Screening of Antibacterial, Anti-Biofilm, Cell Proliferation Inhibition, and Synergistic Effects of Biogenic Synthesized Silver Nanostructures Using *Trillium govanianum* with Antibiotics

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Summary: The aim of study is to develop the economic plant-based antibacterial agents to improve the treatment strategies for bacterial infections at nano-scale. In current research silver nanostructures synthesis was carried out using *Trillium govanianum* aqueous extract. Characterizations were done using UV–Visible spectrophotometer, scanning electron microscopy, X-ray diffraction and Fourier transform infrared spectroscopy. Antibacterial efficacy of green-synthesized silver nanostructures and aqueous extract was evaluated *via* agar well diffusion method, crystal violet assay (antibiofilm analysis), tetrazolium salt assay (cell viability assay), Direct agar overlay technique, and TLC- spot screening against clinical bacterial pathogens. Synergistic effect of synthesized nanostructures, aqueous extract along with antibiotics was determined by agar disc diffusion method. Minimum inhibitory concentration was also evaluated using agar well diffusion method. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was used for screening of antioxidant potential effect at various concentrations (0.5, 1 and 1.5 µl). The indication of major phytochemical constituents was determined using both qualitative methods and Thin Layer Chromatography (TLC). Absorbance in UV region (295 nm) corresponds to the out of plane quadrupole Plasmon-resonance while at 350 nm corresponds to in-plane dipole resonance. SEM images shows prepared the morphology of Ag nanostructures TGAgNSs is not exactly spherical while XRD analysis shows that highly crystalline TGAgNSs have been prepared with average crystallite size of 27.94 nm. The FTIR spectrum represents sharp peaks of aldehyde, amide I, aromatic rings, and polysaccharides. Minimum inhibitory concentration showed the increased bactericidal effect with increase in concentration of silver nanostructures. Maximum inhibition of *E. coli*, *S. epidermidis*, *S. pyogenes*, and *K. pneumonia* was recorded at 0.12 mg/ml concentration of TGAgNSs with 7.6.0±0.0 mm, 7.6±0.0 mm, 6.6±0.0 mm, and 7.0±0.0 mm zones of inhibition. Similarly, TGAgNSs showed maximum inhibition of *E. coli*, *S. pyogenes*, and *S. marcesscens* at 0.14 mg/ml concentration (8.3±0.0 mm, 7.0±0.0 mm, 7.0±0.0 mm), respectively. Cell viability, antibiofilm assay, TLC-spot screening, and TLC-bio-autography proves the potential antibacterial effect of TGAgNSs. Synergistic effect showed greater antibacterial effect. Glycosides, saponins, flavonoids, proteins, terpenoids, alkaloids, phenols, and tannins were detected. The scavenging potential of TGAgNSs was calculated as 74% at 1.5 µl. It was concluded that green synthesized nanostructures have efficient antibacterial potential and would be used in medical devices to cure infections in near future.

Keywords: *Trillium govanianum*, Silver nanostructures, Green nanotechnology, Synergistic effect, Photochemical screening, T\LC Bioautography, Antibiofilm assay, MTT assay, Minimum inhibitory concentration.

Introduction

The leading cause of premature deaths throughout the world are infectious diseases, killing almost 50,000 people every day; especially the burns remain a large public health issue and secondary infections are reported to be the main cause of hospital deaths in 50-75% cases [1]. Variety of bacterial agents such as *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus* are responsible for infectious diseases. Most of human pathogenic bacteria from all over the world are drug-resistant [2]. The continuous use of antibiotics, make microorganism resistant to drug and also cause adverse effects on host which include hypersensitivity, immunosuppressant and allergic reactions [3]. This has created immense clinical problems in the treatment of infectious diseases [4]. In the last three decades, number of new antibiotics has been produced by many pharmacological industries, but the microbial resistance to these drugs has been increased day by day. Some bacteria have the genetic ability to attain resistance against these therapeutic drugs [5].

Increasing microbial resistance against antibiotics is a burning health problem in the developing countries nowadays. Thus, there is an

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urgent need to improve the existing drugs to overcome this problem. The therapeutic applicability of silver and medicinal plants in bacterial infections treatment is well known [6, 7]. In recent times, silver nanoparticles synthesis using medicinal plants is attempted; secondary metabolites present in the medicinal plants are used to reduce silver to nano-size up to 100 nm, exhibit improved properties compared to the larger particles [8]. The silver compounds have been well known for their antibacterial activity since ancient times [9] and it has been demonstrated that silver is nontoxic to human cells at low concentration [10]. In the field of high sensitivity bimolecular detection and diagnosis of different diseases silver nanoparticles have been used tremendously [11], antimicrobials and therapeutics [12], catalysis [13], and microelectronics [14]. Silver have inhibitory effect on many microbes commonly present in medical and industrial processes [15]. The significant use of silver and silver nanoparticles is to prevent infections against burn and open wounds [16].

Many human diseases can be treated by using active constituents from medicinal plants and also have the capacity to produce a large number of organic phytochemicals that is known as secondary metabolites [17]. Both infectious and non-infectious diseases are treated by medicinal plants and these are the source of various pharmacological products and as a natural composite that acts as a new anti-infectious agent [18]. Huge number of secondary metabolites has been reported from various medicinal plants for their antimicrobial activity and their demands have been increased in both developed and developing countries. These natural products are easily biodegradable and have lowest environmental hazards [19]. The natural products of medicinal plants may give a new source of antibacterial, antifungal and antimicrobial agents [20, 21]. Nowadays green synthesized nanoparticles have great importance in the field of medicine to cure the infectious agents [22]. Various plants such as *Medicago sativa, Tritium vulgare, Bergenia ciliata, Artimesia vulgaris, Tamarindus indica, Cinnamomum camphora, Avena sativa, Azardirachta indica, Emblica officinalis, Aloe vera, Coriandrum sativum, Carica papaya,* and *Acanthella elongate* [23-28].

In current research, silver nanostructures were synthesized using *Trillium govanianum* (family *Trilliaceae)*. This medicinal plant is mostly dispersed in South Asia, from Pakistan to Bhutan (at an altitude of 2700-3800 m) [29]. The phytochemical constituents of *Trillium* are used in cancer treatments [30]. In folk medicine, *T. govanianum* is used to cure dysentery, inflammation, sepsis, menstrual, sexual disorders and also used in wound healing [31]. The powdered of the plant is used as body and sexual tonic [32]. The aim of research was to assess the antibacterial effect of green synthesized silver nanostructures using *Trillium govanianum* aqueous extract to cure the bacterial infections.

Experimental

Collection of medicinal plants

Rhizomes of *Trillium govanianum* were collected from district Neelum, Azad Jammu and Kashmir, Pakistan. The plant was identified by ethnobotanist, Department of Botany, Faculty of Science, University of Azad Jammu and Kashmir, Muzaffarabad, Pakistan. Rhizomes were rinsed with running tap water to remove soil, and air-dried under shaded area at room temperature (20 ºC ± 2) for few weeks and dried material was crushed into fine powder using pestle and mortar.

Preparation of extract

Fine powder was used for the preparation of aqueous extract *via* standard protocol of maceration [33]**.** Five gram of powder was added to distilled water (100 ml) and stirred at 60℃ for 1 h. After heating, the mixture was cooled and filtered with Whatman paper number 1 to collect filtrate.

Green synthesis of nanostructures

Silver nitrate (1 mM) was used for the nanoparticle synthesis in amber bottle to prevent the auto-oxidation of silver. For the synthesis of silver nanostructures, 10 ml of aqueous extract (TGaqu) was mixed with 90 ml of 1 mM silver nitrate solution and boiled at 100℃ up to color changed. The synthesized nanostructures (TGAgNSs) were purified by centrifugation for 30 min at 15,000 rpm. The nanostructures were washed with distilled water to remove stabilizer residue. These nanostructures were washed thrice with distilled water then dried in an incubator at 37℃ for overnight [34-36]. After incubation, dried TGAgNSs were collected, weigh, and dissolved in DMSO for further processes. The pH of silver nanostructures was recorded as 5.6.

Characterization of nanostructures

Characterization of green synthesized nanostructures (TGAgNSs) was done using UV–Visible spectrophotometer (Perkin Elmer, LAMBDA 950 spectrometer). Absorption spectra was recorded

between 200-800 nm. The shape and size of the synthesized nanostructures were determined using scanning electron microscope (SEM: Jeol JSM-6510LV). The pellets of TGaqu and TGAgNSs were subjected to FTIR for functional group determination (Perkin Elmer Spectrum 100 series). FTIR analysis was carried out by Perkin Elmer spectrum 100 series spectrometer by using solid-phase Potassium Bromide (KBr) pellets method in the range 400 cm^{-1} to 4000 cm^{-1}. The structural analysis was carried out by X-Ray Diffraction technique (X'Pert PRO 3040/60).

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration of TGaqu and TGAgNSs was performed by agar well diffusion method against 7 pathogenic bacteria such as *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Serratia marcesscens, Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Streptococcus pyogenes* [37, 38]. Agar dilution method was used for the MICs values. The MICs values (0.02 mg/ml, 0.04 mg/ml, 0.06 mg/ml, 0.08 mg/ml, 0.10 mg/ml 0.12 mg/ml and 0.14 mg/ml) of TGaqu and TGAgNSs were used to evaluate the antibacterial effect. Nutrient agar (NAM; Oxide CMOO3) and Nutrient broth media (NBM; CM1) were used for bacterial culture. The overnight culture (10^7 colony forming units (cfu)/ml) was mixed with freshly prepared nutrient agar medium (NAM) at 45°C and was poured into sterilized Petri dishes. All Petri dishes were kept at room temperature in laminar flow for solidification. 5 mm wells were formed using a 1 ml of sterilized micropipette tip and sterilized needle was used for the removal of agar plug. Approximately 30 µl of each (TGaqu, and TGAgNSs), $AgNO_3$, and negative control (DMSO) were poured in each prepared wells and placed at 37 °C for 24 h. Microbial growth inhibition was determined by measuring the diameter of the zone of inhibition after 24 h in millimeter [39]. The diameter of the clear zones (if greater than 5 mm) around each well was measured with the help of scale [40]. The growth sensitivity tests were expressed as 0 for no sensitivity, 1-3 mm for low sensitivity, >3-6 mm for moderate sensitivity and >6-25 mm for high sensitivity.

Synergistic effect

Sensitivity test of various antibiotics such as ampicillin (10 µg/ml), tobramycin (10 µg/ml), and trimethoprim (10 µg/ml) against all tested bacterial strains was assessed by agar disc diffusion method and used as positive control [41]. Synergistic effect of synthesized TGAgNSs, and TGaqu along with antibiotics was also determined by the agar diffusion technique with slight modification [42]. For the screening of synergistic effect antibiotic discs were permeated with 10 µl TGAgNSs and TGaqu, and placed at room temperature up to dry. Later permeated discs were placed on the solidified agar at equal distance. All these plates were kept at 37°C for 24-48 h. After incubation the zone of inhibition was measured and compared [43].

Antibiofilm assay

Crystal violet assay used for antibiofilm activity with slight modifications [44]. Bacteria were grown in Borosilicate tubes (Minitek, USA) containing 2 ml of nutrient broth medium and 30 mg/ml concentration of TGaqu and TGAgNSs for overnight at 37 °C. Silver nitrate and Chloramphenicol used as control. After incubation broth medium was removed and attached cells were stained by adding 125 µl of a 0.1% crystal violet. Borosilicate tubes were incubated at room temperature for 10-15 min and rinsed with water to remove excess unattached cells and dye. After staining, crystal violet was solubilized by adding 30% acetic acid and incubated at room temperature for 10-15 min. Solubilized crystal violet was quantified at 550 nm using spectrophotometer. 30% acetic acid in water used as the blank control.

MTT assay

MTT assay was used for estimating the viability of bacterial cells [45]. For this, 0.2 mg/ml MTT (Genebase, China) was dissolved in DMSO and incubated at room temperature for 1-4 h. Bacterial cells (100 µl) were grown at 37°C in NBM (3 ml) for overnight. Next day bacterial cells (100 µl) from overnight culture were grown at 37°C in NBM (1 ml) for 3 h (exponentially growing cultures) by adding 100 µl of each test sample. To initiate the reduction reaction, 10 µl MTT stock solution was added to, and the mixture was incubated at 37°C for 2-4 h (without shaking) with the tube cap open. Purple color appears which indicated the formation of formazan crystals. To dissolve the crystals 500 µl of DMSO was added. The formazan crystals were dissolved at room temperature. The absorbance of each sample was measured at 570 nm in a spectrophotometer using DMSO as control.

DPPH free radical scavenging activity

DPPH [(di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium] free radical scavenging activity of

extract and silver nanostructures was determined with slight modifications [46]. Half ml of MeOH (0.1 mM) solution of DPPH was mixed with 20-150 µl of extracts, mixed with vortex vigorously and left for 30 min at 37°C in an incubator. The volume was made up to 2 ml by adding methanol. Methanol was used as a baseline control. One milliliter of 0.1 mM DPPH (in ethanol) was added to different concentrations (0.5 ml, 1 ml, and 1.5 ml) of TGAgNSs and TGaqu. The reaction mixture was shaken and incubated in the dark for 30 min. The absorbance at 517 nm was measured. Ascorbic acid was used as the standard and the control was prepared as described above without a sample. The lower absorbance of the reaction mixture indicated a higher percentage of scavenging activity The absorbance was recorded at 517 nm. Water (20-150 µl) was used as a control and the percentage scavenging activity was calculated by formula: %=[(Ao-Ai)/Ao] ×100; where Ao is the absorbance of control and Ai is the absorbance with extracts.

Phytochemical screening

The phytochemical screening was carried out on the aqueous extract of *Trillium govanianum* and Silver nanostructures for glycosides, flavonoids, tannins, saponins, proteins, terpenoides, alkaloids, and phenols using standard procedures to identify the constituents as described by [47-50].

Thin layer chromatography

The indication of major phytochemical constituents was further confirmed by TLC using precoated Silica gel 60F264 plates with some modifications of used protocols [51, 52]. In order to get better resolution of the components, different screening systems (SS) were used such as SS1 {Chloroform: Ethyl acetate: acetic acid (5:5:1)}, SS2 {Acetone: acetic acid: Distilled water (8:2:10)}, SS3 {acetic acid: distilled water (8:2)} SS4 {Butanol: Acetone: Distilled water (10:5:20)}and SS5{Chloroform: acetone: distilled water (8:2:10)}. TLC plates were kept in tank without touching the baseline and left for development. The solvent front was marked and the developed plates were dried, and observed under visible as well as UV light (734 nm). Various colors of spots like red, brown, green, blue, yellow, and reddish were also recorded. R_f value of each spot was calculated as R_f = Distance traveled by the solute /distance traveled by the solvent. After the development of chromatogram on TLC plates, various chemical sprays were used for the detection of phytochemicals [53]. For flavonoids, $AlCl_3$ was used (yellow, green and blue fluorescence in longer wavelength UV light (360 nm) indicated positive results). Fast blue B reagent spray was used for the detection of phenols (red spots appear), 1% ninhydrin spray for amino acids (reddish spots appear), 37% formaldehyde in $H_2SO_4$for alkaloids and urea in HCl for sugars (pink spots, ketoses in blue spots). 0.05% of DPPH solution in methanol was sprayed on the surface of developed TLC plates and incubated for 10 min at room temperature. The antioxidant constituents were detected as yellowish-green spots produced via bleaching of DPPH on the TLC plates.

TLC-Bioautography

To measure the bio-autography, direct agar overlay technique was used with slight modifications as demonstrated [54, 55]. TLC-bioautography was done against all tested bacterial pathogens. The TGaqu (100 µl of 0.12 mg/ml) and TGAgNSs (100 µl of 0.12 mg/ml) were used to spot on the chromatograph silica gel plates. The developed chromatogram was placed in sterilized Petri plates. Further, the fresh overnight grown culture of tested bacteria was mixed with nutrient agar and then poured over the chromatogram as a thin layer. The plates were left at room temperature for 5 min, then for incubation at 37°C for overnight to record the zones of growth inhibition around the active chromatogram spot.

Spot Screening

TLC spot screening was performed using modified protocol of [56]. Spots on the preparative silica gel plates were scratched with the help of clean and dry spatula and collected in beaker containing 70% ethanol and left overnight. The content in the beaker was stirred and filtrated through Whatman No. 1 filter paper. The filtrate was collected in clean and dry beaker via centrifugation at 15000 rpm for 1 min. The filtrate containing active compound was used for the determination of antimicrobial effect through agar-well diffusion method [57].

Statistical analysis

Each experiment was repeated in triplicates. Mean ± Standard Deviation from absolute data was calculated using online calculator (http://easycalculation.com/statistics/standard-deviation.php).

Results and Discussion

Preliminary Synthesis of TGAgNSs

The extract was pale yellow in color before addition of $AgNO_3$ and this was changed to dark brownish color on boiling, suggesting the rapid

formation of silver nanostructures. The change of color indicates the preliminary biosynthesis of silver nanostructures (Fig. 1.A). The reduction and stabilization of silver ions is due to combination of plant biomolecules such as proteins, amino acids, enzymes, polysaccharides, alkaloids, tannins, phenolics, saponins, terpinoids, *vitamins* and some other metabolites like antioxidants [58]. Similarly, in current research flavonoids, alkaloids, amino acids, and sugars were detected. Biological organisms were utilized as reducing agents during nanoparticle synthesis [59]. Similarly, hydroxyl and carboxylic groups of TGAgNSs could be act as stabilizing and reducing agents in the synthesis of nanostructures [60]. Some other functional groups (phenolics, flavonoids, and alkaloids) are also involved in the capping and stabilizing of nanostructures [61]. The reduction mechanism also capable to control the size and the formation of stable bonding between nanoparticles and phytochemicals [62].

Two absorption bands were observed, one in UV region (295 nm) corresponds to the out of plane quadrupole Plasmon-resonance and other at 350 nm corresponds to in-plane dipole resonance (Fig. 1.A). SEM image depicted that the morphology of nanostructures not exactly spherical. The crystalline size is measured by XRD- analysis as 27.94 nm size (Fig. 1.B.I and 1.B.II). All the peaks belong to pure Ag and the average crystallite size is estimated by Debye Scherrer formula, $D=K\lambda/\beta\cos\theta$ [63](Fig. 1.B.II). Our results are also in agreement with the findings of previously synthesized green nanoparticles [64-67]. They showed the sizes of nanoparticles range from 5 nm to 100 nm using *Coleus aromaticus*, *Petroselinum crispum*, peanut seeds, and *Justicia adhatoda*. The FTIR spectrum was recorded for both AGAgNPs (Fig. 1.C.I) and AGaqu (Fig. 1.C.II). The FTIR spectrum of crude *Trillium govanianum* extract (TGaqu) showed absorbance peaks at 3408 cm^{-1}, 2341 cm^{-1}, 1037 cm^{-1}, 1605 cm^{-1}, 1554 cm^{-1}, and 1402 cm^{-1}. The band at 3408 cm−1 was responsible for O-H stretching. The band 2341 cm^{-1} was due to the presence of aldehyde C-H stretching and was shifted to lower frequency (2011 cm^{-1}) in TGAgNSs, when compared with the TGaqu. The band 1605 cm^{-1}in TGaqu was due to the presence of amide I vibrations and this band was shifted to 1608 cm^{-1} in TGAgNSs because of the proteins that possibly will bind to AgNPs through the amine groups. The bands 1554 cm^{-1} and 1402 cm^{-1} are responsible for C-O-C stretching, which could be attributed to the reduction of Ag+, because the band was shifted to 1390 cm^{-1} and 1075 cm^{-1}. FTIR spectrum of TGAgNSs shows absorption bands at 3393 cm^{-1}, 2011 cm^{-1}, 1608 cm^{-1}, 1543 cm^{-1}, 1390 cm^{-1}, 1075 cm^{-1}, and 1010 cm^{-1}. The absorption bands at 3393 cm^{-1}represent the OH group stretching and sp2 C-H (alkane) vibration respectively, 2860 cm-1 band represent presence of alkyne functional group. The absorption at 1608 $cm^{-1}$and 1543 cm^{-1} was attributed to the C─C═C symmetric stretching vibration of aromatic rings. The absorption band at 1390 cm-1 was attributed to the C─N stretching vibration. The absorption band at 1075 cm^{-1} and 1010 cm^{-1} might have contributed by the C─O group of the polysaccharides in the TGAgNSs. FTIR analysis indicated the possible naturally occurring organic molecules, taken part in stabilization and capping of the Ag nanoparticles. These peaks could be ascribed to O–H group in polyphenols or proteins/enzymes or polysaccharide [68-69]. The shifting of bands may be due to binding of functional groups with the nanoparticles. These findings are consistent with the results of [70].

Characterization

Agar well diffusion assay

Results of MICs revealed that the antibacterial efficacy of TGAgNSs is increased with increasing the concentration against all tested pathogens. Maximum inhibition of *E. coli*, *S. epidermidis*, *S. pyogenes*, and *K. pneumonia* was recorded at 0.12 mg/ml concentration of TGAgNSs with 7.6±0.50 mm, 7.6±0.0 mm, 6.6±0.0 mm, and 7.0±0.0 mm zones of inhibition. Similarly, TGAgNSs also showed maximum inhibition of *E. coli*, *S. pyogenes*, and *S. marcesscens* at 0.14 mg/ml concentration (8.3±0.0 mm, 7.0±0.0 mm, 7.0±0.0 mm). Maximum inhibition of *S. epidermidis*, and *K. pneumonia* with 7.0±0.0 mm and 7.0±0.0 mm zone of inhibition was also recorded in case of TGAgNSs at 0.10 mg/ml. While TGAgNSs showed moderate inhibition of *P. aeruginosa* on all tested concentrations. On the other hand, TGaqu showed low or even moderate antibacterial effect at all concentrations against all tested pathogens, Both TGAgNSs and TGaqu showed lowest inhibition of *S. marcesscens* at all concentrations except 0.14 mg/ml (Table-1). It was observed that TGAgNSs had strong antibacterial effect compared to the TGaqu. Current research is tuned with earlier reported studies [71-73]. They showed that Ag nanoparticles had antibacterial effect on fish pathogen and other microbes. According to [74] regulation of proper transport via plasma membrane may be affected by the silver nanoparticles which leads to the cell death. Sulaiman *et al*. [75] reported that phosphorous and Sulphur containing enzymes or proteins of DNA of

bacterial cell may affected by the silver nanoparticles which lead to the death of cells via enzymatic inhibition. Other researchers also reported that silver nanoparticles may attached to the bacterial cell surface, penetrated into the cell, destroy the cytoplasmic enzymes and killed the bacteria [74]. In current research green synthesized silver nanostructures proves the antibacterial efficacy against clinical bacterial pathogens and have various advantages such as low cost, overcome the resistance, reduce toxicity compared to the conventional antibiotics used. Our findings are agreed with the previous literature [76, 77]

Antibiogram assay and synergistic effect

Agar disc diffusion assay indicated that ampicillin and trimethoprim had no antibacterial effect against all tested bacterial pathogens while tobramycin showed the maximum inhibition of *E. coli* (10.0±0.0 mm), *K. pneumonia* (10.0±0.0 mm), *S. epidermidis* (10.0±0.0 mm) and *S. pyogenes* (8.0±0.0 mm). Synergistic effect of TGAgNSs and TGaqu with conventional antibiotics was done against seven pathogens at the concentration of 0.12 mg/ml because these pathogens are strongly inhibited at this concentration (Fig. 2). Tobramycin showed maximum inhibition of *E. coli* (10.0±0.0 mm), *S. marcescens* (7.0±0.0 mm) and *S. pyogenes* (8.0±0.0 mm) when combined with the TGaqu extract. On the other hand, Tobramycin indicated the maximum inhibition of *K. pneumonia* (10.0±0.0 mm), *P. aeruginosa* (7.0±0.0 mm), and *S. epidermidis* (10.0±0.0 mm) when combined with TGAgNSs compared to antibacterial effect of antibiotics used. Low or even no antibacterial effect was recorded when ampicillin and trimethoprim were used in combination with TGAgNSs and TGaqu extract. It was observed that the synergistic effect may be due to the presence of phytochemical constituents which interact with the antibiotics. In current research, saponins, tannins, alkaloids, free amino acids, quinones, phenols, steroids, glycosides, terpenoids, flavonoids were found in both TGAgNSs and TGaqu. Our results are agreed with the previous literature [78]. They showed that synergistic effects occurred when phytochemicals and antibiotics combined. Shaaban *et al.* [79] illustrated the synergistic/antagonistic effect between antibiotics and some essential oils against pathogenic and food-spoiler microorganisms. Some medicinal plants have synergistic antimicrobial effect when combine with different antibiotics [80].

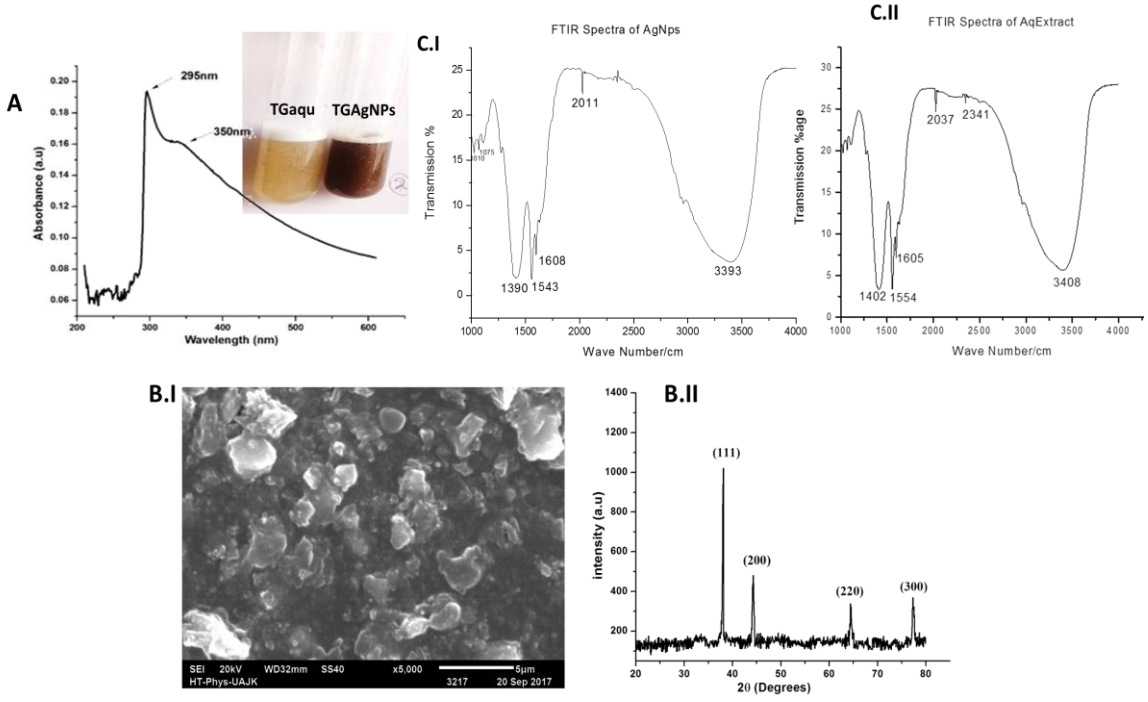

Fig. 1: Preliminary Synthesis and characterization of green synthesized silver nanoparticles (TGAgNSs). A) UV-Vis spectra analysis, B) Scanning electron microscopy and XRD-analysis of synthesized silver nanoparticles; C) FTIR of Green synthesized nanoparticles.

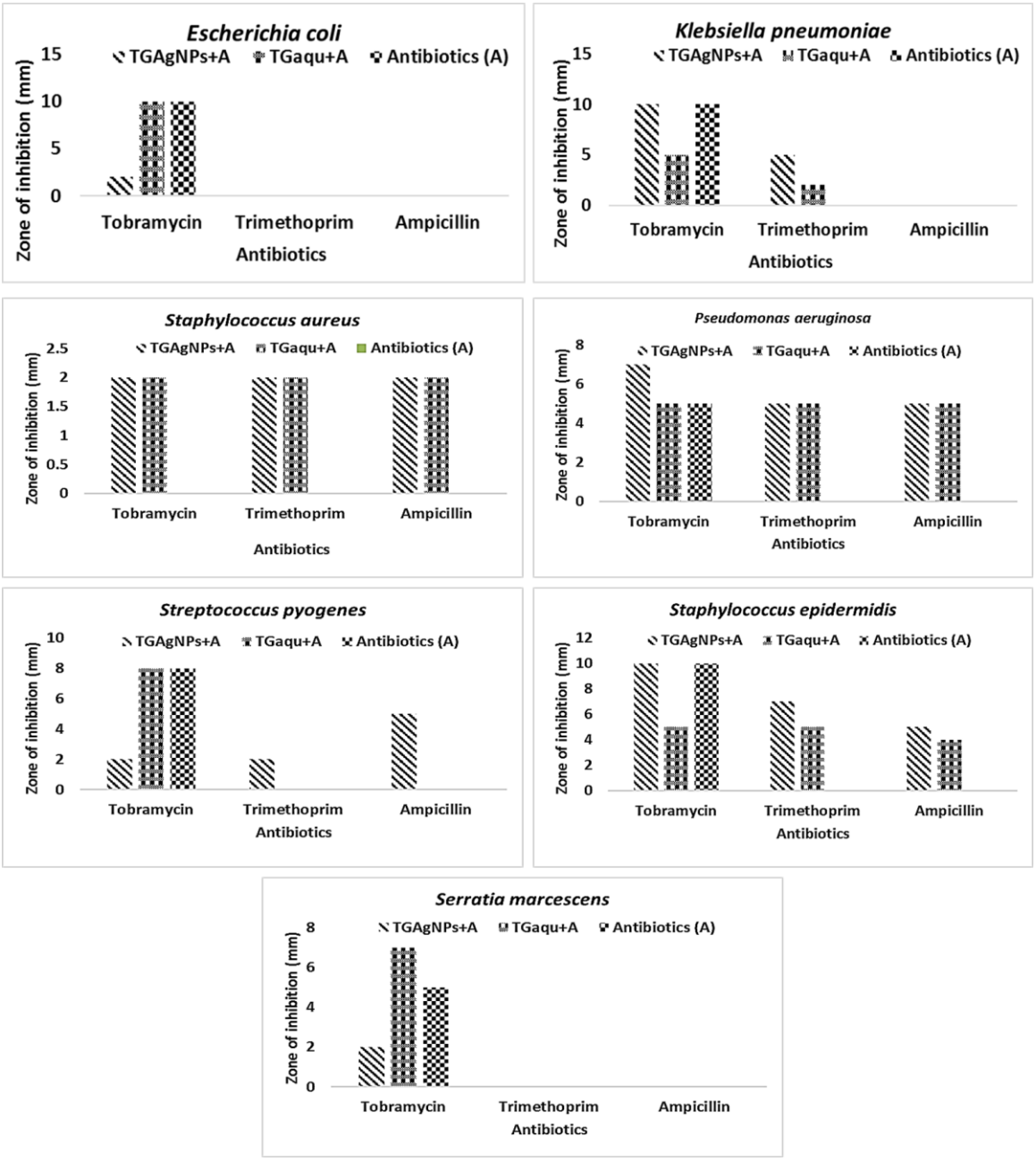

Fig. 2: Synergistic activity of green synthesized silver nanoparticles (TGAgNSs) and extract (TGaqu) against bacterial pathogen.

Table-1: Antibacterial activity of green synthesized silver nanoparticles (TGAgNSs) and extract (TGaqu) of *Trillium govanianum* against bacterial pathogens.

Pathogens	Zones of Inhibition in (mm) at various concentrations (mg/ml)													
	0.02		0.04		0.06		0.08		0.10		0.12		0.14	
	TGaqu	TGAgNSs	TGaqu	TGAgNSs	TGaqu	TGAgNSs	TGaqu	TGAgNSs	TGaqu	TGAgNSs	TGaqu	TGAgNSs	TGaqu	TGAgNSs
E. coli	0.0±0.0	2.0±0.0	2.0±0.0	5.0±0.0	1.0±0.0	4.0±0.0	0.0±0.0	5.0±0.0	1.0±0.0	5.0±0.0	0.0±0.0	7.6±0.0	0.0±0.0	8.3±0.0
S. epidermidis	0.0±0.0	7.0±0.0	0.0±0.0	7.0±0.0	0.0±0.0	6.3±0.0	0.0±0.0	7.6±0.0	0.0±0.0	7.0±0.0	0.0±0.0	7.6±0.0	0.0±0.0	5.0±0.0
S. pyogenes	0.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	2.0±0.0	4.0±0.0	2.0±0.0	5.0±0.0	0.0±0.0	4.0±0.0	2.0±0.0	6.6±0.0	0.0±0.0	7.0±0.0
S. aureus	2.0±0.0	2.0±0.0	2.0±0.0	4.0±0.0	2.0±0.0	2.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	2.0±0.0	5.0±0.0	0.0±0.0	5.0±0.0
K. pneumonia	5.0±0.0	5.0±0.0	5.0±0.0	5.0±0.0	5.0±0.0	5.0±0.0	5.0±0.0	5.0±0.0	5.0±0.0	7.0±0.0	5.0±0.0	7.0±0.0	1.0±0.0	5.0±0.0
P. aeruginosa	2.0±0.0	5.0±0.0	2.0±0.0	4.0±0.0	0.0±0.0	4.0±0.0	2.0±0.0	5.0±0.0	5.0±0.0	5.0±0.0	2.0±0.0	5.0±0.0	1.0±0.0	2.0±0.0
S. marcescens	2.0±0.0	2.0±0.0	2.0±0.0	2.0±0.0	2.0±0.0	2.0±0.0	2.0±0.0	3.0±0.0	2.0±0.0	2.0±0.0	0.0±0.0	5.0±0.0	0.0±0.0	7.0±0.0

The growth sensitivity tests were expressed as 0 for no sensitivity, 1-3 mm for low sensitivity, >3-6 mm for moderate sensitivity and >6-25 mm for high sensitivity.

Cell viability assay and Biofilm inhibition effect of TGAgNSs

The substantial reduction in the bacterial cells viability was recorded when TGAgNSs and TGaqu used compared to control samples (DMSO and pathogen alone) at the concentration of 0.12 mg/ml. TGAgNSs showed the significant reduction of *E. coli*, *S. marcescens*, and *K. pneumonia* compared to chloramphenicol. The reduction in cell viability relay on the concentration used (Fig. 3.A). Antibiofilm assay of silver nanostructures (TGAgNSs) and TGaqu showed the significant reduction in biofilm of all tested pathogens compared to the control samples (DMSO and pathogen alone) (Fig. 3.B). TGAgNSs showed the significant reduction of *E. coli*, *S. epidermidis*, *S. pyogenes*, *K.pneumonia* and *S. marcescens* compared to TGaqu extract. These results support the antibacterial effect against above-tested pathogens *via* agar well diffusion method at 0.12 mg/ml concentration. Our results are consistent with the earlier outcomes that the antibiofilm activity of AgNPs was confirmed against antibiotic-resistant microbes [81]. They reported that the AgNPs effectively reduced the biofilm of the tested bacteria. Chloramphenicol showed the reduction of biofilm formation and used as positive control. Our findings suggest that TGAgNSs are able to prevent biofilm formation and also it could be used against clinical pathogenic infections. Current outcomes are also agreed with the results of [82] they described that silver neither interfere with the growth cycle nor affect the cell viability, but only diminishes biofilm formation property. Our results are also confirmed by [83] suggested that the biosynthesized AgNPs have ability to inhibit 80–90 % biofilm formation [84].

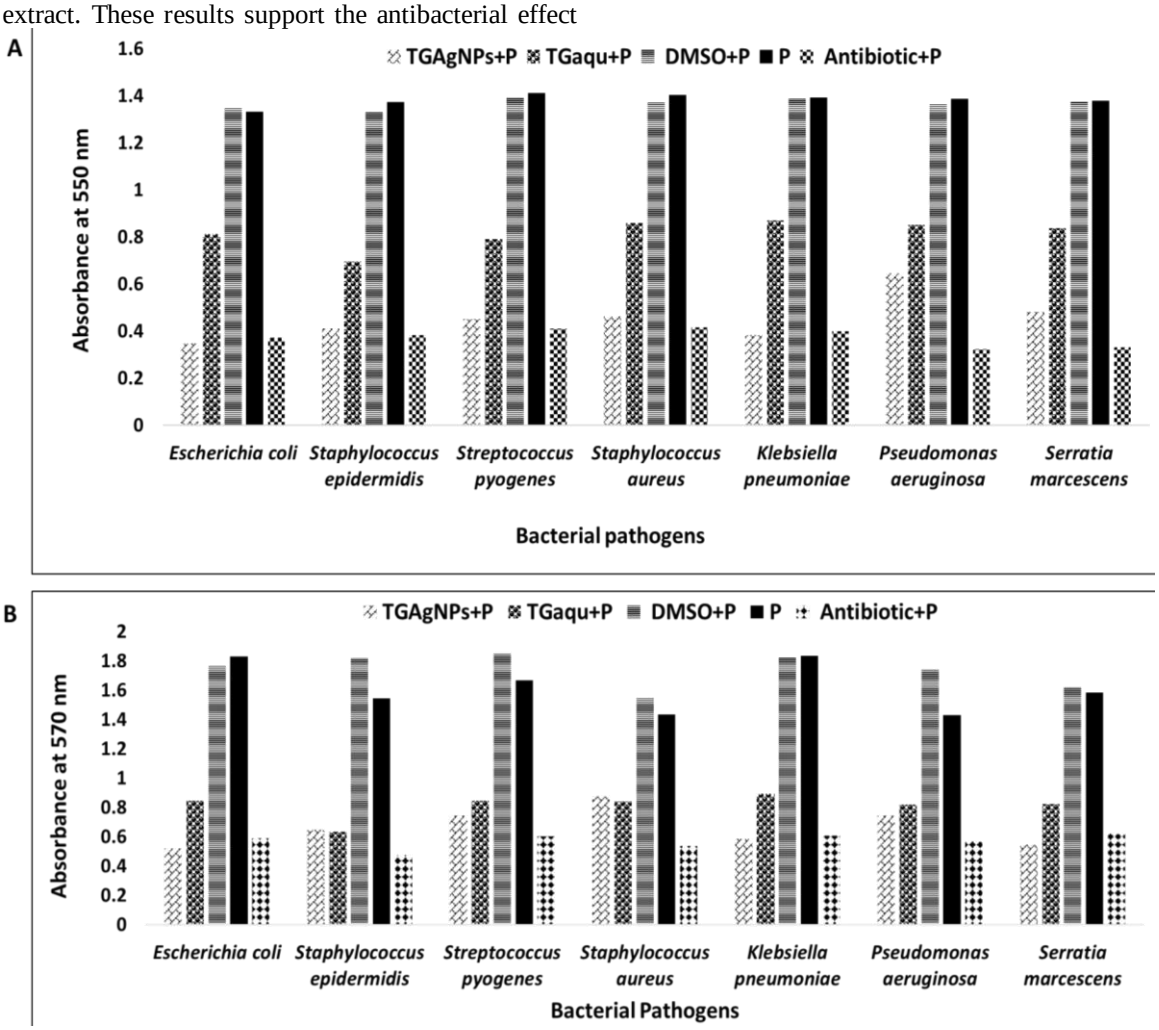

Fig. 3: Biological activities of green synthesized silver nanoparticles against clinical bacterial pathogens. (A) Cell viability assay; (B) Biofilm inhibition effect of TGAgNSs.

TLC-profiling and TLC-bioautography and spot Screening

TLC profiling of TGAgNSs and TGaqu gives remarkable finding that directing towards the presence of phytochemical constituents as mentioned above. Different absorbing bands/Spots of various colors (yellow, green, dark green, brown, and dark brown) were observed on Thin layer chromatographic plates under ultra-violet light at 254 nm and 336 nm, and indicates the presence of glycosides, phenols, flavonoids, and alkaloids. Various phytochemicals give different R_f values of TGaqu 0.9, 0.821, 0.914, 0.312 and 0.832 and TGAgNSs 0.85, 0.935, 0.854, 0.8907 and 0.909 in different solvent system. The presence of flavonoids, alkaloids, amino acids, and sugars was further confirmed by spraying the different chemicals on the TLC-developed plates. Direct bioautography against seven clinical pathogens expressed the antibacterial activity as zone of inhibition around the active chromatogram spot. TLC-plates showed the spots of the zone of inhibition clearly when treated with MTT. Solvent systems that had no inhibition effect in the direct bioautography. So they didn't influence the detection of biological activity. A characteristic zone of inhibition of ketones/ oligosaccharides, flavonoids, antioxidants, amino acids, terpenes, steroids and phenols diffused radially were observed around the TLC plates having separated components on it against tested bacterial pathogens. Spot screening indicated that phytochemicals exhibited the strong antibacterial activity. Results revealed that antioxidants and ketones had maximum antibacterial effect against *S. epidermidis*, *P. aeruginosa* and *E. coli* with 7.0±0.0 mm, 7.0±0.0 mm and 6.0±0.0 mm (In case of ketones) and 7.0±0.0 mm (in case of antioxidants) as shown in (Table-2). Amino acids, antioxidants, and flavonoids showed the moderate inhibition of *S. pyogenes*, *S. aureus*, and *K. pneumonia* while had low antibacterial effect against *S. marcescens*. On the other hand, flavonoids had no effect on the growth of *E. coli*. The biological activity against bacteria is due to presence of different phytochemicals separated on chromatogram. Our results are confirmed by [85] and [86] suggested that the growth of many bacteria was inhibited by tannins. Okwu and Josiah [87] reported that Terpinoids have anti-inflammatory properties, and antimicrobial properties [88]. Mann *et al*., [89] reported that steroids have been inhibited growth of many bacteria. The glycosides in various plant displayed the antimicrobial properties [90- 92].

Table-2: Spot screening of TLC-developed plates of TGAgNSs and TGaqu against bacterial pathogens via agar well diffusion method

Phytochemicals → Pathogens↓	Zones of Inhibition in (mm)			
	Ketones	Amino Acid	Antioxidant	Flavonoids
E. coli	**7.0±0.0**	**5.0±0.0**	**5.0±0.0**	**0.0±0.0**
S. epidermidis	**6.0±0.0**	**4.0±0.0**	**7.0±0.0**	**0.0±0.0**
S. pyogenes	**5.0±0.0**	**5.0±0.0**	**5.0±0.0**	**5.0±0.0**
S. aureus	**2.0±0.0**	**5.0±0.0**	**5.0±0.0**	**4.0±0.0**
K. pneumonia	**5.0±0.0**	**5.0±0.0**	**5.0±0.0**	**5.0±0.0**
P. aeruginosa	**7.0±0.0**	**5.0±0.0**	**5.0±0.0**	**2.0±0.0**
S. marcescens	**5.0±0.0**	**2.0±0.0**	**2.0±0.0**	**2.0±0.0**

Antioxidant activity

Reactive oxygen species have valuable effects on the cellular responses and immune function, but at high levels free radicals and oxidants can damage cell structures including lipids, proteins, and DNA by generating oxidative stress [50]. In current research, results proved that TGAgNSs and TGaqu possess hydrogen donating capabilities and act as an antioxidant. It was observed that TGAgNSs showed highest antioxidant capacity than TGaqu extract. The scavenging potential of TGAgNSs (0.12 mg/ml) was calculated as 36%, 49% and 74% at concentrations (0.5, 1 and 1.5 µl) compared to TGaqu (0.12 mg/ml) which was calculated as 29%, 32% and 69% (Fig. 6.A). Our results indicated that TGAgNSs have more antioxidant potential than TGaqu and the percentage increased with the concentration of TGAgNSs and TGaqu. Kharat and Mendhulkar [93] studied the antioxidant activity of green synthesized nanostructures using DPPH assay and suggested that photosynthesized NPs could be used as a potential free radical scavenger. The natural plants have antioxidant free radical scavengers which help in preventing pathologies like heart disease, cancer, arthritis, and liver disease [94, 95]. Phytochemicals present in our plant are responsible for antioxidant activity, this is confirmed by many researchers; Terpenoids, steroids, phenols, tannins have been found to possess antioxidant in various studies [96-104]. Glycosides present in plant extract also exhibit antioxidant properties [105-108]. The present investigation strongly recommends the usage of AgNSs as useful natural antioxidants for health preservation against progressive diseases.

Conclusion

Green technology was adopted to synthesize silver nanostructures using *T. govanianum* rhizomes extract. The synthesized silver nanostructures showed efficient antibacterial effect against all the tested bacterial pathogens. This eco-friendly method could be a cheap, inexpensive and easy approach that used

for silver nanoparticle synthesis and thus has a potential to be used in biomedical applications.

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