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Research Article

ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED FROM *CLAUSENA ANISATA* (WILLD.) HOOK F EX BENTH (RUTACEAE)

ARSIA TARNAM Y*

Department of Biotechnology, Jamal Mohamed College, Tiruchirappalli - 620 020, Tamil Nadu, India. Email: arsia.sherif@gmail.com

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ABSTRACT

Objective: In this study, the antibacterial activity of silver nanoparticles (SNPs) synthesized from *Clausena anisata* was analyzed using agar well diffusion method and disc diffusion method. *C. anisata*, a medicinal plant belonging to the family Rutaceae, is represented by 20 species available in India and used traditionally for the treatment of several ailments, but there is a requirement to identify its phytoconstituents, its target, mode of action and treatment using plant products either alone or in combination with synthetic drugs.

Methods: *C. anisata* leaves and roots were procured from Manamettupatti, a village in Pudukottai District, Tamil Nadu. The shade dried leaf and root were powdered and extracted using ethanol by maceration method. The extracts of leaf and root were further subjected to synthesis SNPs, characterized and the antibacterial activity was studied against *Bacillus subtilis*, *Staphylococcus aurues*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa,* and *Provedencia rettgeri*.

Results: The SNP leaf and root extracts showed antibacterial activity in the concentration ranging from 50 µg to 150 µg/ml. All the extracts showed antibacterial activity against all the tested micro-organisms. In which, the SNP leaf extract showed maximum zone of inhibition against *P. aeruginosa* (12±0.5) ($p<0.0001$), followed by *B. subtilis* ($p<0.0001$), *S. aurues* ($p<0.0001$), *P. vulgaris* ($p<0.0001$), and *P. rettgeri* ($p<0.001$) with each 10 mm in diameter and *E. coli* with 8±0.6 mm in diameter, where standard showed 15±0.57 mm in diameter against *E. coli.* The maximum zone of inhibition for SNP root extract was observed against *E. coli* ($p<0.01$) and *P. retttgeri* ($p<0.01$) with 15 mm in diameter each, followed by *P. aeruginosa* ($p<0.0001$), *B. subtilis* ($p<0.0001$), *S. aureus* ($p<0.0001$) and *P. vulgaris* ($p<0.0001$) with 14±2.5 mm, 12±1.52, 12±2.1 and 9±0.57 mm zone of inhibition, respectively, and standard showed 20±1.52 mm zone of inhibition against *P. rettgeri.*

Conclusion: The phytoconstituents of *C. anisata* SNP root extract may be acting synergistically or independently in exerting an overall antibacterial action in this study and that should be chemically analyzed, and their chemical structure should be understood to develop an effective antibacterial therapeutic agent in future.

Keywords: *Clausena anisata*, Agar well diffusion method, Disc diffusion method, Zone of inhibition, Ethanol, Maceration and silver nanoparticles.

INTRODUCTION

Medicinal plants are the plants that have been alleged to have a medicinal property, which is effects that relate to health, or which have been proven to be useful as drugs by western standards or which contains constituents that are used as drugs [1]. India has about 45000 plant species, of which several thousands have claimed to possess medicinal properties. The World Health Organization reported that nearly 80% of the world's population depends on traditional medicine of plants to meet their health care needs. These medicinal plants fall into two distinct areas, the traditional system of medicine and modern system of medicine [2].

Due to the increased resistance of micro-organisms to antimicrobial drugs, it become necessary to search for new cost-effective antimicrobial drugs either natural or synthetic [3]. The plants secondary metabolite serves as defense mechanism against predation by micro-organisms, insects, and herbivores. Terpenoids give plant their odors, quinones and tannins are responsible for plant pigment, simple phenols, terpenoids, essential oil, alkaloids, lectin, polypeptides and phenolic acids are shown to be toxic to micro-organisms [4].

Phytoalexins are antimicrobial compounds in which after elicitation of biosynthetic pathway requires the *de novo* expression of the enzymes. Low molecular weight antimicrobial compounds that are produced after infection are called phytoanticipin. They both are used as antimicrobial agents in human medicine [5]. Plant-mediated synthesized NPs are biodegradable, non-toxic, and biocompatible that show quick action by entering into cell membrane and act as an alternative system of herbal medicine to treat infections [6].

Clausena anisata of family Rutaceae is locally known as "Kattu-karveppillai," is deciduous shrubs or small tree that grows up to 10 m, found in India, Tropical and South East Asia, which is indigenous in South Africa. Leaves are pinnately compound with 10-17 alternate leaflets and a terminal leaflet. The leaves are densely dotted with pellucid glands and have a strong scent when bruised. Flowers are small and attractive, white with orange-yellow stamens and having gynophores [7]. In mozambique, it is locally known as "Horsewood." In *C. anisata,* the iron content was found to be >1000 µg/day and this may be useful for the persons with micronutrient deficiency. The dry mass per unit area of *C. anisata* is 3.24, nitrogen concentration per unit dry mass is 22.3 and water concentration per unit dry mass is 2.5 [8]. They have the ability to withstand heavy pruning, easy to grow and free of pests and diseases. Nearly, about 9 out of 23 known species of *Clausena* genus were explored and identified for chemical and biological studies.

With this background, this research study has been initiated with an overall objective to analyze the possible effects of silver nanoparticles (SNPs) from *C. anisata* for its antibacterial activity.

METHODS

Plant collection

The fresh leaves and roots of *C. anisata* were procured during the months of October to November 2013 from Manamettupatti, Viralimalai Taluk (Pudukottai District, Tamil Nadu). The botanical identity of the plant specimen was identified by Dr. S. John Britto, Director, The Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph's College (Campus) Tiruchirappalli - 620002

and authenticated as *C. anisata* (Willd.) Hook f. ex. Benth. The leaf and root parts were washed with tap water followed by distilled water and shade dried in a well-ventilated room. The dried materials were powdered, stored in an airtight container, and used for further solvent extraction.

Preparation of the ethanol extract

The powdered leaf and root were extracted using ethanol in the ratio of 1:10 by maceration method. After 48 hrs, the extracts were filtered through Whatmans No: 1 filter paper. The leaf and root material were then macerated again with ethanol and the combined filtrate obtained from the first and the second maceration was then concentrated to dryness under controlled temperature 40-50°C using Rota evaporator (Yamato, Japan (Model-RE801)). The extract was preserved for synthesizing SNPs.

Optimization and synthesis of SNPs [9]

Different concentrations of leaf and root ethanolic extracts (1, 3 and 5 ml) were taken in a conical flask separately. The volume was made up to 10 ml using 1 mM $AgNO_3$ solution, stirred, and exposed under different conditions such as sunlight irradiation, direct boiling, and several short burst of microwave irradiation at a frequency of 2.45 GHz in a domestic microwave oven (Samsung, G2739N) at a power output of about 100 W in a cyclic mode (on 10s, off 10s) to prevent overheating as well as aggregation of metals, ultraviolet (UV) irradiation, and room temperature. The color change of the solution was checked periodically.

Bulk production and recovery of SNPs by centrifugation

About 1 ml was chosen for bulk production as 10 ml leaf and root extracts in 100 ml of 1 mM $AgNO_3$. After bioreduction, the solution consisting of SNPs was subjected to centrifugation at 10,000 rpm for 15 minutes, and the supernatant was discarded. The pellet formed was air dried, lyophilized (Penguin classic plus, Lark India), and stored for further characterization.

Characterization studies

The biosynthesized SNPs were characterized by the following methods.

Visual observation

A change of color from green to reddish brown for leaf and from beige to dark brown for root was observed in the solution after visible irradiation.

UV spectrophotometric analysis

The formations of leaf, root extract mediated SNPs were confirmed by the spectral analysis. The UV spectra of the biosynthesized SNPs were recorded using Lambda 35, Perkin Elmer Spectrophotometer by continuous scanning from 190 to 1100 nm and distilled water was used as the reference for the baseline correction.

Fourier transform infrared spectroscopy (FTIR) analysis

The functional groups in the biosynthesized SNPs of ethanolic leaf and root extracts were analyzed by FTIR spectroscopy. These measurements were carried out using a Perkin Elmer spectrum RX I FTIR instrument with a wavelength range of 4000-400/cm. The results were compared for shift in functional peaks.

Field emission scanning electron microscope (FESEM) analysis

FESEM was used to characterize the mean particle size, morphology of the SNPs. A small drop of biosynthesized SNPs solution was placed on glass slide and allowed to dry. The samples were analyzed using FEI Quanta 200 FEG machine at a low vacuum in the range 10-20 Kv.

Energy dispersive X-ray spectroscopy (EDS) analysis

The elemental composition of the synthesized SNPs was analyzed with energy dispersive spectroscopy coupled to scanning electron microscope.

X-ray diffraction (XRD) analysis

The SNPs solution thus obtained was purified by repeated centrifugation at 10,000 rpm for 20 min. The purified SNPs were dried. The structure and composition of SNPs were studied by XRD (XPERT-PRO machine). The data were collected in the 2θ range. The average size of SNPs was calculated.

Determination of antibacterial activity

Microorganisms

In vitro antimicrobial activity was examined for all the extracts of SNP leaf and SNP root. Micro-organisms were obtained from American Type Culture Collection and maintained at 4°C on nutrient agar slants. Among ten micro-organisms investigated four Gram-positive bacteria were *C. perfingers* (ATCC - 13124), *S. aureus* (ATCC - 25923), *S. epidermis* (ATCC - 12228), and *Bacillus subtilis* (ATCC - 6051), whereas five Gram-negative bacteria were *K. pnuemoniae* (ATCC - 27736), *Proteus vulgaris* (ATCC - 6380), *Pseudomonas aeruginosa* (ATCC - 10148), *Escherichia coli* (ATCC - 25922), and *Provedencia rettgeri* (ATCC - 25932).

Agar well diffusion method [10]

About 100 ml of nutrient broth was inoculated with the test organisms and incubated at 37°C for overnight. By using a sterile pipette, 0.6 ml of the broth culture of each test organism was added to 60 ml of molten agar, which was cooled. Mixed well and poured into a sterile Petri plate. Mueller-Hinton agar plates of each test organism were prepared. A required number of wells were prepared in the plates with the help of a cork-borer (0.85 cm). SNP extracts (leaf and root) 50 µg/ml, 100 µg/ml, and 150 µg/ml of sample and silver nitrate 100 µl were introduced into the wells. The plates were incubated overnight at 37°C. Microbial activity was determined by measuring the diameter of zone of inhibition using a ruler in millimeter. The results were compared with the standard ciprofloxacin (1 mg/ml).

Disc diffusion assay method [11]

This method was carried out to screen the antimicrobial activity. Sterile Mueller-Hinton agar plates were prepared by pouring 15 ml into each plate. The plates were allowed to solidify for 5 minutes and 0.1% inoculum suspension was swabbed over the surface and was allowed to dry for 5 minutes. The extracts at a concentration of 1 mg/ml were loaded on 6 mm sterile disc. The loaded disc was placed over the surface of the medium and the extract was allowed to diffuse for 5 minutes and incubated overnight at 37°C. The zones of inhibition were measured using a ruler, and the average was taken from the mean of triplicates.

Statistical analysis

All experiments were carried out in triplicates. A statistical analysis was performed using PRISM Software Package (Version 5.0). Results were expressed as mean±standard deviation and the significances by differences between mean values were determined by one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* analysis. $p<0.01$, $p<0.001$, and $p<0.0001$ were considered statistically significant.

RESULTS

Among various concentrations and methods used for synthesis of SNPs, room temperature (10-15 minutes), and sunlight irradiation (10 minutes) were found to be effective for ethanolic leaf and root extracts, respectively. 1 ml of the homogenized extracts was shown maximum synthesis of SNPs.

Visual observation

A change in color from green to reddish brown for leaf (Fig. 1a-f) and from beige to brown for root (Fig. 2a-f) was observed in the solution after respective irradiation.

Characterization of SNPs

The synthesized SNPs from ethanolic leaf and root extracts were characterized to confirm its synthesis. The different characterization methods were as follows.

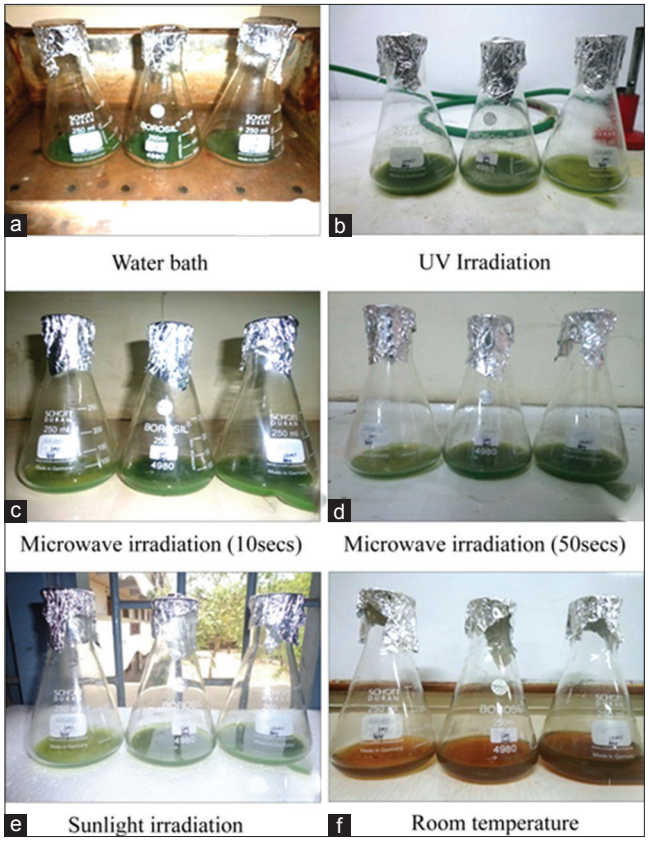

Fig. 1: (a-f) Optimization and synthesis of silver nanoparticles using *Clausena anisata* ethanolic leaf extract

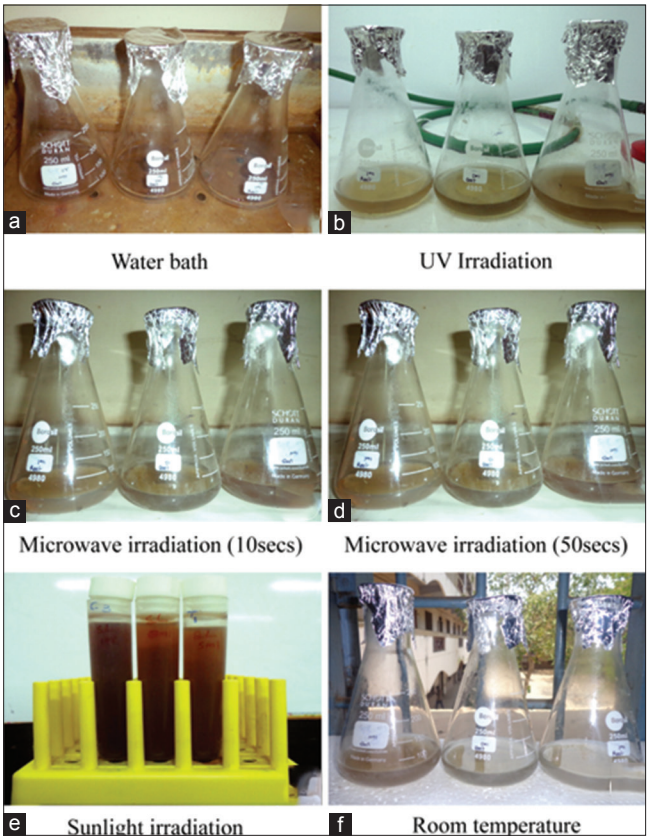

Fig. 2: (a-f) Optimization and synthesis of silver nanoparticles using *Clausena anisata* ethanolic root extract

UV spectrophotometric analysis

The color change of leaf and root ethanolic extracts of *C. anisata* arised due to the excitation of SPR with the SNPs. The SPR of SNPs produced a peak centered near to 488.99 nm (Fig. 3) and 432.97 nm (Fig. 4) for leaf and root, with absorption 0.86693 and 1.5557, respectively. These results indicated the successful conversion of silver nitrate (Ag^+) to silver (Ag^0).

FTIR analysis

FTIR analysis was used to identify the functional groups of the active components in the extracts that are responsible for reducing and capping the synthesized SNPs. Eight IR bands were identified for SNP extracts which were tabulated. The absorption peak of 1631.77 cm^{-1} and 1630.84 cm^{-1} for SNP leaf and SNP root in the infrared region of the electromagnetic spectrum exhibited the binding of amide linkage with SNPs that may be assigned to the carbonyl stretch in proteins and clearly indicated the presence of protein as capping agent for SNPs.

FTIR spectral data for the biosynthesized SNP leaf extract are attributable to -C≡C-H, C-O, N-O symmetric stretch, N-H bend, -C≡C-, RCO_2H and O-H/H-bonded stretch exists in the region of 667.22/cm, 1015.41/cm, 1360.46/cm, 1631.77/cm, 2083.51/cm, 2833.19/cm, 3432.71/cm and 3464.31/cm respectively (Fig. 5) (Table 1).

FTIR spectral data for the biosynthesized SNP root extract were attributed to the N-H stretch at 3432.97/cm, H-C=O stretch exists in the region of 2832.62/cm, peaks at 663.20/cm, 774.15/cm, 1015.96/cm, 1361.75/cm and 1630.84/cm can be assigned to C-H bend, C-Cl stretch, C-O stretch, N-O symmetric stretch and RNH_2 stretch (Fig. 6) (Table 2).

FESEM analysis

This analysis was performed to measure the size and shape of the SNPs. The synthesized SNPs showed spherical form and with a size distribution ranging from 50 nm to 66 nm, with an average size 60.67 nm for SNP leaf. The size distribution ranging from 23 nm to 44 nm, with average size 32.75 nm was found to be for SNP root (Fig. 7).

EDS analysis

The EDS analysis was performed to know the percentage of silver in the sample at 3 KeV. The EDS spectra showed the different types of elements with their weight percentage such as silver (55.24%), carbon (18.97%), chlorine (13.43%), oxygen (9.61%) and copper (2.76%) in SNP leaf (Fig. 8) and in SNP root it was found to be silver (24.72%), carbon (38.78%), oxygen (28.25%), chlorine (6.37%) and calcium (1.88%), respectively (Fig. 9).

XRD analysis

XRD analysis was used to confirm the crystalline nature of the SNPs. Different diffraction intensities were recorded from 20° to 80° in 2θ angles. This was compared with the standard ICDD files. The biosynthesized SNPs using extracts of *C. anisata* was further confirmed by the characteristic peaks observed in the XRD image at 2 θ angles with minimum and maximum range at 27.48°-76.44° for SNP leaf (Fig. 10) and 27.64° - 76.53° for SNP root (Fig. 11).

Determination of antibacterial activity

An antibacterial activity for the SNP leaf and SNP root extracts of *C. anisata* was determined using agar well diffusion method and disc diffusion method. The data pertaining to the antibacterial potential of the extracts were presented. The SNP leaf and root extract showed antibacterial activity against all the organisms tested in this study.

The SNP leaf and root extracts showed antibacterial activity in the concentration ranging from 50 µg to 150 µg/ml. All the extracts showed antibacterial activity against all the tested micro-organisms. In which, the SNP leaf extract showed maximum zone of inhibition against *P. aeruginosa* (12±0.5) ($p<0.0001$), followed by *B. subtilis* ($p<0.0001$), *Staphylococcus aurues* ($p<0.0001$), *P. vulgaris* ($p<0.0001$), and *P. rettgeri* ($p<0.001$) with each 10 mm in diameter and *E. coli* with 8±0.6 mm in

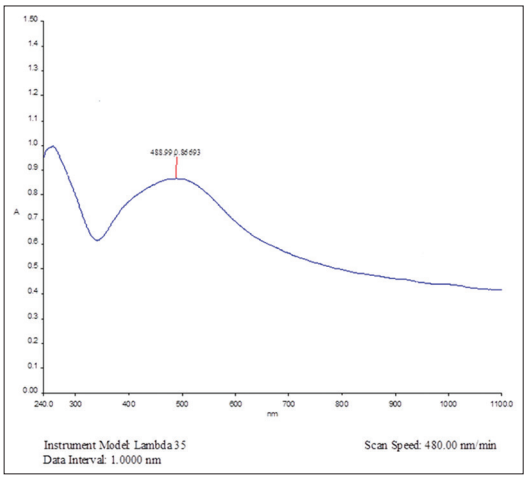

Fig. 3: Ultraviolet-visible profile for ethanolic silver nanoparticles leaf extracts of *Clausena anisata*

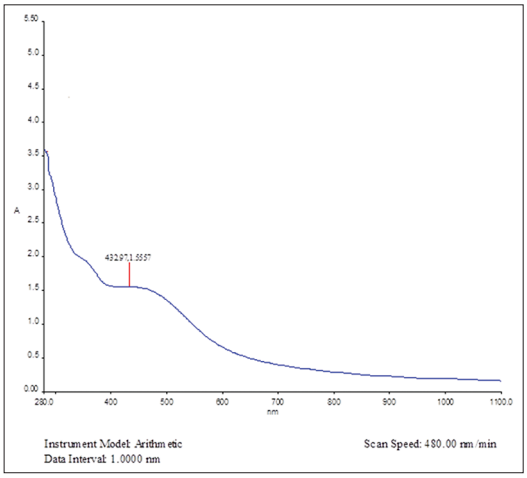

Fig. 4: Ultraviolet-visible profile for ethanolic silver nanoparticles root extracts of *Clausena anisata*

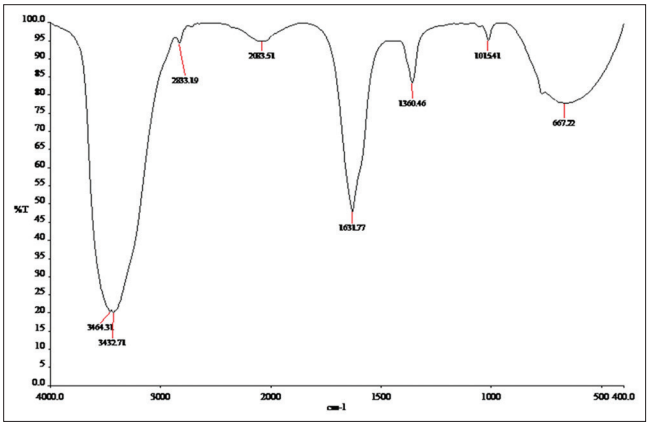

Fig. 5: Fourier transform infrared spectroscopy spectrum of *Clausena anisata* ethanolic silver nanoparticles leaf extracts

diameter, where standard showed 15±0.57 mm in diameter against *E. coli* (Fig. 12) (Table 3).

The maximum zone of inhibition for SNP root extract was observed against *E. coli* (p<0.01) and *P. retttgeri* (p<0.01) with 15 mm in diameter each, followed by *P. aeruginosa* (p<0.0001), *B. subtilis* (p<0.0001),

Table 1: Functional groups in SNP leaf extracts of *C. anisata*

S. No	Peak value cm^{-1}	Bond	Functional groups
1	667.22	-C≡C-H	Alkynes
2	1015.41	C-O Stretch	Alcohol, ester, ether
3	1360.46	N-O symmetric stretch	Nitro compounds
4	1631.77	N-H bend	Primary amine
5	2083.51	-C≡C- Stretch	Alkynes
6	2833.19	RCO_2H	Carboxylic acid
7	3432.71	O-H stretch/H- bonded	Alcohol, phenol
8	3464.31	O-H stretch/H- bonded	Alcohol, phenol

SNP: Silver nanoparticles, *C. anisata: Clausena anisata*

Table 2: Functional groups in SNP root extracts of *C. anisata*

S. No	Peak value cm^{-1}	Bond	Functional groups
1	663.20	C-H bend	Alkynes
2	774.15	C-Cl Stretch	Alkyl halide
3	1015.96	C-O stretch	Alcohol
4	1361.75	N-O symmetric stretch	Nitro compounds
5	1630.84	RNH_2	Amines
7	2832.62	H-C=O	Aldehyde
8	3432.97	N-H stretch	Amine, amide

SNP: Silver nanoparticles, *C. anisata: Clausena anisata*

S. aureus (p<0.0001) and *P. vulgaris* (p<0.0001) with 14±2.5 mm, 12±1.52, 12±2.1 and 9±0.57 mm zone of inhibition, respectively, and standard showed 20±1.52 mm zone of inhibition against *P. rettgeri* (Fig. 13) (Table 4).

DISCUSSION

Plants are used to cure human diseases due to the presence of phytoconstituents, which are medicinally significant. The phytoconstituents present in the plants are non-nutritive and responsible for the physiological actions in humans. The development of drugs from this phytoconstituents is becoming important in recent years for several ailments. Hence, this study was conducted to evaluate the antibacterial of *C. anisata* extracts SNP leaf and SNP root.

The phytoconstituents in plants play an important role as reducing and capping agents that stabilize the SNPs to prevent agglomeration and increase in particle size [12]. The mechanism of synthesis of *C. anisata* SNPs under sunlight irradiation may be due to solar photos that hit the NPs present in the solution during exposure under sunlight. The dissolved oxygen molecules in the solution will react with the excited electrons at the particle surface and get converted into oxygen anion radicals [12]. The change in color of the reaction was due to the splitting of $AgNO_3$ to Ag^+ and NO^-_3, as time progress. The reduction in Ag^+ ions into Ag may be due to the metabolites in the leaf and root extracts acted as an electron donor. Due to the excitation of surface plasmon vibrations, the formation of SNPs was indicated by brown color of the aqueous solution.

Similar to this study, [13] reported the synthesis of SNPs using aqueous garlic extract by exposing under bright sunlight for 15 minutes and it was found to be stable for a very long period and retained their bactericidal potential. The biosynthesis of SNPs from aqueous leaf extract of *Synedrella nodiflora* under sunlight irradiation was also reported by [14] that produced a peak centered near 428.7 nm.

UV-visible spectroscopy is an indirect method for determining the reduction of silver ions to SNPs. The optical phenomenon is due to the plasmon resonance in silver and gold metals. The SPR band is due to the free electrons in the conduction band due to small particle size [15]. Due to the combination of proteins, amino acids, enzymes,

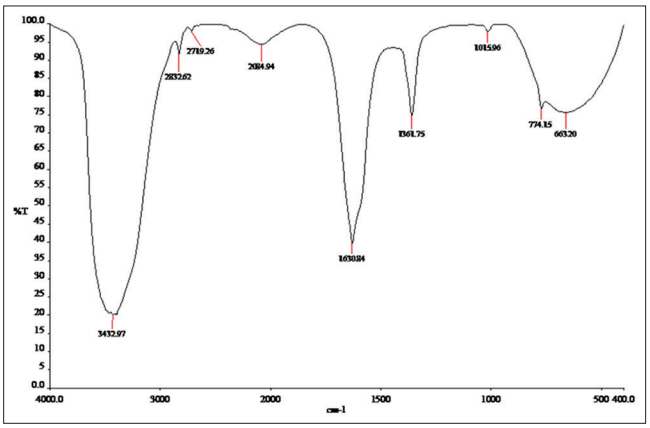

Fig. 6: Fourier transform infrared spectroscopy spectrum of *Clausena anisata* ethanolic silver nanoparticles root extracts

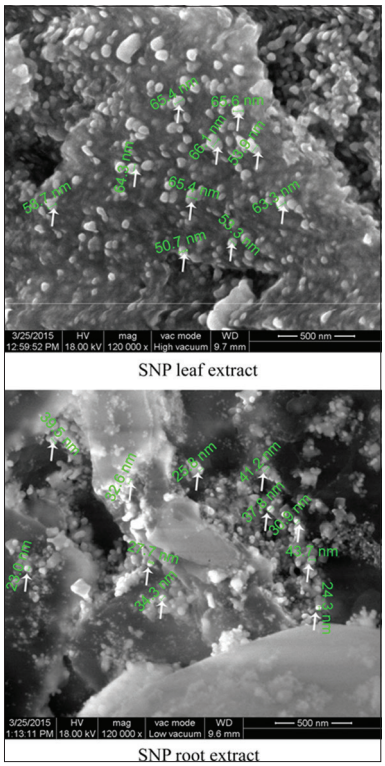

Fig. 7: Field emission scanning electron microscope analysis of *Clausena anisata* silver nanoparticles (SNP) leaf and SNP root extract

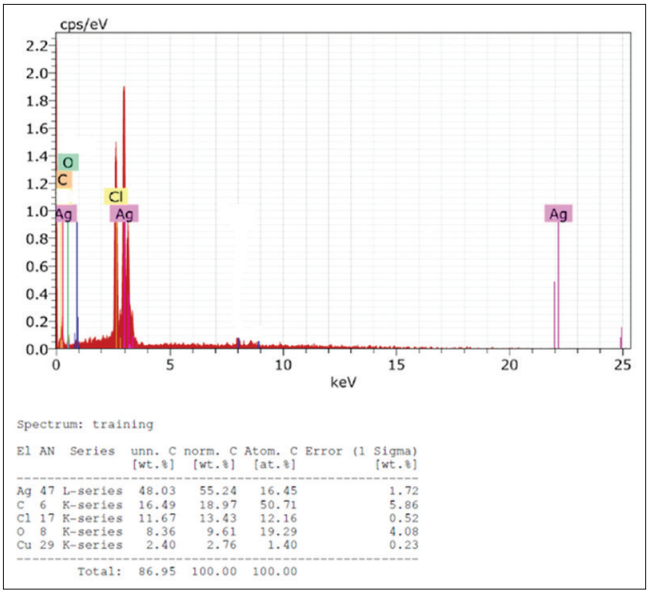

Fig. 8: Energy dispersive X-ray spectroscopy analysis of *Clausena anisata* silver nanoparticles leaf extract

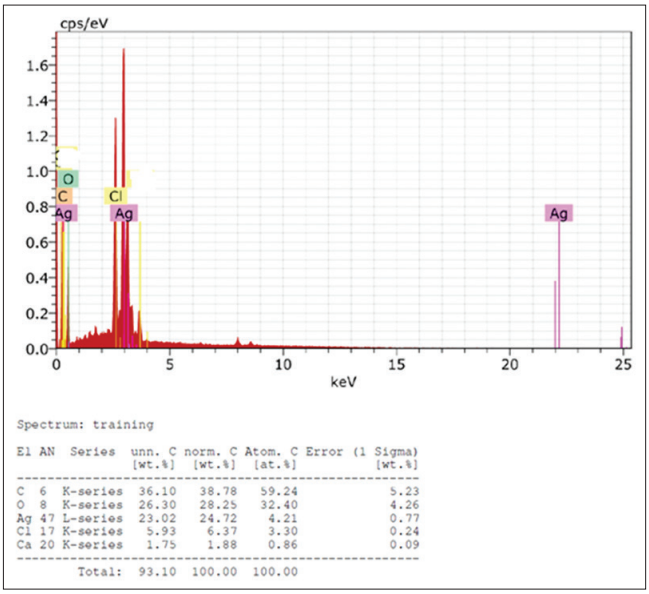

Fig. 9: Energy dispersive X-ray spectroscopy analysis of *Clausena anisata* silver nanoparticles root extract

polysaccharides, alkaloids, tannins, phenolics, saponins, and terpenoids may be responsible for the reduction and stabilization of silver ions [16]. Proteins that have stronger binding affinity to SNPs will increase the stability of synthesized NPs.

The absorbance peaks obtained for *C. anisata* leaf and root extracts was in line with [17] who investigated the synthesis of SNPs from *Musa balbisiana, Azadirachta indica* and *Ocimum tenuiflorum* extracts, which possessed a characteristic absorption peaked in the range of 425-475 nm confirmed the reduction of silver ions due to SPR. Another study carried out by [18] on the extracts of *Securinega leucopyrus* showed SNPs with absorbance peaks within the range of 470-490 nm, which was corroborated with this study.

An earlier report suggested that peaks denoting –C-N stretching vibrations of amine, C-O-C, ether linkage, -C-O-, germinal methyl, -C=C- groups were from aromatic rings and alkyne bonds and responsible for compounds such as alkaloid, flavonoid, and terpenoids that may be act as capping and stabilizing agent for SNPs [19].

In this study, the stability of SNPs may be due to the interaction of hydrogen bond and electrostatic interaction between the capping molecules bound to the SNPs. The SNPs were not in direct contact that indicates that capping agents have stabilized the NPs. The larger SNPs may be due to the aggregation of the smaller ones.

The same type of results was found in SNPs synthesized from stem bark extract of *S. alternifolium* that showed particle size ranging from 4 to 48 nm sized spherical shaped particles [20]. The SNPs synthesized from leaf extract of *Bixa orellana* showed size ranging from 35 to 65 nm [21]. The SEM results are also consistent with those of [22] who reported that synthesis of SNPs from aqueous leaf extract of *Phyllanthus amarus* showed particle ranging between 32 and 53 nm.

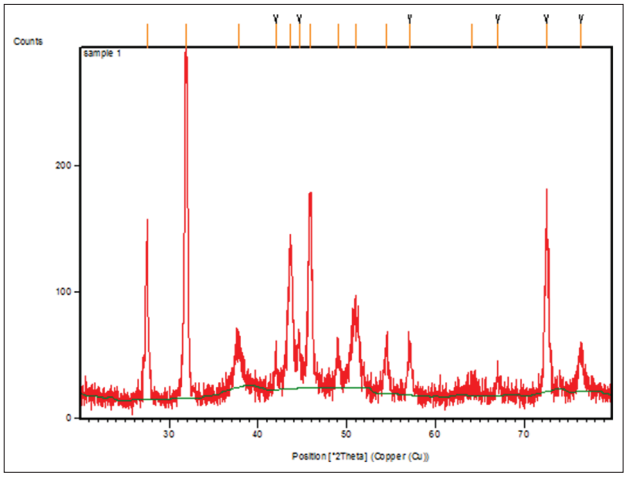

Fig. 10: X-ray diffraction analysis of *Clausena anisata* silver nanoparticles leaf extract

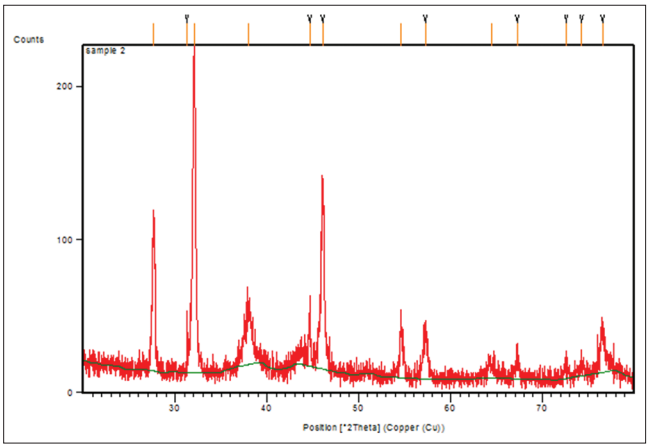

Fig. 11: X-ray diffraction analysis of *Clausena anisata* silver nanoparticles root extract

In this study, the other weak signal corresponding to O_2, Cl, and Ca which could have derived from the plant extract and due to biomolecules bound to the surface of the SNPs. Carbon and copper peaks may be due to the presence in the grid. It was reported that NPs synthesized using plant extracts may have a thin layer of capping organic material surrounding the NPs that was obtained from the plant leaf or root extracts. Here, the several Braggs reflection peak was pointed toward crystal structure of silver. The few unassigned Braggs peak might be due to capping agent stabilizing the NPs with were consistent with the earlier report [23].

The antibacterial activity for crude extracts and SNPs synthesized using extracts of *C. anisata* was similar to previous report of [24] who revealed that SNPs from aqueous leaf extract of *M. emarginata* was active against *P. seruginosa, E. coli, B. cereus, S. aureus* and *E. coli* where highest MIC was found for *B. cereus* with 100 µg/ml and [25] carried out a similar study on antibacterial activity of leaf acetone extract of *C. anisata* that was active against both Gram-positive and Gram-negative bacteria with MIC 0.1 mg/ml - 0.5 mg/ml. The dichloromethane extract of the bark was active against *S. aureus, E. coli, Streptococcus pyogens* with MIC 5 mg/ml. The aqueous extract did not show any activity against the tested organisms.

The secondary metabolites in plants are most widely used in agriculture, medicine, pharmaceutical industry and also act as natural antibiotic that inhibit the growth of microorganisms. The phytoconstituents such as coumarins, quinines, terpenoids, essential oil, tannins, flavonoids, alkaloids, phenanthrene, phenolic acids, polyphenols and

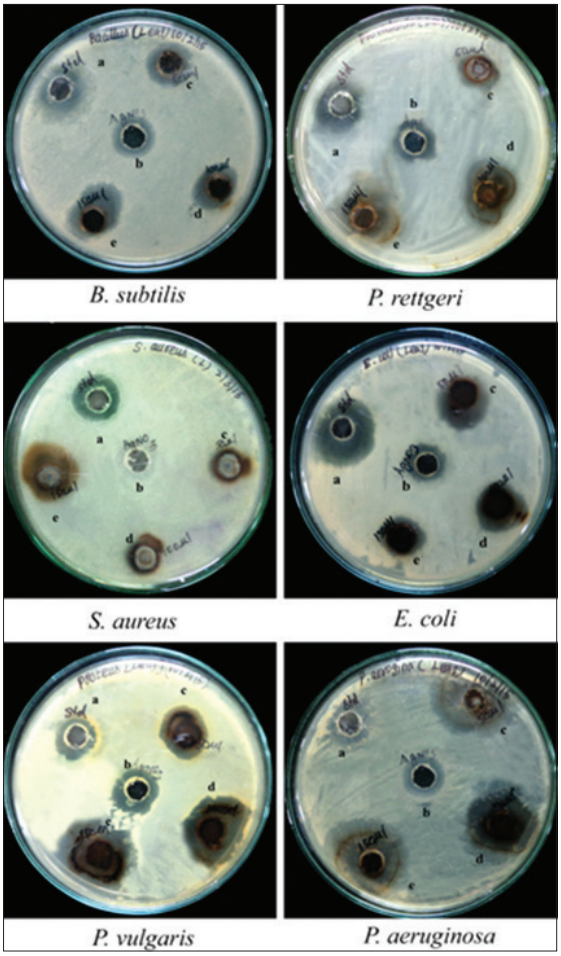

Fig. 12: *Clausena anisata* ethanolic silver nanoparticles leaf extracts showing zone of inhibition against test organisms

other aromatic compounds act as defense mechanisms against several microorganisms, insects, and herbivores [26]. The organisms used in this was selected based on the previous reports of [27] who isolated the presence of *P. aeruginosa*, *E. coli*, *S. aureus, Klebsiella pneumonia* and *P. vulgaris* out of 157 organisms from the diabetic patients with foot lesions and Gram-negative organisms was found to be predominantly present in the lesions. Several studies showed the presence of *Clostridium perfringers, Staphylococcus epidermis,* and *B. subtilis* along with other Gram-positive and Gram-negative organisms in diabetic wound.

The difference in the activities of the antibacterial test, for Gram-positive and Gram-negative bacteria is due to the morphological difference in the cell wall and membrane organization. Due to this difference, the Gram-positive bacteria are more susceptible to phytoconstituents compared to Gram-negative bacteria, in which a combined phytoconstituents is needed to provide a synergistic antibacterial activity against Gram-negative organisms. Depending on their solubility or polarity of the solvents, different solvents have the ability to extract different phytoconstituents. In this study, the ethanolic extracts might have the higher solubility rate for phytoconstituents that leads to highest antibacterial activity.

In the case of SNPs, silver itself having antimicrobial activity against wide range of bacteria, fungi and viruses. When silver is used in nitrate form, it induces the antimicrobial effect. But when SNPs are used, high surface area will be available for the microbes that are exposed to [28]. Gram-positive bacteria are less susceptible to Ag^+ than Gram-negative bacteria that may be due to the thick peptidoglycan layer in Gram-positive bacteria that bears negative charge than Gram-negative

Table 3: Antibacterial activity of *C. anisata* ethanolic SNP leaf extracts

Test organisms	Zone of Inhibition (mm)				
	Standard (ciprofloxacin)	$AgNO_3$	50 µg/ml	100 µg/ml	150 µg/ml
Gram-positive					
B. subtilis (ATCC-6051)	5±0.57	4±0.6	7±0.5*	9±0.57**	10±1.15***
S. aurues (ATCC-25923)	5±1	4±0.57	6±0.5*	8±0.57**	10±0.5***
Gram-negative					
E. coli (ATCC-25922)	15±0.57	5±0.5	7±0.6	8±1	8±0.6
P. vulgaris (ATCC-6380)	5±1.15	4±1	6±1*	9±0.6**	10±1.5***
P. aeruginosa (ATCC-10148)	5±0.57	4±0.6	6±0.57*	8±1**	12±0.5***
P. rettgeri (ATCC - 25932)	5±0.58	5±1	8±0.5*	9±1.15**	10±1.15**

A statistical significance (p) calculated by one-way ANOVA between the extracts and standard followed by Tukey's *post-hoc* test of significance. *p<0.01, **p<0.001, ***p<0.0001: Between extract and standard. SNP: Silver nanoparticles, *B. subtilis*: *Bacillus subtilis*, *S. aurues*: *Staphylococcus aurues, E. coli*: *Escherichia coli, P. aeruginosa*: *Pseudomonas aeruginosa, P. vulgaris*: *Proteus vulgaris, C. anisata*: *Clausena anisata*

Table 4: Antibacterial activity of *C. anisata* ethanolic SNP root extracts

Test organisms	Zone of Inhibition (mm)				
	Standard (ciprofloxacin)	$AgNO_3$	50 µg/ml	100 µg/ml	150 µg/ml
Gram-positive					
B. subtilis (ATCC-6051)	5±1.15	4±1	7±1.5**	10±1.15***	12±1.52***
S. aurues (ATCC-25923)	5.03±1.98	5±1.52	5±1.15	10±0.98***	12±2.1***
Gram-negative					
E. coli (ATCC-25922)	17±1.154	5±1.15	8±1.15	10±1.15	15±1.9*
P. vulgaris (ATCC-6380)	5±1.53	4±0.57	7±1**	8±1**	9±0.57***
P. aeruginosa (ATCC-10148)	5±0.58	6±1*	10±1.2**	12±1**	14±2.5***
P. rettgeri (ATCC – 25932)	20±1.52	5±1.15	9±1.1	12±1.53	15±1*

Statistical significance (p) calculated by one-way ANOVA between the extracts and standard followed by Tukey's *post-hoc* test of significance. *p<0.01, **p<0.001, ***p<0.0001: Between extract and standard. SNP: Silver nanoparticles, *B. subtilis*: *Bacillus subtilis*, *S. aurues*: *Staphylococcus aurues, E. coli*: *Escherichia coli, P. aeruginosa*: *Pseudomonas aeruginosa, P. vulgaris*: *Proteus vulgaris, C. anisata*: *Clausena anisata*

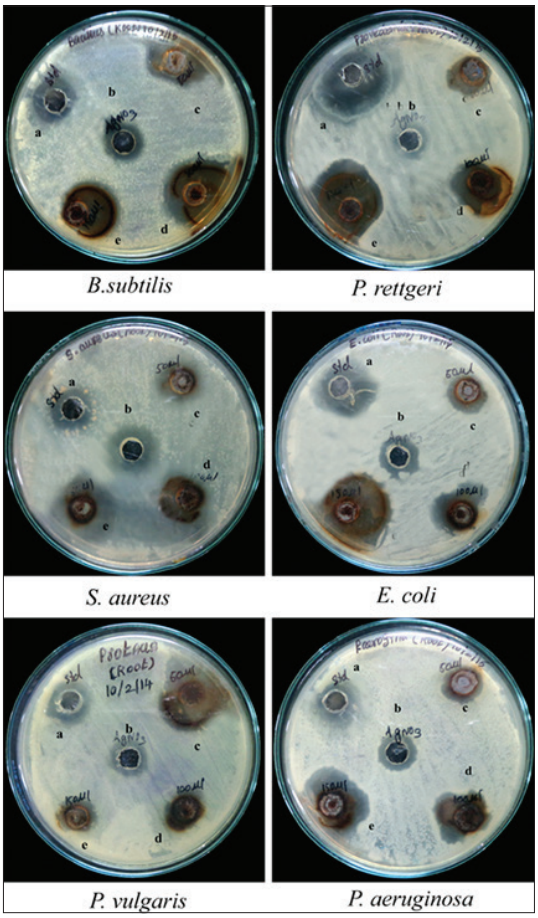

Fig. 13: *Clausena anisata* ethanolic silver nanoparticles root extracts showing zone of inhibition against test organisms

bacteria. In this study, SNP showed efficient antimicrobial activity that may be due to the extremely large surface area that provides better contact with test organisms.

CONCLUSION

In conclusion, on the result of this study among SNP leaf and SNP root extract, the SNP synthesized using the root extract of *C. anisata* showed maximum potent of antibacterial that may be due to the active phytoconstituents which were responsible for the activities. The SNPs synthesized from *C. anisata* ethanolic extract was characterized by UV-Vis, FTIR, XRD, EDS, and FESEM that supports the stability of SNPs. The extracts may be acted as a reducing and capping agent. The antibacterial activity of SNP root extract showed wider activity against Gram-negative than Gram-positive organisms. The phytoconstituents of *C. anisata* SNP root extract may be acting synergistically or independently in exerting an overall antibacterial action in this study, and that should be chemically analyzed, and their chemical structure should be understood to develop an effective antibacterial therapeutic agent in future.

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