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Original Article

SYNTHESIS, CHARACTERIZATION, ANTI BACTERIAL ACTIVITY AND ECOTOXICITY OF SILVER NANOPARTICLES FROM CALLUS EXTRACT OF *JUSTICIA JENDARACIA* (BURM.F)

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ABSTRACT

Objective: Synthesis of silver nanoparticles from callus extract of *Justicia jendaracia (*Burm.f) and the anti bacterial activity,ecotoxicity of the synthesized particles has been studied in the present study.

Methods: The callus was induced in Murashige and Skoog (MS) medium supplemented with growth regulators and evaluated for nanoparticles synthesis. Anti bacterial activity was studied against human pathogenic bacteria *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) was done by well diffusion assay.Ecotoxicity study was studied against *Vigna mungo* under pot assay.

Results: The extract from the callus showed ability of synthesis of nanoparticles with plasmon absorption maxima at 430 nm, spherical particles with the size range of 50–65 nm. The antibacterial activity with these synthesized nanoparticles showed distinct effect on the all the tested strains. Ecotoxicity study reveals that nanoparticles did not cause any distinct effect on soil macronutrients, soil enzyme activity, soil microbial population and plant growth parameters of *Vigna mungo*.

Conclusion. Present study would suggest possible utilization of biogenic silver nanoparticles from the callus extract of *Justicia jendaracia* as the therapeutic agent without causing any undesirable effects to the environment.

Keywords: Callus, *Justicia Jendaracia*, Silver Nanoparticles,Anti Bacterial Activity,Eco Toxicity.

INTRODUCTION

Nanotechnology is currently employed as a tool to explore the darkest avenues of medical sciences in several ways like imaging,sensing, targeted drug delivery, gene delivery systems and artificial implants [1,2]. Hence, nanosized organic and inorganic particles are finding increasing attention in medical applications due to their amenability to biological functionalization. Based on enhanced effectiveness, the new age drugs re-nanoparticles of polymers, metals or ceramics, which can combat conditions like cancer and fight human pathogens like bacteria[3].Silver nanoparticles (Ag-np) are among the most commercialised nanoparticles due to their antimicrobial potential[4].Production of silver nanoparticles can be achieved through different methods[5].Silver nanometal has been used in many consumer applications, mostly because of its recognized inhibitory effect towards many bacterial strains & micro organisms commonly present in medical & industrial process.Production of nanoparticles can be achieved through different methods. Chemical approaches are the most popular methods for the production.However, some chemical methods cannot avoid the use of toxic chemicals in the synthesis protocol [6].Since, noble metal nanoparticles such as silver, gold nanoparticles are widely applied to human contacting areas, there is a growing need to develop environmentally friendly processes of nanoparticles synthesis that do not use toxic chemicals(.Biological systems such as microorganisms and plants have a unique ability to control the structure phase and nano structural topography of the inorganic crystals[7,8].Use of plants for the fabrication of nanoparticles has drawn attention of researchers as a rapid, low cost, eco friendly and a single step method for the biosynthesis of nanoparticles for the first time demonstrated synthesis of gold nanoparticles inside a live plants[9,10]. Similarly alfalfa sprouts were also used for the synthesis of silver nanoparticles [11,12].Research on biosynthesis of metal nanoparticles has been carried out using different plant species and microorganisms has been reported [13-16].

There is now a wider debate about the risks and benefits of the many manufactured nanomaterials and consumer products and this includes consideration of risks to the environment [17]. The unique properties of nanoparticles such as high specific surface area, abundant reactive sites on the surface as a consequence of a large fraction of atoms located on the exterior rather than in the interior of nanoparticles as well as their mobility, could potentially lead to unexpected health or environmental hazards [18,19]. Therefore, organisms, and especially those that interact strongly with their immediate environment such as algae, plants, and fungi, are expected to be affected as a result of their exposure to ENPs. Developmental phytotoxicity of nanoparticles is a critical knowledge gap because nanoparticles entering wastewater streams may predominantly be incorporated intosewage sludge and applied to agricultural fields [20].The present study highlighted synthesis of silver nanoparticles from callus extract of Justicia jendaracia and their anti bacterial activity,ecotoxicity.

MATERIALS AND METHODS

Callus induction

Young healthy shoots of *Justicia jendaracia* were collected during the month between June-July from 3 year old plants growing at the herbal garden maintained by the Department of Biotechnology, Sathyabama University, Chennai, India. Fresh leaves were first washed in running tap water to remove debris and soil from the surface and soaked in 5% (v/v) liquid detergent (Tween 20, Himedia, India) for 10 minutes, then washed under running tap water. Those leaves then surface sterilized with 0.05% (w/v) $HgCl_2$ for 12-15 min and thoroughly rinsed four or five times with sterile distilled water. The explants (10 mm) were excised and placed on solid medium. The basal medium used in all experiment was MS's (Murashige and Skoog) medium with 3% (w/v) sucrose and 0.8 % (W/v) agar (Himedia, India). Depending on the experiment, the basal medium was supplemented with various plant growth regulators as

required.The pH of the media was adjusted to 5.8 ± 0.02 and dispensed in 20 l aliquots into culture tubes (15 x 2.5 cm, Borosil, India) and capped with plugs of non-adsorbent cotton prior to autoclaving at 121º C for 15 minutes. Cultures in all experiments were incubated in the culture room maintained under 16/8 hrs (light/dark) photoperiod at 25 ± 1 º C under cool white fluorescent tubes (Philips, India) at an intensity of 50µ mol $m^{-2}s^{-1}$ and 75-80 % relative humidity. Each experiment was repeated thrice with 10 replicates per treatment.

Preparation of callus extract and synthesis of silver nanoparticles

Synthesis of silver nanoparticles from callus culture was done by modified method of Mude *et al* [21].The callus of one month old was washed with sterile distilled water and 25g. of washed callus was crushed in 100 ml of sterile distilled water using pestle and mortal. The crushed material was finely filtered through whatman No. 1 filter paper and used as source for the synthesis of silver nanoparticles.About 10 ml of the filtered callus extract was added to 90 ml of silver nitrate solution (0.1M) and incubated at 30 C for about 24 hours.

Characterization of silver nanoparticles

Silver nanoparticles synthesis by the callus extract was confirmed by the colour change of the reaction mixture to brown from green color.Nanoparticles were recovered from the reaction mixture by centrifugation at 10000 rpm for 10 minutes (5 times).The collected particles were washed with sterile distilled water and the suspension was subjected for the optical measurements by using UV-Vis spectrophotometer. Scanning electron microscopy was used to record the micrograph images of synthesized nanoparticles.SEM study was carried out in SAIF (Sophasticated analytical instrument facility),Indian institute of technology(IIT),Chennai,Tamiln Nadu,India

Antibacterial activity of synthesized nanoparticles

The anti bacterial activity against drug resistant *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) was done by well diffusion assay. Both the strains were obtained from American type culture collection (ATCC) and maintained on Trypticase soy agar slants. A loop ful of slant culture was inoculated into trypticase soy broth and incubated at 37ºC for 12-16 hrs toreach mild log phase. The respective broth culture was uniformly spread with sterile cotton swabs on sterile Mueller Hinnton Agar Media (Hi-media, India). The wella were made using cork borer and aliquots of silver nanoparticles (aliquots of 50 and 100µg were prepared from concentrated silver nanoparticles) was loaded into the wells. The plates were incubated at 37ºC for 24 hrs.

Dynamic growth curve assay

Tryptic soy broth (Hi media) was used for inocula preparation of the bacterial strain. Cultures was inoculated from fresh slopes and incubated in 100 ml of sterile tryptic soy broth with the different concentration of silver nanoparticles (5,25,50,75 and 100 µg/ml prepared in deionized water)under shaking at 37 ºC and every 5 hour interval, the broth withdrawn from the flask and the optical density was measured at 640nm

Minimum Inhibitory concentration

To determine minimum inhibitory concentration, different aliquots of Ag silver nanoparticles (aliquots were prepared from final concentrated synthesized nanoparticles as 25, 50, 75, 100 µg/ml) was added into 12 hrs trypticase broth culture and incubated at 37ºC for 24 hrs. The tubes were observed for turbidity after the incubation period.

Eco toxicity studies

Effect on soil heterotrophic microbial population

Soil sample collection

Soil sample was collected from Paddy field from Porur, Chennai, Tamil Nadu, India in sterile polythene bag, kept in ice box and brought to the laboratory

Treatment

The collected soil sample was finely sieved through nylon mesh and 100g of the sieved soil sample was transferred to 250ml of sterile plastic container. 100µg of synthesized nanoparticles was added to the soil sample and mixed well using sterile glass rod. Incubated at 37ºC for 1 month. Control and 3 replicates were maintained. After the incubation period 1 g of well homogenised treated soil was dissolved in 99ml of sterile distilled water kept under shaking condition for 30 mins serial dilution was carried out from this homogenised soil sample. 0.1ml aliquot of the sample was spread plate on nutrient agar and potato dextrose agar. The seeded plates were incubated at 37ºC (bacteria) for 24 hrs, fungi 28ºC for 5 days. The colony count was recorded after the incubation period

Table 1- Effect of plant growth regulators as MS supplements on callus induction from the leaf explants of *Justicia gendarussa* after 6 weeks.

Sl.No.	Different concentration and combination of PGRS used	Percentage of Response	Nature of callus
1.	MS + 0.5 mg/l NAA + 1.0	68.2 ± 0.8	C, G, Md
2.	mg/l BA	74.7 ± 0.2	C, G, Lg
3.	MS + 1.0 mg/l NAA + 1.0	84.2 ± 0.7	C, G, Lg
4.	mg/l BA	87.5 ± 0.4	C, G, Md
5.	MS + 1.5 mg/l NAA + 1.0	80.3 ± 0.5	C, G, Md
6.	mg/l BA	78.6 ± 0.3	C, G, Lg
7.	MS + 2.0 mg/l NAA + 1.0	66.4 ± 0.6	C, P, Lg
8.	mg/l BA	72.4 ± 0.5	C, G, Lg
9.	MS + 2.5 mg/l NAA + 1.0	68.6 ± 0.3	C, P, Md
10.	mg/l BA	72.8 ± 0.9	C, G, Lg
	MS + 3.0 mg/l NAA + 1.0 mg/l BA		
	MS + 1.0 mg/l NAA + 2.0 mg/l BA		
	MS + 1.5 mg/l NAA + 2.0 mg/l BA		
	MS + 2.0 mg/l NAA + 2.0 mg/l BA		
	MS + 2.5 mg/l NAA + 2.0 mg/l BA		

Values are mean ± SD Lg – Large, Md – Moderate, C- Compact, G-Greenish White, P – Pale green.

Effect on soil macronutrients

Effect of respective nanoparticles on soil macronutrients total nitrogen, total phosphoroua and total potassium was studied adopting standard soil testing method.

Effect on soil enzyme activity

Toxic effect of nanoparticles on soil enzyme mainly alakaline phosphatase and urease activity was studied with spectrophotometric assay using soil homogenate [22].

Effect of nanoparticles on plant growth parameters of Vigna mungo

Collection of seeds and Nanoparticle treatment

Healthy seeds of *Vigna mungo* were collected from Agriculture department in sterile polythene bags and all seeds were cleaned thrice in sterile distilled water and immersed in the synthesized Nanoparticle suspension for 1 hr. After the treatment, the treated seeds were allowed to shade dry.

Pot assay

The pots of 14 cm diameter and 12 cm in height were filled with the fertile loam soil upto ¾ th height of the pot. The treated seeds were seeded in the respective pots. Control and replications were maintained in each treatment. Water was sprinkled on the pots regularly. The seedling emergence was observed periodically and the shoot length and leaf surface area were noted after periodic time intervals.After 30th day of treatment, the amount of chlorophyll present in the leaves was estimated by standard method [23].

RESULT AND DISCUSSION

Callus induction

Leaf explants when cultured on MS medium supplemented with α-naphthalene acetic acid (NAA) or indole-3-butyric acid (IBA) or N^6-benzyl adenine (BA) produced no callus. At 1.5-2.5 mg/L BAP, moderate amount of callus was obtained. In MS medium augmented with 1.5 mg/L and 2.0 mg/L BA, leaf explants with midrib initially showed callus formation but after 8 weeks it gradually turned black and died. High frequency callus formation was obtained from leaf explants on MS medium supplemented with NAA-BA and NAA-KIN. The callus was compact, nodular and pale green. High frequency callus formation was obtained from leaf explants on MS medium supplemented with 2,4-D and BA and also with 2,4,5-T and BA. The leaf callus when transferred MS medium with 2 mg/L 2,4-D and 0.75 mg/L BA or 2.5 mg/L 2,4,5-T and 0.5 mg/L BA produced friable callus. The compact callus, when sub cultured on to B_5 medium with 0.75 mg/L 2,4-D and 0.2 mg/L NAA generated highest frequency of friable callus (Table 1,Figure 1).

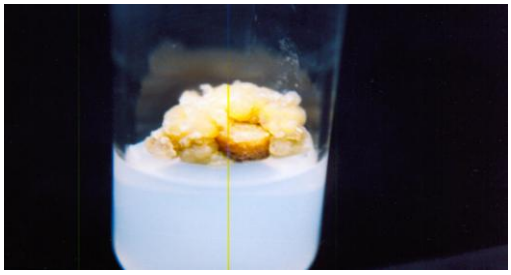

Fig.1: Callus culture of *Justicia gendarussa* grown in MS media

Synthesis and anti bacterial activity of silver nanoparticles

This is the first report of synthesis of silver nanoparticles from callus extract of *Justicia jendaracia.*Synthesis of silver nanoparticles was primarily confirmed by colour change of the reaction mixture to brown from green colour which was observed during eighth hour of incubation.Further synthesized silver nanoparticles were characterized by UV-Vis spectrophotometer, a strong broad surface Plasmon peak located at 430 nm (fig.2), The surface plasmon band remains in the range of 420 -440nm throughout the reaction period that suggesting that the particles are dispersed in the aqueous solution with no evidence for aggregation after complete of the reaction.The solution was extremely stable even for several weeks after reaction, it is known that silver cations area higly reactive and tend binds strongly to electron donar groups containg sulphur, oxygen or nitrogen.The primordial assay of silver nanoparticles was performed by EDAX and SEM. (fig.3) shows the EDAX spectrum of the silver nanoparticles. Strong signals from the silver particles were observed (42.44% in mass), while weaker signal from C, O and S atoms are also recorded. The SEM micrograph at 30000 times magnification was shown in (fig: 4). In this micrograph observed spherical nanopartilces in the size range of 50-60nm

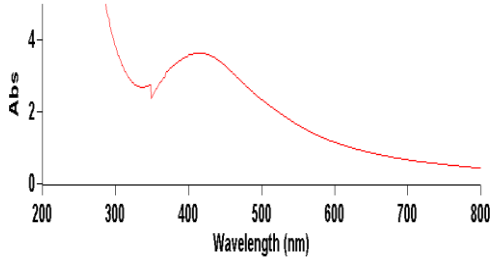

Fig.2:UV-Visible spectra of synthesized silver nanoparticles

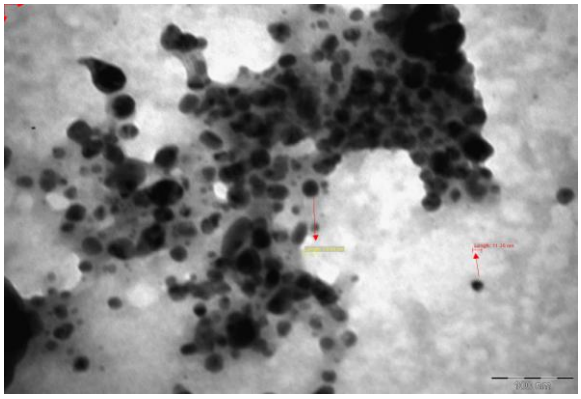

Fig.3: Scanning electron micrograph of nanoparticles

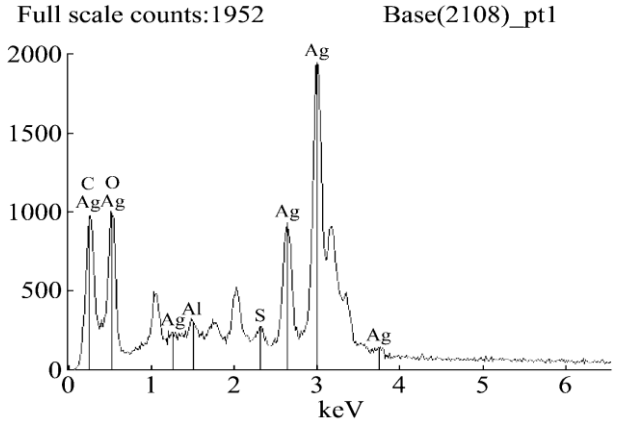

Fig.4: EDAX spectra of synthesized nanoparticles

.Reduction of silver into silver nanoparticles by the callus extract due to the presence of variable bioactive compounds. Polyphenols like tannic acids are efficient reducing agent in the synthesis of silver nanoparticles. plants are generally rich in polyphenolic compounds Thus phenolic derivatives may determine the nanoparticle synthesis by the plants [24].*Justica gendarussa* (Burm. f) (Family- Acanthaceae, common name Black adusa) is a evergreen shrub found throughout India and also in all Asian countries like Malaysia, Indonesia, Sri Lanka and Bangladesh. The plant is used in traditional medicinal practice for chronic rheumatism, inflammations, bronchitis, vaginal discharges, dyspepsia, eye diseases and fever [25,26]. *Justicia* has been found to contain lignans, naturally occurring phenolic dimers and triterpenoids which might be responsible for reduction of silver ions into silver nanoparticles. Similar work in callus mediated synthesis of silver nanoparticles has been reported from *Lycopersicon esculentum* [24], *Carica papaya* [21], *Costus speciosus* [27].

Synthesized particle were evaluated against pathogenic bacteria *Escherichia coli (ATCC 25922*) and *Staphylococcus aureus (ATCC 29213*) adopting well diffusion assay with 50 and 100 µg/ml concentration. The antibacterial activity with these synthesized nanoparticles showed distinct effect on both the tested strains. Zone of inhibition against *E.coli* was found to be 17.0 and 21.0mm at 50 and 100 µg/ml whereas 15.0 and 20.3 mm was recorded in *Staph.aureus* (Table 2).and minimum inhibitory concentration (MIC) was found to be 10 and 11.3 µg/ml for *E.coli* and *Staph.aureus* (Table 3). Synthesized silver nanoparticles showed distinct effect on dynamic growth curve of all the tested bacteria during all the tested time periods and more effect was observed in high concentration (Table 4,5). Increasing concentration of nanoparticles progressively inhibited the growth of all the tested bacteria The lag phase of all the tested bacteria in the nanoparticles treatment was found to be more prolonged than the control which reveals the stability of the nanoparticles used in this study.

Table 2. Inhibitory zone (mm) with *S.aureus* and *E. coli* with different concentration of silver nanoparticles

S.No	Concentration (µg/ml)	Zone of inhibition (mm)	
		Staph.aureus	*E.coli*
	125	10.0	11.0
	250	12.0	15.0
	375	15.0	17.0
	4100	20.3	21.0

Table 3.Minimum inhibitory concentration (MIC) of silver nanoparticles against tested bacteria

S.No	Tested bacteria	MIC (µg/ml)
1	E.coli	10.0
2	Staph.aureus	11.3

Table 4.Optical density of E.coli grown in LB broth containing different concentration of silver nanoparticles

S.No	Concentration	optical density at Different time period (Hourss)				
	(µg/ml)	0	5	10	15	20
1	Control	0.0	0.14	0.23	0.42	0.34
2	5	0.0	0.11	0.14	0.19	0.17
3	10	0.0	0.07	0.12	0.15	0.16
4	25	0.0	0.06	0.08	0.13	0.14
5	75	0.0	0.02	0.03	0.05	0.07
6	100	0.0	0.01	0.02	0.04	0.06

Table 5. Optical density of Staph.aureus grown in LB broth containing different concentration of silver nanoparticles

S.No Concentration	optical density at different time period (hourss)				
(µg/ml)	0	5	10	15	20
1Control	0.0	0.17	0.28	0.42	0.43
25	0.0	0.14	0.21	0.24	0.20
310	0.0	0.10	0.12	0.15	0.16
425	0.0	0.07	0.09	0.11	0.13
575	0.0	0.03	0.05	0.07	0.09
6100	0.0	0.02	0.04	0.05	0.07

Eco toxicity studies

Soil microbial population, macronutrients and soil enzyme activity

Concersn over the potentially harmful effects of such nanoparticles has stimulated the advent of nanotoxicology as a unique and significant research discipline. However, the majority of the published nanotoxicology articles have focused on mammalian cytotoxicity or impacts to animals and bacteria, and only a few studies have considered the toxicity of MNMs to plants. Developmental phytotoxicity of NMS is a critical knowledge gap because nanoparticles entering wastewater streams may predominantly be incorporated intosewage sludge and applied to agricultural fields [28]. In the present study, biogenic silver impact on soil parameters, plant growth parameters of *V.mungo* has been studied.Soil microbial population in nanoparticles treatment did not show any distinct effect and there was no significant difference was observed between control and treatment(Table 6)As in microbial population enzyme activity was not affected. In the nanoparticles treatment, alkaline phosphatase and urease reveals similar activity as in control(Table 7)

Table 6. Effect of biogenic nanoparticles on soil heterotrophic microbial population (CFU/g)

S.No	Microorganism	Treatment	Colony count (CFU/g) Pre Treatment	after treatment
1	Bacteria	Control	$14.4X10^4$	$10.5X\ 10^7$
2		Ag NPs	$12.5X\ 10^4$	$10.0X10^7$
3	Mold	Control	$7.5X\ 10^3$	$10.7X\ 10^6$
4		AgNPs	$6.9X\ 10^3$	$11.0X\ 10^6$
5	Yeast	Control	$23.4X\ 10^2$	$41.4X\ 10^4$
6		AgNPs	$24.0X\ 10^2$	$46.5X10^4$

Table 7. Effect of nanoparticles on soil enzyme activity (U/ml)

.No	Treatment	Enzyme activity (U/ml)	
		Pre Treatment	after treatment
		APU	APU
1	Control	5.12.5	7.73.3
2	Ag NPs	5.72.8	6.03.5

AP- alkaline phosphatase, U- urease

Similar observation could be observed in soil macronutrients.(Table 8). Eco toxic effect of various nanoparticles and nanodevices such as photoactive ZnO or TiO2 [29], bactericide Ag, hydrophobic Carbon nanotubes [30] and fullerenes or Cadmium oxide particles, Au and Iron oxide NPs [31] reported.

Phytotoxicity of the synthesized nanoparticles has been studied with *Vigna mungo*. As compared with algae and fungi, plants might also be exposed to nanoparticles in atmospheric and terrestrial environments. Airborne nanoparticles will be attached to leaves and other aerial parts of plants whereas roots will interact with waterborne or soil-material-associated nanoparticles. Therefore, one can expect that plant communities with higher leaf area indexes (LAI) will also have a higher interception potential for airborne ENPs [33]. The phytotoxicity of NPs was evaluated by the seed germination technique. The germination index has been extensively used as an indicator of phytotoxicity in soils [22]. In the present study, 96.0 % seed germination was recorded in the nanoparticles treatment. Similar report of biogenic silver nanoparticles effect on seedling emergence and growth parameters of economic important plants reported by Karthick Raja Namasivayam and Chitrakala [34].Plant growth parameters of green gram was not affected in nanoparticles treatment. The shoot length, leaf surface area, number of new branches emerged and total chlorophyll content of the test plants were measured at periodic time interval(Table 9) It was observed that all the tested plant growth parameters of the plants were increasing continuously from the day of seedling emergence as in control.

CONCLUSION

silver nanoparticles from callus of *Justicia jendaracia* (Burm.f) induced in MS media supplemented with 2,4-D and BA and also with 2,4,5-T and BA induced callus and the extract derived from the callus reduced silver into silver nanoparticles and showed distinct anti bacterial activity against human pathogenic bacteria. Ecotoxicity study revealed less toxic effect on soil parameters which would suggests the possible utilization of biogenic silver as the therapeutic agent without causing any undesirable effects to the environment.

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