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ORIGINAL ARTICLE

Preliminary Phytochemical screening and evaluation of Antimicrobial activity of Silver nano-particles of *A. cadamba* bark (AgNPsC)

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ABSTRACT
In recent times, green biosynthesis of nanoparticles has generated wide interest due to their stability, rapidity, cheaper cost and eco-friendly behaviour. In present study Anthocephalus cadamba bark extract based silver nanoparticles (AgNPsC) are synthesized and size was analyzed by dynamic light scattering (DLS) using Zeta sizer nano (ZS), Malvern, instrument, UK. These nanoparticles were screened for antimicrobial activity against E. coli, B. subtilis, S. aureus and P. aureginosa and compared with antimicrobial activity of silver nitrate and hydro-methanolic bark extract of A. cadamba. Result suggested that AgNPsC exhibit much higher zone of inhibition with respect to $AgNO_3$ and hydro-methanolic A. cadamba bark extract. Among various bacterial strains E. coli and S. aureus were found to be more sensitive against AgNPsC. Thus these AgNPsC can be a good alternative therapeutic approach in future and can be used as an effective antimicrobial material.
***Keywords**: AgNPsC, antibacterial activity, hydro-methanolic extract*

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INTRODUCTION

Anthocephalus cadamba belongs to family Rubiaceae, is a tropical tree species that is native to South Asia and Southeast Asia. It is found in Bangladesh, Nepal, India, Myanmar, Sri Lanka, China, Australia, Philippines and Indonesia, Thailand and east-ward in Malaysian archipelago to Papua New Guinea acknowledged by various vernacular names at different places [1].

It is a large deciduous tree with a broad umbrella-shaped crown and straight cylindrical bole. The tree usually reaches a height of 45 meter with a stem diameter of 100-160 cm, generally has a thin plank buttresses at the base. *A. cadamba* is associated closely with Lord Krishna in Indian mythologies hence secured a spectacular position in Hindu dharma. Radha and Krishna are supposed to have conducted their love play in the hospitable and sweet-scented shade of the Kadamb tree [2]. The plants growing on arid land contain some functional components that protect them from their stress habitat in which the endophytes may also possess novel strategies for their survivability. Plant is commonly known as Kadamb.

Ancient medicinal system if India, Charak Sanhita, Sushruta Sanhita and Ayurveda describe several herbal formulations of different parts of Kadamb and their applications also. These herbal formulations of Kadamb has been used as traditional medicine in the treatment of eye infection, skin disease, dyspepsia, stomatitis, cough, fever, anaemia, wound, inflammation, ulcers, blood disorders, stomach pain and also in the treatment of snake venom (not as antidote), in different Indian sub-continents [3].

Phytochemical investigation of different parts of *A. cadamba* showed the presence of some active biological components such as indole alkaloids, terpenoids, saponins, sapogenins, terpenes, steroids, fats, reducing sugar, glycosides, and flavonoids [4]. Due to the presence of these phytochemicals *A. cadamba* holds some pharmacological activities; antioxidant, hypolipidemic [5], hepatoprotective [6], antimicrobial

[7], analgesic, antipyretic, anti inflammatory [8], antidiabetic [9], immunomodulatory [10], diuretic and laxative [11], anticancerous [12], anthelmintic [13] and used in the treatment of various ailments.

Nanotechnology is a concept of modern science used for numerous physical, biological and pharmaceutical applications. Nanoparticles are clusters of atoms with specific size ranges from 1-100 nm. Physical and chemical processing of the biosynthesis of nanoparticles is costly and also has adverse effect in the medicinal applications, thus green synthesis of nanoparticle biosynthesis is preferred by researchers, recently [14, 15]. Plant produced nanoparticles are more stable than others. Many biomolecules in plants such as proteins, amino acids, polysaccharides, alkaloids, alcoholic compounds and vitamins are supposed to be involved in formulation, stabilization and bioreduction of nanoparticles by reducing ions [16].

Silver nanoparticles (AgNPs) are one of the most commonly used metal nanomaterial because it's a safe and effective bactericidal metal which is highly toxic to bacterial cells and non-toxic to animal cells [16]. These silver nanoparticles possess some distinctive physic-chemical properties; high electrical and thermal conductivity, surface enhance Raman scattering, chemical stability, catalytic activity and non linear behaviour. Due to these properties AgNPs have potential values in inks, microelectronics and medical imaging. They are also popular in costumer products including, plastics, soaps, pasts, food and textiles because of their batericidal and fungicidal properties. Silver nanoparticles are used as antimicrobial agents due to their extremely large surface area which provides better contact with microorganisms [14, 17]. Considering the fact present study has been designed to study preliminary phytochemical screening of hydro-methanolic extract of *A. cadamba* bark as well as comparative analysis of antibacterial activity of *A. cadamba* bark and its silver nano particle.

MATERIAL AND METHODS

Plant material and extract preparation

The stem bark of *A. cadamba* was collected from Vrindavan, Mathura (UP) in January 2020.The bark was identified and authenticated by Dr. Ashok Kumar, BSA Collage, Mathura. The stem bark of *A. cadamba* was dried in shade and grinded in victimisation dry grinder by which 20 gm of coarse powder was collected. This coarse powdery stem bark of the *A. cadamba* was packed in soxhlet equipment and subjected for continuous extraction with hydro-methanolic (Distilled water: methanol within the magnitude relation 80:20) solution at 45-50^{o} C until complete extraction. Extracted solution was then poured in petri plate and kept in hot air oven for drying. Obtained crystalline methanolic extract was brown in colour, 2.5 gm in weight (i.e is 12.5%yeild) and kept at 4^{o}C for further use.

Phytochemical screening

Standard protocols were followed for the quality analysis of phytochemiclas; alkaloids, carbohydrates, glycosides, tannins, saponins, reducing sugars, proteins, flavonoides, triterpenes, phenols and steroids.

Alkaloids-

Hager's test: 2 mg of bark extract was taken in a test tube. A few drops of hager's reagent was added. Formation of yellow colour ppt. indicated the presence of alkaloid.

Wagner's test: 2 mg of bark extract was acidified with 1.5 % v/v HCl and few drops of wagner's reagent was added. Yellow or brown ppt. indicates the presence of alkaloids.

Mayer's test: 2 mg of bark extract was taken in a test tube. A few drops of mayer's reagent was added. Formation of white or pale yellow colour confirms the presence of alkaloid.

Carbohydrates-

Anthrone reagent: 2 mg of bark extract was shaken with 10 ml of water in a test tube after this, filtration is done using filter paper. To this filtrate 2 ml anthrone reagent was added. Formation of green or blue colour confirms the presence of carbohydrates.

Benedict's test: 2 mg of bark extract dissolved in 10 ml of water in a test tube followed by filtration using filter paper. To this filtrate 2 ml Benedict solution is added and boiled for 5 minutes. Formation of brick red colour ppt. confirms the presence of carbohydrates.

Fehling's test: 2 mg of bark extract was shaken with 10 ml of water in a test tube after this, filtration is done using filter paper. To this filtrate 1 ml of equal volume of fehling A and fehling B is added and boiled for 5 minutes. Formation of brick red colour ppt. confirms the presence of carbohydrates.

Molisch test: 2 mg of bark extract was shaken with 10 ml of water in a test tube after this filtration is done using filter paper. To this 20% of alcoholic solution of alpha naphthol is added along with 2 ml of conc. HCl. Formation of violet-red ring at the junction indicates the presence of carbohydrates.

Glycosides-

Molisch test: 2 mg of bark extract was shaken with 10 ml of water in a test tube after this, filtration is done using filter paper. To this 2-3 drops of Molisch reagent is added and mixed after this conc. $H_2SO_4$is

added drop by drop carefully from the side of test tube. Formation of violet-red ring indicates the presence of glycosides.

Keller-Kiliani test: 4 ml of glacial acetic acid along with few drops of 2 % $FeCl_3$ is added to 10 ml of filtrate. To this 1 ml of H_2SO_4 is added from the side of test tube. Appearance of brown ring indicate the presence of glycosides.

Tannins- 2 ml bark extract filtrate is taken in a test tube and 2 ml of $FeCl_3$ was added to it. Formation of blue-black ppt.indicate the presence of tannin.

Saponins- 5 ml of bark extract was taken in a test tube. To this a drop of sodium bicarbonate was added. Then test tube was shaken vigorously and then test tube is left for 3-4 minutes. Appearance of honeycomb froth confirms the presence of saponins.

Reducing Sugar-

Benedict's test: 2 mg of bark extract was shaken with 10 ml of water in a test tube after this filtration is done using filter paper. To this filtrate 2 ml Benedict solution is added and boiled for 5 minutes. Formation of brick red colour ppt. confirms the presence of reducing sugar.

Fehling's test: 2 mg of bark extract was shaken with 10 ml of water in a test tube after this filtration is done using filter paper. To this filtrate 1 ml of equal volume of Fehling A and Fehling B is added and boiled for 5 minutes. Formation of brick red colour ppt. confirms the presence of reducing sugar.

Proteins-

Biuret test: Two-three drops of 2% $CuSO_4$ was added to 2 ml of bark extract (1%) followed by addition of 1 ml of 95% ethanol and excess of potassium hydroxide solution. Formation of pink colour in ethanol layer indicates the presence of protein.

Millon's test: Few drops of Millon's reagent were added to 2 ml bark extract. Formation of white ppt. indicates the presence of protein.

Flavonoids-

Alkaline reagent test: 2 ml NaOH (2%) is added in 2 mg of bark extract as a result yellow colour is produced. To this few drops of dil HCl is added. Decolourisation of yellow extract will indicate the presence of flavonoids.

Triterpenes-

Extract were treated with chloroform and filtered. To the filtrate few drops of conc. H_2SO_4 is added. Appearance of golden yellow colour indicates the presence of triterpenes.

Phenols-

For the identification of phenol, ferric chloride test is employed. Few drops of ferric chloride are added to the test tube containing bark extract, presence of violet-blue colour indicate the presence of phenol.

Steroids-

To 1 ml of extract, 10 ml of chloroform were added followed by addition of 10 ml of conc. H_2SO_4. Change of colour violet to blue /green indicate the presence of steroids.

Plating technique

Sterilized nutrient agar medium for bactericide assay and Sabouraud dextrose agar medium for antifungal assay was prepared and poured sterilely in separate Petri plates and allowed to solidify under aseptic conditions.

Microorganism

Escherichia coli, Bacillius subtilis, Pseudomonas aeruginosa, *Staphylococcus aureus* and *Salmonella typhi* were taken from micro-biological lab of biotechnology department (GLA-University, Mathura). The cultures were maintained on medium slants. They were kept at 4^oC and sub-cultured after every 15–20 days to check the viability of the cells.

Antimicrobial Activity Assay

Antimicrobial activity was carried by disk diffusion method. Following long incubation, the medium is examined for zone of inhibition. The zone of inhibition was measured. Sterilized agar medium was poured into 80 mm diameter sterile Petri dishes to a depth of 4 mm and allowed to solidify. Subsequently with the assistance of sterile measuring device, micropipette 10 µl of various microorganism culture was kept within the central position of plates. Colonies were spread uniformly on the surface of the media with the help of sterile glass spreader. The inoculated plates were then allowed to dry for couple of minutes. With the assistance of sterile extractor antimicrobial disks unfit in numerous formulation concentration (0.25mg/ml, 0.50mg/ml, 0.75mg/ml and 1mg/ml) were placed on the surface of their several media at specific corner that were antecedent tagged. The anti-bacterial activity of silver nanoparticles of *A. cadamba* bark extract (AgNPsC) with concentration (0.4 mg/ml) were tested against *E. coli, B. subtilis, P. aeruginosa* and *S. aureus*. The disk was then pressed down on the medium. Plates were then kept in

refrigerator for 15 minutes following the incubation at 37°C for 24 hours. After 24 hours, diameter of the inhibition zone was measured.

Preparation of silver nanoparticles of *A. cadamba* bark extract (AgNPsC)

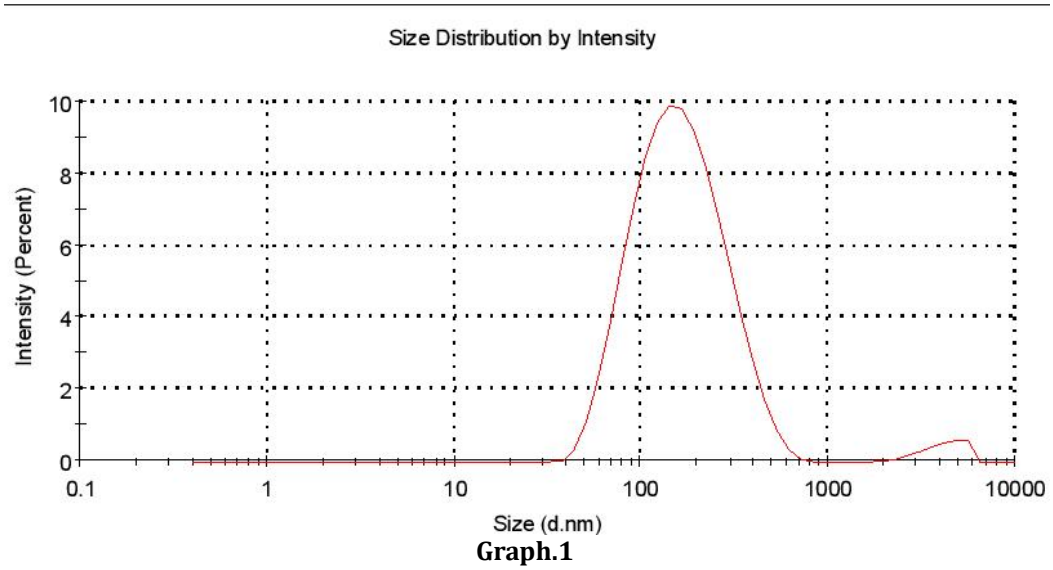
Preparation of silver nanoparticles of *A. cadamba* bark extract was prepared according to *Ankamwar et al.* with slight modification, 25 gm bark was crushed and accessorial into a flask containing a 125 metric capacity unit of milliQ water. Flask was then heated at 100°C in victimization water tub for 5 minutes. Resulting solution was filtered to get clear filtrate of bark extract. Take 90 ml of 1 mM AgNO3 solution, followed by addition of 10 ml bark extract for biosynthesis of silver nanoparticles. Keep it with magnetic bar on magnetic stirrer for 12 hours. The colourless solution turned brown indicating the formation of silver nanoparticles. Collected sample was centrifuged at 11000 rpm for 45 minutes. The supernatant obtained from previous step was additional centrifuged at 16000 rpm for 60 minutes (AgNPs). The pellets obtained from each centrifugal step were washed twice with Milli-Q water and reserved for further characterization.

Characterization Method

A. cadamba bark silver nanoparticles formed were characterised by UV-Vis-NIR spectrum analysis, by measuring the absorbance at 190–1100 nm by a spectrophotometer. Transmission electron Microscopy (TEM) was performed at associate fast voltage of 200 kV for morphological analysis of silver nanoparticles. Carbon coated TEM grids were loaded with a drop of mixture silver nanoparticles answer followed by drying within a vacuum appliance. The sample loaded grid was then scanned by TEM. To check differing types of useful teams, Attenuated Total Reflection Infra-Red (ATR-IR) measurements of extracts and silver nanoparticles were performed by employing a Platinum ATR photometer. To check the crystal structure of silver nanoparticles Powder XRD (Rigaku Ultima-IV X-ray diffractometer) was performed.

Results

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	142.1	Peak 1:	179.9	97.3	101.1
PdI:	0.249	Peak 2:	4241	2.7	990.3
Intercept:	0.927	Peak 3:	0.000	0.0	0.000
Result quality :	Good				



Graph.1

RESULTS AND DISCUSSION

Phytochemical screening of *A. cadamba* bark confirms the presence of alkaloids, flavonoids, glycosides, steroids, tannins, saponins, phenols, tritepens, carbohydrates and reducing sugars while proteins are absent as given in Table1.

Table-1 Preliminary phytochemicals test

	Test	Inference
1.	Alkaloids	Positive
2.	Flavonoids	Positive
3.	Glycosides	Positive
4.	Steroids	Positive
5.	Tannins	Positive
6.	Saponins	Positive
7.	Phenols	Positive
8.	Triterpenes	Positive
9.	Carbohydrates	Positive
10.	Reducing sugars	Positive
11.	Protein	Negative

Hydromethanolic extract of *A. cadamba* bark exhibit significant antibacterial activity against *E. coli, B. subtilis, S. aureus* and *S. typhi. A. cadamba* bark extract at 1.0 mg/ml concentration shows higher zone of inhibition extract against *E. coli* (14 mm) & *S. aureus* (14 mm) in comparison with *B. subtilis* (11 mm) and *S. typhi* (10 mm) as shown in Table 2. Dose dependent activity of *A. cadamba* bark extract was found against all test organisms. Streptomycin and normal saline were used for positive control and negative control, respectively.

Table 2 Results of antmicrobial activity *A. cadamba* bark extract

S.No	Conc.	*E. coli*	*B. subtilis*	*S. typhi*	*S. aureus*
1.	1.0 mg/ml	14 mm	11 mm	10 mm	14 mm
2.	0.75 mg/ml	13 mm	10 mm	9 mm	12 mm
3.	0.50 mg/ml	9 mm	5 mm	9 mm	11 mm
4.	0.25 mg/ml	8 mm	0 mm	4 mm	0 mm
5.	Streptomycin (10 µg/disc) (+ve control)	18.5 mm	30 mm	19 mm	22 mm
6.	Normal saline (-ve control)	0 mm	0 mm	0 mm	0 mm

Moreover antibacterial activity of $AgNO_3$ (0.017 mg/ml), AgNPsC (0.4 mg/ml) and hydromethanolic extract of *A. cadamba* bark (1mg) was carried out against *B. subtilis*, *E. coli*, *S. aureus* and *P. aureginosa*. Amongst the test organisms used, *E. coli* (15 mm) was found to be most sensitive. *S. aureus* (14.5 mm) came next followed by *B.* subtilis (12 mm) and then *P. aureginosa* (8.8 mm) against AgNPsC as shown in Table 3. Results suggested that AgNPsC shows highest antibacterial activity against all test organisms in comparison to $AgNO_3$ and *A. cadamba* bark extract.

Table 3 Antibacterial activity of Silver nitrate ($AgNO_3$), AgNPsC & *A. cadamba* bark extract

Bacterial species	$AgNO_3$ (0.017 mg/ml)	AgNPsC (0.4 mg/ml)	*A. cadamba* bark extract (1mg/ml)
B. subtilis	5 mm	12 mm	11 mm
E. coli	6 mm	15 mm	14 mm
S. aureus	8 mm	14.5 mm	14 mm
P. aureginosa	7.5 mm	8.8 mm	5 mm

Antibacterial activity of bark extract of *A. cadamba* was due to presence of some bioactive compounds like alkaloids, flavanoids, steroids, terpenes, glycosides, tannins, saponins. These bioactive compounds can easily penetrate the cell membrane of bacteria hence entre the cell and ensure antibacterial activity. Antibacterial activity of silver nanoparticles of *A. cadamba* bark extract (AgNPsC) supposed to depend on the growth phase of organism, media component, environment of nanoparticles and metabolism of the organism [18]. Earlier study showed that AgNPs have potent antibacterial activities against certain micro-organisms [19]. Silver nanoparticles contain silver ions which act on bacterial plasma or cytoplasmic membrane and releases K^+ ions from bacteria, moreover silver ions deposited into the vacuoles and in cell wall as granules, can interact with nucleic acids, although their lethal action is unclear [20]. Present study signifies the enhancement in antimicrobial activity by using nanoparticles.

CONCLUSION

Silver nanoparticles synthesized using *A. cadamba* bark extract showed significant antimicrobial activity even higher than that of bark extract and silver nitrate. Concentration of nanoparticles has been observed to significantly affect the growth of micro-organisms and shows antibacterial activity against *E. coli, B. subtilis, S. aureus, P. aureginosa.* Study indicates that silver nanoparticles of *A. cadamba* bark extract can be used as effective antibacterial substance and can be effectively used in preparation of pharmaceuticals.

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