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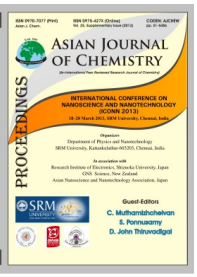

Green Synthesis of Crystalline Silver Nanoparticles Using *Indigofera aspalathoides*-Medicinal Plant Extract for Wound Healing Applications†

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A simple biological green synthesis of silver nanoparticles for wound healing applications was developed using medicinal plant *Indigofera aspalathoides*. The exploitation of various medicinal plant materials for the biosynthesis of nanoparticles is considered a green technology as it does not involve any harmful chemicals. In the present study an aqueous leaf extract of *Indigofera aspalathoides* was used to synthesize silver nanoparticles for wound healing applications. Bioreduction of silver nitrate were used to synthesize silver nanoparticles using aqueous plant extracts. Water-soluble organics present in the plant materials are responsible to reduce the silver ions to nano-sized Ag particles. The green synthesized nanoparticles were characterized by UV-visible spectroscopy. Scanning electron microscopy, energy dispersive X-ray analysis and Fourier transform infrared spectroscopy. The kinetics of reduction of aqueous silver ions during reaction with the *Indigofera aspalathoides* crude extract was performed by UV-visible spectroscopy. The SEM analysis showed that aqueous silver ions, when exposed to extract were reduced and resulted in the biosynthesis of silver nanoparticles in the size range of 20-50 nm. This eco-friendly approach for the synthesis of nanoparticles is simple, can be scaled up for large-scale production and will be tested for wound healing applications following excision wound method in animal models.

Key Words: Nanoparticles, Bioreduction, *Indigofera aspalathoides*, Wound healing.

INTRODUCTION

Indigofera aspalathoides Vahl. (Papilionaceae) commonly known as Shivanarvembu is spread throughout the southern states of India[1]. In the traditional medicinal system, the leaves, flowers and tender shoot are said to have cooling and demulcent effect[2]. They are used in the form of decoction for treating leprosy and cancer. *Indigofera aspalathoides* Vahl isan under shrub which is widely distributed, but is mostly found in South India and Sri Lanka. In the traditional medicinal system, the leaves, flowers and tender shoot are said to be cooling and demulcent; they are used in the form of decoction for leprosy and cancerous affections[3]. The leaves are also applied to abscesses. The whole plant is used in edematous tumors and the ashes are used in preparations for dandruff's. The methanol extract of *Indigofera aspalathoides* also have hepatoprotective acvity[1,4]. *Wedelia calendulacea* (compositae) is commonly known as 'Bhimraj' in Bengali, which is widely, distributed in Bengal, Assam, Myanmar and plains districts of Tamilnadu. In traditional system of medicine, the leaves are considered as tonic, alterative and useful in cough, cephalalgia, skin disease and alopecia. An infusion of the plant is given in Indo China for swellingabdomen. The plant is very specific for viral hepatitis[1,2,4-6].

The leaves are also applied to abscesses. The whole plant is used in edematoustumors and the ashes are used in preparations for dandruff's (The Wealth of India, 1959). The methanol extract of *Indigofera aspalathoides* also possess hepatoprotective activity[7]. From previous study[8] results proved the responsible role of secondary metabolites as active compounds isolated from plants to prove the wound healing in animal models. The histopathological examination also showed the wound healing process of the wounded tissue in *Indigofera aspalathoides* leaf extract treated mice was comparably equivalent to the reference drug used as positive control to treat the mice. No healing was observed in negative control group. Granulation tissue mainly contains fibroblasts, collagen fibers, very less oedema and newly formed blood vessels which were also observed in the plant extract treated mice. The histopathological examination provided added evidence for the

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experimental wound healing studies which was based on the contraction value of wound area and to measure tensile strength[8].

Also in one of our studies, we have fabricated a wound dressing byelectro spinning method and incorporate *Indigofera aspalathoides* plant extracts[9]. The electro spun poly capro lactone nanofibers as skin tissue engineering substrates. The blending electro spinning technique makes it possible to combine the advantages of herbal medicines and electro spunnanofibers. Our results proved the electro spun *Indigofera aspalathoides* nanofibers would be a novel substrate for skin tissue engineering[9].

The silver sol gel has some specific advantages over regular cosmetic skin care or burn and wound care products, So the make the indigofera as palathoides extracts to be applied to the wound area directly. To strengthen the biomimetic approaches in wound healing, recently plants have been employed a major role in the synthesis of green nanoparticles by bioreduction of silver nanoparticles. The polyol components and the water-soluble heterocyclic components with different shape have attributes for both protective and reductive biomolecules.

In this paper we have synthesized the silver nanoparticles for *Indigofera aspalathoides*. The stable green synthesized silver nanoparticles (GSNP's) were analyzed by UV-visible, Fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), energy dispersive X-ray (EDAX) spectra. The synthesized and well-studied green nanoparticles will be used as a hydrogel dressing without any preservative which will be most efficient for cuts, new burns and dry wounds.

EXPERIMENTAL

Collection of plants: The plants *Indigofera aspalathoides* were collected from Thanjavur, Tamil Nadu, India and the herbarium was prepared for authentication and taxonomic identification was done by Dr. Jayaraman, Madras Christian College, Tambaram, Chennai, Tamil Nadu. The voucher specimen was numbered and kept in our research laboratory for further reference.

Preparation of aqueous extract: The leaves of *Indigofera aspalathoides* were washed and air and shade at room temperature for at least two weeks, cut into small pieces and powdered in a mixer and sieved using a 20 µ mesh sieve to get uniform size range and used for further studies. The 20 g of the sieved leaf powder was added to 100 mL of sterile distilled water in a 500 mL Erlenmeyer flask and boiled for 5 min. The flasks were kept under continuous dark conditions at 30 ºC. The extract was filtered and were stored in an airtight container and protected from sunlight for further use[10].

Phytochemical activity: The qualitative phytochemical analysis of *Indigofera aspalathoides* extracts were performed following the method of Parekh, and Chanda[11] to determine the presence of alkaloids (Mayer's, Wagner, Dragendorff's), flavonoids (alkaline reagent, Shinoda), phenolics (lead acetate, alkaline reagent test); triterpenes (Liberman-Burchard test), saponins (foam test), tannins (gelatine)[12]. The results were qualitatively expressed as positive (+) or negative (-)[13].

Synthesis of silver nanoparticle: Silver nitrate ($AgNO_3$) from Sigma-Aldrich, USA and the aqueous leaf extract of *Indigofera aspalathoides* were used for the bioreduction synthesize of nanoparticles. 5, 10 and 15 mL of aqueous leaf extract of *Indigofera aspalathoides* were carefully added to 10 mL of 1 mM aqueous $AgNO_3$ solution in 250 mL Erlenmeyer flasks and incubated in a rotary shaker at 150 rpm in dark. The colour change in the colloidal solutions occurred pointing out forming silver nanoparticles[14,15].

UV-visible absorbance spectroscopy analysis: The bioreduction (by $AgNO_3$) of nanoparticles was recorded periodically using UV-visible 3000+ double beam spectrophotometer which has slit widths of 0.5, 1.0, 2.0 and 5.0 nm. The samples were diluted with 2 mL deionized water and measured by UV-visible spectrum at regular different time intervals[16]. The deionized water was used as a blank for background correction of all UV-visible spectra. All samples were loaded into a 1 cm path length quartz cuvette for sampling. The UV-visible spectrometric readings were scanned from 200 to 800 nm and recorded at a scanning speed of 0.5 nm interval. The UV-visible spectra were fit with Gaussian curves correcting for a cubic background for full-width at half maximum (FWHM) and wavelength of maximum absorbance measurements. The Gaussian fits to the UV-visible spectra all had goodness of fit values (χ^2 *ca.* 1), showing accurate curve analysis[14].

SEM analysis of silver nanoparticles: The prepared silver nanoparticles were characterized using high resolution SEM analysis (JEOL, JSM-5600LV). The samples were prepared by simple drop coating of suspend silver solutions on to an electric clean glass and allowing the solvent (water) to evaporate. The samples were left to dry at room temperature[21-23].

FTIR spectroscopy analysis of dried biomass after bio reduction: To identify the biomolecules present in the leaf extract of *Indigofera aspalathoides* and the biomolecules within the silver nanoparticles after the synthesis. A carefully weighed quantity of the synthesized nanoparticles were subjected to FTIR analysis (Perkin Elmer RX1)[19]. The bioreduced chlorauric and silver solutions were centrifuged at 10,000 rpm for 15 min and the pellet was washed three times with 20 mL of deionized water[20]. The resulting purified suspension was dried and ground with KBr pellets and analyzed by FTIR. The FTIR was recorded in the range of 4000-400 cm^{-1}. To obtain good signal and noise ratio, 512 scans were recorded[21].

EDAX spectrum measurements: The elemental composition of the synthesized nano particles by *Indigofera aspalathoides* were dried; drop coated on to carbon film and tested using EDAX analysis (S-3400N; Hitachi, Tokyo, Japan)[22,23].

XRD measurement: To characterize the purified silver nanoparticles XRD measurements were conducted using XRD-6000 X-ray diffractometer (Shimadzu, Japan) operated at a voltage of 40 kV and 30 mA with CuK_α radiation in θ-2θ configurations. The crystallite domain size was calculated from the width of the XRD peaks by assuming that they were free from non-uniform strains using the following Scherer formula[24].

$$D = \frac{0.94\lambda}{\beta\cos\theta}$$

where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half maximum (FWHM) and θ is the diffraction angle[15,25,26]. To expel the added instrumental broadening, the FWHM was corrected using the FWHM from a large-grained Si sample.

$$\beta \text{ corrected} = (FWHM^2_{sample} - FWHM^2_{Si})^{1/2}$$

This modified formula is valid only when the crystallite size is smaller than 100 nm[19].

RESULTS AND DISCUSSION

Phytochemical screening: Methanol extract of *Indigofera aspalathoides* was evaluated for the presence of various phytoconstituents by performing different qualitative chemical tests. It showed the presence of various phytochemicals that is shown in Table-1. From Table-1, we conclude the methanol extract of *Indigofera aspalathoides* mainly contains amino acids, carbohydrates, terpenoids, tannins, alkaloids, flavonoids, saponins, glycosides and lipids.

TABLE-1
PHYTOCHEMICAL SCREENING OF METHANOL EXTRACT OF *Indigofera aspalathoides*

Phytochemical	*Indigofera aspalathoides*
Amino acids	+
Carbohydrates	+
Terpenoids	+
Tannins	+
Alkaloids	+
Steroids	-
Flavonoids	+
Saponins	+
Glycosides	+
Lipids	+

Key: + Present, - Absent

UV-visible spectrophotometer: An immediate reduction of silver ions within 20 min may be because of the presence of water soluble phytochemicals like alkaloids, phytosterols, tannins, flavonoids, triterpines in the *Indigofera aspalathoides* plant extract. It was observed the reduction of silver ions occur rapidly and more than 90 % of reduction of silver ions is complete within 8 and 14 h, respectively, after adding the aqueous plant extract to the metal ion solutions[10]. The characteristic absorption peak at 420 nm in UV-visible spectrum (Fig. 1) confirmed forming silver nanoparticles. Surface plasmon resonance (SPR) patterns, characteristics of metal nanoparticles strongly depend on particle size, stabilizing molecules or the surface adsorbed particles and the dielectric constant of the medium. The nanoparticles showed an absorption peak around 420 nm after 8 h of reaction, which is a characteristic surface plasmon resonance band of silver nanoparticles possibly because of exciting longitudinal plasmon vibrations in silver nanoparticles in the solution[27,28].

SEM images of silver nanoparticles: The inspection of SEM images clearly suggests the faint thin layer of other material on the surface silver nanoparticles because of capping silver ions. The SEM analysis of bio reduced silver nanoparticles confirmed the size of the metal particles are in the

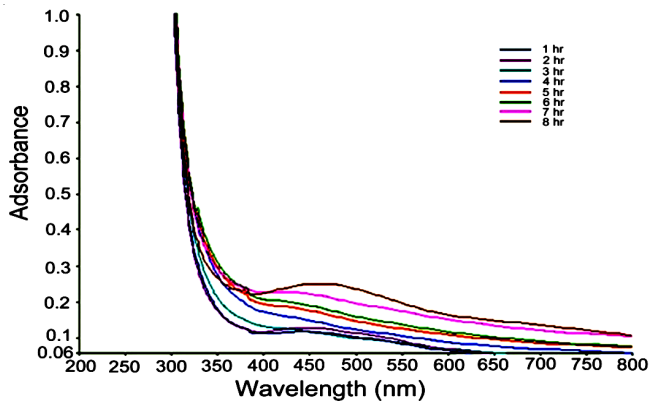

Fig. 1. Time dependant absorption spectra of silver nanoparticles after the bioreduction of silver in the aqueous plant extract of Indigofera aspalathoides

nano-range and are roughly square in shape. The size of silver nanoparticles are in the range of 45 and 69 nm after 24 h and the representative SEM image is shown in Fig. 2.

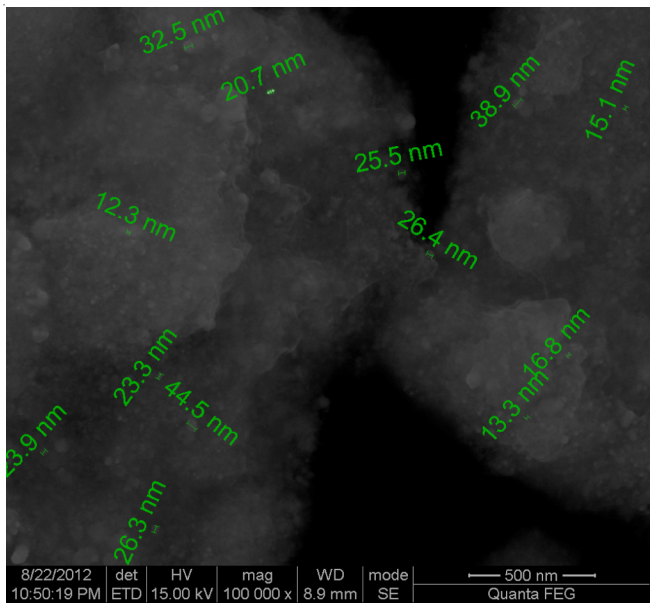

Fig. 2. SEM image of green silver nano particles synthesized by reduction of aqueous $AgNO_3$ ions using *Indigofera aspalathoides* extract

EDAX for silver nano particles: The analysis through energy dispersive X-ray spectrometers confirmed the presence of elemental silver signal of the silver nanoparticles as shown in Fig. 3. The vertical axis displays the number of X-ray counts while the horizontal axis displays energy in KeV. The identification lines for the major emission energies for silver are displayed and corresponding with peaks in the spectrum, thus giving confidence that silver has been correctly identified. The EDAX spectrum clearly confirms that 93.8 % was silver. The weak signals of arise at 0.5 KeV corresponds to proteins/enzymes that are either bound to the silver Nanoparticle, also a strong signal of 0.24 KeV for C atom, which is because of the functional compounds present in aqueous plant extract. The formation of individual square shaped silver nanoparticles using *Indigofera aspalathoides* are in the range of 2-4 keV. The similar signal energy peaks were also obtained previous researchers[29].

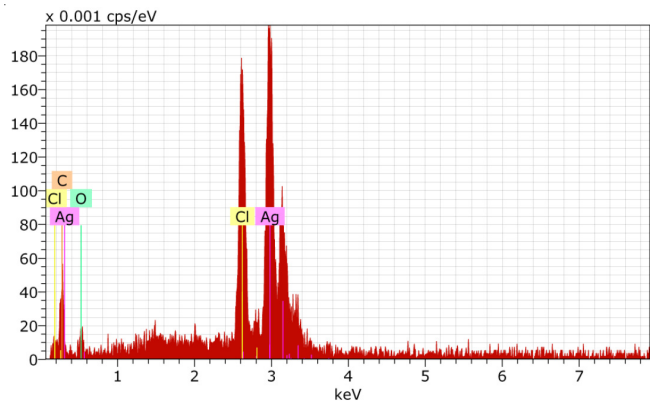

Fig. 3. Energy dispersive X-ray spectrum equipped with SEM of silver nanoparticles resulting from the experiment using 15 mL of extract

FTIR of silver nano particles: FTIR measurements were carried out to identify the potential biomolecules in the *Indigofera aspalathoides* aqueous plant extract responsible for reducing the chloroaurate ions. The size distribution and characterization of green synthesized silver nano particles was further verified by FTIR using *Indigofera aspalathoides* synthesized silver nanoparticles are shown in Fig. 4. The interaction of nanoparticles with phytochemicals of *Indigofera aspalathoides* showed intense peaks at 2884, 1600, 1507, 1387, 1074 and 1335 cm^{-1} relative shift in position and intensity distribution were confirmed with FT-IR.

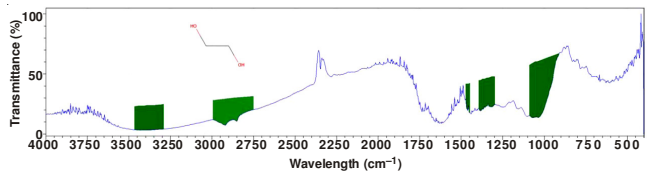

Fig. 4. FTIR spectrum of silver nanoparticles

XRD analysis of green silver nano particles: The X-ray structural diffraction pattern of the green synthesized silver nanoparticles produced using the leaf extracts were further proved and confirmed by the characteristic peaks observed in the XRD image for silver (Fig. 5). The average grain size of the silver nanoparticles formed in the bioreduction were determined using Scherr's formula, $d = (0.9\lambda \times 180°)/\beta \cos \theta\pi$ and estimated as 65 nm. The XRD pattern clearly explains the crystalline structure of the silver nanoparticles formed in by green biosynthesis.

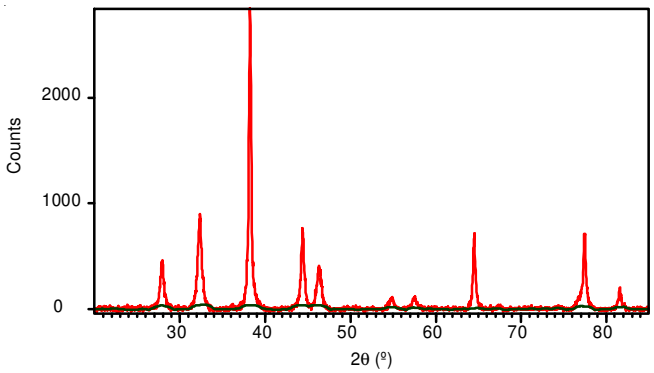

Fig. 5. XRD Spectrum of green synthesized silver nanoparticles

Conclusion

Present results described a simple and eco-friendly time-dependent method to biosynthesize green crystalline silver nanoparticles in metal solution using medicinal plant extracts which does not need special physical conditions. This research also explained that the *Indigofera aspalathoides* can be an excellent bioreductant and easily available less expensive plant source for the synthesis of silver nanoparticles. The *Indigofera aspalathoides* aqueous leaf extract be environmentally, friendly and therefore this protocol could be used for the rapid production of silver nanoparticles.

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