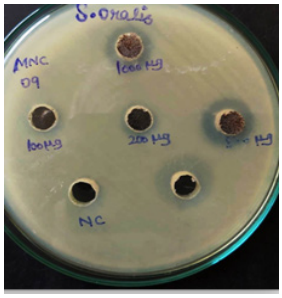


Figure 4(g) ***S. oralis***

Figure 4(h) ***S. faecalis***

Figure 4(i) ***P. vulgaris***

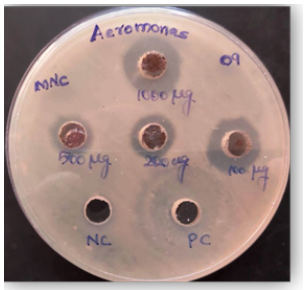


Figure 4(j) ***A. hydrophila***

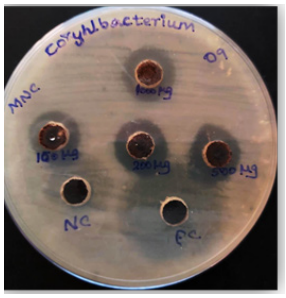


Figure 4(k) ***C. diptheriea***

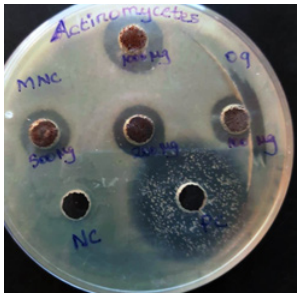


Figure 4(l) ***Actinomycetes Sp.***

Figure 4 (a) - (l) Visible clear zone produced by polyherbal AgNPs against bacterial species.

Conclusions

In the present study, the use of a natural, low-cost biological reduction agent and polyherbal formulation extract can produce metal nanostructures through an effective process of green nanochemistry. The biosynthesized silver nanoparticles of polyherbal formulation have proven excellent antibacterial activity against human pathogens by disc diffusion method. The methodology adopted in this study is a quick, rapid, simple, economic and environmental friendly. Prepared nanoparticles can be used against human pathogens as antimicrobial drug. Due to these applications, this method is potentially exciting for the large-scale synthesis of nanoparticles. In this regard, we can affirm that the AgNP's obtained by green synthesis offer a potential tool to combat against increasing antibiotic resistance.

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