

Green synthesis of silver nanoparticles using *Arnebia nobilis* root extract and wound healing potential of its hydrogel

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The present study reports wound healing potential of silver nanoparticles (AgNPs) hydrogel using *Arnebia nobilis* (*A. nobilis*) root extract. It makes a convenient method for the green synthesis of AgNPs and evaluated for its wound healing activity. Silver has been used for the treatment of medical ailments for over 100 years due to its natural antibacterial and antifungal properties. The synthesized nanoparticles were characterized using UV-visible spectrophotometer; transmission electron microscopy, X-ray diffraction (XRD), scanning electron microscopy, and Fourier transform infra-red spectrometry. The nanoparticles were found to be mostly spherical in shape. XRD study shows that the particles are crystalline in nature with face centered cubic geometry. The synthesized AgNPs exhibited good antibacterial potential against both Gram-positive and Gram-negative bacterial strain as measured using well diffusion assay. The recent emergence of nanotechnology has provided a new therapeutic modality in AgNPs for use in wounds. We investigated the wound-healing potential of AgNPs hydrogel using *A. nobilis* root extract in an excision animal model. The study showed that hydrogel of AgNPs using *A. nobilis* root extract exert positive effect due to their antimicrobial potential. The results provide insight into the mechanism of actions of AgNPs and have provided a novel therapeutic direction for wound treatment in the clinical practice.

Key words: Antibacterial potential, *Arnebia nobilis*, Fourier transform infra-red, silver nanoparticles, wound healing, X-ray diffraction

INTRODUCTION

Plant extracts utilization for the formation of nanoparticles have gained tremendous importance in the past 2 decades due to the enhancement of chemical, physical, and biological properties of the particles formed by this green process. Nanotechnology has dynamically developed as an important field of modern research with potential effects in electronic and medicine.^[1-3] Silver nanoparticles (AgNPs) have proven useful in antibacterial clothing, burn ointments and as coating for medical devices because of their mutation-resistant antimicrobial activity.^[4] In recent years, the biosynthetic method using plant extracts has received more attention than chemical and physical methods, and even more than the use of microbes, for the nanoscale metal synthesis due to the absence of any requirement to maintain an aseptic environment. Synthesis of nanoscale metals using plant materials

has been reported earlier.^[5,6] Plant leaf extract of onion,^[7] *Syzygium cumini*,^[8] basil,^[9] *Saraca indica*,^[10-12] and *Piper nigrum*^[13] had been used for the synthesis of gold and AgNPs, which lead to formation of pure metallic nanoparticles of silver and gold and can be used directly. The chemical methods are extremely expensive and use toxic chemicals, which may pose potential environmental and biological risks.

It has been known for a long time that silver compounds are very effective antibacterial agents against both aerobic and anaerobic bacteria. The use of silver in nanoparticle form (when compared to its ionic form) seems to have reduced cellular toxicity, but not antibacterial efficacy. It has been demonstrated clearly that the superior antibacterial properties of AgNPs, are due to the formation of free radicals from the

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DOI:
10.4103/0973-8398.134925

surface of Ag.^[14] AgNPs acted against those bacteria, which were found to be antibiotic resistant.^[15,16] Furthermore, the addition of antibiotics to AgNPs has been shown to have synergistic effects against microorganisms.^[17-20] It has been demonstrated that modification of silver sulfadiazine using dendrimers increased the anti-bacterial efficacy.^[21] Apart from being an excellent anti-bacterial agent, AgNPs appears to have anti-inflammatory properties as well. The effect of AgNPs using a porcine model of contact dermatitis was explored. Here, it was confirmed that AgNPs had direct anti-inflammatory effects and improved the healing process significantly when compared with controls.^[22] Addition of AgNPs reduced the production of proinflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α) and interferon-gamma (IFN- γ), although the intracellular pathways involved still remains largely not elucidated.

The use of silver in the past has been restrained by the need to produce silver as a compound, thereby increasing the potential side effects. Nanotechnology has provided a way of reducing pure AgNPs. The ultimate goal for wound healing is a speedy recovery with minimal scarring. Wound healing proceeds through an overlapping pattern of events including coagulation, inflammation, proliferation, matrix, and tissue remodeling. The availability of AgNPs has ensured a rapid adoption in medical practice. Their application can be broadly divided into diagnostic and therapeutic uses. In terms of therapeutics, one of the most well-documented and commonly used applications of AgNPs is in wound healing. Compared with other silver compounds, many studies have demonstrated the superior efficacy of AgNPs in time required for healing. It was shown that in wounds treated with AgNPs, there was better collagen alignment after healing when compared to controls, which resulted in better mechanical strength, although the exact mechanisms for these biological effects have not yet been elucidated.^[23]

Ratanjot (*Arnebia nobilis* [*A. nobilis*] Rech.f) has been used to extract dye for its application on various textile substrates such as cotton, wool, silk, nylon, polyester, and acrylic. The dyed polyester shows pink color, nylon shows blue and all other substrates acquire a purple hue under similar dyeing conditions. The dye shows excellent wash, rub and perspiration fastness; however, light fastness is found to be poor.^[24] The roots of *A. nobilis* have traditionally been used as a colorant in food and cosmetic preparations. The deep red color obtained is attributed to the presence of shikonin and its isomer alkannin and their derivatives. The major component is alkannin β,β -dimethylacrylate [5,8-dihydroxy-2- (1'- β,β -dimethylacryloxy-4'-methylpent-3'-enyl)-1,4-naphthoquinone], accounting for nearly 25% of the total coloring matter. Alkannin acetate [2-(1'-acetoxymethylpent-3'-enyl)-5,8-dihydroxy-1,4-naphthoquinone] made up ca. 8% and shikonin [(5,8-dihydroxy-4'-methylpent-3'-enyl)-1,4-naphthoquinone] contributed ca. 6% of the coloring matter.^[25]

In this article, we have reported the microwave assisted rapid green synthesis of stable AgNPs using *A. nobilis* root extract, antibacterial potential and effect on wound healing and reduction in scar appearance through excision rat model. The antibacterial potential of the synthesized AgNPs was assessed against both Gram classes of bacteria. The potential benefits of AgNPs in all wounds can therefore be enormous.

EXPERIMENTAL

Synthesis of silver nanoparticles using aqueous extract of *Arnebia nobilis* roots

Arnebia nobilis roots were purchased from the local market and washed with deionized water to remove any impurities, dried in dark to completely remove the moisture and powdered in a mixer grinder and then sieved using a 20-mesh sieve to get uniform size range. The final sieved powder was used for all further studies. For the production of extract, 5 g powder was added to a 500 ml Erlenmeyer flask with 100 ml sterile distilled water and then exposed to microwave for 3 min. Then the raw extract obtained was filtered in hot condition with Whatman filter paper to remove fibrous impurities. The resultant clear extract was used for the synthesis of AgNPs. For the reduction of Ag⁺ ions, 20 ml aqueous *A. nobilis* root extract was added to 100 ml of 10⁻³ M aqueous solution of AgNO₃ (Qualigens, India) and the solution mixture was exposed to microwave radiation at a fixed frequency of 2450 MHz and power of 450 W. Periodically, aliquots of the reaction solution were removed and subjected to UV-visible (UV-vis) spectroscopy measurements. The half portion of colloidal solution thus obtained was immediately used for preparation of hydrogel. The remaining colloidal solution was centrifuged at 8000 rpm for 10 min and subsequently re-dispersed in deionized water twice to get rid of any unbound biological molecules.

Preparation of hydroxypropyl methylcellulose hydrogel

The hydrogels were prepared by cold suspension method. Hydroxypropyl methylcellulose (Methocel K4) (Colorcon, India) 3% w/v was slowly dispersed in colloidal solution of AgNPs as prepared in section "Synthesis of AgNPs aqueous extract of *Arnebia nobilis* roots" with continuous stirring until gel was formed. It was kept overnight to swell, then filled in tubes and stored at 4°C until further use, 0.2% w/v Methyl paraben (Qualigens, India) was used as preservative.

Characterization

The progress of AgNPs formation was monitored using UV-vis spectra by employing a UV-vis Shimadzu double beam spectrophotometer 1800, Columbia, USA operated at a resolution of 1 nm with optical path length of 10 mm. Optical density was measured by diluting the colloidal solution 2 times using deionized water.

Scanning electron microscopy (SEM) was performed on gold-platinum coated samples that were previously air dried

on silicon wafers and analysis was done using an analytical scanning electron microscope (SEM ZEISS EVO®HD, Germany).

The Fourier transform infra-red (FTIR) spectrum was recorded using Bruker-FTIR Alpha Instrument, Germany at a resolution of 4/cm.

For X-ray diffraction (XRD) measurements, the synthesized nanoparticles were centrifuged at 8000 rpm for 10 min and subsequently redispersed in deionized water twice to get rid of any unbound biological molecules that were not responsible for bio-functionalization or capping. The pure residue was dried perfectly in an oven overnight at 60°C.

Powder was used for crystallinity study using Bruker X-ray diffractometer (D-2 Phase) with Cu K(α) radiation, Germany.

Analysis of antibacterial activity

Agar well diffusion method was used to evaluate the bactericidal activity of silver nano-colloid solution. Sterile nutrient agar medium was poured into sterile petri plates and allowed to solidify. The petri plates were incubated at 37°C for 24 h to check for sterility. The medium was seeded with the organism culture (1 ml) by pour plate method. Bores were made on the medium using sterile borer. 10, 20, 40, and 80 μ g/ml of AgNPs was added to the respective bores. The petri plates were kept in refrigerator at 4°C for 30 min for diffusion. After diffusion the petri plates were incubated at 37°C for 24 h and zone of inhibition were observed and measured.

Animal experiment

Animals

Albino rats (Wistar) weighing 150-200 g of either sex were used for the study. They were procured from the animal house of NIET, Greater Noida. The animals were acclimatized for 1 week under laboratory conditions. They were housed in polypropylene cages and maintained at $27 \pm 2^\circ\text{C}$ < 12 h dark/light cycles. They were fed with standard rat feed and water *ad libitum* was provided. The litter in the cages was renewed thrice a week to ensure hygienicity and maximum comfort for animals. Ethical clearance for handling the animals was obtained from the Institutional Animals Ethical Committee prior to the beginning of the project work bearing the protocol number 1121/ac/CPCSEA/07/NIET/IAEC.

Method

Excision wound model

The experimental animals were grouped into three groups containing six animals each and treated as follows:

- Group I: Control-Received no treatment
- Group II: Standard-Received standard marketed silver sulfadiazine cream
- Group III: Composition (test)-Received test composition, which is synthesized AgNPs using aqueous extract of *A. nobilis* root.

A circular wound of about 500 mm² full thickness of a predetermined area was made on the depilated back of the rat. The preparation was topically applied once a day, starting from the day of wound made, until complete epithelialization. The parameters studied were wound closure and epithelialization time. The wound were traced on mm² graph paper on days 3, 6, 9, 12, 15, and 18 and thereafter on alternate days until healing was complete. The percentage of wound closure was calculated. The period of epithelialization was calculated as the number of days required for falling of the dead tissue remnants of the wound without any residual raw wound.

RESULTS AND DISCUSSION

The color change of the colloidal solution into reddish brown indicated the formation of AgNPs. The intensity of the color of reaction mixture increased evenly with time of microwave exposure, thus confirming the formation of AgNPs. It was due to the excitation of the surface Plasmon vibration in metal nanoparticles. The reduction of pure Ag⁺ ions to Ag⁰ was monitored by measuring UV-vis spectrum of the reaction media at regular intervals. The metal ions reduction occurred very rapidly and reduction of most of the Ag⁺ ions was completed in 225 s. Absorption spectra of AgNPs formed in the reaction mixture at different wavelength (nm), i.e., 250-550 nm was recorded. The particles showed sharp absorption maximum peak at 410 nm which gradually decreased as the nanometer increased [Figure 1a].

Figure 1b shows the change in absorbance with time on exposure of colloidal solution of AgNPs to microwave. The absorbance gradually increased with time up to 225 s of exposure after which a fall in absorbance was observed. Absorbance intensity increases steadily as a function of reaction time and it was observed that the surface Plasmon peak occurs at 420 nm with a slight shift in the vertex of the peak toward shorter wavelength (blue shift) and fixed at 405 nm,^[12] but in the present study we observed a decrease in absorbance after prolonged exposure to microwave. The probable reason for the same could be attributed to the aggregation of particles.

Figure 2 shows the XRD patterns of AgNPs synthesized from root extract of *A. nobilis*. A number of Bragg's reflection with 2θ values of 38.30, 44.50, 64.60, and 77.50 corresponding to 111, 200, 220, and 311 set of lattice planes are observed (JCPDS, silver file no. 04-0783). The XRD analysis showed diffraction peaks corresponding to fcc structure and crystallinity of AgNPs.

The particle size and shape is confirmed with SEM. The particles are almost in spherical shape with diameters in the range of 40-70 nm and are well dispersed [Figure 3]. Particles may be aggregated after prolonged exposure to microwaves. This aggregation of particles may be the principle reason to the fall in absorbance.

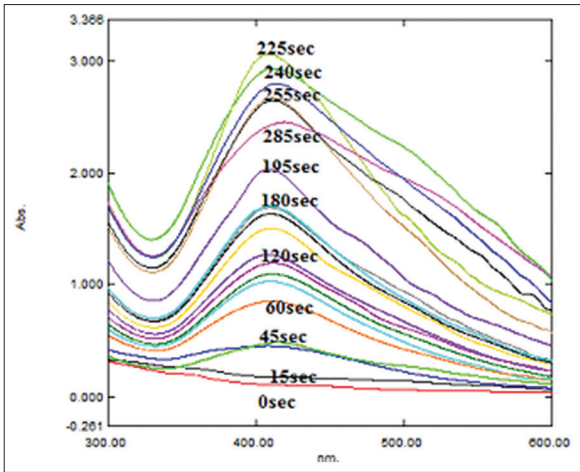


Figure 1a: UV-visible spectra of bio-synthesized silver nanoparticles

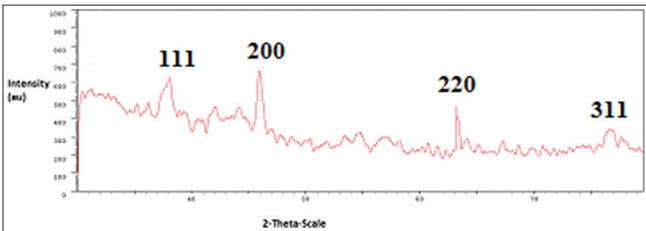


Figure 2: X-ray diffraction spectrum of bio-synthesized silver nanoparticles

It is observed that colloidal AgNP solution is extremely stable for more than 4 months. It has been reported that proteins can bind AgNPs through free amine groups and provide a good protecting environment for metal hydrosols during the growth processes.^[26,27] Figure 4 shows some pronounced absorbance bands at around 3400-3450/cm (N-H stretching), and 1620-1650/cm (aromatic rings), suggest the presence of proteins on the surface of Ag-core particles. The peak around 3400-3450/cm of plant proteins in the nanoparticles shell is much narrower. This can be explained as H-bonds can be formed between the amide groups. As plant molecules get adsorbed onto the AgNPs surface, the amide groups intend to form stronger bonds with Ag atoms, which will break most of the H-bonds between the N-H groups and lead to the narrowing and blue-shifts of the amide bond. It can be concluded that the *A. nobilis* proteins have adsorbed as a layer over the AgNPs, which in turn stabilizes the synthesized nanoparticles.

Antibacterial activity

Nano-silver is active toward Gram-positive as well as Gram-negative bacterial strain. In our experiment, a good zone of inhibition was observed for Gram-negative (*Escherichia coli* [*E. coli*]) bacterial strains than Gram-positive (*Staphylococcus aureus* [*S. aureus*]) bacterial strains. The zone of inhibition increased with increasing concentration of AgNPs [Table 1]. From the data, it is inferred that the synthesized AgNPs were effective for both type of

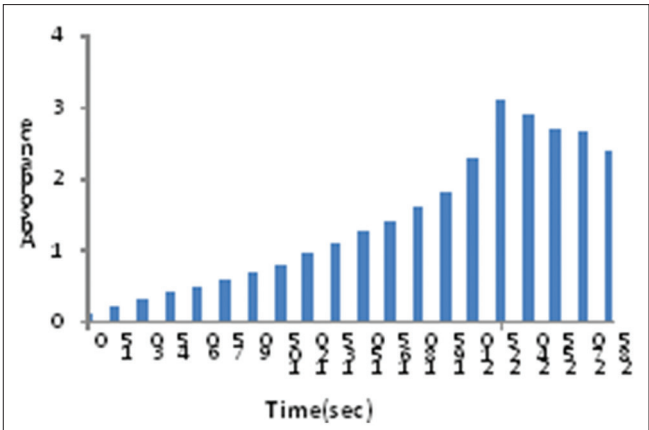


Figure 1b: Histogram depicting absorbance with respect to time(s)

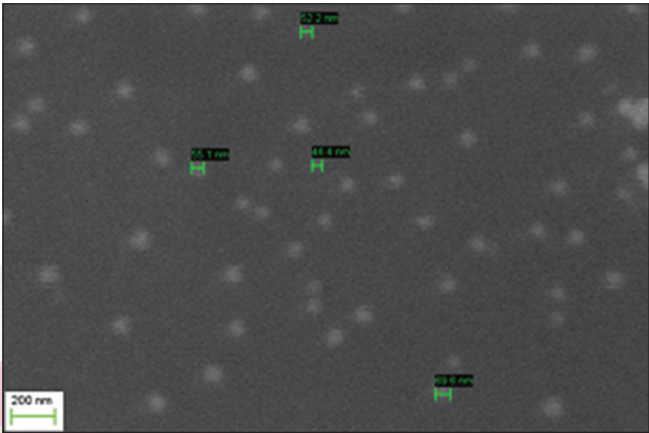


Figure 3: Scanning electron microscopy image of bio-synthesized silver nanoparticles after microwave exposure

Table 1: Antimicrobial activity of silver nanoparticles against *Escherichia coli* and *Staphylococcus aureus*

Concentration (µg/ml)	Average zone of inhibition (mm)	
	<i>E. coli</i>	<i>S. aureus</i>
10	16	15
20	17	16
40	18	17
80	20	19

microbes. Moreover, the zone of inhibition observed around Gram-negative bacterial strains is found to be reproducibly of the bigger size than the Gram-positive bacterial strains. We have noticed that AgNPs synthesized using *A. nobilis* root extract produce sensitivities toward both *E. coli* and *S. aureus*.

Wound healing activity

The results of the present investigations revealed that the hydrogel of AgNPs using *A. nobilis* possess significant wound healing activity in excision wound models. In spite of tremendous development in the field of synthetic drugs during recent era, they are found to have some or other side-effects, whereas plants still hold their own unique

place, by the way of having no side-effects. Therefore, a systematic approach should be made to find out the efficacy of plants against wounds so as to exploit them as herbal wound healing agents. Experimental assessment of the wound healing activity of AgNPs showed an increased rate of wound contraction and epithelialization in treated animals [Figure 5, 6a and b]. Topical application of the AgNPs on excision wounds accelerated wound contraction and reduced epithelialization period in rats. Wound healing involves regeneration of specialized cells by the proliferation of surviving cells and connective tissue response characterized by the formation of granulation tissue.^[28] It is also characterized by hemostasis, reepithelialization and remodeling of the extracellular matrix. Epithelialization, which is the process of epithelial renewal after injury, involves the proliferation and migration of epithelial cells toward the center of the wound, while wound contraction is largely due to the action of myofibroblasts.^[29,30] Thus, the effect of ethanolic extract and the acetone fraction on wound contraction and epithelialization suggest it may enhance epithelial cell migration and proliferation, as well as the formation, migration, and action of myofibroblasts.

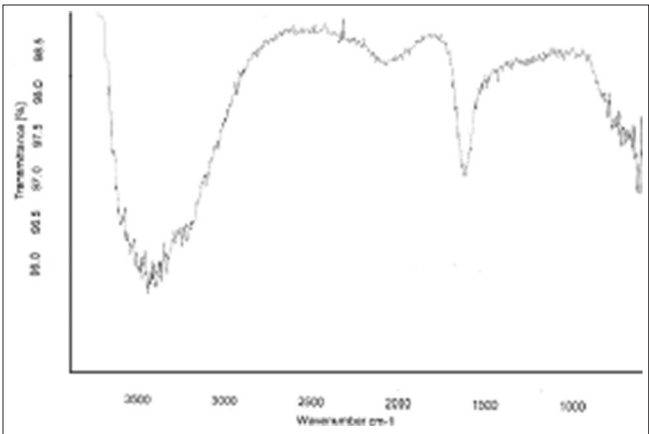


Figure 4: Fourier-transform infrared absorption spectra of biosynthesized silver nanoparticles

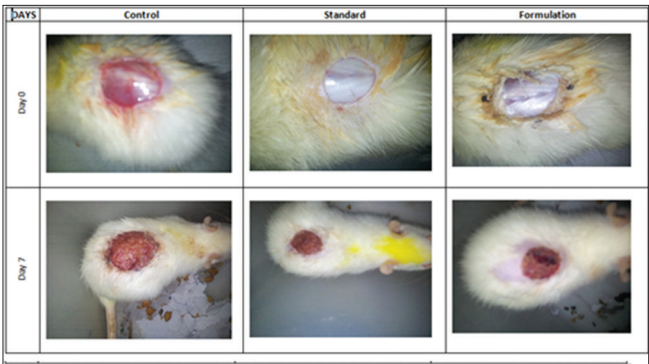


Figure 6a: Macroscopic observation of excision wound for control, standard (silver sulfadiazine cream) and formulation (hydrogel of synthesized silver nanoparticles using aqueous extract of *Arnebia nobilis* root) on day 0,7

It is, likely that in addition to enhancing wound contraction and epithelialization, AgNPs may also stimulate processes associated with tissue regeneration.

Topical application of hydrogel formulation improved wound contraction and closure and the effects were distinctly visible in the 1st and 2nd week.

Standard also showed enhanced wound healing and its effect was more prominent in the 4th week.

The preparations improved wound contraction and epithelialization. The percentage wound healing for formulation was 9.34% more than that of standard at day 14; however, the standard showed 1.78% higher healing on day 21.

Reduction mechanism

The major compound present in *A. nobilis* is naphthoquinones, whereas the alkanin β,β -dimethyl acrylate [5,8-dihydroxy-2

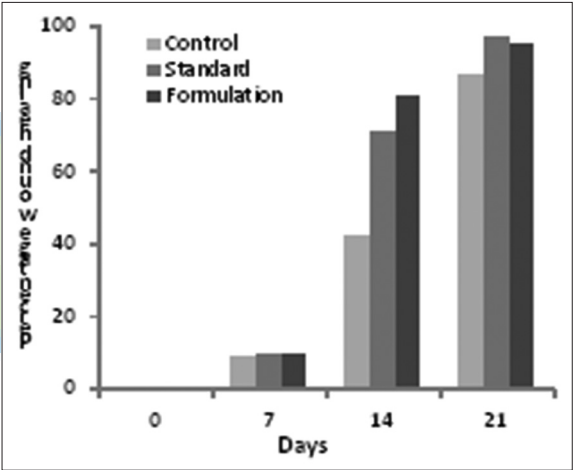


Figure 5: Percentage wound healing after at days 0, 7, 14, and 21 for control, standard (silver sulfadiazine cream), and formulation (hydrogel of synthesized silver nanoparticles using aqueous extract of *Arnebia nobilis* root)

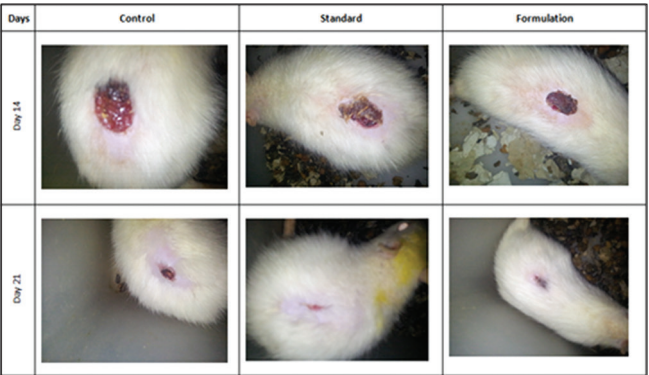


Figure 6b: Macroscopic observation of excision wound for control, standard (silver sulfadiazine cream), and formulation (hydrogel of synthesized silver nanoparticles using aqueous extract of *Arnebia nobilis* root) on day 14 and 21

(1'- β , β -dimethylacryloxy-4'-methylpent-3'-enyl)-1,4-naphthaquinone] was identified as the major component followed by alkanin acetate [2-(1'-acetoxy-4'-methyl pent-3'-enyl)-5,8-dihydroxy 1,4-naphthoquinone] and shikonin [5,8-dihydroxy-4'-methylpent-3'-enyl)-1,4-naphthoquinone].^[25] The two hydroxyl groups present in the benzene ring are participating in the reduction reaction. In the probable reaction mechanism [Figure 7], Ag^+ first forms an intermediate silver complex and then finally silver ion and quinone. These free silver ions are reduced to AgNPs using free electrons or nascent hydrogen produced in the process. AgNPs have negative zeta potential value in their pure form.^[31] The possible capping mechanism of polyphenolic compound is given in Figure 8, where, the interaction of hydrogen atom of the polyphenolic compound of root extract with Ag nanoparticles has been shown.

CONCLUSION

The following work demonstrated the capability of using aqueous extract of plant products (*A. nobilis* roots) toward the synthesis of AgNPs, its antibacterial potential and wound healing potential of hydrogel. The experiments were carried out by using *A. nobilis* root extracts, which is environmentally benign and renewable, act as reducing and capping agents. Green synthesis of AgNPs using *A. nobilis* root extract is a simple and efficient route to produce spherical shaped, fcc structured nanoparticles

with diameter range of 20-80 nm. No chemical reagent or surfactant was required in this synthesis. Color change was observed due to surface Plasmon resonance during the reaction with the ingredients present in the plant root extract results in the formation of AgNPs which was confirmed by UV-vis, XRD, FTIR, and SEM AgNPs, so obtained, were stable for more than 4 months. Investigation of the antibacterial effect of nano-sized silver colloidal solution against *E. coli* and *S. aureus* reveals high efficacy

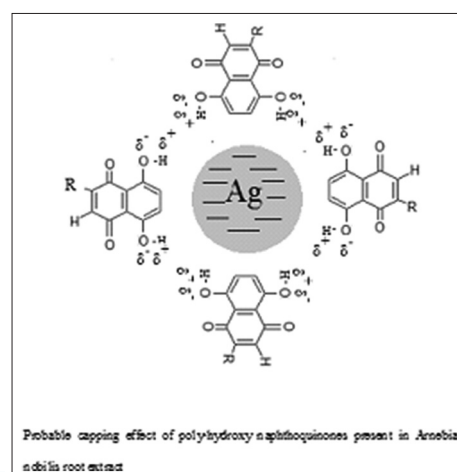


Figure 8: Probable capping effect of poly-hydroxynaphthoquinones present in *Arnebia nobilis* root extract

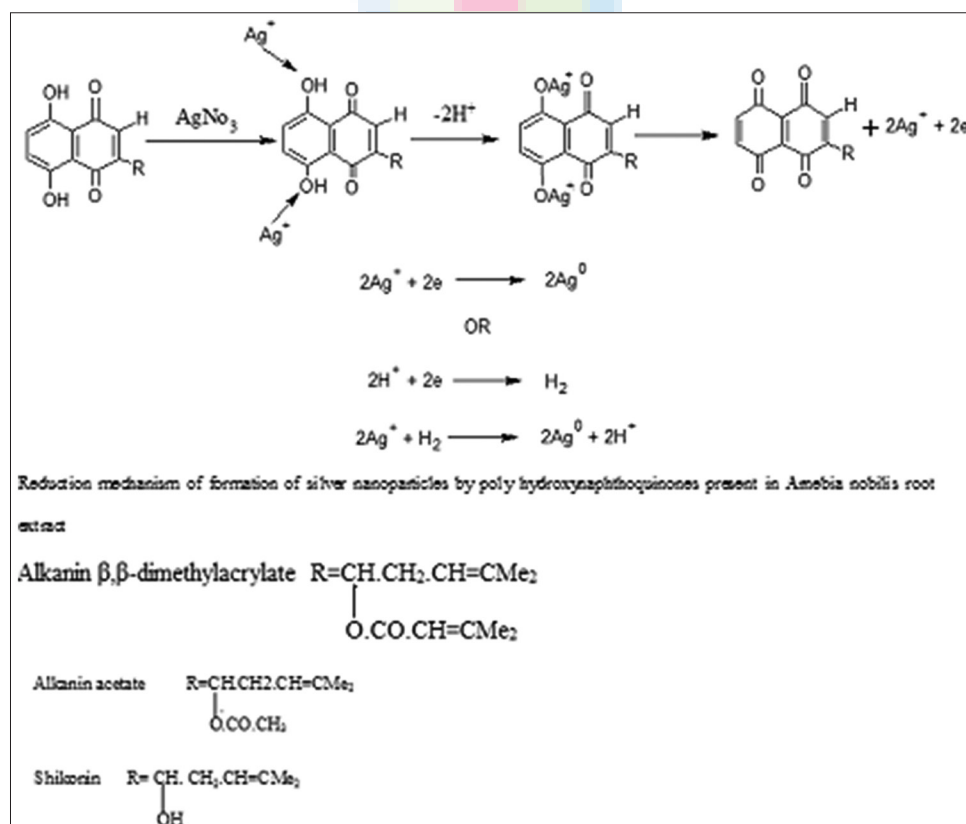


Figure 7: Reduction mechanism of formation of silver nanoparticles by poly-hydroxynaphthoquinones present in *Arnebia nobilis* root extract

of AgNPs as a strong antimicrobial agent. AgNPs using *A. nobilis* revealed higher microbicidal activity indicating that they are not only eco-friendly, but also yield enriched turn over compared with chemically synthesized particles. The excision wound model showed comparable wound healing with that of standard.

ACKNOWLEDGMENT

We duly acknowledged Dr. A. K. Chauhan and Dr. Balvinder Shukla of Amity University, Noida for their support and providing facilities for the fulfillment of this study. We specially thank to Dr. G. S. Chakraborty of NIET, Greater Noida for his valuable support in animal studies.

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How to cite this article: Garg S, Chandra A, Mazumder A, Mazumder R. Green synthesis of silver nanoparticles using *Arnebia nobilis* root extract and wound healing potential of its hydrogel. *Asian J Pharm* 2014;8:95-101.

Source of Support: Nil. **Conflict of Interest:** None declared.