

Invitro efficacy of green synthesized silver nanoconjugates of *Valeriana wallichii* methanolic root extract against MDR strain of *Acinetobacter baumannii*

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Abstract

Background

Antimicrobial resistance is peaking up all over the world and requires immediate attention. In quest of finding a sustainable solution to the present threat of future epidemics which is going to be caused by *Acinetobacter baumannii* (*A. baumannii*) as per the WHO report (2020), this study has been used to pave a path for finding an herbal solution to it. The novelty of this study is that *Valeriana wallichii* (*V. wallichii*) has been analyzed for its methanolic extract/nano-conjugate against *A. baumannii*.

Results

HPLC of the extract has shown the presence of polyphenols- Quercetin, Rutin, and Tannic acid in high concentration. This fraction on nano-conjugation with silver has shown its equivalence to tetracycline antibiotics in restricting the growth of *A. baumannii*. Further, insilico studies taking crucial death receptors (Amp C, Fab1, RstA, LpxA, Aspartokinase, and shikimate dehydrogenase) of *A. baumannii* have shown binding energy ranging from - 11.6 to -13.3 kcal/mol for Tannic acid and close to this for Rutin and Linalool. It signifies that the fraction is potent enough to be a potential drug candidate for restricting this bacterium. The results were amazing when *V. wallichii* silver nanoconjugate (Vw-AgNCs), size range 1-200nm validated by DLS, AFM, and SEM-EDX on green synthesis has many times substantially enhanced restriction of *A. baumannii* as compared to only extract of *V. wallichii*. Keeping in view, its application to mankind, the cytotoxicity of the extract was evaluated on the human Fibroblast cell line (L929), showing an LC50 value of 368ug/ml. Many types of research have shown the time-dependent cytotoxicity of antibiotics as low as 5-50ug/ml providing us with a huge therapeutic window for the Vw-AgNCs.

Conclusion

Vw-AgNCs exhibited strong antimicrobial activity than the only extract and chemically synthesized nanoparticles so they can be a potential antimicrobial agent against *A. baumannii* in the future.

Background

Nearly 50,000 people die from infectious diseases each day, making them the major cause of death worldwide. The treatment of infectious diseases with antimicrobial agents shows a significant increase in bacterial resistance to several antibiotics (1). The development of Antimicrobial Resistance (AMR) in bacteria is neither an unexpected nor a new phenomenon and with the continuous use of antibiotics, resistant microorganisms have emerged as a great threat (2). *Acinetobacter baumannii* (*A. baumannii*) is one of the most important drug-resistant pathogens and in recent years, the World Health Organization (WHO)(3) has red Flagged *A. baumannii* and has put it on the priority list for new antibiotics search. The bacteria has presented several drug-resistant mechanisms including drug efflux pumps, drug-inactivating enzymes, and drug target mutations (4). *A. baumannii* is resistant to a number of available classes of antimicrobial drugs and easily picks up resistance to other classes as well. Additionally, it is exceedingly resistant to desiccation and can exist for months on inert surfaces. These traits make it particularly successful in the hospital environment and have contributed to the spread of multidrug-resistant (MDR) *A. baumannii* clones worldwide (5).

There have been few new antibiotics introduced by the pharmaceutical industry in the past ten years, and none of them have enhanced the efficacy against multidrug-resistant bacteria, despite the desire of many scientists to invent antibiotics working *via* novel modes of action (6). The search for new and potent antimicrobial agents derived from natural resources has become necessary due to the rising ineffectiveness of currently available antibiotics. Drugs have been primarily

derived from medicinal plants for many years. Ayurveda, Siddha, homeopathy, and other reformers all refer to the use of plant-based medications for both preventive and therapeutic purposes in ancient Indian medicine. The first protection for managing health and treating many ailments is plant-based therapeutics or medicines, which are used by around 88% of the world's population. (7) Plant-based drugs have increased in popularity.

since they have minimal adverse effects and the ability to be efficient in recent years. The secondary metabolites that plants utilize as weapons against infection by various microorganisms include tannins, terpenes, terpenoids, alkaloids, flavonoids, coumarins, polysaccharides, glycosides, gum, and phenols. (8), New chemical drugs are considered to be less tolerable to the human body than drugs obtained from plants. (9) The antibacterial, antifungal, and antioxidant properties of plant extracts are excellent. Synthesizing nanoparticles, which have proven antibacterial properties, can significantly increase these activities.(10)

In the current situation, nanotechnology presents an opportunity to re-examine the biological characteristics of antimicrobial compounds that are already well-known. So, the development of novel applications in this field makes them an attractive alternative to antibiotics. (10) Nanoparticles have unique characteristics such as a large surface-to-volume ratio and due to its extensive use in the field of physiochemical and biological processes, the emphasis on synthesizing nanomaterials steadily grew. Several metals like silver (11), gold (12), and copper(13) were used for the synthesis of nanoparticles. Silver has long been considered to have antibacterial activities, and silver nanoparticles (AgNPs) are considered to be non-toxic and eco-friendly antimicrobial substances with the potential for broad-spectrum efficacy and a much lesser propensity to develop bacterial resistance than antibiotics. As a result, there has been an increase in interest in the research and development of AgNPs in recent years.(14). The biocidal efficacies of silver-based compounds against several pathogenic bacteria (15) were studied. AgNPs are commercially used for wound dressing, drug delivery(16), biomolecular detection, and diagnostic studies (17). Chemical, physical, and photochemical methods can be used to synthesize nanoparticles, which pose numerous risks to both humans and the environment. (18),(19) Due to its ease, low cost, safety, environment-friendliness, and capability for deep penetration, the green synthesis of nanoparticles using various plant parts/components has recently drawn a lot of attention. AgNPs have been synthesized using various plant extracts, according to reports in the literature. (20) Plant extracts contain various phytoconstituents which possess various functional groups, (21) which act as reducing agents without the addition of synthetic chemicals to produce stable nanoparticles or nanoconjugates (22). The conjugation of plant polyphenols with metal nanoparticles exhibits the advantageous properties of both materials (23). Additionally to working as a reducing and capping agent during the formation of metallic nanoparticles, polyphenols also increase the biological activity of nanoparticles, whereas conjugated silver nanoparticles (AgNCs) increase polyphenol stability in extreme situations (such as heat, light, *etc.*)

In this study, AgNCs were synthesized using a methanolic extract of *Valeriana wallichii* (*V. wallichii*) root popularly known as Indian Valerian (or Tagara) and belongs to the family Valerianaceae. It can be found in India, Bhutan, Myanmar, Pakistan, and Afghanistan and is a native of the temperate Himalayas. The roots and rhizomes are the main plant components used for therapeutic purposes. It is used in anxiety, insomnia, epilepsy, failing reflexes, hysteria, neurosis, and sciatica (24). The plant's therapeutic properties are attributed to a class of compounds called valepotriates. The valepotriates are a group of monoterpenoids of iridoid type having an epoxy group and β -acetoxy isovaleric acids (25). Many aspects of the plant have been explored however, appropriate research on this plant's antibacterial properties, particularly with regard to the MDR strain, is lacking. Thus, For the first time, the current study aimed to evaluate the antibacterial potential of AgNCs synthesized using methanolic root extract of *V. wallichii* as a possible potent antibiotic drug against the MDR strain of *A. baumannii*.

Killing Mechanism of *A. baumannii*

A. baumannii uses three mechanisms by which it shows antimicrobial resistance. The first one is when efflux pumps are present or when the membrane's permeability is decreased, and it stops the antibiotic from reaching the target area where

it performs its activity. The second mechanism includes the inactivation of antimicrobial drugs, which might occur when they are hydrolyzed or altered in some way structurally. Many enzymes, mainly carbapenemases, are implicated in this mechanism. The third way it causes resistance is by altering the target site that the antibiotic is meant to target which can be done by post-transcriptional alterations to different proteins or by genetic mutations at particular locations. (26),(27). A wide range of antimicrobials, including beta-lactams, aminoglycosides, fluoroquinolones, macrolides, oxazolidinones, tetracyclines, rifamycin, and polymyxins are generating resistance as a result of this last mechanism.(28). To combat *A. baumannii-resistant* strains, it is important to understand this mechanism.

Shape, size, aggregation behavior, surface coating, surface/volume ratio, and colloidal stabilization are some of the morphological and physicochemical factors that contribute to the bactericidal activity of silver. When these factors are properly configured, they may help develop a broad-spectrum inhibitory response against a variety of infectious bacteria. (29), (30).

A broad surface area of interaction with the membrane and microbial cell wall is offered by AgNPs with a spherical shape, which functions very efficiently (31, 32). The bacterial cell wall, membranes, and nucleic acids undergo structural alterations and deformations as a result of the silver ions' binding to negatively charged proteins and nucleic acids. The AgNPs also damage membranes and induce the release of reactive oxygen species (ROS), forming free radicals with a powerful bactericidal action (33). Once the cell wall and cell membrane, the first two lines of defence for microorganisms, have been fully destroyed, silver ions or tiny AgNPs can easily enter the microbial body and harm the organism's inner components. As a consequence, ribosomes may be denatured with inhibition of protein synthesis, as well as translation and transcription can be blocked by binding with the genetic material of the bacterial cell(34, 35) AgNPs engage further with organelles like mitochondria, causing irreparable harm to the cellular machinery of microbes by interacting with macromolecules like proteins and DNA(36) (37, 38).

Results And Discussions

Multidrug-resistant bacterial infections pose a significant problem for both hospitals and clinics. According to a recent report published by the U.N. agency (WHO), carbapenem-resistant *A. baumannii* is considered to be the world's most dangerous superbugs, and the bacteria is listed as the priority-1 (critical) pathogens (Scientific American, 2017). High doses of antibiotics are frequently used to treat resistant microorganisms, although this causes significant side effects. The existence of different secondary metabolites determines the efficacy of therapeutic plants in preventing microbial development. Nano-size silver materials represent a novel antimicrobial approach. the plant metabolites such as polyphenols, terpenoids, alkaloids, etc mediate the bioreduction of silver ions to nanoconjugates.

For the first time, this study is aimed at evaluating the methanolic extract of the root of *V. wallichii* as a reducing agent for synthesizing Nanoconjugates. This extract could function as a capping and stabilizing agent because it contains significant secondary metabolites and biological constituents including proteins and ethylene groups. Their various activities were investigated using qualitative and quantitative methods including HPLC. Further, nanoconjugates were characterized using DLS, AFM, and SEM–EDX. Its antimicrobial activity was studied.

Phytochemical Screening

The methanolic root extract of *V. wallichii* has shown the presence of saponins, tannins, flavonoids, alkaloids, phenols, terpenoids, inulin, glycosides, carbohydrates, and reducing sugars as mentioned in Table 1.

As per another study done by Babu *et al.*, (39), *Valeriana Jatamansi* (*V. jatamansi*) showed the presence of alkaloids and reducing sugars in the hydro-alcoholic extract of the plant while steroids and saponin were found in the hexane extract of the plant.

Table 1
Phytochemical screening of methanolic *V. wallichii* root extract

Extract Name	Methanolic Valeriana wallichii extract
Test Name	
Saponins	++
Tannins	+
Steroids	-
Terpenoids	+
Flavonoids	++
Napthoquinone	-
Inulin	+
Carbohydrates	+
Alkaloids	+
Phenol	+
Starch	+
Amino acid	-
Glycosides	+
Reducing Sugar	++
Phlobtannins	+
*Note: “++”: present in higher amount; “+”: present; “-”: absent	

Quantification Of Phytochemical Constituents

None of the scientific papers has reported the presence of antioxidant and polyphenolic content in the methanolic root extract of *V. wallichii* though some researchers have studied some of these activities for *V. Jatamansi* which is a completely different species to be considered.

So, for the first time, Total Phenolic, Flavonoid, Terpenoid & Tannin Contents, FRAP, Reducing Power Assay & DPPH antioxidant activity is being reported for this extract.

Total Phenolic Content (Tpc)

TPC in the extract was measured using the Folin-Ciocalteu (F-C) technique with gallic acid used as the standard. TPC was estimated for the extract and represented as mg of gallic acid equivalents (GAE) per g of sample in dry weight (mg/g) using the regression equation of the calibration curve ($Y = 0.0087x$; $R^2 = 0.9917$) (Graph 1, Supplementary). The mean TPC value observed in the extract was 28.536 ± 0.253 mg GAE/g (Table 2). The result was found to be significant at $p < .05$ i.e., $p = 0.01$.

As per the study done by Rawat *et al.*, (40), the TPC of *V. jatamansi* was maximum in methanolic extract (14.37 mg GAE/g dw). Also, According to Thusoo *et al.*, (41), the highest amount of phenolic compound was observed in the methanolic extract of *V. jatamansi* root (187.13 ± 6.8 mg GAEs/g) followed by aqueous extract (77.66 ± 2.1 mg GAE/g).

Numerous studies have shown that the presence of phenolic compounds in plants is related to their antioxidant activities. This association is most likely caused by the plants' redox characteristics, which enable them to function as reducing agents, hydrogen donors, and singlet oxygen quenchers.

Table 2
Total phenolic content (TPC) in the methanolic root extract of *V. wallichii*

S. No	Samples	100 (µg/ml)	200(µg/ml)	300 (µg/ml)	400 (µg/ml)	500 (µg/mL)	Mean TPC value (GAE/g)
1.	Root	16.582 ± 0.236	28.153 ± 0.236	37.846 ± 0.329	27.233 ± 0.236	32.856 ± 0.236	28.536 ± 0.253

Total Flavonoid Content (Tfc)

The TFC of the extract was estimated using the regression equation of the calibration curve ($Y = 0.0047x$; $R^2 = 0.9955$) (Graph 2, supplemental) and represented as mg quercetin equivalents (QE) per g of sample in dry weight (mg/g). The mean TFC value obtained for the extract was 21.568 ± 0.4199 mg QE/g (Table 3). The result was found to be significant at $p < 0.0001$.

In a finding by Thusoo *et al.*, (41), the Aqueous extract of *V. jatamansi* had a relatively high amount of flavonoids (452.30 ± 12.4 mg QE/g) followed by methanolic extract (257.69 ± 9.8 mg QEs/g). In another study maximum flavonoid content was found in the Acetone extract of *V. jatamansi* (6.24 ± 0.01) followed by ethanol (6.15 ± 0.01) and methanol (5.58 ± 0.02) (40).

Table 3
Total flavonoid contents (TFC) in the methanolic root extract of *V. wallichii*.

S. No	Samples	500 (µg/ml)	600 (µg/ml)	700 (µg/ml)	800 (µg/ml)	900 (µg/ml)	1000 (µg/ml)	Mean TFC value (QE/g)
1.	Root	18.063 ± 0.347	19.411 ± 0.437	20.546 ± 0.437	22.815 ± 0.437	23.666 ± 0.437	25.015 ± 0.437	21.586 ± 0.4199

Determination Of Total Terpenoid Content (Ttc)

The total terpenoid content of the extract was estimated using a standard calibration curve ($y = 0.0038x$, $r^2 = 0.9913$) (Graph 3, supplementary) of Linalool and expressed in mg linalool equivalents/g of extract. The mean TTC value (LE/g) was found to be 26.315 ± 0.324 (LE/g) as presented in Table 4. The result was found to be significant at $p < 0.0001$. It is being reported for the first time in this extract.

Table 4
Determination of total terpenoid content in the methanolic root extract of *V. wallichii*.

S. No	Samples	0.5 (mg/ml)	1 (mg/ml)	2 (mg/ml)	3 (mg/ml)	4 (mg/ml)	Mean TTC value (LE/g)
1.	Root	11.070 ± 1.749	17.736 ± 3.727	26.684 ± 2.263	37.912 ± 1.102	38.175 ± 0.754	26.315 ± 0.324

Estimation Of Total Tannin Content (Ttc)

The total tannin content of the extract was estimated using a standard calibration curve ($y = 0.0218x$, $r^2 = 0.992$) (Graph 4, supplementary) of gallic acid and expressed in mg gallic acid equivalents/g of extract. The results show that the extract had a mean TTC of 4.690 ± 0.032 mg gallic acid equivalents/g (GAE/g) of extract as depicted in Table 5. The result was found to be significant at $p < 0.0001$. We have evaluated this activity for the first time in our extract.

Table 5
Total Tannin Content in the methanolic root extract of *V. wallichii*.

S. No	Samples	100 (µg/ml)	200 (µg/ml)	300 (µg/ml)	400 (µg/ml)	500 (µg/ml)	Mean Reducing Power value (GAE/g)
1.	Root	1.472 ± 0.044	3.091 ± 0.067	4.673 ± 0.061	6.124 ± 0.033	8.091 ± 0.016	4.690 ± 0.032

Ferric Reducing Antioxidant Power (Frap)

The ability of the extract to convert ferric (III) iron to ferrous (II) iron was used to estimate the antioxidant activity as determined by the FRAP technique. It was seen in the extract using a standard calibration curve ($y = 0.0023x$, $r^2 = 0.998$) (Graph 5, supplementary) of FeSO₄ (100–1000 µg). The results were presented as mg Fe (II) equivalents per g of the extract's dry weight. The mean antioxidant activity observed with this method was 70.343 ± 0.117 mg FeSO₄/g. (Table 6). The result was found to be significant at $p < 0.0001$.

A study reported maximum antioxidant activity by FRAP assay in acetone extract of *V. jatamansi* (6.70 ± 0.20), followed by Methanol (6.47 ± 0.29) (40).

Table 6
Ferric Reducing Antioxidant Power (FRAP) in the methanolic root extract of *V. wallichii*.

S. No	Samples	50 (µg/ml)	100 (µg/ml)	200 (µg/ml)	300 (µg/ml)	400 (µg/ml)	Mean FRAP value (FeSO ₄ /g)
1.	Root	15.989 ± 0.128	37.927 ± 0.128	68.552 ± 0.128	102.072 ± 0.128	127.177 ± 0.077	70.343 ± 0.117

Reducing Power Assay

The Reducing Power Assay of the extract was estimated using a standard calibration curve ($y = 0.0145x$, $r^2 = 0.989$) (Graph 6, supplementary) of Ascorbic acid and expressed in mg ascorbic acid equivalents/g of extract. The reducing power of the extract has shown the mean reducing power, 32.117 ± 0.141 AAE/g (Table 7). The properties of the reducing power can be an indicator of the antioxidant potential of the compound evaluated. The result was found to be significant at $p < 0.0001$. This is being estimated by us for the first time in this extract.

Table 7
Reducing Power Assay in the methanolic root extract of *V. wallichii*.

S. No	Samples	100 (µg/ml)	200 (µg/ml)	300 (µg/ml)	400 (µg/ml)	500 (µg/ml)	Mean Reducing Power value (AAE/g)
1.	Root	23.354 ± 0.141	30.043 ± 0.141	30.273 ± 0.141	34.871 ± 0.141	42.043 ± 0.141	32.117 ± 0.141

Dpph Free Radical Scavenging Assay

The antioxidant potential of the extract was analyzed by DPPH free radical scavenging assay. The standard was ascorbic acid and its IC₅₀ value was found as 0.817 µg/ml (Graph 7, supplementary). The IC₅₀ value of the extract evaluated in this study was found to be 32.253 ± 0.319 µg/mL as shown in Table 8. (Graph 8, supplementary) The result was found to be significant at $p < 0.0001$.

Thusoo *et al.*, (41) reported the methanolic extract of *V. jatamansi* as the most potent antioxidant (IC₅₀ 78 ± 2.9 µg/mL). Rawat S. *et al.*, (2016) revealed almost similar antioxidant capacity in different solvent extracts of *V. jatamansi* studied by him in the range of 9.82 ± 0.08 to 10.88 ± 0.07 mM AAE/100 g dw.

Table 8
DPPH radical scavenging activity of methanolic root extract of *V. wallichii*.

Concentration (µg/ml)	% Inhibition	IC ₅₀
10	17.032 ± 0.395	32.253 ± 0.319
20	34.785 ± 0.308	
30	49.096 ± 0.250	
40	61.961 ± 0.549	
50	71.744 ± 0.636	

High-pressure Liquid Chromatography (Hplc)

Polyphenolic flavonoids like quercetin and rutin and Tannins like tannic acid were determined in the methanolic root extract of *V. wallichii* root by the HPLC method. The retention time of the standard quercetin, rutin, and tannic acid was found to be 3.147 min, 3.817 min, and 3.093 min respectively as shown in Fig. 2 and the retention time of the extract was also found similar to the standards *i.e.*, 3.015 min, 3.110 min., and 3.085 min respectively as compared in Fig. 2 which indicates the presence of both polyphenolic flavonoids and tannins in the extracts. The identified compounds and their corresponding concentrations are listed in Table 9.

In a previous study, Rawat *et al.*, (40) reported Six phenolic compounds: gallic acid, catechin, hydroxyl benzoic acid, caffeic acid, chlorogenic acid, and p-coumaric through HPLC in *V. jatamansi* but in our study, we have focused on the concentration of quercetin, rutin, and tannic acid.

Table 9
Concentrations of Polyphenolic flavonoids & Tannins against their respective standards in the methanolic root extract of *V. wallichii*

Standards				
Peak ID	Ret Time	Peak Height	Peak Area	Concentration
Quercetin	3.147	329519.219	3898018	100
Rutin	3.817	176880.094	6469150	100
Tannic Acid	3.093	566871.625	3475821	100
Valeriana wallichii methanolic root extract				
Peak ID	Ret Time	Peak Height	Peak Area	Concentration
Quercetin	3.015	39301.43	2091349.375	99.1981
Rutin	3.11	15727.441	585420.625	98.9848
Tannic Acid	3.085	4846.437	27028.447	100

Molecular Docking Studies

One of the current and efficient ways of assessing therapeutic compounds for potential targets of *A. baumannii*'s drug resistance is computer-aided screening. The KEGG database searches indicated a few potential drug targets that could be putative therapeutic targets for the development of antibiotics based on the functional involvement of *A. baumannii* in the amino acid biosynthesis pathway.

In the docking analysis, the binding affinities of the identified compounds (Fig. 3) were studied against the *A. baumannii* receptors as mentioned in Table 10. The docking analysis revealed that tannic acid depicted the maximum binding affinity in the range of – 11.6 kcal/mol to – 13.3 kcal/mol with all the receptors studied. After Tannic acid, rutin and linalool also have shown comparatively higher affinities with the receptors. Details of the binding energy and the active amino acid residues of all the ligands which are interacting against the crucial receptors are mentioned in Table 10. The docked complexes were further visualized for their molecular interactions using the Discovery Studio 2021 client. 2D and 3D structures of the best-docked molecules against all the receptors are shown in Fig. 3.

Table 10

The binding potential (ΔG (Kcal/mol)) of secondary metabolites present in the methanolic root extract of *V. wallichii* against 4 receptors retrieved from PDB and 2 modeled receptors of *A. baumannii* using AutoDock Vina

Receptor	PDB ID	Ligand	Docking Score (kcal/mol)	Amino acid residue
Hydrolysing Class C Extended- Spectrum –Lactamase (AmpC): The major gene code for β-lactamase known as amp C from <i>A. baumannii</i> was a prioritized drug target by database search.	4QD4	Gallic Acid	-5.3	Met295, Val294, Glu291, Ser290, Asn289, Asn345, Arg342, Cit402
		Quercetin	-6.1	Cit402
		Ascorbic Acid	-4.9	Tyr224
		Linalool	-7.9	Phe123, Pro124, Pro218, Gln122, Tyr224, Cit402, Ser317
		Rutin	-8.3	Ser317, Arg342, Asp125, Pro218, Val127, Tyr224, Gln122
		Tannic Acid	-11.6	Glu291, Val294, Ser290, Asn289, Asn345, Arg342, Met295, Cit402
NADH-dependent enoyl-ACP reductase (FabI): FabI has been reported as an important drug target in both methicillin-resistant <i>Staphylococcus aureus</i> and MDR <i>Mycobacterium tuberculosis</i> . Enoyl-[acyl-carrerir protein] reductase I (FabI), which is involved in the metabolism of biotin and fatty acids, was identified as a possible therapeutic target using the KEGG pathway.	4ZJU	Gallic Acid	-5.9	Lys9, Ser237, Gln141, Cys143, Arg140, Asp88
		Quercetin	-6.0	Lys9, Cys236, Arg186, Pro238, Asp88, Arg140
		Ascorbic Acid	-4.5	Pro238, Cys143, Arg186
*Note- values with bold & underlined are depicting the highest binding scores of the ligands on the receptors.				

Receptor	PDB ID	Ligand	Docking Score (kcal/mol)	Amino acid residue
		Linalool	-7.5	Asp88, Cys236, Arg186
		Rutin	-7.9	Gly142, Gln141, Asp88, Arg186, Pro238, Arg140, Lys9
		Tannic Acid	-13.3	Ala192, Tyr149, Ile195, Ser93, Ser21, Ala68, Asp66, Val67, Cys65, Gly95, Ile94, Ile22, Lys166
Response regulator BfmR (RstA): Recent studies have revealed that RstA in <i>A. baumannii</i> has been validated as a potential antimicrobial target. The transcriptional regulatory protein-RstA, one of the main outer membrane proteins involved in this bacteria's two-component system, was thought to be the therapeutic target.	5E3J	Gallic Acid	-4.5	Glu33, Ile10, Glu50
		Quercetin	-5.4	Ile10, Lys9, Glu50
		Ascorbic Acid	-4.7	Lys107, Leu19, Pro108, Val109, Pro111
		Linalool	-6.3	Arg123, Asp53, Leu122, Pro8, Arg119
		Rutin	-7.2	Ala10, Leu97, Glu98, Arg74, Arg71, His78, Asp102
*Note- values with bold & underlined are depicting the highest binding scores of the ligands on the receptors.				

Receptor	PDB ID	Ligand	Docking Score (kcal/mol)	Amino acid residue
		Tannic Acid	-12.3	Glu50, Arg45, Arg46, Arg42, Lys9, Ile10, Val36, Gln24
UDP-N-acetylglucosamine acetyltransferase (LpxA): LpxA is conserved in almost all prokaryotes and a crucial enzyme in the biosynthetic pathway of the lipopolysaccharides and reported as a potential therapeutic drug target for many pathogenic Gram-negative bacteria. LpxA (one of the major proteins involved in the amino acid sugar and nucleotide metabolism) was prioritised as probable drug target.	4E6U	Gallic Acid	-4.8	Glu84, Ile85, Ile55, Thr36
		Quercetin	-5.0	Ile35, Gln57, Asn87, Gly86, Glu84
		Ascorbic Acid	-4.2	Thr36, Arg54
		Linalool	-6.8	Glu84, Lys109, Asp130, Asn87, Ser112
		Rutin	-7.8	Thr36, Ile55, Gly38, Glu84, Lys109, Asn87, Ser112
		Tannic Acid	-13.3	Lys178, Gly259, Arg262, Ilea260, Ser228
Aspartokinase: Lysine, methionine, and threonine are three essential amino acids, and aspartokinase (EC 2.7.2.4), an enzyme, catalyzes the first stage of their production. The absence of this gene in animals makes this enzyme a key target for antimicrobials	Homology Modeling	Gallic Acid	-5.6	Lys7, Val210, His212, Gly209, Gly10, Ser41
		Quercetin	-5.9	Ser41, Gly151, Ser153, Asp154
		Ascorbic Acid	-4.5	Gly151, Gly152, Ser153
*Note- values with bold & underlined are depicting the highest binding scores of the ligands on the receptors.				

Receptor	PDB ID	Ligand	Docking Score (kcal/mol)	Amino acid residue
		Linalool	-8.1	Arg150, His212, Gly151, Gly209, Thr173, Tyr172, Asp174, Gly10, Lys7, Asp154, Thr47
		Rutin	-8.8	Gly10, Ser41, Asp154, Asp174, Lys7, Lys45
		Tannic Acid	-12.2	Tyr222, Asp115, Ile116, Lys165, Ala162
Shikimate dehydrogenase: In <i>Pseudomonas aeruginosa</i> , <i>Pseudomonas putida</i> and <i>Helicobacter pylori</i> , shikimate dehydrogenase (EC 1.1.1.25) have been identified as an interesting target for antimicrobial drugs.	Homology Modeling	Gallic Acid	-6.1	Ser17, Asp102, Gln243
		Quercetin	-6.4	Gln243
		Ascorbic Acid	-4.6	Met214, Tyr216, Leu240, Thr62, Val7
		Linalool	-8.1	Ser192, Val63, Ala130, Lys66
		Rutin	-8.5	Ser15, Tyr216, Ser194, Thr62, Asp102, Met239, Lys66, Met214, Val63, Ala130, Pro11

***Note** values with bold & underlined are depicting the highest binding scores of the ligands on the receptors.

Receptor	PDB ID	Ligand	Docking Score (kcal/mol)	Amino acid residue
		Tannic Acid	-12.7	Arg155, Gly129, Asn150, Arg151, Ala127, Lev200, Ser196
*Note- values with bold & underlined are depicting the highest binding scores of the ligands on the receptors.				

According to the study reported by S. Skariyachan *et al.*, (42) the binding potential of Limonin, Ajmalicine & Strictamin was studied against the same receptors as evaluated by us in the current study as shown in Table 10.

They showed that Limonin (7,16-Dioxo-7,16- dideoxylimondiol), a natural limonoid commonly present in Citrus sps. demonstrated promising interaction towards modeled aspartokinase with a binding energy of – 8.7 kcal/mol. Likewise, it illustrated a significant binding towards the probable targets such as AmpC and FabI, with binding energies of – 8.4 and – 8.1 kcal/mol respectively. Ajmalicine ((19 α)-16, 17- didehydro-19-methyloxayohimban-16-carboxylic acid methyl ester) exhibited promising interaction with Fab1 and aspartokinase with a binding energy of -7.5 and – 7.1 kcal/mol respectively. Strictamin (Akuammilan-17-oic acid methyl ester) displayed a good binding with modeled shikimate dehydrogenase and AmpC with binding energies of – 7.8 and – 7.4 kcal/mol respectively.

However, in our study, the binding energy is quite enhanced compared to the previous study reported, which qualifies natural compounds close to these found in our extract by HPLC or quantitative phytoconstituent methods as potent antimicrobial agents.

Table 10- The binding potential (ΔG (Kcal/mol)) of secondary metabolites present in the methanolic root extract of *V. wallichii* against 4 receptors retrieved from PDB and 2 modeled receptors of *A. baumannii* using AutoDock Vina

Cytotoxicity and anti-cancerous activity of methanolic root extract of *V. wallichii*

The extract was evaluated for its toxicity and anticancerous property using an MTT assay. The extract has shown anticancerous efficacy against Cervical cancer cells (Hep 2C) and has not shown any toxicity on Human Fibroblast cells (L929). In Hep2C cells, the IC₅₀ value was found to be 298.70 $\mu\text{g/ml}$, however, in L929 cells, the IC₅₀ value was found to be 368.53 $\mu\text{g/ml}$, as shown in Fig. 4 The extract has shown a selectivity Index of 1.234, which can be effectively used for antiproliferative activity against tumor cells.

None of the researchers have reported the activity of *V. wallichii* methanolic root extract on the cancerous cell line, however many studies have shown the anticancerous activity of the pure compound isolated from *V. wallichii* and *V. jatamansi*. So, this is being reported for the first time in our study as per our knowledge.

Previous studies have reported that valepotriates isolated from *V. wallichii* D. C. and *V. jatamansi* exhibited potent cytotoxic and antitumor activities (43, 44). According To Lin *et al* (2014),(45) 4 valepotriates isolated from *V. jatamansi* showed strong activity against metastatic prostate cancer (PC-3M) cells with IC₅₀ values of 1.66, 6.82, 5.75, and 6.22 μM . Wang *et al.* in 2017(44) evaluated the cytotoxicity of iridoids of *V. jatamansi* against two human cancer cell lines- lung adenocarcinoma (A 549) and gastric carcinoma cells (SGC 7901) and reported no cytotoxicity effect or very weak cytotoxicity with only two of the compounds. Zhu *et al* (2019)(46) showed that F3 (a novel fraction isolated from *V. jatamansi* Jones) exhibited significant growth inhibitory activities on human breast cancer cell lines, MDA-MB-231 cells (IC₅₀ 2.91 $\mu\text{g/ml}$) and MCF-7 cells (IC₅₀ 4.23 $\mu\text{g/ml}$). Han *et al.*,(47) demonstrated for the first time that valeric acid suppresses liver cancer development. Three liver cancer cell lines with the lowest IC₅₀ were reported (HepG2: 0.948 mM;

Hep3B: 1.439 mM: and SNU-449: 1.612 mM). However, IC₅₀ values for liver cancer cell lines SNU-449, Hep3B, and HepG2 were 1.92, 2.15, and 3.27 times higher, respectively, than values for the liver normal cell line THLE-3 (3.097).

The cells were evaluated under an Olympus inverted microscope using a 10X objective lens (magnification 100X). Scale bar: 100 µm.

Characterization of Green Synthesis of Silver Nanoconjugates of *V. wallichii* methanolic root extract (Vw-AgNCs)

Uv-visible Spectrophotometry

Using a UV-Vis spectrophotometer, the reduction of silver ions into nanoconjugate by *V. wallichii* methanolic root extract was detected by recording the absorption spectra between the wavelengths of 200–800 nm. AgNC development in the solution was verified by UV-Vis spectral analysis, which revealed that the highest absorption was at 430 nm. (Fig. 5).

Foujdar *et al.*, (23) showed in the spectral analysis that Punica granatum Peel's PolyphenolsBased Silver nanoconjugates have maximum absorbance near about 420 nm. Another study also reported similar results that the UV-Vis absorption spectrum of five aqueous leaf extract-mediated silver nano-conjugates has shown a single peak at a wavelength ranging between 402.0 nm to 429.0 nm (48).

Dynamic Light Scattering (DLS)

The DLS technique was used to determine the particle size of the synthesized nanoconjugates. According to the DLS graph of Vw-AgNCs (Fig. 6), the particle size range was between 100 to 120 nm.

The size of nanoparticles obtained in this study is supported by the results of Ghosh *et al.*, (48), who indicated the size of five leaves of aqueous extract-mediated silver nano-conjugates varies from 18.17 nm to 105.7 nm.

Atomic Force Microscopy (AFM) Pattern

AFM was used to determine the parameters of the surface morphology of nanoconjugates. The well-dispersed, nearly spherical shape of the nanoconjugates was confirmed by AFM scans. Figures 7A and 7B show the 2D and 3D pictures of synthesised Vw-AgNCs. The size obtained from Vw-AgNCs was around 12nm (Fig. 7C). The maximum profile of peak height was 69.6573 nm and the average surface roughness was 2.0033 nm. The observed difference in AFM and DLS may be due to the clustering of nanoconjugates in solution form.

A study done by Mishra *et al.*, (49) reported the maximum profile of peak height at 73.43 nm and the average surface roughness at 55.10 nm in green synthesized silver nanoparticles using ethanol leaf extract of *Nyctanthes arbor-tristis*.

Scanning electron microscopy (SEM)

The results of the SEM investigation are shown in Fig. 8 below at magnifications of 30000X and 50000X. The size of the Vw-AgNCs ranged between 70 and 700 nm (Fig. 8). The data obtained demonstrate that the bioactive constituents from the *V. wallichii* methanolic root extract on the AgNCs, which is indicated by the formation of clusters at several points. The samples used to examine the morphology of Vw-AgNCs may have been agglomerated, therefore showing larger particle sizes than that obtained from DLS & AFM.

Proposed antibacterial mechanism of Vw-AgNCs against *A. baumannii*

The SEM micrographs indicate deformity in *A. baumannii* cells treated with Vw-AgNCs resulting in ruptured cells, where in some cases, the outer cell membrane was intact but in others, membrane distortion was observed and the cells have lost

their shape and structural integrity. Additionally, interactions with the exposed peptidoglycan caused by these spherical Vw-AgNCs causes the N-acetyl glucosamine (NAG) and N-acetyl muramine (NAM) to lose their integrity (NAG). This insertion and penetration of synthesized Vw-AgNCs lead to oozing out of the cytoplasmic constituents, resulting in cell death and complete killing (Fig. 8). The grey dots in the background display green synthesized AgNPs.

Several studies have reported that most biogenic AgNPs have a spherical geometry, with a size range of 5 to 500 nm with antimicrobial activities reported against them (50, 51).

Energy Dispersive X-ray Spectrum (Edx)

Silver elements were detected in the sample, according to the EDX analysis of the Vw-AgNCs. The binding energies of silver, whose weight and atomic percentage are 7.62 and 1.31%, respectively, are represented by the peaks about 3 keV. The result shows the peaks as depicted in Fig. 9 for the presence of the silver element. The high atomic and weight percentages of organic elements, such as C and O, revealed the possible presence of organic compounds, such as polysaccharides, phenols, and proteins; The occurrence of other peaks (Ca, Si, Mg, Na, Al, Cl, etc) was presumed to relate with the glass underneath, which held the sample and Au was because of Gold Sputtering. Also, different elements detected by EDX analysis, such as Si, Al, and Cl, were reported to act as capping agents of the biogenic AgNPs according to a previous study (52, 53).

Another study also reported a similar result after the synthesis of biogenic AgNPs using *Ferula asafoetida* that demonstrated the presence of Ag peaks at 3 keV with weight and atomic percentages of 11.6% and 7.7%, respectively. Also, high atomic and weight percentages of organic elements, C, N, and O, were present in their study as well (50). A.K. Mishra *et al*, (49) reported similar results in the study done by them and revealed the existence of metallic silver in biosynthesized nanoparticles with a sharp peak at 3 keV. They also showed the occurrence of other peaks (Ca, Si, Mg, and Na) as were in our study. Also, He *et al*, (54) revealed the presence of silver element in synthesized Ag NPs using *A. katsumadai* seed aqueous extract (AKSE) with a peak at 3 keV.

Antimicrobial Activity

Using the well diffusion technique, the potential antibacterial efficacy of Vw-AgNCs against MDR- *A. baumannii* was investigated. The findings were compared to those obtained for AgNPs synthesised using the chemical method. The Vw-AgNCs were showing higher zones of inhibition than chemical AgNPs which proved it to exhibit good antibacterial activity against it. The highest zone of inhibition was observed to be 21 mm $\pm$ 0.450 at 20 μ l of Vw-AgNCs. Among standard antibiotics tested, Ampicillin and Vancomycin were found to be resistant and the zone of inhibition of Tetracyclin and Gentamicin was 22mm & 14mm respectively, which was similar to the green synthesized AgNPs. Vw-AgNCs have proved to be the potential drug candidate against the clinical strain of MDR- *A. baumannii* as they have shown remarkably promising results as compared to standard antibiotics as using Vw-AgNCs may be one way for combating microbial resistance. Unlike standard antimicrobial agents, low doses of silver nanoconjugates can act as more potent dosages in the treatment of diseases as shown in our results too (Table 11, Fig. 10) (55). Plant extract by itself, on the other hand, lacked any antibacterial effect.

However, it is to be assumed that the plant's root extract utilized has antibacterial properties as the capped methanolic root extract of *V. wallichii* is getting enhanced by silver as shown in the results.

Our study was in accordance with the findings of Abootalebi *et al*, (50) which revealed that the antimicrobial potential of biogenic NPs (Fer@AgNPs) was significantly higher than chemical AgNPs. Peiris *et al*, (56) reported green synthesized silver nanomaterials exhibited antibacterial efficiency & an inhibition zone diameter of 12 mm against *A. baumannii*. The diameter of the zone of inhibition of biosynthesized nanoparticles using ethanol leaf extract of *Nyctanthes arbor-tristis*

against *A. baumannii* was 13-15mm, which increased with the increase in the concentration of nanoparticles as per the study done by Mishra *et al.* (49).

The conjugation of NPs with the antibacterial components of the extract may be the cause of the improved antimicrobial impact of the green synthesized AgNCs compared to chemical AgNP. The antimicrobial mechanism of green synthesized AgNCs is still unknown but several studies propose that the interaction of silver nanoconjugates with microorganisms gives rise to the release of silver ions which are potential destroyers of microbial cells due to their ability to deactivate enzymes in microbial cells and membrane permeability disruption leading to lysis and apoptosis (57). Furthermore, Sulfur and phosphorus, two essential elements of DNA, interact with silver ions in ways that affect DNA replication, cell growth, and even microbial cell death. They can prevent protein production by denaturing ribosomes in the cytoplasm (58). Moreover, it has the potential to harm human cells and has significant side effects. As a result, in the current investigation, we additionally assessed the extract's impact on a normal human cell line as well as a malignant human cell line as a measure of its cytotoxic effect and potential as an anticancer drug. Several studies have proven that phytoconstituents like polyphenolic compounds are safe for healthy cells while showing cytotoxicity against cancerous cells.

Table 11
Zone of inhibition (mm) as measured by the Agar Well-Diffusion method

S.No.	Components	Zone of inhibition (mm)
		MDR- <i>A. baumannii</i>
1	TGMC-50 (100 µl)	-
2	AgNO ₃ -5mM (100 µl)	-
3	D.W. (100 µl)	-
4	Blank-media (100 µl)	-
5	Chem AgNP (50 µl)	-
6	S5 (100 µl)	-
7	5A TG SNP- 100 µl	17.4 mm ± 0.600
8	5A TG SNP- 75 µl	17.5mm ± 0.200
9	5A TG SNP- 50 µl	16.5mm ± 0.300
10	5A TG SNP- 25 µl	17mm ± 0.400
11	5A TG SNP- 20 µl	21 mm ± 0.450
12	5A TG SNP- 15 µl	20 mm ± 0.450
13	5A TG SNP- 10 µl	19.5 mm ± 0.100
14	5A TG SNP- 5 µl	18 mm ± 0.500
15	5A TG SNP- 4 µl	14mm ± 0.350
16	5A TG SNP- 3 µl	16mm ± 0.250
17	5A TG SNP- 2 µl	15.5mm ± 0.150
18	5A TG SNP- 1 µl	11.5mm ± 0.200
19	Ampicillin	-
20	Vancomycin	9mm ± 0.30
21	Gentamycin	14mm ± 0.25
22	Tetracycline	22mm ± 0.15

Note: TGMC-50: *V. wallichii* methanolic root extract 50mg/ml; 5A TG SNP: 5mM synthesized silver nanoconjugate of *V. wallichii* methanolic root extract; D.W.: Distilled water; S5: Chem AgNP 5mM.

Figure 10a) Negative Control- Mac Conkey Control- Mac Conkey b) Bacterial Control- MDR- *A. baumannii*. c) Positive Control: Plate having wells with I) TGMC-50: 50 mg/ml *V. wallichii* methanolic root extract.; II) AgNO₃-5: 5mM AgNO₃.; III) D.W.: Distilled Water.; IV) Blank: Empty well, showing no Zone of Inhibition. d) Positive Control: Plate having wells with I) Chem AgNPs: Chemically synthesized AgNPs.; II) S5- 100% Methanol; e) Vw-AgNCs: Volume- 25, 50, 75 & 100 µl; **f)** Vw-AgNCs: Volume- 5, 10, 15 & 20 µl; **g)** Vw-AgNCs: Volume- 1, 2, 3 & 4 µl; h) Standard Antibiotics: Ampicillin, Vancomycin, Tetracycline, and Gentamicin.

Conclusion

The present study focuses on an eco-friendly, rapid, and cost-effective approach to synthesizing silver nanoconjugate by utilizing *V. wallichii* extract (Vw-AgNCs) which has exhibited excellent and effective antimicrobial property better than the crude extract against the MDR strain of *A. baumannii*. The results have been validated with the help of nanoconjugate characterization and further comparative analysis along with a crude extract of *V. wallichii*. Therefore, this study has demonstrated that a value-added product can be developed from *V. wallichii* root extract for the biomedical, pharmaceutical, biotechnological, and nanotechnology-based Industries. This study also summarizes that Vw-AgNCs could be a potential source for the production of highly effective antimicrobial coating in Intensive care units and various surfaces in hospitals preventing the spread of multidrug-resistant *A. baumannii* infection during post-surgery recovery periods and reducing a load of hospital-borne infection in the population.

Materials And Methods

Collection of Plant Material

The Medicinal plant (*V. wallichii*) root was collected from, Khari Baoli, Kucha Challan, Chandni Chowk, Old Delhi. The plant root was identified by CSIR-National Institute of Science Communication and Information Resources Council of Science & Industrial Research Ministry of Science & Technology, Gov. of India.

Preparation Of Plant Material

The dried roots were cleaned, disinfected, and washed with distilled water. Then it was crushed into a powder sample using an electronic blender. Plant root powder was used for methanolic extraction by using the Soxhlet apparatus. The filtrates obtained were filtered and dried in Hot Air Oven. After that, the final concentration was prepared and stored at 4 °C till further use.

Qualitative Phytochemical Screening

The methanolic root extract of *V. wallichii* was subjected to qualitative phytochemical testing and screened for the presence of different chemical groups of compounds like- saponins, tannins, Steroids, terpenoids, flavonoids, Naphthoquinone, Insulin, carbohydrates, alkaloids, Phenol, Starch, Amino acid, glycosides, reducing sugars, Phlobtannins as per the standard protocol (59).

Quantification Of Phytochemical Constituents

Estimation of Total Phenolic Content (TPC)

Folin-Ciocalteu colorimetric technique was used to measure the total phenolic contents (TPC) in the extracts with few modifications.(60) From the standard solution (1 mg/ml), various gallic acid solutions (10–50 µg/ml) in methanol were prepared. In the same way, Various concentrations of the plant extract were also prepared. 5 ml of 10% Folin-Ciocalteu reagent and 4 ml of 7.5% Sodium carbonate were added to each 1 ml concentration to create the final volume of 10 ml. After fully mixing, the resulting blue liquid was set aside at room temperature for 30 minutes in the dark. The absorbance at 765 nm was then determined. The average absorbance readings at varied gallic acid concentrations were used to create the calibration curve. The experiments were carried out in triplicate. The total phenolic content was expressed as mg gallic acid equivalents (GAE) per g of extract in dry wt. (mg/g). (61)

Determination Of Total Flavonoid Content (Tfc)

The total flavonoid content of the extracts was determined using an aluminum chloride colorimetric test. Various concentrations of Quercetin (1 mg/ml) were prepared. In the same way, different concentrations of plant extracts were also prepared and 0.5 ml of extract at each concentration was added to the test tube. The mixture was then given 0.1 ml of 10% Aluminum Chloride and 0.1 ml of 1 M Potassium Acetate. 2.8 ml of distilled water was added to the mixture to make its final volume 3.5 ml. 30 minutes of room temperature incubation were given to the mixture. At 415 nm, the absorbance was measured. The average absorbance value from three readings was used to estimate the total amount of flavonoids present. Quercetin equivalent (mg QE/g) was used to express the flavonoid content using the standard calibration curve. (61)

Determination Of Total Terpenoid Content

The Ghorai *et al.* procedure was used to calculate the total terpenoid content (TTC) of the extracts. (62). 1 ml of the extract with different conc., and 2 ml chloroform were added. After vortexing, the sample mixture was kept at room temperature for 3 minutes. After that, 200 µl of concentrated sulfuric acid was added, and the mixture was left to sit at room temperature in the dark for 1.5 to 2 hours. A reddish-brown precipitate developed in the mixture during incubation. Following the decantation of the supernatant, 3 ml of 100% methanol was added to the precipitate and thoroughly vortexed to dissolve it. A visible spectrometer was used to measure absorbance at 538 nm. The experiment was carried out in triplicates The TTC of the extracts was calculated as mg of linalool per g (mg LE/g) of extract (dry weight, DW)(63).

Estimation Of Total Tannin Content

The tannins were determined by the Folin-Ciocalteu method as per the previous procedure (64) with some modifications. In a mixture of 3.75 ml of distilled water, 0.25 ml of Folin-Ciocalteu phenol reagent, and 0.5 ml of 35% sodium carbonate solution, 0.5 ml of the extract was added. The mixture was kept at room temperature for 30 min after mixing well. Different concentrations of standard solutions of gallic acid (20–100 µg/ ml) were prepared for this test. Absorbance was measured against the blank at 700 nm. The experiment was carried out in triplicate. The tannin content was expressed as mg of gallic acid equivalents/ g (mg GAE/g) of the dried sample.(65)

Estimation Of Ferric Reducing Antioxidant Power (Frap)

According to the procedure described by Sudha *et al.*, the Ferric reducing antioxidant power assay was performed with minor modifications (66). 10 mM TPTZ (2,4,6-Tripyridyl-s-Triazine) solution in 40 mM HCl, 20mM FeCl₃ .6H₂O, and 300 mM acetate buffer (pH 3.6) in the ratio of 10:1:1 was present in the FRAP reagent. It was freshly prepared. 3ml FRAP reagent was mixed with 1 ml extract with different concentrations. At 593 nm, the absorbance of the reaction mixture was measured after 15–30 min of incubation at 37°C. The experiment was carried out in triplicates and FRAP was expressed as mg of FeSO₄/g of dry sample. (67)

Determination Of Reducing Power Assay

The method was performed as described by Hue *et al.* (68) with some modifications. 0.5ml of extract at various concentrations was added with 0.5ml of 1% potassium ferricyanide and 0.2M phosphate buffer (pH 6.6) each. The mixture was incubated in the water bath for 20 minutes at 50°C. After adding 0.5 ml of 10% trichloroacetic acid, the mixture was centrifuged at 3000 rpm for 10 minutes at room temperature. 0.1ml of 0.1% Ferric Chloride and 0.5ml of deionized water were added to the supernatant (0.5ml). At 700 nm, absorbance was measured. Readings were taken in triplicates and Reducing power was expressed as mg of Ascorbic acid equivalent (AAE/L). (67)

Dpph Free Radical Scavenging Assay

With slight modifications, the DPPH radical scavenging activity was calculated in accordance with Moraes-de-Souza *et al.* (69). The 0.5 ml of extract at various concentrations, 0.3 ml of a 0.4 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) sol. in methanol and 3 ml of methanol, were present in the mixture. Absorbance was measured at 517 nm following a 30-minute incubation in the dark at room temperature. The concentration of the samples that showed 50% DPPH radical scavenging activity was known as the inhibitory concentration (IC₅₀). A lower IC₅₀ value, therefore, suggested higher antioxidant activity. The following equation was used to measure the antioxidant activity. (67)

$$\%inhibition = \left[\frac{A_{control} - A_{sample}}{A_{control}} \right] \times 100$$

A_{control} = Absorbance of Control; A_{sample} = Absorbance of the sample

High-pressure Liquid Chromatography (Hplc)

By using HPLC, the prepared extracts were evaluated for the presence of phenolic compounds and alkaloids against standards (quercetin, rutin, and tannic acid). For the analysis, a C-18 reversed phase column with a 4.6 mm internal diameter and a 250 mm length (Phenomenex, Gemini 5) was employed. Quercetin, rutin, and tannic acid were separated using the mobile phases of methanol: 0.1% orthophosphoric acid (77:23) and methanol: water (50:50), respectively, at a constant flow rate of 1 ml/min. The temperature of the column was 38°C, and the injection volume was 2 µl. For the detection of quercetin, rutin, and tannic acid, the wavelengths were set at 254 nm, 370 nm, and 280 nm, respectively. The retention time was observed by using HPLC.(70) (59)

Molecular docking studies of Phytoconstituents (Ligands) with A. baumannii Receptors

The study of receptor-ligand interactions was predicted by molecular docking. AutoDock Vina v1.1.2 created the 3D coordinates file for the grid box for each of the chosen receptors in accordance with the prescribed protocol (71). The number of interacting residues, cluster RMS, hydrogen bonds, and minimum binding energy (kcal/mol) was taken into consideration while choosing the best-docked poses from a wide range of conformations (72).

Three-dimension SDF files of Gallic acid (Pubchem ID- 370), Quercetin (Pubchem ID- 5280343), Ascorbic acid (Pubchem ID- 54670067), linalool (Pubchem ID- 6549), Rutin (Pubchem ID- 5280805) and Tannic acid (Pubchem ID- 16129778), were taken from the PubChem database. (<https://pubchem.ncbi.nlm.nih.gov/>). PDB files of Hydrolysing Class C Extended-Spectrum –Lactamase (AmpC) (PDB ID: 4QD4), NADH-dependent enoyl-ACP reductase (FabI) (PDB ID: 4ZJU), Response regulator BfmR (RstA) (PDB ID: 5E3J), UDP-N-acetylglucosamine acetyltransferase (LpxA) (PDB ID: 4E6U) were downloaded from Protein data bank (<https://www.rcsb.org>). Aspartokinase and Shikimate dehydrogenase was not found in the PDB data bank so these were modeled using Swiss Model for their structure. Discovery Studio 2021 was used to visualize the interactions of amino acids with molecules and the 2-D interactions images.

Cytotoxicity and anti-cancerous activity of methanolic root extract of V. wallichii

Cytotoxic activity of the extract was evaluated using a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) assay on Hep2C and L929 cells(73). The cells were seeded (~ 1× 10⁴ cells/well) into 96-well culture plates with a flat bottom, and they were incubated for 24 hours at 37°C with 5% CO₂ and 95% air. Hep2C cells and L929 cells were treated with the extract (50–400 µg/mL). 10 µl of MTT reagent (5 mg/mL) was added to the mixtures after 48 h of incubation, and they were again re-incubated for 3 h. Using 100 µl DMSO, the resultant formazan was solubilized. Finally, an automated microplate reader was used to measure the absorbance at 570 nm (Bio-Rad, Illinois, USA). The experiments were

performed in triplicates. By comparing the absorbance between the samples and the blank, the cell *viability* (%) was estimated. The % inhibition was calculated using the formula below:

$$\%inhibition = \left[\frac{100 - \text{MeanAbsorbanceof test compound} - \text{MeanAbsorbanceof negative control}}{\text{MeanAbsorbanceof Positive control} - \text{MeanAbsorbanceof negative control}} \right] \times 100$$

Green synthesis of silver nanoconjugates of *V. wallichii* methanolic root extract (Vw- AgNCs)

For the synthesis of AgNCs, a solution of silver nitrate was made. Co-precipitation with methanolic plant root extract of *V. wallichii* results in the synthesis of AgNCs from silver nitrate solution., whose secondary metabolites act as reducing and capping agent. 5 mM solution of silver nitrate was prepared in an Erlenmeyer flask. 10 ml of plant extract (10mg/ml) was added. In order to reduce silver nitrate's photoactivation at room temperature, this system was incubated in a dark room. The transformation of the solution's colour from colourless to yellowish-brown confirmed that Ag⁺ had been reduced to Ag⁰. Its formation was also confirmed by using UV-Visible spectroscopy(74). (49)

Characterization Of Synthesized Vw-agncs

Vw-AgNCs were characterized using UV-Vis spectrophotometer, Dynamic light scattering (DLS), Atomic Force Microscopy (AFM), Scanning electron microscopy (SEM), and energy dispersive X-ray spectrum (EDS).

The synthesis of Vw-AgNCs was monitored by measuring the optical density of the reaction mixture with the help of a double-beam UV–Vis spectrophotometer (Shimadzu UV-2600). The absorbance of the reaction mixture was recorded between 200 and 800 nm. DLS was used to determine the average size of Vw-AgNCs by using MALVERN Data Sizer. AFM (NDMDT solver pro P47 model) was used to evaluate the surface morphology, 2D topography, and 3D topography of the Vw-AgNCs. A single drop of the liquid sample was evenly distributed in the middle of the tiny glass slide. The sample was fixed on the glass slide by heating it for more than 15 minutes at 60°C.

The Vw-AgNCs' SEM and EDS analyses were performed on Ultra 55 Model-II Carl Zeiss SEM equipped with an EDS instrument. By using a very small quantity of the sample, a thin film was developed on a glass slide. The presence of elemental silver in Vw-AgNCs was determined by using EDX (51 N-1000–EDX System, Oxford).(49, 74) (75)

Determination Of Antimicrobial Potential Of Vw-agncs

Bacterial strain: MDR- *A. baumannii* was obtained from Batra Hospital. It was subcultured freshly in Luria Broth (LB) medium and cultured on a MacConkey Agar plate for further analysis.

Antibacterial activity was assessed using the agar well diffusion method using a cell suspension (1.5 10⁶ CFU/ml) and the Macfarland turbidity standard No. 0.5. Standardization of the suspension was accomplished by adjusting the optical density to 0.1 at 600 nm (Shimadzu, UV-VIS Spectrophotometer). On the surface of the agar plate, 100µl of inoculum was spread, and an aseptic well with a diameter of 6 to 8 mm was punched. All the Extracts and standard antibiotics (ampicillin, vancomycin, gentamycin, and tetracycline) at desired concentration were introduced into the well and were incubated at 37°C for 24–48 hrs. Antibacterial activity was observed by measuring its zone of inhibition. The assay was performed in triplicate and the mean diameter was recorded (76) (77).

Statistical Analysis

The mean and standard deviation are used to express all experimental results. Microsoft Excel and GraphPad Prism were used to analyze the data. Every experiment was performed three times, then averaged. Statistical significance was defined as a P value less than 0.05.

Abbreviations

A. baumannii- *Acinetobacter baumannii*

MDR- Multidrug-resistant

V. wallichii - *Valeriana wallichii*

TPC- Total Phenolic Content

TFC -Total Flavonoid Content

FRAP -Ferric Reducing Antioxidant Power

DPPH-2,2-diphenyl-1-picrylhydrazyl

TTC - Total terpenoid content

HPLC - High-pressure liquid chromatography

MTT -3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium

Vw- AgNCs-*V. wallichii* silver nanoconjugates

DLS - Dynamic light scattering

AFM - Atomic Force Microscopy

SEM - Scanning electron microscopy

EDX - Energy dispersive X-ray spectrum

LB- Luria Broth

WHO- World Health Organisation

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article (and its supplementary information files).

Conflict of interests

The authors declare that they have no competing interests

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Author contributions statements: **MK-** Methodology, Formal analysis, Validation, Data curation, Investigation, Writing - Original Draft, Software. **MA-** Methodology, Formal analysis, Validation, Data curation, Investigation, Writing - Original Draft, Software. **ANVLK-** Software, Investigation, Resources, Visualization. **PM:** Writing - Review & Editing, Resources. **OPS:** Writing - Review & Editing, Resources, Visualization. **KB:** Conception, Design, Methodology, Supervision, Data curation, Writing - review & editing, Project administration. All authors read and approved the final manuscript.

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Supporting Information: All the Standard curve graphs for quantitative estimation of *V. wallichii* methanolic root extract phytoconstituents are given in a separate Supplementary file mentioning its regression equation and R square value.

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Figures

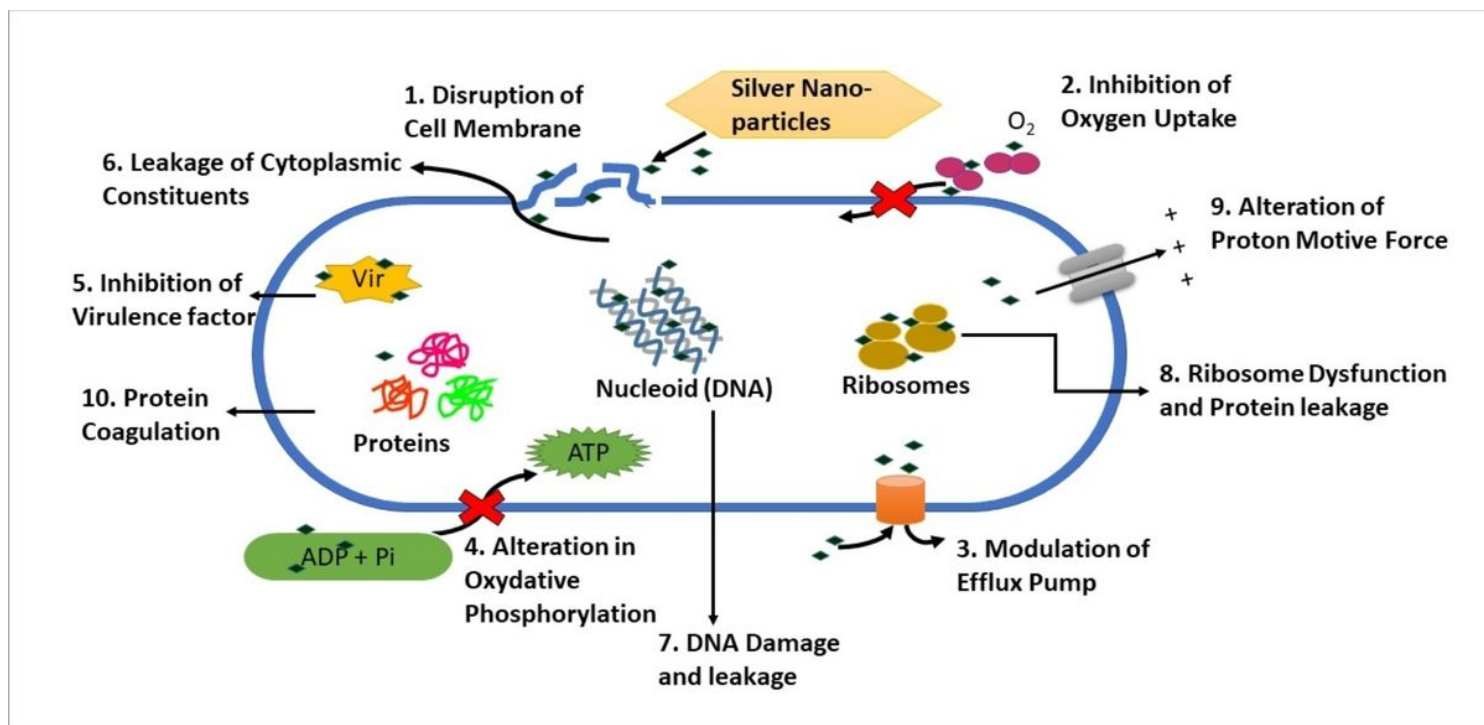


Figure 1

Mechanism of action of AgNP against *A. baumannii*.

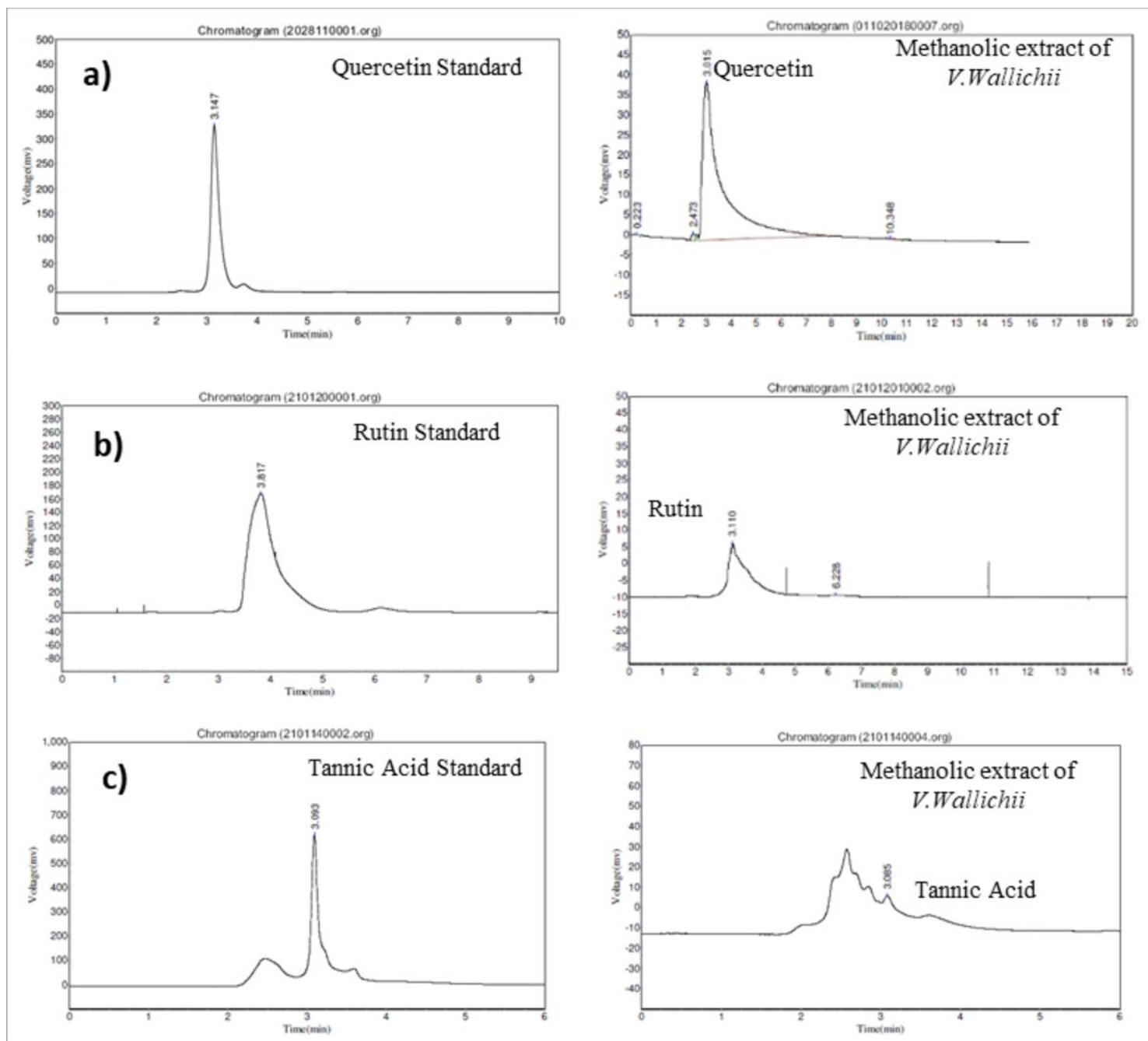


Figure 2

HPLC Chromatogram showing methanolic root extract of *V. wallichii* in reference to a) Quercetin standard; b) Rutin standard and c) Tannic Acid standard.

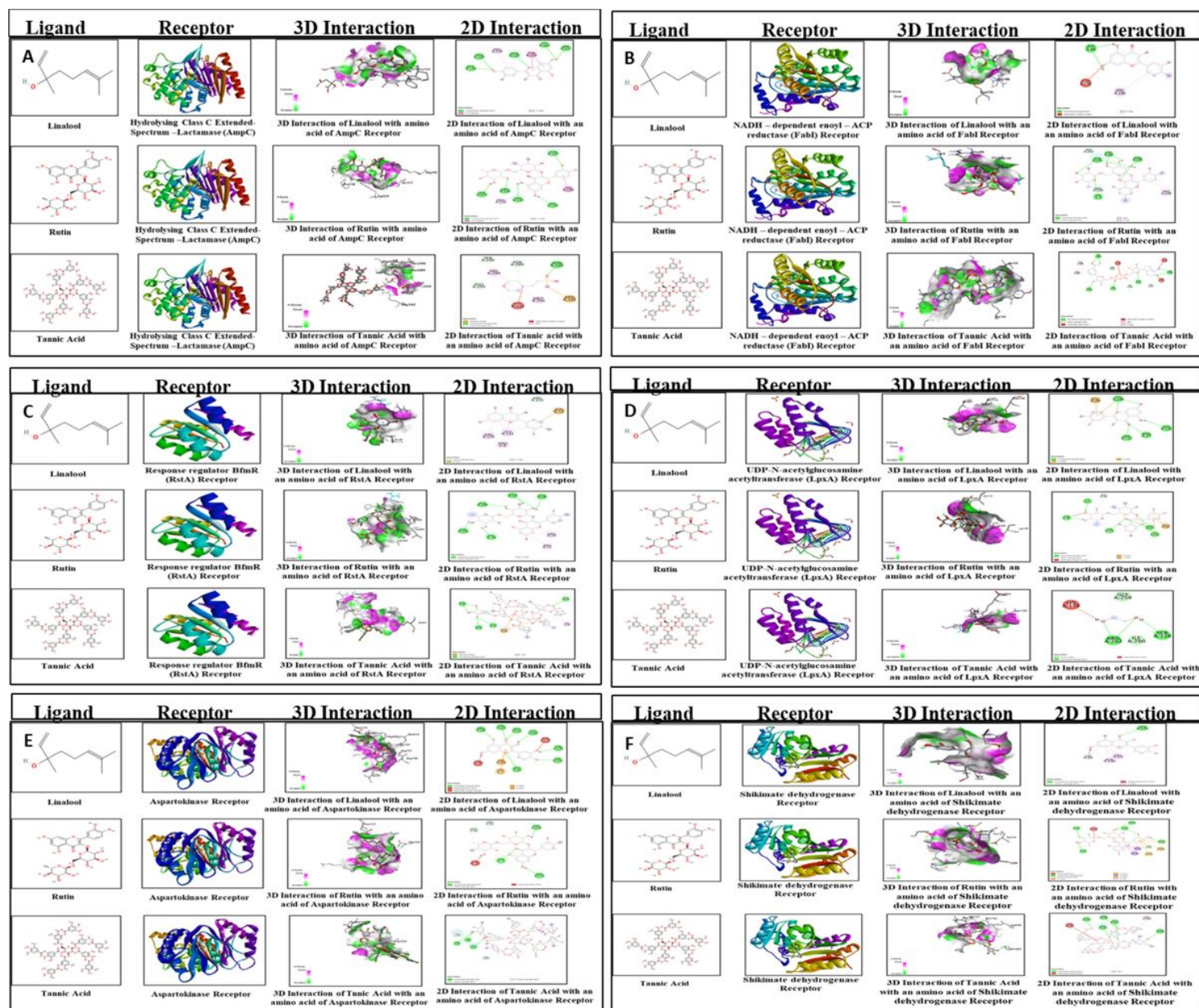


Figure 3

Molecular docking of Linalool, Rutin, and Tannic acid, their 3D and 2D Interactions with amino acids of *A. baumannii* receptors- A) AmpC; B) FabI; C) RstA; D) LpxA; E) Aspartokinase and F) Shikimate dehydrogenase.

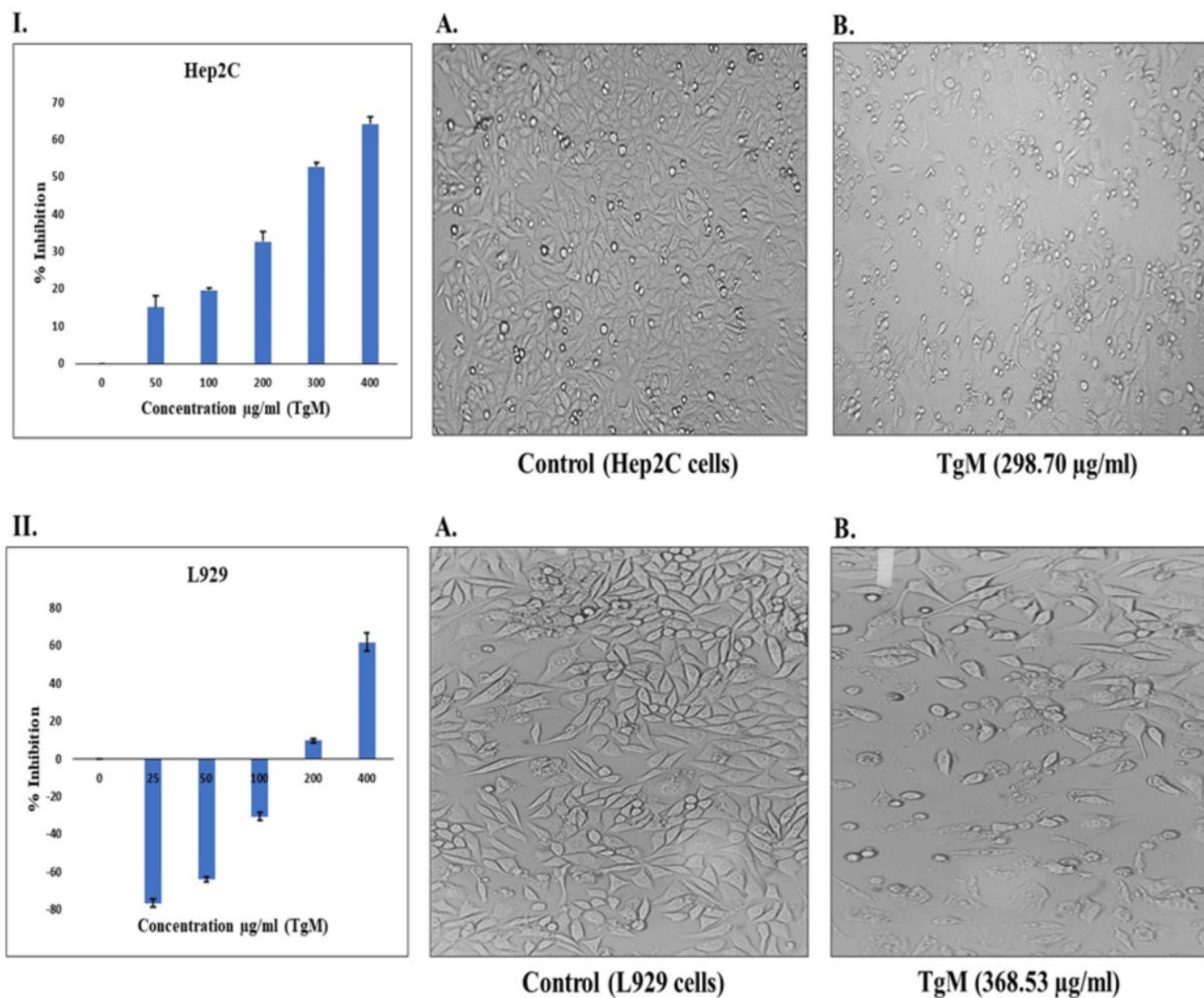


Figure 4

Cytotoxicity and anticancerous activity of methanolic root extract of *V. wallichii* where, I- Graph showing IC₅₀ value of the extract when treated in the range of 50–400 $\mu\text{g/ml}$. IA- cervical cancer control cells (Hep2C); IB- Hep2C cells after the treatment with extract over 48 h; II- Graph showing IC₅₀ value of the extract when treated in the range of 25–400 $\mu\text{g/ml}$; IIA- human fibroblast control cells (L929); IIB- L929 cells after the treatment with extract over 48 h.

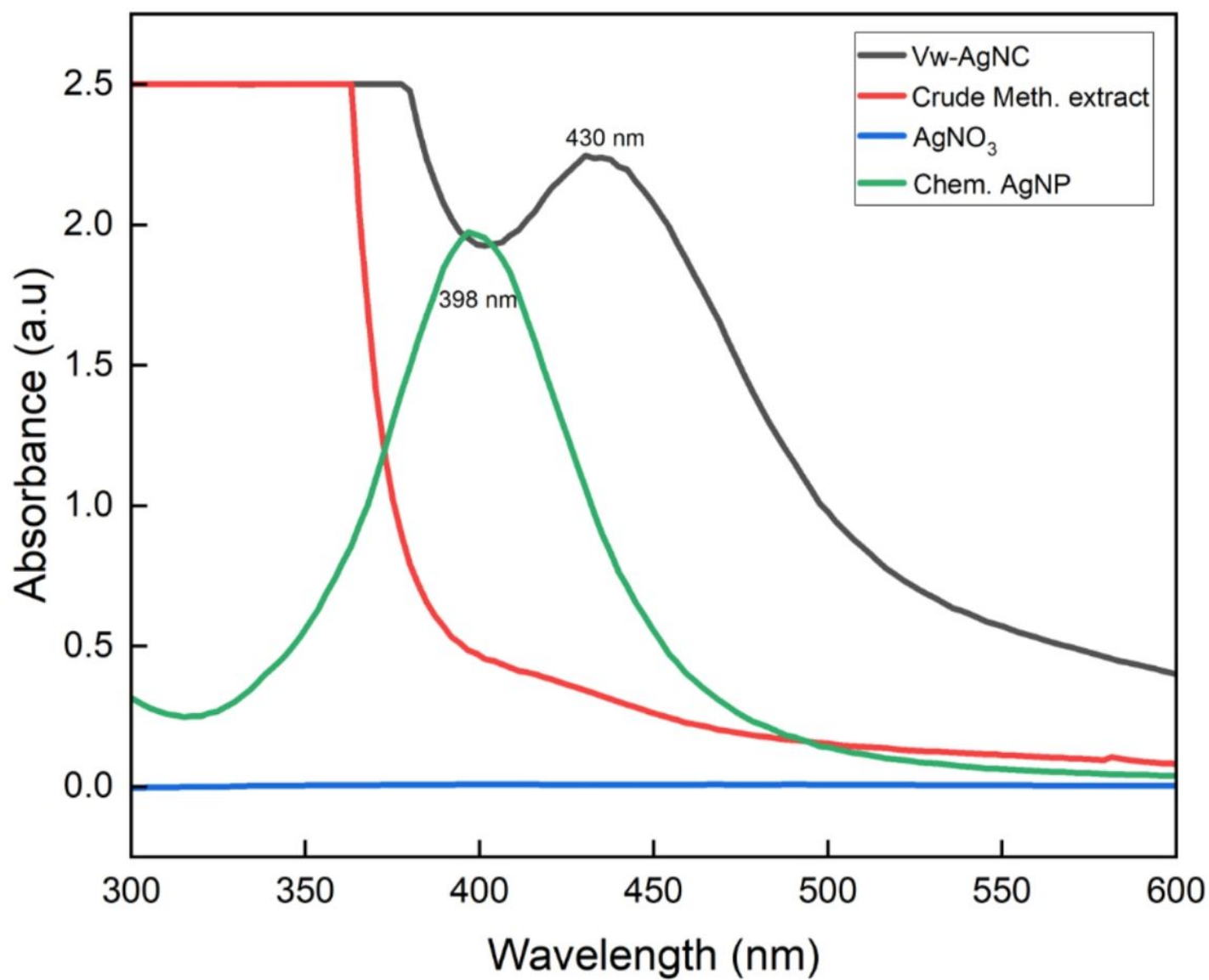


Figure 5

UV-Vis Spectral Images of A- *V. wallichii* methanolic root extract; B- Vw-AgNCs { λ max=430 nm} and C- Chemically synthesized Silver Nanoparticles

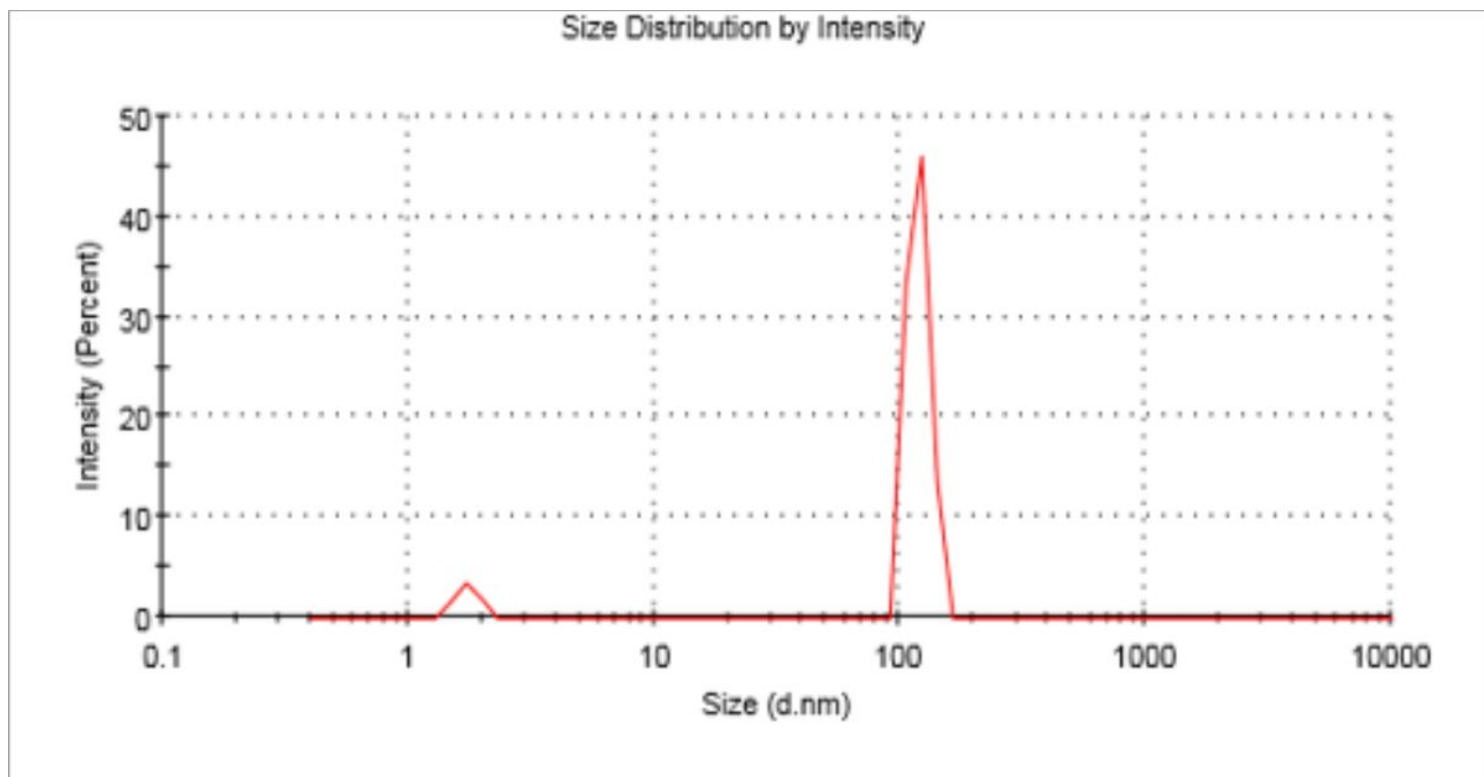


Figure 6

DLS Images of Vw-AgNCs

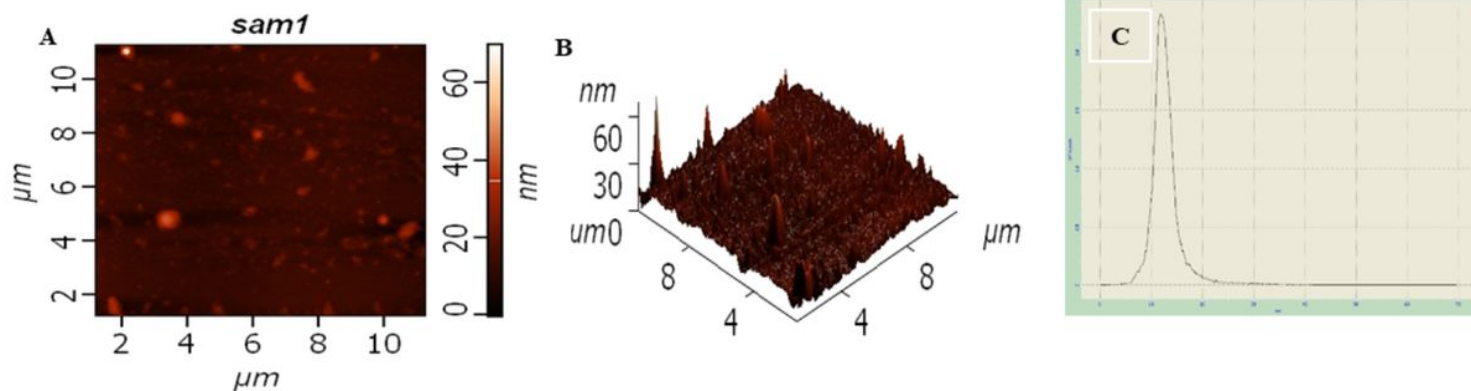


Figure 7

A- AFM images of Vw-AgNCs in a 2D view; **B**- 3D view; **C**- graph showing size of synthesized Nanoparticles (12nm).

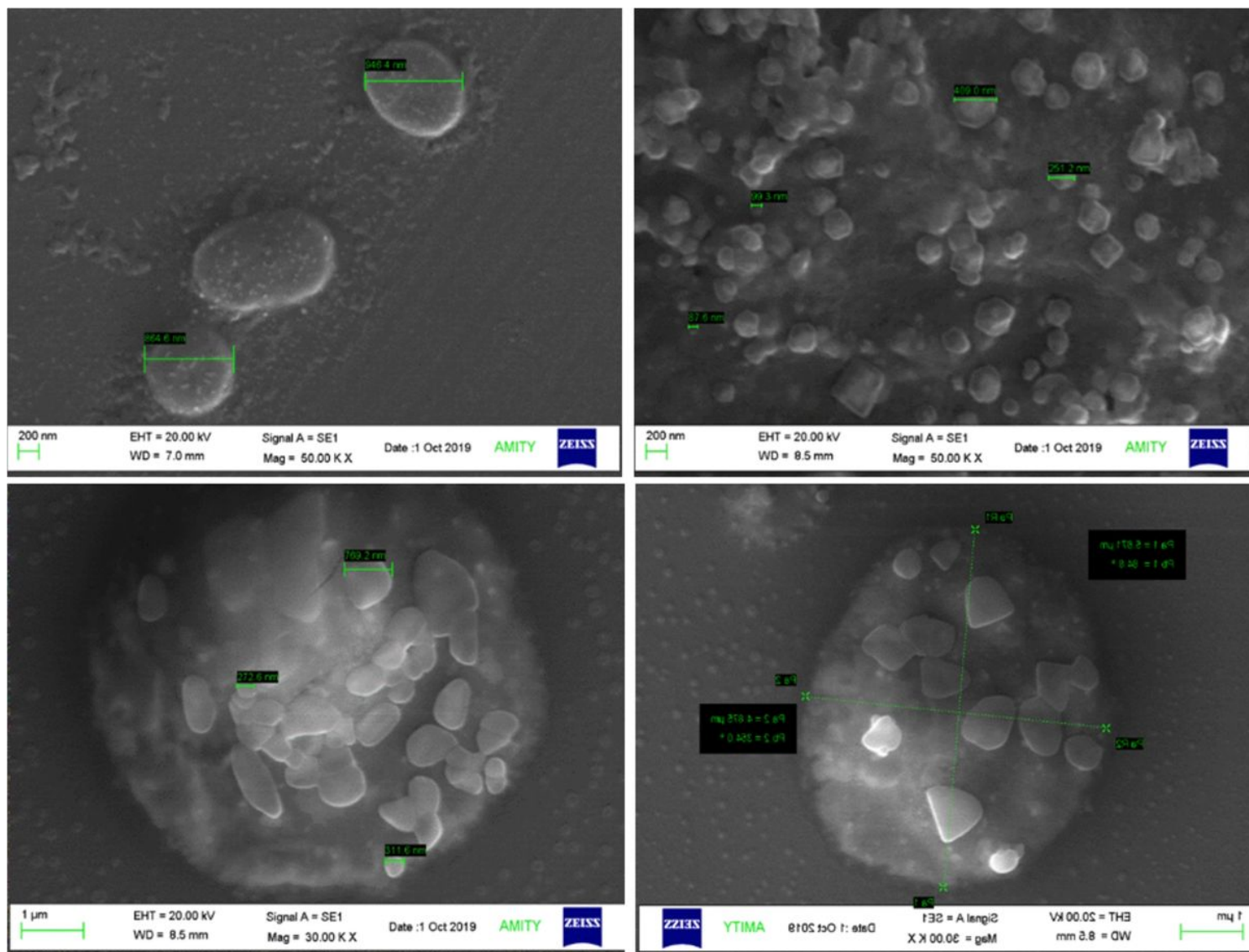


Figure 8

SEM Images of Vw-AgNCs.

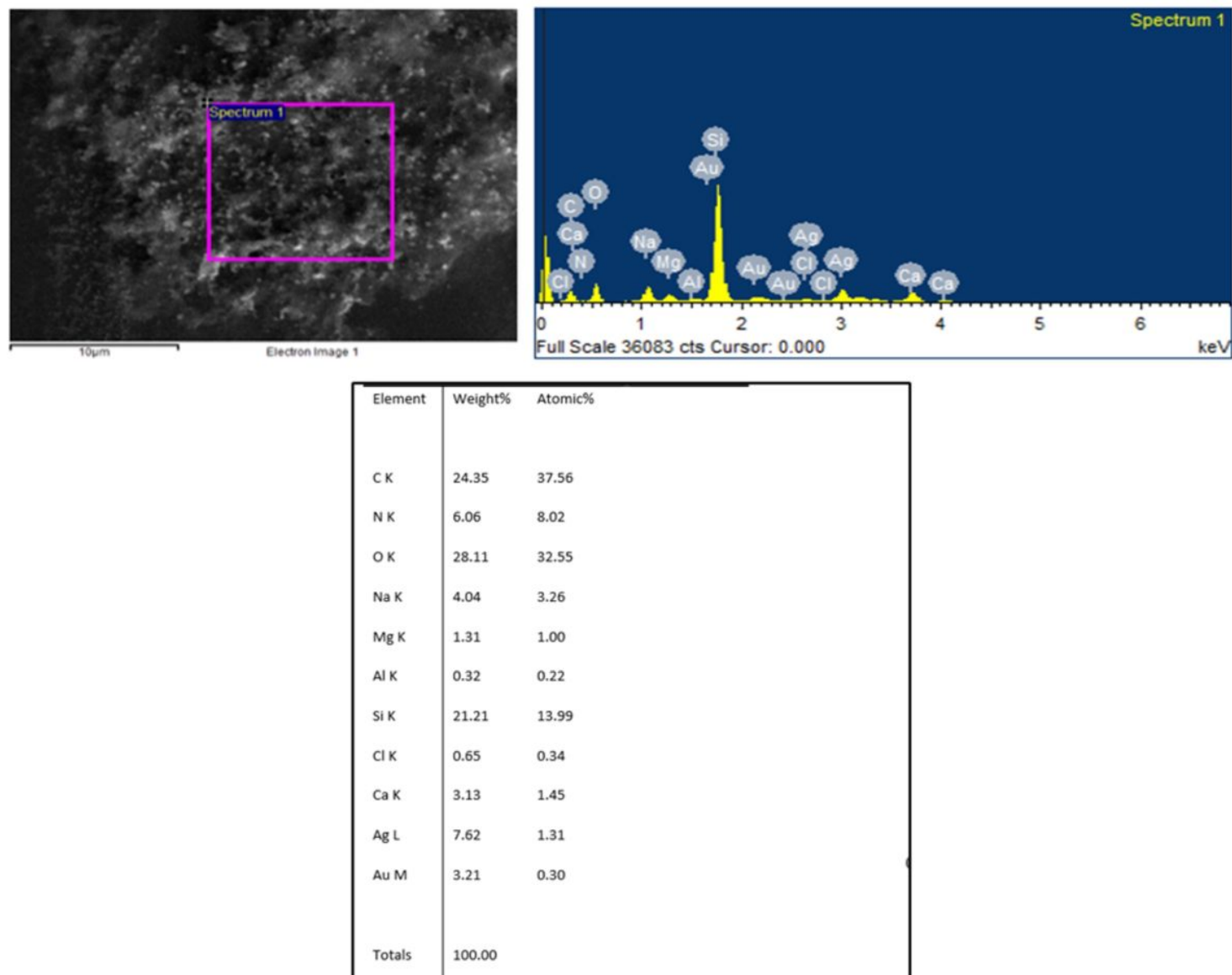


Figure 9

Elemental compositional analysis with EDX of Vw-AgNCs showing the presence of the silver element.

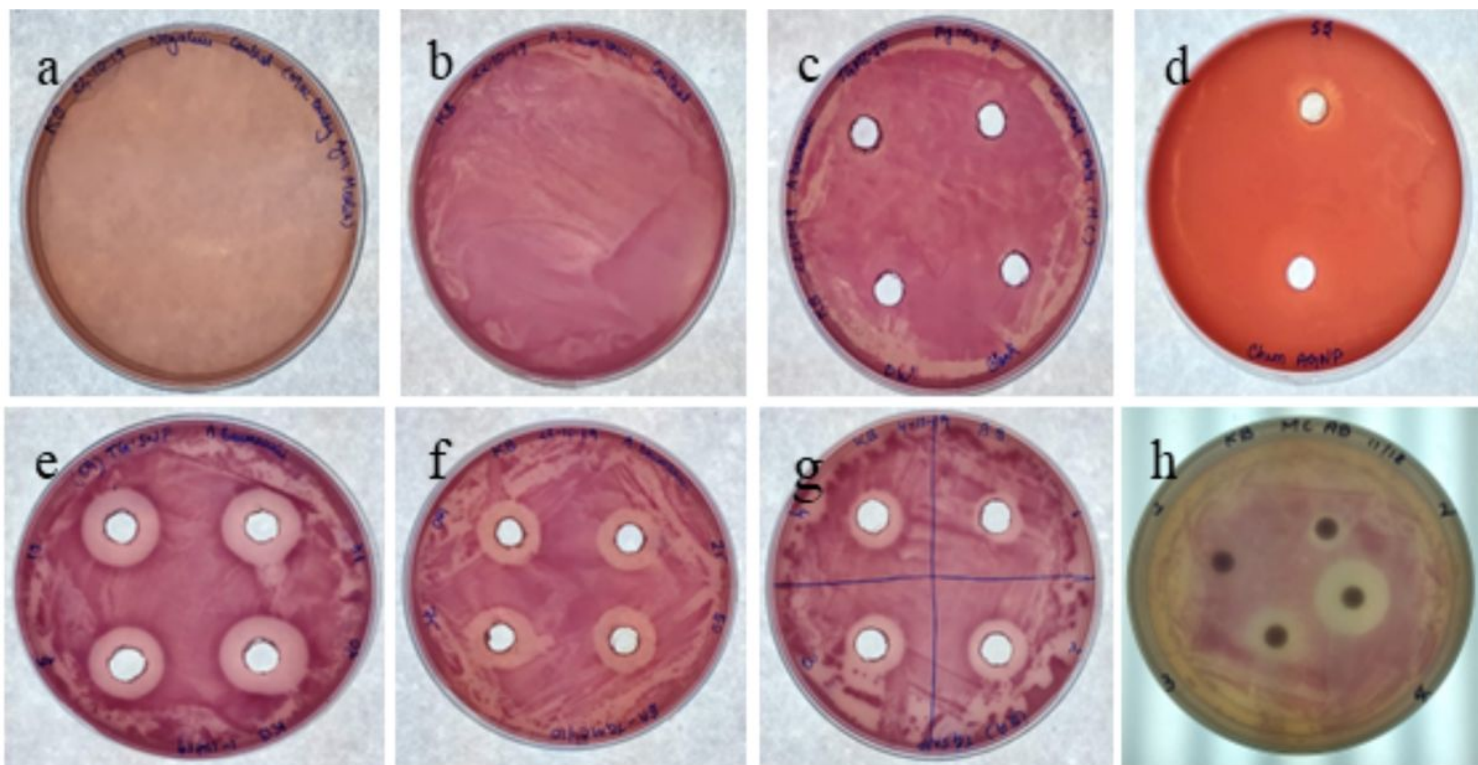


Figure 10

a) Negative Control- Mac Conkey Control- Mac Conkey **b)** Bacterial Control- MDR- *A. baumannii*. **c)** Positive Control: Plate having wells with I) TGMC-50: 50 mg/ml *V. wallichii* methanolic root extract.; II) AgNO_3 -5: 5mM AgNO_3 .; III) D.W.: Distilled Water.; IV) Blank: Empty well, showing no Zone of Inhibition. **d)** Positive Control: Plate having wells with I) Chem AgNPs: Chemically synthesized AgNPs.; II) S5- 100% Methanol; **e)** Vw-AgNCs: Volume- 25, 50, 75 & 100 μl ; **f)** Vw-AgNCs: Volume- 5, 10, 15 & 20 μl ; **g)** Vw-AgNCs: Volume- 1, 2, 3 & 4 μl ; **h)** Standard Antibiotics: Ampicillin, Vancomycin, Tetracycline, and Gentamicin.

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