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ANTI-ESKAPE ACTIVITY OF GREEN SYNTHESIZED SILVER NANOPARTICLES FROM *PICRORHIZA KURROA* ROYLE EX BENTH.

Muhammad Musthafa [*1], Kuppannan Gobianand [1] and M. Manohar [2]

Department of Microbiology [1], Vivekanandha College of Arts and Sciences for Women, Namakkal - 637205, Tamil Nadu, India.
Department of Microbiology [2], Sadakkathullah Appa College, Tirunelveli - 627011, Tamil Nadu, India.

Keywords:

ESKAPE pathogens, Antimicrobial resistance, Silver nanoparticles, Green synthesis, *Picrorhiza kurroa*

Correspondence to Author:
Muhammad Musthafa

Research Scholar,
Department of Microbiology,
Vivekanandha College of Arts and Sciences for Women, Namakkal - 637205, Tamil Nadu, India.

E-mail: pmusthu@gmail.com

ABSTRACT: The facts like developing resistance in bacterial pathogens against conventional antibiotics, emergence of new infectious agents *etc.* have been leading the scientists to tedious research activities to find out antimicrobial drugs with novel modes of action. Among the multidrug-resistant (MDR) bacterial species that pose threat to human life, the IDSA has identified a group pathogens known as ESKAPE pathogens which show extensive drug resistance through a wide range of mechanisms. Silver nanoparticles (AgNPs) have been identified as very effective and to avoid the chemical contamination in preparation of them, green synthesis is being used. In the present study, AgNPs using *Picrorhiza kurroa* Royle ex Benth were synthesized using reduction method. The green synthesized AgNPs were characterized by UV–Vis, DLS-zeta potential and FESEM analysis. Anti-ESKAPE activity of the synthesized nanoparticles was also performed. FESEM studies revealed that the nanoparticles were spherical and nanosize in shape. The UV–Vis spectral studies confirmed the surface plasmon resonance of green-synthesized silver nanoparticles. The particle size was found to be 267 nm and the stability (Zeta potential) was observed to be −50.6 mV. The biosynthesized AgNPs were found to have a pronounced antibacterial activity against *E. coli*, *K. pneumonia*, *P. aeruginosa*, *S. aureus*, *A. baumanii* and *E. aerogenes*. Hence, the developed green synthesized AgNPs can be used as an effective antimicrobial alternative to conventional antibiotics for the significant treatment of infections caused by multidrug-resistant ESKAPE pathogens.

INTRODUCTION: Even though the discovery of antibiotics was one of the ones the greatest achievements in the medical history [1], the increasing number of bacterial pathogens resistance to the antibiotics, the emergence of new infectious agent made the World Health Organization to warn that the 21st century may be seeing the beginning of a pre-antibiotic era, posing the biggest threat to health, food security, and development [2], and it requires the development of alternative therapeutic options [3]. Many bacteria can resist lethal concentrations of antibiotics using a number of mechanisms [1] and become multidrug-resistant (MDR) strains, which is according to The European Society for Clinical Microbiology and Infectious Diseases (ESCMID) is pathogens that exhibit resistance *in-vitro* against three or more classes of antibiotics [4].

Apart from other common terms like extensively drug-resistant (XDR), pan drug-resistant (PDR), *etc.* used while explaining antimicrobial drug resistance, an acronym dubbed by the Infectious Diseases Society of America – IDSA is receiving

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more attention as 5 as the bacterial members in this term represent pathogens that are associated with the highest risk of mortality thereby resulting in increased health care costs and hence, the WHO included them in the group of 12 pathogenic bacteria against which new antibiotics are urgently needed [6]. The acronym is "ESKAPE," and the member pathogens are *Enterococcus faecium* (E), *Staphylococcus aureus* (S), *Klebsiella pneumoniae* (K), *Acinetobacter baumannii* (A), *Pseudomonas aeruginosa* (P) and Enterobacter species/ *Escherichia coli* (E), which are behind two-third of all healthcare-associated infections (HAIs) [7], responsible for the most of the nosocomial infections [10] and environment forming the most common multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria [8].

They are notorious in managing to "escape" the action of commonly available antibiotics and ultimately lead to increased mortality and spread to the community [9]. The Gram-negative members of the ESKAPE are the major reservoirs of resistance, and they transmit the resistance throughout the community, with very few new antimicrobial agents in the "pipe-lines" to tackle them [5, 9]. According to Richard P. Feynman (1959), who introduced the concept, nanoparticles (NPs) are those who are having at least one dimension and size between 1 nm and 100 nm, and it was Norio Taniguchi who put forward the term "nano-technology" in 1974 [10]. Now, it is widely used in treatment as nano-medicine. There are different metal nanoparticles with a broad range of uses as antimicrobial agents, and these properties are because of their unique physco-chemical characteristics [6].

Silver nanoparticles (AgNPs) synthesized using physical, chemical, or biological (green synthesis) protocols have displaced novel anti-microbial properties among all metal nanoparticles with less chances of resistance development in bacteria due to the multi-target approach of AgNPs [6, 12, 13]. The higher ratio of surface area to volume [14], natural antimicrobial activities, and numerous modes of action NPs they possess are other plus points [11]. There are different mechanisms of AgNPs action, including damaging cell wall by electron transport transduction pathway by penetrating as a result of Ag^+ ions released and react with intracellular target and cause ultimate toxicity destruction of structures like cell wall, cell membrane, DNA, proteins *etc.* [6, 11, 15, 16, 17]. As the AgNPs are excellent candidates in antibacterial properties, as therapeutics, drug delivery agents, etc. their demand is increasing. To use in bioprocess, the silver nanoparticles should be biocompatible and free from toxic chemicals [18]. Many of the recent studies have illustrated that the application of NPs is a potential way to kill pathogenic bacteria, including MDR strains, and the chances for drug resistance are very less [11, 19].

For the best usage of AgNPs in biological applications, AgNPs should be free from toxic chemicals that may be produced as a by-product during their synthesis. For this, the biosynthesis of AgNPs is recommended, in which the Silver nanoparticles are prepared by reducing to metal state using plant biomolecules. This method is otherwise called green synthesis and is very simple, accurate, and cost-effective [17, 18]. *Picrorhiza kurroa* is one among the medicinal herb, grows in the hilly regions of India and Nepal. It is a small perennial herb. Roots are about 5-10 cm long and commonly known in India as kardi / karu / katukhurohani. *Picrorhiza kurroa* contains apocynin, a powerful anti-inflammatory agent that reduces platelet aggregation. Traditional methods utilizing *Picrorhiza kurroa* used to treat liver and upper respiratory conditions in India and China [20]. Hence, the present study, analyze the ability of silver nanoparticle synthesized using *Picrorhiza kurroain* controlling the growth of ESKAPE pathogenic species and the characterization of green synthesized AgNPs. The AgNPs using *Picrorhiza kurroa* Royle ex Benth were synthesized using the reduction method. The green synthesized AgNPs were characterized by UV–Vis, DLS-zeta potential, and FESEM analysis. The anti-ESKAPE activity of the synthesized nanoparticles was also performed.

MATERIALS AND METHODS:

Collection of the ESKAPE Pathogenic Strains: The ATCC (LYFO-DISK) cultures of ESKAPE pathogens were purchased from Microbiologics, USA, and were processed as per the directions provided by the supplier.

Collection of the Herb for Green Synthesis of AgNPs: The herb *Picrorhiza kurroa* Royle ex Benth was purchased from local medicinal plant market at Narikkuni, Kozhikode Dist., Kerala on September 30th, 2017 and identified (MVRAMC /RSBK1/PAN-5/2017) by Dr. Shamna, Department of Pharmacology, MVR Ayurvedic Medical College, Kannur, Kerala, India.

Extraction and Synthesis of Green AgNPs from Plant Extract: The fresh and mature stems of the plant were collected, and about 20 gm was thoroughly washed four times with de-ionized water to remove dust particles and air-dried at room temperature. Then it was finely chopped into small pieces and added to 100 ml of deionized water, stirred for 20 min at 60 °C. After boiling, the leaf extract was cooled at room temperature and filtered conferring 75 ml of leaf broth, which was stored at 4 °C. In the green synthesis of AgNPs, 0.01 M of an aqueous solution of $AgNO_3$ (99.99%) was used. About 5 mL of leaf broth was added to 45 ml of 0.01 M $AgNO_3$ aqueous solution and allowed at the ambient condition to react. After different time intervals, the color change of reaction mixture is observed from transparent yellow to dark brown indicates that the formation of AgNPs. The AgNPs solution was allowed to centrifuge, and the excess liquid was removed by evaporation in a dryer yielding black colored silver nano-powder. The AgNPs were collected and stored in dry place [24].

Characterization of the Green Synthesized AgNPs: The structural morphology of the green synthesized AgNPs was examined and measured by Field Emission Scanning Electron Microscopic (FESEM) using TM-1000, Hitachi, Japan. The UV-visible absorption of the AgNPs was measured by using a UV-Vis spectrophotometer (Shimadzu 2400) at a resolution of 1 nm under room temperature conditions. The particle size of the developed nanoparticles was analyzed by Particle Size Analyzer (PSA). Zeta potential is an essential parameter for the characterization of stability in aqueous nanoparticle suspensions.

Anti-ESKAPE Activity of AgNPs: Anti-ESKAPE activity of the AgNPs was evaluated. Sterile Nutrient Agar (Composition g/L: Peptone: 5 g; Yeast extract: 5 g, Beef extract: 3 g, Sodium chloride: 5 g, Agar 15 g; Final pH (7.0 ± 0.2) plates were prepared and allowed to solidify. About 0.1% inoculum suspensions of the test organisms were swabbed uniformly over the agar surface.

Under sterile conditions, 6mm wells were cut on the agar surface of each NA plates. About 50 µl each of AgNP was loaded into the well, and the plates were incubated at 37 ºC for 24-48 h. The antibacterial activity was evaluated in terms of zone of inhibition around the wells of each extract in all the inoculated NA plates. The clear inhibition zones were measured and recorded in millimeters.

RESULTS:

Topographical Analysis of the Green Synthesized AgNPs: The FESEM observation was carried out for the green synthesized silver nanoparticles. The topological analysis showed the surface morphology of the developed green synthesized AgNPs. The NPs were even shape and spherical nature. This result strongly confirms that *Picrorhiza kurroa* extracts might act as a reducing and capping agent in the production of silver nanoparticles. FESEM image of the green synthesized AgNPs is shown.

UV-Vis Spectrum Analysis of the Green Synthesized AgNPs: Silver nanoparticles were synthesized by the green synthesis method using *Picrorhiza kurroa* plant extract. UV- visible spectrum of the aqueous medium containing silver nanoparticles showed an absorption peak at around 278 nm. The pattern clearly shows the many peaks at 2θ corresponding to the different planes, respectively. Compared with JCPDS (file no: 89-3722), the typical pattern of green-synthesized AgNPs is found to possess an FCC structure.

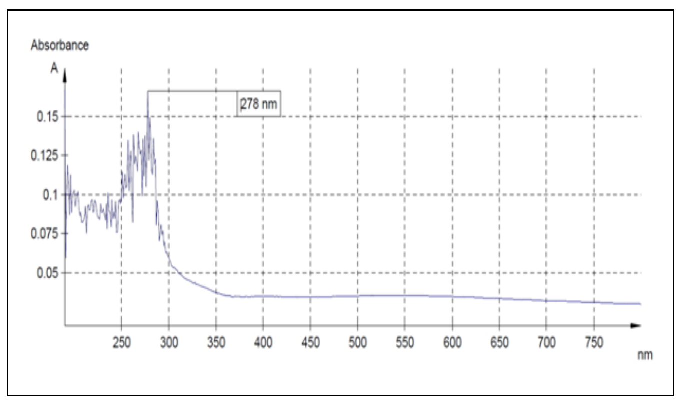


FIG. 1: UV–VISIBLE SPECTRUM OF THE GREEN SYNTHESIZED SILVER NANOPARTICLES

Particle Size and Stability of the Green Synthesized AgNPs: The size distribution image of biosynthesized silver nanoparticles was analyzed.

The calculated average particle size distribution of AgNPs is 267 nm. The zeta potential of the green synthesized AgNPs was found as a sharp peak at −50.6 mV **Fig. 2**.

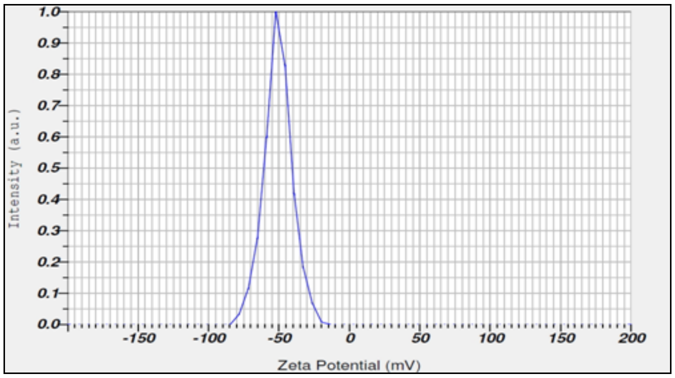
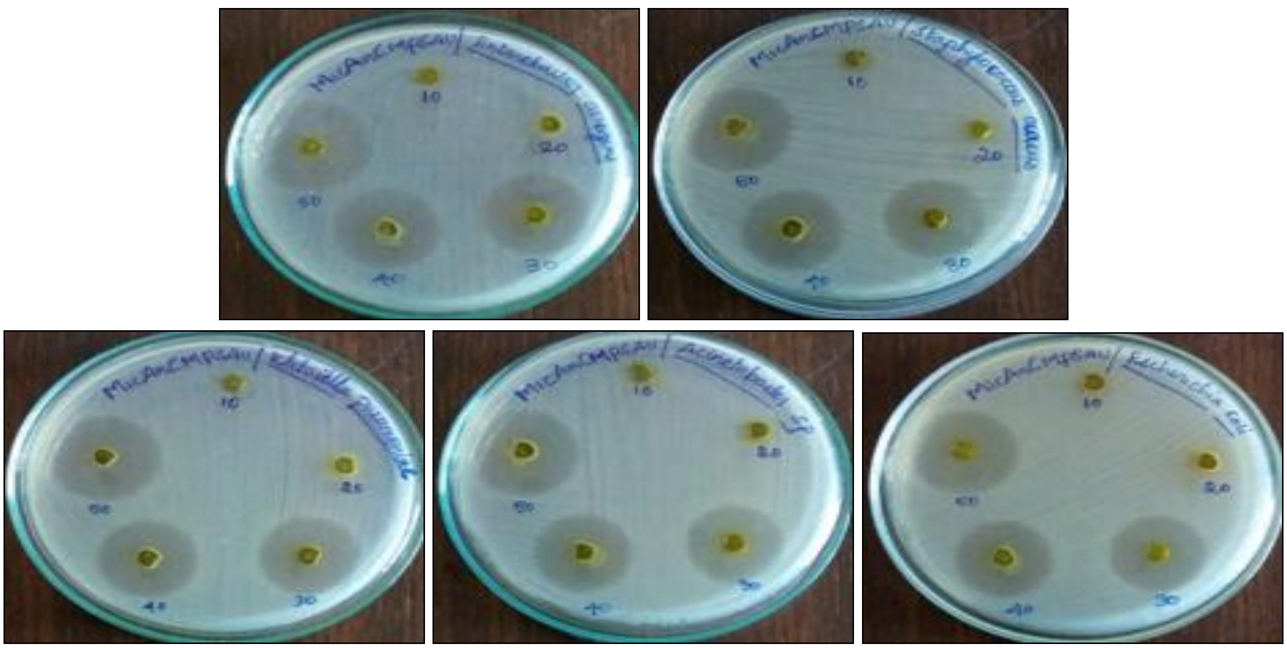


FIG. 2: ZETA POTENTIAL OF THE GREEN SYNTHESIZED AGNPS

It is suggested that the surface of the nanoparticles is negatively charged and dispersed in the medium. The negative value confirms the repulsion among the particles and proves that they are very stable.

Anti-ESKAPE Activity of the Green Synthesized AgNPs: Antibacterial activity was observed against all the ESKAPE pathogens. About 8 ± 0.0 mm, 10.66 ± 1.15 mm and 15.33 ± 1.15 mm of inhibitory zones were obtained for *E. aerogenes*, *S. aureus*, and *K. pneumoniae*. Inhibitory zones against *A. baumanii*, *P. aeruginosa*, and *E. coli* were observed to be 8 ± 0.0 mm, 11.33 ± 1.15 mm and 20 ± 0.0 mm. Highest inhibitory zones were obtained against *E. coli*. Thus, the order of organisms based on inhibitory zones was found to be *E. coli*. > *K. Pneumonia* > *P. aeruginosa* > *S. Aureus* > *A. baumanii* > *E. aerogenes*. The antibacterial activity of the AgNPs against ESKAPE pathogens is shown in **Pictures 1-5** and the diameters of zone of inhibition in **Table 1**.



PICTURES 1-5: THE ZONES OF INHIBITION FORMED BY DIFFERENT CONCENTRATIONS OF AGNPS AGAINST DIFFERENT ESKAP EMEMBERS *E. AEROGENES*, *S. AUREUS*, *K. PNEUMONIAE*, *A. BAUMANII* AND *E. COLI* RESPECTIVELY

TABLE 1: ANTIBACTERIAL ACTIVITY OF THE GREEN SYNTHESIZED AGNPS AGAINST ESKAPE PATHOGENS

S. no.	Bacteria	Diameter of Zone of Inhibition (in mm)
1	*E. aerogenes*	8 ± 0.0
2	*S. aureus*	10.66 ± 1.15
3	*K. pneumoniae*	15.33± 1.15
4	*A. baumanii*	8 ± 0.0
5	*P. aeruginosa*	11.33± 1.15
6	*E. coli*	20 ± 0.0

*values are calculated in Mean ± SD

DISCUSSION: *Picrorhiza kurroa* plant extracts are used for the synthesis of the silver nanoparticles. The main advantage of using plant extracts to synthesize silver nanoparticles is easy accessibility, Safe and, in most cases, non-toxic plants, which can help to reduce silver ions. They have different types of metabolites that can help in the reduction of silver ions and synthesizing faster. The chemicals of herbal like terpenes, flavones, ketones, aldehydes, amides, and carboxylic acids are directly involved in the reduction of ion and the formation of silver nanoparticles. Moreover, the green synthesis method is eco-friendly, of low cost, and capable of producing AgNPs at room temperature [25]. The formation of the AgNPs during the reduction process is indicated by a change in the color of the reaction solution from colorless to dark brown, which can be visually observed. The developed nanoparticles were observed under Fields Emission Scanning Electron microscope (FESEM). Similar results were also reported for phyto-synthesized silver nanoparticles [26]. The nanoparticles were found to be in nanosize and spherical in shape. Metal nanoparticles have free electrons, which yield a surface plasmon resonance (SPR) absorption band due to the mutual vibration of electrons of metal nanoparticles in resonance with light waves. The appearances of the peaks in the UV-Vis spectrum show the characteristics of surface plasmon resonance of green synthesized silver nanoparticles. It clearly indicates that *Picrorhiza kurroa* extracts might act as a reducing and capping agent in the synthesis of AgNPs. The particle size of AgNPs from the PSA was found to be 267 nm. The zeta potential of the green synthesized AgNPs was found as a sharp peak at −50.6 mV. This indicates that the developed nanoparticles were highly stable.

The antimicrobial properties of silver have dependent on the amount of silver and ration of released silver. Silver is inactive in the form of metal, but it reacts with moisture in the skin and wound fluids and ionized. The silver of ion is highly reactive and attaches to tissue proteins and leads to structural changes in the cell wall of the bacteria and the membrane of the nucleus and ultimately led to the cell of deformation and death. The strong antimicrobial effects in alternative sources are very important because the resistance of many pathogens to antibiotics is one of the major problems of medical science. Antibiotic resistance is recognized as one of the most important concerns in the public health that the reason is that antibiotic overuse or incorrect [27]. Increases antibiotic resistance result in raising the cost of health care due to prolonged treatment, including admission and recuperation, need to the making of new antibiotic agents, and applying effective and widespread methods of infection control in order to prevent the spread of pathogens resistant to antibiotics. Another problem when using antibiotics in treatment is the occurrence of lethal and dangerous side effects such as hypersensitivity reactions (Anaphylactic shock), growth inhibition of hematopoietic stem cell, liver, and kidney failure in some of the patients. Although these complications may occur in the small percentage of patients, but these are important due to dangerous and lethal. With the advancement of nanotechnology science and the production of silver nanoparticles and also a demonstration of the antimicrobial properties of these nanoparticles, their use has increased dramatically in medicine and related sciences [28].

Thus, in the context of developing drug-resistant pathogens, there is a search for various new antimicrobials. Infections due to ESKAPE pathogens are high at risk. In the present work, the green synthesized AgNPs showed significant antimicrobial activity against ESAKPE pathogens. The highest inhibitory zones were obtained against *E. coli*. Thus, the order of organisms based on inhibitory zones was found to be *E. coli*. > *K. pneumonia* > *P. aeruginosa* > *S. aureus* > *A. baumanii* > *E. aerogenes*. These biosynthesized nanoparticles are widely used in cancer therapy, wound healing, antimicrobial activity, water paints, cotton fabrics, and textiles, *etc*. The green synthesis of AgNPs has also paved a better methodological approach in the medical field.

CONCLUSION: In the present work, *Picrorhiza kurroa* leaf extracts were prepared by using water as a solvent. The green synthesized AgNPs were characterized by UV–Vis, DLS-zeta potential, and FESEM analysis. The anti-ESKAPE activity of the synthesized nanoparticles was also performed. FESEM studies revealed that the nanoparticles were spherical and nanosize in shape. The UV–Vis spectral studies confirmed the surface plasmon resonance of green-synthesized silver nano-particles. The particle size was found to be 267 nm, and the stability (Zeta potential) was observed to be −50.6 mV. The biosynthesized AgNPs were found to have a pronounced antibacterial activity against *E. coli*, *K. pneumonia*, *P. aeruginosa*, *S. aureus*, *A. baumanii* and *E. aerogenes*. Hence, the developed green synthesized AgNPs can be used as an effective antimicrobial as an alternative to conventional antibiotics for the significant treatment of infections caused by multidrug-resistant ESKAPE pathogens.

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CONFLICTS OF INTEREST: Authors declare no conflicts of interest.

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