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RESEARCH ARTICLE MICROBIOLOGY

GREEN SYNTHESIS OF SILVER NANOPARTICLES USING LEAVES OF LEPISANTHES TETRAPHYLLA AND EVALUATION OF THEIR ANTIBACTERIAL ACTIVITY AGAINST DRUG RESISTANT CLINICAL ISOLATES.

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ABSTRACT

The silver nanoparticles (AgNPs) synthesized using aqueous leaf extract of *Lepisanthes tetraphylla* as the reducing agent were evaluated for antibacterial activity against drug resistant bacterial isolates. The nature of AgNPs synthesized were analysed by UV-Vis spectroscopy, X-ray diffraction and Scanning electron microscopy. The silver nanoparticles were with an average size of 30nm, spherical and poly-dispersed. The antibacterial potential of synthesized AgNPs were compared with that of crude methanolic leaf extract of Lepisanthes tetraphylla by well diffusion method. The AgNPs at 30-50µg(0.03-0.05mg) concentration significantly inhibited bacterial growth against multi drug resistant *Staphylococcus aureus* , *Escherichia coli*, *Pseudomonas aeruginosa and Acinetobacter species*. The study revealed that crude methanolic leaf extract of L.tetraphylla inhibited the growth of only *Escherichia coli*, at 1mg/ml concentration. Thus AgNPs showed broad spectrum antibacterial activity at lower concentration may be a good alternative therapeutic approach in future.

KEYWORDS

Lepisanthes tetraphylla, antibacterial activity, silver nanoparticles, well diffusion method.

INTRODUCTION

Infectious diseases are the foremost leading cause of premature deaths. In recent years, drug resistance to human pathogenic bacteria has been commonly reported. The emergence of multidrug resistant strains complicated the treatment of infectious diseases in immunocomprimised patients. This potentiates the search for new antimicrobial substances from other sources. The use of medicinal plants for treatment has been in practice for a very long time since plants are known to produce certain chemicals which are naturally toxic to bacteria[1]. Medicinal plants have been used for several purposes and are known to inhibit the growth of microbes, including fungi.

Screening of compounds obtained from plants has resulted in the isolation of innumerable therapeutic agents, representing its molecular diversity engineered by nature. In the present scenario, there is a vast area of research in phyto-medicine, which increases the global tendency for reinforcement and documentation of traditional system of medicine. In the present study, we have selected Lepisanthes tetraphylla leaf extract and silver nano particles synthesized using it as a reducing agent to screen the antibacterial activity against multidrug resistant clinical isolates such as Methicillin resistant *Staphylococcus aureus* (MRSA), ESBL producing *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter* species. The spectrum of beta lactamase producing organisms are a breed of drug resistant pathogens that are rapidly becoming important globally in the area of hospital acquired infections. Extended spectrum betalactamases (ESBL) are transferable plasmid encoded, mutated beta lactamases enzymes, that have the capability to hydrolyze third generation cephalosporins. These enzymes are found in a variety of Enterobacteriaceae, most often in Escherichia coli and Klebsiella pneumonia[2,3]. Slime producing isolates are reported to have a high rate of resistance to antibacterial agent [4]. The emergence of multidrug resistant organisms raises the problem of untreated bacterial infections[5].To overcome the problem of antibiotic resistance, medicinal plants may be an alternative for treatment. The new infection fighting strategies also involve the action of silver nanoparticles against drug resistant bacteria.

Plant mediated synthesis of silver nanoparticles is an increasing commercial demand due to its wide applicability in medicine. Nanoparticles include ultra fine particles of metals, metal oxides, nonmetals and ceramics .Silver has long been recognized as having inhibitory effect on microbes . New applications of nanoparticles and nano-materials are emerging rapidly[6,7]. The present investigation was conducted to study the antibacterial activity of crude extracts of *Lepisanthes tetraphylla* and silver nanoparticles synthesized using it as a reducing agent against various drug resistant bacterial isolates.

MATERIALS AND METHODS

Plant description

Lepisanthes tetraphylla, commonly called as Nekota (Tamil name), is a shrub growing in India which belongs to the family Sapindaceae. The drupes contain saponins which are the natural surfactant. L.tetraphylla has been historically used in folk remedies as a mucolytic agent, emetic agent and contraceptive. Though they exhibit antimicrobial properties, the effectiveness of

these folk remedies have not been subjected to extensive scientific scrutiny.

Figure.1(a)
Leaves of L.tetraphylla

Figure.1(b)
Methanolic leaf extract of L.tetraphylla

Plant extraction

Fresh leaves of L.tetraphylla were collected from Herbal society, Pondicherry, Tamilnadu India. The identity of the plant was confirmed at Center for Advanced Studies, Botany, University of Madras. The plant materials were cleaned, shade dried and homogenized to a fine powder and stored in air tight container.

The crude leaf extracts of the plant were obtained through direct extraction with different solvents. The solvents used were water, methanol and ethyl acetate. The fresh leaves were collected, washed, shade dried and homogenized to fine powder.50gm of ground material was extracted with 500ml of solvent in a conical flask in shaking condition for 48-72hrs.The solvent containing extracts were then decanted and filtered through filter paper and the process were repeated twice using fresh solvent. The filtrate was finally separated from the solvent using Soxhlet apparatus.

Identification of drug resistant isolates

Bacterial strains used in the study were isolated from clinical samples such as urine, pus, blood, throat swabs and ear swabs. The test microorganisms included about ten isolates each of Methicillin resistant *Staphylococcus aureus* (MRSA), Extended spectrum beta lactamase (ESBL) producing Escherichia coli (*E.coli)*, Multidrug resistant(MDR) *Pseudomonas aeruginosa and Acinetobacter species* . All the clinical strains were isolated and identified by appropriate cultural methods and biochemical reactions and antibiotic susceptibility testing was carried out by Kirby Bauer disc diffusion method accoding to CLSI guidelines.

ESBL bacterial isolates were identified by Double-disc synergy test, .The procedures and interpretation of Double disc synergy test was described previously [8]. The MRSA strains of *Staphylococcus aureus* were identified on basis of its resistance to Cefoxitin[9, 10].

Biogenesis of Silver nanoparticles

The leaves of L.tetraphylla weighing 25 g were thoroughly washed in distilled water, dried and cut into fine pieces and was crushed in 100 ml of sterile distilled water and filtered through what man No.1 filter paper. The filtrate was again filtered through 0.6µm sized filters. They were stored at 4 °C used within a week.

Ten millilitre of the suspension of leaf extract was added to 90 ml aqueous solution of silver nitrate (1mM) for reduction into silver ions and incubated at room temperature for about 24 hours. The primary detection of silver nanoparticles was carried out in the reaction mixture by observing the colour change of medium from greenish to dark brown (Fig.2a & Fig.2b). After incubation the silver nanoparticles were isolated and concentrated by repeated centrifugation (4-5times) at 10,000 rpm for 10 minutes. The supernatant was replaced with distilled water each time and suspension stored as lyophilised powder for optical measurements [11, 12]

Figure.2(a)

Aqueous extract of L.tetraphylla before synthesis of silver nanoparticles

Figure.2(b)

L.tetraphylla –colour change of aqueous extract to golden yellow after synthesis of Ag nanoparticles.

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CHARACTERIZATION OF SILVER NANOPARTICLES

UV-visible spectra analysisThe reduction of pure silver ions was monitored by measuring the UV-vis spectrum of the reaction medium at 5 hours after diluting a small aliquot of the sample into distilled water. UV spectral analysis was done by using UV –Vis spectrophotometer. The absorption spectrum of aqueous Ag nitrate solution exhibited maximum at 440 nm.

X-ray diffraction analysis

X-ray diffraction pattern of the silver nanoparticles were obtained by measurement of angles at which X-ray beam is diffracted by crystalline phases in specimen. X-ray diffraction (XRD) measurement of bioreduced silver nitrate solution drop coated on glass substrate biofilm was carried out on XRD instrument operating at 40kv and a current of 30mA.

Scanning Electron Microscopic (SEM) Analysis

Thin films of the sample were prepared on a carbon coated copper grid by just dropping a

very small amount of the sample and then the film on the grid were allowed to dry by putting it under a mercury vapour lamp for 5 min for detecting the size and shape of silver nanoparticles using scanning electron microscope.

ANTIBACTERIAL EFFICACY OF SILVER NANOPARTICLES AND CRUDE LEAF EXTRACT OF LEPISANTHES TETRAPHYLLA

Silver nanoparticles synthesized from leaves of Lepisanthes tetraphylla were tested against multidrug resistant strains of both Gram positive cocci (*Staphylococcus aureus*-MRSA), Gram negative bacilli (ESBL *E.coli*, MDR *Pseudomonas aeruginosa* and *Acinetobacter species*) by well diffusion method. Pure cultures were subcultured in Muller Hinton broth and incubated for 24 hrs at 37˚C. Wells of 6mm diameter were made on Muller Hinton agar plates using sterile stainless steel cork borer. Using sterile microtips 30-50µl (0.03-0.05mg) of the sample of nanoparticle solution was poured onto wells. Like wise, crude leaf extract (0.1-1mg) solution was placed onto wells and incubated at 35˚C for 24 hrs. The different levels of zone of inhibition were measured.

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RESULTS AND DISCUSSION

Reduction of silver ions present in the aqueous solution of silver nitrate during the reaction with the ingredients present in the *Lepisanthes tetraphylla* leaf extract have been visualized by colour change from green to yellowish-brown which indicated the formation of silver nanoparticles. The silver nanoparticles were analysed by UV-Vis spectroscopy. Maximum absorbance was seen at 445nm, indicated the formation of spherical silver nanoparticles in majority[13]. **T**he biosynthesized silver nanoparticles using the leaf extract of *Lepisanthes tetraphylla* was further demonstrated and confirmed by the characteristic peaks in the XRD image and the structural view under the scanning electron microscope (Fig.3 & Fig.4). The XRD pattern showed intense peaks in the whole spectrum of 2theta value ranging from 10-80. The SEM image (Fig.3) showed relatively spherical silver nanoparticles formed with diameter ranging from 20-40nm. Similar results were expressed in plant extracts of *Boswellia ovalifoliolata, Shorea tumbuggaia, Euphorbia hirta*.[13,14]

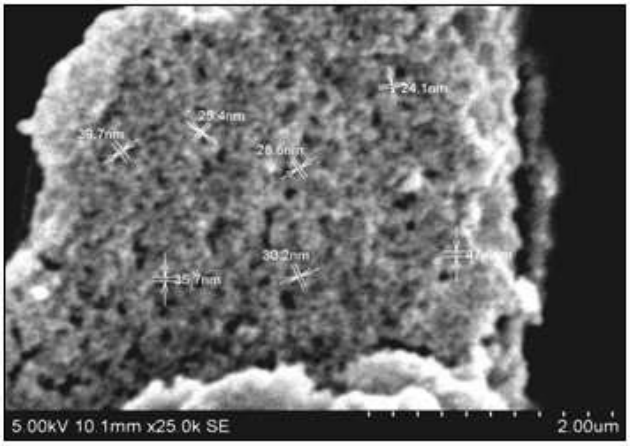

Figure.3
SEM image of Silver nanoparticles formed *L.tetraphylla*

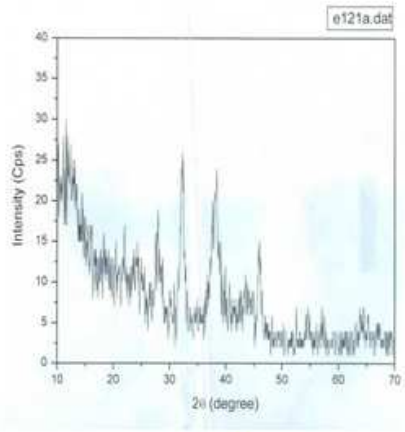

Figure.4
XRD image of Ag nanoparticles synthesized . Intense peak at 32degree-2theta values ranging from 10-80

The antibacterial activity of silver nanoparticles was investigated against pathogenic Gram positive bacteria (*Staphylococcus aureus)* and Gram negative bacteria (*E.coli, Pseudomonas aeruginosa and Acinetobacter species*) by well diffusion method (Fig.6). The inhibition zones of silver nanoparticles was also compared with those of crude methanolic leaf extract of *L.tetraphylla*. The diameter of the inhibition zones around wells with silver nanoparticles and wells with crude extract is represented in table I. The silver nanoparticles at 0.03-0.05mg (30-50µg) concentration significantly inhibited bacterial growth against Methicillin resistant *Staphylococcus aureus* (MRSA), Extended spectrum beta lactamase producing *Escherichia coli*, Metallo bata lactamase producing *Pseudomonas aeruginosa and Acinetobacter species*, whereas the crude methanolic leaf extract of L.tetraphylla inhibited the growth of only ESBL E.coli at the concentration of 1mg/0.1ml among the tested clinical isolates (Fig.5). The other solvent extracts (aqueous and ethyl acetate extracts of leaves of *L.tetraphylla*) exhibited no activity against the drug resistant bacterial isolates [15] . Several proteins mainly cell wall bound enzymes with amino groups are responsible for the synthesis of nanoparticles and biological synthesis and characterization of silver and gold nanoparticles are different size ranging from 8-40nm[16]. Nanoparticles of various sizes and properties may be obtained by further tapping the plant bioresources of diverse type.

Figure.5(a)
Antibacterial activity of crude extract of L.tetraphylla with varying concentrations by well diffusion method.

Figure.5(b)
Antibacterial activity of Silver nanoparticles by well diffusion method at 0.03-0.05mg (30-50µg)

Table 1
Antibacterial activity of crude methanolic leaf extract of L.tetraphylla and silver nanoparticles

S.NO	Name of bacterial species	Control (Commercially available antibiotics) Zone of inhibition(mm)	Methanolic leaf extract of L.tetraphylla @1mg Zone of inhibition(mm)	Ag Nanoparticle @0.03mg Zone of inhibition (mm)
1	MRSA (Methicillin resistant Staphylococcus aureus.)	Amikacin(30µg) ≥17mm	<10mm	17±0.5
2	ESBL E.coli.(Extended spectrum beta lactamase producing Escherichia coli)	Piperacillin-Tazobactam (100/10 µg) >21mm	16±0.5	20±0.5
3	MDR(Multidrug resistant) Pseudomonas aeruginosa	PolymyxinB(300units) ≥12mm	No zone	15±0.5
4	MDR(Multidrug resistant) Acinetobacter species	PolymyxinB(300units) ≥12mm	≤10mm	14±0.5

CONCLUSION

In conclusion, the bioreduction of aqueous silver ions using the leaf extract of Lepisanthes tetraphylla has been demonstrated. This study supports the reduction of metal ions through leaf extracts leading to the formation of silver nanoparticles of fairly well defined dimensions. This green chemistry approach towards silver nanoparticle synthesis is economically viable and can be easily produced. Silver metal and silver dressings when used in reasonable amounts, has no negative effects on human body and it has natural antimicrobial activity[17,18]. The study found that AgNPs synthesized using the leaf extract of Lepisanthes tetraphylla showed broad spectrum activity and should be explored further for antimicrobial applications .

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