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Green synthesis of silver nanoparticles using aqueous leaf extract of *Andrographis paniculata* and the evaluation of their antibacterial efficacy

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Abstract
Multidrug resistant strains of microorganisms are the major threat to the treatment of infectious disease. *Andrographis paniculata* leaf extract and its mediated synthesized silver nanoparticles were evaluated against *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Proteus vulgaris* and *Pseudomonas aeruginosa.* Antibacterial activity was studied using disc diffusion method using sterile discs. The bactericidal efficacy was recorded as zone of inhibition measured after overnight inhibition with specific concentration of extracts. The green synthesized silver nanoparticles were characterized using UV-Spectroscopy and Scanning Electron Microscopy (SEM). The alcoholic extract shows moderate activity against the tested organisms when compared with aqueous extract however, a significant zone of inhibition of 12mm to 17mm was recorded with reference to 10µg/ml concentration of green synthesized silver nanoparticles. The zone of inhibition recorded was higher than the positive control amoxicillin. To conclude, *Andrographis paniculata* mediated synthesized silver nanoparticles shows better efficiency as bactericidal agent when compared with ethyl acetate and aqueous extract of the tested plant and the silver nanoparticles coated with bioactive compounds of the plant material can be function as a better alternative to control the drug resistant bacteria in place of synthetic medicines used to control or treat the bacterial infection.

Keywords: *Andrographis paniculata*, Silver nanoparticles, antibacterial, phytochemistry

Introduction
Infectious diseases are the major cause of death around the world. Microbial infections pose a serious consequences in health and welfare of the public especially when the disease caused by multidrug resistant organism. Bacterial infections pose a serious public health concern, when an infectious disease caused by a multi drug resistant organism. *Escherichia coli* and *Klebsiella pneumoniae* are the prominent multi drug resistant pathogenic organisms isolated from clinical samples [1]. *Pseudomonas aeruginosa* an opportunistic pathogen can cause infections in immune compromised hosts [2]. *Staphylococcus aureus* is commonly isolated from urinary tract infections. Antibiotics can impose stress and generate various cell responses in microorganisms [3]. Antibiotic induced mutations are one of the reasons behind the development of resistance among organisms [4]. Alteration in the cell structure and metabolism of microbes makes them more susceptible towards the drugs [5]. The antibiotics were classified on the basis of their target and resultant effect that leads to the death of the organisms [6]. The antibacterial drug designing mainly focuses on blockage of essential cellular functions [7]. Development of drug resistance among the pathogenic micro organisms becomes a major obstacle in the diagnosis and treatment of infectious diseases [8]. Multidrug resistance bacteria can compromise the clinical utility of major chemotherapeutic antimicrobial agents, and these organisms possess distinct molecular mechanism to overcome the resistance [9].
Prevalence of more number of drug resistant bacteria results in the search of novel drug with different mode of action to kill the pathogenic microorganisms. Biosynthetic methods employing either biological microorganisms or plant extracts have emerged as a single and viable alternative to chemical synthetic procedure and physical methods. Green chemistry leads to the invention of chemical products from natural resources, which are non-hazardous to the public as well as the environment [10]. Exploration of new antimicrobial agents from natural resources with novel mode of action is the need of the hour.
Silver ions known for its strong bactericidal effect [11]. Synthesis of silver nanoparticles (AgNP's) by employing biological methods will be a viable alternative over chemical and physical methods. The green synthesized nanoparticles will reduce the health hazards caused by the synthetic particles. Silver nanoparticles are generally smaller than 100nm containing 20-15000 silver atoms and exhibit unusual physical, chemical and biological properties [12].

AgNP's with the diameter of 10-15nm has increased the stability, biocompatibility and antimicrobial activity [13]. Silver nanoparticles adhere to the cell membrane and penetrate into the cell and cause damages to the vital functioning of the cell [14]. The interaction of AgNP's with the ribosomes leads to the denaturation and resulted in the inhibition of translation and protein synthesis [15]. Silver ions and silver nanoparticles interfere with disulfide bonds and blocks the binding sites of protein leading to functional damages in the organisms [16]. AgNP's and DNA interaction may cause the denaturation of the later and interruption in cell division [17]. Transcription of genes in the microorganisms was interrupted due to the intercallation of AgNP's in the DNA helix [18]. The DNA molecule becomes condensed and loss its replication potential due to the action of silver nanoparticles. [19]. Silver nanoparticles possess the ability to anchor to the cell wall and penetrating it by which it causes structural changes in the cell membrane results in the death of the organism. Formation of free radicals by nanoparticles is considered to be the mechanism by which the cell dies [20].

Andrographis paniculata an annual branched, erect and herbaceous plant belongs to the family Acanthaceae is native to India and Sri Lanka. The plant is traditionally used to treat various ailments. It shows antidiabetic activity [21]. Antibacterial activity [22]; Antioxidant activities [23]. The aqueous leaf extract of *Andrographis paniculata* was used as a reducing agent for the reduction of silver ions and to synthesize the nanoparticles. The present study intended to analyze the antibacterial activity of green synthesized silver nanoparticles against disease causing microorganisms.

Materials and Methods

Chemicals and reagents

Silver nitrate, nutrient broth, Muller-Hinton agar, and other chemicals were purchased from Himedia Laboratories, Mumbai, India.

Collection of plants

Fresh and disease free leaves of *Andrographis paniculata* was collected from the garden of Presidency College, Chennai, India and the plant was identified and authenticated with the help using herbarium at the Plant Biology and Biotechnology of Presidency College, Chennai, India.

Preparation of plant extract

The fresh leaves were washed thoroughly with sterile water to remove the dirt particles adhere to it and into small pieces. The pieces of leaves were allowed to shade dry at the temperature 25-30 ˚C for a period of 96 hours. The plant was cleaned regularly for their moisture content and to find out the leaves were disease free. The dried plant leaves were made into coarse powder using mixer and blender with the help of a sieve. The fine powder was collected and stored in an air tight container. The powdered material was subjected to soxhlet method for the extraction of phytocompounds. In a soxhlet apparatus the samples were loaded in the chamber are different solvents such as aqueous and ethyl acetate and the cycle was continuation for a period of 8hours or till the complete extraction of the compound. The extracts collection using soxhlet apparatus were concentrated using vacuum evaporator and the concentrated extracts were stored at 4 ˚C till further use.

Phytochemical analysis

Qualitative estimation of secondary metabolites was performed using the method [24] of Harborn. The aqueous and ethyl acetate extracts were subjected to this analysis.

Synthesis of silver nanoparticles

The matured, undamaged and diseases free leaves of *Andrographis paniculata* were collected and thoroughly washed with tap water to remove soil and finally with double distilled water. The leaf broth was prepared by adding 3 grams of chopped leaves to 100 ml of sterile distilled water and boiled for 5 minutes and cooled. Then the solution was filtered with Whatman filter paper then 10 ml of filtrate was added to 90 ml of 1 mM aqueous silver nitrate for the reduction of silver ions. After adding the colorless silver nitrate solution turns intense brown color which indicates the formation of silver nanoparticles.

Characterization of silver nanoparticles

The reduction of silver ions were monitored by measuring UV-Vis spectrum of reaction medium from the wavelength of 200-800 nm by using distilled water as blank. The AgNPs were subjected to Scanning Electron Microscopy (SEM) to observe the surface morphology of the particles.

Collection and Preparation of Inoculums

The bacterial strains were obtained from Microbial Metabolite lab culture collection, CAS in Botany, University of Madras, Guindy campus, Chennai, Tamil Nadu, India. The bacterial strains of *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Proteus vulgaris* and *Pseudomonas aeruginosa.* Stock cultures were maintained at 4 ˚C on slants of nutrient agar. Active culture were prepared by transferring a loop full of culture from the stock culture to conical flasks with 100 ml of nutrient broth the bacteria, were incubated with agitation for 24 hrs at 37 ˚C on an shaking incubator at 110 rpm. Each test organism suspension was subsequently stroked out on Muller Hinton Agar media Incubated at 37 ˚C for 24 hrs.

Antibacterial assay

Antibacterial assay was carried out with the help of disc diffusion assay. The sterile agar disc was loaded with different concentration of extract of about 25µg/ml, 50µg/ml, 75µg/ml and 100µg/ml and standard solution of amoxicillin (25µg/ml/Distilled water). Petriplates loaded with overnight culture was used for the study. Inoculated samples were used to study the antibacterial assay. The extract loaded disc were placed on the plate and laid down firmly to mark. The zone of inhibition (mm in diameters) was measured and taken as the activity against the test pathogens.

Results

Bioactive compounds present in the plant extract plays the major role in the control of infectious organisms. A qualitative estimation of phytoconstituents available in the aqueous and ethyl acetate extracts was presented in Table 1. The primary screening of bioactive compounds reveal the presence of steroids, tannins, coumarins, alkaloids, diterpenoids, cardiac glycosides, carbohydrates and flavonoids in the ethyl acetate mediated solvent extract. However, moderate amount of the bioactive compounds were present with reference to aqueous extract.

Antibacterial efficacy of the green synthesized silver nanoparticles and aqueous and ethyl acetate mediated leaf extracts was tested against Gram positive (*Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus*) and Gram negative (*Escherichia coli, Proteus vulgaris* and *Pseudomonas aeruginosa*) organisms. The antibacterial activity was expressed has average diameter of the zone of

inhibition of bacterial growth around the disc. The aqueous leaf extract of the tested plant shows moderate activity against the tested organism when compared with that of standard antibiotic. The Ethyl acetate leaf extract of *Andrographis paniculata* shows a potential bactericidal activity against *Staphylococcus aureus* and the zone of inhibition was recorded as 13mm with reference to 100 µg/ml concentrations. The growth of *Pseudomonas aeruginosa* and *Bacillus subtilis* were also inhibited marginally by the action of ethyl acetate extract. *Bacillus cereus, Escherichia coli* and *Proteus vulgaris* were not responded to the plant extract and they were show resistance against the antibiotic tested as well as the plant extracts. The green synthesized silver nanoparticles shows maximum inhibitory activity against both gram positive and gram negative bacteria. The zone of inhibition observed with reference to *Pseudomonas aeruginosa* and *Bacillus subtilis* were 17mm and 15mm with 100 µg/ml concentration. Followed by *Staphylococcus aureus* and *Escherichia coli* with the zone of inhibition 13 mm diameters. The resistant strains *Bacillus cereus, Escherichia coli* and *Proteus vulgaris* were shown to be affected by the green synthesized silver nanoparticles. The zone of inhibition observed with reference to silver nanoparticles suggesting the role of green synthesized silver nanoparticles as potential bactericidal activity.

Table 1: Phytochemical analysis of leaves of *Andrographis paniculata*

Phytochemical Compounds	Aqueous	Ethyl acetate
Steroids	+	+++
Tannins	+	+
Saponins	-	-
Coumarins	+	++
Alkaloids	+	++
Enodins	-	-
Diterpenoids	+	+
Cardiac glycosides	+	++
Carbohydrates	+	+
Flavonoids	++	+++
Phenols	-	-
Anthocyanins	-	-
Proteins	-	-
Leucoanthocyanins	-	-

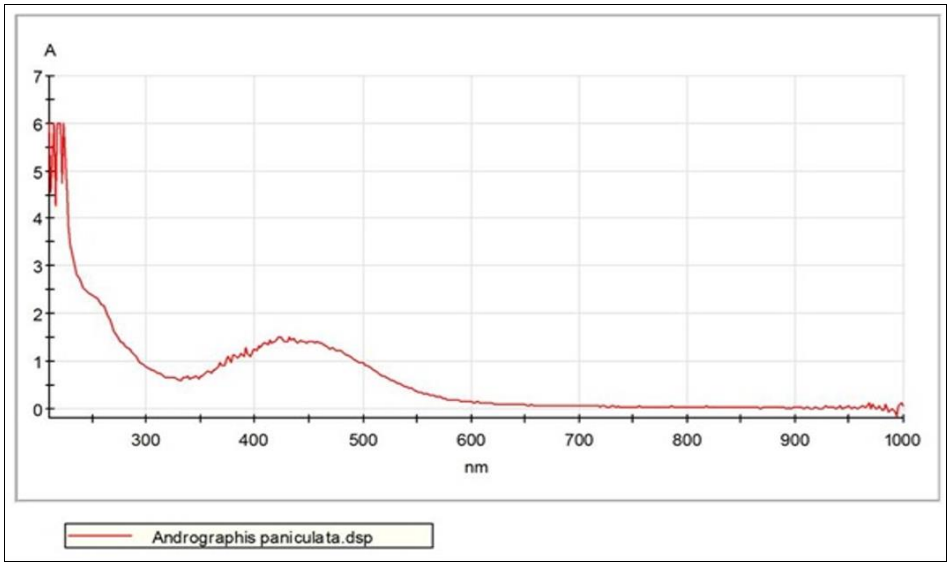

Fig 1: UV-Vis spectrum of silver nanoparticles of *Andrographis paniculata*

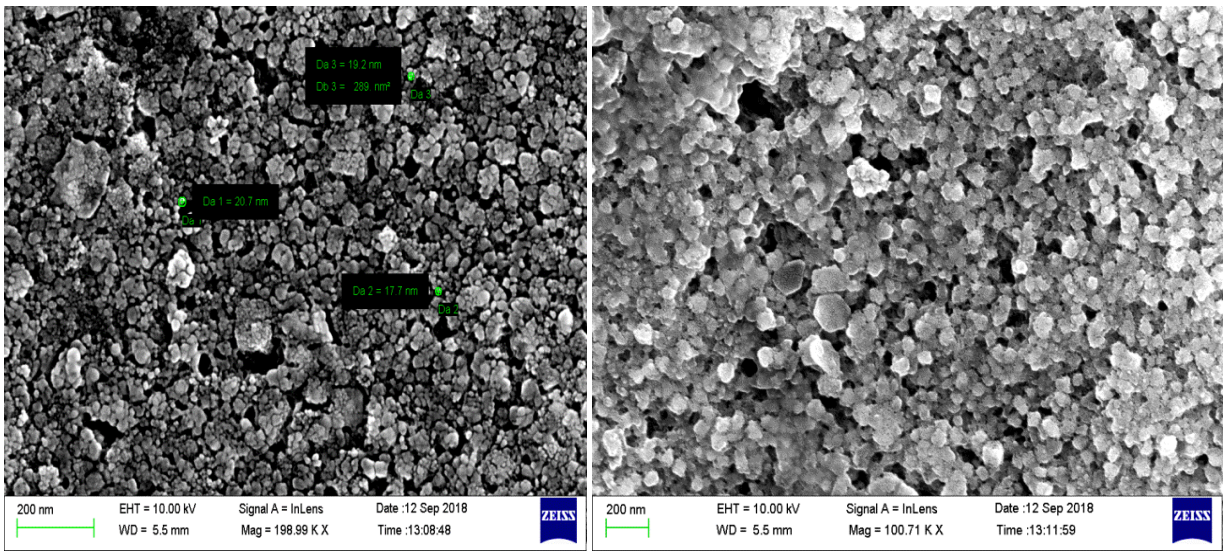

Fig 2: Scanning electron microscopic images of synthesized nanoparticles using aqueous leaf extract of *Andrographis paniculata*

Table 2: Antibacterial activity (Zone of inhibition) of green synthesized silver nanoparticles of *Andrographis panicculata*

Organisms	Zone of inhibition (mm)					
	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	100 µg/ml	Amoxicillin
Bacillus cereus	8	9	9	10	12	13
Bacillus subtilis	10	11	11	12	15	14
Staphylococcus aureus	9	10	10	11	13	13
Escherichia coli	9	9	9	10	13	15
Proteus vulgaris	8	8	9	10	12	11
Pseudomonas aeruginosa	13	14	14	15	17	16

4. Discussion

Emergences of drug resistant bacterial strains are the most serious global threats to treatment of infectious diseases. The problem of emerging bacterial resistance traditionally has been solved by the discovery of new antimicrobial drugs. It is important to develop an appropriate and efficient antimicrobial drug with a novel mode of mechanism using the natural resources which can act on synthetic drug resistant organisms. *Andrographis paniculata* explores such a possibility to utilize it as a principle active medicine to reduce the multi drug resistance problem. In this present study the ethyl acetate leaf extract of *Andrographis paniculata* shows potential activity against gram positive bacteria. The study also reveals the presence of various bioactive compounds such as alkaloids, flavonoids, steroids, tannins, carbohydrates and cardiac glycosides. Bhalodia and Shukla (2011) [25] proposed that there is a direct relationship between the concentration of extract and the antibacterial efficacy. It evident from the results that a dose dependent increase in the antibacterial efficacy of the ethyl acetate extract was observed. Moreover, the alcoholic extract shows potential bactericidal activity when compared with aqueous extract due to the presence of various chemical constituents in the ethyl acetate mediated extract. The above findings are in agreement with the observations of Mustafa *et al*., [26] (2018) suggesting the role of chemical constituents and volatile compounds and their relation with the potential antibacterial activity. The alkaloids are nitrogenous compounds that the functions in the defense of plants against herbivorous and pathogens due to their potential biological activities [27]. Tannin the metal ion chelators, protein precipitating agents has potential effect on biological system [28]. It is evident from the results that the alcoholic extract of *Andrographis paniculata* shows potential activity against pathogenic organisms when compared with aqueous extracts due to the presence of tannins and alkaloids. The combination of secondary metabolites present in the alcoholic extracts attaches with various targets of the bacterial cell and thereby causing damages to the cell wall of bacteria resulting in the death of the organism. The green synthesized silver nanoparticles were more effective against both gram positive and gram negative organism when compared with crude extracts. Ag nanoparticles significantly increase the cell permeability and affect the proper transport through plasma membrane the contact of cells to the particles were more efficient with silver nanoparticles measuring less than 80nm results in the intracellular bioavailability of silver [29]. The present results were justified with the above findings that silver nanoparticles were more effective in damaging the cell wall of the bacteria and there by killing the organisms. Silver in a form of an ionic or nanosilver were highly toxic to the microorganisms [30]. Silver has usually been used in opposition to numerous diseases within the beyond it found use as an antiseptic and antimicrobial against gram positive and gram negative bacteria [31, 32]. The present study reveals that the plant mediated synthesized silver nanoparticles to be a better alternative source to synthetic drugs. Hence, the nanoparticles loaded with medicinal plant extracts will play the major role in the control of drug resistance bacteria.

5. References

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