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ORIGINAL ARTICLE

Evaluation of Antibacterial Properties of Biogenically Synthesized Silver Nanoparticles from Rajat and Spahtika Bhasma Against Multidrug-Resistant Bacteria

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ABSTRACT
The emergence of antimicrobial resistance is a primary global healthcare concern, emphasizing the need to investigate alternative strategies for treatment. Traditional medicinal practices, such as bhasma in Ayurvedic medicine, are being reconsidered as potential sources of new antimicrobial agents. Bhasma's unique properties make it a promising material for synthesizing silver nanoparticles with potent antimicrobial properties. This study isolated Enterococcus faecalis and Klebsiella pneumoniae from clinical samples using Hicrome UTI agar plates. Pure bacterial cultures were obtained and coded as KP30 and EF22, followed by molecular identification using 16S rRNA sequencing. Rajat and Spahtika bhasma, procured locally, were used as reducing agents for synthesizing silver nanoparticles (AgNPs) from a 1 mM $AgNO_3$ solution. The nanoparticles were synthesized by the green synthesis technique and characterized by UV-Vis spectroscopy and scanning electron microscopy (SEM). The antimicrobial efficacy of the synthesized silver nanoparticles (AgNPs) was tested against KP30 and EF22 by well diffusion assay at different concentrations. The synthesis of silver nanoparticles using Rajat and Spahtika bhasma was confirmed by a color change. Rajat bhasma AgNPs turned dark brown, while Spahtika bhasma AgNPs changed to a brownish hue within 24 hours. UV-Vis spectroscopy showed distinct absorption peaks at 340.3 nm for Rajat bhasma AgNPs and at 341.2 and 500 nm for Spahtika bhasma AgNPs, indicating successful nanoparticle formation. SEM analysis revealed spherical nanoparticles with average sizes of 95.36 ± 3.4 nm for Rajat and 83.26 ± 8.3 nm for Spahtika, although some aggregation was noted. Antibacterial testing demonstrated that Spahtika bhasma AgNPs exhibited a larger zone of inhibition (24 mm at 100 µg) against KP-30, while Rajat bhasma AgNPs displayed consistent inhibition across both bacterial isolates. These findings suggest that Spahtika bhasma AgNPs possess superior antibacterial activity compared to Rajat bhasma AgNPs.
Keywords- *Antimicrobial resistance, Bhasma, Silver nanoparticles (AgNPs), Antibacterial activity, Scanning electron microscopy (SEM)*

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INTRODUCTION

The growing prevalence of drug-resistant microbes endangers the global healthcare system by reducing the efficacy of existing medical treatments. The rapid emergence of multidrug-resistant microorganisms represents a significant threat to the implementation of effective therapeutic strategies for treatment. The rise of antimicrobial resistance has emerged as a critical concern for healthcare practitioners and policymakers, highlighting the urgent need to investigate alternative methodologies for addressing these harmful microorganisms [1,2]. In this context, traditional medicinal practices, such as using Bhasma in Ayurvedic medicine, have garnered renewed interest as potential sources for novel antimicrobial agents.
Bhasma, a traditional Ayurvedic formulation, has been employed for centuries to address various medical issues and conditions. It has a longstanding history of application in traditional Indian medicine, which

has been employed to manage diverse pathological conditions and enhance overall physiological well-being[1,2]. The distinctive properties of bhasma, including its ability to act as a reducing agent, make it a promising material for synthesizing silver nanoparticles with potent antimicrobial properties.

Among the various natural sources, bhasma, a traditional Indian Ayurvedic preparation, can be a promising approach for synthesizing silver nanoparticles by environment-friendly methods [3]. Bhasma is a unique class of Ayurvedic medicines produced by calcinating metal sheets with medicinal plant extracts, forming nanoparticles with enhanced therapeutic potential [4]. The synthesis of AgNPs using bhasma involves the reduction of silver ions by the various phytochemicals present in the medicinal plant extracts, which act as reducing and stabilizing agents, leading to the formation of stable and biocompatible silver nanoparticles [5,6].

The antimicrobial properties of AgNPs have been widely explored, as they exhibit potent activity against a broad spectrum of bacteria, including both Gram-positive and Gram-negative strains [3]. The antibacterial efficacy of silver nanoparticles synthesized using bhasma is attributed to their multifaceted mechanism of action, which includes disrupting the bacterial cell membrane, generating ROS that cause oxidative stress, and inhibiting various cellular processes [3,5,6].

This study aims to synthesize and characterize silver nanoparticles using Rajat Bhasma and Sphatika Bhasma as reducing agents. It aims to evaluate their antimicrobial activity against clinically significant, multidrug-resistant bacteria, including *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

MATERIALS AND METHODS

Isolation and identification of *K. pneumoniae* and *E. faecalis* from clinical samples

The bacterial isolates of *Klebsiella pneumoniae* (Gram-negative) and *Enterococcus faecalis* (Gram-positive) were isolated from clinical samples on Hicrome UTI agar plates (Himedia- M1353). The pure cultures of the isolates were obtained by four-way streaking and were coded as KP 30 (*K. pneumoniae*) and EF 22 (*E. faecalis*). Both isolates were tested for antibiotic sensitivity. Further molecular identification of KP 30 and EF 22 was performed using 16S rRNA sequencing, and the obtained sequences were submitted to the GenBank database.

Collection of Bhasma

In this research study, our objective was to conduct a comprehensive comparative analysis of two distinct bhasma samples, Rajat bhasma (a metal-based bhasma) and Spahtika bhasma (a mineral-based bhasma), both procured from the local Patanjali store in Chandrapur, as a reducing agent in the synthesis of silver nanoparticles.

Synthesis of Silver Nanoparticles from bhasma

One mM of $AgNO_3$ (Hi media) aqueous solution was prepared to synthesize silver nanoparticles. The Rajat and Spahtika bhasma suspensions in 1 mg/ml concentrations were prepared by vigorous vortexing. The $AgNO_3$ solution and bhasma were mixed in a ratio of 9:1 and kept undisturbed for 24 hours [7]. The reaction was performed in a room temperature dark chamber to minimise silver nitrate's photo-activation. The color change and plasmon resonance were observed at 0 hrs. and 24 hrs. respectively to validate the formation of silver nanoparticles. The synthesized silver nanoparticles were then centrifuged at 10000 rpm for 2 hours, the supernatants were discarded, pellets were washed twice with distilled water [8]. The obtained powder of nanoparticles was dried and used for further analysis.

Physiochemical Characterization of Silver Nanoparticles.

The synthesized silver nanoparticles were characterized using advanced analytical techniques to confirm successful synthesis and evaluate their physicochemical properties. The structural and optical characteristics of the synthesized AgNPs were systematically examined. Characterization was performed using UV-visible (UV-Vis) spectroscopy to assess the optical properties and confirm nanoparticle formation, while scanning electron microscopy (SEM) was employed to evaluate the nanoparticles' morphology, particle size.

Antibacterial Activity Evaluation of Silver Nanoparticles via Agar Well Diffusion

The synthesized AgNPs were tested for their antibacterial properties against two multidrug-resistant bacterial isolates, KP30 and EF22, using the Kirby Bauer well diffusion technique [9]. The dried silver nanoparticles were dispersed in DMSO at 1 mg/ml concentration and sonicated for 30 minutes to ensure even dispersion. The bacterial cultures' turbidity was adjusted to a 0.5 MacFarland turbidity standard and then spread evenly on sterile Muller Hinton agar (Himedia- M173) plates using a swab. Three wells were bored on the plates with an 8 mm cork borer, and three different concentrations of the AgNPs suspension (25 µl, 50 µl, and 100 µl, corresponding to 25 µg, 50 µg, and 100 µg, respectively) were loaded into the wells. [10]. The plates were incubated at 37°C for 24 hours, and the zone of inhibition was measured in millimetres using a zone measuring scale.

RESULT AND DISCUSSION

This research carried out a study on the isolation, identification, and antibacterial activity of bacteria against two types of silver nanoparticles.

Isolation and identification of *E. faecalis* and *K. pneumoniae* from clinical samples

The morphological and cultural characteristics of KP 30 and EF22 are outlined in Table 1. Based on morphological culture as well as 16S rRNA study, it was confirmed that isolate KP 30 is *Klebsiella pneumoniae* and EF 22 is *Enterococcus faecalis* the 16S rRNA studies confirmed the same.

Table 1- Morphological and cultural characteristics of isolates

Organism	Morphological Characteristics	Cultural characteristics
KP 30	Gram Staining- Gram-negative rod shape bacteria	Blue mucoid colonies were observed on UTI agar plates
	Motility – non motile	
EF 22	Gram Staining	Light green to blue small colonies were observed on UTI agar plates.
	Motility	

Sequence results of 16S rRNA

Molecular identification techniques, such as 16S rRNA gene sequencing, have become indispensable tools in the field of microbiology, enabling researchers to accurately classify and delineate the evolutionary relationships of bacterial isolates [11]. The study presents the phylogenetic analysis of the bacterial strain KP30 based on its 16S rRNA sequence. The 16S rRNA sequence obtained for the KP30 strain was analyzed and compared to sequences deposited in public databases to infer its taxonomic placement [12]. Phylogenetic reconstruction using the 16S rRNA data revealed that the KP30 strain closely clusters with members of the *Klebsiella pneumoniae* species. The phylogenetic characteristics of *K. pneumoniae*, as observed in the present study, are consistent with previous findings reported [13,14] Fig 1.

GGCGGGGGCTACCATGCAAGTCGGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGG
TGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAATG
TCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGG
TGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAG
ACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCG
TGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGRTRAGGTTAATAACCTTGT
TTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCG
TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCA
ACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGT
GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTG
CGGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGT
GAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAA
ATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACC
TGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCAT
GGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGC
CAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGGTGGGGATGACGTCAAGTCATCAT
GGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGGATACAAAGAGAAGCGACCTCGCGAGAGCAAGC
GGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGT
AATCGTGGATCAGAATGCCACGGTGAATACGT

16S rRNA Gene Sequence of Bacterial Isolate KP30

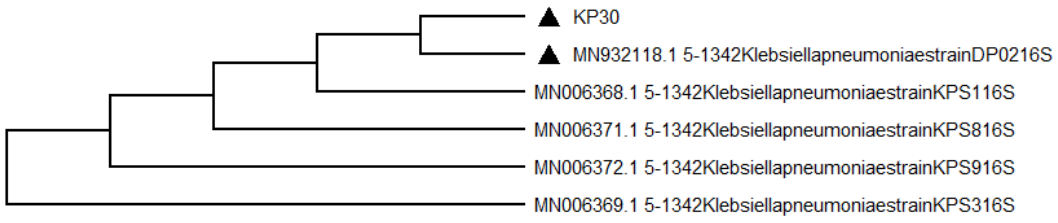

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Fig 1: Phylogenetic tree showing the evolutionary relationship of bacterial isolate KP30 based on 16S rRNA gene sequences.

The 16S rRNA sequence analysis revealed that the EF22 isolate closely resembles *Enterococcus faecalis*, a commensal organism that has a significant hospital-associated pathogen. *Enterococcus faecalis* is known to thrive in the hospital setting. Its robust phenotypic traits and capacity to acquire antimicrobial resistance can lead to severe nosocomial infections and associated morbidity and mortality.

The phylogenetic analysis of the 16S rRNA sequence of EF22 confirmed its close relatedness to *Enterococcus faecalis*. This finding is consistent with previous studies that have characterised a typical *Enterococcus faecalis* strain, such as those isolated from different sources [15,16] Fig 2..

The obtained 16S rRNA gene sequences for isolates KP30 and EF22 were deposited in GenBank under accession numbers OR517291 and OR517292, respectively.

GGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGCTTCTTTCCTCCCGAGTGCTTGC
ACTCAATTGGAAAGAGGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCATCAGAGGGGGATAA
CACTTGGAAACAGGTGCTAATACCGCATAACAGTTTATGCCGCATGGCATAAGAGTGAAAGGCGCTTTCG
GGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGAT
GCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACCGGGAGGCAGCAGT
AGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCG
TAAAACTCTGTTGTTAGAGAAGAACAAGGACGTTAGTAACTGAACGTCCCCTGACGGTATCTAACCAGAA
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGG
CGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTG
GAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATAT
ATGGAGGAACACCAGTGGCGAGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTC
AGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCT
TGACATCCTTTGACCACTCTAGAGATAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCAT
CATTTAGTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCAT
CATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCTAGACCGCGAGGTC
ATGCAAATCTCTTAAAGCTTCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGAGCCGGAATCGCTAGT
AATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAG
TTTGTAACACCCGAAGTCGACCTTTTTGGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGTGAAGTC
GTAACAAC

16S rRNA sequence of EF22

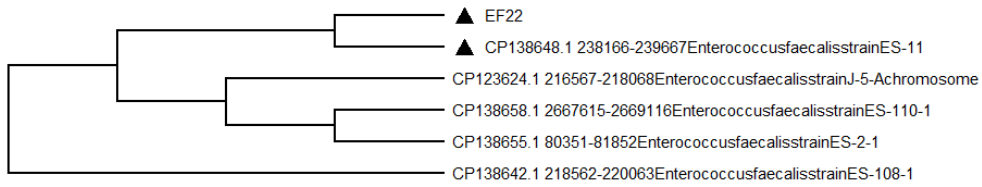


Fig 2: Phylogenetic tree showing the evolutionary relationship of bacterial isolate EF22 based on 16S rRNA gene sequences, demonstrating its close association with *Enterococcus faecalis* reference strains

Synthesis of Silver Nanoparticles from bhasma.

Visual observations and plasmon resonance preliminarily did the detection of synthesized silver nanoparticles. Color change in 24 hours was observed in both the nanoparticles subjected to synthesis using Rajat and Spahtika bhasma as a reducing agent. The Sphatika Bhasma-mediated silver nanoparticle solution exhibited a color change from milky white to brownish, indicating nanoparticle formation. In contrast, the solution prepared using Rajat Bhasma changed from light grey to dark brown within 24 hours, further confirming the progression of nanoparticle synthesis. The change in color intensity observed in silver nanoparticles can be attributed to two potential mechanisms: the excitation of Plasmon Resonance or the reduction of silver nitrate. Comparable color changes have been reported in previous studies, including those by Gandhi and Khan [17], Rao and Savithramma [18], and Gudikandula

and Charya [19], where various biological materials were employed as reducing agents for the biosynthesis of silver nanoparticles.

Physiochemical Characterization of Silver Nanoparticles

UV visible spectroscopy- The formation of silver nanoparticles can be reliably verified using UV-Visible spectroscopy, which detects the characteristic surface plasmon resonance (SPR) phenomenon associated with these nanomaterials. The typical absorption peak observed for silver nanoparticles falls in the range of 400-460 nm [20], although the exact wavelength can vary depending on factors such as particle size, shape, and agglomeration state [20,21,22]. In the present study the peak for Rajat Bhasma AgNPs was observed at 340.3 nm and for Shpatika bhasma AgNPS it was observed at 341.2 and 500 nm. Previous research conducted by Vigneshwaran et al. [23], Bindhu and Umadevi [24], AbdelRahim et al. [25], and Saxena et al., [26] strongly indicates that silver nanoparticles produce surface plasmon resonance due to the presence of free electrons. This was prominently observed at wavelengths of 320nm, 339nm, 420nm, and 480nm in their respective studies.

Scanning Electron Microscopy

The scanning electron microscopy analysis provided valuable insights into the morphological characteristics of the synthesized silver nanoparticles. The results confirmed the formation of spherical, small silver nanoparticles, with the mean particle size 95.36 ± 3.4 nm for Rajat bhasma AgNPs and 83.26 ± 8.3 nm for Sphatika bhasma AgNPs. However, the particle size recorded in the current study is in the higher range for the nanoparticle scale [27,28]. Furthermore, the SEM images revealed an uneven particle size and structure distribution, as well as a high degree of particle aggregation. (Figure- 3A and 3B)

The observed formation of small, spherical silver nanoparticles is in agreement with prior investigations, which have reported comparable morphological characteristics in silver nanoparticles synthesized using diverse phytochemical and microbial-mediated methods. These findings support the consistency of biosynthetic approaches in producing nanoparticles with defined size and shape parameters. [27,28,29,30]. The silver nanoparticles produced in this study lay within the size range documented in existing literature, although they tend to be larger. [27,28]. The observed particle aggregation and uneven size distribution may be attributed to the complex nature of the biomolecules involved in the reduction and stabilization of the silver nanoparticles [27,29]. Baishya et al., (2012) emphasized a reliable size range of silver nanoparticles produced with *Bryophyllum pinnatum* serving as a reducing agent. [31]. In a similar vein, Ghaffari-Moghaddam et al., (2014) documented the production of larger silver nanoparticles using various plant extracts, which aligns with the results observed in the current study [32].

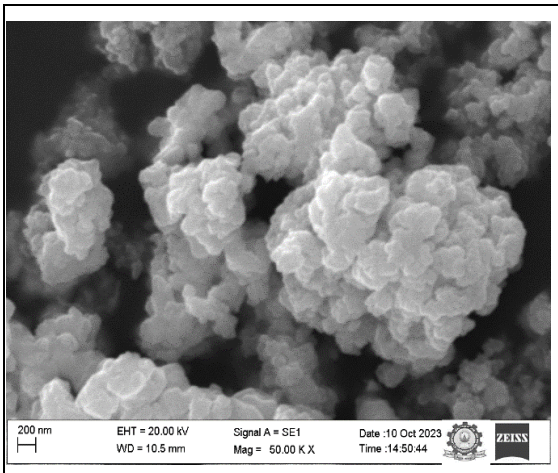


Figure 3A- Rajat Bhasma AgNPs

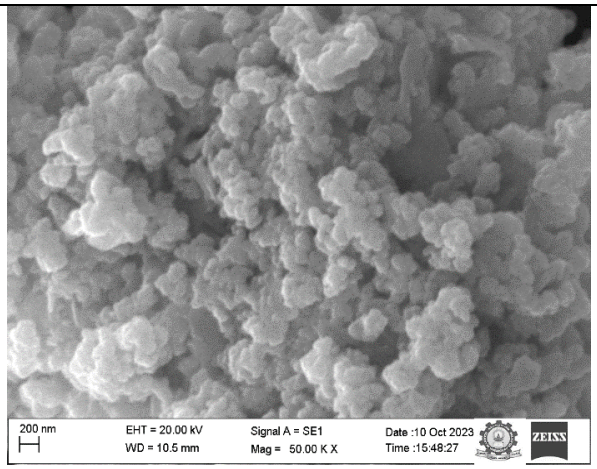


Figure 3B- Spahatika Bhasma AgNPs

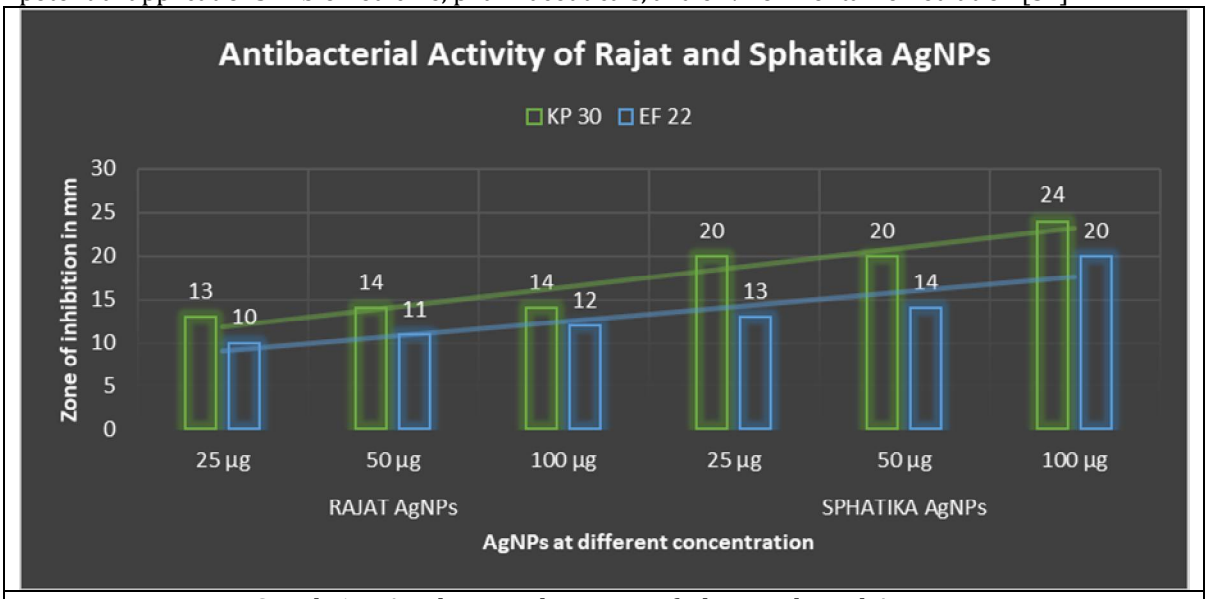
Figure 3- Scanning Electron Microscopy Images of Silver nanoparticles

Antibacterial Activity Evaluation of Silver Nanoparticles via Agar Well Diffusion .

The research conducted in this study aimed to assess the antibacterial properties of Rajat bhasma AgNPs and Sphatika Bhasma AgNPs against two bacterial isolates, KP-30 and EF-22. Rajat and Sphatika bhasma synthesized nanoparticles demonstrated antibacterial activity against the tested bacterial isolates. Sphatika bhasma AgNPs exhibited the highest zone of inhibition against the KP-30 isolate at 100 µg concentration (24 mm). In comparison, the lowest zone of inhibition was observed with 25 µg of Rajat

bhasma AgNPs against the EF-22 isolate. Similar results of broad-spectrum bactericidal activity were observed in the study of Sadeghipour *et al.,* [33] in which they tested silver nanoparticles against both Gram-positive and Gram-negative species. Rai et al., [34], in their study, described the broad-spectrum activity of silver nanoparticles, which is in line with the findings of the current study. It was observed that Sphatika bhasma AgNPs showed substantial antibacterial activity against the KP-30 isolate compared to Rajat bhasma AgNPs. Rajat bhasma AgNPs displayed a similar range of inhibition zones against both bacterial isolates at all three tested concentrations. (Graph 1- and Figure 4A and 4B).

The bactericidal activity of the synthesized nanoparticles was attributed to the silver nanoparticles' ability to disrupt the bacterial cell membrane, interfere with cellular functions, and generate reactive oxygen species, ultimately leading to bacterial cell death [35,36]. These findings are consistent with previous research on the antibacterial properties of biogenic silver nanoparticles. The study suggests that these nanoparticles could serve as a sustainable substitute for traditional antimicrobial agents, with potential applications in biomedicine, pharmaceuticals, and environmental remediation [37].



Graph 1- - Antibacterial activity of Bhasma-based AgNPs

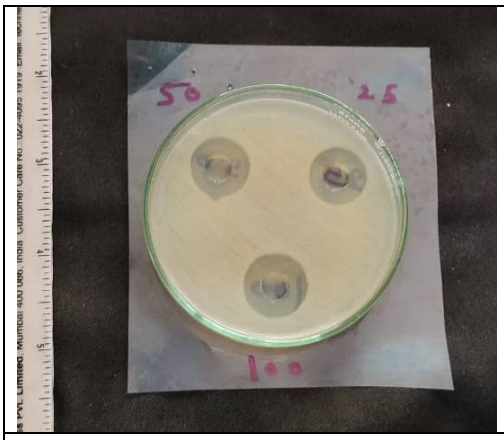


Figure 4A- KP-30 Sphatika Bhasma AgNPs

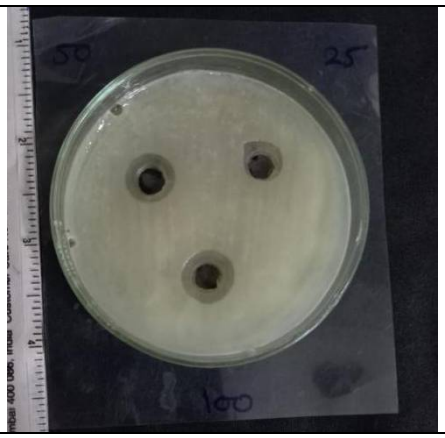


Figure 4B- EF 22 Rajat Bhasma AgNPs

Figure 4- Antibacterial activity of Bhasma-based AgNPs

CONCLUSION

The present study isolated and identified *Klebsiella pneumoniae* (KP30) and *Enterococcus faecalis* (EF22) from clinical samples and synthesized silver nanoparticles using Rajat and Sphatika bhasma. Molecular identification was confirmed through 16S rRNA gene sequencing. The AgNPs exhibited colorimetric changes and UV-Vis absorption peaks, with SEM confirming spherical nanoparticles sized at 95.36 ± 3.4 nm for Rajat bhasma and 83.26 ± 8.3 nm for Sphatika bhasma, along with some observed aggregation. Antibacterial tests demonstrated that Sphatika bhasma AgNPs were particularly effective against *K. pneumoniae*, while Rajat bhasma AgNPs showed consistent inhibitory effects. These results highlight the potential of biogenic AgNPs as antimicrobial agents. In conclusion, bhasma-based AgNPs are promising for clinical use due to their antibacterial properties and eco-friendly synthesis, warranting further research on their stability and cytotoxicity.

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