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Novel biogenic synthesis of silver nanoparticles and their therapeutic potential

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1. ABSTRACT

Here, we explored the medicinal uses of the novel biogenic silver nanoparticles of *Pterospermum acerifolium* (PaAgNPs) as a cost effective, eco-friendly, reducing and stabilizing compounds. The formation of PaAgNPs was confirmed by changing its color from colorless to yellowish brown, with maximum absorbance at 417 nm. FTIR spectrum of PaAgNPs suggested the presence of polycyclic compound similar to betulinic acid which plays as a capping agent and provided stability to PaAgNPs. FESEM and HRTEM images depicted the spherical shape of synthesized biogenic silver nanoparticles with an average particle size range of 10-20 nm. The EDX spectrum of the solution confirmed the presence of elemental silver signals. The crystalline nature of PaAgNPs was identified by XRD technique and its stability was recorded using Zeta potential analyzer. The antioxidant potential was assayed using diphenyl-β-picrylhydrazyl (DPPH). Maximum free radical scavenging action of PaAgNPs was 69.52% as compared to 63.53% for PALE. Using a model of carrageenan-induced paw edema in rats, PaAgNPs showed two-fold enhanced anti-inflammatory activity *in vivo*.

2. INTRODUCTION

Plants are enriched with alkaloids, glycosides, terpenoids, polyphenols and steroids which can be used for the treatment of patients in various ailments. The antioxidant and anti-inflammatory properties possessed by these plant bioactive constituents prevents oxidative stress and degenerative diseases such as atherosclerosis, ischemic heart disease, aging, inflammation, diabetes, immunosuppression and cancer in cells (1,2). Although the management of these inflammation is taken care

by steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) but it is often associated with various side effects such as gastric hemorrhage (3-6).

In the present investigation, we exploited the potential of *Pterospermum acerifolium* for the treatment of cellular damage caused due to oxidation and inflammation. *Pterospermum acerifolium* belongs to the family (Sterculiaceae), commonly known as 'Kanak Champa' in India. The traditional system of Indian medicine had described the importance of this plant in ayurvedic perspective as an anti-cancer agent when the mixture of its flowers with sugars are applied locally (7). The barks and flowers of this plant were scalded and then mixed to be utilized for the treatment of smallpox. For the treatment of a headache, flowers of *Pterospermum acerifolium* were converted into a paste with rice water and applied locally (8). The presence of taraxerol, friedelin, 1-friedelen-3-one and b-sitosterol-3-O-b-D-glucose have been reported in the leaves of *Pterospermum acerifolium* (9).

However, till date their exists no report on the synthesis of silver nanoparticles using *Pterospermum acerifolium* leaves extract (PALE) to enhance its therapeutic potential. Earlier, researchers have used biogenic nanoparticles of noble metals which have gained significant attention due to their potential applications in biomedicine, drug delivery, imaging, photonics and catalysis (10, 13). These biogenic nanoparticles are generally synthesized by using chemical reduction, ultrasonic fields, lithography, photochemical reduction, ultra-violet irradiation and microwave assisted techniques (14, 18).

Recently, the biosynthesis of metal nanoparticles using natural plant extracts are of keen interest due to their catalytic, optical, and magnetic properties (19, 20). The noble metals such as platinum, palladium, selenium, lead, gold and silver are usually utilized for preparing metallic nanoparticles. Among these, silver has been widely exploited in the synthesis of ecofriendly nanoparticles. Silver has a crucial role in the reduction of oxidative stress in biological systems with enviable size, shape and stability (21, 25). Hence, a simple biosynthetic method using green chemistry approach has been addressed for synthesizing *Pterospermum acerifolium* silver nanoparticles (PaAgNPs) for their antioxidant, and anti-inflammatory potential.

3. MATERIALS AND METHODS

3.1. Chemicals

Silver nitrate ($AgNO_3$) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH), were obtained from Sigma-Aldrich, St. Louis, USA while ascorbic acid, Carrageenan, and carboxymethyl cellulose (CMC) were procured from Hi-Media Laboratories Pvt. Ltd. Mumbai, India. All the glasswares were treated with aqua regia (HCl: HNO3 = 3:1) for 30 minutes, then thoroughly washed with Milli-Q water (Milli-Q plus system, Millipore Co.) having high water resistivity (18.2 MΩ-cm) and finally dried in hot air oven for the period of 5 h prior to use.

3.2. Plant material

Fresh young leaves of *Pterospermum acerifolium* were collected locally from Bhopal ($23^{o}25'N$ $77^{o}41'E$) Madhya Pradesh, India. It was identified and authenticated by Dr. Zia Ul Hasan, Head of the Department, Department of Botany, Saifia Science College, Bhopal, with specimen voucher no. 458/Bot/Saifia/14.

3.3. Extract preparation

Freshly collected *Pterospermum acerifolium* leaves were shade dried for 15 days, then crushed into a fine powder and sieved (Sieve number 80). Powdered leaves (5g) were then mixed with distilled water (100ml) in an Erlenmeyer flask (250ml). The obtained solution was heated at 60°C for 20 min followed by the filtration of extract with Whatman paper no. 1 and kept at 4°C for further analysis (26).

3.4. Biogenic synthesis of PaAgNPs

Biogenic synthesis of novel PaAgNPs was carried out by adding 10 ml of PALE into 90ml $AgNO_3$ (2mM) solution at 27°C with continuous stirring (120 rpm) for 2h. The change in color of the reaction mixture from colorless to yellowish brown indicated the formation of PaAgNPs. Synthesized PaAgNPs were washed (thrice) with Milli-Q water and centrifuged at 15,000 rpm for 10 minutes. The PaAgNPs were collected and redispersed in Milli-Q water for characterization (26).

3.5. Phytochemical screening

Qualitative analysis of PALE and synthesized silver nanoparticles (PaAgNPs) were performed using standard protocols (27) for determining, the presence of alkaloids (Dragendorff's reagents), flavonoids (Lead acetate test), tannins (Ferric chloride test), glycosides (Killer-Killani test), anthraquinones, saponins (foam test), steroids (Liebermann- Burchard test), terpenoids (Nollers test), and coumarins (UV filter paper test).

3.6. Characterization of PaAgNPs

3.6.1. Spectroscopic analysis

The optical absorbance of the PaAgNPs dispersion was taken between 300-800 nm with a UV-vis spectrophotometer (Shimadzu model no. 1700) at a resolution of 1nm to investigate the bioreduction of silver ions by PALE extract. FTIR (PerkinElmer spectrometer) analysis of the dried PaAgNPs was performed using potassium bromide (KBR) pellet (FTIR grade) and the spectrum was recorded in the range of 4000-400 cm^{-1} at a resolution of 4 cm^{-1}.

3.6.2. Microscopic analysis

Aqueous PaAgNPs dispersion was prepared for High-resolution transmission electron microscopy (HRTEM) by placing a drop of it on a copper grid and allowing the water to evaporate completely. Histogram and size distribution were calculated using 'Image J 1.49' software by measuring the diameters of at least 100 particles. The elemental silver composition of the silver in biogenic synthesized PaAgNPs was analyzed using energy dispersive X-ray analysis (EDX). The selected area electron diffraction (SAED) pattern in High resolution transmission electron microscopy (HRTEM, Tecnai, G2 20) was used to identify texture and discrimination of nanocrystalline from amorphous phases of nanoparticles. To study the surface morphology of PaAgNPs one drop of prepared PaAgNPs was coated with a thin layer of gold using a sputter coater (Biotech SC005) for the 60s and observed under Field emission scanning electron microscopy analysis (FESEM, ZEISS ultra plus 55).

3.6.3. XRD analysis

Biogenic synthesized PaAgNPs were air dried and collected on a glass slide. The XRD was carried out on a Brucker D-8 Advance X-ray diffractometer system operated at a voltage of 45 kV and a current of 40mA with Cu kα radiation over the 2θ value range from 0-80. The XRD data were analyzed using high score software origin, 8.1 (28).

3.6.4. Hydrodynamic size (DLS) and zeta potential analysis

Zeta potential and DLS measurement were used for identifying surface charge, hydrodynamic size of biogenic synthesized nanoparticles. PaAgNPs diluted sample were placed in a folded capillary cell supported with platinum electrodes and inserted into the sample holder of the zeta potential analyzer Malvern Zetasizer Nano ZS90 at 25°C.

3.7. Antioxidant activity

3.7.1. Antiradical (DPPH) assay

The antiradical effect of PaAgNPs and PALE was performed using diphenyl-β-picrylhydrazyl (DPPH) method (29). Varying concentrations of PALE and PaAgNPs ranging from (100-500 µg/ml) were mixed individually with DPPH (3ml; 0.1mM) which were kept in the dark for 30 minutes. The absorbance of samples were taken at 517 nm using a UV-visible spectrophotometer. Ascorbic acid was used as standard and DPPH methanol reagent without sample used as a control. Antiradical activity (%) were calculated using the following formula:

$$\textit{Antiradical activity}\ (\%) = \frac{A_0 - A_1}{A_1} \times 100$$

(Where, A_0 = Absorbance of control and A_1 = Absorbance of samples)

3.8. Anti-inflammatory activity

3.8.1. Experimental animals

The anti-inflammatory test was performed on Wister albino rats (male and female) in the age group of 4 months (120 to 160 g). Rats were procured from animal house facility, Sapience Bio-analytical Research Lab, Bhopal, (M.P.) India. The temperature (25±2°C) with relative humidity 44–56% and dark: light (12:12 hours) cycles were maintained in the cross ventilated animal house. During the experiment, animals were fed with standard pallet diet and water *ad libitum*. The experiment was approved by the institutional animal ethics committee (IAEC) as per CPCSEA guidelines (approval no. 1413/PO/a/11/CPCSEA).

3.8.2. Acute oral toxicity studies

As per OECD guidelines (425), the oral acute toxicity study was evaluated in Wistar albino rats. Three animals were selected for a maximum tolerable dose (2000 mg/kg body weight) of biogenic synthesized PaAgNPs. The sample were given by gavage using oral canula. Animals were observed individually for any toxicity signs like convulsion, tremor, circling, depression, and mortality up to 24 h after dosing. All observations were systematically recorded with individual records maintained for each animal.

3.8.3. Carrageenan-induced rat paw edema

Carrageenan with 1% w/v saline (0.1ml) was injected into the sub-plantar region of the left hind paw of each experimental rat causing acute inflammation. The paw volume was measured plethysmometrically at the time intervals of 1 h (0-4 h) after the carrageenan injection. The inhibition of paw edema (%) was estimated by the following equation:

$$\textit{Inhibition of paw edema (\%)} = 100\left(1 - \frac{vt}{Vc}\right)$$

Where Vc is the edema volume in the control group and Vt is the edema volume of tested groups (30, 31). Grouping and dosing of the experimental rats were performed as follows; A total of 30 rats was used. The rats were divided into 5 groups comprising of 6 rats in each group: Group I**:** Rats treated with 1ml/100gm, sterile distilled water as a control; Group II: Rats treated with Diclofenac sodium, (10 mg/kg, p.o.); Group III: Rats treated with PALE (300mg/kg, p.o.); Group IV: Rats treated with 2mM of PaAgNPs dispersion (200 mg/kg, p.o.); Group V: Rats treated with 2mM PaAgNPs dispersion (300 mg/kg, p.o.). The rats were injected with a single shot of their respective doses after every 24 h for 7 days. On the 7th day, the experimental rats were injected with carrageenan (0.1 ml).

3.8.4. Statistical analysis

All the samples were evaluated three times and the values are expressed as a mean ± standard deviation. One way analysis of variation (ANOVA) and posthoc Tukey-Kramer multiple comparison tests were

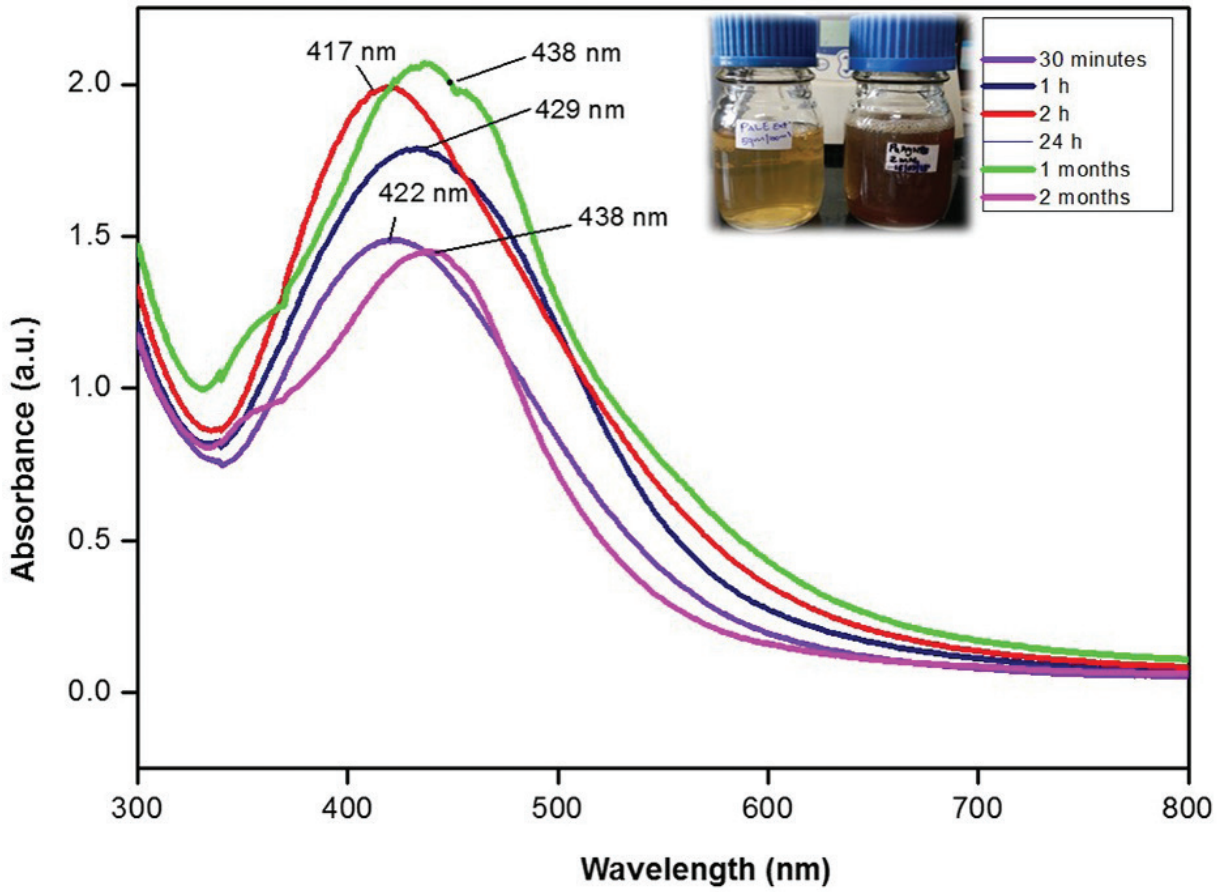

Figure 1. UV-visible absorbance spectra of PaAgNPs.

done with the aid of statistical software, GraphPad InStat 3. Differences between groups were considered significant at $P < 0.05$ levels.

4. RESULTS AND DISCUSSION

The aqueous PALE which act as a novel reducing, capping and stabilizing agents was utilized for the biogenic synthesis of PaAgNPs. The color change of aqueous PALE during PaAgNPs synthesis from colorless to yellowish brown was observed due to surface plasmon resonance phenomena. The stable peak of synthesized PaAgNPs was recorded at 417 nm (Figure 1). Synthesized PaAgNPs exhibited strong absorption of electromagnetic waves in the visible range owing to their optical resonant property, which could be due to collective oscillation of conduction electrons, combined with the incident light. The stability of the PaAgNPs was determined periodically by UV- visible spectroscopy and the solution were found to be stable for more than 60 days.

At present the exact mechanism of biogenic synthesis of silver nanoparticles is still in its infancy. Though, researchers have proposed different hypotheses regarding mechanistic aspects of nanoparticles biosynthesis (32). The biogenic synthesis of nanoparticles is a complex process involving certain phytochemicals such as vitamins, enzymes/proteins, organic acids (citrates, amino acids), and polysaccharides which act as reducing and capping agents. It has been reported that the alkaloids, terpenoids, flavonoids, polyphenols present in the plant extract are the key secondary metabolites having hydroxyl, carbonyl, and amine functional groups in them (33). The FTIR analysis of these functional groups depicts their reaction with metal ions and reduced their size in nano range. These mechanisms also provide stability as well as biocompatibility to synthesized nanoparticles by capping around them (29, 33).

Presence of phytochemical constituents namely coumarins, tannins, saponins, steroids and terpenoids were recorded in PALE as well as PaAgNPs (Table 1). The qualitative phytochemical analysis is an important factor for the bulk extraction and identification of phytoconstituents. These phytoconstituents present in the leaf extract influences the synthesis of PaAgNPs

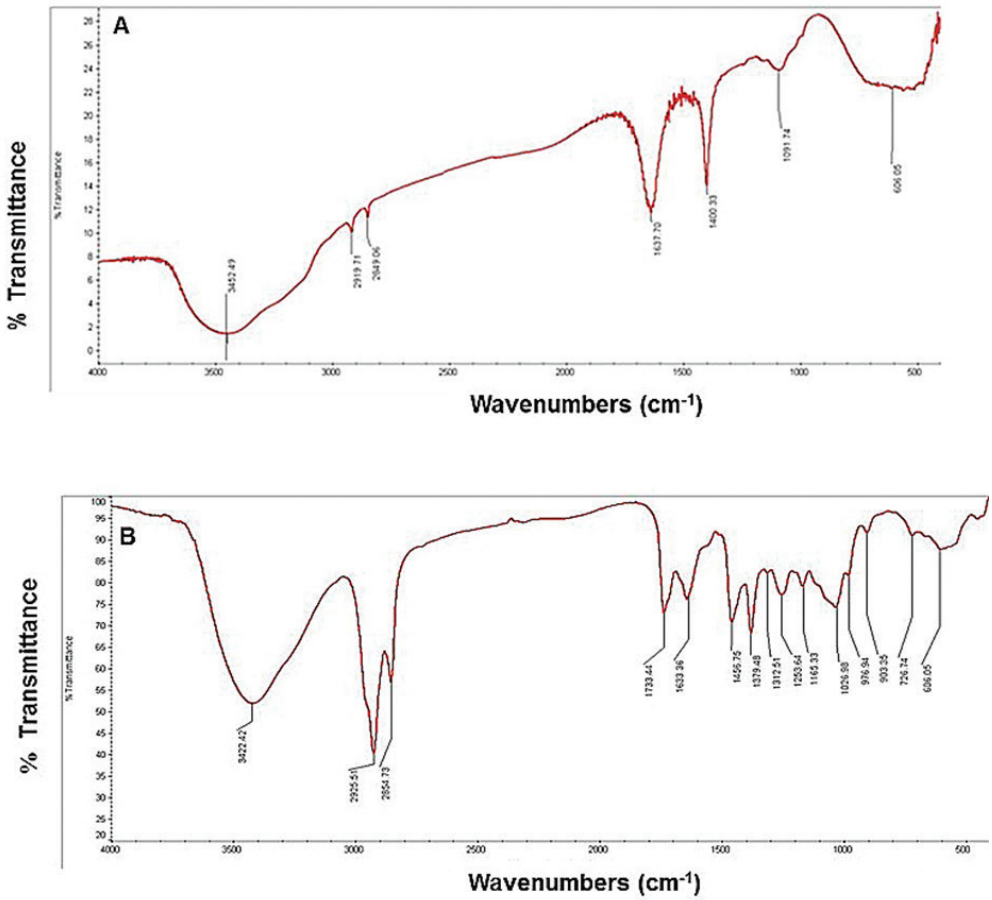

Figure 2. FTIR spectra of A. PALE and B. Biogenic synthesized PaAgNPs.

Table 1. Phytochemical screening of PALE and PaAgNPs

S.No	Phytochemicals	Screening	
		PALE	PaAgNPs
1.	Alkaloids	-	-
2.	Coumarins	+	+
3.	Anthraquinones	-	-
4.	Tannins	+	+
5.	Glycosides	-	-
6.	Flavonoids	-	-
7.	Saponins	+	+
8.	Steroids	+	+
9.	Terpenoids	+	-
(+) sign and (-) sign represent the presence and absence of phytochemicals, respectively			

at room temperature, which was confirmed by FTIR analysis. FTIR spectrum showed variations in the transmittance due to the interaction of nanoparticles with phytoconstituents (29). The IR spectrum confirmed that the stabilization of PaAgNPs was primarily due to the presence of betulinic acid. The IR spectrum of PALE (Figure 2A) showed peaks at 3452 cm^{-1} (Broad peak), 2919 cm^{-1}, 2849 cm^{-1}, which corresponds to the hydroxyl group of polyphenols and C-H stretching respectively, while peak at 1637 cm^{-1} represented C=O stretching (carbonyl) and peak at 1091 cm^{-1} showed C-O stretching (alcohols). The carboxylic acid group peak was reflected at 1400 cm^{-1} in the spectra. The comparative analysis of FTIR spectra of PALE with synthesized PaAgNPs showed sharp signals (intense peaks), which clearly indicated the presence of betulinic acid acting as a capping agent (Figure 2B).

FESEM of synthesized PaAgNPs showed it to be spherical in shape (Figure 3 A, B). However, the synthesized particles were polydisperse in nature when observed using HRTEM with SAED and the histogram of PaAgNPs indicated the particles to be 10 to 20 nm size with an average diameter of 13.85 ± 1.8 nm (Figure 4 A-C). The data obtained from EDX showed

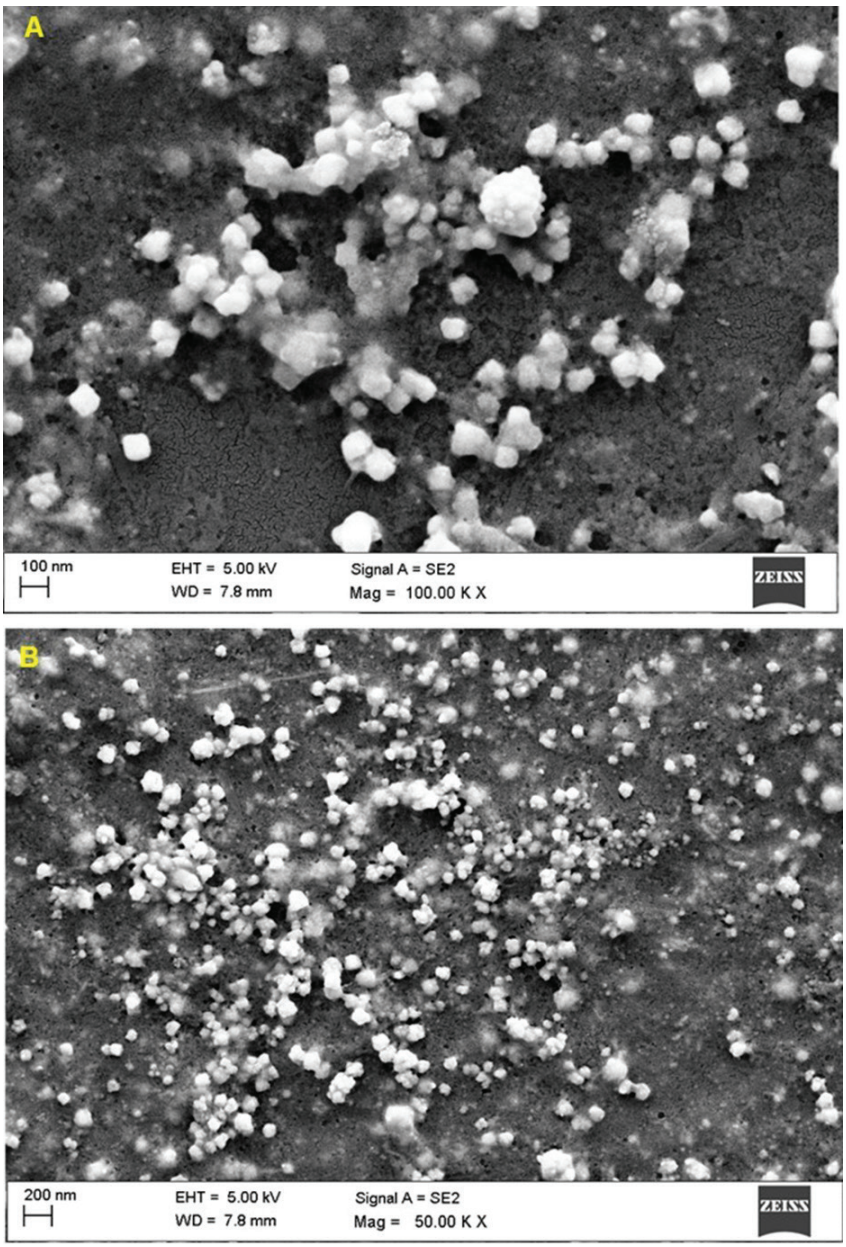

Figure 3. FESEM micrograph of PaAgNPs at different magnification (A) 100 KX and (B) 50 KX.

strong signals of silver and weak signals of oxygen, sulfur, carbon in synthesized PaAgNPs (Figure 4D). Crystalline nature of synthesized nanoparticles (PaAgNPs) was identified by X-ray diffraction studies. XRD spectrum showed three characteristic diffraction peaks at 2θ = 38.0^{o}(111), 44.1^{o}(200) and 64.0^{o}(220) which could be indexed to the crystallographic planes of pure silver (Figure 5). XRD diffraction patterns analyzed by PANalytical X'Pert High Score Plus software showed the synthesized PaAgNPs were cubic crystal structure which matched with the Joint Committee on Powder Diffraction standards; JCPDS no. 04-0783. Similar results were reported by Krishnaraj *et al*, 2010 while using the silver nanoparticles obtained from *Acalypha indica* leaf extracts (30). Zeta potential measurements showed the surface charge value to be -18.1 mV which indicated that the synthesized PaAgNPs were stable (Figure 6A). The size distribution of the PaAgNPs dispersion was measured by dynamic light scattering (DLS) and the results suggested that Z- Average value (d. nm) to be 104.5 (Figure 6B). Evidently, synthesized PaAgNPs particle size in DLS was higher than the HR-TEM and FE-SEM analysis which could be due to the hydrodynamic radius probed with DLS. These results

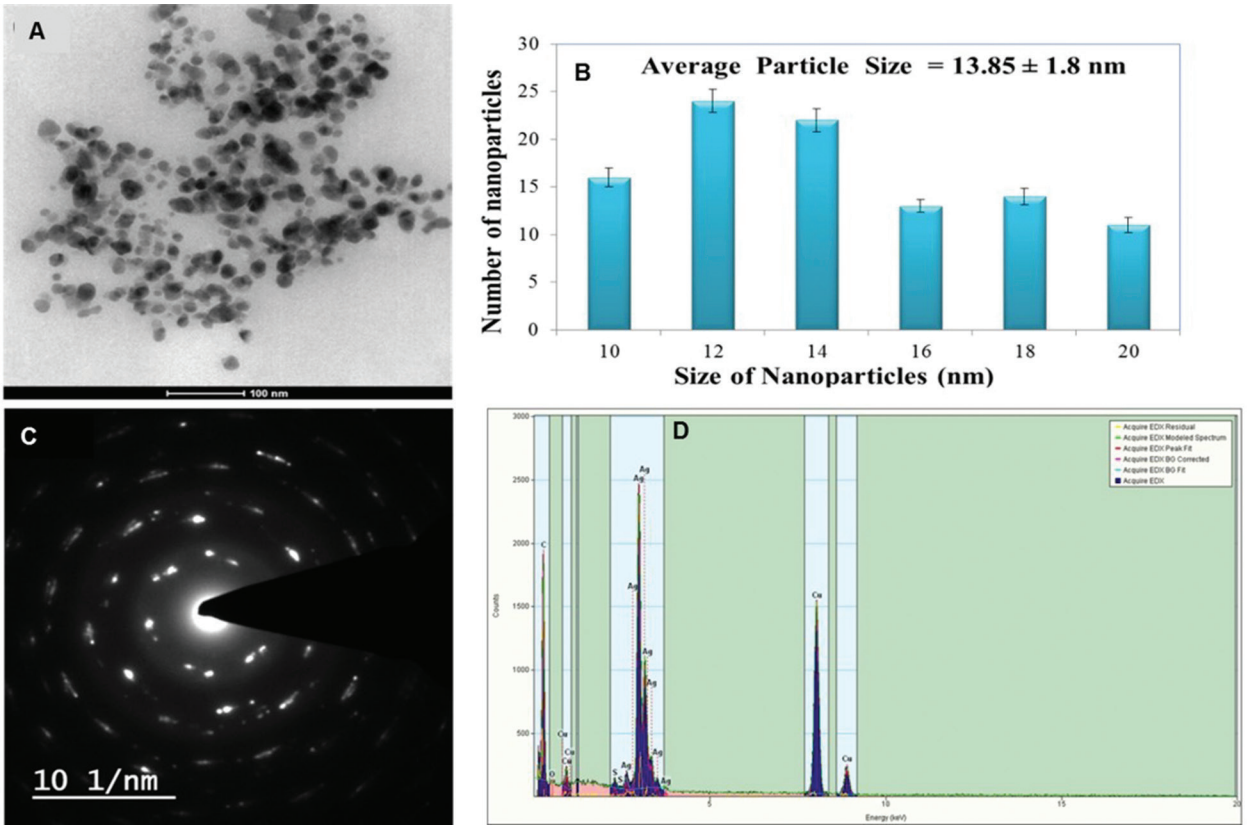

Figure 4. A. HRTEM micrograph of PaAgNPs, B. Diameter distribution histogram of 2mM biogenic PaAgNPs nanoparticles, C. SAED pattern indicated the polydispersed nature of synthesized PaAgNPs, D. Energy-dispersive spectroscopy (EDX) spectrum of PaAgNPs.

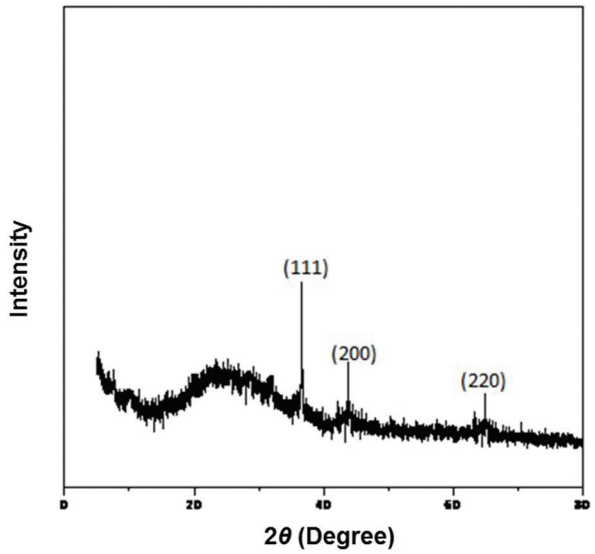

Figure 5. XRD pattern of the biogenic PaAgNPs.

are in agreement with biogenic synthesis of nanoparticles reported by other researchers (34).

The antioxidant potential assay using DPPH showed a maximum free radical scavenging action of synthesized PaAgNPs to be 69.52% as compared to 63.53% of PALE (Figure 7). Earlier, investigators stated that the selenium, platinum and silver nanoparticle can improve scavenging action of DPPH which accepts hydrogen or electrons from silver nanoparticles (35-37). The enhanced antioxidant activity of PaAgNPs could be attributed due to the functional groups and phytochemicals adhered to them which were originated from the PALE extract.

The acute inflammation studies done were demonstrated by the standard experimental model of Carrageenan-induced rat paw edema. The non-antigenic nature of Carrageenan, which is devoid of apparent systemic effects was preferred as phlogistic agent for testing anti-inflammatory drugs. Acute toxicity studies performed using biogenic synthesized PaAgNPs on rats indicated no mortality at a dose level of 2000 mg/kg. Data showed higher reduction in PaAgNPs (39.68%) as compared to PALE (20.13%) at 300 mg/kg concentration.

However, standard drug (Diclofenac sodium) reduced maximum paw edema by 47.61% (Figure 8).

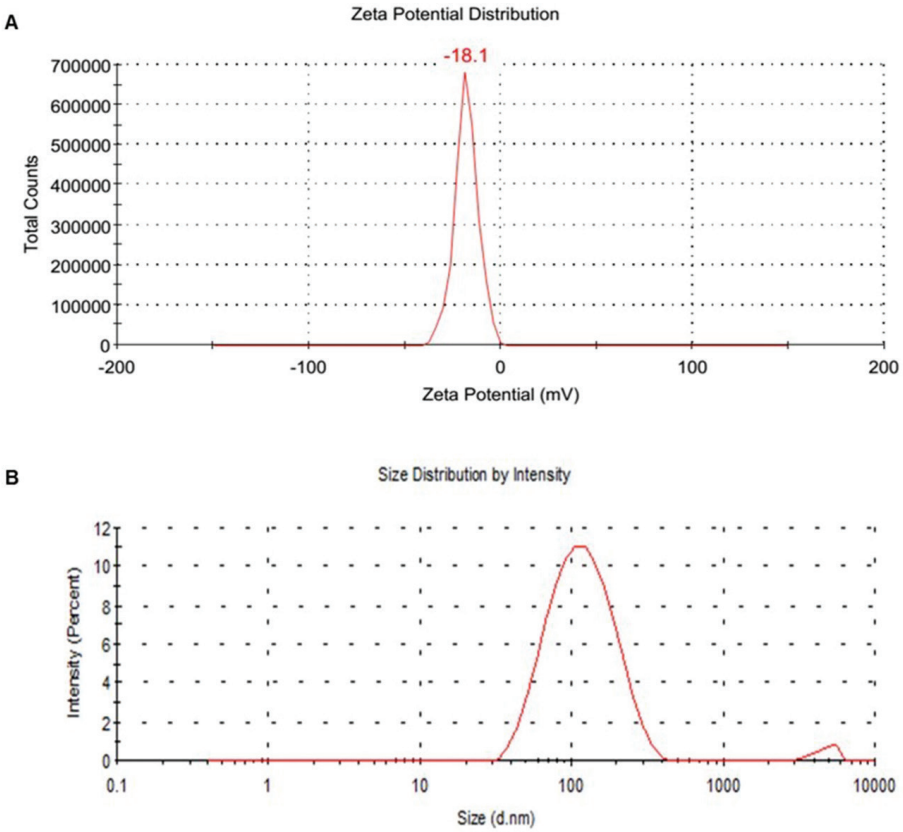

Figure 6. A. Zeta potential analysis of biogenic synthesized PaAgNPs, B. DLS size distribution pattern of PaAgNPs using PALE.

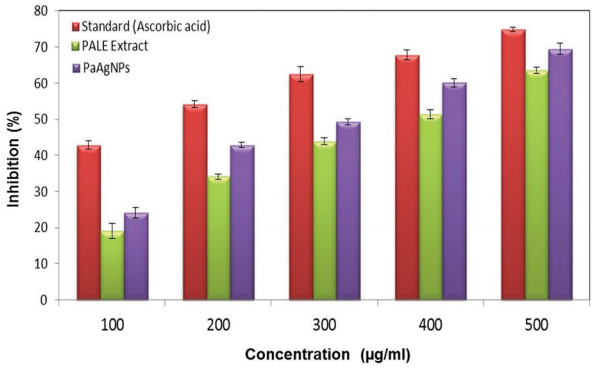

Figure 7. DPPH assay of PaAgNPs, PALE extract and ascorbic acid at different concentrations.

Data revealed a twofold enhanced anti-inflammatory potential of the synthesized PaAgNPs as compared to aqueous PALE extract. Furthermore, a high degree of reproducibility was exhibited by the experimental model (38). The non-responsiveness to inflammation of the available synthetic drugs in the market has given impetus to the development of effective ayurvedic nanoformulations. In this regard, biogenically PaAgNPs have emerged as potential candidates for wound healing, antimicrobial dressings and as anticancer agents (39).

5. CONCLUSION

Nontoxic, highly stable novel biogenic PaAgNPs were synthesized from aqueous PALE using green chemistry approach. Phytochemical analysis of PALE and PaAgNPs showed the presence of saponins, steroids, tannins, coumarins and terpenoids which play a vital role in the synthesis and stabilizing PaAgNPs. The biogenic synthesized PaAgNPs showed excellent antioxidant potential when tested using antiradical (DPPH) assay. Two fold increased anti-inflammatory activity of PaAgNPs was reported which showed a dose-dependent reduction in rats paw edema. Current investigation revealed the potential of synthesized PaAgNPs in the development of new herbal formulations with improved antioxidant, anti-inflammatory and anticancer properties, thereby contributing in opening up new avenues for exploring alternative nanomedicine.

6. DECLARATION OF CONFLICTING INTEREST

The authors declare that there is no conflict of interest.

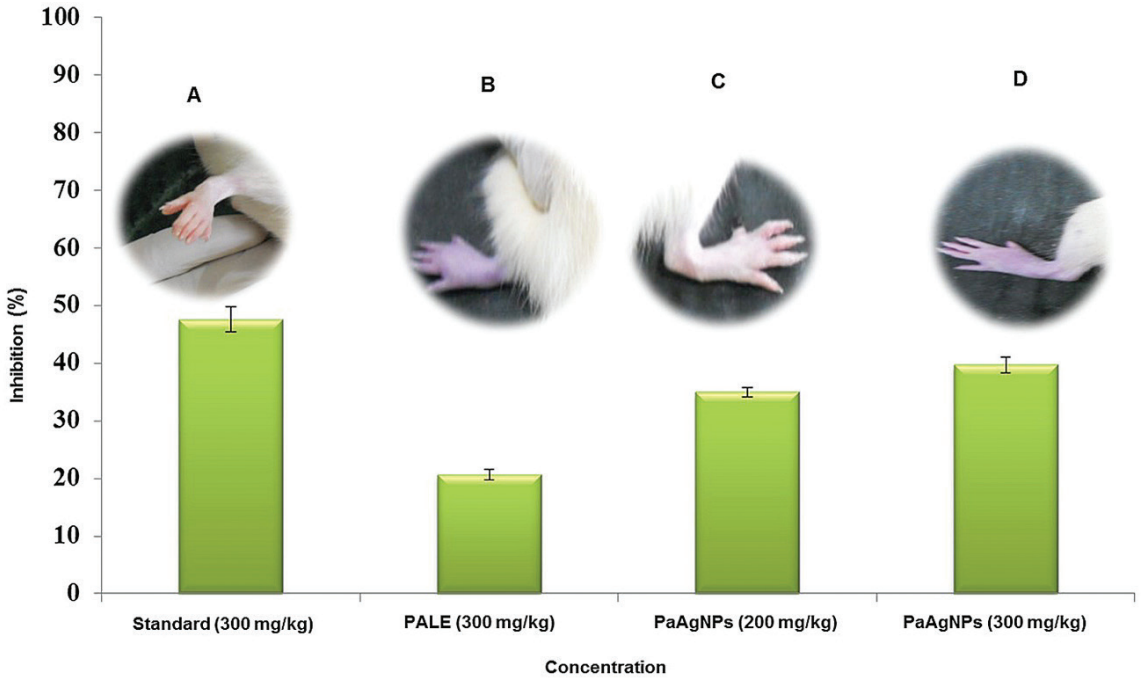

Figure 8. Inhibition (%) in rats paw edema A. Standard, B. PALE, C. PaAgNPs at 200mg/kg and D. PaAgNPs at 300mg/kg, (P<0.0.5) concentrations.

7. ACKNOWLEDGEMENTS

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