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BACTERIOSTATIC ACTIVITIES OF ANTIOXIDANT SILVER NANOPARTICLES PREPARED FROM LEAVES OF *AZADIRACHTA INDICA* AND *PSIDIUM GUAJAVA*

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ABSTRACT

Silver nanoparticles, developed from leaf of *Azadirachta indica* and *Psidium guajava* were examined for their antioxidant and antimicrobial properties. Among the five standard antioxidant assay protocols (viz. ABTS radical decolorization assay, DPPH radical decolorization assay, assay for total phenolis content, FRAP assay and hydroxyl radical scavenging assay) used in the present study, nanoparticles showed equivalent antioxidant activities with the aqueous leaf extracts. A likely chelation of the less polar biomolecules with Ag^+ ion during nanoparticle formation was also indicated. Nanoparticle formation also improved the bacteriostatic properties in terms of zone of inhibition against all the five bacteria (viz. *Escherichia coli*, *Klebsiella aerogens*, *Staphylococcus aureus*, *Salmonella typhi* and *Bacillus cereus*) tested in the study. Improvement in bacteriostatic activities of the nanoparticles against the above mentioned microorganisms clearly indicated potential use of the nanoparticles against food borne bacteria for application in favor of humans.

KEYWORDS: Antioxidant, Bacteriostatic, Nanoparticles, *Azadirachta indica*, *Psidium guajava*.

INTRODUCTION

Herbal drugs, the gift of nature constitute a major part in all traditional systems of medicines., Mankind has exploited nature since ancient times for all kind of useful products and enjoyed the colors, flavors and fragrances of flowers, fruits and other plant parts.[1] Medicinal plants also embody a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many effective and influential drugs.[2] Medicinal plants are the essence of Ayurveda and Ayurvedic treatments. In India, the salutary use of herbs dates back to the vedic period.[3] The Rigveda has documented about 67 medicinal plants, Yajurveda documented 81 species and Atharvaveda 290 species. Today, about 70,000 to 80,000 plant species are used for medicinal or aromatic purposes globally. Some common traditional medicinal plants are *Ocimum tenuiflorum* (tulshi), *Justicia adhatoda* (basak leaves), *Andrographis paniculata* (kalmegh), *Nyctanthes arbour-tristis* (shiuli leaves), *Azadirachta indica* (neem) and *Psidium guajava* (guava leaves). Silver nanoparticles were proven to be most efficient as they possess good antimicrobial and antioxidant activities. Plant extract was also used for the synthesis of metallic nanoparticles because it was proved to be less toxic and also need less refinement as compared to chemical methods.[4]

The field of nanotechnology is one of the most active research areas in modern materials science. Nanoparticles exhibit new or improved properties based on specific characteristics such as size, distribution and morphology. The term "nanoparticles" is used to describe a particle with size in the range of 1 nm-100 nm, at least in one of the three possible dimensions. In this size range, the physical, chemical and biological properties of the nanoparticles changes in fundamental ways from the properties of both individual atoms/molecules and of the corresponding bulk materials.[5] Silver nanoparticles are of interest because of the unique properties (e.g., size and shape depending optical, electrical and magnetic properties) which can be incorporated into antimicrobial applications, biosensor materials, composite fibers, cryogenic superconducting materials, cosmetic products and electronic components.[6] Recently, nanoparticle synthesis is among the most interesting scientific areas of inquisition, and there is growing awareness to produce nanoparticles using environmentally friendly methods (green chemistry).

Azadirachta indica (Neem) is a fast growing evergreen and popular tree widely cultivated in India. It has been used for the treatment of various diseases and disorders as mentioned in Ayurvedic medicine for 4000 years.[7] Bioactive principles present are azadirachtin, meliacin, gedunin, salanin, nimbin, valassin and many others.[8] Extracts of *Psidium guajava* (Guava) leaves, pulp and seeds are used to treat respiratory and gastrointestinal

disorders and as an antispasmodic, anti-inflammatory, as a cough sedative, anti-diarrheic, in the management of hypertension, obesity and in the control of diabetes mellitus.[9] In the present study, an approach was planned to synthesize silver nanoparticles from leaves of the two medicinal plants viz. *Azadirachta indica* (Neem) and *Psidium guajava* (guava), grown by organic farming. The ensuing silver nanoparticles were analyzed for any differences in antioxidant potential vis-a-vis water extract of the leaves. The antimicrobial activity of the silver nanoparticles vis-a-vis leaf extracts were also delineated against the five different food borne pathogenic bacteria viz. *Escherichia coli, Klebsiella aerogenes, Staphylococcus aureus, Salmonella typhi* and *Bacillus cereus*.

MATERIALS AND METHODS

Preparation of leaf extract

Fresh leaves of *Azadirachta indica* (Neem) and *Psidium guajava* (guava) were collected from local farms engaged in organic farming only. The leaves (designated as AI and PG, respectively) were inspected for any irregularity and washed with distilled water thoroughly. 5 gms of wet leaves were chopped into small pieces and soaked with 50 ml of water. Then the mixture was warmed at 50^{o}C for 5 minutes. After cooling, the leaves were macerated in a mortar and pestle and the admixture was filtered through Whatman 1 filter paper. The filtrates were stored at 4^{o}C for further studies.

Preparation of silver nanoparticles

Ten ml of the leaf extract was added to 90 ml of aqueous solution of different concentrations of silver nitrate solution ($AgNO_3$) for reduction of silver nitrate into metallic silver and maintained in the incubator (28±2^{o}C) in static condition and the completion of the reaction was carried out for a period of 2 h. Optimum extract-to-silver ratio was ascertained by examining the UV-Vis spectra of the ensued products. The colourless silver nitrate solution is changed from pale yellow to dark red and finally dark brown colour which indicated formation of silver nanoparticles.

UV-Vis spectroscopic analysis

Double beam UV-Vis spectrophotometer (Model - Systronics 2202) was used to obtain the absorption spectra of the silver nanoparticles formed in the solution. The sample was scanned for wavelength range from 320nm to 680nm. The bio reduction of silver ions in the solution was monitored by sampling the aqueous component at different time intervals. A control reaction mixture was also maintained with only leaf extracts and was scanned with identical parameters.

ABTS radical decolorization assay

The assay was performed using a previously described procedure.[10] The oxidant was generated by persulfate oxidation of 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid. The oxidant solution was mixed with the sample/standard solutions in such a way that total volume of the solution reached 1 ml. The absorbance at 734 nm in a Systronics spectrophotometer (model – 2202) was read at room temperature, 4 minutes after mixing. The results were expressed as Gallic acid equivalents.

DPPH radical decolorization assay

The assay was performed using a previously described procedure.[10] DPPH solution (0.1 mM) was mixed with sample/standard solution and the decrease in absorbance of the mixture after 20 minutes of incubation in the dark was monitored at 517 nm in a Systronics spectrophotometer (model – 2202). The results were expressed as Gallic acid equivalents.

Estimation of total phenolics content

Total phenolics compound contents were determined by the Folin-Ciocalteau method.[11] The samples/standards were mixed with Folin-Ciocalteau reagent (1:10 diluted with distilled water) for 5 min and aqueous sodium carbonate (1 M) was then added. The absorbance of the reaction mixture was then measured at 765 nm in a UV-Vis spectrophotometer (model – Systronics 2202). Gallic acid was used as standard. The results were expressed in terms of gallic acid equivalent/gm sample.

Ferric reducing antioxidant power: FRAP

Ferric reducing antioxidant power of the samples was estimated with a previously described procedure.[11] Briefly, a maximum of 100 µl of extract solution or standard was mixed with 1.9 ml of FRAP reagent and incubated at 37^{o}C for 30 mins. FRAP reagent was prepared by mixing 0.1 M aqueous acetate buffer (pH 3.6), TPTZ solution and ferric chloride solution. After the stipulated time period, absorbance was measured at 593 nm in a UV-Vis spectrophotometer (model – Systronics 2202). Gallic acid is used as standard. Results were expressed as Gallic acid equivalents (GAE).

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging potentials of the samples were estimated with a previously described procedure.[12] Briefly, 10 mM each of $FeSO_4.7H_2O$, EDTA, 2-deoxy-D-ribose and H_2O_2 solutions were prepared in water. Each solution of above four with sample/standard solution was mixed in a test tube to get a final volume of 1 ml and incubated at 37^{o}C for 90 mins. H_2O_2 solution was added last. After the incubation, 2.8% (w/v) aqueous TCA solution and 1% (w/v) aqueous TBA solution were added to the reaction mixture and kept at boiling water bath for 20 mins. Development of the pink chromophore was measured at 532 nm in a UV-Vis spectrophotometer (model – Systronics 2202). Results were expressed as Gallic acid equivalents (GAE).

Antimicrobial activity

The antibacterial activity was measured by agar well diffusion method.[13] Each bacterial isolates was previously grown on sterile Muller Hinton Agar (HiMedia M173) plate at 35°C for 24 hours. After that,

each of the isolates was inoculated with 100µl of standardized inoculums of each bacterium (in triplicates) and spread with sterile cotton swabs. Wells are 6 mm sizes were made with sterile borer into agar plates containing the bacterial inoculums. Different working dilutions of the leaf extracts as well as the silver nanoparticles synthesized from the extracts were prepared in sterile water. From these different dilutions, 50µl solution was poured into the wells of the respective culture plates. The plates thus prepared were left at room temperature for ten minutes allowing the diffusion of the extract into the agar. After incubation for 24 hrs at 35°C, the plates were observed. If antibacterial activity was present on the plates, it was indicated by an inhibition zone surrounding the well containing the different dilutions of extracts and nanoparticles. The zone of inhibition was measured and expressed in millimeters. Antibacterial activity was recorded if the zone of inhibition was greater than 6 mm. The antibacterial activity results were expressed in term of the diameter of zone of inhibition and <9mm zone was considered as inactive; 9-12mm as partially active; while13-18mm as active and >18mm as very active.

RESULTS AND DISCUSSION

Silver nanoparticles exhibit yellowish brown color in aqueous solution due to excitation of surface Plasmon resonance.[14] On mixing the light colored leaf extracts with aqueous solution of the aqueous Ag-ion complex, a change in the color from lighter colors to darker colors were observed (Fig. 1). It was due to the reduction of Ag^+ which indicates the formation of silver nanoparticles.[15]

(a) (b)

Fig. 1- Formation of silver nanoparticles with (a) AI and (b) PG leaf extracts, as observed by visual confirmation.

The formation of the silver nanoparticles was further confirmed by UV-visible spectroscopy. UV-visible spectra shows absorption in the range of 300-700 nm for the plant extract and the plant-derived silver nanoparticles show a distinct absorption at that range. Absorption spectra at different wave lengths ranging from 300-700 nm exhibited peaks at around 357.6 nm for AI leaves and PG leaves. However after nanoparticle formation, the peaks were shifted (red shift) to 440 nm after 30 mins and 1 hour, respectively for AI extracts. PG extracts showed red shift to 439.2 nm after nanoparticle formation. Earlier studies also reported synthesis of silver nanoparticles by indication with red shifts in UV visible spectrum.[16] Faster bio-reduction methods were also reported to produce silver nanoparticle within an hour of reaction.[17]

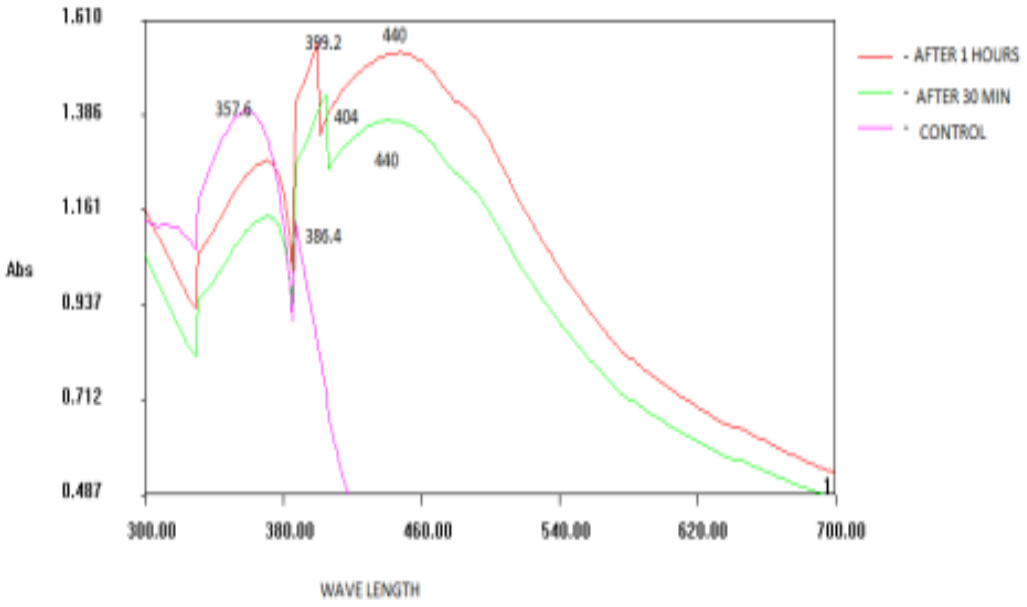

Fig. 2 - UV-Vis spectra of leaf extract of AI (control) and silver nanoparticles prepared thereof after 30 mins and 1 hour.

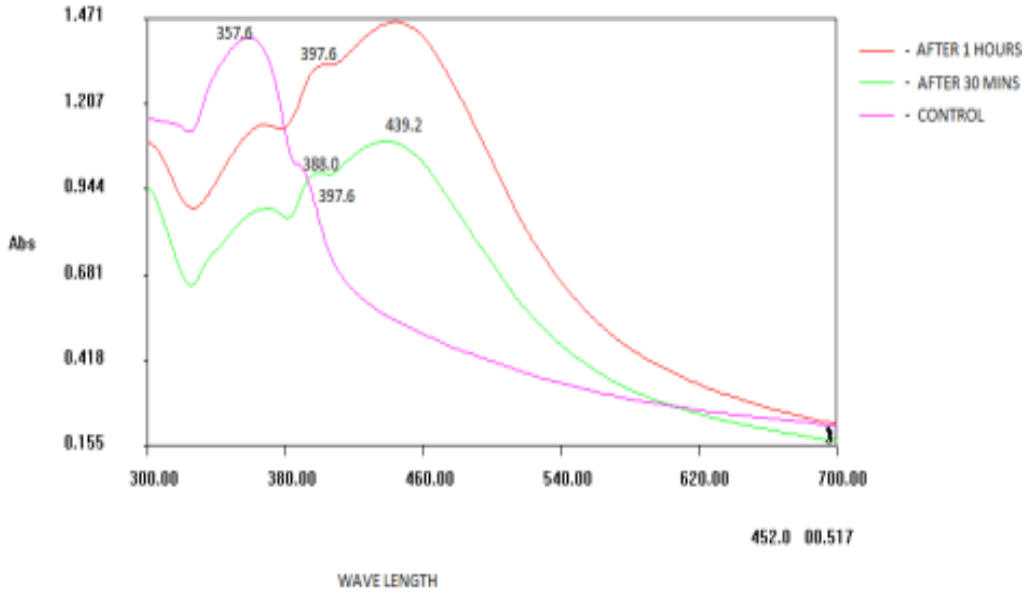

Fig. 3 - UV-Vis spectra of leaf extract of PG (control) and silver nanoparticles prepared thereof after 30 mins and 1 hour.

The results of antioxidant assays were furnished in Table 1. It was observed that DPPH radical neutralization potential of the control deteriorated after nanoparticle formation, although in the ABTS assay, it improved radically. The radical scavenging activities like ABTS assay and DPPH assay is generally used to indicate antioxidant potential of plant extracts. However, ABTS assay is performed in aqueous medium (i.e. aqueous buffer), whereas DPPH assay is based on non-aqueous less polar medium (i.e. alcohol).[18] The extracts prepared in the present study might contain both polar and non-polar antioxidant biomolecules as indicated by the results. It was also observed that DPPH radial scavenging potential deteriorated, indicating probable chelation of the less polar biomolecules with Ag^{+} ion to reduce it to metallic silver. This has been agreed with the results of TPC determination and FRAP assay.

Table 1 - Antioxidant profiles of leaf extracts of Neem (AI) and Guava (PG) in comparison to nanoparticles prepared thereof. Results are expressed of mean±SD of Gallic acid equivalents (GAE).

Plant	Treatment	GAE (μg/gm)				
		ABTS assay	DPPH assay	TPC	FRAP assay	Hydroxyl radical scavenging assay
AI	Control	369.41±24.63	2025.00±217.33	556.12±28.46	1128.50±79.72	291.99±29.64
	Np	786.03±16.76	1158.97±143.39	566.80±14.51	1143.34±20.12	807.66±14.18
PG	Control	147.75±13.72	1118.75±61.27	411.81±14.44	3132.05±183.26	407.02±29.93
	Np	811.86±15.86	1165.44±96.03	378.52±11.57	2619.89±130.98	825.05±21.31

Nanoparticles prepared from the leaf extracts showed highly improved hydroxyl radical scavenging abilities. Silver nanoparticles are nowadays used in various physiological adverse conditions, sometimes in encapsulated forms.[19] The nanoparticles prepared in the present study can be used to scavenge the most deleterious free radical of the physiological system, hydroxyl radical, in proper dosage forms.

Antimicrobial activities of the leaf extracts and the nanoparticles were furnished in the antibiogram of Table 2. The results indicated that the water extract of the leaves inhibited the growth and multiplication of the tested bacterial strains - *Escherichia coli, Salmonella typhi, Staphylococcus aureus, Klebsiella aerogenes* and *Bacillus cereus.* Water extract of Neem (AI) leaves showed higher zone of inhibition against *E.coli* at 10,20,40,60,80 and 100mg/ml concentration than the other bacteria.

Table 2 - Bacteriostatic activities of Neem (AI) against common food borne bacteria. LE - Water extract of leaf, NP - nanoparticles prepared from the water extract.

Concentration (mg/ml)	Sample	Mean diameter of zone of inhibition (mm)				
		E.coli	***B.cereus***	***S.typhi***	***S.aureus***	***K.aerogenes***
100	LE	11.66	11.33	11.66	10.66	12.66
	NP	17.66	15.66	17.33	16.66	18.00
80	LE	10.33	10.33	10.66	8.00	10.66
	NP	15.66	14.33	15.33	14.66	16.66
60	LE	9.00	8.66	8.00	-	9.66
	NP	14.00	14.00	14.33	11.66	13.66
40	LE	-	7.66	-	-	8.00
	NP	10.66	11.33	10.66	10.00	11.33
Control	$AgNO_3$	9.00	9.33	10.66	-	-

Water extract of Guava (PG) leaves showed higher zone of inhibition against *E.coli* at 40,60,80 and 100mg/ml concentration than the other bacteria (Table 3). Inhibitory effects against *E.coli* and *B.cereus* were better than *S.aureus*. Also the leaves extract is well effective against *S.aureus* at 100 and 80 mg/ml concentrations.

Table 3 - Bacteriostatic activities of Guava (PG) against common food borne bacteria. LE - Water extract of leaf, NP - nanoparticles prepared from the water extract.

Concentration (mg/ml)	Sample	Mean diameter of zone of inhibition (mm)				
		E.coli	***B.cereus***	***S.typhi***	***S.aureus***	***K.aerogenes***
100	LE	16.33	24.33	11.66	19.66	11.66
	NP	19.33	12.33	16.33	22.33	14.33
80	LE	15.33	20.66	10.33	18.33	9.33
	NP	17.66	11.33	15.33	18.66	11.66
60	LE	12.00	17.33	9.33	15.00	-
	NP	13.66	9.00	12.66	17.33	-
40	LE	9.66	13.66	-	12.00	-
	NP	11.33	-	-	12.66	-
Control	$AgNO_3$	8.66	9.33	10.66	-	-

CONCLUSION

It can be concluded from the present study that biological synthesis of silver nanoparticles using leaf extracts of organically farmed *Azadirachta indica* and *Psidium guajava* provided environmental friendly, simple and efficient route for synthesis of silver nanoparticles. Formation of silver nanoparticles were achieved at room temperature. The extracts prepared in the present study might contain both polar and non-polar antioxidant biomolecules as indicated by the antioxidant assays. Nanoparticles prepared from the leaf extracts showed commended improvement in hydroxyl radical scavenging abilities. Bacteriostatic activities of the nanoparticles were also improved, clearly indicating potential use of the nanoparticles against food borne bacteria for application in favor of humans.

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