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Antibacterial and Antibiofilm Activity of Therapeutic Herbs Stabilized Green Synthesis Silver (Ag) Nanoparticles against Drug Resistant Clinical Pathogen

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ABSTRACT

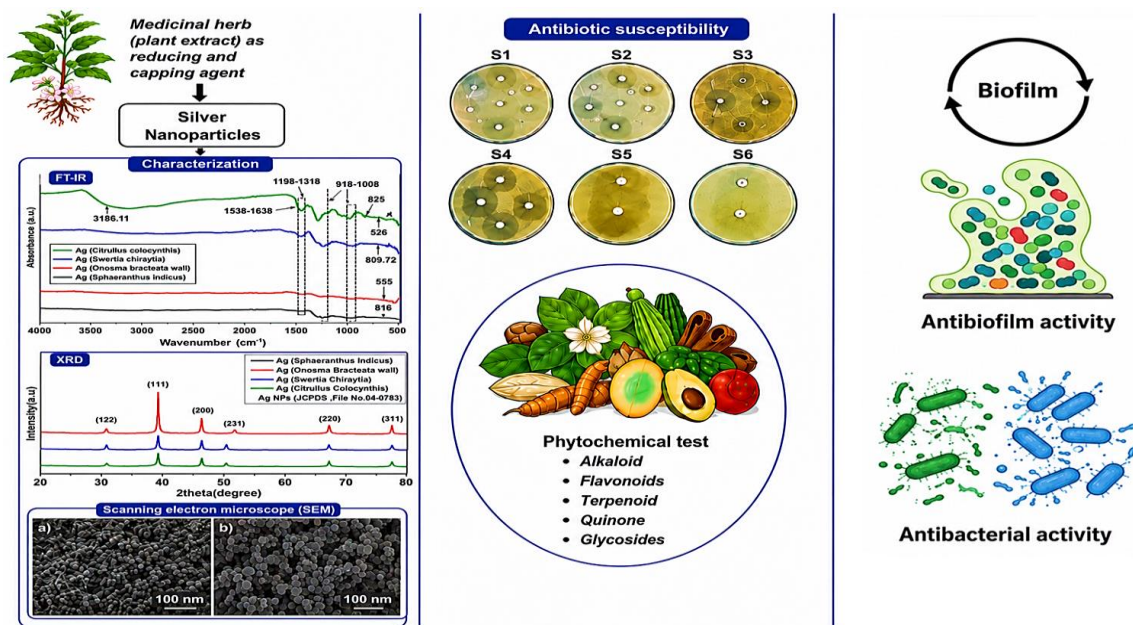
Different therapeutic herbs are abundantly present in the southern region of Punjab, Pakistan. In the current study, four medicinal herbs, *Onosma bracteata* Wall., *Citrullus colocynthis*, *Sphaeranthus indicus*, and *Swertia chirata*, were selected for the green synthesis of silver nanoparticles. The synthesized AgNPs were characterized using scanning electron microscopy, Fourier transform infrared spectroscopy, and X-ray diffraction analysis. SEM analysis showed that the particle size of the synthesized AgNPs was in the range of 75–100 nm, while XRD confirmed a face-centered cubic crystalline structure with an average crystallite size of 24 nm. FTIR analysis indicated the presence of functional groups that may contribute to the reduction, capping, and stabilization of the nanoparticles. The antibacterial and antibiofilm activities of the synthesized AgNPs evaluated against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus*. Ciprofloxacin and linezolid used as standard antibiotics for comparison against *E. coli* and *S. aureus*, respectively. Among the four herbal AgNPs, *Swertia chirata*-derived AgNPs showed the strongest antibacterial activity, with maximum inhibition zones of 42.13 ± 0.17 mm against *E. coli* and 47.44 ± 0.34 mm against *S. aureus* at 50 mg/mL. The lowest MIC and MBC values also observed for *Swertia chirata* AgNPs. Against *E. coli*, the MIC and MBC were 78.125 µg/mL and 156.25 µg/mL, respectively, while against *S. aureus*, the MIC and MBC were 39.0625 µg/mL and 78.125 µg/mL, respectively. In the antibiofilm assay, *Swertia chirata* AgNPs showed the highest biofilm inhibition, reaching 85.77% against *E. coli* and 71.26% against *S. aureus*. Phytochemical screening confirmed the presence of bioactive constituents in the herbal extracts, which may have contributed to AgNP synthesis. These findings suggest that green-synthesized herbal AgNPs, particularly those derived from *Swertia chirata*, have promising antibacterial and antibiofilm potential against drug-resistant bacterial pathogens.

Keywords: Antibacterial, Anti-biofilm, Macromolecules, Silver nanoparticles, MIC, MBC.

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GRAPHICAL ABSTRACT



INTRODUCTION

In order to support the development of personalized medical treatments, nanotechnology focuses on designing and assembling new materials by precisely arranging atoms and molecules to form innovative molecular structures that target individual cells, organelles, or even smaller components. Nanotechnology is the study and manipulation of matter at the nanoscale, whose unique properties enable groundbreaking applications [1, 2], since they are 100-10,000 times smaller than human cells, offer new ways to interact with biomolecules both inside and outside of cells [3]. Antibiotics offered a more focused method of treating infections, the use of silver in medicine decreased in the 20th century. Nonetheless, interest in silver as a substitute or supplement to traditional antibiotics has increased recently due to the rise in antibiotic-resistant pathogens. Late in the 20th century, Ag NPs gained attention again as a result of the advancement in nanotechnology. The potential of Ag NPs as strong antibacterial agents against a range of pathogens, including antibiotic-resistant strains, shows that Ag NPs can be utilized to fight multidrug-resistant (MDR) bacteria, a growing health care concern [4, 5].

Although Ag NPs are non-bioactive, they release silver ions that have biological activity when they slowly dissolve in

aqueous solution under physiological condition. Because of their gradual oxidation and posterior corrosion, this enables precise and prolonged controlled release, offering long-term protection even at low concentrations of NPs. Ag NPs function as an analogous to a drug delivery system, where a concentrated form of the silver ion, an active specie, is carried to and released close to biological target sites [6-8]. One feature that distinguishes AgNPs is localized surface plasmon resonance (LSPR), which is conducted on by the coherent oscillation of conduction-band electrons in response to input electromagnetic radiation. Because of the localized surface Plasmon resonance (LSPR) effect, Ag NPs can be used in a variety of ways, such as targeted drug delivery, nanomedicine, antibacterial formulations, environmental cleanup, therapeutic interventions, and improved sensor technologies [9, 10]. One of the main causes of chronic diseases and mortality is bacterial infections [11].

The majority of bacteria live as biofilms, which are frequently made up of several species that interact with their surroundings and one another. A mature biofilm forms as a result of the fast bacterial multiplication and cessation of flagellum production when they settle. At this point, the bacteria are firmly united, creating a barrier of defense that is resistant to antibiotic therapy and may be a factor in

persistent systemic infections [12]. Ag NPs are such good local heat conductors, and can efficiently transmitting heat to nearby tissues, this can lead to the death of bacterial biofilms when coupled with hyperthermia [13]. Mitochondria plays a central role in reactive oxygen species (ROS) production, so the disruptions in their function such as inhibition of the electron transport chain can significantly elevate ROS levels. Thus, ROS production is closely linked to mitochondrial activity [14]. Ag NPs can damage mitochondria both directly and indirectly by triggering various cellular reactions, suggesting that mitochondria are a major intracellular target of Ag NPs. This mitochondrial damage can accumulate over time, leading to increase ROS production and ultimately causing apoptotic cell death in bacteria [15, 16]. Green synthesis produces biocompatible and environmentally safe nanomaterials by reducing and stabilizing metal ions during MNPs synthesis by using phytochemicals rather than dangerous chemicals. Nanomedicine is one of the current imminent fields to intensify the curative action of phytochemical agents and enabling the efficient drug delivery system of nanoparticles [17]. Phytochemicals in plant extracts play a crucial role by donating electrons and capping of nanoparticles, imparting significant biological and therapeutic qualities to Ag NPs [18]. Antibiotics are strong medications used to treat diseases that were once fatal. Like any potent drug, antibiotics have major side effects. After taking antibiotics excessively, there is an increase susceptibility to side effects. Antibiotics also damage the configuration of the infectious agent, which causes bacterial adaptation or mutations and, ultimately, the emergence of new strains that are resistant to the current antibiotic regimen. Bacteria develop several ways to avoid the effects of antibiotics, hence resistance develops. The diffusion of antibiotic resistance genes is a rising current ecological and public health problem. Resistant bacterial strains may neutralize the components of antibiotics thus make it ineffective. Other may be able to change their outer structure and receptors to prevent antibiotics from attaching to them, while others may be able to exchange the antibiotics out of the bacteria. Because of these mechanisms, some bacteria may be able to withstand against the antibiotics and develop a resistance that they can then pass on to other bacteria as they propagate. Mutations in genetic material compels bacteria to be more resistant [19, 20]. Infectious diseases are commonly treated with herbal medicines that have been proven effective by scientific studies. The extracts of four medicinal herbs *Citrullus colocynthis*, *Sphaeranthus indicus*, *Onosma bracteatum*, and *Swertia chirata* have been employed in the

current study for ethno medical practices in treating disorders as an anti-inflammatory, antibacterial analgesics, antirespiratory, and anticancer. These herbs are particularly valued in the rural southern region of Punjab, Pakistan for the treatment purpose. This region is particularly prone to antibacterial infections due to factors such as contaminated water sources, inadequate hygiene practices, and constant exposure to animal and subtropical environment. Due to high rates of illiteracy, people are unaware to the proper antibiotic treatment in a way misuse. Incomplete courses and subtherapeutic doses of antibiotics as a result generating antibiotic resistance pattern in them against pathogenic bacterial strains. These circumstances add on the high chronicity of infections in this area even after availing the treatment facilities. *E. coli* and *S. aureus* are the selected resistant strains in this present research study due to their high prevalence in contaminated drinking water, unhygienic conditions and severe climatic changes as well. Based on this literature background, the goal and novelty of this research study is a green synthesise of Ag NPs from these often used medicinal herbs categorically and deems the potential and significance of each herb as a strong antibiotic and antibiofilm activity by comparing them conjointly against the selected gram negative and gram positive bacterial strains in order to treat various resistant antibacterial infections and antibiofilm activity that become an outbreak in this rural, illiterate region.

MATERIALS AND METHODS

Collection of medicinal herbs

Fresh *Citrullus colocynthis* was collected from its plant located in the Nawabpur area, of Multan district Pakistan. The rest of other medicinal herbs such as *Onosma bracteatum*, *Sphaeranthus indicus*, and *Swertia chirata* were brought from the Al Hakeem market in chowk ghanta ghar in Multan, Pakistan. The herbs were authentically identified by their botanical characteristics as they are not freshly available in the region except *Citrullus colocynthis*. The local names of the herbs are *Citrullus colocynthis* (*Indrayan*), *Onosma bracteatum*, (*Gul e gaozuban*), *Sphaeranthus indicus*, (*Gorakh Mundi*), and *Swertia chirata* (*Chirata Talkh*).

Medicinal herbal extraction

The selected herbs were cleansed with deionized water (DIW) to get rid of dust particles, and to eliminate any remaining moisture, the herbs were then let to air dry. The sample was then ground into a coarse powder using a normal grinder, 15 g of freshly ground herbal powder was dissolved in 500 mL of highly pure DIW for 30 minutes at

100°C using a magnetic stirrer. After passing through Whitman paper 41, the mixture was centrifuged for 30 minutes at 4000 rpm. The extract is then refrigerated for use in the subsequent phases of silver nanoparticle production and phytochemical analysis.

Green synthesis of silver nanoparticles

During the preparation, a modified version of the standard protocol was used. First, 2 g of silver nitrate were dissolved in 25 ml of distilled water using a magnetic stirrer set at 600 rpm for 30 minutes. After that, 25 ml of each of the four selected herbal plant extracts were gradually added while being constantly stirred for an hour at 80°C. During this process, a precipitate formed, turning the fluid black. The precipitate was separated by centrifugation, which involved spinning for 30 minutes at a high speed (5000 rpm). In order to gradually eliminate various compounds, the supernatant containing the unbound phytochemicals was repeatedly rinsed with ethanol and distilled water after the AgNPs settled to the bottom of the centrifuge tube. Ultimately, the gathered precipitates were dried for four hours at 85°C-100°C in a furnace [21-23]. Drops of 0.1 N NaOH were added to the solution to alkalize it until a basic pH of 9 to 10 was reached. This optimization of silver nanoparticles modulate their surface plasmon resonance which can increase the stability of silver nanoparticles by influencing the rate of reduction of silver ions [24, 25].

XRD characterization and crystallite size calculation

The crystalline structure of the synthesized silver nanoparticles was analyzed using X-ray diffraction. The diffraction intensities of AgNPs recorded over a 2θ range of 20°–80° using Cu-K α radiation with a wavelength of 1.5406 Å. The average crystallite size of the synthesized AgNPs calculated using the Debye–Scherrer equation:

$$D = k\lambda / \beta \cos \theta$$

Where **D** represents the average crystallite size, **k** is the shape factor or correction factor equal to **0.9**, **λ** is the wavelength of Cu-K α radiation, **β** is the full width at half maximum, and **θ** is the Bragg angle.

Phytochemical screening

Following the standard procedures, qualitative phytochemical analysis was carried out to confirm the presence of phytochemicals in the herbal extracts, including terpenoids, flavonoids, alkaloids, quinones, glycosides, and saponins [26-28]. 2 ml of the extract and three drops of 1% hydrochloric acid, the mixture was filtered and steam-boiled. After adding 6 ml of Mayer-Wagner reagent to 1 ml of the heated solution, a cream-colored or brownish-red precipitate formed, signifying the presence of alkaloids. About 0.5 ml of the extract were combined with 5 ml of

distilled water, and the mixture was then shaken violently, production of persistent foam confirms the detection of saponins, 200 mg of the herbal extract was dissolved in 10 ml of ethanol and then filtered. 2 ml of the filtrate were then treated with magnesium ribbon and strong hydrochloric acid, flavonoids were indicated by the formation of a crimson or pink tint. A yellowish color was produced when 0.5 ml of the extract was combined with 1 ml of distilled water and sodium hydroxide (NaOH) confirms the presence of glycosides. When 1 ml of sulfuric acid was mixed with about 2 ml of the extract, a red precipitate appeared, which indicated the presence of quinones.

0.5 ml of the herbal extract was combined with 2 ml of chloroform and then 3 ml of sulfuric acid. The emergence of a reddish-brown tint indicated the presence of terpenoids.

Selected bacterial strains

The Rasco Pharmaceutical Industry's R&D lab in Lahore, Pakistan, provided the clinically isolated bacterial strains *Escherichia coli* (ATCC 6538) and *Staphylococcus aureus* (ATCC 8739) for the present study.

Disc diffusion method for bacterial antibiotic susceptibility profile

3 ml of saline solution was combined with bacterial colonies to create the bacterial suspension. The Shimadzu UV-Visible spectrophotometer (UV-1800) was used to measure the optical density (OD) of the resultant suspension at 600 nm. The suspension was suitably diluted in line with the OD values using a 10-fold serial dilution method. In the meantime, Petri dishes were filled with sterile Mueller Hinton Agar. Using sterile cotton swabs, 100 μ L of the diluted bacterial suspension was evenly distributed across each plate once the medium had solidified. To preserve aseptic conditions, antibiotic discs were then carefully placed on the agar surface next to a flame using sterile forceps. For a whole day, the plates were incubated at 37°C. Following incubation, a Vernier caliper was used to measure the zones of inhibition surrounding the antibiotic discs in millimeters.

Qualitative antibacterial assays

Agar well diffusion test

To produce an optimally diluted sample with a concentration of 10⁸ CFU per 100 μ L, a 10-fold serial dilution procedure was employed. Once the agar plates were inoculated, 100 μ L of the bacterial suspension was put to each Petri dish and equally distributed with sterile cotton swabs. Sterile pipette tips were then used to make wells in the agar and was used to fill each well with 100 μ L of nanoparticle solutions at concentrations of 50 mg/mL, 25 mg/mL, 12.5 mg/mL, and 6.25 mg/mL respectively.

Standard antibiotics were used as positive controls, and pure medicinal plant extracts with concentration 100 μ L, were used as blank controls to compare with silver nanoparticles samples. The plates were incubated at 37°C for a whole day. After that, the zones of inhibition were measured, which were correlated with the controls.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of all four selected medicinal herbal extracts and their silver nanoparticle composites were determined against both isolated bacterial species, using 96-well flat-bottom microtiter plates (Polystyrene, 96 TM BD, USA). First, 100 μ L of sterile saline was added to each well. Pure extracts and their solutions containing silver nanoparticles were then serially diluted twice, with concentrations ranging from 312.5 μ g/mL, 156.25 μ g/mL, 78.125 μ g/mL, 39.0625 μ g/mL, 19.53125 μ g/mL, 9.7652 μ g/mL, 4.8826 μ g/mL, 2.4413 μ g/mL, 1.2206 μ g/mL, and 0.6103 μ g/mL. Each concentration was added in wells 1 to 10 at a volume of 100 μ L. Wells 11 and 12 were negative and positive controls, respectively. Following the serial dilutions, 100 μ L of a bacterial suspension at a concentration of 10^8 CFU/mL was added to wells 1 to 10 and also in positive control wells, while the herbal silver nanoparticles samples were added to the negative control wells. The plates were then incubated at 37°C for 24 hours. After incubation, observations were recorded, and for further confirmation of MIC and MBC, 10 μ L aliquots from the respective wells were plated onto the agar plates to observe the bacterial growth [29, 30].

Anti-biofilm assay

A modified tube approach method was used to qualitatively evaluate the antibiofilm activity of herbal silver nanoparticles and optical density measurements to determine the percentage of biofilm inhibition. Each six sterile glass tubes contain two milliliters of tryptic soy broth (TSB), were inoculated with a bacterial suspension at a concentration of 10^8 CFU/mL. MIC concentration of herbal silver nanoparticles and a standard antibiotic were subsequently added in the tubes. Pure plant extracts did not exhibit substantial antibiofilm activity as they showed no activity in the MIC and MBC assay. One of the six tubes contained only inoculated TSB as a positive control. Each tube contain 2 mL of sterile TSB supplemented with 2% glucose, the tubes were then incubated at 37°C for 24 hours. Following the incubation period, the contents in the tubes were properly disposed of and non-adherent cells were

removed and washed out with phosphate-buffered saline (PBS). After that, 2 mL of 99% methanol was added to the inner walls of tubes in order to fix the residual biofilm, then let it stand for 15 minutes. The tubes were heat-dried after being fixed. After drying, a 2% iodine solution was used to dye the fixed biofilms. Subsequently, 2 mL of 33% glacial acetic acid was added to each tube to detach the stained biofilm from the inner walls, allowing it to dissolve into the solution. The optical density of each resulting solution was measured using a Shimadzu UV/Vis Spectrophotometer (UV-1800). The percentage of antibiofilm assay for each herbal silver nanoparticle solution and the standard antibiotic as a positive control, were then calculated by using the appropriate formula [31, 32].

Statistical analyses

To ensure accuracy and consistency, every experiment was run in technical triplicates except antibiofilm assay. An independent t-test was used to analyze the inhibitory zones observed for *S. aureus* and *E. coli*. The data was given a 95% confidence interval, and p-value criteria of ≤ 0.05 was used to determine statistical significance.

RESULTS AND DISCUSSION

Morphology analysis

The morphology and purity of the synthesized silver nanoparticles (Ag-NPs) were examined through scanning electron microscope (SEM) as shown in Figure 1 (a, b). Both (a, b) were taken from top view of the same sample with different resolution and scale. The surface morphology of AgNPs was circular, hexagonal and agglomerated in shape confirmed from Figure 1 (a, b). The agglomerated and cluster shape of these NPs confirm the polycrystalline in character. Also, the size/diameter were recorded from Figure 1 (a, b) that laid in the range of 75-100nm approximately.

Fourier transform infrared (FTIR) spectroscopy

The AgNPs were synthesized through green synthesis technique using four different herbs (*Citrullus colocynthis*, *Swertia chirata*, *Onosma Bracteata wall* and *Sphaeranthus indicus*). The chemical nature, surface chemistry and functional groups of all four samples were analyzed through FTIR technique. As AgNPs were laid down in the range below 100nm that produced dangling bonds on their surfaces. The dangling bonds were increased with increase the surface to volume ratio compared to the bulk counterpart that effect on their biotic properties and enhance their applications. KBr tablets of all four samples were prepared to characterize them through FTIR technique as shown in Figure 2. In Figure 2, the spectrum range of FTIR technique

was taken in the range of 500-4000 cm^{-1} .

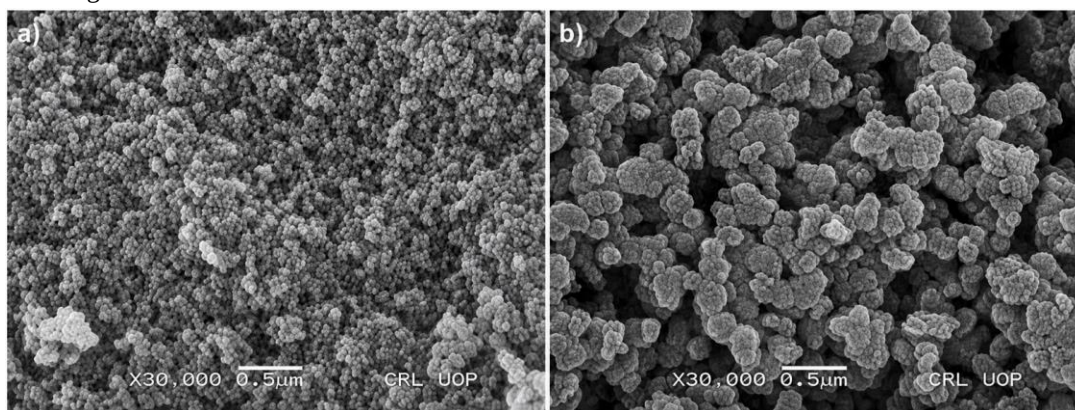


Figure 1: (a, b) SEM micrograph of Ag NPs at two different scales.

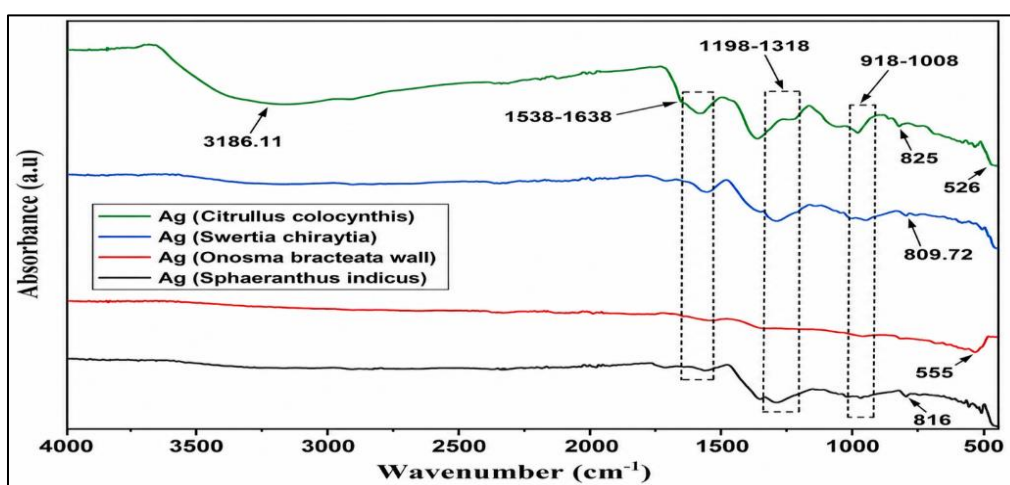


Figure 2: The FTIR results of AgNPs using four different herbs (*Citrullus colocynthis*, *Swertia chiraytia*, *Onosma bracteata wall* and *Sphaeranthus indicus*).

The peaks morphology of all four silver oxide NPs (synthesis with different herbs) were approximately identical but greater numbers of broad and sharp peaks were observed in AgNPs synthesis with “*Citrullus colocynthis* and *Swertia chiraytia*” herbs compared to the other two “*Onosma bracteata wall* and *Sphaeranthus indicus*”. In the range of (2970-3650) cm^{-1} , broad peak of absorption was observed only in AgNPs that was synthesized from *Citrullus colocynthis* herb but no single peak was observed in the remaining three herbs of Ag-NPs. That absorption range represent the presence of abundance of hydroxyl group in a form of stretching (OH) bond [33, 34]. At the range of 1538-1638 cm^{-1} , four different bonds were observed; at 1577 cm^{-1} , 1615 cm^{-1} , 1632 cm^{-1} and 1638 cm^{-1} linked with N-O asymmetric stretching bound, C=O bond, alkanes and N-H stretching vibration bonds respectively [33, 35]. But these absorption peaks were clearly seen in *Citrullus colocynthis*

and *Swertia chiraytia* herbs but very minor intensity beeps were found in the remaining two herbs. In the range of 1198-1318 cm^{-1} C-O stretching bond of alcohol, ether and carboxylic acid were detected as the bumps in this range also represent steroids, terpenoids and alkaloids as mentioned and discuss by Venugopal, Alagesan and Ramzan et al [34, 36]. In the range of 918-1008 cm^{-1} , the absorption peaks were attributed the stretching of cellulose vibration, vibrational bond (C-H) of the methyl group and salt of carboxylate group as recently mention in the literature [37, 38]. At 825 cm^{-1} sharp peak was observed only in *Citrullus colocynthis* herbs that linked with C-H vibrational bending bond. Whereas the twist bond of CH₂ was detected at 816 cm^{-1} only in *Sphaeranthus indicus* herb and C-C was observed in only in *Swertia chiraytia* herbs at 809.72 cm^{-1} as shown in Figure 2. The N-H vibrational bond as reported in earlier studies is confirmed by a few extremely low peaks

seen in the 500 cm^{-1} region [39]. The contribution of different functional groups acting as a stabilizing agent by lowering the surface energy of the NPs to stabilize them was demonstrated by the FTIR analysis based on experimental data and earlier studies.

Structural analysis of silver nanoparticles

The XRD patterns of AgNPs synthesized using four different herbs, *Citrullus colocynthis*, *Swertia chirata*, *Onosma bracteatum* wall and *Sphaeranthus indicus*, are shown in Figure 3. Four prominent Bragg reflections were observed at 2θ values of approximately 38.12° , 44.76° , 64.50° , and 77.22° , corresponding to the (111), (200), (220),

and (311) planes, respectively [41–43]. These diffraction peaks confirmed the face-centered cubic crystalline structure of AgNPs. The results were also compared and confirmed with standard JCPDS data 89-3722, 87-0720, 04-0783, 84-0713, and 14-0072 [44]. Compared with the other herbal AgNPs, *Sphaeranthus indicus* AgNPs showed relatively less intense peaks at the major diffraction angles. The average crystallite size of the synthesized AgNPs was calculated as 24 nm. A small additional peak around $2\theta = 32.15^\circ$ was observed only in *Sphaeranthus indicus* AgNPs, which may indicate the presence of silver oxide, Ag_2O , as reported previously [41].

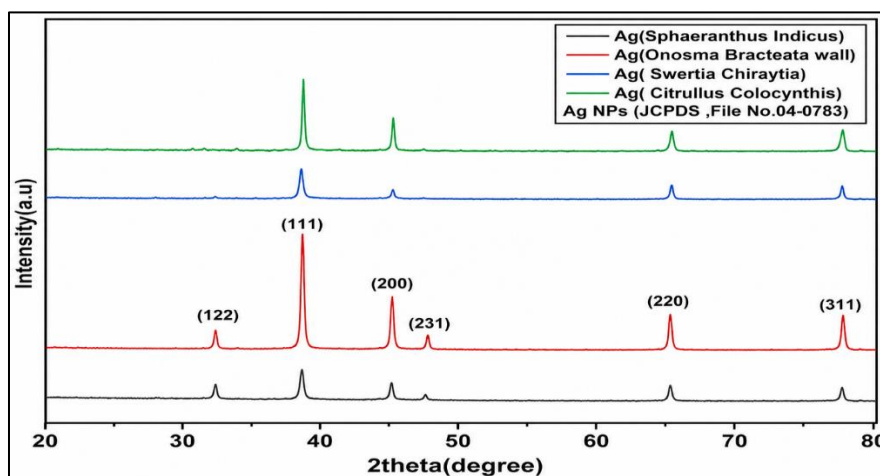


Figure 3: The XRD pattern of AgNPs using four different herbs (*Citrullus colocynthis*, *Swertia chiraytia*, *Onosma bracteata* wall and *Sphaeranthus indicus*).

Phytochemical analysis

After 30 to 60 minutes of adding the silver nitrate salt solution to the extract, surface plasmon resonance (SPR) vibrations caused a color shift from rust orange to dark brown during the continuous stirring phase. The phytochemicals present in the selected medicinal herbal extracts were thought to be responsible for the reduction of the silver to Ag-NPs. The reduction of Ag^+ ions, which

led to the effective synthesis of AgNP, was confirmed by this color shift. The presence of different phytochemicals was analyzed listed in Table 1.

According to Hemlata et al, silver nanoparticles synthesized from *Cucumis prophetarum* exhibited superior antibacterial effects when unite with the aqueous extracts of herbs due to the presence of different phytochemicals bioactive compounds [45, 46].

Table 1: Different phytochemicals in the medicinal herbs.

Medicinal herbs	Phytochemicals					
Names	Alkaloid	Saponins	Flavonoids	Glycosides	Terpenoids	Quinones
<i>Citrullus Colocynthis</i>	+	+	+	+	+	-
<i>Onosma Bracteatum</i>	-	-	+	-	-	+
<i>Sphaeranthus Indicus</i>	+	-	+	-	+	-
<i>Swertia chirata</i>	+	+	+	+	+	-

Subculture of selected microorganisms

Staphylococcus aureus and *Escherichia coli* bacterial strains

were subcultured and grown on nutrient Agar, then stored at 4°C in a refrigerator for subsequent assessment of the

antibacterial and antibiofilm activity of the medicinal herbal extracts and their silver nanoparticles.

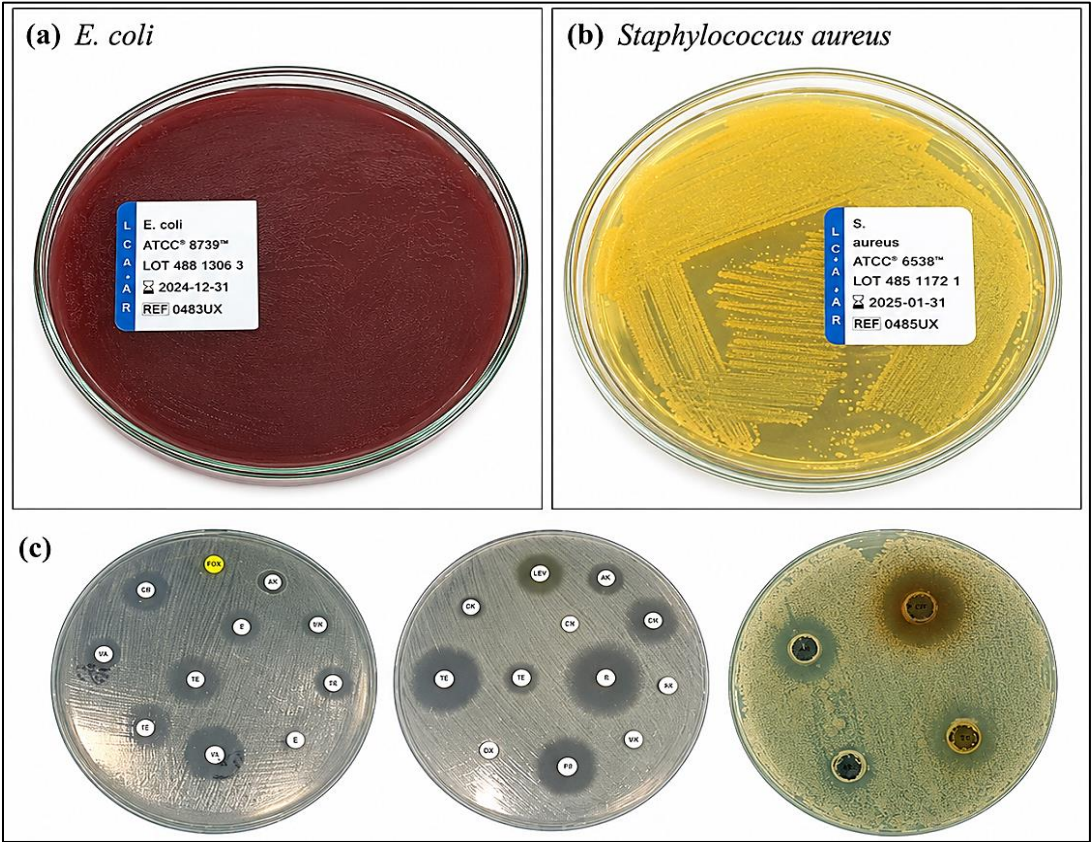
Antibacterial susceptibility profile

The antibacterial susceptibility profile indicated that the selected bacterial strain of *Escherichia coli* and

Staphylococcus aureus was sensitive and resistance to some antibiotics as given in Table 2 and illustrated in Figure 4 (a-d). Ciprofloxacin and Linezolid was selected as the standard antibiotic for *E. coli*, and *S. aureus*, respectively.

Table 2: Antibiotic susceptibility testing of pathogens.

<i>Escherichia coli</i>			<i>Staphylococcus Aureus</i>		
Antibiotic	Zone of Inhibition (mm)	Status	Antibiotic	Zone of Inhibition (mm)	Status
Cefixime (CFM)	00	Resistant	Teicoplanin (TEC)	12	Sensitive
Trimethoprim (SXT)	00	Resistant	Trimethoprim (SXT)	00	Resistant
Cefalexin (CN)	00	Resistant	Chloramphenicol (C)	12	Sensitive
Amoxicillin (AUG)	15	Resistant	Fusidic acid (FC)	18	Sensitive
Ciprofloxacin (CIP)	00	Resistant	Clindamycin (CD)	15	Sensitive
Levofloxacin (LEV)	13	Resistant	Azithromycin (AZM)	11	Sensitive
Ceftriaxone (CRO)	00	Resistant	Cefoxitin (FOX)	12	Sensitive
Meropenem (MRP)	30	Sensitive	Vancomycin (VA)	13	Sensitive
Imipenem (IMI)	23	Sensitive	Linezolid (LZ)	00	Resistant
Tazobactam (TZP)	23	Sensitive	Tetracycline (TE)	11	Sensitive
Tigecycline (TGC)	19	Sensitive	Ceftaroline (CPT)	20	Sensitive
Ceftazidime-avibactam (CZA)	28	Sensitive			



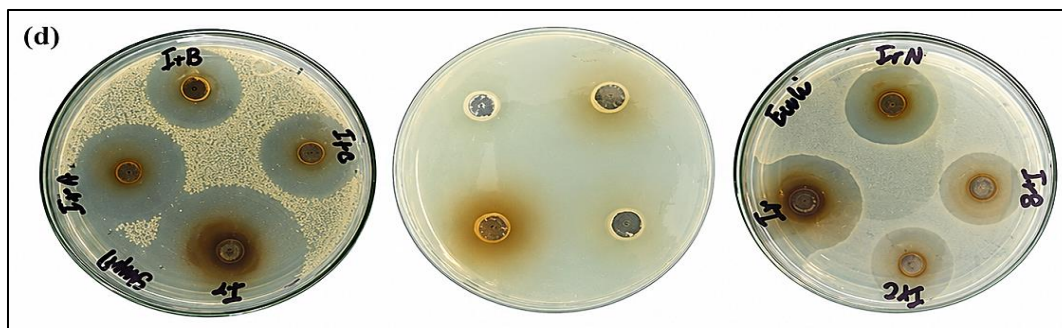


Figure 4: (a, b) Culturing of selected bacterial strains of *E. coli* and *S. aureus*, (c, d) Antibiotic susceptibility test of pathogens *E. coli* and *S. aureus*.

Agar well diffusion assay

Agar well diffusion assay revealed the, significant zone of inhibition at various concentrations in Table 3. However, the four pure herbal extracts did not produce the zone of inhibitions thus showed nill antibacterial activity against these harmful bacterial strains. The standard antibiotics used in the study reflecting limited potency, suggesting resistance in

antibacterial activity against the selected bacterial strain. Among the herbal silver nanoparticles, *Swertia chirata* exhibited the slightly high zones of inhibition against *S. aureus* and *E. coli*, measuring 47.44 ± 0.34 mm and 42.13 ± 0.17 mm more specifically at the highest concentration of 50 mg/mL. It is reported that the zone of inhibition expanded as the concentration of nanoparticles increased [50].

Table 3: Tabular representation of average zone of inhibitions of herbal silver nanoparticles.

	Conc.	<i>Citrullus colocynthis</i> nanoparticles	<i>Sphaeranthus indicus</i> nanoparticles	<i>Onasoma bracteate</i> nanoparticles	<i>Swertia chiraytia</i> nanoparticles
	50 mg/ml	46.42 ± 0.40	40.51 ± 0.43	36.81 ± 0.27	47.44± 0.34
<i>Staphylococcus aureus</i>	25mg/ml	41.86± 0.58	36.02 ± 0.23	33.08± 0.48	39.63± 0.52
	12.5mg/ml	34.15 ±0.39	29.20 ± 0.31	20.83 ± 0.88	38.08 ± 0.42
	6.25mg/ml	32.23± 0.61	24.67 ± 0.30	19.79 ± 0.16	34.81 ± 0.56
	50 mg/ml	33.63± 0.38	33.22± 0.30	36.86± 0.52	42.13 ± 0.17
<i>Escherichia coli</i>	25mg/ml	30.46± 0.47	28.45± 0.84	25.30± 0.29	36.25 ± 0.28
	12.5mg/ml	26.05± 0.56	23.58± 0.42	21.98± 0.65	34.52± 0.59
	6.25mg/ml	23.32± 0.27	22.46± 0.56	20.65± 0.87	29.88± 0.29

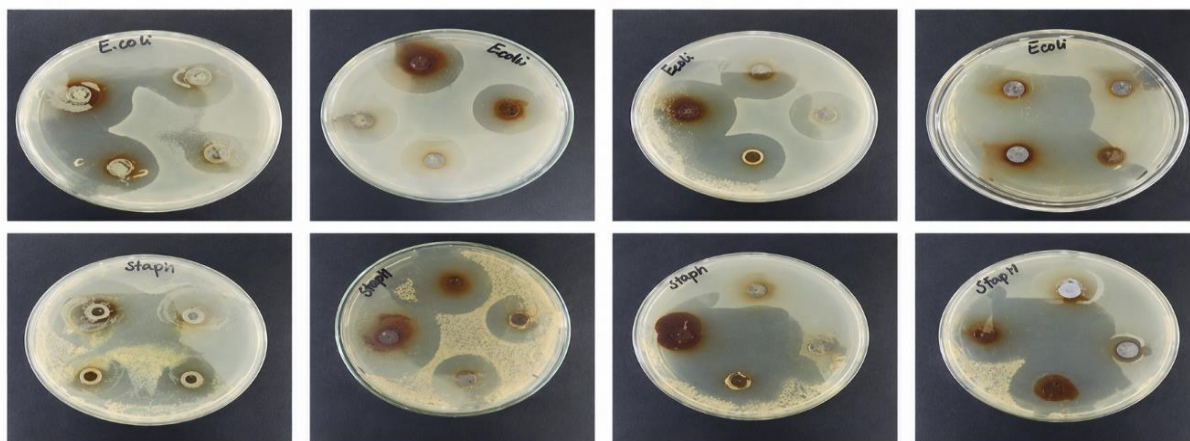


Figure 5: (a) Zone of inhibition of *Onosma bracteatum*, *Sphaeranthus indicus*, *Citrullus colocynthis* and *Swertia chirata* against *E. coli*. (b) Zone of inhibition of *Onosma bracteatum*, *Sphaeranthus indicus*, *Citrullus colocynthis* and *Swertia chirata* against *Staphylococcus aureus*.

The remaining medicinal herb silver nanoparticles also showed significant zones of inhibition at all the concentrations against both bacterial strains respectively. This is related to the fact that these herbal synthesized silver nanoparticles exhibit strong antibacterial activity against *S. aureus* and *E. coli*. Even at the lowest

concentration of 6.25 mg/mL, in Figure 5 (a, b) and Figure 6 (a-d).

The inhibition zones of all four herbal silver nanoparticles showed a statistically significant relation ($p < 0.05$) with each other according to the independent T test as shown in Table 4.

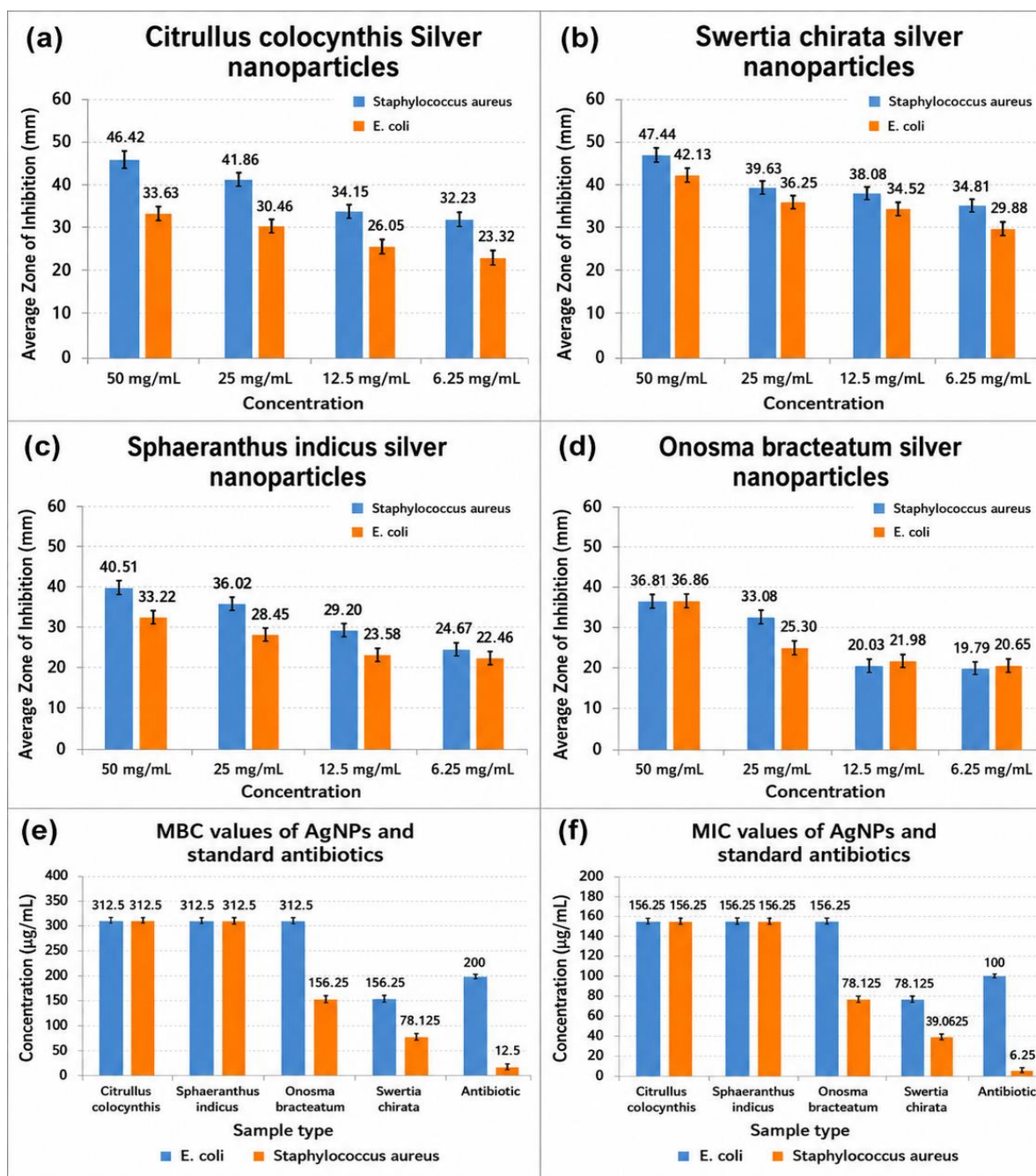


Figure 6: (a-d) Graphical presentation of average zone of inhibitions of herbal AgNPs against *E. coli* and *St. aureus*, (e, f). Graphical representation and comparison of MIC and MBC values of standard antibiotics and Nanoparticles against *E. coli* and *S. aureus*.

Table 4: Independent T-test on average zone of inhibition in silver nanoparticles of herbal extracts showed ($P < 0.05$) proves statistically significant results.

		N	Correlation	Sig.
Pair 1	<i>Onosma bracteatum</i> AgNPs <i>Sphaeranthus indicus</i> AgNPs against <i>E. coli</i> .	4	.956	.044
		N	Correlation	Sig.
Pair 1	<i>Onosma bracteatum</i> AgNPs <i>Sphaeranthus indicus</i> AgNPs against <i>Staphylococcus aureus</i>	4	.974	.026
		N	Correlation	Sig.
Pair 1	<i>Citrullus colocynthus</i> AgNPs and <i>Swertia chirata</i> AgNPs against <i>E. coli</i>	4	.930	.042
		N	Correlation	Sig.
Pair 1	<i>Citrullus colocynthus</i> AgNPs and <i>Swertia chirata</i> AgNPs <i>Staphylococcus aureus</i>	4	.957	.043

The correlation and independent T test cannot be computed in case of pure herbal extracts because the standard error difference is 0 and nil zone of inhibition and significant relation was observed according to numerous published research studies silver nanoparticles had mild antibacterial activity against Gram positive bacteria as compared with the Gram negative bacteria. Interestingly, both Gram-positive and Gram-negative bacteria were strongly inhibited by AgNPs synthesized from *Carduus crispus*, specifying that differences in the composition of bacterial walls do not

impede the antibacterial strength of herbal silver nanoparticles [47, 49]. These findings are strongly correlated with our present research study.

MIC and MBC assay

A comparison of the MIC and MBC values revealed a significant potency of antibacterial activity, when all four herbal aqueous extracts were combined with Ag nanoparticles at particular concentrations against *E. coli* and *Staphylococcus aureus* as given in Table 5 and Figure 6 (e, f).

Table 5: Tabular representation and comparison of MIC and MBC values of standard antibiotics and AgNPs against *E. coli* and *Staphylococcus aureus*.

	<i>Escherichia coli</i>				<i>Staphylococcus Aureus</i>			
	Original Extracts		Nano Composites		Original Extracts		Nano Composites	
	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC
<i>Citrullus colocynthus</i>			312.5µg/ml	156.25µg/ml			312.5µg/ml	156.25µg/ml
	--	--			--	--		
<i>Sphaeranthus indicus</i>	--	--	312.5µg/ml	156.25µg/ml	--	--	312.5µg/ml	156.25µg/ml
<i>Onosma bracteates</i>	--	--	312.5µg/ml	156.25µg/ml	--	--	156.25µg/ml	78.125µg/ml
<i>Swertia chiraytia</i>	--	--	156.25µg/ml	78.125µg/ml	--	--	78.125µg/ml	39.0625µg/ml
Antibiotic standards	Ciprofloxacin		200µg/ml	100µg/ml	Linezolid		12.5 µg/ml	6.25 µg/ml

The pure herbal extracts, regardless of concentration, were found ineffective in inhibiting or killing bacterial cells. *Swertia chirata* silver nanoparticles exhibited the notable lowest MIC value 78.125 µg/mL and 39.062 µg/mL against *Staphylococcus aureus* and MBC values ranging 156.25 µg/mL and 78.125 µg/mL against *E. coli* amongst the four

herbal based silver nanoparticles. According to P. Parvekar study, the MIC and MBC of nanocomposites were found to be significant at 625 µg/mL against *Staphylococcus aureus* only [29].

Antibiofilm assay

The significant and potent antibiofilm assay of all four herbal

silver nanoparticles (AgNPs) was observed against *Escherichia coli* and *Staphylococcus aureus*, as illustrated in Figure 7.

Swertia chirata AgNPs revealed slightly highest percentage 85.77% at MIC concentrations of 78.125 µg/mL against *E.coli* and 71.26% at concentration of 39.062 µg/mL against *S. aureus* as reported in Table 6 and Figure 8.

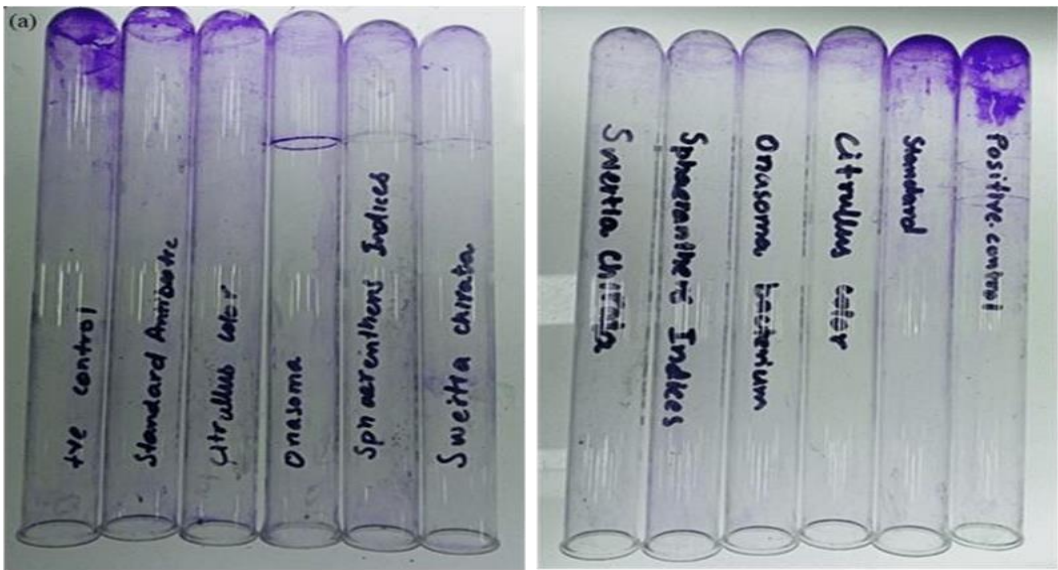


Figure 7: Stained biofilm of bacterial growth, *Staphylococcus aureus* at right side and *E.coli* on left side, showing antibiofilm activity of nano particles and standard antibiotics.

Table 6: Tabular representation and comparison of antibiofilm activity of standard antibiotics and nanoparticles.

Sample	<i>Escherichia coli</i>		<i>Staphylococcus Aureus</i>	
	Bacterial OD	Percentage Inhibition	Bacterial OD	Percentage Inhibition
Positive Control	0.675	NA	0.4650	NA
<i>Citrullus colocynthis</i>	0.150	77.77%	0.2453	47.24%
<i>Sphaeranthus indicus</i>	0.116	82.81%	0.1738	62.62%
<i>Onosomabracteate</i>	0.122	81.92%	0.2250	51.61%
<i>Swertia Chiraytia</i>	0.096	85.77%	0.1336	71.26%
Antibiotic Standards	0.509	24.59%	0.4278	8.00%

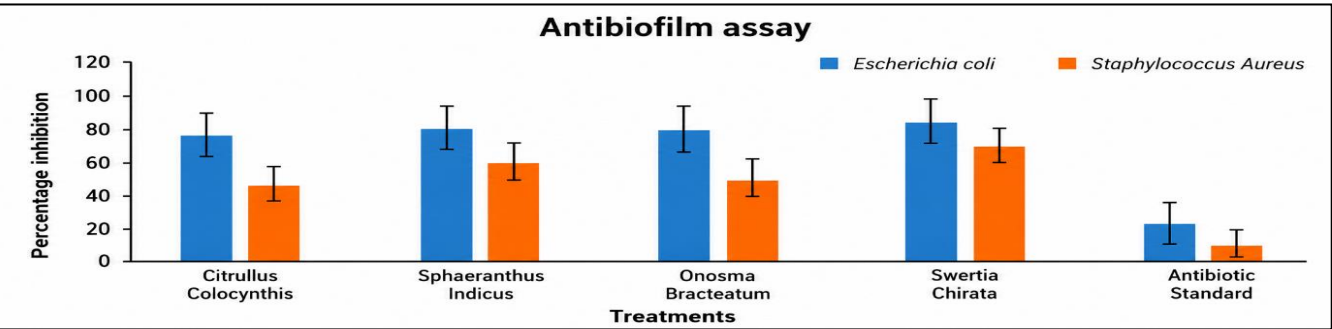


Figure 8: Percentage inhibition of biofilm formation by herbal AgNPs and standard antibiotic against *E. coli* and *S. aureus*.

Mohanta YK and Goswami et al. also reported the use of herbal AgNPs to eradicate biofilms. At a concentration of 15 mg/ml in *S. aureus* by 89% and in *E. coli* by 75%. The current study's findings demonstrate that silver nanocomposites combined with herbal extracts have strong antibiofilm activity against biofilms formed by the gram-positive bacterium *S. aureus* and the Gram-negative bacteria *P. aeruginosa* and *E. coli* at relatively low concentrations [51, 52]. Gram-negative bacteria may be less susceptible as a result of the positive charge of AgNPs being trapped and blocked by lipopolysaccharide, as proposed by Loo et al [53]. But contrary to that our herbal Ag nanoparticles showed slightly high antibiofilm activity against gram negative bacteria *E.coli*. Silver nanoparticles of *Phyllanthus niruri* decreased *S. aureus* biofilms by more than 40% and inhibited *E. coli* biofilms by 64.70% at sub-inhibitory levels [54, 55].

CONCLUSION

This study demonstrated the successful green synthesis of silver nanoparticles using four medicinal herbs: *Citrullus colocynthis*, *Onosma bracteatum*, *Sphaeranthus indicus*, and *Swertia chirata*. SEM analysis showed AgNP sizes in the range of 75–100 nm, while XRD confirmed their crystalline face-centred cubic structure with an average crystallite size of 24 nm. The synthesized AgNPs exhibited significant antibacterial and antibiofilm activity against drug-resistant *Escherichia coli* and *Staphylococcus aureus*. Among all samples, *Swertia chirata*-derived AgNPs showed the strongest activity, with maximum inhibition zones of 42.13 ± 0.17 mm against *E. coli* and 47.44 ± 0.34 mm against *S. aureus*, along with the highest antibiofilm inhibition of 85.77% and 71.26%, respectively. These findings suggest that herbal-mediated AgNPs, particularly those synthesized using *Swertia chirata*, may serve as promising antibacterial and antibiofilm agents against resistant bacterial pathogens. Further studies should investigate their molecular mechanism of action, cytotoxicity, and in vivo therapeutic potential.

Credit authorship Contribution Statement

S. Ayyaz: Writing – original draft, Conceptualization, review, and finalized. **K. Ahmad, M. W. Khalid, Z. Javeed, B. Nadia, A. Ashraf, M. M. Shah:** Editing and proofreading in nanoparticle synthesis. **H. Aali, R. Ahmad, M. I. Khan, M. Abbas:** Proofreading, editing, and writing – review. **S. Khan:** Supervision, Conceptualization, review & editing, funding acquisition.

Declaration of generative AI and AI assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI tools/services in reviewing and editing the submitted manuscript and take(s) full responsibility for the content of the publication.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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