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Anti-Angiogenic, Cytotoxic and Antimicrobial Activity of Plant Mediated Silver Nano Particle from *Tragia involucrata*

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ABSTRACT

The biosynthesis of silver nanoparticles is a cost effective and environmental friendly alternative to chemical and physical methods. In the present study, we have studied the green synthesis of silver nanoparticles from *T. involucrata* leaf extract and their biological properties. Silver nano particles (AgNPs) were characterized using UV-Visible spectrophotometer (UV-VIS), scanning electron microscope, X-ray diffraction (XRD), fourier transform infrared spectroscopy (FTIR). The AgNPs were tested for cytotoxic, antiangiogenic, antimicrobial as well as DNA diffusion assays. These biogenic nanoparticles showed significant cytotoxic activity against MOLT-4 cell lines, antiangiogenic and antimicrobial activity. Diffusion of DNA was comparatively higher in AgNPs treated cells than the control. Hence, *T. involucrata* leaf extract mediated AgNPs can be exploited in the development of novel drug. The unique properties of this AgNPs can be put to great use for human betterment.

Key words: Green synthesis, silver nano particles, *Tragia involucrata,* cytotoxicity, antimicrobial

INTRODUCTION

Nanotechnology is one of the promising fields of research and generating new avenues and applications in medicine (Vaidyanathan *et al*., 2009). The most important and distinct property of nano particles is that they exhibit larger surface area to volume ratio (Vankar and Bajpai, 2010). Nanoparticles can be produced using gold, silver, iron etc. The most effectively studied nanoparticles today are those made from noble metals, in particular silver, platinum, gold and palladium (Gurunathan *et al*., 2009; Jain *et al*., 2009).

Silver nanoparticles (AgNPs) have become the focus of intensive research owing to their wide range of applications in areas such as catalysis, optics, antimicrobials and biomaterial production (Parashar *et al*., 2009; Liu and Lin, 2004). Silver nanoparticles exhibit new or improved properties depending upon their size, morphology and distribution (Vorobyova *et al*., 1999; Bae *et al*., 2002). The application of silver nanoparticles (AgNPs) is burgeoning day by day in all forms of human life (Mandal *et al*., 2006). The silver nanoparticles are produced by the reduction of silver nitrate either by chemical, physical or biological methods (Basavaraja *et al*., 2008; Kowshik *et al*., 2003). The use of high temperatures and toxic chemicals to synthesize AgNPs in physical and chemical methods prompted the scientists to look for the alternative methods which are cost effective, with less toxicity (Keki *et al*., 2000; Jha and Prasad, 2010). Green synthesis is the method of choice for many

researchers as this method is not having the limitation of using high temperature as well as toxic chemicals (Yu, 2007). Various approaches using plant extract have been used for the synthesis of metal nanoparticles (He *et al*., 2007).

Plant and plant derived products were the basis of many traditional systems since ages and they are still providing the remedies for various ailments (Su *et al*., 2010). About 60% of the world population is dependent on traditional medicine (Shameli *et al*., 2012). The use of traditional medicines derived from plants is common in developing and developed countries (Seth and Sharma, 2004).

Tragia involucrata (Euphorbiaceae) is one such medicinal plant widely found in the Indian subcontinent (Dhara *et al*., 2000; Ribeiro, 2008). The efficacy of this plant is well known in Indian traditional medicine and it is used for treatment of eczema, wounds and headache (Samy *et al*., 1998; Savithramma *et al*., 2011). The anti-microbial, anti-inflammatory, antitumor, cytotoxic and antifertility activity of *T. involucrata* has been reported (Samy *et al*., 2006a, b; Joshi *et al*., 2011a, b; Joshi and Gopal, 2011; Nazeema and Sugannya, 2014). Even though this plant has been extensively studied for various medicinal properties, no work has been carried out on the AgNPs synthesis and the biological activities.

So, the aim of the present study was to synthesis the silver nanoparticle from *T. involucrata* and to evaluate the cytotoxic activity using acute lymphoblastic leukaemia cell lines (MOLT-4), antiangiogenesis, DNA diffusion using lymphocytes and antimicrobial efficacy.

MATERIALS AND METHODS

Chemicals and cell lines: MOLT-4 cell line was obtained from the repositories of National Centre for Cell Sciences (NCCS), Pune, India. DMEM, RPMI-1640, penicillin and streptomycin were purchased from Sigma chemicals Co., USA. Dimethylsulfoxide (DMSO) was from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. All other chemicals used in the study were obtained commercially and were of analytical grade.

Synthesis of silver nanoparticles: *Tragia involucrata* leaves (Fig. 1) were collected from Sulya, Karnataka, India. Silver nanoparticles synthesized using the method explained by Awwad *et al*. (2013). Fresh leaves of *T. involucrata* (10 g) were homogenized, centrifuged at 5000 rpm for 15 min and filtered. Leaf extract (10 mL) was added drop wise to 40 mL of 20 mM aqueous $AgNO_3$ solution for the reduction of Ag^+ ions.

Characterization of silver nanoparticles: The reduction of Ag^+ ions was monitored visually as well as by UV-visible spectroscopic measurement. The UV-visible spectrophotometric measurement of the reduced solution was recorded on OPTIMA UV-Vis spectrophotometer (wavelength range of 350-700 nm). The FTIR was carried out to identify the biomolecules responsible for reduction, capping and efficient stabilization of silver nano particles. X-ray diffractometer was operated at a voltage of 40 kV and a current of 30 mA with Cu kα radiation in a θ-2θ configurations. The crystallite domain size was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherrer formula. $D = 0.94\ \lambda/\beta\ \text{Cos}\ \theta \rightarrow (1)$, Scanning Electron Microscopic (SEM) analysis was done using Hitachi S-4500 SEM machine.

Cytotoxicity assay by trypan blue method: Trypan blue is a widely used colorimetric dye that distinguishes live cells from dead ones by a dye exclusion method. Live cells exclude the dye and

Fig. 1: Arial parts of *Tragia involucrata*

remain clear and translucent in the microscopic field. In contrast, trypan blue penetrates membranes of dead cells and stains them a dark blue colour. About 50 µL of cell suspension (MOLT-4) to which 50 µL of 0.4% trypan blue was added and mixed well. The suspension was counted using automated cell counter (Countees cell counter Invitrogen)

Antimicrobial activity: The antibacterial activity of silver nanoparticles from plant extract was determined by using standard well diffusion method against pathogenic bacteria like *Streptococcus* and *Pseudomonas* species. Nutrient agar was used for cultivation of the bacteria. The bacterial cultures were swabbed on the plates. The nanoparticle solution was poured into each well on all the plates. The plates were incubated at 37°C for 24 h and zone of inhibition was measured (Saxena *et al*., 2010).

DNA diffusion assay: The "DNA diffusion" assay is a simple, sensitive and rapid method for estimating apoptosis in single cells. The assay involves mixing cells with agarose and making a microgel on a microscopic slide, then lysing the embedded cells with salt and detergents (to allow the diffusion of small molecular weight DNA in agarose) and finally visualizing the DNA by a sensitive fluorescent dye, ethidium bromide (Singh, 2004).

Angiogenesis assay: Fertilized eggs were randomly divided into two groups; the control group and experimental group (treated with concentration of 200 µg mL^{-1} AgNPs) and then incubated at 37°C and 55-65% humidity in the incubation system (Ribatti, 2010). On day eighth of incubation, shells were opened in laminar hood and filter discs (6 mm) were dipped in the extract, dried and placed on the chorioallantoic membrane and incubated at 37°C. On the 12th day of incubation, all the cases were photographed using a research photostereomicroscope (Ziess, India).

Statistical analysis: All values were expressed as Mean±SD. Comparison between the control and sample were performed by analysis of variance (ANOVA) with Tukey's multiple comparison test using Graphpad Prism v3.0 software. $p>0.05$ were considered as significance.

RESULTS

The present study was aimed at green synthesis of silver nanoparticle from *T. involucrata* leaves and assessing its biological activity. A distinct color change was observed after 24 h as the solution turned dark yellow to black color suggesting formation of silver nanoparticles (Fig. 2). The reduction of Ag^+ was confirmed from the UV-Vis spectrum of the solution and silver surface plasmon resonance band at 430 nm as observed (Fig. 3).

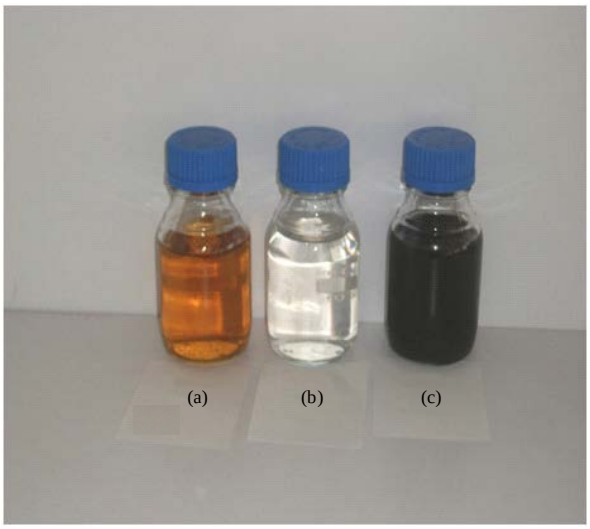

Fig. 2(a-c): Green synthesis of AgNPs, (a) Aqueous leaf extract of *T. involucrata*, (b) 20 mM aqueous $AgNO_3$ and (c) Colour changed from yellowish to black after adding 20 mM aqueous $AgNO_3$

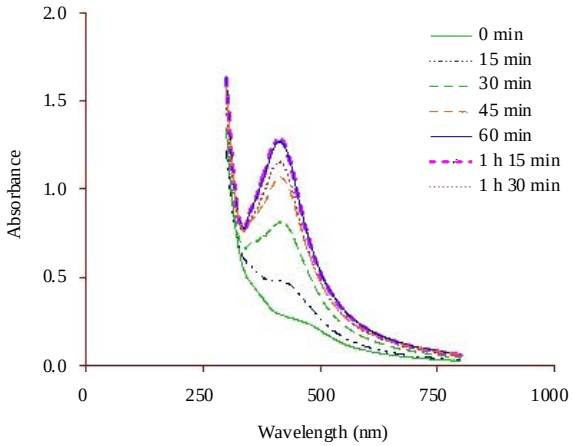

Fig. 3: UV-Vis spectra of reduction of silver ions to silver nanoparticles at different time interval

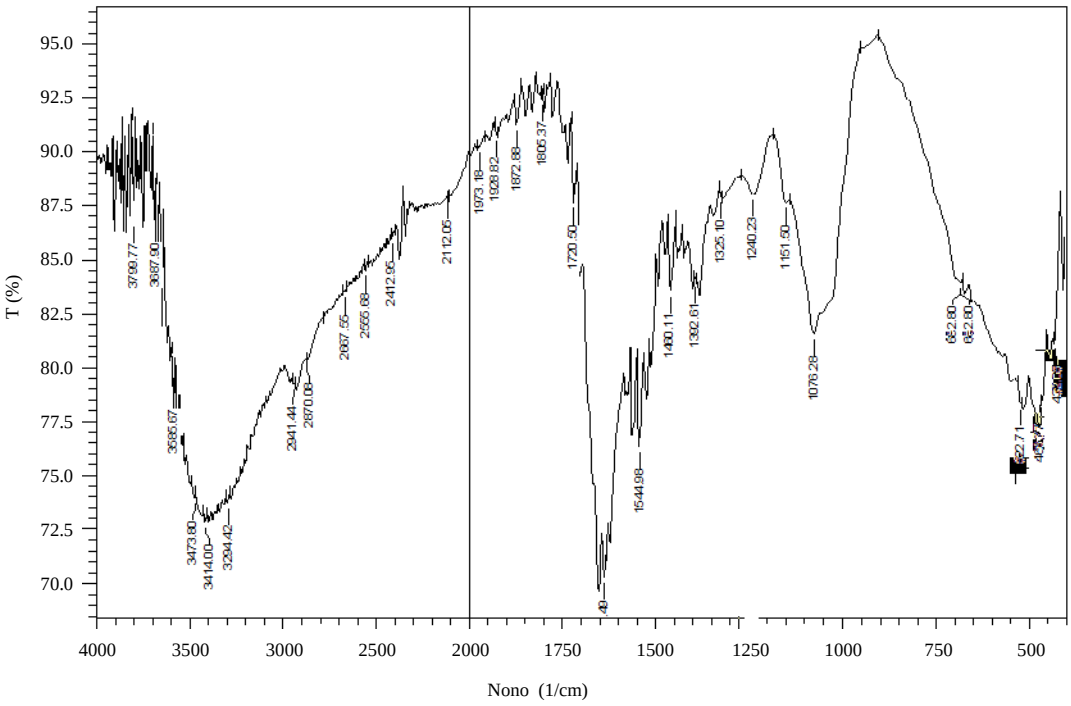

Fig. 4: The FTIR spectra of *T. involucrata* AgNPs

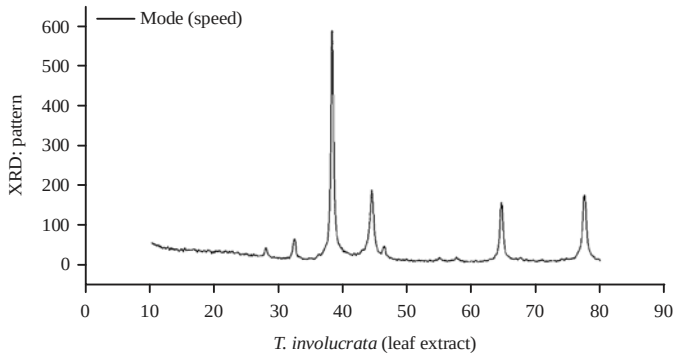

Fig. 5: The XRD patterns recorded from drop-coated films on glass substrate of silver nanoparticles synthesized by *T. involucrata* leaf extract with $AgNO_3$

The band intensities in different regions of spectrum were analyzed by FTIR. The FTIR spectra of *T. involucrata* samples containing silver nanoparticle was depicted in Fig. 4.

The X-ray diffraction pattern of the biosynthesized silver nanostructure produced by the leaf extract was further demonstrated and confirmed by the characteristic peaks observed in the XRD image (Fig. 5). The XRD pattern showed four intense peaks (38.50, 44.50, 65 and 76°) in the whole spectrum of 2θ value ranging from 10-80 and indicated that the structure of silver nanoparticles is Face Centered Cubic (FCC).

The SEM measurements were carried out to determine the morphology and shape of AgNPs. The SEM micrograph (Fig. 6) revealed that, the AgNps were rod shaped and well dispersed without agglomeration. The particle sizes of AgNPs synthesized by *T. involucrata* leaf extract were within 100 nm.

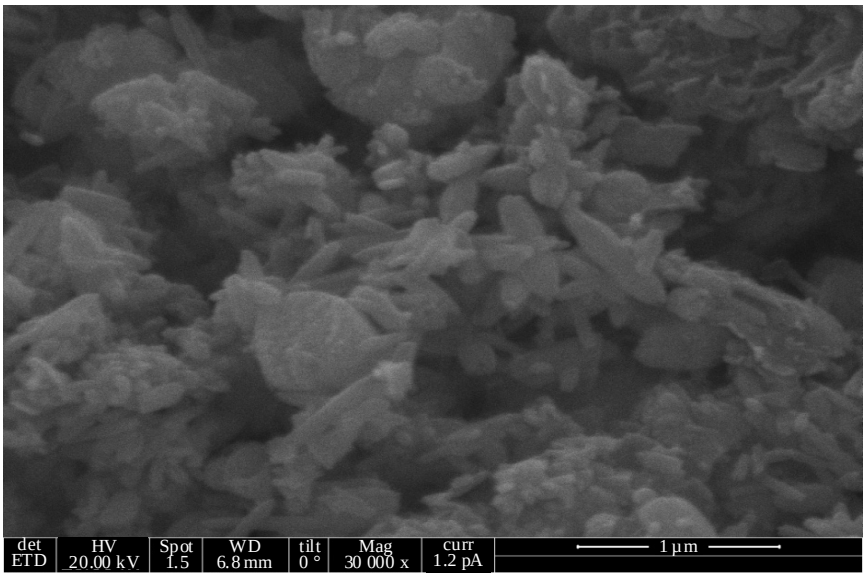

Fig. 6: Scanning microscope image of medium sized AgNPs synthesized by *T. involucrata* leaf extract with $AgNO_3$

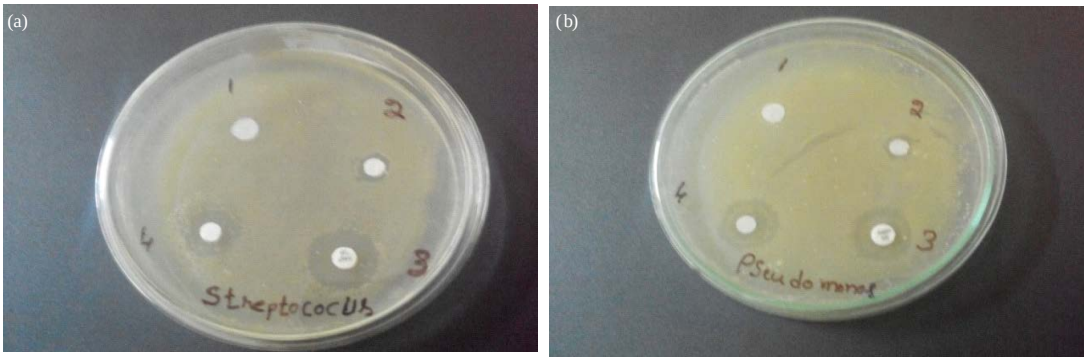

Fig. 7(a-b): Antibacterial activity of *T. involucrata* AgNPs (a) *Streptococcus aureus* and (b) *Pseudomonas aeruginosa*

The antimicrobial activity of AgNPs was tested against two pathogenic bacteria. The AgNPS showed significant antibacterial activity against tested organisms and the extent of antibacterial activity was comparable to that of standard drug (Fig. 7). Antimicrobial effect of AgNPs was found to be dose dependant. The clear inhibitory zone was appeared against *Streptococcus* and *Pseudomona*s at 50 and 100 µg concentration of sample (Fig. 8). This suggests that the synthesized nanoparticle showed good antibacterial activity against human pathogens (Table 1).

Tragia involucrata AgNPs showed slightly increased diffusion of DNA (140.09±18.45) compared to control (111.22±8.78). Apoptotic DNA shown in Fig. 9.

In vitro cytotoxicity effect of *T. involucrata* silver nanoparticle was assessed against MOLT-4 (Human leukemia) cell lines in different time intervals of 24, 48 and 72 h. Four different concentration of extract showed good activity against MOLT-4 (Fig. 10).

Total number of blood vessels was decreased in case of *T. involucrata* nanoparticle (14.0±0.86) compared to control (17.0±1.05). Decreased blood vessels were shown in Fig. 11.

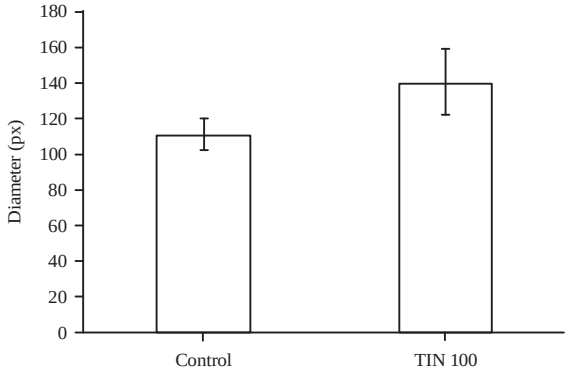

Fig. 8: DNA diffusion assay of AgNPs synthesized by *T. involucrata* leaf extract with $AgNO_3$

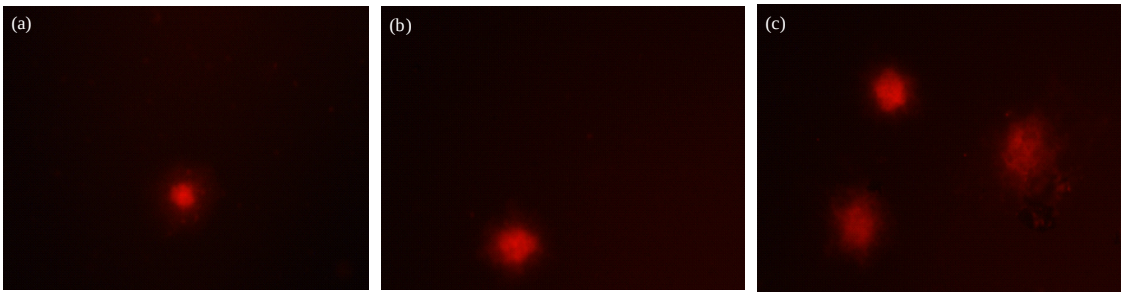

Fig. 9(a-c): Depicts of images of apoptotic cells, (a) Normal cell, (b-c) Apoptotic cells

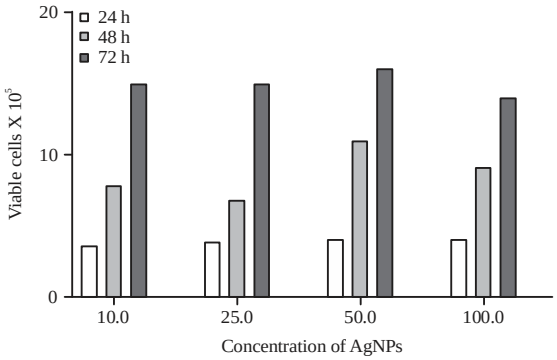

Fig. 10: Percent viability measured on MOLT-4 cells after treatment with AgNPs for 24, 48 and 72 h, by trypan blue method

Table 1: Antibacterial activity of NPs synthesized from *T. involucrata* leaf extract

Organisms and concentration of AgNPs (μg mL^{-1})	Zone of inhibition (mm)	
	AgNPs	Ampicillin (10 μg mL^{-1})
Streptococcus aureus		
50	6.5±0.11	15.0±1.72
100	12.0±1.23	
Pseudomonas aeruginosa		
50	5.0±0.90	14.0±1.88
100	11.5±0.24	

N = 3, Values are expressed as Mean±SD

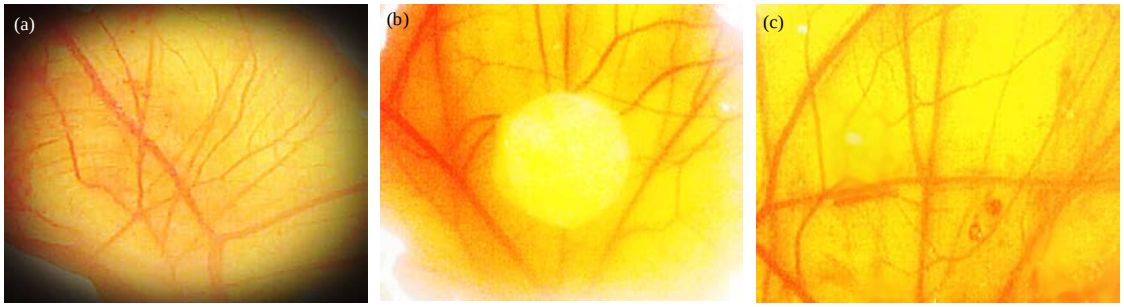

Fig. 11(a-c): Angiogenesis, (a) Control, (b) *T. involucrata* leaf AgNPs on chorioallantoic membrane and (c) CAM with reduced blood vessels

DISCUSSION

The development of easy, reliable and eco-friendly methods for the synthesis of nanoparticles helps to increase interest in study of applications of nanoparticles that are beneficial for mankind (Bhattacharya and Gupta, 2005; Vankar and Bajpai, 2010). Biosynthesis of nanoparticles by plant extracts is currently under extensive exploitation to use them in therapeutics. Medicinal plants possessing either immunomodulatory or antioxidant properties show anticancer activities as well (Nazeema and Sugannya, 2014).

In this study, AgNPs were synthesized from *T. involucrata* extract by the reduction of Ag^+ ions. Yellow colored reaction mixture turned to black color after 24 h of reaction suggesting the formation of AgNPs. This is due to surface plasmon resonance phenomenon. Maximum absorbance at 420 nm is due to the synthesis of silver nanoparticles. Similar results are reported in previous studies in various medicinal plants such as *Citrullus colocynthis*, *Phyllanthus amarus* and *Allium cepa* (Savithramma *et al*., 2011; Satyavani *et al*., 2011; Annamalai *et al*., 2011).

Characterization of nanoparticle was done by using scanning electron microscope. About 50-100 nm rod shaped silver nanoparticle were synthesized and analysed using XRD. Nanoparticles were mostly dispersed and rod shaped. It resemblance to the study of *Cinnamomum zeylanicum* bark powdered extract mediated silver nanoparticle (Sathishkumar *et al*., 2009).

X-ray diffraction was carried out to confirm the chemical composition and crystalline structure of synthesised sliver nanoparticle. In the present study, the XRD pattern showed four intense peaks (38.50, 44.50, 65 and 76°) in the whole spectrum of 2θ value ranging from 10 to 80 and indicated that the structure of silver nanoparticles is Face Centered Cubic (FCC). The formation of silver nanoparticle was confirmed by comparing with the standards. Awwad *et al*. (2013) have reported the similar pattern in silver nanoparticles produced from carob leaf extract.

In the present the study, *T. involucrata* silver nanoparticle exhibited by significant antibacterial activity against *Streptococcus* and *Pseudomonas*. The exact mechanism which silver nanoparticles employ to cause antimicrobial effect is not clearly known (Shameli *et al*., 2012). Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. There is formation of 'pits' on the cell surface and accumulation of the nanoparticles on the cell surface (Prabhu and Poulose, 2012).

Human leukemia cell lines (MOLT-4) were used to evaluate the toxicity of nanoparticle. Cytotoxicity was evaluated by trypan blue assay in different time intervals. MOLT-4 cells were

sensitive to AgNPs at 10 μg mL^{-1} and the viability was increased with the time of incubation. Similar trends were observed for the different doses tested. Toxicity effects of silver nanoparticles reduced by various plants and chemical reductions were investigated against various cells.

Angiogenesis, which is required for physiological events, plays a crucial role in several pathological conditions, such as tumor growth and metastasis. Angiogenesis, which is the formation of new blood vessels from pre-existing ones, is regulated by the balance of many stimulating and inhibiting factors (Otrock *et al*., 2011). The chorioallantoic membrane (CAM) assay has been proved as a reliable *in vivo* model to study angiogenesis and many inhibitors and stimulators of angiogenesis have been examined by this common method (Ribatti, 2010). The AgNPs of *T. involucrata* leaf extract showed significant antiangiogenic effect in CAM assay. The mechanism of action of AgNPs in preventing the angiogenesis is not known. The AgNPs may hamper the blood vessel formation either by up regulating the inhibitors or down regulation of the stimulators. Further studies of AgNPs at molecular level may help in finding out the mechanism by which the AgNPs act on angiogenesis process.

DNA diffusion assay described as a simple, sensitive and rapid method for estimating apoptosis in single cells. Genotoxicity testing plays a crucial role in the evaluation of potential human toxicity, so that hazards can be prevented (Ribeiro, 2008). In this study, DNA diffusion was slightly increased in AgNPs synthesized using *T. involucrata* leaf extract with $AgNO_3$ compared to control. This is the first study in which genotoxic potential of silver nanoparticle was assessed.

CONCLUSION

Tragia involucrata leaf mediated silver nanoparticle showed significant anticancer activity against MOLT-4 cells lines, anti-angiogenic anti bacterial as well as genotoxic potential. Hence, these nanoparitcles can be exploited to produce novel therapeutic agent against various ailments. The study of the unique properties of this substance as well as the mechanism of action can be put to great use for human betterment.

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