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Green Synthesis of Silver Nanoparticles using *Curculigo orchoides* and Evaluation of its Antimicrobial Activity Against ESBL Organism

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Abstract: Extended-spectrum beta-lactamases (ESBL) enzyme producing bacteria are a growing concern in healthcare as these strains are resistant to multiple classes of antibiotics. Development of effective antimicrobial agents against ESBL strain is the major concern of researchers worldwide. Currently nanoparticles have been proposed as a potential treatment of ESBL- producing bacteria. Green synthesis of nanoparticles has several advantages for chemical synthesis. In current study, effect of *Curculigo orchoides* mediated silver nanoparticles (AgNP) was tested against ESBL producing organisms. The bioactivity of *C. orchoides* was confirmed by qualitative phytochemical analysis. The synthesized silver nanoparticles were subjected to a series of characterization to confirm its structure and composition. UV visible spectroscopy revealed a strong peak at 390 nm which confirmed the presence of silver nanoparticle. The current FTIR results strongly suggested that the reduction of Ag²⁺ to Ag⁰⁺ in the aqueous rhizome extracts was due to a combination of biomolecules. The SEM and TEM analysis revealed that the size and shape was nanoparticle to be 35-39 nm and spherical in shape. The XRD analysis revealed the silver nanoparticle to be crystalline in nature. The antibacterial activity of biosynthesized silver nanoparticles was tested against *Escherichia coli* and *Staphylococcus aureus*. The ESBL organisms were resistant to the standard antibiotic ampicillin. Instead, the organisms were susceptible to the silver nanoparticle. The efficiency increased with increase in concentration. This study revealed that several strains had more sensitivity to nanoparticles than others, as seen by the greater zone of inhibition for Gram-positive strain (*S. aureus*) than for Gram-negative bacteria (*E. coli*). The *C. orchoides* synthesized silver nanoparticle is alternative to existing antibiotics which are ineffective in clinical treatments.

Keywords: Extended-spectrum beta-lactamases, *Curculigo orchoides*, Silver nanoparticles, Antibiotics, Antibacterial

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Introduction

A significant new technology, the production of nanomaterial, with implications in several industrial areas has emerged in the recent past. Different chemical and physical processes may be used to produce metal nanoparticles, but there is an increasing need to create a technique that is easy to use, affordable, environmentally friendly, and size-controlled (Marchiol, 2012). Utilizing biological resources for the synthesis of nanoparticles has recently emerged as an alternate method for nanotechnologists (Singh *et al.*, 2016). In terms of systems that may be used to synthesized nanomaterial, biological resources are the most abundant, diverse, and extensive (Ramimoghdam *et al.*, 2013). Examples include bacteria, fungus, and plants, each of which has benefits over more traditional processes (Shah *et al.*, 2015). In comparison to the physical and chemical methods, the plant-mediated green nanoparticle synthesis is more rapid, cheaper, more environmentally benign, and only requires a single step (Mitiku and Yilma, 2018). Silver nanoparticles are a compelling alternative to antibiotics due to their well-documented antibacterial action, which stimulated the creation of innovative applications in this sector. It has frequently been discovered that the silver nanoparticles (AgNPs) exhibit broad-spectrum antibacterial action against human and animal diseases (Anju *et al.*, 2021).

The most often utilized nanomaterial is silver nanoparticles (AgNPs). Silver has mostly been used in medicine up to this point as a component of antibacterial medications with a wide spectrum of antimicrobial action, such as collargol, protargol, AgNO_3 , and $\text{C}_{10}\text{H}_9\text{AgN}_4\text{O}_2\text{S}$ (silver sulfadiazine), in the form of metal, metal-protein complexes, and salts (Gusev *et al.*, 2016). Numerous analysts have observed the biogenesis of nanoparticles and their uses in the fields of medicine, drug delivery, photonics, electronics, chemical sensing, biosensing, and catalysis (Nath and Banerjee, 2013). Our knowledge of living cells and molecular interactions can be improved through nanotechnology (Shemetov *et al.*, 2012).

A popular traditional medicinal plant called *Curculigo orchoides* Gaertn rhizomes have long been utilized as a tonic. In India, the rhizomes of *C. orchoides* are used as an aphrodisiac (Chauhan *et al.*, 2007) and a treatment for piles, diarrhoea, jaundice, Scolic, asthma and gonorrhea (Asif, 2012). Glycoside, polysaccharides (hemicelluloses and other polysaccharides), tannin, starch, mucilage, resin, fat, and calcium oxalate were also said to be present in the rootstock (Jagtap, 2016). Its extract has been shown to have hepatoprotective, immunostimulant, and antimicrobial properties as well as sexual stimulation and osteoporosis preventive properties (Srivastava and Pandey, 2015). Contrary to popular belief numerous new antibacterial medications have been developed, bacteria resistance to them has evolved and is becoming a worldwide problem as we are rapidly running out of treatment choices (Coates *et al.*, 2002).

Since late 1980s, infections caused by ESBL-producing microbes have been reported worldwide. 1–8 Patients infected with an ESBL-producing bacterium may have poor outcomes due to delays in acquiring effective antibiotic medication and limited treatment alternatives. ESBL-positive strains are resistant to third-generation cephalosporins, penicillins, and monobactam (Rice, 1999). The major objective of the current study was to investigate the bioactive compounds present in the rhizome of *C. orchoides*. Based on the bioactivity, silver nanoparticles were synthesized using rhizome aqueous extracts, and assess the antibacterial efficacy of AgNPs against the ESBL strain.

Materials and Methods

Plant Collection:

C. orchoides rhizome was gathered from Thuraiyur (11° 8' 29.2380" N, 78° 35' 40.1100" E). Fresh rhizome was properly cleaned for 20 min under running water, then rinsed several times in sterile distilled water. The rhizome was then

chopped into small pieces and pulverized with a mortar and pestle (Brintha *et al.*, 2017).

Plant Extraction:

The powdered rhizomes were extracted in a soxhlet extraction equipment using water (Hot extraction). 100 g of the dried, gritty rhizomes of *C. orchioides* were extracted using water. By filtering the extract as it remained hot and distilling the subsequent filtrates under reduced pressure and vacuum, the solvent was completely eliminated from the mixture. After drying, it was desiccated and maintained there for experimentation (Madhavan *et al.*, 2007)

Qualitative Phytochemical Analysis:

The presence of phytoconstituents or secondary metabolites, such as alkaloids, glycosides, saponins, phytosteroids, phenolic compounds, and flavonoids, tannins responsible for the therapeutic benefits of the drug was qualitatively investigated in *C. orchioides* using standardized protocol (Aloysius *et al.*, 2020).

Green Synthesis of Silver Nanoparticles:

90 ml aqueous extracts of rhizome were mixed with 100 ml of 1 mM silver nitrate solution (AgNO_3), and the mixtures were then let to sit at room temperature for 3 h with continuous agitation in magnetic stirrer. The mixture's light yellowish color changed into a brown solution, indicating the synthesis of AgNPs. The colour transition was seen visually and documented with photographs. The reaction mixture was also centrifuged for 10 min at 8,000 rpm. Silver nanoparticle pellet suspension was put into two separate petri plates and dried at 60°C overnight after the pellets were thoroughly suspended in double-distilled water and centrifuged for three times (Venkatachalam *et al.*, 2017).

Characterisation of Silver Nanoparticles:

UV-Vis spectroscopy is used therefore to characterize the synthesized nanoparticles solution. A sample of 3 ml is placed in a cuvette and scanned in double beam UV-Vis spectrophotometer over the wavelength range of

300 nm to 700 nm. The outcomes of the graphical analysis were documented (Kiran *et al.*, 2019). Then it was examined using an advanced power X-ray diffractometer (XRD) that used copper microwave band (CuK α) radiations at a voltage of 9 KW and a current of 30 mA. By scanning AgNPs in KBr pellets with a resolution of 4 cm⁻¹ (Kayalvizhi *et al.*, 2016), Fourier transform infrared spectroscopy (FTIR) analysis was used to collect data on the functional groups that are present in the AgNPs (Ghaseminezhad *et al.*, 2012). Utilizing a Hitachi S-4500, scanning electron microscopic (SEM) examination was carried out. The samples were made into thin films using a copper grid that had been coated with carbon. The materials were then scrutinized using the SEM apparatus (Banu and Balasubramanian, 2015). Through the use of transmission electron microscopy (TEM), the appearance, structure, and size of nanoparticle were also studied (TEM CM200; Philips, USA). The samples were put onto the copper grids for TEM investigation, where they were then dried further under IR light. At 120 kV, the loaded sample grid analysis was carried out. By using the minimum and maximum particle sizes in the picture, the average particle size was then manually computed (Sankaranarayanan *et al.*, 2017).

Antimicrobial Activity Against ESBL Strain:

The clinically isolated ESBL microorganisms *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 33591), were used to test *in vitro* antibacterial activity of the synthetic AgNPs. The cultures were cultivated for 24 h at 37°C in nutrient broth. The McFarland turbidity standard was used to quantify the concentration of microbial inoculums, which was adjusted to 2.5×10^5 colony-forming units (CFUs) per ml using a spectrophotometer (600 nm). After that, sterile cotton swabs were used to wipe the cultures onto the test media, and gel punctures were used to create wells on Muller-Hinton agar plates that were about 6 mm in diameter. AgNp synthesized using *C. orchioides* were added to the wells at different concentrations (250, 500, 1000 $\mu\text{g/ml}$) to



Fig. 1: Aqueous extraction of plant sample.

Table 1: Soxhlet extraction of *Curculigo orchiodies*

S. No.	PLANT	TYPE OF EXTRACT	COLOUR	CONSISTENCY	YIELD PER CENT (%)
1	<i>Curculigo orchiodies</i>	Aqueous	Brown	Greasy	14.11

analyze its antibacterial activity against ESBL strain. Each plate's zone of inhibition was assessed after the plates were incubated at 37°C overnight and represented in millimeter (Awadelkareem *et al.*, 2022).

Results and Discussion

Extraction Yield:

C. orchiodies rhizomes had been using in Indian traditional medical systems, the study's goal was to assess qualitative amount of secondary metabolites present in these tuberous components. The most potent extracts and the plant's medicinal capabilities were discovered through the phytochemical screening of extracts. The fact that this plant's rhizomes contain flavonoids, phenols, alkaloids, tannins, terpenoids, and cardiac glycosides suggests that they have both physiological and medicinal qualities (Madhavan, 2015). The extract obtained by soxhlet extraction (Fig. 1) was weighed, and the

yield percentage was estimated using the air-dried powdered raw material. The 14.11% yield of aqueous extracts from *Curculigo orchiodies* powdered root tubers are given in Table 1.

Phytochemical Analysis:

The preliminary phytochemical qualitative study of the tuberous rhizome is given in Table 2 and represented in Figure 2. The plants aqueous extract reveals the presence of flavonoid, saponin, terpenoid, alkaloid, phytosteroid, and alkaloid. The existence of organic components is thought to be the catalyst for therapeutic activity. According to a study conducted by Anandakirouchenane *et al.* (2013), the methanolic extract was found to contain flavonoids, phenols, glycosides, saponins, terpenoids, and sterols, but not alkaloids or tannins. Similar it showed that cardiovascular glycosides were only found in chloroform and ethanol extracts, while saponins and steroids were absent from chloroform and ethanol extracts (Anandakirouchenane *et al.*, 2013). In contrast, the

Table 2: Qualitative phytochemical analysis of *Curculigo orchidies*

PHYTOCHEMICAL ANALYSIS	Phenol	Alkaloid	Flavonoid	Tannin	Glycosides	Saponin	Terpenoid	Steroid
<i>Curculigo orchidies</i>	++	+	++	-	-	++	+	+

+ present; ++ appreciable amount; –absent

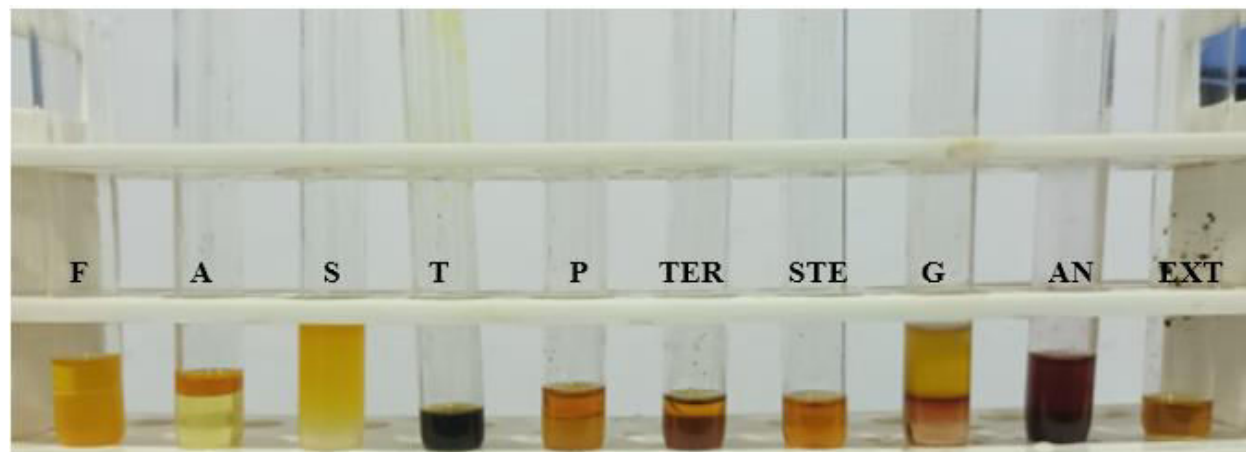


Fig. 2: Qualitative Phytochemical analyses of *Curculigo orchidies*. **F-** Flavonoids; **A-** Alkaloids; **S-** Saponins; **T-** Tannins; **P-** Phenols; **Ter-** Terpenoids; **Ste-** Steroids; **G-** Glycosides; **AN-** Anthraquinone; **Ext-** Extract.

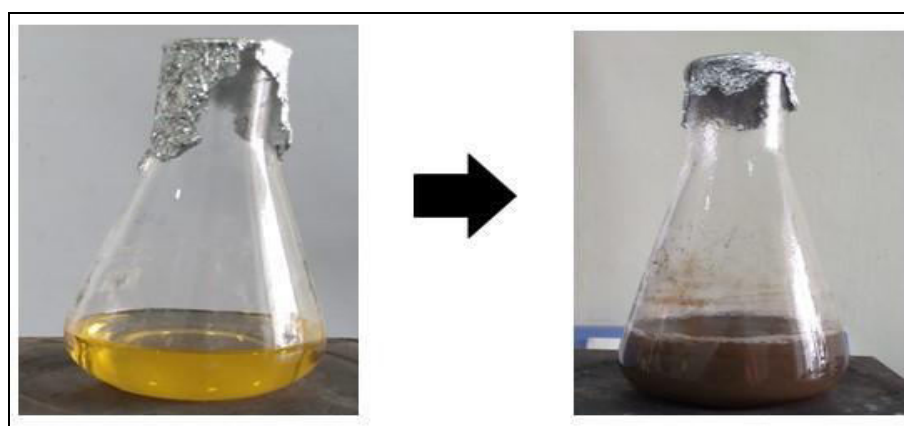


Fig. 3: Biosynthesis of silver nanoparticles.

current investigation revealed that aqueous extract contained primary phytochemicals like phenols, alkaloids, flavonoids, Saponin, terpenoid and steroids. From previous study of Jagtap *et al.* (2016) it was evident that alkaloids were present in methanolic extracts but not saponins in chloroform extracts. Similarly to our study, analysis conducted by Brintha *et al.* (2017) revealed that *C. orchidies* rhizome's confirms the presence of flavones, saponins, triterpenes, and diterpenes in water extract (Brintha *et al.*, 2017).

From the previous analysis reported it is evident that the presence of phytochemicals varied with different extracts used.

Green Synthesis of Silver Nanoparticle:

Green synthesis of silver nanoparticle using *Curculigo orchidies* was discussed in the current study. The transition of color from yellow to dark brown revealed the formation of silver nanoparticles utilizing rhizome extract which is represented in Figure 3. When rhizome extract

was exposed to a 0.001M aqueous AgNO₃ solution, extracellular silver nanoparticle production took place. It was instantly apparent that the silver ions had completely decreased. During the incubation phase, the reaction mixture's color (brown) changed as the outcome of the synthesis of silver nanoparticles. The appearance of dark brownish particles strongly suggests that silver nanoparticles formed after the addition of the rhizome extract. The previous studies conducted by other researchers revealed that the color change was caused by the stimulation of SPR (surface plasmon resonance) vibrations in conjunction with the metal silver nanoparticles (Kumar *et al.*, 2012; Kalaiaarasi *et al.*, 2015).

Characterization of Nanoparticle:

One crucial method for determining the production of metal NPs (nanoparticles) is a UV-VIS spectrum. The synthesis of AgNPs is evidenced by the greatest absorption peak at 390 nm (Fig. 4). The existence of the board resonance suggests that the AgNPs in the solution have aggregated. The SPR of nano-sized silver metal has been correlated to the spectrum containing bands in this range, demonstrating the presence of silver nanoparticles in the solution after exposure to UV radiation. Many phytochemicals found in the leaf extract may play a major role in the conversion of the ionic form of silver to the metallic nanoparticle (Velmurugan *et al.*, 2015). The results of the current investigation closely matched the reports of the UV-VIS absorption spectra of the AgNPs produced in previous research studies (Sankaranarayanan *et al.*, 2017; Aref and Salem, 2020).

The bioreduction of Ag⁺ ions to silver nanoparticles is caused due to the reduction by rhizome extract, according to FT-IR analysis. Through FTIR spectroscopy analysis, the presence of biomolecules that are responsible for encapsulating silver nanoparticles was discovered. The FTIR tests revealed infrared bands, respectively, at 3449, 2723, 1635, 1383, 1119, 915, 754 and 617 cm⁻¹ (Fig 5). Sharp absorption peaks were seen at 3,449 cm⁻¹, 2, 723, 1, 635 and

1, 383 cm⁻¹, respectively (Fig. 5). Peaks at 3,449 cm⁻¹ are attributed to OH stretching in alcohols and phenolic compounds (Das *et al.*, 2006). The C-N stretching discovered was linked to the absorption peak centered at 2, 072 cm⁻¹. The infrared spectra seen at 1,635 cm⁻¹ is similar to that observed for natural proteins, indicating that proteins may interact with produced nanoparticles during the silver ion reduction procedure without changing their secondary structure (Raja *et al.*, 2015). The occurrence of the peak at 1383 cm⁻¹ denotes the presence of the C-N stretching band of the protein's aromatic functional group (Gopinath *et al.*, 2012). Additionally, the carbonyl group of amino acid residues has a strong ability to cover metal nanoparticles and it acts as a capping agent to provide stability for synthetic metallic silver nanoparticles. Additionally, saponins content of the *C. orchioide* plant included amide and OH groups, according to the FTIR study. The AgNPs synthesized utilizing *A.spicifera* showed intense peak at 3351.28 cm⁻¹, 2633.71 cm⁻¹, 2083.50 cm⁻¹, 1637.18 cm⁻¹, 1082.87 cm⁻¹, and 712.34 cm⁻¹. The main FTIR bands at 3351.20 cm⁻¹, 2633.74 cm⁻¹, and 712.34 cm⁻¹ were absorbed. They represent the presence of alcohols and phenols (O-H), carboxylic acids and their derivatives (C=O), and chloroalkanes (CX) respectively (Seidel, 2012).

Based on SEM examination, spherical-shaped nanoparticles with a typical size of 35-39 nm were found to be non-aggregated, evenly distributed. Figure 6 represents the arrangement of the aggregates. Additionally, studies utilizing *Bauhinia variegata* extract for the synthesis of silver nanoparticles have shown aggregated nanoparticles with a size range of 20-80 nm (Chinnappan *et al.*, 2018), providing support for the current findings.

The appearance, structure, and size of the produced nanoparticles were investigated by TEM. According to the observed TEM image (Fig. 7), most of the particles are spherical, while only a small number have unusual shapes. The presence of certain organic molecules around the silver

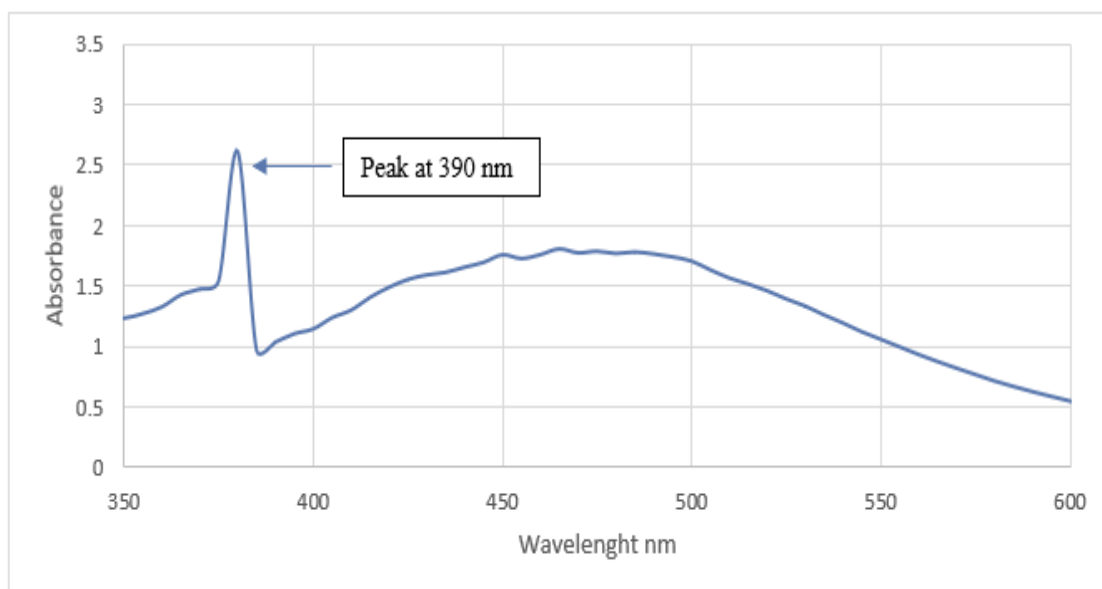


Fig. 4: UV- visible spectroscopy of silver nanoparticles.

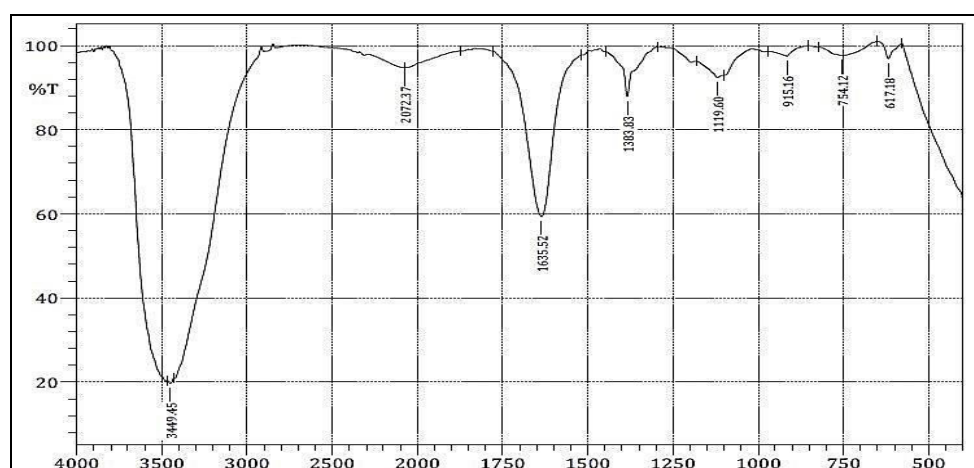


Fig. 5: FTIR analysis of silver nanoparticles.

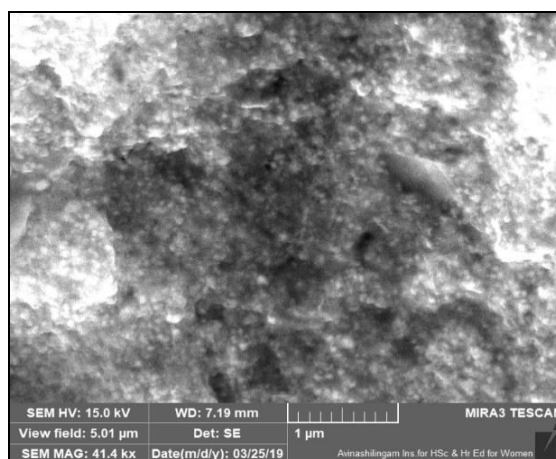


Fig. 6: SEM analysis of silver nanoparticles.

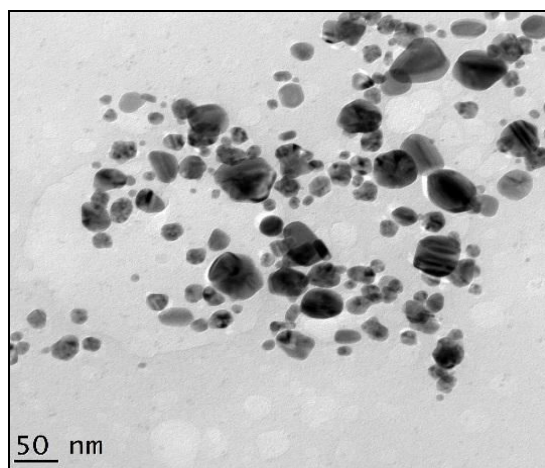


Fig. 7: TEM analysis of silver nanoparticles.

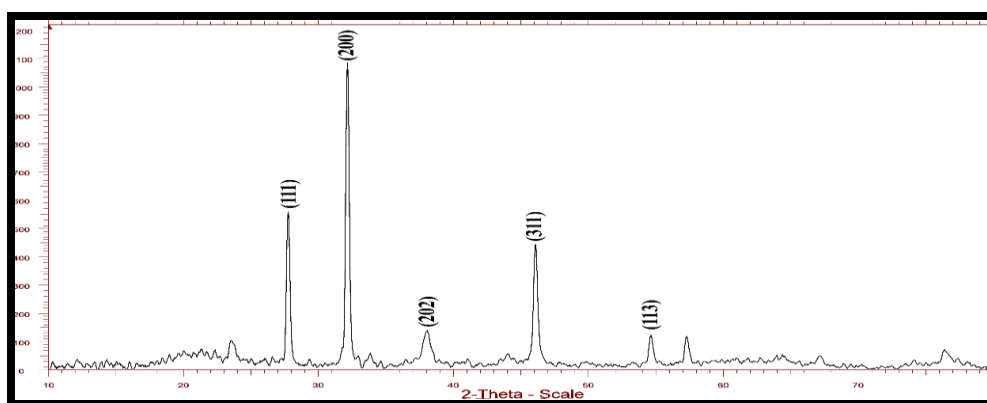


Fig. 8: XRD analysis of silver nanoparticles.

nanoparticles makes them extremely stable. The particles size is determined to be 36.25- 39.23 nm, with an average particle size of 36 nm based on the current data. According to TEM analysis, the size distribution of the AgNPs from *Elaeagnus latifolia* was between 30 and 50 nm (Phanjom *et al.*, 2012).

The XRD patterns of silver nanoparticles generated from *Curculigo orchiodies* extract showed a series of Bragg reflections with 2θ values of 27.5° , 32.3° , 39.7° and 56.19° corresponding sets of lattice planes to the 111, 200, 202 and 311, respectively, represented in Figure 8. They are categorized as the band for silver face-centered cubic formations. Hence, it was evident from the result that the reduction of crystalline Ag^+ ions results in the formation of silver nanoparticles (Shankar *et al.*, 2003). Previous study conducted by Zargar *et al.* (2011)

revealed that the silver nanoparticles exhibit XRD peaks at 38.17° , 44.31° , 64.44° , 77.34° , and 81.33° corresponding to the face-centered cubic (FCC) planes 111, 200, 220, 311, and 222, respectively.

Antimicrobial Activity Against ESBL Strain:

Resistance to antimicrobials lowers the number of treatment options through increasing the cost and making it more difficult to eradicate microorganisms due to increased infection severity (O'Neill, 2016). AgNPs were evaluated for their antibacterial efficacy against ESBL organism by agar well diffusion assay. The zone of inhibition (ZOI) around each well was measured, and the average inhibitory zone was calculated and presented in Table 3 and diagrammatically represented in Figures 9 and 10. This study revealed that several strains were more sensitive to nanoparticles than others, as seen by the greater zone of inhibition for Gram-positive strain

Table 3: Zone of inhibition against ESBL organism

CONCENTRATION (μg)	<i>Curculigo orchoides</i> (Aqueous extract)(mm)	
	<i>E. coli</i> (ESBL)	<i>S. aureus</i> (ESBL)
250	14	16
500	17	19
1000	19	21
Ampicillin (30 μg)	-	-

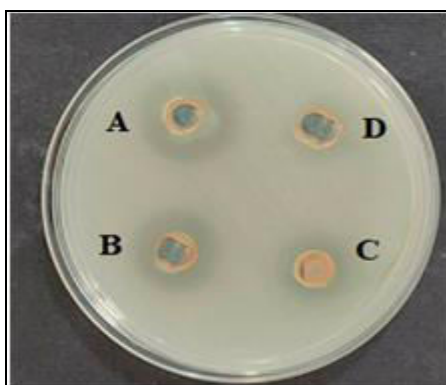


Fig. 9: Antibacterial activity against *E.coli* (ESBL). A- AgNP (1000 μg); B - AgNP (500 μg); C- AgNP (250 μg); D- Ampicillin.

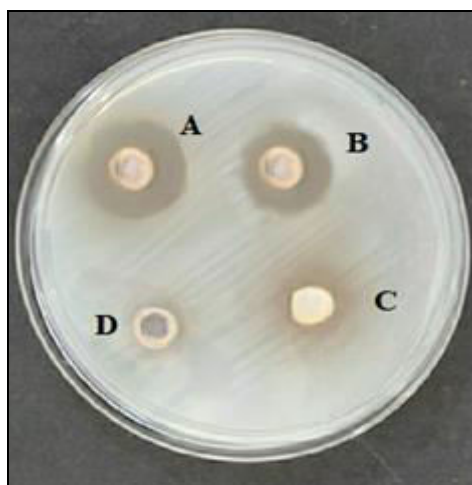


Fig. 10: Antibacterial activity against *S.aureus*. A- AgNP (1000 μg); B - AgNP (500 μg); C- AgNP (250 μg); D- Ampicillin.

(*S. aureus*) than for Gram-negative bacteria (*E. coli*). The rhizome extract mediated AgNPs were found to be highly toxic to ESBL organism. The ESBL strains were resistant to standard drug

ampicillin. According to reports, silver nanoparticles bind to negatively charged microorganisms through electrostatic attraction in the cell wall membrane and interact with thiol

groups in the cell wall, which causes the production of ROS (reactive oxygen species) and rupture of the bacterial cell. It has been established that the AgNPs' ionic attachment to the bacteria's surface causes the growth inhibition (Nikaido and Vaara, 1985; Bindhu and Umadevi, 2015). According to previous reports, biosynthesized silver nanoparticles with a small particle size are more efficient antibacterial agents because of high contact volume such that a large proportion of silver atoms are in direct proximity with their environment (Chen and Schluesener, 2008). AgNP's methanol and aqueous extract of *T. arjuna* bark, antibacterial efficacy against human bacterial pathogens showed varied degrees of zone of inhibition. Using AgNP's, the highest zone of inhibition (25 mm) was seen in *E. coli*, *P. aeruginosa* followed by *A.baumannii* (23 mm), *P. mirabilis*, *S. typhimurium*, and MRSA (20 mm) (Akther *et al.*, 2019). A zone of inhibition of 15 mm, 13 mm, and 9 mm in diameter for *E. coli*, *Klebsiella* spp., and *Proteus* spp., respectively, is produced by *Rhizopus stolonifer* silver nanoparticles against resistant ESBL-strains (Banu *et al.*, 2011). The previous results quoted above are in accordance with current study.

Conclusion

In conclusion silver nanoparticles have shown promising antibacterial activity against ESBL-producing organism at increasing concentration. However, further research is needed to fully understand the action of silver nanoparticle and their potential toxicity to human cells. Nonetheless, the development of silver nanoparticle-based antibacterial agent could lead to more effective and safer treatments against ESBL producing organism. The future prospects of silver nanoparticles based antibacterial agents include optimization of nanoparticle size and concentration.

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