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Synthesis of antimicrobial silver nanoparticles by callus cultures and *in vitro* derived plants of *Catharanthus roseus*

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This study highlights the development of a biological method to synthesize silver nanoparticles using the aqueous leaf, callus, and root extracts of *in vitro* derived plants of *Catharanthus roseus*. The antibacterial activity of synthesized silver nanoparticles was assessed against different clinical samples such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Candida albicans*. The highest antibacterial activity was noticed with stabilized silver nanoparticles against all the tested pathogens. Silver nanoparticles synthesized in this process are quiet fast and low cost. Therefore, current study justified the development of eco-friendly process for the synthesis of silver nanoparticles using plant extracts which is an important step in the field of application of nanotechnology. This green chemistry has many advantages such as, quick production, cost effective and easy way to produce nanoparticles.

Key words: Botany, Clinical samples, Dharwad, Green chemistry, India, Karnataka, Nanoparticles, Pharmacy, Pathogens.

Green nanotechnology is gaining more and more attention due to its wide applications in the pharmaceutical and biomedical field (Malabadi *et al.* 2012a, 2012b, 2012c; Shanker *et al.* 2003a, 2003b; Hettiarachchi and Wickramarachchi, 2011; Zhang *et al.* 2008; Song and Kim, 2009;Gardea-Torresdey *et al*. 2002, 2003; Shankar *et al*. 2003a, 2003b, 2004a, 2004b; Nabikhan *et al.* 2010; Geethalakshmi and Sarada, 2010; Farooqui *et al.* 2010; Elumalai *et al.* 2010; Khandelwal *et al.* 2010; Saxena *et al.* 2010; Sharma *et al*. 2007). The advantage of green nanoscience over traditional chemical methods is that they do not use toxic chemicals during synthesis and easily scaled up at the larger scale (Malabadi *et al*. 2012a, 2012b, 2012c; Ghosh *et al.* 2012; Xia *et al.* 2010;

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Zhang *et al.* 2008; Thirumurgan *et al.* 2009, 2010). However, toxicity of using metal oxide during synthesis of nanoparticles is one of the major concerns on the human health (Malabadi *et al.* 2012a, 2012b, 2012c). There has been an extensive research on the chemical synthesis of nanoparticles, but these particles have several potential hazards also, including carcinogenicity, genotoxicity, cytotoxicity, and general toxicity (Song and Kim, 2009 Malabadi *et al.* 2012a, 2012b, 2012c). Engineered nanomaterials like the nanoparticles are so small that they can pass through the skin, lungs and intestinal tract with unknown effects to human health (Song and Kim, 2009; Malabadi *et al.* 2012a, 2012b, 2012c). Metal-based nanoparticles have been linked to both environmental and animal toxicity in a variety of studies (Xia *et al.* 2010; Malabadi *et al.* 2012a, 2012b, 2012c). One of the best example is the use of 500 nm TiO2 particles have some ability to cause DNA strand breakage (Xia *et al.* 2010). Silver has long been recognized as having inhibitory effect on bacterial strains and other microorganisms present in medical and industrial process (Song and Kim, 2009; Malabadi *et al.* 2012a, 2012b, 2012c). The most important application of silver and silver nanoparticles is in medical industry such as topical ointments and creams containing silver to prevent infection against burns and open wounds (Song and Kim, 2009; Geethalakshmi and Sarada, 2010; Sharma *et al.* 2007; Gardea-Torresdey *et al.* 2002, 2003). Another widely used applications are medical devices and implants prepared with silver-impregnated polymers. Several salts of silver and their derivatives are commercially manufactured as antimicrobial agents (Song and Kim, 2009 Malabadi *et al.* 2012a, 2012b, 2012c). In addition to this silver containing consumer products such as colloidal silver gel and silver-embedded fabrics are now used in sporting equipment (Song and Kim, 2009; Nabikhan *et al.* 2010; Malabadi *et al.* 2012a, 2012b, 2012c; Gnanadesigan *et al.* 2011; Shankar *et al.*2003a, 2003b; Geethalakshmi and Sarada, 2010; Mukunthan *et al.* 2011; Warisnoicharoen *et al.* 2011; Vankar and Shukla, 2012; Ghosh *et al.* 2012).

The Madagascar periwinkle, *Catharanthus roseus* belonging to *Apocynaceae* plant family, is a medicinally important plant that produces more than 130 mono-terpenoid indole alkaloids (MIA's) in different organ (Asolkar *et al.* 1992; Jordan *et al.* 1991; Laflamme *et al.* 2001; Mahroug *et al.* 2006; Malabadi *et al.* 2009). It is also grown in many gardens as an ornamental plant. Pharmaceutically valuable secondary metabolites known as terpenoid indole alkaloids have been extracted from the leaves of *C. roseus* plants. The leaves and stem are the source of dimeric alkaloids, vinblastine and vincristine, which are indispensable drugs for a number of anticancer chemotherapies (Chopra *et al.* 1956; Kulkarni *et al.* 1999; Dutta *et al.* 2005; Malabadi *et al.* 2009). On the other hand, roots accumulate the antihypertensive alkaloids, ajmalicine and serpentine (Dutta *et al.* 2005; Malabadi *et al.* 2009). It is cultivated as a medicinal plant for its leaves and roots, which contain anticancer and antihypertensive alkaloids respectively (Rastogi and Mehrotra, 1991; Jordan *et al.* 1991; Kulkarni *et al.* 1999; Dutta *et al.* 2005; Mahroug *et al.* 2006; Malabadi *et al.* 2009). The present study was aimed to investigate the antibacterial activity of silver nanoparticles synthesized from the callus, leaf and root extracts of *in vitro* derived plants of *Catharanthus roseus* by preliminary disc diffusion assay screening. The antimicrobial activity of silver nanoparticle was tested against clinical samples such as *Bacillus subtilis Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Candida albicans*.

Materials and methods

Source of plant material

Catharanthus roseus seeds were collected from Namdhari Seeds Pvt Ltd, Bangalore, Karnataka, India (Malabadi *et al.* 2009). Experimental plants were grown in the Botanical Garden, Karnatak University, Dharwad (Malabadi *et al.* 2009). Germinated seedlings were

grown for at least 2 to 3 months so that we should have enough plant material for the establishment of *in vitro* cultures (Malabadi *et al*. 2009).

Induction of embryogenic tissue

Shoot tips of both experimental plots and field grown plants of *C. roseus* were collected from the Botanical Garden, Karnatak University, Dharwad, India. Shoot tips (0.5-0.8 cm) harvested from mother plants were carefully washed in distilled water (Malabadi *et al*. 2009). They were surface decontaminated sequentially with 0.1% streptomycin (20sec), 70 % (v/v) ethanol (50sec) and 0.1% (w/v) $HgCl_2$ (2 min) and thoroughly rinsed with sterilized double distilled water (Malabadi *et al*. 2009). Transverse- thin sections of 1-2 mm thick were cut from shoot tips and these sections were cultured on MS (1962) basal medium with 3.0% sucrose (Hi media, Mumbai), 0.7% agar (Sigma), 0.5gl^{-1} meso inositol, 1.0gl^{-1}casein hydrosylate, 0.5gl^{-1} L-glutamine, 250 mgl^{-1} peptone, 0.2gl^{-1} p-aminobenzoic acid, and 0.1gl^{-1} biotin (Malabadi *et al*. 2009). The medium was supplemented with 2.0 µM 2, 4-D and TRIA 5 µM as per our published protocol (Malabadi *et al*. 2009). The cultures were raised in 25mm X 145mm glass culture tubes (Borosil) containing 15ml of the medium and maintained in dark for 4-6 weeks at 25± 3^oC with a relative humidity of 55-60%. Nutrient medium without TRIA served as control (Malabadi *et al*. 2009). The pH of the medium was adjusted to 5.8 with NaOH or HCl before Gellan gum was added (Malabadi *et al*. 2009). The media were then sterilized by autoclaving at 121^oC and 1.08 Kg/cm^2 for 15min. L-glutamine, p-aminobenzoic acid and TRIA were filter sterilized and added to the media after it had cooled to below 50^oC (Malabadi *et al*. 2009). All the cultures of *C. roseus* were examined for the presence of different developmental stages of somatic embryos by morphological and cytological observations of callus (Malabadi *et al*. 2009). The cultures showing oval, round, heart shaped embryos were identified and subcultured on the initiation medium for further two weeks for the better development of somatic embryos. The full strength inorganic salts MS basal medium supplemented with 2.0 µM 2, 4-D and 5 µM TRIA (induction medium I) was used as an effective induction medium for producing the embryogenic tissue (Malabadi *et al*. 2009).

Maintenance of embryogenic tissue

The embryogenic tissue showing various developmental stages of somatic embryos was maintained on full strength inorganic salts MS basal medium supplemented with 2.0 µM 2, 4-D and 5 µM TRIA for the proliferation of callus (maintenance medium) (Malabadi *et al*. 2009). The embryogenic tissue was subcultured for every 15 days. All the cultures were maintained under a cool white fluorescent light (100µmol m^{-2} s^{-1}) at 25±3^oC with a relative humidity of 55-60% (Malabadi *et al*. 2009).

Somatic embryo maturation

The white- friable- embryogenic tissue showing different developmental stages of somatic embryos was transferred to maturation medium to induce cotyledonary embryo development (Malabadi *et al*. 2009). The full strength (inorganic salts) MS basal medium supplemented with 3.0% sucrose, 5 µM ABA and 0.8% agar (maturation medium) was used for this purpose (Malabadi *et al*. 2009). All the cultures were again maintained in the dark for 4 to 6 weeks. Microscopic observation was conducted to ensure the development of somatic embryos (Malabadi *et al*. 2009).

Germination and recovery of plantlets

After maturation, the cotyledonary somatic embryos were taken from the cultures for germination. The germination medium used was half strength (inorganic salts) MS basal medium with 0.7% agar without any growth regulators (germination medium) (Malabadi *et al*. 2009). Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlet was based on the presence of an epicotyl. After 2 weeks on germination medium, the plantlets were directly transferred to vermiculite (Malabadi *et al*. 2009). Plantlets were placed in a growth room under a 16 hr photoperiod (50µ mol m^{-2} s^{-1}) for hardening (Malabadi *et al*. 2009). These harvested roots, leaf explants from *in vitro* derived plants and callus cultures were utilized for the synthesis of silver nanoparticles as per our previous published protocols (Malabadi *et al*. 2012a, 2012b, 2012c).

Synthesis of silver nanoparticles

The callus, leaf and roots of *in vitro* derived plants of *Catharanthus roseus* was oven dried at 50° C and ground to make a fine powder. Further, 25 grams of powdered callus, leaf and roots were taken separately in two 250ml beaker containing 100ml of sterile distilled water. Mixture was boiled for 10 min at 100°C (Malabadi *et al.* 2012a, 2012b, 2012c). The callus, leaf and root extracts were collected separately in two beakers by a standard filtration (Whatman filer paper) method. 1mM $AgNO_3$ (silver nitrate) solution was prepared and stored in amber colour bottle (Malabadi *et al.* 2012a, 2012b, 2012c). 10ml of callus, leaf and root extracts were taken in beaker separately and 50ml of 1mM $AgNO_3$ solution was added to the beaker drop wise with constant stirring at 50-60° C and color change was observed (Malabadi *et al.* 2012a, 2012b, 2012c; Linga Rao and Savithramma, 2012). The color change was checked periodically and the beakers were incubated at room temperature for 40 hours (Malabadi *et al.* 2012a, 2012b, 2012c; Linga Rao and Savithramma, 2012). The color change of the callus, leaf and root extracts from yellow to brown indicated the presence and synthesis of silver nanoparticles. The extract contents were then centrifuged at 10,000 rpm for 20 min (Malabadi *et al.* 2012a, 2012b, 2012c; Linga Rao and Savithramma, 2012). The silver nanoparticles were isolated and concentrated by repeated (4-5 times) centrifugation of the reaction mixture at 10, 000 g for 10 min. The supernatant was replaced by distilled each time and suspension stored for the further use. The supernatants were used for the spectrometric UV analysis and for the evaluation of antibacterial activity (Malabadi *et al.* 2012a, 2012b, 2012c; Linga Rao and Savithramma, 2012). The spectrometric analyzed results highlighted the presence and reduction of silver ions in the tested samples. The reduction of silver ions was monitored by measuring the absorbance of the reaction mixture in a range of wavelength from 300 to 600 nm using spectrocolorimeter to find the absorbance peak (Malabadi *et al*. 2012a, 2012b, 2012c).

Stabilization of silver nanoparticles

The protocol for the stabilization of silver nanoparticles has been adopted from previous published papers (Gardea-Torresdey *et al*. 2002, 2003; Shankar *et al*. 2003a, 2003b, 2004a, 2004b; Sharma *et al*. 2007; Malabadi *et al.* 2012a, 2012c) with slight modifications in our experiments. In this method, 0.9 g of polyvinyl alcohol (PVA) was dissolved in 100ml sterile distilled water at 100° C by vigorously stirring to form homogenous solution (Nabikhan *et al.* 2010; Malabadi *et al.* 2012a, 2012c). An aqueous solution of 20 ml of silver nanoparticles synthesized by callus, leaf and root extracts was added with PVA solution (Nabikhan *et al.* 2010). This solution was then allowed to stir in a flask for about 10 min, and then the solution was purged with nitrogen. A fresh solution of $5x10^{-3}$ M was prepared and introduced drop by drop into PVA-$AgNO_3$

solution (Nabikhan *et al.* 2010; Malabadi *et al.* 2012a, 2012c). The solution was then stirred for 15min under inert atmosphere at room temperature of 28^{o}C. Silver nanoparticles were also similarly prepared in the absence of PVA. The color intensity at 420 nm was measured separately for callus, leaf and root extracts stabilized with and without PVA (Nabikhan *et al.* 2010; Malabadi *et al*. 2012a, 2012c).

Screening of antibacterial activity

The screening of antibacterial activity was conducted according to our previously published protocols (Malabadi, 2005; Malabadi *et al*. 2005, 2007, 2010; Malabadi and Vijayakumar, 2005, 2007, 2008; Malabadi *et al.* 2012a, 2012b, 2012c). The test pathogenic organisms used in the investigations of antibacterial activity were *Bacillus subtilis* and *Staphylococcus aureus*, and the gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae* and fungal strain *Candida albicans* (Malabadi, 2005; Malabadi *et al*. 2005, 2007, 2010; Malabadi and Vijayakumar, 2005, 2007, 2008; Malabadi *et al.* 2012a, 2012b, 2012c). Tenfold serial dilution of overnight MH broth cultures were prepared and 100 µl of each dilution were spread onto MH agar plates using a glass spreader (Malabadi, 2005; Malabadi *et al*. 2005, 2007, 2010; Malabadi and Vijayakumar, 2005, 2007, 2008; Malabadi *et al.* 2012a, 2012b, 2012c). The plates were incubated overnight at 37^{o} C. Following the assumption that each living bacterial cell will grow into a separate colony on the plate, the number of cells present per milliliter of the original overnight cultures was calculated (Malabadi, 2005; Malabadi *et al*. 2005, 2007, 2010; Malabadi and Vijayakumar, 2005, 2007, 2008; Malabadi *et al.* 2012a, 2012b, 2012c). The optical density (OD) at 600 nm for each dilution was determined using spectrophotometer, and used to indicate numbers of bacterial cells in cultures for the antibacterial screening and MIC determination (Malabadi, 2005; Malabadi *et al.* 2012a, 2012b, 2012c).

The antibacterial activity was done by disc-diffusion assay method (Malabadi, 2005; Malabadi *et al*. 2005, 2007, 2010; Malabadi and Vijayakumar, 2005, 2007, 2008; Malabadi *et al.* 2012a, 2012b, 2012c). Filter paper discs (Whatman No 3 and 6 mm in diameter) were sterilized by autoclaving. In this method, 1000 µl of silver nanoparticles prepared from callus, leaf and root extracts was mixed in 1.0 ml of sterile distilled water and then applied to sterile paper discs of 5mm diameter (Whatman Filter papers) (Malabadi *et al.* 2012a, 2012b, 2012c). These discs were air-dried under sterile conditions. Similarily 1000 µl nanocomposite of silver nanoparticle and PVA prepared from callus, leaf and root extracts was mixed in 1.0 ml of distilled water and applied to sterile paper disc. These discs were then placed on Muller Hinton Agar swabbed with clinical strains of bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*, and the gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae* and fungal strain *Candida albicans* at a concentration of 10^{6} bacteria/ml for bacteria (Malabadi, 2005; Malabadi *et al.* 2005, 2007, 2010; Malabadi and Vijayakumar, 2005, 2007, 2008; Malabadi *et al*. 2012a, 2012b, 2012c). The plates were incubated at 37^{o} C for overnight. The zone of inhibition was measured in millimeter after 24 h of incubation and recorded. Each extracts of callus, leaf and root were tested in quadruplicate (four discs per plate), with a silver sulphadiazine (1mg ml^{-1}) disc as a reference or positive control (Malabadi, 2005; Malabadi *et al*. 2005, 2007, 2010; Malabadi and Vijayakumar, 2005, 2007, 2008; Malabadi *et al.* 2012a, 2012b, 2012c). The ratio between the diameter of the inhibition zones (mm) produced by callus, leaf and root extracts and the inhibition zone around the disc with silver sulphadiazine (mm) was used to express antibacterial activity. The activity of Silver Sulphadiazine was included in this equation to adjust for plate-to-plate variations in the sensitivity of a particular bacterial strain (Malabadi, 2005).

The antibiotic silver sulphadiazine was included as standard in each assay. Extract-free solution was used as blank control (Malabadi, 2005; Malabadi *et al.* 2005, 2007, 2010; Malabadi and Vijayakumar, 2005, 2007, 2008). The microplates were incubated overnight at 37^{o} C (Malabadi *et al.* 2012a, 2012b, 2012c). As an indicator of bacterial growth, 40µl p-iodonitrotetrazolium violet (INT) (Sigma) dissolved in water were added to the microplate wells and incubated at 37^{o} C for 30 min (Eloff, 1998a, 1998b). MIC values were recorded as the lowest concentration of extract that completely inhibited bacterial growth (Malabadi, 2005; Malabadi *et al.* 2005, 2007, 2010; Malabadi and Vijayakumar, 2005, 2007, 2008; Malabadi *et al.* 2012a, 2012b, 2012c). Since the colorless tetrazolium salt is reduced to a red colored product by biologically active organisms, the inhibition of growth can be detected when the solution in the well remains clear after incubation with INT (Eloff, 1998a, 1998b).

Results and Discussion

Antimicrobial activity of root, leaf and callus cultures of *in vitro* derived plants of *Catharanthus roseus* extracts were determined and results were presented in table-1. During the process of synthesis of silver nanoparticles, the color change was observed from green to brown, by the addition of the aqueous solution of the silver nitrate. This color change was mainly due to the reduction of silver ion to silver which indicated the formation of stable silver nanoparticles. Silver nanoparticles exhibit dark yellowish-brown color in aqueous solution due to the surface Plasmon resonance phenomenon (Ponarulselvam *et al.* 2012). Furthermore, stabilization of silver nanoparticles has played an important role in the antimicrobial activity. In the present study, the highest antimicrobial activity was recorded with stabilized nanoparticles against non-stabilized silver nanoparticles. Therefore, stabilization with polyvinyl alcohol (PVA) has increased the efficiency of antimicrobial activity against tested clinical samples (Table-1) which also confirms with our previous findings of *C. speciosus* and *C. ternatea* (Malabadi *et al.* 2012b, 2012c). The silver nanoparticles prepared are found to be stable over a period of one month at room temperature and showed no signs of aggregation. This might due to stabilization of silver nanoparticles. Generally metal nanoparticles aggregate among themselves and progressively grow into larger clusters and eventually precipitates, deviating from nanoscale (Hettiarachchi and Wickramarachchi, 2011; Malabadi *et al.* 2012b, 2012c). This avoids the effectiveness of synthesized nanoparticles and discourages its applications (Hettiarachchi and Wickramarachchi, 2011; Malabadi *et al.* 2012b, 2012c). Coalescence may be prevented by adding a cluster stabilizer. Synthetic polymers such as polyvinyl chloride, polyvinyl alcohol, and polyvinyl pyrrolidone are already in usage as stabilizing polymers of silver nanoparticles (Hettiarachchi and Wickramarachchi, 2011; Malabadi *et al.* 2012b, 2012c). Due to non-biodegradability and toxicity of synthetic polymers, at presently a considerable attention has been drawn on the use of natural polymers to stabilize silver nanoparticles (Hettiarachchi and Wickramarachchi, 2011; Malabadi *et al.* 2012b, 2012c). Furthermore, especially in medical applications, additional steps are necessary to remove the synthetic polymer after synthesis, which makes the synthesis process much complex and less economic. Once the stabilizer is eliminated there is a tendency to diminish the stability of synthesized silver nanoparticles, altering the effective particle size (Hettiarachchi and Wickramarachchi, 2011; Malabadi *et al.* 2012b, 2012c). Therefore, natural polysaccharides being a environment benign, biodegradable, highly abundant and low cost, they are more favorable to be utilized as a stabilizer for synthesized silver nanoparticles (Hettiarachchi and Wickramarachchi, 2011; Malabadi *et al.* 2012b, 2012c). Recently in another report by Ponarulselvam *et al.* (2012), the leaves of *C. roseus* can be good source for synthesis of silver nanoparticle which shows

antiplasmodial activity against malaria parasiste *Plasmodium falciparum*, and hence this study justified the development of value added products from the medicinal plants *C. roseus* for biomedical and nanotechnology based industries (Ponaruselvam *et al*. 2012). Mukunthan *et al.* (2011) reported successful synthesis of silver nanoparticles using aqueous leaves extracts of *C. roseus*. Therefore, development of a green method to synthesize silver nanoparticles is desired. Furthermore, these green methods are very low cost, fast, efficient and generally lead to the formation of crystalline nanoparticles with a variety of shapes (spheres, rods, prisms, plates, needles, leafs or dendrites), with sizes between 1 and 100nm (Kouvaris *et al*. 2012).

Table 1: Antimicrobial activity of *in vitro* derived plants, and callus cultures of *Catharanthus roseus* on the clinical samples by disc diffusion method

Clinical samples	**Diameter of inhibition zone** (mm)					
	Plant extracts	Silver nanoparticles	Silver Nitrate ($AgNo_3$)	Methanol	Ethanol	Water
Bacillus subtilis	**Root**	**Root**				
	+PVA	20				
	-PVA	10	11	7	1	0
	Callus	**Callus**				
	+PVA	19				
	-PVA	11				
	Leaf	**Leaf**				
	+PVA	24				
	-PVA	7				
Staphylococcus aureus	**Root**	**Root**				
	+PVA	22				
	-PVA	10	9	8	1.5	0
	Callus	**Callus**				
	+PVA	21				
	-PVA	8				
	Leaf	**Leaf**				
	+PVA	21				
	-PVA	10				
Escherichia coli	**Root**	**Root**				
	+PVA	21				
	-PVA	10	11	6	1.5	0
	Callus	**Callus**				
	+PVA	20				
	-PVA	9				
	Leaf	**Leaf**				
	+PVA	23				
	-PVA	12				
Klebsiella pneumoniae	**Root**	**Root**				
	+PVA	21				
	-PVA	10				
	Callus	**Callus**	10	3	1	0
	+PVA	20				
	-PVA	11				
	Leaf	**Leaf**				
	+PVA	21				
	-PVA	12				
Candida albicans	**Root**	**Root**				
	+PVA	20				
	-PVA	11	8	3	1	0
	Callus	**Callus**				
	+PVA	17				
	-PVA	8				
	Leaf	**Leaf**				
	+PVA	24				
	-PVA	13				

These features mainly depend on the process parameters, such as the nature of the plant extract and the relative concentrations of the extract and metal salts reaction, pH, temperature and time of reaction, as well as the rate of mixing of plant extract and metal salts (Kouvaris *et al*. 2012). In our present study, leaf extract derived silver nanoparticles showed the highest toxicity as compared against root and callus extracts. This might be due to the presence of dimeric alkaloids, vinblastine and vincristine in leaf and stem extracts of *C. roseus* which are indispensable drugs for a number of anticancer chemotherapies (Kulkarni *et al.* 1999; Dutta *et al*. 2005; Malabadi *et al.* 2009). On the other hand, roots accumulate the antihypertensive alkaloids, ajmalicine and serpentine (Dutta *et al*. 2005; Malabadi *et al*. 2009). The antimicrobial activity in terms of inhibition zone significantly varied with test microbes and the type of the extracts. This differential antimicrobial activity of silver nanoparticles can be attributed to their differential sizes and shape: the antimicrobial activity increases with decreasing size of the silver nanoparticles (Nabikhan *et al*. 2010; Malabadi *et al.* 2012a, 2012b, 2012c). The results of this study also clearly indicated that silver nanoparticles synthesized from leaf, root and callus extracts of *C. roseus* has many pharmaceutical applications for the control of deadly pathogens (Malabadi *et al.* 2012). The highest antibacterial activity of *C. roseus* are probably due to the presence of mono-terpenoid indole alkaloids (MIA's) in different organ (Jordan *et al*. 1991; Laflamme *et al*. 2001; Mahroug *et al.* 2006; Malabadi *et al.* 2009). In another example, silver nanoparticles were synthesized from calli cells of *Citrullus colocynthis* (Satyavani *et al.* 2011). The callus extract reacted with silver nitrate solution confirmed silver nanoparticle synthesis through the steady change of greenish colour to reddish brown (Satyavani *et al.* 2011). They also confirmed that plant based silver nanoparticles possess considerable anticancer effect compared with commercial nanosilver (Satyavani *et al.* 2011). The reduction of the metal ions through the callus extracts leading to the formation of silver nanoparticles of fairly well defined dimensions. Use of silver nanoparticle should emerge as one of the novel approaches in cancer therapy and when the molecular mechanism of targeting is better understood, the applications of silver nanoparticles are likely to expand further (Satyavani *et al.* 2011). The silver nanoparticles show efficient antimicrobial activity compared to other salts. Therefore, silver is ideally suited for effective control of germs, molds and fungus. Its benefit over the use of antibiotics can be used as a powerful strategy to combat the increasing spread of multidrug resistance resulting from broad use of antibiotics. Therefore, clinical efficiency of antibiotics has been compromised (Ghosh *et al*. 2012). The main biomolecules responsible for nanoparticle synthesis were polyphenols or flavonoids (Ghosh *et al.* 2012). In one of our recent study, the synthesis of silver nanoparticles using whole plant extracts of *Clitoria ternatea* has been reported (Malabadi *et al.* 2012). Antibacterial activity of silver nanoparticles was assessed by using disc diffusion method against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*, since *Bacillus* species and *S. aureus* strains may cause diarrhoea and an enteropathogenic form of *E. coli*, and *Klebsiella* species may cause food poising (Malabadi *et al.* 2012). The results of this study also clearly indicated that silver nanoparticles synthesized from plant extracts of *Clitoria ternatea* has many pharmaceutical applications for the control of deadly pathogens (Malabadi *et al.* 2012). The significant and higher antibacterial activity of *Clitoria ternatea* are probably due to the presence of flavonoids in the plant (Malabadi *et al.* 2005; Malabadi *et al.* 2012). Shanker *et al*. (2003) demonstrated the rapid synthesis of stable silver nanoparticles in high concentration using proteins/enzymes extracted from *P. graveolens* leaf. The reduction of metal ions and stabilization of the silver nanoparticles is believed to occur by an enzymatic process (Shankar *et al*. 2003).

Biological properties of nanoparticles is largely influenced by size, distribution and morphology (Satyavani *et al.* 2011; Malabadi *et al.* 2012a, 2012b; Farooqui *et al*. 2010). There are many applications of nanoparticles in medicine and they play an important role in drug delivery (Malabadi *et al*. 2012a, 2012b). Reduction of Ag (I) to Ag (o) can be achieved by chemical, electrochemical, and phytochemical reduction as well as thermal, ultrasound, microwave, gamma and electron irradiation (Hettiarachchi and Wickramarachchi, 2011). Silver nanoparticles are novel silver compounds composed of clusters of silver atoms developed using nanotechnology. Recently, silver nanoparticles have been used in agriculture to extend the maintenance of asparagus leaves (from 2 to 21 days) and to increase the ascorbate, chlorophyll, and fiber contents of leaves, and decrease seed abscission in borage (*Borago officinalis* L.) (Seifsahandi *et al*. 2011). However, chemical methods of synthesizing silver nanoparticles are simpler than physical methods; they have several disadvantages over physical methods (Hettiarachchi and Wickramarachchi, 2011). Chemical reduction leaves the residual reducing agents and hence the final product needs further purified. Furthermore, chemical methods may associate with environmental toxicity and biological hazards (Hettiarachchi and Wickramarachchi, 2011). Biological methods of nanoparticle synthesis using microorganisms, enzymes, and plant or plant extract has been considered as one of the alternatives to chemical and physical methods. The mechanism for the antimicrobial action of silver ions is not properly understood; however, the effect of silver ions on microbe can be observed by the structural and morphological changes (Vankar and Shukla, 2012). Ghosh *et al*. (2012) reported successful synthesis of silver nanoparticles using *D. bulbifera* tuber extract followed by an estimation of its synergistic potential for enhancement of the antibacterial activity of broad spectrum antimicrobial agents. In another development, preparation of silver nanoparticles have been reported using aqueous extract of lemon leaves (*Citrus limon*) which acts as reducing agent and encapsulating cage for the silver nanoparticles (Vankar and Shukla, 2012). These silver nanoparticles have been used for durable textile finish on cotton and silk fabrics. Remarkable antifungal activity has been observed in the treated fabrics (Vankar and Shukla, 2012). The antimicrobial activity of silver nanoparticles derived from lemon leaves showed remarkable enhancement in activity due to synergistic effect of silver and essential oil components of lemon leaves (Vankar and Shukla, 2012). This investigation demonstrated the extracellular synthesis of highly stable silver nanoparticles by biotransformation using the extracts of lemon leaves by controlled reduction of the Ag^{+} ion to Ag^{O} (Vankar and Shukla, 2012). Further the silver nanaparticles were used for antifungal treatment of fabrics which was tested by antifungal activity assessment of textile material by agar diffusion method against *Fusarium oxysporum* and *Alternaria brassicicola* (Vankar and Shukla, 2012). *Aloe vera* plants have been exploited for the synthesis of silver nanoparticles and gold nanotriangles (Chandran *et al.* 2006). Further tamarind leaf extract have been utilized for the synthesis of gold nanotriangles and studies their potential application in vapor sensing (Ankamwar *et al*. 2005). The mechanism for the antimicrobial action of silver ions is not properly understood; however, the effect of silver ions on microbe can be observed by the structural and morphological changes (Vankar and Shukla, 2012). The silver nanoparticles show efficient antimicrobial activity compared to other salts due to their extremely large surface area, which provides better contact with microorganisms. Therefore, silver is ideally suited for effective control of germs, molds and fungus. Its benefit over the use of antibiotics can be used as a powerful strategy to combat the increasing spread of multidrug resistance resulting from broad use of antibiotics. Therefore, clinical efficiency of antibiotics has been compromised (Ghosh *et al*. 2012).

The antimicrobial activity of silver nanoparticles from lemon leaves showed enhancement in activity due to synergistic effect of silver and essential oil components of lemon leaves (Vankar and Shukla, 2012). Silver is inherently anti-microbial and antibacterial substance. Silver has been widely utilized for thousands of years in human history. Its applications include jewels, utensils, currency, dental alloy, photography and explosives (Mukunthan *et al.* 2011). Among the silvers many applications, its disinfectant property is being exploited for hygienic and medicinal purposes, such as treatment of mental illness, nicotine addiction and infectious diseases like syphilis and gonorrhea (Mukunthan *et al.* 2011). It is also suggested that when DNA molecules are in relaxed state the replication of DNA can be effectively conducted. But when DNA is in condensed form it loses its replication ability hence, when the silver ions penetrate inside the microbial cell the DNA molecules turns into condensed form and loses its replication ability leading to cell death (Vankar and Shukla, 2012). In another report, it has been stated that heavy metals react with proteins by getting attached with thiol group and the proteins get inactivated (Vankar and Shukla, 2012). Further silver at low concentrations does not enter cells, but it is adsorbed onto the bacterial surface just as silver tends to adsorb to other surfaces. Thus, silver ions resist dehydrogenation because respiration occurs across the cell membrane in bacteria rather than across the mitochondrial membrane as in eukaryotic cells (Ghosh *et al.* 2012). Therefore, silver nanoparticles and antibiotics kill the bacteria with different mechanisms (Ghosh *et al.* 2012). Thus, synergistic effect can act as a powerful tool against resistant microorganisms (Ghosh *et al.* 2012). A bonding reaction between antibiotics and silver nanoparticles by chelation ultimately increases the concentration of antimicrobial agents at specific points on the cell membrane (Ghosh *et al*. 2012). This may be attributed due to the selective approach of silver nanoparticles towards the cell membrane that consists of phospholipids and glycoprotein (Ghosh *et al.* 2012). Therefore, silver nanoparticles facilitate the transport of antibiotics to the cell surface acting as a drug carrier (Ghosh *et al.* 2012). As the silver nanoparticles bind to the sulfur-containing proteins of the bacterial cell membrane, the permeability of the membrane increases, facilitating enhanced infilteration of the antibiotics in the cell (Ghosh *et al.* 2012). Recently it was also shown that silver nanoparticles are composed of silver (0) atoms. Silver nanoparticles are larger in size than a silver ion, which makes them react with more molecules, leading to more antimicrobial activity (Ankamwar *et al*. 2005; Chandran *et al.* 2006; Sharma *et al.* 2007; Elumalai *et al*. 2010; Khandelwal *et al.* 2010; Ghosh *et al.* 2012; Mukunthan *et al.* 2011; Geethalakshmi and Sarada, 2010; Gnanadesigan *et al*. 2011; Kouvaris *et al.* 2012; Nabikhan *et al*. 2010; Prabhu *et al*. 2010; Savithramma *et al*. 2011a, 2011b; Saxena *et al.* 2010; Shankar *et al.* 2003a, 2003b, 2004a; Thirumurgan *et al*. 2009, 2010). Therefore, green nanotechnology have potential applications in the biomedical field in terms of cost effectiveness, easy scale up which is an added advantage in controlling the deadly pathogens.

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