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Research Article
Characterization of Silver Nanoparticles Synthesized Using the Extract of the Leaves of *Tridax procumbens*

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Abstract
The synthesis, characterization and application of biologically synthesized nanomaterials have become the prime area of study in nanotechnology. Green synthesis of nanoparticles using plant extracts is being explored globally owing to the absence of disadvantages associated with conventional methods. This study reports the synthesis of silver nanoparticles using the extract of *Tridax procumbens* Linn. leaves, characterization of the synthesized nanoparticles and the evaluation of blood clotting activity of the nanoparticles. Ultraviolet-Visible (UV-Vis) spectroscopy confirmed the synthesis of nanoparticles. X-Ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) studies revealed the characteristics of the nanoparticles synthesized. The nanoparticles have been found to reduce the time taken for blood clotting significantly ($p = 0.01$) and thus proved to be efficient hemostatic agents. *Tridax procumbens* leaves exhibit hemostatic activity by promoting blood clotting. The nanoparticles synthesized using extract of the leaves of *T. procumbens* were found to promote blood coagulation better than the plant extract. The hemostatic potential of the nanoparticles can be further validated *in vivo*.

Key words: Nanosilver, green synthesis, haemostatic activity, clotting time, X-ray diffraction, ultraviolet-visible

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nanoparticles, especially inorganic nanoparticles have been a subject of research for few years, because of their versatile applications and advantages. Gold and silver nanoparticles have a broad spectrum of applications particularly in the medical field. The therapeutic uses of these nanoparticles are being researched. The availability, compatibility and capability of these nanoparticles to function as effective tools for medical imaging as well as treatment are being established (Xu *et al*., 2006; Priya *et al*., 2014). The biological method of synthesis of nanoparticles have proved to be a better method than the chemical methods as the former has prominent advantages like low capital involved in production and less energy consuming. The use of hazardous chemicals such as hydrazine eliminates the method from being an eco-friendly one while, green synthesis using plant extracts is environmentally benign. Moreover, nano-crystalline silver colloids produced by such aqua-chemical routes exhibit aggregation with time, thereby compromising with the size factor upon storage (Kalimuthu *et al*., 2008; Mittal *et al*., 2013).

Green synthesis of nanoparticles is environment friendly. Due to slower kinetics, they offer better manipulation and control over crystal growth and their stabilization. Green synthesis is advantageous over chemical and physical method as it is cost effective, easily scaled up for large scale synthesis and in this method there is no need to use high pressure, energy, temperature and toxic chemicals (Forough and Farhadi, 2010; Logeswari *et al*., 2013). It sidelines various undesired deleterious effects of the chemical synthesis process and is thus environment friendly. Also, the active constituents of the plants used for green synthesis of nanoparticles adhere to or cap the nanoparticles and thereby improve their therapeutic efficacy. Nanosilver has been extensively used in wound management, treatment of ulcers and lesions, healing of skin-graft sites and preventing infection (Demling and DeSanti, 2002; Philip and Unni, 2011).

Tridax procumbens Linn. is a specie of flowering plant and a local weed, which occurs in tropical as well as subtropical regions in all the seasons. This is a perennial herb with a creeping stem; leaves are simple, ovate-elliptical and coarsely serrate or lobed. Flowers are yellow, in heads each on a long stalk and fruits are small. *Tridax procumbens* is traditionally known for its antiviral and antioxidant efficacies, wound healing activity, insecticidal and anti-inflammatory activity. It also possesses significant medicinal properties against blood pressure, malaria, dysentery, diarrhoea, stomach-ache and headache (Nia *et al*., 2003). The extracts of the leaves of *T. procumbens* have been proved to decrease the time taken for blood clotting and thus helps in hemostasis. The objective of this study was the biosynthesis of silver nanoparticles using the extract of *T. procumbens* leaf. The biologically synthesized silver nanoparticles were tested for blood coagulation activity.

MATERIALS AND METHODS

All chemical and reagents used were of analytical grade. Silver nitrate was procured from Himedia. Liquiceilin-E was procured from Tulip Diagnostics Pvt Ltd., India.

Collection of plant material: *Tridax procumbens* L. plant leaves were collected from the campus of Loyola College. The plant was identified and duly authenticated by Prof Jayaraman Director, Institute of herbal Botany plant Anatomy Research Centre (Voucher No. PARC/2012/1440).

***Tridax procumbens* extract preparation:** A 10 g portion of thoroughly washed fresh *T. procumbens* leaves were cut into fine pieces and 200 mL of sterile distilled water was added and exposed to microwave irradiation for 3 min to subdue the active plant constituents (Nadagouda *et al*., 2011). The solution was then filtered in hot condition. The clear filtrate which is the extract of the leaf was used for nanoparticles synthesis.

Preparation of silver nitrate solution: Analytical grade silver nitrate procured from Hi Media labs (RM 638) was prepared as 10^{-3} and 10^{-6} M solutions and used in this experiment.

Synthesis of silver nanoparticles: For the synthesis of silver nanoparticles using *T. procumbens*, 10 mL of plant extract was added to 50 mL of silver nitrate. The solution was then subjected to microwave irradiation for 3 min and the colour change was observed from light yellow to brown colour. The solution was then centrifuged at 3000 rpm for 20 min. The silver nanoparticles were isolated and concentrated by repeated centrifugation of the reaction mixture at 3,000 g for 20 min. The supernatant was replaced with distilled water each time. The nanoparticles were washed well to remove any residue particles that were not the capping agents. The suspension was dried and stored as a crystalline powder for characterization studies and biological assay.

Characterization of silver nanoparticles: The physical characterization of silver nanoparticles was done at SAIF, IIT Madras.

Ultraviolet-visible spectra analysis: The UV-Vis spectrum of the synthesized nano-silver particles was studied with Cary 5E UV-Vis spectrophotometer from 300-800 nm.

X-ray diffraction analysis: The X-Ray Diffraction (XRD) pattern of the silver nanoparticles synthesized was studied by Bruker Kappa AXE XII with Cu Kα (l = 1.54 Å) radiation, scanning range from 10°-90°.

Fourier transform infra-red spectroscopy analysis: The Fourier Transform Infra-Red Spectroscopy (FTIR) of the dried nanoparticles was studied using Perkin-Elmer Spectrum-One instrument. The 256 scans of silver nanoparticles were taken in the range of 400-4000 cm^{-1} and the resolution was kept as 4 cm^{-1}.

Scanning electron microscopic analysis: Scanning Electron Microscopic (SEM) analysis was done using Quanta 200 FEG scanning electron microscope. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

Blood coagulation study: Blood samples were collected from healthy volunteers and were anticoagulated using 3.8% tri-sodium citrate. The sample was immediately centrifuged at 4000×g for 15 min. The plasma was separated and was stored at 4°C until, further use. In a series of test tubes, 0.1 mL of the plasma sample and liquiceilin-e was added and thoroughly mixed. The tubes were placed at 37°C for 20 min for incubation. Pre-warmed calcium chloride solution (0.1 mL) was added to the plasma and reagent mixture. Then, 1 mL of aqueous extract and different concentrations of nano silver were added to different tubes and kept at 37°C. The contents in the tubes were mixed until clot formation. Coagulation time was then calculated. Normal saline served as negative control and 50 mg mL^{-1} of commercial heparin was used for the positive control.

RESULTS

Synthesis and characterization of silver nanoparticles: In this study, the silver nanoparticles were synthesized and studied using the extract of the leaves of *T. procumbens*. To fasten the process, heating using microwaves was adopted. The formation of homogenous silver nanoparticles was best achieved by an exposure to microwave heating for 3 min.

Visual inspection: Upon addition of *T. procumbens* leaf extract to silver nitrate solution, a light yellowish colour was observed, which gradually changed to dark brown colour on subjecting to microwave irradiation. This colour change indicated that there was reduction of silver particles. The same treatment was given to silver nitrate solution without the addition of plant extract and no change in colour was observed.

Ultraviolet-visible spectra analysis: In the present study, a single peak at 430-460 nm was observed with the silver nanoparticles synthesized with both 10^{-3} and 10^{-6} M $AgNO_3$. Thus a lower concentration of silver nitrate is sufficient to synthesize silver nanoparticles (Fig. 1a-c).

X-ray diffraction analysis: X-ray diffraction study confirmed the presence of silver nanoparticles. The Bragg reflections at 2 h = 38.18, 44.37, 64.48 and 77.63 can be indexed to the (111), (200), (220) and (311) orientations, respectively, confirmed the presence of silver nanoparticles. Spurious diffractions due to crystallographic impurities were absent (Fig. 2).

Fourier transform infrared spectroscopy analysis: The representative spectra of stabilized silver nanoparticles obtained using *T. procumbens* and the leaf extract of *T. procumbens* alone has been given in Fig. 3b.

Scanning electron microscope: The silver nanoparticles are clearly seen at high magnification (100,000x) in the micrograph. The particles are predominantly spherical in shape ranging in size from 26-30 nm (Fig. 4).

Blood coagulation activity of AgNPs: The effect of silver nanoparticles on prothrombin time was observed for each blood sample. Observations of clotting time obtained from *T. procumbens* leaves extracts are depicted in Table 1.

Table 1: Effect of silver nano particles on prothrombin time

Normal PT (sec)	TPE-PT (sec)	10^{-3} M $AgNO_3$-PT (sec)	NP TP-PT (sec)
12±2*	20±2	20±1	10±1*

*p = 0.01, All values are expressed as Mean±Standard error, PT: Prothrombin time, TPE: Extract of *Tridax procumbens* Leaves, NP TP: Silver nanoparticles synthesized using *Tridax procumbens* leaf extract

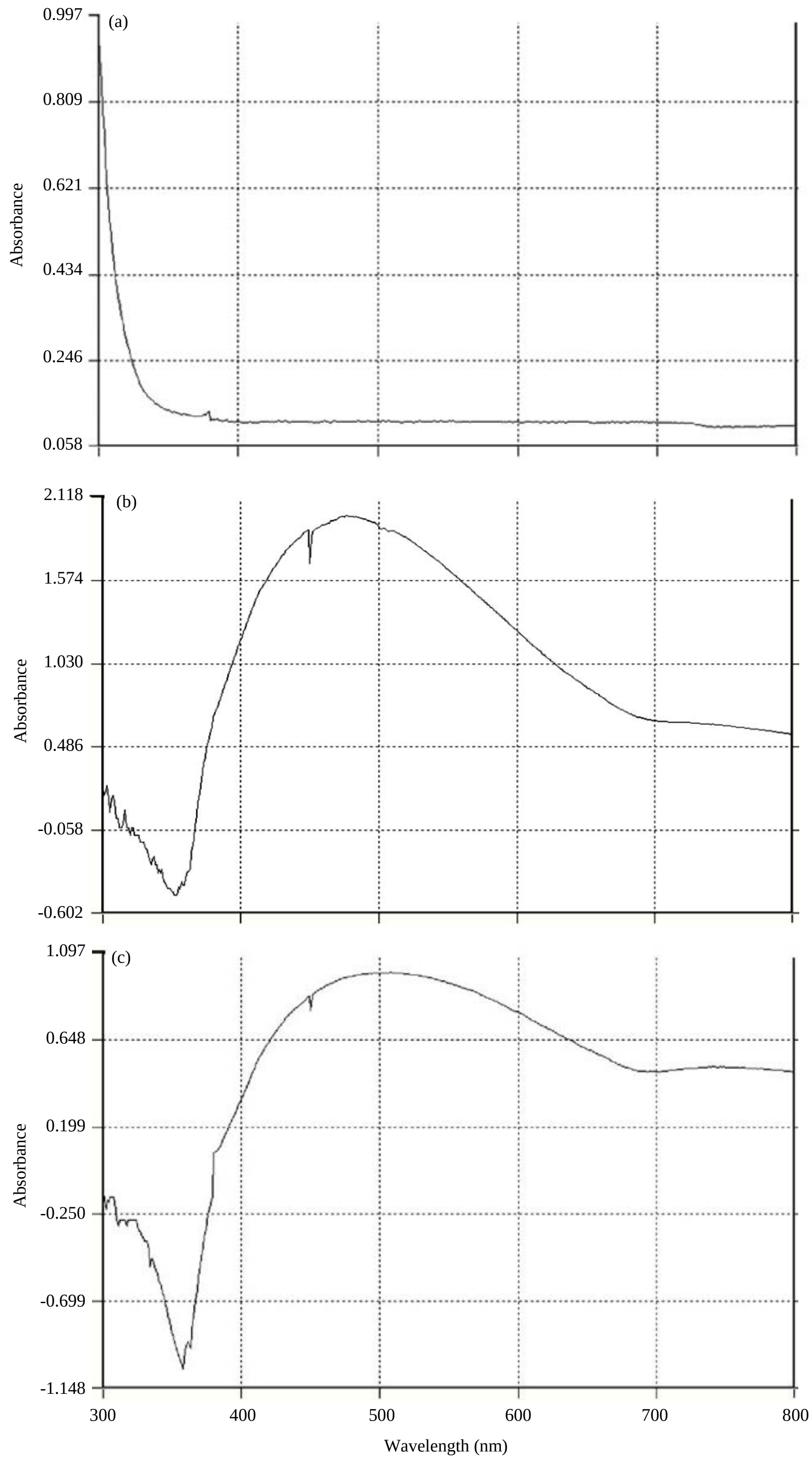

Fig. 1(a-c): UV-Vis spectrum (a) Colloidal silver nitrate without plant extract, (b) Silver nanoparticles synthesized using *T. procumbens* leaf extract and 10^{-6} M $AgNO_3$ and (c) Silver nanoparticles synthesized using *T. procumbens* leaf extract and 10^{-3} M $AgNO_3$

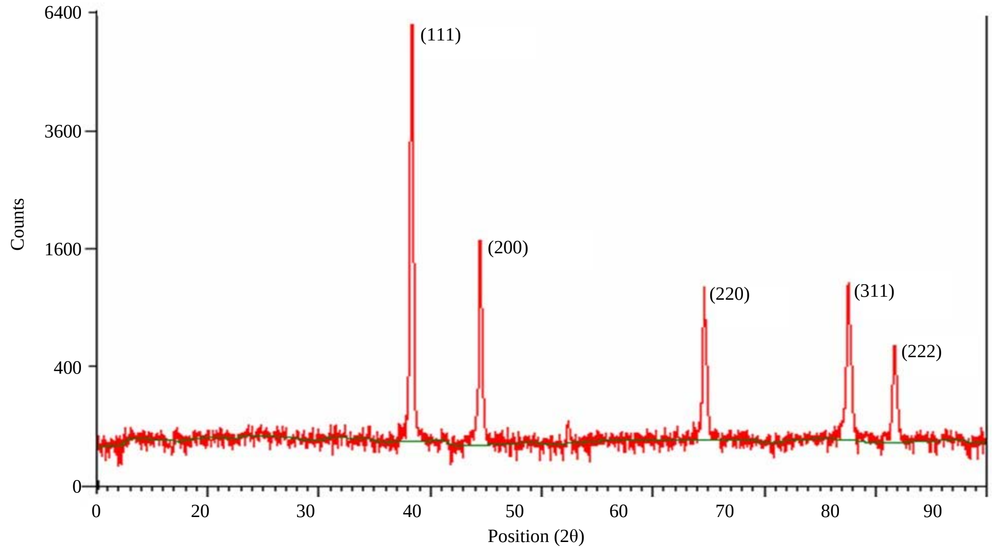

Fig. 2: X-ray diffraction patterns of silver nanoparticles synthesized using *T. procumbens* leaf extract

DISCUSSION

There has been an increasing demand for ecofriendly synthesis of nanoparticles that do not have any toxic effects so as to avoid adverse effects in medical applications. Nanoparticles have a wide range of applications such as catalysis, electronics, photonics, optoelectronics, sensing and pharmaceuticals. There has been growing need for environment friendly metal nanoparticles.

The physical and chemical methods of nanoparticles synthesis involve top-down or bottom-up approach and these methods produce enormous quantity of byproducts which are hazardous to health. Taking into consideration the therapeutic benefits of the phytochemicals, vitamins, minerals and fibers present in *T. procumbens* leaves, the present study involved the green synthesis of silver nanoparticles. The leaves of *T. procumbens* have been used traditionally to treat wounds, cuts and reports on the blood clotting ability of the leaves are available (Saxena and Albert, 2005). The current method of biosynthesis of silver nanoparticles has a time-related advantage over conventional methods (Bhati-Kushwaha and Malik, 2013). In this study, the formation of homogenous silver nanoparticles is best achieved by an even heat transfer provided by microwave heating.

The colour change to dark brown indicated that there was reduction of silver particles. The absence of change in colour with the silver nitrate solution devoid of plant extract showed that the reduction of silver was by the plant extract and not by the microwaves. The microwave-assisted method is much faster than the earlier conventional studies. The time required for the conventional synthesis of silver nanoparticles using plant extracts ranged from several minutes to few hours and thus are rather slow. This observation is supported by Nadagouda *et al*. (2011), who have reported the microwave assisted green synthesis of silver nanoparticles.

This is a commonly used technique for structural characterization of silver nanoparticles. The position and shape of plasmon absorption of silver nanoparticles are strongly dependent on the particle size, dielectric constant of the medium and surface adsorbed species. The colour developed in the solution as a result of formation silver nanoparticles was observed with a Surface Plasmon Resonance (SPR) peak in the UV-vis spectroscopy. The appearance of single peak for nanoparticles synthesized using both high and low concentrations of silver nitrate. Thus a lower concentration of silver nitrate is sufficient to synthesize silver nanoparticles. Further studies were done only with nanoparticles synthesized with 10^{-3} M $AgNO_3$. According to Mie's theory, only a single SPR band is expected in the absorption spectra of spherical nanoparticles, whereas, anisotropic particles could give rise to two or more SPR bands depending on the shape of the particles (Nithiya and Sangeetha, 2014). The absence of a peak in the 400-500 nm region analysed with the colloidal silver nitrate solution, which was devoid of *T. procumbens* leaf extract indicated the absence of nanoparticles and thus confirmed the role played by the plant extract in the synthesis of nanoparticles. Similar kind of results was reported by Sosa *et al*. (2003).

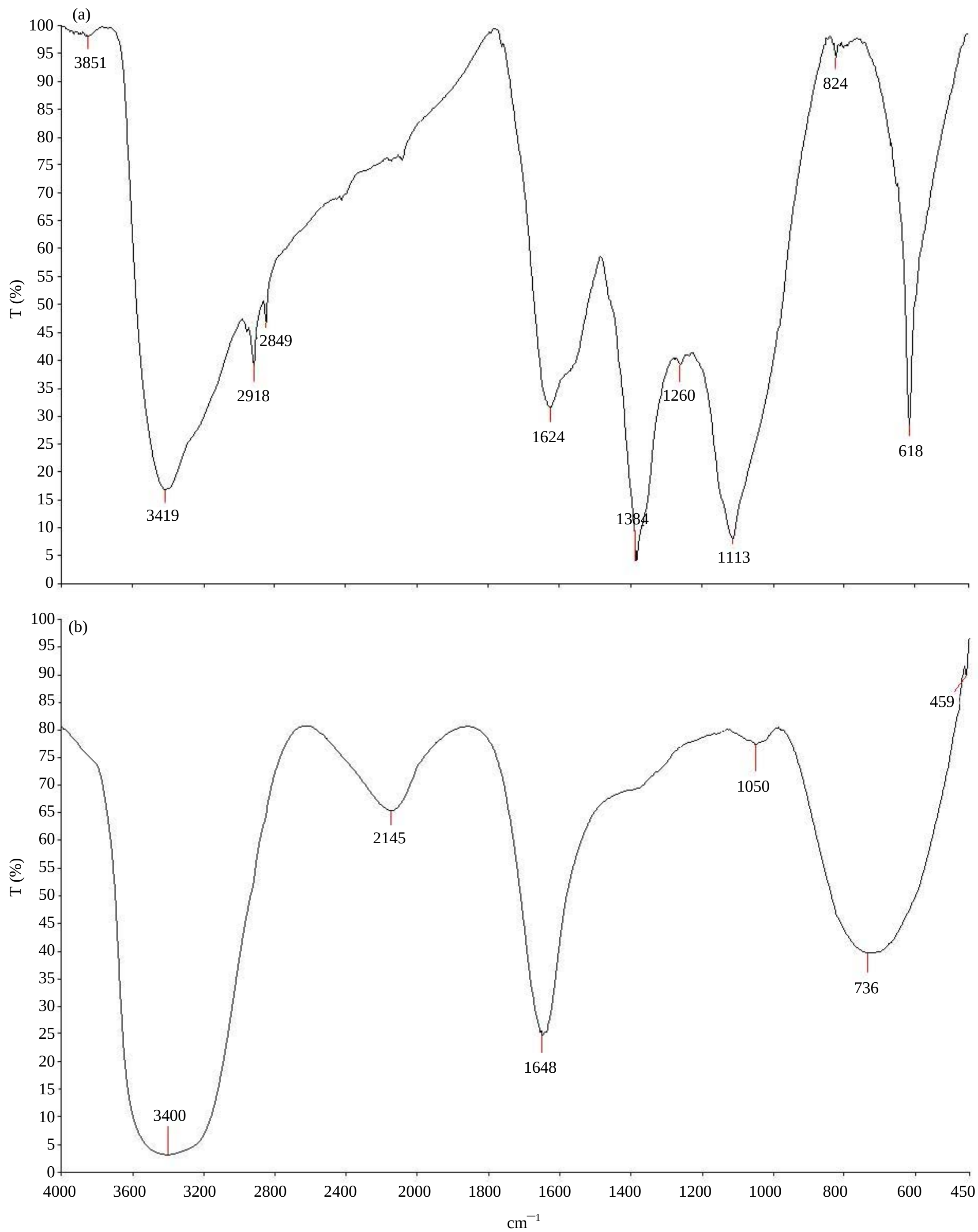

Fig. 3(a-b): FTIR spectrum analysis (a) Silver nanoparticles synthesized using *T. procumbens* leaf extract and (b) *T. procumbens* leaf extract

X-ray diffraction study confirmed the presence of silver nanoparticles. The Bragg reflections at 2 h = 38.18, 44.37, 64.48 and 77.63 can be indexed to the (111), (200), (220) and (311) orientations, respectively, confirmed the presence of silver nanoparticles. Spurious diffractions due to crystallographic impurities were absent. The intensity of peaks reflects the high degree of crystallinity of the silver nanoparticles. The diffraction peaks were broad indicating that the crystallite size is very small. The XRD shows that silver nanoparticles formed are crystalline (Sivakumar *et al.*, 2011). X-ray diffraction spectra of pure crystalline silver structures have been published by the Joint Committee on Powder

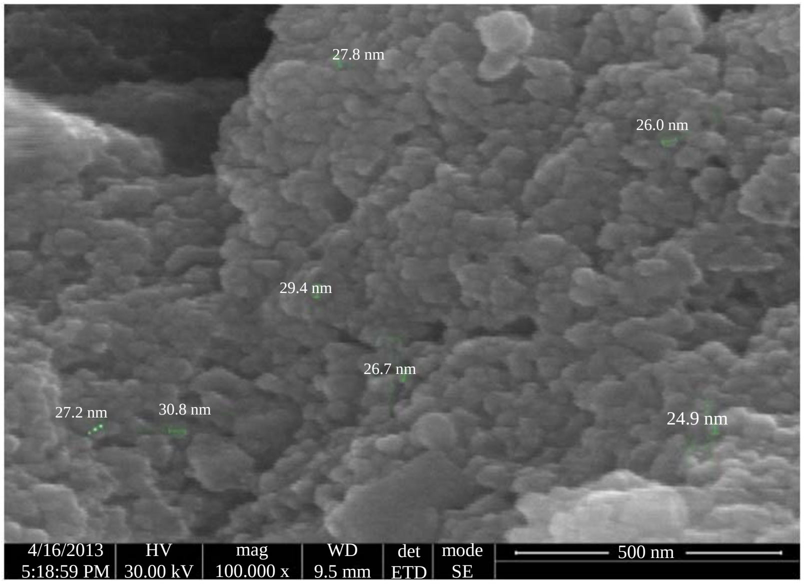

Fig. 4: Scanning electron microscopic image of silver nanoparticles synthesized using leaf extracts of *Tridax procumbens*

Diffraction Standards (file No. 04-0783). Similar studies showed a peak at 20° with min and max range 19.4-23.33° (Bhati-Kushwaha and Malik, 2014). The FT-IR spectroscopic studies were carried out to study the plausible mechanism behind the formation of these silver nanoparticles and provide information regarding the functional groups present on the surface of the nano particles. The very strong absorption peaks at 1634 and the strong absorption peaks at 1384 represents the presence of NO_2, which may be from $AgNO_3$ solution, the metal precursor involved in the silver nanoparticles synthesis process.

Appearance of broad peak at wavenumber 3400 in the nanoparticle spectrum indicates presence of phenolic hydroxyl group (OH) which represent the stretching vibration because of presence of hydrogen bonds belonging to flavonoids and tannins. This along with the peak of 736 cm^{-1} which represents the aromatic ring C-H vibrations, indicates the involvement of free catechin. This suggests the attachment of some polyphenolic components on to silver nanoparticles. This means the polyphenols attached to silver nano particles may have atleast one aromatic ring. Among them, the absorption peak at 1050 cm^{-1} can be assigned as absorption peaks of C-O-C- or -C-O- or C-N stretching vibrations of aliphatic amines. The peak at 1648 cm^{-1} represent carbonyl groups (C = O) from polyphenols such as catechin gallate, epicatechin gallate and theaflavin (Krishnan and Maru, 2006; Satyavani *et al*., 2011). Studies have confirmed the fact that the carbonyl group form amino acid residues and proteins has the stronger ability to bind metal. The proteins could therefore, form a layer over the metal nanoparticles to prevent agglomeration. The size and structure of silver metal nanoparticles was further characterized using SEM analysis. The analysis showed clear spherical morphology of silver nanoparticles of size from 26-30 nm.

The clotting time for nanoparticles synthesized using *T. procumbens* was less than that for *T. procumbens* extract in blood samples obtained from all the subjects. The nanoparticles significantly shortened the clotting time of blood samples of all the subjects by 2 min. Clotting time determination is a routine test performed to check deficiency in clotting factors. The flowers, leaves and aerial parts possessed anti-septic, insecticidal, parasiticidal, hepatoprotective and wound healing properties (Mundada and Shivhare, 2010; Saxena and Albert, 2005; Ravikumar *et al*., 2005). The clotting time of silver nanoparticles of *T. procumbens* was reduced in all blood samples suggesting that it possess haemostatic activity. In a similar study, the clotting time of 10 human volunteers with various extracts of the leaves of *T. procumbens* was studied by employing Lee-White's method to understand haemostatic activity. The ethanolic extract showed positive activity when compared to fresh leaf juice and petroleum extract. It has been discussed that the plant leaves are used to check hemorrhage from cuts, bruises and wounds (Diwan *et al*., 1982).

CONCLUSION

The green synthesis of silver nanoparticles has various advantages over conventional synthesis. In this study, green synthesis of nanoparticles of silver was achieved using the extract of *T. procumbens* leaves. The synthesized nanoparticles were characterized and analysed for blood coagulation activity. The studies ascertained the presence of plant secondary metabolites on the surface of nanoparticles. Thus these silver nanoparticles can be used to promote blood clotting.

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