CARIPILL MEDIATED SYNTHESIS OF SILVER NANOPARTICLES FOR ANTIBACTERIAL CREAM AGAINST WOUND MICROBES.

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Abstract: Here, a single step approach to produce the silver nanoparticles (Ag NPs) has been developed. The nanoparticles were synthesized under heating conditions using caripill as reducing and capping agent. The Ag NPs were characterized using Fourier transform infrared spectroscopy (FTIR), Raman, Transmission electron microscopy (TEM), Scanning electron microscopy (SEM) and X-ray Diffraction Spectroscopy (XRD). The nanoparticles exhibited the size around 40-45 nm and their antimicrobial activity was tested against bacteria isolated from the animal wounds and the results indicated that the nanoparticles exhibited bacterial killing efficiency. The Ag NPs cream was made and their antimicrobial efficiency was successfully tested. Concluding, this report has provided new simple and efficient method for the design of drug capped Ag NPs.

Keywords: Nanoparticles; silver nanoparticles, nanosize; caripill; antimicrobial studies

Introduction: The nanoparticles based anti-microbial formulation offered more feature due to their size and wide range of anti-bactericidal activities against several bacteria [1-5]. The silver nanoparticles have been widely used as an antibacterial, antifungal, antiviral, anti-inflammatory and anti-cancerous agent. The silver nanoparticles release the free radicals; induced reactive oxygen species (ROS) and it causes oxidative injury inside the cells. The silver nanoparticles exhibit a wide range of antimicrobial activities. Recent years several methods were reported to produce the silver nanoparticles such as laser ablation, gamma irradiation, electron irradiation, chemical reduction, photochemical methods, microwave processing, green synthesis, and the biological methods [6-15]. The antibiotic encapsulated nanoparticles were reported by several research groups [16-20], but in this work we developed caripill drug mediated silver nanoparticles using single pot approach. The caripill plays a dual role such as reducing as well as capping agent. The advantage of using caripil and silver ions together is that if bacteria have resistance against one of the components, a further component could kill them in a different manner. The caripill drug was formulated using carica papaya leaf extract and it's contained riboflavin, thiamine, and ascorbic acid. For the synthesis, initially the silver nitrate solution was prepared and it was mixed with caripill tablets and the reduction process was accelerated by using heat. The ascorbic acid present in the drug act as a milder reducing agent to convert silver nitrate to silver nanoparticles and it was confirmed by taking the absorbance of the resultant colloidal solution. The physicochemical characterizations of the synthesized nanoparticles were confirmed by using SEM, TEM, EDX, XRD and FTIR. To study their antimicrobial properties the synthesized nanoparticles were incubated with microbial cultures and their antimicrobial efficacy was determined. The silver nanoparticles cream was made and their bacterial killing efficiency was tested in this work.

Experimental: Reagents: Nutrient Broth and Muller Hinton Agar were procured from Himedia, PEG(Polyethylene glycol) 3350, and PEG 400 were procured from Sigma, Glucose (Fischer Scientific), DMEM (Gibco), caripill (Micro labs) and Silver nitrate (MP Biomedicals).

Preparation of biological Silver Nanoparticle

For silver nanoparticles synthesis, 100 mL of 1 mM $AgNO_3$ was taken in a conical flask, mixed with 2 g of caripill and heated for 20 min at 70°C with stirring. After 20 min, the nanoparticles were collected by centrifugation at 8500 rpm for 15min.

The chemical composition of the nanoparticles and their crystalline structures were examined by using SEM- EDX (Quanta 200 FEG), FTIR (Perkin Elmer Spectrum1 FT-IR instrument), Raman spectra (Bruker RFS 27: Stand-alone FT-Raman Spectrometer), XRD (Bruker) at the Sophisticated Analytical Instrumentation Facility (SAIF), Indian Institute of Technology, Chennai using established methods.

Determination of the Anti- microbial property

For anti-microbial studies, two different bacterial isolates, one each belonging to *Escherichia coli* and *Staphylococcus spp,* isolated from dog wound surfaces and identified were used. Agar well Diffusion method was adopted to study the antibacterial activities. For this study 100 µL of 10^8 CFU/mL of *E.coli* and *Staphylococcus* were taken and it was spread plated on the Muller Hinton Agar plates. The wells were bored and filled with 50 µL nanoparticles of different concentrations (1~ 100 µg/mL). The plates were incubated at 37°C for 24 h. After overnight incubation, the zones of inhibition for *E.coli* and *Staphylococcus* were noted (all experiment were performed in triplicate).

Preparation and Formulation of silver nanoparticles cream: 2 g of Poly (ethylene glycol) 400, 8 g of Poly (ethylene glycol) 3350 was mixed in a 100 mL beaker and it was heated at 75ºC in oven for 1 h. And 1 g of dried Ag NPs was mixed with poly ethylene glycol polymers. The sample was mixed and it was poured it plastic jars.

Assessment of toxicity: All in vivo experiments were conducted according to the guidelines of Ethical Committee of the TANUVAS, IAEC approval No.2172/DFBS/2013 at Chennai. The study was performed following Using Balb/c mice under.(5 male and 5 female, 12 to 14 weeks old, with healthy intact skin and acclimatized to laboratory conditions for 7 days) were used. Approximately 24 h before application, the hair of each mouse was closely clipped with an electric clipper to expose the back from the scapular to the lumbar region. Ag NPs cream (0.1 mg/g) was applied uniformly at the dose level of 2000 mg/kg and held in contact with the skin with a porous gauze patch. After the exposure period of 24 h, observations on mortality, intoxication, body weight, and necropsy were recorded for a 14 day period.

Results and discussion: In this report we produced the Ag NPs using caripill as a reducing agent. The caripill contained ascorbic acid, and other water soluble vitamins. The ascorbic acid mainly acts as mild reducing agents against $AgNO_3$. During synthesis, the reaction medium showed acidic pH and it induce the Ag NPs formation from the silver nitrate solution without using any stabilizing agents and it may be due to presence of other traces minerals and vitamins. The ascorbic acid reducing ability was increases with an increasing reaction temperature. The colour of the solution was changes from colourless to brownish, within 20 min at 80°C. The formed silver nanoparticles were confirmed by using spectrophotometry and it showed the UV bands at 460 nm. Initially we tried to synthesis the Ag nanoparticles at room temperature, but the UV-Vis experiments indicated that the Ag^+ ions reduction needed long time at room temperature and forms the particles after 24 h. So the effect of the reaction temperature was investigated by adjusting the temperature ranges from 50 to 90°C. The SEM results indicated that, 50°C forms the nanoparticle size ranges from 18~20 nm within 60 min and the UV bands showed the peaks at 475 nm. The 60 °C forms the particle size range from 30 ~35 nm within 30 min and the UV bands showed the peaks at 460 nm. The 70 °C showed the uniform size range from38~40 nm within 20 min. the UV bands showed the peak at 460 nm. In higher temperatures (more than 70°C), the particle formation is too quick and the size also bigger (more than 100 nm) so the higher temperature over 70°C was not considered in this work (Figure 1).

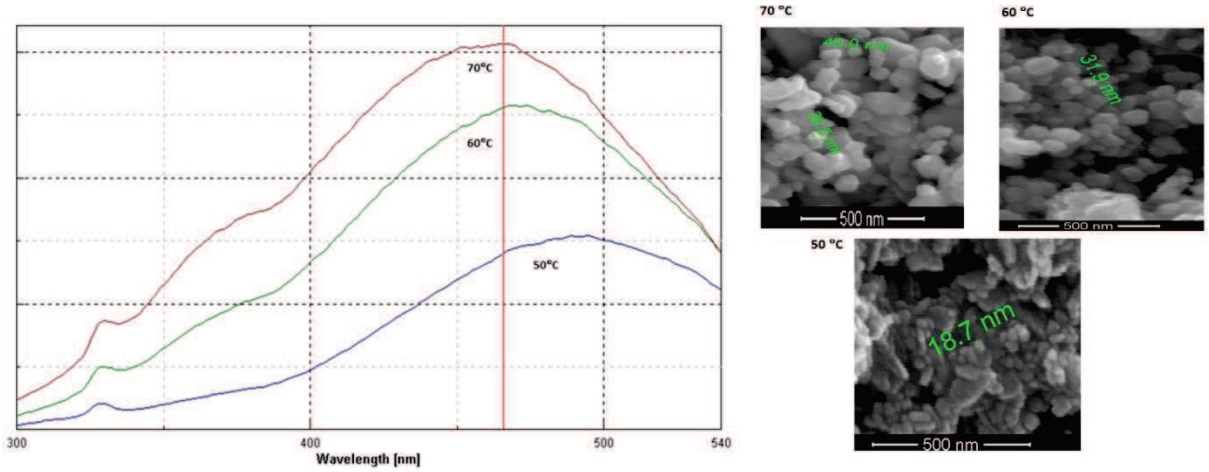

Figure 1. The size measurement and absorption spectra results of the silver nanoparticles synthesized at 50~70 °C.

In this study, we fixed 70 °C as an optimum temperature, other experimental parameters, such as the concentration of the silver ions remain constant. To minimize the volume effect of the reactants, the total volume of the proposed system was basically kept at 100 mL by adjusting the concentration of reducing agent. The nanoparticles morphology was studied by using SEM and TEM. These images confirmed that the synthesized particles are spherical and their sizes were around 40 ± 2 nm (Figure 2)

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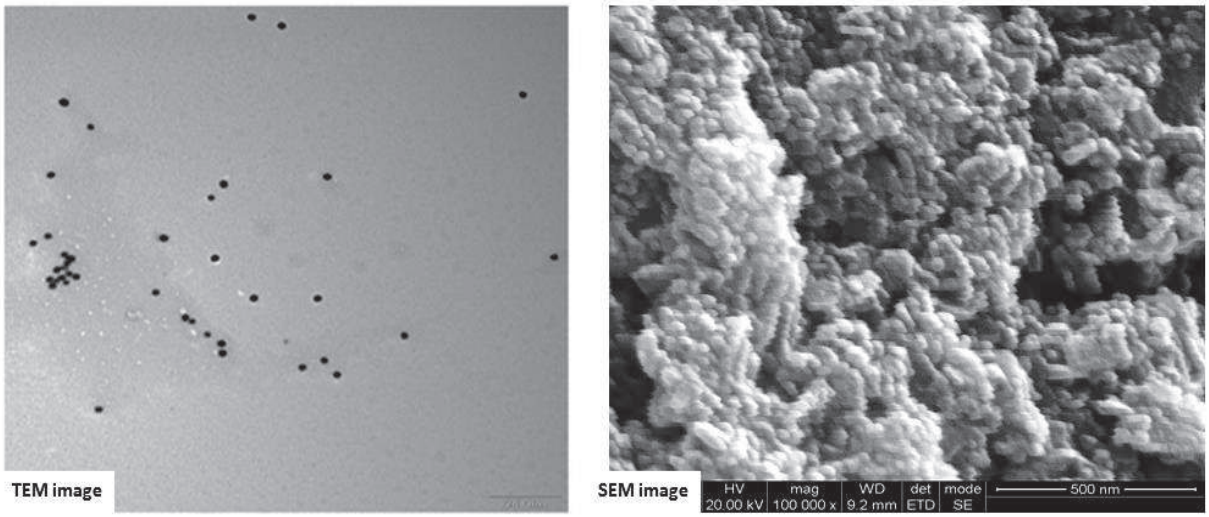
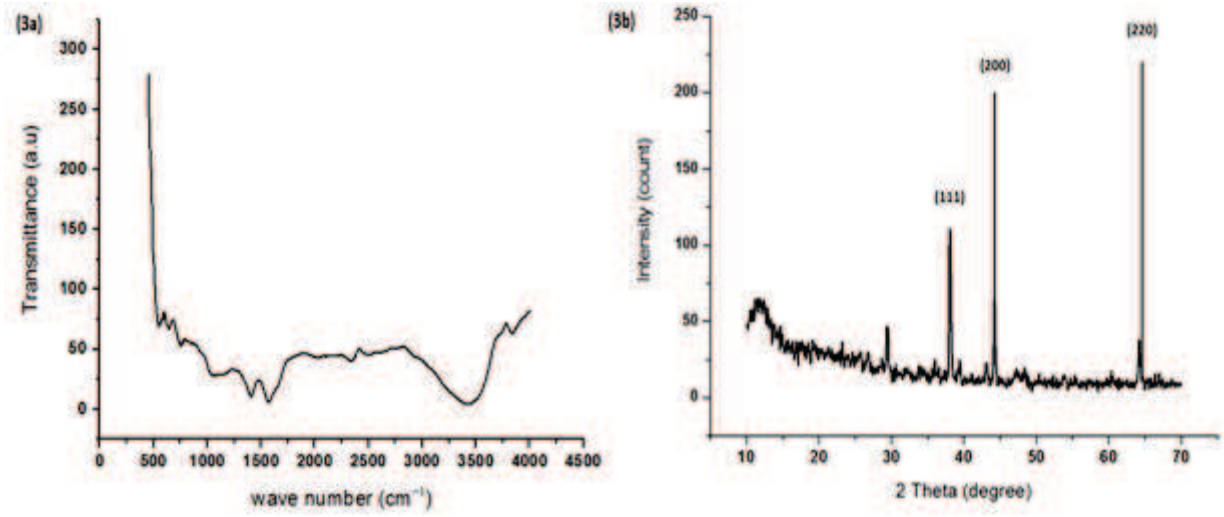


Figure 2. The size measurement results of silver NPs by Transmission electron microscope (TEM) and Scanning electron microscope (SEM).

The elemental compositions, crystal structure and the functional group of the silver nanoparticles were confirmed by using EDX, XRD and FTIR spectra. The silver nanoparticles showed peaks at 3430 cm^{-1}(Strong stretching from $AgNO_3$), 2924 cm^{-1}(OH plane), 1066 and 1630 cm^{-1} which represent amide linkages groups, the spectral band at 501 and 654 cm^{-1} due to the presence of Ag ions,1351 cm^{-1} due to (NO_2) in FTIR. (Figure.3a). The XRD pattern of results of Ag NPs showed the diffraction peaks at $2\theta = 38.08°$, 44.49°, and 64.20° assigned to the (111),(200) and (220) planes. The diffraction pattern highly matched the database of Joint Committee on Powder Diffraction Standards (JCPDS) file No. 04-0783. The average grain size of the silver nanoparticles formed in the reduction process was determined using Debye–Scherrer formula, $D = K\lambda/\beta\cos\theta$ where 'D' is particle diameter size, K is a constant equal 0.94, 'λ' is wavelength of X-ray source (0.1541 nm), 'β' is the full width half maximum (FWHM) and 'θ' is the diffraction angle corresponds to lattice plane (111). For the particle size calculations, K=0.94, λ=0.154 nm, β is calculated as 0.0039 radians (β= B1 (observed)-B2 (resolution) × 3.14/180), 2θ = 64.24 so the θ = 32.12. The D was calculated as D=0.94 × 0.154/0.0039 × cos 32.12, so D = 45.02. The D value of 2θ = 38.08 was calculated as 37.35 nm. The mean size of silver nanoparticles with respect to (111) of Bragg's reflection gave the size is about 37.35 nm (Figure 3b). The EDX detector indicated that the prepared nanoparticles contains major amount of Ag and trace amount of O, Cu and C is present. The carbon and the copper peaks correspond to the TEM holding grid. Throughout the scanning range of binding energies, no obvious peak belong to impurity is detected. The result indicates that the synthesized product is composed of high purity Ag (Figure 3c).



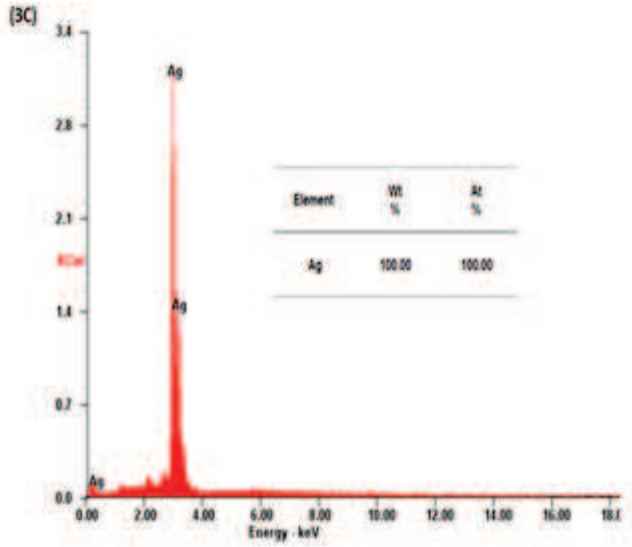


Figure 3. Physico chemical characterization silver nanoparticles

(3a) FTIR spectra analysis of synthesized Ag nanoparticles: The analysis was carried out using potassium bromide back ground, for the analysis the samples were pelleted with potassium bromide.
(3b). XRD pattern of Ag nanoparticles.
(3c). the elemental analysis results of synthesized silver nanoparticles and it was carried out in EDX

To study the antimicrobial efficacy, the *E.Coli* was used and it was incubated with different concentration of silver nanoparticles in agar medium. After 24 h, the zone of inhibition was noted and the results indicated that the inhibition purely depended upon the AgNP concentration (Figure 4) and 100% killing efficacy was recorded after~20µg/mL for *E.Coli*. We also study the antimicrobial efficacy (%) against *Staphylococcus sp*. and the results indicated that the nanoparticles also showed antibacterial activity against *Staphylococcus sp.*

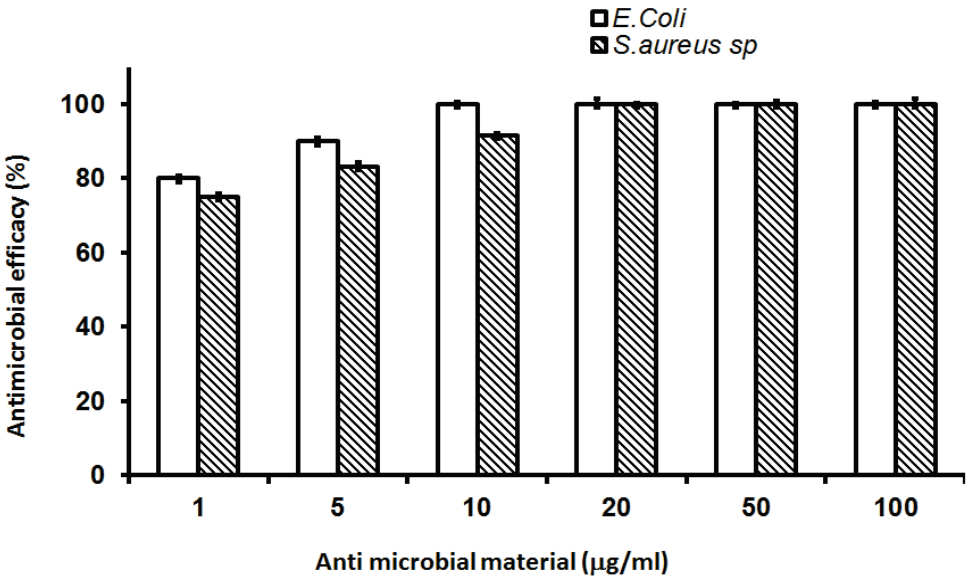
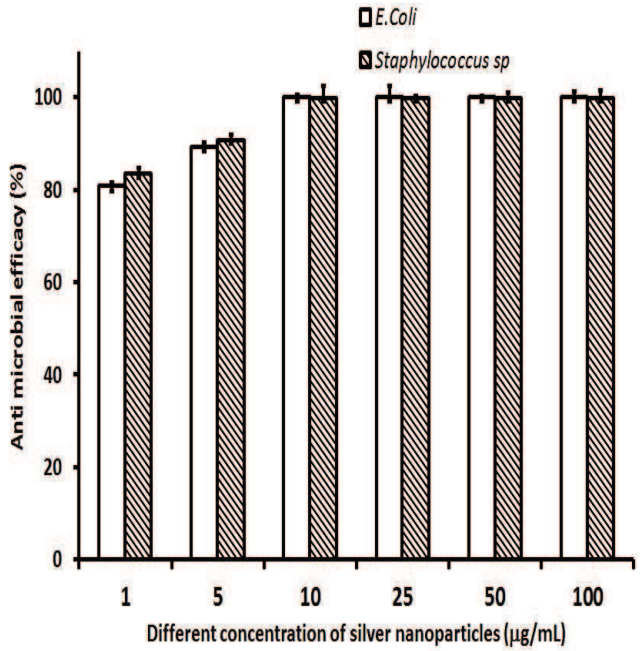


Figure 4.

Antimicrobial activity determined through the agar well diffusion method for the different concentrations of Ag nanoparticles against (A) *E.coli* (B) *Staphylococcus sp.* The results were expressed as the mean percentage ± SD of three independent experiments each performed in triplicate.

Initially we formulated cream with different concentration of Ag NPs (1 ~100 µg/mL) and PEG base cream and their antimicrobial studies indicated

20 µg/mL of Ag concentration is enough to prepare the cream (Figure 5). The antimicrobial study of the Ag nanoparticles cream was compared with free Ag nanoparticles. For this study, 500 mg of cream contained 10 µg of silver nanoparticles was loaded onto whatman filter paper 42 and it was cut into circular shape (0.3 × 0.3 cm). For the comparison, the same quantity of the silver nanoparticles in solution was loaded into the filter paper and their anti-microbial effect was studied using *E.coli* as a model organism.



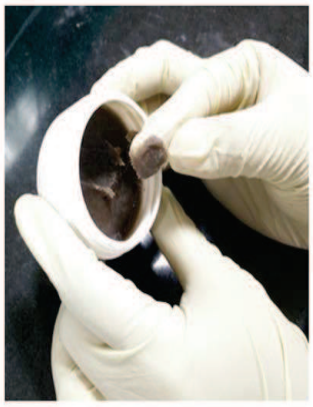


Figure 5.

Comparison of the antimicrobial activity of Ag NPs cream prepared with different concentration of Ag nanoparticles and the images of the cream.

that the PEG based white ointment base cream helps in the dispersion of the nanoparticles in the medium. The toxicity studies were performed in order to determine the safety of the Ag cream. An in vivo experiment on the determination of LD50 for the cream was performed in mice. The acute dermal LD50 value of mice, both male and female, was found gained body weight. No mortality or signs of intoxication or any adverse skin reactions were observed in the treated male and female mice during the observation period of 14 days following application. Gain in body weight of treated animals was normal in the first and second weeks after treatment. At termination of the study, animals were sacrificed and necropsy was performed on all animals. Also, no pathological abnormalities were seen in any of the organs of sacrificed animals.

The results indicated that, the Ag cream showed the inhibition zone (mm) values around 10 ± 0.07 mm, but the free Ag nanoparticles showed the values around 8.1 ± 0.15 mm. These observations suggest

Conclusion: In conclusion, we have described a new preparation method for Ag NPs synthesis using caripill tablets. The synthesized nanoparticles were successfully characterized and their antimicrobial activity was tested against wound microbes. The anti-microbial cream was made with the nanoparticles and it was tested against wound microbes. The *in vivo* studies indicated that the cream is safe. In conclusion, the method reported here is very easy, cost effective, and highly suitable for bactericidal applications.

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