Journal of Phytology 2021, 13: 64-071
doi: 10.25081/jp.2021.v13.7026
http://updatepublishing.com/journal/index.php/jp

Research Article

ISSN: 2075-6240

Synthesis, physicochemical characterization and biological activity of synthesized Silver and Rajat Bhasma nanoparticles using *Clerodendrum inerme*

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ABSTRACT

Bhasma is Metallo-medicine and made from metals and minerals. Rajatbhasma or Silver Bhasma belongs to a group of nanoparticles that have medicinal values and are used in Ayurveda as Drugs against various ailments. *Clerodendrum inerme*a traditionally well-accepted plant is used extensively in ayurvedic therapeutic formulations, but till date no major steps have been carried out to validate the scientific relevance of synthesized nanoparticles from Rajatbhasma using *C. inerme*. Therefore, in the present study biosynthesized nanoparticles were characterized by UV–Vis spectroscopy, SEM, FESEM and EDX analysis whereas, a comparative study has also been made to check the antioxidant and antimicrobial activity of synthesized silver and rajatbhasma nanoparticle. The SEM and FESEM analysis revealed that the synthesized nanoparticles are well shaped and the average particle size ranges between 30–90 nm and 10-50 nm respectively. In the case of EDX analysis, the highest peak at ~3Kev in the case of synthesized silver and rajatbhasma nanoparticle supports the formation of silver nanoparticles. Subsequently, antioxidant and antimicrobial activities of the synthesized nanoparticles showed excellent results when compared to the standard. The obtained results may provide support in the field of therapeutics and drug delivery and might prove beneficial as a novel drug candidate against bacterial infection in the future.

Received: March 09, 2021
Revised: May 02, 2021
Accepted: May 04, 2021
Published: May 15, 2021

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KEYWORDS: *Clerodendrum inerme*, Rajat Bhasma, SEM, FESEM, DPPH, *Staphylococcus aureus*

INTRODUCTION

Ayurveda means "The Science of Life" in Sanskrit and probably be the oldest healing science. It has been developed in Vedic culture and Ayurveda therapies have been mentioned in various Indian mythology. These therapies are typically based on complex, herbal compounds, minerals, metal substances, etc. Indian system of medicine use metals in the formulations since time immemorial and also referred to in Chinese and Egyptian civilization way back in 2500 B.C (Pal *et al*., 2014). Bhasma is Metallo-medicine and made from metals and minerals. The process of Bhasmikarana is used to transform metals and minerals into bioassimilable form, *i.e*., Bhasmas. The metals and minerals obtained from ore have to undergo extensive oxidation under intense heat to prepare bhasma following the process of Shodhana and Marana. A section of the Ayurveda deals with various metal and non-metal formulations mixed with herbs called Bhasmas. It is a unique ayurvedic medical practice known as ayurvedic knowledge that passes from generation to generation verbally (Vayalil *et al*., 2002). Among the Bhasmas, one popular preparation is Rajatbhasma [calcined silver (ARGENTUM) particles] (RB) (Sharma *et al*., 2016). Medicinally Rajatbhasma (RB) is very important and RB is used as Ayurvedic Drug against various diseases like antistress, anti-anxiety, neuroprotective activity, etc. This Ayurvedic drug is also used in eye diseases, cough, jaundice, anemia, psychological diseases, abdominal diseases, liver and kidney problems (Pal *et al*., 2014).

A nanoparticle is typically defined as an ultra-fine particle of matter ranges between 1-100 nanometres in diameter (Mao *et al*., 2018). In recent days, silver nanoparticles (AgNPs) have become the most promising nanomaterials for biological applications

particularly as novel anti-microbial agents (Burdușel *et al*., 2018). However, silver nanoparticles have lethal effects on humans by inducing reactive oxygen species (ROS) mediated stress responses (Mao *et al*., 2018). One alternative therefore could be the use of RB for making nanoparticles (Sharma *et al*., 2016). It has been widely reported that plant secondary metabolites like carbohydrate, polysaccharides, alkaloids and flavonoids are highly effective in the case of synthesis of the nanoparticle. *Clerodendrum inerme* (L.) Gaertn. (Syn. *Volkameria inermis* L.) is a perennial shrub commonly known as lanjai or garden quinine belonging to the family Lamiaceae and is used in many of the herbal preparations of Siddha and Ayurveda in India (Sasikala *et al*., 1995). Ethno medicinally, the leaves of *C. inerme* are used to cure different ailments like skin disease, elephantiasis, asthma, epilepsy, topical burns, etc. (Sasikala *et al*., 1995). The proper chemical profiling of synthesized nanoparticles from Rajatbhasma by using *C. inerme* extract still remain unaddressed. Therefore, in the present study, a comparative investigation has been made to check the antioxidant activity and antimicrobial efficacy of synthesized silver nanoparticle (SNP) and rajatbhasma nanoparticle (RBNP).

MATERIALS AND METHOD

Procurement of Rajatbhasma

Rajat Bhasma is known as an Ayurvedic Drug for the treatment of various diseases, manufactured by Baidyanath Group, India.

Preparation of Plant Extract

Clerodendrum inerme (L.) Gaertn (Syn. *Volkameria inermis* L.) fresh leaves were collected from NBU campus, Darjeeling (West Bengal). Fresh and disease-free leaves of the plant were washed twice with double-distilled water; shade dried at room temperature for 21 days and pulverized into fine powder by using a mechanical grinder. Powdered leaves of the plant (10g each) were extracted in a Soxhlet apparatus using absolute methanol (the ratio of plant material to solvent was 1:10 m/v) for 6-7 hours. The extracts were then concentrated under reduced pressure and controlled temperature (40-50 °C) using a rotary evaporator (Buchi Rotavapor R-3, Switzerland). The extracts were further lyophilized using Eyela Freeze Dryer (FDU-506, USA) to obtain dry powder and stored at 4°C until further use. During the experiment, we have used 100 mg/ml concentration by dissolving plant extract in distilled water.

Synthesis of Nanoparticle from Rajatbhasma

Synthesis of Rajat Bhasma (RB) aqueous extract was conducted through the decoction method. In this process, 0.5 g RB and 30 ml distilled water were taken in a flask. The solution was heated for 15–20 min at 80 °C using a magnetic starrier for constant starring. Simultaneously, the plant extract (PE) (250 µl) was added until the colour of the solution changed from yellow to reddish-brown. After 24 hours the samples were centrifuged at 6000 rpm for 20 min at room temperature. After centrifugation, the samples were air-dried and stored at 4 °C for further use.

Synthesis of Nanoparticle from Silver Nitrate ($AgNO_3$)

For the synthesis of silver nanoparticle, 0.7g of $AgNO_3$ (SN) was added with 40 ml distilled water and plant extract was added simultaneously (maintaining the same condition as mentioned above) and gradually the colour changed from yellow to reddish-brown. After 24 hours the samples were centrifuged at 6000 rpm for 20 min at room temperature. After centrifugation, the samples were air-dried and stored at 4°C for further use.

Characterization of Synthesized Silver Nanoparticle (Snp) and Rajat Bhasma Nanoparticle (Rbnp)

UV-Visible spectra analysis

Characterization of biogenically synthesized nanoparticle was done by UV-Visible spectroscopy after 24hr of experiment and the graph was also plotted.

Scanning electron microscopy (SEM) and field emission scanning electron microscope (FESEM)

SEM analysis was performed using JEOL model Smart Coater: DII 29030 SCTR, JEOL Solutions for Innovation, Tokyo, Japan. The powdered samples were mounted on copper mesh and a 3 nm gold coating was done by a gold sputtering unit. These samples were observed under the scanning electron microscope (JEOLJSM-IT100InTouchScope™ Scanning Electron Microscope, JEOL Solutions for Innovation, Tokyo, Japan FESEM analysis was done to record the surface morphology of AgNPs with Carl Zeiss at an accelerating voltage of 5 kV and at 100000× magnification.

Energy dispersive X-ray spectroscopy (EDX)

To determine the presence of elemental Ag in biogenically synthesized nanoparticle EDX analysis was done using Oxford-EDX instruments that use 80 mm^2 SDD detectors that detect elements under high resolution (Das *et al*., 2019).

Determination of In-vitro Antioxidant Activity

A total of eight antioxidant assays were performed by previously reported methods using silver Nano, rajatbhasma nanoparticle and plant extract (Dutta *et al*., 2018; Kar *et al*., 2019).

Antimicrobial Activity

Test bacteria

Four reference strains of clinically important pathogenic test bacteria used in this study include; two Gram-positive; *Bacillus subtilis* (MTCC-121) and *Staphylococcus aureus* (MTCC-3160) and two Gram-negative; *Escherichia coli* (MTCC-1698) and *Klebsiella pneumonia* (MTCC-103). The bacteria were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India. These bacterial cultures were maintained by regular sub culturing on

nutrient agar slants and stored at -20°C using 10% glycerol as a preservative.

Preparation of test solutions

The synthesized rajatbhasma and silver nanoparticles were dissolved in double distilled water to give a working concentration of 10 mg/ml and 2.5 mg/ml. The nanoparticles were sterilized by filtration through a 0.2-micron syringe filter.

Anti-microbial activity

Antibacterial activity of the synthesized rajatbhasma and silver nanoparticles was carried out according to the agar well diffusion method of Perez *et al*., (1990) with slight modifications. A loopful of bacterial culture were aseptically inoculated into 10 ml of pre-sterilized Mueller-Hinton broth (HIMEDIA M391-100G, India) followed by 5 hr incubation at 37°C in a shaking condition. These actively growing broth culture suspensions prior to antimicrobial assay were adjusted turbid metrically to 0.5 McFarland standards with specified pre-sterilized broth to yield a bacterial suspension of 1-2 × 10^8CFU/ml.

Agar well diffusion method

The antimicrobial activities of the synthesized rajatbhasma and silver nanoparticle were evaluated employing the agar-well diffusion assay. Twenty millilitres of the Mueller-Hinton molten agar (45 °C) was aseptically mixed with 1000µl of a bacterial suspension (1-2× 10^8CFU/ml) and poured into sterile Petri plates and kept for solidification. Once the agar was hardened, wells of 8.0 mm diameter were punched aseptically into the agar medium using a sterile cork borer and the wells were filled with 100 µl of the synthesized rajatbhasma and silver nanoparticle (2.5 and 10 mg/ml).The plates were then incubated for 24 hr at 37°C in an incubator. Penicillin (10units/ml) (Pfizer) and Streptomycin (10µg/ml) (Abbott) served as positive controls for Gram-positive and Gram-negative bacteria respectively. The diameters of the resulting zone of inhibition (ZOI) were measured in the nearest millimetres (mm) range. Zone of inhibition less than 9.0 mm was not considered. Solvent control distilled water was included in every experiment as negative control. All samples were tested in triplicate and the zone of inhibition results shown are the average.

Statistical Analysis

For reproducibility, all data were prepared as the mean ± SD of six measurements. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Dunnett's test using KY Plot version 5.0 (32 bit) for windows. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

UV-Visible Spectroscopy

The transition of colour of the reaction mixture from colourless to yellow then to reddish-brown was the visual evidence of Ag^+ ion reduction into nanoparticle (Figure 1) (Bose *et al*., 2015). Further formation of silver nanoparticles was confirmed by UV-Visible spectra. It was reported that the presence of surface Plasmon resonances (SPR) spectra within the wavelength of 400-500nm confirms the synthesis of nanoparticle (Das *et al*., 2019). In this study, characteristic SPR bands of synthesized silver and rajatbhasma nanoparticles were observed at 430 nm. These results indicate that nanoparticle was synthesized successfully.

Scanning Electron Microscopy (SEM) and Field Emission Scanning Electron Microscope (FESEM)

From SEM image of biogenically synthesized silver and rajatbhasma nanoparticle, it was found that nanoparticles were spherical and cubical in shape (Figure 2a and 2b). The particle size ranges between 30–90 nm respectively. In the case of FESEM analysis Figure 3a and 3b) it was found that nanomaterials were also spherical and cubical in shape, supporting the result obtained by SEM analysis. The particle size ranges between 10-50 nm. In some cases nanoparticles were bulked may be due to cross linking or evaporation of solvent during sample preparation (Das *et al*., 2019).

Energy Dispersive X-ray Spectroscopy (EDX)

To know both qualitative and quantitative information regarding elements present in the nanoparticle sample, EDX analysis was done for both biogenically synthesized and rajatbhasma nanoparticle (Figure 4a and 4b). Several elements such as C (carbon), S (sulfur), and Si (silicon) were present but silver (Ag) shows the highest peak at ~3Kev and the percentage of Ag shows 80.00 % in the case of nanoparticle synthesized from *C. inerme* and 37.30% in rajatbhasma nanoparticle. This result supports the formation of silver nanoparticles (Bhakya

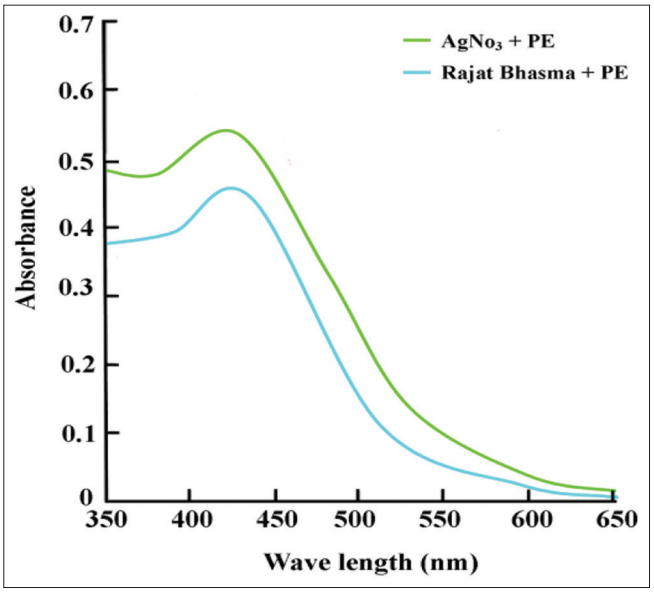

Figure 1: UV-visible spectra of Ag nanoparticle, synthesized by reducing AgNO3 and rajatbhasma using *Clerodendrum inerme* leaf extract

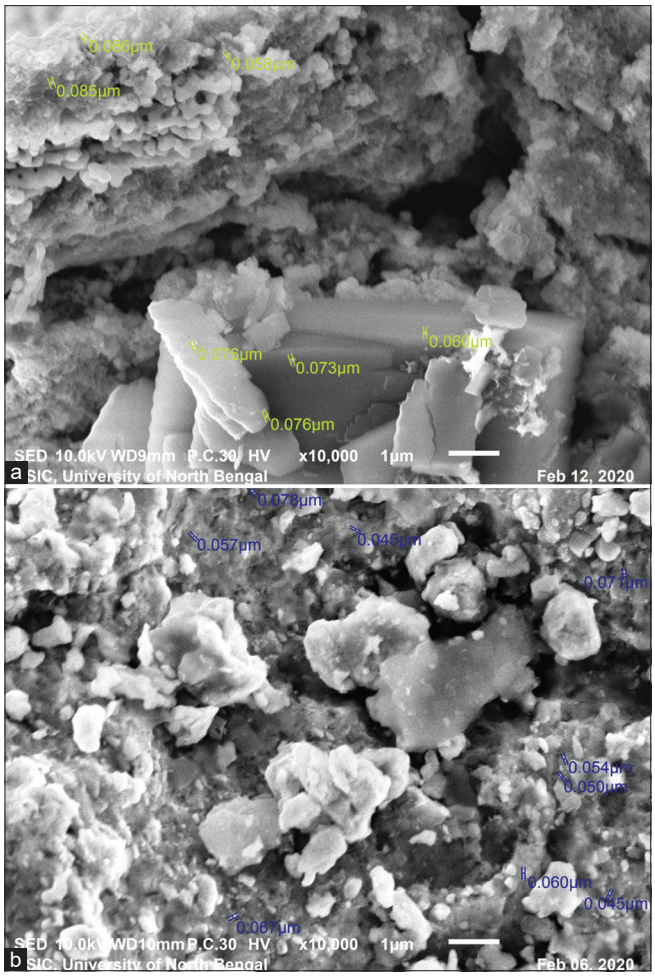

Figure 2: SEM micrograph analysis of biosynthesized (a) silver and (b) rajatbhasma nanoparticles

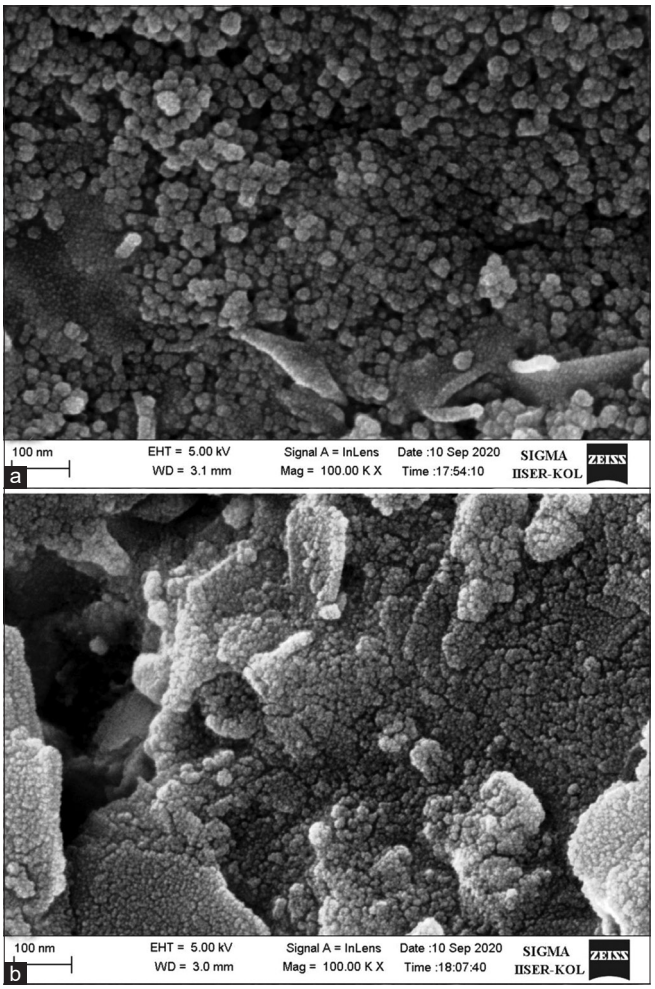

Figure 3: FESEM micrograph analysis of biosynthesized (a) silver and (b) rajat bhasma nanoparticles

et al. 2016) and also the presence of carbon and oxygen in the results supports the idea of bioreduction of metallic silver into elemental silver where the alkyl chain agent act as a stabilizing agent (Das *et al*., 2019).

Antioxidant Assay

It is hard to conclude about anti-oxidant or free radical scavenging property based on a single experimental model. Therefore, several experiments have been designed to establish the anti-oxidant properties of silver and rajatbhasma nanoparticles. Human beings always encountered various types of free radicals like H_2O_2, superoxide, nitric oxide (NO), etc. directly or indirectly via the environment. Biosynthesized silver and rajatbhasma nanoparticles exhibit significant dose-dependent scavenging activity against all the free radicals. The functional groups of leaf extract that are involved in the reduction of silver ion for nanosilver formation are mainly responsible for its antioxidant activity. In the present study, rajatbhasma nanoparticle (RBNP) showed the highest percent of inhibition (42.48 ± 0.16 at 200µg/ml) when compared to SN (silver nitrate), SNP (silver nanoparticle) and RB (rajat bhasma) respectively (Figure 5a). The property of DPPH is to accept an electron or hydrogen radical to attain stability, which changes the colour of the solution due to the presence of natural antioxidants (Bhakya *et al*., 2016). For superoxide anion (Figure 5b), hypochlorous acid (Figure 5c) and total antioxidant (Figure 5d) scavenging assay, RBNP showed significant radical scavenging activity when compared with respective standard. During metabolic reactions in peroxisome and mitochondria superoxide anion ($O_2^{\cdot-}$) are formed which undergoes spontaneous dismutation and generates singlet oxygen and damages various biological molecules (DNA and Protein) (Das *et al*., 2019). In case of cellular inflammation, activated phagocytic cells (neutrophils) release hydrogen peroxide and generate potent reactive oxygen species (ROS) like Hypochlorous acid (HOCl) by oxidation of Cl^- in the presence of myeloperoxidase enzyme (MOP) (Valentao *et al*., 2002; Pedraza-Chaverrí *et al*., 2007). Ferric reducing power assay is a high throughput method for the determination of antioxidant levels in biological samples (Bhakya *et al*., 2016). In this study at the highest concentration (200µg/ml) of RBNP showed better reducing power activity than the standard BHT (Figure 6a).

On the other hand, Nitric oxide (NO) takes part in the inflammatory pathway and acts as an inflammatory mediator. Calcium independent isoform of NOS (iNOS) activates by lipopolysaccharide (LPS) during chronic inflammation and produces NO. The NO is directly associated with destructive consequences (Gimenez-Garzó *et al.*, 2015; Sehitoglu *et al.*, 2015). In this study biogenic ally synthesized RBNP showed good scavenging activity at the highest concentration (32.96 ± 0.0 at 200µg/ml) (Figure 6b). Hydrogen peroxide (H_2O_2) is formed in peroxisomes from superoxide anion (O_2^{-}) in the presence of superoxide dismutase (SOD). H_2O_2 accumulates in cells and converts into Hydroxyl radical (OH•) when it comes in contact with other transition metals like Fe^{2+}, Cu^{2+}etc, that may cause lipid peroxidation and DNA damages (Matés & Sánchez-Jiménez, 2000; Ray & Husain, 2002). Here RBNP showed the highest Hydrogen peroxide (Figure 6c) and hydroxyl radical (Figure 6d) activity at the concentration of 200µg/ml when compared to the respective standards. Thus, Present findings reveal that RBNP might be instrumental in the recovery of oxidative stress-related disorders.

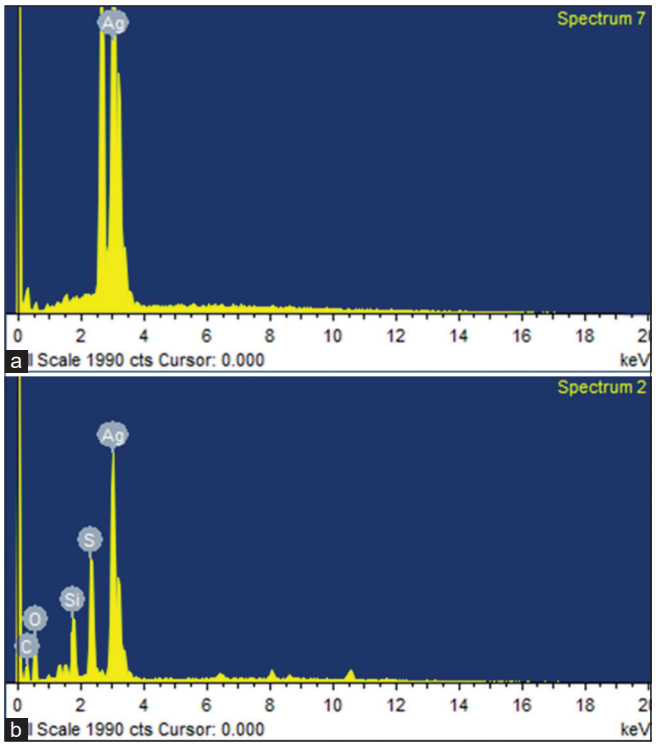

Figure 4: EDX spectra and elemental profile of biosynthesized (a) silver and (b) rajat bhasma nanoparticles

Antimicrobial Activity of the Synthesized Nanoparticles

Antibacterial activity of the synthesized silver and rajatbhasma nanoparticle was investigated against two Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) and two Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*) pathogenic bacterial strains growing on Mueller-Hinton agar medium using agar well diffusion method (Perez *et al.*, 1990). The present study revealed that the highest tested concentration of both synthesized nanoparticles exhibited promising antibacterial efficacy concerning Gram-positive bacteria namely, *S. aureus* and *B. subtilis* (Table 1; Figure 7). However, a marginal decrease in the inhibitory effect of both the nanoparticles was noticeable towards Gram-negative bacteria, *E. coli* and *K. pneumonia* (Table 1; Figure 8). Further, in comparison, the Gram-positive bacteria were recorded to be more susceptible

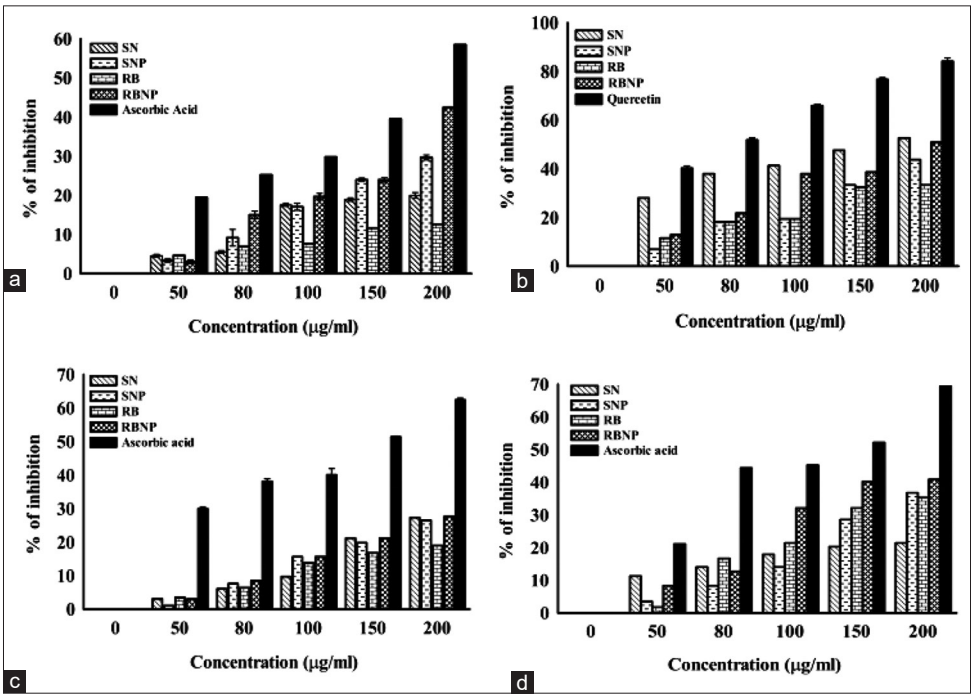

Figure 5: Antioxidant activity of synthesized silver and rajatbhasma nanoparticle (a) DPPH activity (b) Superoxide radical (c) Hypochlorous acid (d) Total antioxidant scavenging activity [SN: silver nitrate, SNP: silver nanoparticle, RB: rajat bhasma and RBNP: rajat bhasma nanoparticle

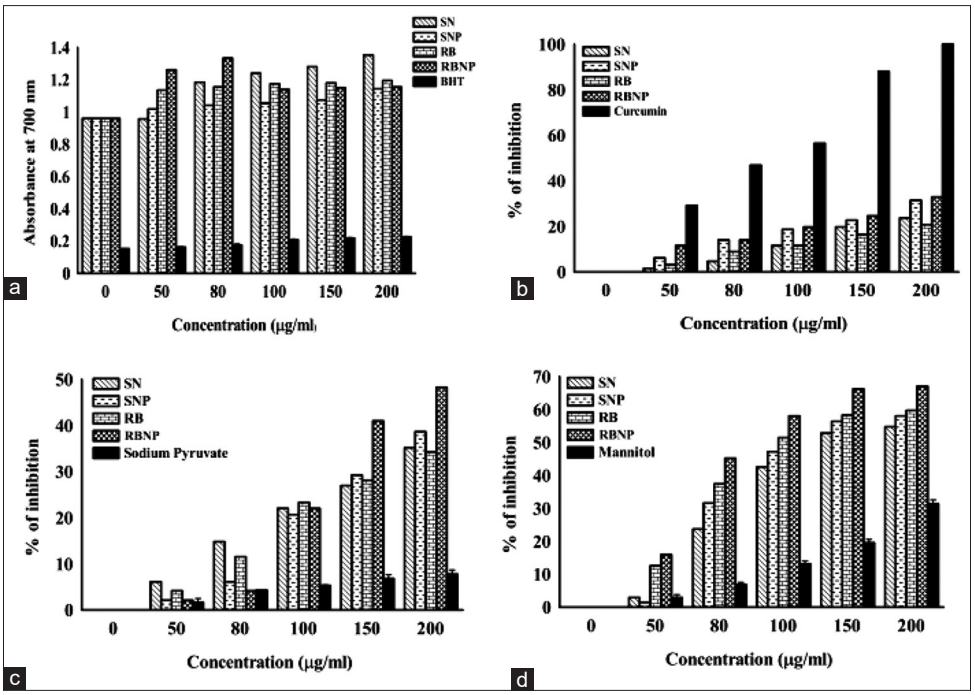

Figure 6: Antioxidant activity of synthesized silver and rajatbhasma nanoparticle (a) Reducing power assay (b) Nitric oxide (c) Hydrogen peroxide (d) Hydroxyl radical scavenging assay [SN: silver nitrate, SNP: silver nanoparticle, RB: rajat bhasma and RBNP: rajat bhasma nanoparticle]

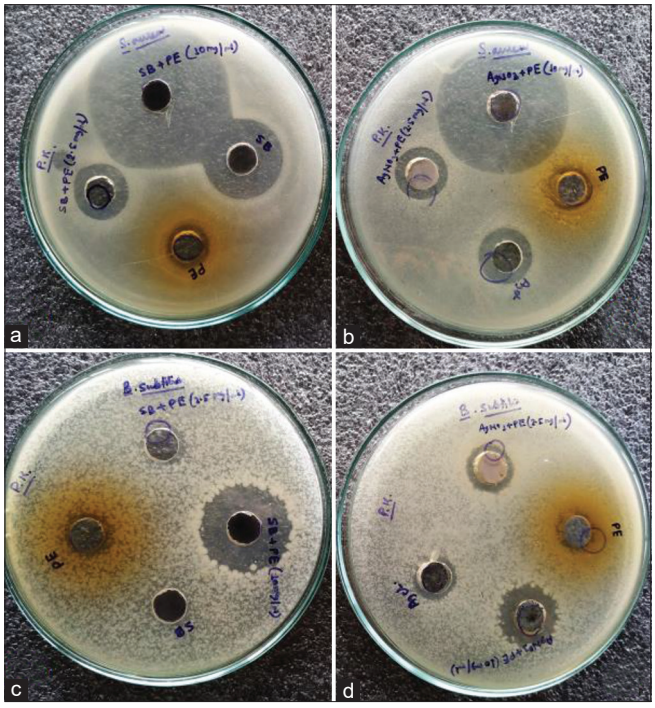

Figure 7: Antimicrobial activity of synthesized rajatbhasma and silver nanoparticle. (a, c) RBNP (rajat bhasma nanoparticle) and (b, d) SNP (silver nanoparticle) against gram positive bacteria *S. aureus* and *B. subtilis*

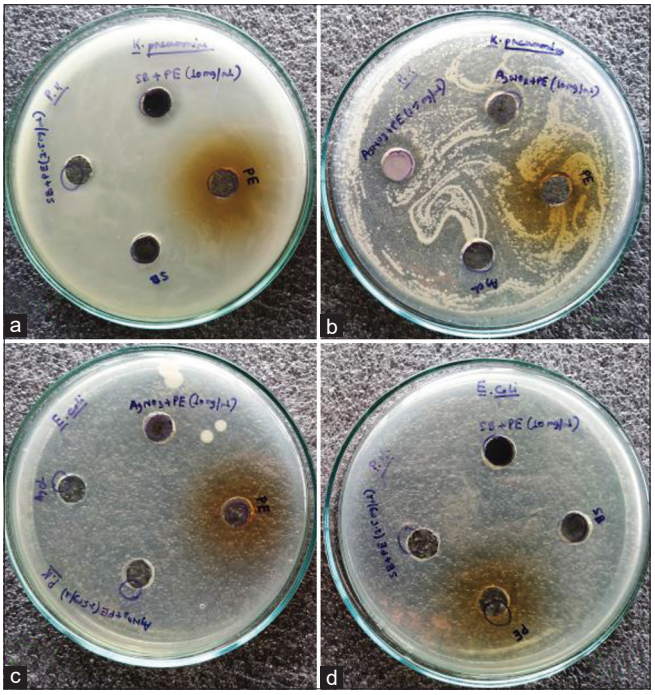

Figure 8: Antimicrobial activity of synthesized rajatbhasma and silver nanoparticle. (a, c) RBNP (rajat bhasma nanoparticle) and (b, d) SNP (silver nanoparticle) against gram negative bacteria *K. pneumonia* and *E. coli*

than Gram-negative bacteria. This may be due to the binding ability of metal at higher concentrations onto the surface of Gram-positive bacteria which is believed to be the primary step for penetration into the bacteria (Beveridge & Fyfe, 1985). Once penetration is completed the synthesized silver and rajatbhasma nanoparticles bring about the killing of the bacterial cells by interacting with phosphorous and sulfur-containing biomolecules such as DNA and proteins (Baker *et al.*,

Table 1: Antimicrobial activity of rajat bhasma and silver nanoparticle.

Sample	Diameter of zone of inhibition (mm)			
	S. aureus	*B. subtilis*	*E. coli*	*K. pneumonia*
RBNP (10 mg/ml)	49	28	10	17
RBNP (2.5 mg/ml)	20	14	10	10
RB	29	10	10	10
PE	14	11	10	10
SNP (10 mg/ml)	43	21	10	20
SNP (2.5 mg/ml)	19	16	10	18
AgCl	14	12	10	11
PE	9	13	10	10

SNP: silver nanoparticle, RB: rajat bhasma, RBNP: rajat bhasma nanoparticle, AgCl: silver chloride and PE: plant extract.

2005). However, at lower concentrations, a decrease in efficiency as an antibacterial agent could be attributed to the presence of thick layers of peptidoglycan chiefly made up of linear polysaccharide chains cross-linked by short peptides. This makes the cell wall of Gram-positive bacteria a highly rigid structure that impedes silver and rajatbhasma nanoparticles to penetrate the bacterial cell wall (Ibrahim, 2015). In the case of Gram-negative bacteria, peptidoglycan makes up a minor constituent of the cell wall, due to which silver nanoparticles could easily release silver ion thereby damaging the cell membrane leading to bactericidal activity (Flores-López *et al*., 2016). But in the present study, the Gram-negative bacteria were found to be less susceptible to the synthesized nanoparticles which might be due to resistance exhibited by the test organisms. In general, the present findings align with similar studies of Jagtap & Bapat (2013) and Maiti *et al*. (2014). AgNPs production through natural biogenesis processes using indigenous plant extract suggests that there exists an unambiguous parallelism between ethnopharmacological use and the experimental outcome.

CONCLUSION

The use of metallic nanoparticles in the treatment of various ailments is a relatively recent development in the history of medical sciences. However, the Indian system of medicine uses metal ash (Bhashma) for ages. In this context, the present study aimed to compare the use and efficacy of silver nanoparticles and rajatbhasma for the handling of various ailments. *Clerodendrum inerme* is a plant of Lamiaceae family well documented from its traditional use has been used for the production of nanomedicine. It is apparent from the study that rajatbhasma by no means is inferior to synthetic silver nanoparticles and could be more effective against pathogenic bacteria. Thus, information from the present analysis in combination with existing knowhow promises to facilitate the development of new drugs in modern medicine. Although the present approach involves an *in-vitro* practice, further assessment in *in-vivo* models as a drug delivery system might confer new directions in nanotechnology and ethnomedicine.

Authors' Contribution

AS and PK conceived the idea. PK, SB, AS designed the experiments. PK and SB performed the biochemical tests. AC performed the antimicrobial work. All the authors contributed in writing the manuscript and finalized it.

Conflict of Interest

The authors declare that they have no conflict of interest.

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