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Dhaniyel MG
Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences Mannuthy, Thrissur, Kerala, India

Dr. Aziz Zarina
Associate Professor, Department of Veterinary Physiology, College of Veterinary and Animal Sciences Mannuthy, Thrissur, Kerala, India

Corresponding Author
Dhaniyel MG
Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences Mannuthy, Thrissur, Kerala, India

Assessment of antibacterial potential of silver nanoparticles biosynthesis using trikatu

Dhaniyel MG and Dr. Aziz Zarina

Abstract
Nanotechnology has become one of the most important forefront fields in biology, physics and engineering. Plant-based extracts are believed to be green and effective way in the synthesis of silver nanoparticles. The present study focused on the green synthesis of silver nanoparticles using trikatu extract and evaluation of their antibacterial activity against common pathogens including *Escherichia coli* and *Staphylococcus aureus*.
Phytochemical analysis of the trikatu extract indicated that trikatu is a source of various phytochemicals like alkaloids, glycosides, flavonoids and diterpenes. Silver nanoparticles were prepared by mixing trikatu extract with varying concentration of silver nitrate solution.
Presence of Silver nanoparticles was confirmed by Ultaviolet-visible spectroscopy. The functional groups of the biomolecules were analysed using Attenuated total reflection spectroscopy. X- ray diffraction analysis of AgNPs indicated that the particles were crystalline in nature and were face centered cubic structure. Scanning electron microscope analysis revealed that the silver nanoparticles synthesised using trikatu were spherical shaped and the size was around 0.7 micrometer (70 nanometer).
The antibacterial potential of green synthesised silver nanoparticles was tested against the common pathogens; the Gram-negative *Escherichia coli* and Gram-positive *Stephylococcus aureus* and was shown to have good antibacterial potential against both organisms.

Keywords: Nanotechnology, trikatu, silver nanoparticles, green synthesis, antibacterial

1. Introduction
Nanotechnology deals with the various structures of matter having size less than 100 nm. It comprises very small sized structures and materials of dimensions in the range of few nanometers to less than 100 nanometers [1, 2]. In recent years nanotechnology has become one of the most important and exciting forefront fields in biology, physics and engineering [3]. Nanoparticles show size and shape-dependent features [4] which include applications ranging from biosensing and catalysts to optics, antimicrobial activity, computer transistors, electrometers, chemical sensors, and wireless electronic logic and memory schemes [5]. These particles also have many applications in different fields such as medical imaging, nanocomposites, filters, drug delivery, and hyperthermia of tumors [6, 7].
Nanoparticles can be synthesised in different methods including chemical, physical and biological ways [8]. Rather than the physiochemical synthesis of nanoparticles, synthesis of nanoparticles by biological ways using plant extracts, micro-organisms or enzymes have been proved to be a better ecofriendly alternative [9, 10]. Chemical and physical techniques of nanoparticle synthesis involve the need for toxic solvents and can cause chemical contamination in nanoparticle production [11], hence it does not allow the active use in biomedical fields. Biosynthesis of metal nanoparticle ensures "green" non- toxic way of nanoparticle synthesis and can be confidently used in wide variety of industries. Silver nanoparticles have been shown in numerous studies to display antibacterial properties [12]. Nanoparticles such as gold and silver have been shown to be very effective against different types of microorganisms including Gram positive and Gram-negative bacteria [13]. The synthesis of silver nanoparticle involves reduction of silver ions (Ag^{+}) to metallic silver (Ag^{o}) [14] and agglomeration and stabilization forms the oligomeric clusters of colloidal silver nanoparticles.
Trikatu is an ayurvedic blend formed by mixture of three valuable spices, such as black pepper (*Piper nigrum*), long pepper (*Piper longum*), and rhizomes of ginger (*Zingiber officinale*) [15, 16, 17]. It has got a very powerful reducing activity and that is used in the production of silver nanoparticles. Trikatu is extensively used in the biomedical field and consumption of trikatu

churna shows several health benefits by the virtue of their innumerable therapeutic potentials, such as fever, asthama, cold, cough and other general health disorders [18, 19, 20, 21]. The current study, explains the synthesis and characterisation of silver nanoparticles green synthesised using trikatu extract and evaluation of their antibacterial activity against common pathogens (*E. coli* and *S. aureus*) [22].

2. Materials and Methods

2.1 Collection of plant material

Trikatu an ayurvedic blend of equal parts of the fruits of black pepper (Piper nigrum), long pepper (Piper longum), and rhizomes of ginger (*Zingiber officinale*) was collected individually from ayurvedic shop Irinjalakuda, Thrissur. The collected samples were shade dried, pulverised and subjected to aqueous extraction.

2.2 Phytochemical screening of trikatu extract

2.2.1 Preparation of Trikatu extract

Three equal proportions of fruits were dried under shade and ground into fine powder using mixer grinder. About 40 gm of dried powder was soaked into 400 mL distilled water. This mixture was boiled at 80 ^{0}C in the water bath for 15 minutes [23]. The solution was cooled at room temperature and filtered by Whatman No.1 Filterpaper. The filtrate was concentrated to near dryness using rotatory vaccum flash evaporator under reduced pressure at 30 ^{0}C and stored at 4 ^{0}C in airtight containers.

2.2.2 Synthesis of AgNPs

The nanoparticles were synthesised according to the protocol of [24] with slight modifications. About 10 mL plant extract was added to 90 mL of each of the $AgNO_3$ solutions of varying molarities and mixed well (Fig.1). The solutions were then kept under sunlight for about 5 - 10 min. Reduction of silver ions (Ag^+) to metallic silver (Ag^0) was confirmed by the colour change of solution from colourless to brown [25, 26](Fig.2). All the solutions were further incubated overnight in darkness. The solution obtained which contained AgNPs was purified by repeated centrifugation at 8000 rpm for 20 min followed by re- dispersion of the pellet in de-ionized water. The purified solutions obtained were then subjected to lyophilisation. The AgNPs pellet re-dispersed in de-ionized water was frozen at – 120 °C using cold trap and then subjected to lyophilisation in a freeze drier (Operon FDU 7003, Korea).

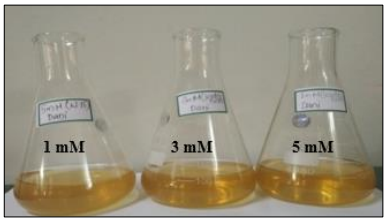

Fig 1: Mixed solution of trikatu extract with varying concentration of silver nitrate solution

Fig 2: Synthesised silver nanoparticles at varying concentration

2.2.3 Phytochemical screening

The aqueous extract isolated from the fruits (black pepper, long pepper and rhizomes of ginger) were tested for the presence of various active chemical constituents [27] namely alkaloids, steroids, saponins, tanins, phenolic compounds, flavonoids, glycosides, diterpenes and triterpenes (Fig.3).

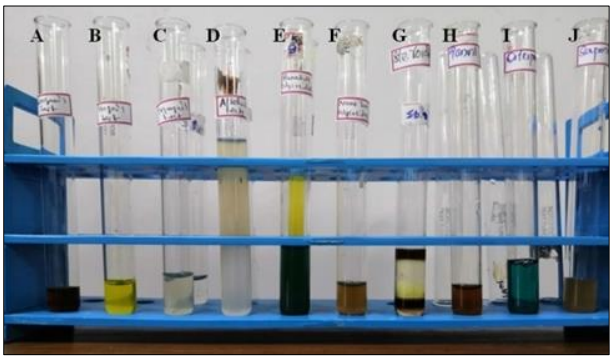

Fig 3: Phytochemical analysis of trikatu aqueous extract (A-Wagner's test, B-Hager's test, C- Mayer's test, D- Alkaloid test, E-Benedict's test, F- NaOH test, G- Steroid test, H- Test for tannins, I-Test for diterpenes, J- Test for saponins)

2.3 Characterisation of the nanoparticles

The formation of AgNPs by reduction of Ag^+ to Ag^0 using trikatu extract was initially confirmed by measuring the surface plasmon resonance peak of the samples using UV-Visible Spectrophotometer [28] (Perkin- Elmer, Lamda 25) by exposing to light of 300-700 nm wavelengths with 1 nm as wavelength resolution. Attenuated Total Reflection spectroscopy used in conjugation with infrared spectroscopy which enables samples to be examined directly in the solid or liquid state. The synthesized AgNPs was determined using the technique of Attenuated Total Reflection (ATR) at Central Instrumentation Laboratory, Mannuthy, Thrissur. It allows very thin sampling pathlength and depth of penetration of the infrared beam into the sample. The molecular and crystal structures of the synthesized AgNPs was determined using the technique of X – ray diffraction spectroscopy (Rigaku X – ray diffractometer, Miniflex, UK) at Centre for Electronics Technology (C-MET) Athani, Thrissur. The AgNPs were studied with CUKα radiation at a voltage of 30 kV and a current of 20mA with a scan rate of 100 2θ/min (θ is the Braggs angle in radians). Surface morphology of biologically synthesized AgNPs was evaluated through SEM. Samples were fixed with double adhesive tape on stubs, splutters coated with gold palladium and examined under SEM installed at Central Instrumentation Laboratory, Mannuthy, Thrissur. The samples were characterized in the SEM at an accelerating voltage of 20 kV upon gold coating for 4 minutes. The pictures were captured.

2.4 Antibacterial activity of AgNPs

The antibacterial potential of the synthesised AgNPs were tested against the common pathogens, *Escherichia coli* and *Staphylococcus aureus* [29]. The type cultures of *E. coli* and *S. aureus* were procured from Microbial Type Culture Collection (MTCC), Chandigarh [30]. The organisms were revived as per the instructions from MTCC in Brain Heart Infusion broth. The identity of both the organisms was confirmed by Gram's staining and biochemical tests including catalase, oxidase and IMViC tests. The cultures were then maintained in selective medium i.e., Eosin Methylene Blue (EMB) agar for E. coli (Fig.5) and Mannitol Salt agar (MSA)

for S. aureus (Fig.4). The inocula were prepared by direct colony suspension method [31]. From the 18-24 hour plates of *E. coli* and *S. aureus* maintained, three to four similar colonies were selected from each and transferred to 5 mL of nutrient broth. The broth was incubated at 37 °C for 2-8 h till light moderate turbidity developed. Turbidity of the inoculums was adjusted to match with that of 0.5 McFarland standards. The well diffusion method [32] was used to compare the antimicrobial activity of the nanoparticles of varying concentrations.

Fig 4: *Staphylococcus aureus* colonies formed in manitol salt agar

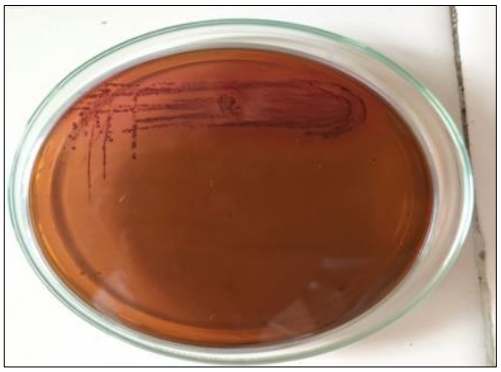

Fig 5: *Escherichia coli* colonies formed in EMB agar

2.5 Antibacterial susceptibility tests

2.5.1 Well Diffusion Test

Two nutrient agar plates were prepared, one for *E. coli* and the other for *S. aureus* respectively, by pouring 15 mL of molten media into sterile petri dishes. The plates were allowed to solidify for 15 min and were incubated at 37 °C for 24 h for checking their sterility. Sterile non-toxic cotton swab were dipped into each standardized inoculum (turbidity adjusted so as to obtain semi confluent growth on the petri plate) and rotated the soaked swab firmly against upper inside wall of the tube to express excess fluid. The broth suspension of the organism was spread evenly on the agar surface in each of the plate three times with swab, turning the plate at 60º angle between each spreading. The inoculum was allowed to dry for 5 min. Four wells of 6 mM diameter were made in the agar using sterile well borer. Fifteen microlitres each of the nanoparticle solutions (1.0, 3.0 and 5.0 mM) as well as 15 µL of a standard antibiotic solution (Ampicillin) were poured into the corresponding wells. The plates were then incubated at 37 °C for 24 h. The diameter of zone of inhibition developed around each well was measured [33]. Among the three samples of AgNPs synthesised using different concentrations of AgNO3, the sample that produced the widest zone of inhibition in the well diffusion test for each bacteria was selected to perform broth dilution test so that the minimum inhibitory concentration (MIC) of the nanoparticle could be determined.

2.5.2 Broth Dilution Test

Test tubes containing sterile 10 mL nutrient broth supplemented with selected AgNPs of varying concentrations (20, 10, 5, 2.5 and 1.25 µg/mL) were inoculated with 0.1 mL inoculum containing 1×106 cells of *E. coli.* The tubes 25 were then incubated in an orbital shaking incubator at 150 rpm for 24 h at 37 °C. Silver nanoparticle-free broth inoculated with the organism was used as negative control while 10 mL nutrient broth supplemented with 20 µg ampicillin inoculated with the bacteria served as positive control. The microbial growth was indexed by measuring the optical density (O.D 600) of all the tubes using UV-Vis spectrophotometer (Perkin-Elmer, Lamda 25). The whole procedure was repeated with *S. aureus* inoculum. Minimum inhibitory concentration is the lowest concentration of an antimicrobial agent that is bacteriostatic that prevents the visible growth of bacteria [34]. The concentrations showing no turbidity were further subjected to test for assessment of minimum bactericidal concentration (MBC) [35].

2.5.3 Assessment of minimum bactericidal concentration (MBC)

The Minimum Bactericidal Concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a bacterium over a fixed period, such as 18 hours or 24 hours, under a specific set of conditions. It was determined from the broth dilution test by sub culturing the concentrations having MIC or above on to nutrient agar plates that did not contain the test agent. The MBC was identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by a pre-determined reduction such as ≥ 99.9%.

3. Results and Discussion

3.1 Phytochemical screening tests

Phytochemical screening showed the presence of chemical constituents namely alkaloids, steroids, saponins, tanins, phenolic compounds, flavonoids, glycosides, diterpenes and triterpenes. Which is responsible for the stabilisation and capping of silver nanoparticles.

3.2 UV-Visible absorption studies

silver nanoparticle synthesised using trikatu extract was confirmed by the spectrophotometric method by checking the peak of the sample at wavelength 300 to 700 nm, at 1 nm resolution. All the samples gave the wavelength in between 382 nm to 386 nm which confirmed the presence of AgNPs. The peak indicate the reduction of silver ions to metallic silver. Similar results were obtained by [36, 37, 9, 1]. They reported the wavelength range of silver nanoparticles absorption peak was in the range of 440 to 460 nm and surface plasmon resonance (SPR) of silver nanoparticles was acting as specific and important optical property of nanoparticle, which depended on refractive index charges, size, shape and distance from each other. In the present study, surface plasmon resonance was responsible for the colour change from slight yellow to brown and a single, strong and broad surface plasmon resonance peak was observed at 380 to 390 nm that confirmed the synthesis of AgNPs (Fig.6).

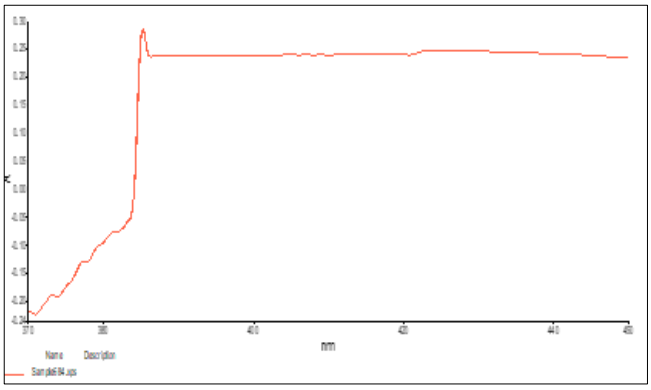

Fig 6: Analysis data of UV-Visible Spectrophotometry of trikatu

3.3 Attenuated total reflection

Silver nanoparticles synthesised with 1 mM, 3 mM and 5 mM silver nitrate solutions produced identical ATR patterns. Analysis of AgNPs by ATR spectroscopy was conducted to identify their functional groups that lead to the reduction of Ag^{+} to Ag^{0} and brought about the capping and stabilisation of AgNPs. The absorption spectra in the range 4000 to 400 cm-1 revealed three bands signifying the presence of amine, carboxyl and hydroxyl functional groups in all the three AgNPs samples. At 1 mM concentration, the peaks were observed at 3339.25, 1634.66 and 510.19. At 3 mM concentration, the peaks were observed at 3349.45, 1634.30, and 499.89 and at 5 mM, the peaks were at 3339.48, 1634.54, and 514.84 (Fig.7).

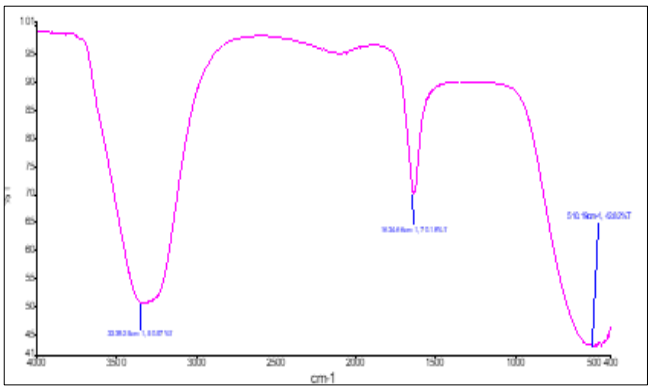

Fig 7: ATR pattern of AgNPs synthesised by trikatu extract

The functional groups of the biomolecules responsible for reduction of Ag+ to Ag0 were analysed using ATR spectroscopy. Three peaks were observed for every single concentration (1 mM, 3 mM, 5 mM). The absorption spectrum lied between 4000 to 400 cm^{-1}. Each peak implied different functional groups like hydroxyl, carboxyl and amine groups present in the sample. The results are in accordance to [38] who reported that the FTR ATR spectra analysis of AgNPs with Neem leaf. The strong and broad peaks at 3330-3350 cm^{-1} were due to boned hydroxyl (-OH) or amine groups, the peak at 1634 cm-1was due to stretching vibration of carboxyl group (-C=O) and the peak at 499-514 cm-1was due to silver nanoparticle banding with oxygen from hydroxyl groups of trikatu extract. These functional groups helped in stabilisation and bio reduction of silver ions to silver nanoparticles [39].

3.4 X- ray diffraction (XRD)

The crystalline structure of synthesised silver nanoparticles was analysed by XRD. The diffracted intensities were recorded at a range of 20º-80º. The XRD analysis for 1 mM, 3 mM and 5 mM AgNPs showed peaks of 27.94º, 32.354º, 46.26º, 54.78º, 57.68º, 76.84º (Fig.8).

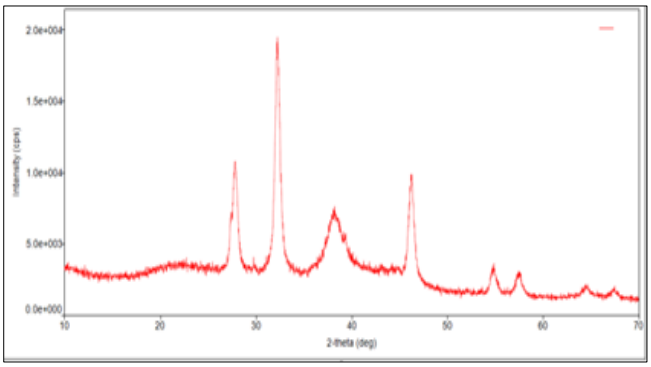

Fig 8: XRD analysis of trikatu

Similar findings were reported by [9]. They synthesised silver nanoparticle using *Urtica dioica* Linn. leaves and the crystalline nature of the nanoparticle were confirmed by x-ray crystallography. The diffracted intensities were started from 20 to 80. Four strong Bragg reflections at 38.45, 46.35, 64.75 and 78.05 corresponding to the planes of (111), (200), (220) and (311) respectively were observed and assorted with respect to the planes of face centered cubic crystal structure of silver (Fig.8). In an earlier study, [1] synthesised silver nanoparticles using *Prunus persica* plant extract. In XRD analysis, the spectrum showed five unique peaks at 38.20, 44.23, 64.33 and 77.40 that were indexed to (111) (200), (220), (311) and (222) reflection planes of face centered cubic structure of silver respectively. XRD spectrum clearly showed that the silver nanoparticles formed by a green approach using *Prunus persica* extract through reduction process were crystalline in nature.

3.5 Scanning electron microscope (SEM)

Silver nanoparticles synthesised from trikatu extract was subjected to scanning electron microscopy to understand the size and other morphological features of the compound. Lyophilised extract of trikatu was examined under SEM. SEM analysis of all three sample concentrations revealed that the AgNPs synthesised using trikatu were spherical shaped and the size was around 0.7 µM (70 nm) (Fig.9). Similar observations were reported by [36, 32, 9]. They observed that all of the silver nanoparticles are spherical in structure with agglomeration and confirmed the average size between a range of 50 to 100 nm [40].

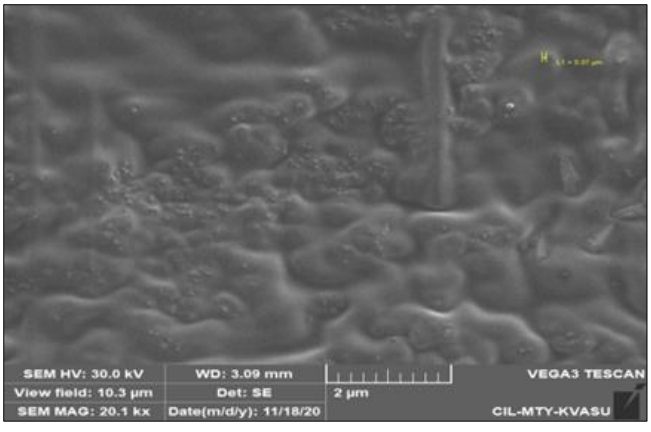

Fig 9: SEM image of silver nanoparticles formed by trikatu extract

3.6 Antimicrobial activity of silver nanoparticles synthesised using trikatu

The antibacterial potential of green synthesised silver nanoparticles was tested against the common pathogens; the Gram-negative *E. coli* and Grampositive *S. aureus*. Type cultures of these organisms procured from MTCC were revived and their identities confirmed by Gram staining and biochemical tests. The well diffusion test was employed to evaluate the antibacterial potential of the nanoparticles by measuring the zone of inhibition. *Escherichia coli* depicted a larger zone of inhibition around the AgNPs synthesised using 5mM AgNO3 (Fig.11) whereas *S. aureus* showed a wider zone of inhibition around those AgNPs synthesised using 3mM AgNO3 (Fig.10). This depict that different strain of bacteria have different inhibition concentration for silver nanoparticles synthesised using trikatu and indicated that the AgNPs formed of 5mM AgNO3 are more effective against Gram negative organisms while those made of 3mM AgNO3 are more effective against Gram positive bacteria. The silver nanoparticle made up of 1mM AgNO3 has least effect on both Gram positive as well as Gram negative organisms. The minimum inhibitory concentration of both the AgNPs as assessed by broth dilution test was found to be 10 µg/mL (Fig.12 and Fig.13). The broth tubes having MIC were further sub cultured onto agar plates that did not contain AgNPs to determine the minimum bactericidal concentration (MBC) which was found to be 20 µg/mL for both Gram positive and Gram negative organisms (Fig.14 and Fig.15). [30] produced silver nanoparticles using onion (*Allium cepa*). The synthesised nanoparticles at concentration 50µg/mL showed complete antibacterial activity against *E.coli* and *Salmonella typhimurium*. [9] produced silver nanoparticle from leaf extract *Urtica dioica Linn*. leaves and checked its antibacterial and synergistic activity against wide range of pathogenic bacteria and found to be very effective. [1] studied the antibacterial activity of bio synthesised silver nanoparticles against two different human pathogens (*E. coli and V. cholera*). It was apparent that the AgNPs showed inhibition zone against two tested organisms. [41] evaluated antibacterial activity of the RcAgNPs against Gram positive and Gram negative bacteria by resazurin reduction method and the results found to be significant. Their activity against bacterial strains showed that RcAgNPs had the potential to be used as antimicrobial formulation. The results of the present study indicate that the aqueous extract of trikatu could be used successfully for the green synthesis of silver nanoparticle. These green synthesised silver nanoparticles using Trikatu exhibited good antibacterial potential against *E. coli and S. aureus*.

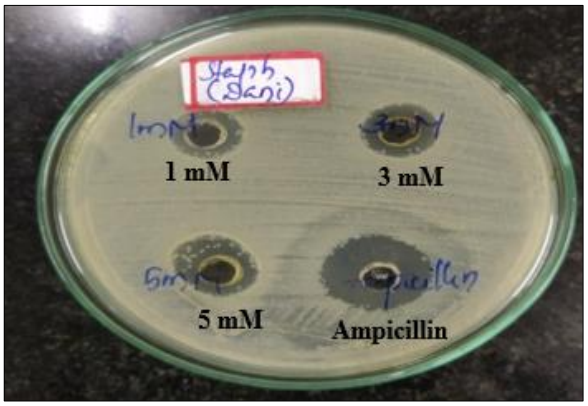

Fig 10: Antibacterial activity of trikatu AgNPs showing zone of inhibition of *Staphylococcus aureus* at 3mM concentration

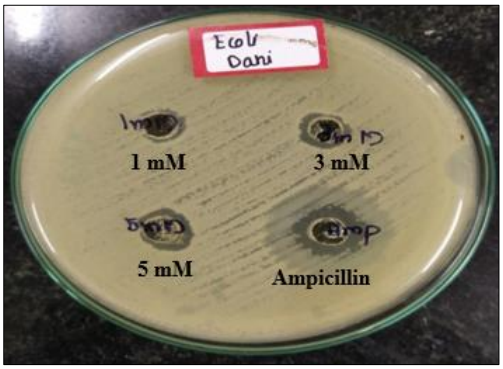

Fig 11: Antibacterial activity of trikatu AgNPs showing zone of inhibition of *Escherichia coli* at 5mM concentration

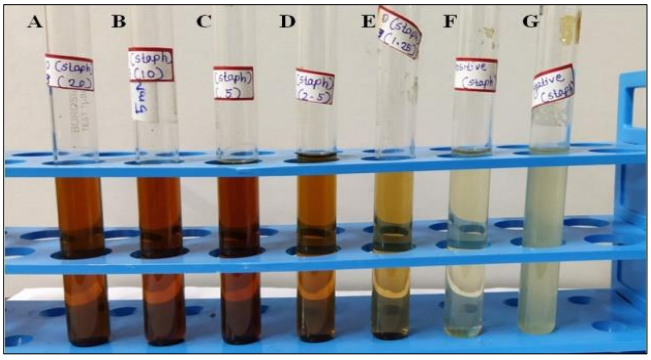

Fig 12: Broth dilution test of *S. aureus* under varying concentrations (A- 20 µg/mL, B- 10 µg/mL, C- 5 µg/mL, D- 2.5 µg/mL, E- 1.25 µg/mL, F- Negative control, G- Positive control)

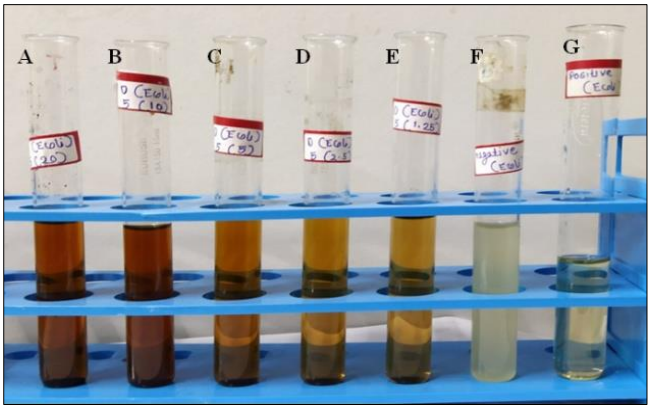

Fig 13: Broth dilution test of *E. coli* under varying concentrations ((A- 20 µg/mL, B- 10 µg/mL, C- 5 µg/mL, D- 2.5 µg/mL, E- 1.25 µg/mL, F- Negative control, G- Positive control)

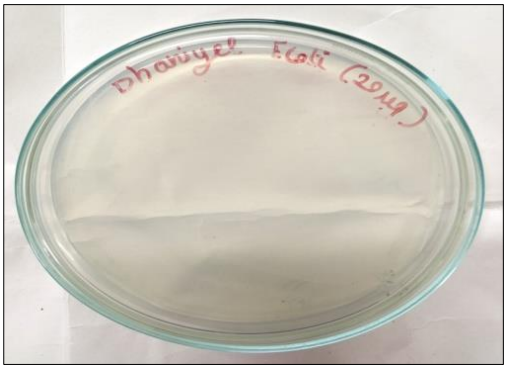

Fig 14: Agar plate streaked with *E. coli* culture containing 20 µg/mL of 5 Mm AgNPs

Fig 15: Agar plate streaked with *S. aureus* culture containing 20 µg/mL of 5 Mm AgNPs

4. Conclusion

Present study focused on the biological synthesis of silver nanoparticle using trikatu extract. Apart from chemical and physical synthesis of nanoparticles, biological synthesis is more ecofriendly and non toxic in nature. Phytochemicals present in the trikatu extract is responsible for the stabilisation and capping of silver nanoparticles also help in the conversion of silver ion to metallic silver. Analytical techniques including UV-Vis spectroscopy, Attenuated total reflection, X – ray diffraction and scanning electron microscopy were used for characterization of silver nanoparticles. The synthesised silver nanoparticles were spherical in shape and have around 70 nm in size and they showed excellent antimicrobial activity against the common pathogens *E. coli and S. aureus*.

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