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Investigating the mode of action of silver nanoparticles stabilized by *Adathoda vasica* targeted against multidrug resistant [MDR] urinary isolates

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ABSTRACT

The present study was designed to evaluate the antibacterial activity of Adathoda vasica against drug resistant uropathogens and to test the efficacy of the plant to stabilize silver nanoparticles [AgNPs]. AgNPs were synthesized using Adathoda vasica leaves. Preliminary formation of nanoparticles was confirmed using UV-Vis spectrum at the absorbance of 420 nm Further it was characterized with FTIR, XRD and SEM analysis. Synthesized AgNPs were then subjected to check the antibacterial activity against a few drug resistant uropathogens like Escherichia coli, Proteus sp., Pseudomonas aeruginosa, S.aureus, Streptococcus sp. and Enterobacter sp. isolated from the patients suffering from urinary tract infection. Also to determine the mechanism of action of the bioactive compound of the plant, docking studies were performed using Schrodinger software. The SEM revealed the sizes of poly-dispersed [50 – 60 nm] nanoparticles in the medium, the silver at nano crystals formation was exhibited by XRD spectrum, and the FTIR analysis represents the biological compounds involved in nanoparticle synthesis. The antibiogram demonstrated that all the uropathogens used in the study were MDR. Antibiotic assay test reveals that the synthesized AgNPs have potent antibacterial activity even in minimum concentration against the uropathogens tested. Docking of the bioactive compound with fimH further demonstrated that the AgNPs exhibited antibacterial activity by preventing bacterial binding. Thus the silver nanoparticles stabilized by Adathoda vasica has shown to possess antibacterial activity and also have the ability to prevent anchorage of bacteria to human cell wall, which may lead to the death of the bacterium.

Key words: *Adathoda vasica*; biofilm, silver nanoparticles, FimH, docking studies

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INTRODUCTION

Urinary tract infection [UTI] is a common infection affecting several annually. Worldwide, about 150 million people are diagnosed with UTI each year, and the cost exceeds 6 billion US dollars [1]. UTI may involve only the lower urinary tract or may involve both the upper and lower tract. The term cystitis has been used to describe lower UTI, which is characterized by a syndrome involving dysuria, frequency, urgency and occasionally suprapubic tenderness. The indiscriminate use of antibiotics has resulted in the emergence of antibiotic resistance, which, has recently become a major problem worldwide [2]. Treatment of infections caused by these resistant bacteria has become very complicated, since they are resistant to many antibiotics. This limits therapeutic options [3]. Therefore, alternative methods of treatment are sought after. The clinical efficacy of many existing antibiotics is being threatened by the emergence of MDR pathogens [4]. Many infectious diseases have been known to be treated with herbal medicines throughout the history of mankind. Such herbal products could be used as plant extracts or pure

bioactive compounds, and they can provide unlimited opportunities for the development of new drugs due to the availability of chemical diversity. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases [5]. The increased failure of chemotherapeutics and emergence of antibiotic resistance exhibited by pathogenic microbial infectious agents have led the researchers to turn their attention to folk medicines, looking for new leads to develop better drugs against microbial infections [6]. This has led to the screening of several medicinal plants for their potential antimicrobial activity [7-11].

Nanoparticles [NPs] differ from traditionally used materials in several properties [12-15]. Their size of less than 100 nm allows them to reach specific target sites within the body and even to be permeable to tissues and cells. Hence, such nanoparticles can deliver the drugs in active forms at sites efficiently than conventional drugs, and thereby may reduce undesirable side effects [16]. Additionally, their high surface to volume ratio make them effective carriers and also minimize toxicity due to the small doses of drug required [17,18]. Since the delivery of the drug should be controlled both in time and in quantity, integrating different coatings with drugs, non-acute and time controlled drug delivery can be achieved. This is very important for treating infectious diseases effectively.

MATERIALS AND METHODS

Chosen plant

Leaves of *Adathoda vasica* were collected from Vayallur road sides at Tiruchirappalli District, Tamilnadu, India and the plant was authenticated by the Botany department of St. Joseph's College and the voucher specimen number is (001). The plant chosen for the study had been washed, macerated and lyophilized. About 500g of all the three plant leaves yielded 33g powder respectively. The procedure was repeated to collect the needed quantity.

Preparation of Silver Nanoparticles from plant extract

Adathoda vasica leaves were washed to remove dust and impurities and shade-dried for a week. The leaves were dried at 37ºC for two days in an incubator to remove any moisture left. They were then ground to fine powder. The powder was added in deionised water to form a suspension. Suspension was thoroughly mixed using a magnetic stirrer at 25 rpm for 10 mins, followed by incubation in shaker incubator for 30 mins at 37ºC. Then the mixture was centrifuged and filtered using 2.5 µm membrane filter [qualitative filter paper – Hi-media] paper. After addition of leaf extract to the silver nitrate solution, instant color change in the reaction mixture was observed from nearly colorless to yellowish brown. The color change occurs due to the reduction of silver ions to elemental form. Complete reduction usually takes 24 hours to occur for the added $AgNO_3$.

Characterization of the synthesized AgNPs

UV- visible spectroscopy analysis [Perkin Elmer lambda 35 model]

The preliminary characterization of the silver nanoparticles was carried out using UV-visible spectroscopy. Noble metals, such as silver [Ag] and gold [Au] nanoparticles exhibit unique and tunable optical properties because of their Surface Plasmon Resonance [SPR], depending upon shape, size and size distribution of the nanoparticles. The reduction of silver ions was monitored by measuring the UV–visible spectra of the solutions after diluting a small aliquot [0.2 mL] of the aqueous component. UV–visible spectroscopy analysis has been done using a Perkin-Elmer Lambda-35 spectrophotometer operated at a resolution of 1 nm as a function of reaction time. The nanoparticles solution was diluted to 20 times with deionized water to avoid errors due to high optical density of the solution. Deionised water was used as a blank.

FT-IR [Fourier Transform Infra Red spectroscopy] analysis [Perkin Elmer spectrum RX 1 model]

FT-IR is most useful for identifying types of chemicals that are either organic or inorganic. It can be utilized to quantify various components of an unknown mixture. It can be applied to the analysis of solids or liquids. The purified liquid of silver nanoparticles in the study was subjected to Fourier Transform Infra Red spectroscopy analysis [FT-IR] for the analysis of functional groups encapsulated on the synthesized nanoparticles. These measurements were carried out on a Spectrum RX 1 model instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1} in potassium bromide [KBr] pellets. For comparison, a drop of 20% *Adathoda vasica* was mixed with KBr powder and pelletized after drying properly. The pellets were later subjected to FTIR spectroscopy measurement and the spectra were recorded in the 4000- 400 nm^{-1} range.

Scanning electron microscopy [SEM] analysis [JEOL-JSM 6390 model]
Silver nanoparticle samples for scanning electron microscopy [SEM] analysis were prepared by adding 25 µl of sample coated with an extremely thin layer [1.5-3.0 nm] of gold or gold – palladium. The surface morphology, size and shape of nanoparticles were studied using SEM.

Energy dispersive X – ray spectroscopy [EDAX] analysis [JEOL-JSM 6390 model]
EDAX analysis was performed on a scanning electron microscope [SEM model, JEOL-JSM 6390, Japan] coupled with an EDAX detector to confirm the biosynthesized AgNPs. EDAX spectrum was measured at 20 kV accelerating voltage. EDAX is used to determine the particular elemental concentration of the sample.

X-Ray Diffraction analysis [XRD]
Crystalline AgNPs were determined by X-ray diffraction analysis. The biosynthesized AgNPs were laid onto the glass substrates on a Phillips PW 1830 instrument operating at a voltage of 40 kV and a current of 30 mA with Cukα radiation. These results were used to predict the average particle size of nanoparticles with the help of JCPDS data.

UTI bacterial pathogens
Urinary pathogens were obtained from a local hospital in Tiruchirappalli, South India. The isolates were subcultured on nutrient agar slants periodically and stored until use.

Antibacterial assay
Disc Diffusion method
The antibacterial assays were also performed by standard disc diffusion method as described in our previous paper [19].

Minimal Inhibitory Concentration [MIC] by broth dilution method
The tube dilution test is the standard method for determining levels of resistance to an antibiotic/test extract. Serial dilutions of the test extracts are made in a liquid medium, which is inoculated with a standardized number of organisms and incubated for a prescribed time, as explained previously [20].

.Determination of cellular toxicity using sheep erythrocytes
The method described by Xian-guo and Ursula [21] was employed to study cellular toxicity. Briefly, 10-fold serial dilutions of the extract were made in phosphate buffered saline. A total volume of 0.8 ml for each dilution was placed in an eppendorf tube. A negative control tube [containing saline only] and a positive control tube [containing plant extract, 5 mg/ml] were also included in the analysis. Fresh sheep erythrocytes were added to each tube, to give a final volume of 1 ml. Solutions were incubated at 37°C for 30 min and all tubes were centrifuged for 5 min and then observed for haemolysis. Complete hemolysis was seen by a clear red solution without any deposit of erythrocytes. Hemolysis was also checked microscopically for presence or absence of intact RBCs.

Biofilm formation by Microtiter plate assay
Quantification of biofilm formation of the isolates under study was done by using microtiter plate method as described by Stepanovic [22].

Docking
Ligand preparation
Chemical structure of the ligand (2-4-but-2-yl phenyl propanoic acid) was obtained from Pubchem (ID:3762)

Protein selection
Three dimensional structures of FimH (PDB ID:4XOC), were retrieved from the Protein Data Bank (PDB), (http://www.pdb.org).

Docking analysis
The docking analysis was carried out by means of the Schrodinger tools. The molecular docking studies performed with the active sites of target protein by the Glide application of Schrodinger [23].

RESULTS AND DISCUSSION

Characterization of silver nano particles of A.vasica

From this study, it has been found that the leaf extract of *Adathoda vasica*, a traditional medicinal plant has the potential to reduce silver nitrate ions to silver nanoparticles. The light brown color of leaf extract of *A.vasica*was changed to yellowish brown after 10 mins of incubation due to the excitation of surface plasmon vibrations in silver nanoparticles.The intensity of the color increased with the incubation time. Nanoparticles size can also be determined by the changes in the color of the reaction solution.

UV-visible spectroscopy analysis

The corresponding UV-Vis absorption spectra for the synthesis of silver nanoparticles using *A.vasica* at 5% concentration for various temperatures [30 °C, 60 °C, 90 °C and 95 °C] are shown in Fig 8. The leaf extract of *A.vasica* [control] shows no evidence of absorption peak in the range in 200 to 900 nm. AgNPs at various temperatures indicated an absorbance peak at 30 °C [448 nm], 60 °C [437 nm], 90 °C [444 nm] and at 95 °C [445 nm] respectively. The spectrum with bands in this range has been associated with the surface plasmon resonance of nano-sized silver metal, confirming the occurrence of silver nanoparticles in the solution after exposure to UV light.

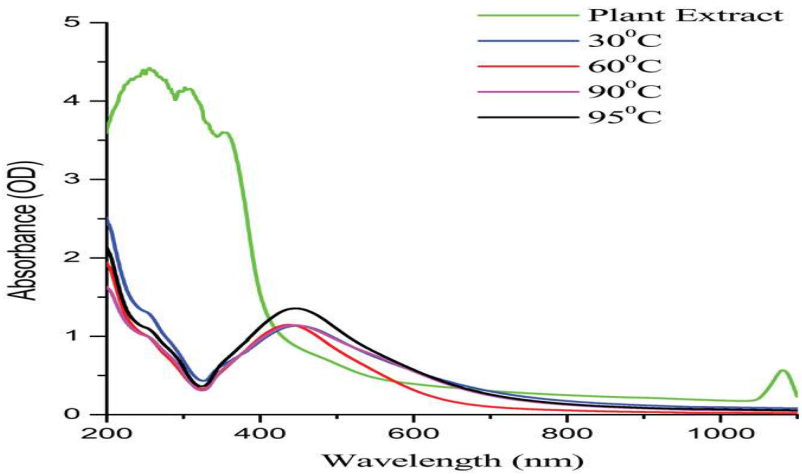


Figure 1: UV-Vis spectroscopy analysis of AgNPs synthesized using *A.vasica*

FT-IR [Fourier Transform Infra Red spectroscopy] analysis

FT-IR [Fourier Transform Infra Red spectroscopy] analysis an infrared spectrum represents a fingerprint of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. Therefore, infrared spectroscopy can result in a positive identification [qualitative analysis] of every different kind of material.

The FTIR spectrum of silver nanoparticles synthesized using *A.vasica* at various temperatures is shown in Fig 2. The band around 3433 cm^{-1} denotes O-H stretches corresponding to amines and alcohols and phenols, 1563 cm^{-1}indicates to α-CH_2bending corresponding to aldehydes and ketones. The peak at 1641 cm^{-1} denotes to NH_2 corresponding to amines and primary amide bends related to carboxylic acids and their derivatives, 1355 cm^{-1} denotes O-H bonding corresponding to alcohols and phenols, 678 cm^{-1} denotes C-H deformation corresponding to alkynes. A peak at 2096 cm-1 indicates that the Silver atoms reduced [Rivallan *et al* 2010].

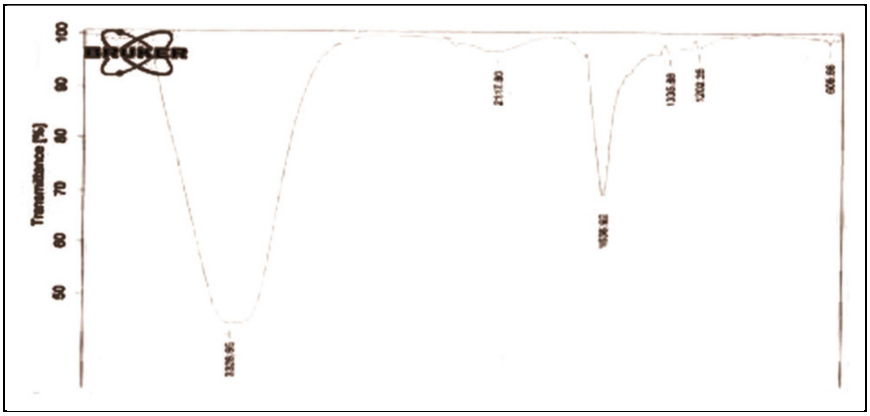


Figure 2: FTIR spectroscopy analysis of AgNPs synthesized using *A.vasica*

X-ray diffraction analysis [XRD]

XRD pattern of biosynthesized AgNPs [Figure 3] shows four intense peaks in the spectrum values ranging from 10 to 80 XRD spectra of pure crystalline silver structures have been published by the Joint Committee on Powder Diffraction Standards [File no.04-0783]. A comparison of our XRD spectrum with the standard confirmed that the silver particles formed in our experiments were in the form of amorphous and crystalline phases coexist. The crystalline peaks at 2θ values of 27.6 °, 32 °, 37.9 °, 44 °, 46 °, 64.3 °, 77°can be indexed as 1 1 1, 1 1 1, 1 1 1, 2 0 0, 1 0 0, 2 2 0, 3 1 1 planes of fcc silver.

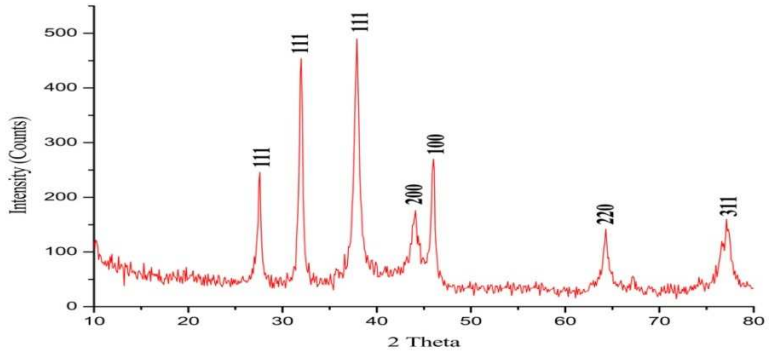


Figure 3: XRD analysis of AgNPs synthesized using *A.vasica*

Scanning electron microscope [SEM] analysis

SEM image shown in Figure 4, [X20,000 magnification] and 12[X30,000 magnification] confirms that the synthesized AgNPs are irregular and spherical in shape and in mono dispersed manner with average diameter range of 50 – 60 nm.

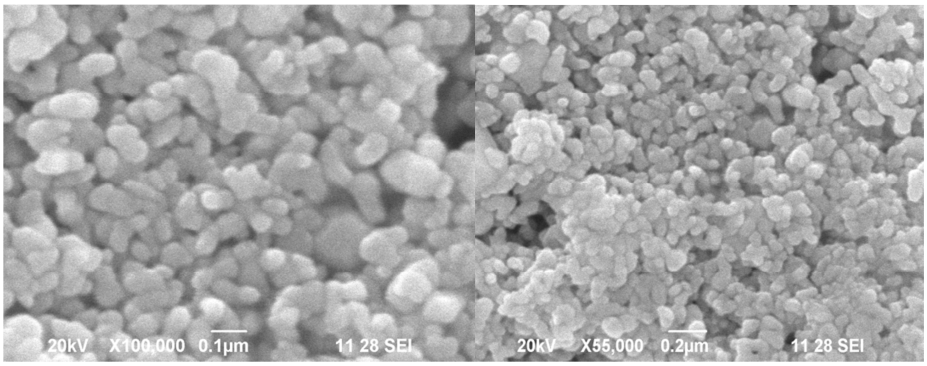


Figure 4: SEM analysis of AgNPs synthesized using *A.vasica*

Screening of Urinary Isolates

Different urinary isolates obtained from various urine samples from patients suffering from urinary tract infection [UTI] were further processed by culturing on MacConkey agar and a special chromogenic UTI agar and were identified by various biochemical tests. The antibiotic profile of the several antibiotics tested against the chosen multi drug-resistant bacterial isolates is as depicted in Table 1. The pattern of the resistances and sensitivities of the chosen bacterial isolates are also noted.

Screening for antibacterial activity by disc diffusion method

Activity of a given extract was determined by measuring the zones of inhibition [ZOI]. Because zones of inhibition were often asymmetrical, experiments were repeated three times and the average was recorded. Tables 2 &3 display the results of the antibacterial activity for the tested samples against the MDR isolates and standard strains respectively. Measured inhibitory zones displayed in the table shows that *Pseudomonas sp*. is highly sensitive exhibiting a zone of 16mm, followed by *S.aureus* and *Klebsiella sp*. It is also seen that the extract had no varied response to Gram reaction.

Table 1: Antibiogram Pattern for selected drug resistant bacterial pathogens

Bact/antibiotics	AMC	AK	CXM	NIT	GEN	NA	NX	CIP	OF	LE
Staphylococcus aureus	R	S	R	R	R	R	S	MS	S	S
Pseudomonas aeruginosa	R	S	R	R	S	R	R	S	S	S
Proteus vulgaris	S	S	MS	R	S	S	S	S	S	S
E.coli	R	S	R	S	S	R	R	R	R	R
Enterobacter	S	S	MS	R	S	R	S	R	R	S
Streptococcus sp.	R	S	R	S	S	R	R	R	R	R

R-Resistant
MS-Moderate Sensitive
S-Sensitive
AMC-Amoxyclav (10mcg)AK-Amikacin (10mcg)CXM-Cefuroxime (30mcg)
NIT-Nitrofurantoin (300mcg)GEN-Gentamycin (50mcg)NX-Norfloxacin (10mcg)
NA-Nalidixic acid (30mcg)CIP-Ciprofloxacin (5mcg)OF-Ofloxacin (2mcg)
LE-Levofloxacin (5mcg)

Table 2: Mean Zones of Inhibition (mm) of the silver nanoparticles synthesized using aqueous extract of *Adathoda vasica* (1%) , plant extract alone (1%) and silver nitrate (1mM) against a few drug resistant bacterial pathogens

Bacteria (MDR clinical isolates)	Zones of inhibition in mm. PE	SNP	AgNO3
Staphylococcus aureus	13.2±1.03	21.21±1.09	5.03±1.1
Pseudomonas aeruginosa	17.97±1.31	22.54±2.03	6.12±1.06
Proteus vulgaris	16.67±1.76	20.67±1.87	5.16±1.78
E.coli	13.71±1.06	18.66±1.44	6.05±1.06
Enterobacter sp.	12.98±1.11	16.89±1.67	5.78±2.05
Streptococcus sp.	13.08±1.34	18.45±1.89	5.61±1.78

Table 3: Minimal inhibitory concentrations (µg/ml) of the silver nanoparticles synthesized using aqueous extract of *Adathoda vasica* (1%), Plant extract alone (1%) and Silver nitrate (1mM) against a few drug resistant bacterial pathogens

Bacteria (MDR clinical isolates)	MIC (µg/ml) PE	SNP	AgNO3
Staphylococcus aureus	500 µg	250 µg	1000 µg
Pseudomonas aeruginosa	500 µg	250 µg	1000 µg
Proteus vulgaris	1000 µg	500 µg	1000 µg
E.coli	500 µg	250 µg	500 µg
Enterobacter	250 µg	125 µg	1000 µg
Streptococcus sp.	500 µg	500 µg	1000 µg

Biofilm Production

The strong biofilm forming capability and antibiotic resistant isolates indicate the powerful contribution in the pathogenesis of urinary infections. The results indicate that the extract caused a significantly higher reduction in

biofilm-forming ability of the isolates (graph 1 & fig 5). The variation of the biofilm production in the control and the extract treated cases suggest that the chosen plant plays an important role in combating urinary infections.

Graph 1: Effect ofthe silver nanoparticles synthesized using aqueous extract of *Adathoda vasica* (1%), Plant extract alone (1%) on biofilm production by a few drug resistant bacterial pathogens

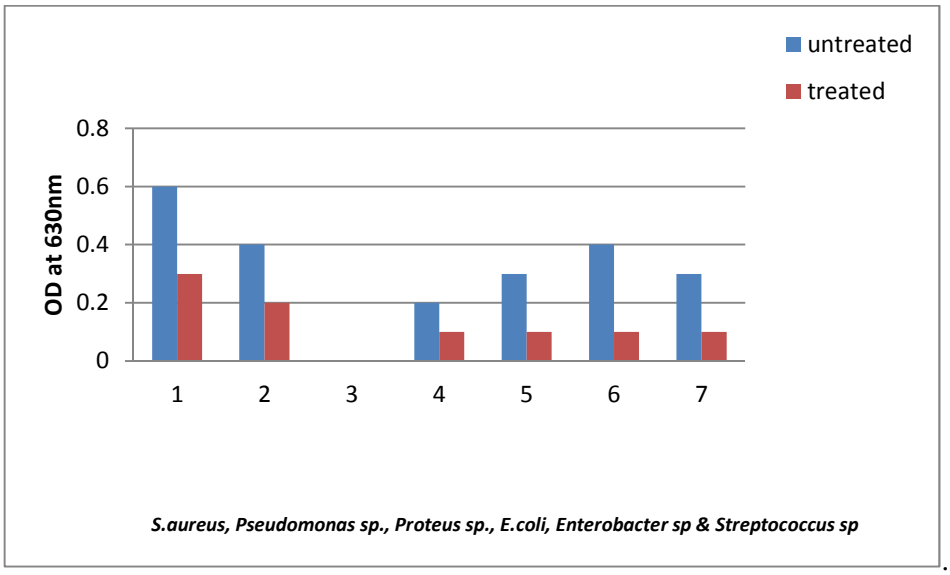
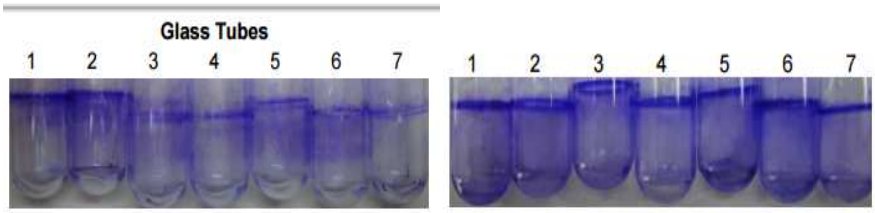


Figure 5:Biofilm formation in glass tubes in the presence of plant extracts and water as control



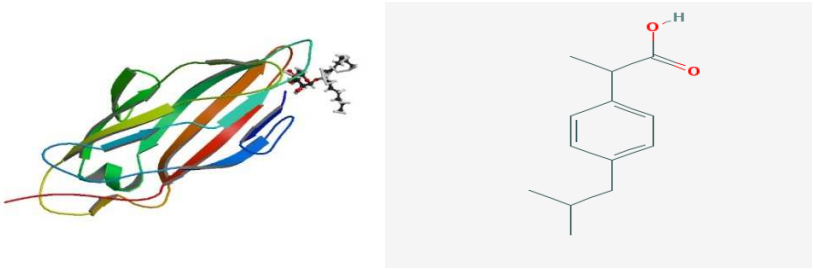
(a)After 12 hours **(b)After 24 hours**

Docking analysis

Receptor FimH is known to play a fundamental role in fimbriae attachment of bacteria to cell surface. Receptor based molecular docking of Fim H against natural compound as ligand (2-4-but-2-yl phenyl propanoic acid) has been carried out. The selected natural compound was docked with the three dimensional structures of FimH (PDB ID: 4XOC), retrieved from the protein data bank using maestro 9.3(Schrodinger).Molecular docking identifies the thermodynamic optimal energy values, types of interactions, potential of bonding and conformation against the receptor protein molecules.Molecular docking results revealed a high docking score value -3.5. Therefore, the interactions identified had hydrogen bonds with a bond length less than three, confirming the stability of the interaction.

Fig.6: a-3D structure of FimH from PDB(4XOC), b-2-4-but-2-yl phenyl propanoic acid Pubchem ID 3672 from *A vasica* & c-Interacting amino acids as predicted and enfolding of compound in the active pocket

a **b**



c

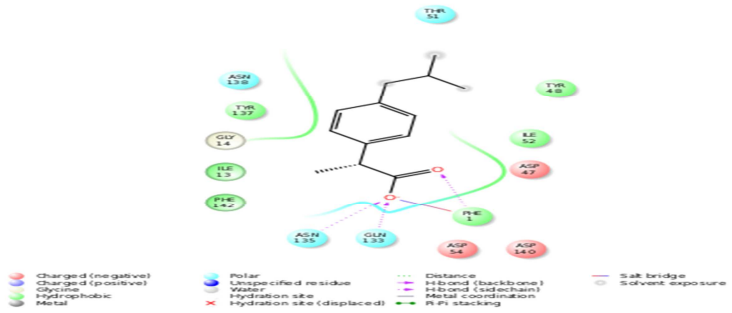


Table 4: The docking score and properties of the compound from *A.vasica*

Title	Docking score	Glide evdw	Glide energy	No of Bonds	Interactive residue	H bond length (Å)
	-3.502	-16.722	-24.629	3	PHE 1- O	2.17
					PHE 1- O-	4.57
					ASN 135-O-	1.96

CONCLUSION

Thus the silver nanoparticles stabilized by *Adathoda vasica* has shown to possess antibacterial activity and also have the ability to prevent anchorage of bacteria to human cell wall, which may lead to the death of the bacterium.

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