J Nanostruct 9(2): 376-383, Spring 2019

RESEARCH PAPER

Ta-AgNps are Potential Antimicrobial Resistance Breakers

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ARTICLE INFO

Article History:
Received 22 October 2018
Accepted 08 January 2019
Published 01 April 2019

Keywords:
Antibacterial Activity
Antioxidant Activity
Phytochemical Screening
T.Arjuna

ABSTRACT

An easy and rapid microwave-assisted green synthesis of silver nanoparticles (AgNPs) was carried out using aqueous bark extract of *arjuna* and their antibacterial and anti-biofilm potential was investigated. In the field of medicine, nanomedicines are gaining importance throughout the world for the treatment of different diseases. The AgNPs were characterized by various techniques .The FTIR data revealed the presence of plant organic constituents and metabolites bound to TA-AgNPs, which contributes for their stability. To elucidate the antibacterial efficacy and biofilm inhibition of Ta-AgNps against multidrug-resistant. Pathogenic *E.coli* harbouring the ESBLs. Treatment of TA-AgNps inhibited the growth of human pathogenic (both gram positive and gram negative) bacterial strains including *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), *Proteus mirabilis* (ATCC 35659), *Acinetobacter baumannii* (ATCC 19606) and *Methicillin-Resistant Staphylococcus Aureus* (MRSA). Further, the clinical isolates of *E.coli* that are resistant and susceptible to antibiotics are utilized to test the efficacy of TA-AgNps. In *E.coli*, ESBLS are responsible for antibiotic resistance. Among, ESBLs CTXM-15 encoding gene is more prevalent in South India. Moreover, docking study with T.arjuna phytochemical constituents confirmed that the phytochemical constituents present in TA-AgNPs interacted well with CTXM-15 and highest interaction was observed with tannic acid. Hence the expression of gene encoding CTXM-15 was screened in clinical isolates. Interestingly, the expression of CTXM-15 was not observed in samples treated with TA-AgNps. The results suggested that AgNPs of *Terminalia arjuna* bark extract can be used to control multidrug-resistant *E.coli* to prevent the antibiotic resistance. To the best of our knowledge, this is the first attempted study to show the effectiveness of Ta-AgNps against multidrug-resistant *E.coli* harbouring CTXM-15.

How to cite this article
Akther T, Priya S, Kumar Sah S, Shahanbaj Khan M, Hemalatha S. Ta-AgNps are Potential Antimicrobial Resistance Breakers. J Nanostruct, 2019; 9(2): 376-383. DOI: 10.22052/JNS.2019.02.019

INTRODUCTION

Nanotechnology is an emerging field in the area of multidisciplinary research especially in biomedicine [1]. The way of nanoparticle synthesis is inferable and application are gaining extreme importance in interdisciplinary research[2]. Silver is a nontoxic inorganic antimicrobial factor and is efficient in the killing of 650 pathogenic microorganisms [3] and is considered as a future generation antimicrobial substitute [4]. Different plant extracts isolated from various parts of the plant such as bark, leaf, fruit pulp, fruit, root and callus [5] have been studied so far for the synthesis of nanoparticles of different sizes and shapes [6]. The T.arjuna belongs to the family Combretaceae, commonly known as Arjuna, is one of the medicinal plants exploited from ancient age for its antidiuretic and cordial property. Along with, cardiac efficacy its bark and leaf extract has been utilized for treating diabetes, bleeding disorders, respiratory disorders, tuberculosis, and cancer [7]. The phytochemical analysis revealed that the extract of T.arjuna bark contains different phytoconstituents like flavonoids, tannins, saponin, and sterols [8]. The present study was carried out to synthesize silver nanoparticles

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from T.arjuna bark extract and characterized by UV-visible spectrophotometer, FT-IR and HRTEM. Furthermore, the efficacy of nanoparticles was investigated against human pathogenic bacterial strains. Beta-lactamases are bacterial enzymes which hydrolyze the beta-lactam ring, usually found in all the beta-lactam antibiotics, resulted in antibiotic resistance [9]. Escherichia coli and Klebsiella species are two major gram-negative bacteria which produce extended-spectrum beta-lactamases (ESBLs) [10]. ESBLs are encoded by blaTEM, blaSHV and blaCTX-M [11]. ESBLs undergo continuous modification and at present, there are more than 300 types of ESBL variants [12]. ESBLs produced by E. coli and Klebsiella species cause urinary tract infections (UTIs) [13]. The ESBL production was evaluated in clinical isolates of E. coli through phenotypic testing. The antibacterial effect and biofilm formation of Ta-AgNPs, against E. coli clinical isolates, was evaluated and correlated with ESBL production especially gene encoding CTX-M-15 which is more prevalent in South India. The nano informatics studies of Ta-AgNPs phytochemical constituents with CTXM-15 revealed that tannic acid interaction score was higher compared to other Phyto-compounds. Additionally, the antibiotic sensitive and resistant clinical isolates of *E.coli* were used to compare the efficacy of TA-AgNPs on growth, biofilm formation and CTX-M15 gene expression.

MATERIALS AND METHODS

Ethical clearance

This study was permitted by the institutional Ethics Committee of School of Life Sciences, B. S. Abdur Rahman Crescent University (Ref. no. BSAU: REG-OFF: 2016/02SLS), Vandalur, Chennai.

Extraction of T.arjuna

The mature bark of T.arjuna was collected from the B.S. Abdur Rahman University, campus, Vandalur, Chennai (TN, India). The bark was washed thoroughly with tap water and air dried in the shade and was ground to fine powder using an electric grinder, which was then stored in airtight container and was kept in dark, dry and cool place for further use.

Phytochemical analysis

Methanolic and aqueous extracts of Terminalia arjuna bark were utilized for qualitative and quantitative analysis. The alkaloids, carbohydrates, saponin, tannin, flavonoids, steroids and terpenoids were estimated by using standard protocols [14].

Free radical scavenging assay

Free radical scavenging activity of aqueous and methanol extracts of the bark of T.arjuna was carried out with 1,1-Diphenyl-2-Picryl Hydrazyl (DPPH) [15].

Green synthesis of silver nanoparticles

Silver nanoparticles from Terminalia arjuna bark were synthesized using a microwave. In a typical reaction procedure, 5mL of aqueous bark extract solution was added to 10 ml of 1 mM aqueous silver nitrate solution with constant stirring. The solution mixture was then exposed to microwave irradiation at a temperature of 90° C with a fixed frequency of 2.45 GHz. After microwave irradiation, the resultant colloidal solutions were naturally cooled to room temperature [16]. The formation of silver nanoparticles from T.arjuna bark extract was confirmed by UV-VIS spectrophotometer (Eppendorf Bio Spectrometer) at 200-800nm wavelengths. The purification of silver nanoparticles was done by centrifugation (9,000 rpm for 20 minutes) and the supernatant was discarded and the pellet was washed two to three times with double distilled water. The AgNPs obtained were allowed to air dry and stored until further use.

Characterization of silver nanoparticles

The FTIR spectra (Perkin Elmer) were used to determine the functional groups bound to the silver nanoparticles. The air-dried AgNPs were used and examined by IR spectrum at the spectral range of 400 – 4000cm-1 Fourier. Morphology, size and composition of T.arjuna AgNPs were determined by scanning electron microscopy (SEM), High-Resolution Transmission Electron Microscopy (HR TEM) (JEOL TEM, USA) and EDAX.

Antimicrobial activity by well diffusion method

The antibacterial activity of silver nanoparticles, aqueous extract, and methanolic extract were determined by the well diffusion method [17] against both gram-positive and gram-negative bacterial strains such as *Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), Salmonella typhimurium (ATCC 14028), Proteus mirabilis (ATCC 35659), Acinetobacter baumannii (ATCC 19606) and Methicillin-Resistant Staphylococcus Aureus(MRSA)*

(ATCC 33591) The bacterial lawn was prepared on sterile nutrient agar plates by using a sterile L rod, wells of approximately 7mm diameter were made by using cork borer. AgNPs, methanol extract and aqueous extract 20µl of each sample and a positive control (Ampicillin, 10µg/ml) were loaded in the wells respectively (1: positive Control, 2: AgNPs, 3: Methanolic extract, 4: Aqueous extract). The plates were incubated overnight at 370C. After, 24 hrs of incubation, zone of inhibition was measured and recorded.

Collection and maintenance of E.coli clinical isolates

A whole set of 83 different clinical isolates of *E. coli* were obtained from Department of Microbiology, Tagore Medical College and Hospital, Chennai, India. The bacterial isolates were identified by following Bergey's manual of bacteriology which includes a morphological appearance of the colonies, staining methods, and biochemical properties [18].The clinical isolates were maintained as glycerol stocks for further use.

Antibiotic susceptibility testing

The clinical strains were tested for antimicrobial susceptibility test by disc diffusion method [19].In brief, each test isolate was seeded on MHA. After incubating overnight at 37°C, one colony from each isolate was inoculated in sterile peptone broth and the OD was adjusted to 0.5 McFarland standard. The suspension was swabbed on surface of MHA plate and left for 5-8 min. For antibiotic susceptibility testing, antibiotic discs such as amoxicillin (10 µg), aztreonam (10 µg), cefotetan (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefepime (30 µg), cefpodoxime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), levofloxacin (5 µg), meropenem (10 µg) were used and complete setup was incubated at 35±2°C for 18-24 hours.

Phenotypic test for ESBL production

All the clinical strains showing the diameter of less than 26 mm for cefotaxime, less than 21 mm for ceftazidime, less than 18 mm for cefepime and less than 19 mm for amoxicillin were selected for ESBL production through phenotypic analysis. Phenotypic ESBL confirmatory test was executed in accordance with CLSI guidelines (CLSI 2012) by using quality control reference strain of *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 700603). All the isolates were selected on the basis of initial screening. A combined disc diffusion test was done by placing a combination of antibiotic discs such as: ceftazidime (CAZ 30 µg) vs ceftazidime/clavulanic acid (CAZ/CA 30/10 µg), cefotaxime (CTX 30 µg) vs cefotaxime/clavulanic acid (CTX/CA 30/10 µg), cefepime (CPM 30 µg) vs cefepime/clavulanic acid (CPM/CA 30/10 µg), amoxicillin (20 µg) vs amoxicillin/clavulanic acid (AMC/CA 20/10 µg) on Mueller Hinton Agar (MHA) plates on which inoculum was spread. The interpretation of test result was done by following CLSI guidelines and an approximate increase of greater than or equal to 5 mm zone of inhibition for the disks containing CTX/CA, CAZ/CA, CPM/CA, AMC/CA versus the comparative and alone CTX, CAZ, AMC disc, was taken as ESBL positive.

Antimicrobial activity of clinical strains of E.coli through well plate method

Antimicrobial activity of Ta-AgNps was evaluated against ESBL positive susceptible and resistant strains of *E. coli* and one ATCC *E. coli* strain by agar diffusion test following CLSI guidelines (CLSI 2012). The inhibition zone was determined by measuring the diameter of the clear zone around each well.

Static biofilm assay

The effect of TA-AgNps on biofilm formation by *E. coli* ATCC and pathogenic strains was carried out [20] . Overnight *E. coli* cultures were diluted in fresh Brain heart infusion broth medium (1:100) in borosilicate glass tubes which contained Ta-AgNps. Tubes without Ta-AgNps served as positive (with bacteria) and negative controls (only broth). All cultures were incubated for 24h. After proper incubation, the medium was discarded and the tubes were washed with double distilled water followed by the addition of 0.1% crystal violet in all tubes. After 15 min of standing, the additional dye was rinsed with PBS. The emergence of biofilm was confirmed by the formation of a violet ring in the tubes.

In-silicostudies-Molecular docking

Molecular docking of 9 different active components of *T.arjuna* was carried out with CTX-M-15, an important enzyme that is produced by ESBL producing *E. coli* strains, giving them resistance to different antibiotics.SYBYL®-X 1.3 (http://www.tripos.com) was used for molecular docking. A total of 9 bioactive compounds from Ta-AgNPswere utilized as ligands for molecular docking studies. The three-dimensional structures

of all the bioactive compounds used were retrieved from PubChem and the final optimized ligands were used for molecular docking. The crystal structure of bacterial target protein CTX-M-15 (PBD ID: 5T66) was retrieved from PDB database (http://www.rcsb.org). Receptor protein was made free from all the crystallographic substructures and water molecules. This was followed by the addition of necessary hydrogen atoms and charges along with Gasteiger - Huckel. Trivial portable operating system (TRIPOS) was employed for minimization process and protein ProtoMol was automatically generated. The final structure was viewed using PyMOL (http://www.pymol.org).

Molecular docking

In this particular study, a total of 9 compounds were docked with CTX-M-15 protein to get the binding affinities, hydrogen bonds and hydrophobic interactions.

RESULTS

Extraction and analysis of phytochemicals

The qualitative analysis of aqueous and methanolic extracts of T.arjuna revealed the presence of carbohydrates, saponin, tannins, flavonoids, alkaloids and terpenoids (Fig. 1c).

DPPH scavenging Activity

The maximum free radicals scavenging activity was observed in methanolic extract at 200µl concentration when compared to aqueous extract (Fig. 1d). The results suggested that T.arjuna bark extract has free radical scavenging activity and this activity could be due to the presence of phytochemical constituents in the extract.

Characterization of silver nanoparticles

The synthesis of sliver nanoparticles resulted in change in the colour of solution from yellowish brown to dark brown due to surface resonance plasmon of nanoparticles. The UV–Visible spectrophotometer analysis of silver nanoparticles showed the maximum absorption in the range of 400-500nm. The maximum absorbance of AgNPs was recorded at 450nm which confirmed the synthesis of silver nanoparticles (Fig. 1a). Results are correlated with the previous studies[21]

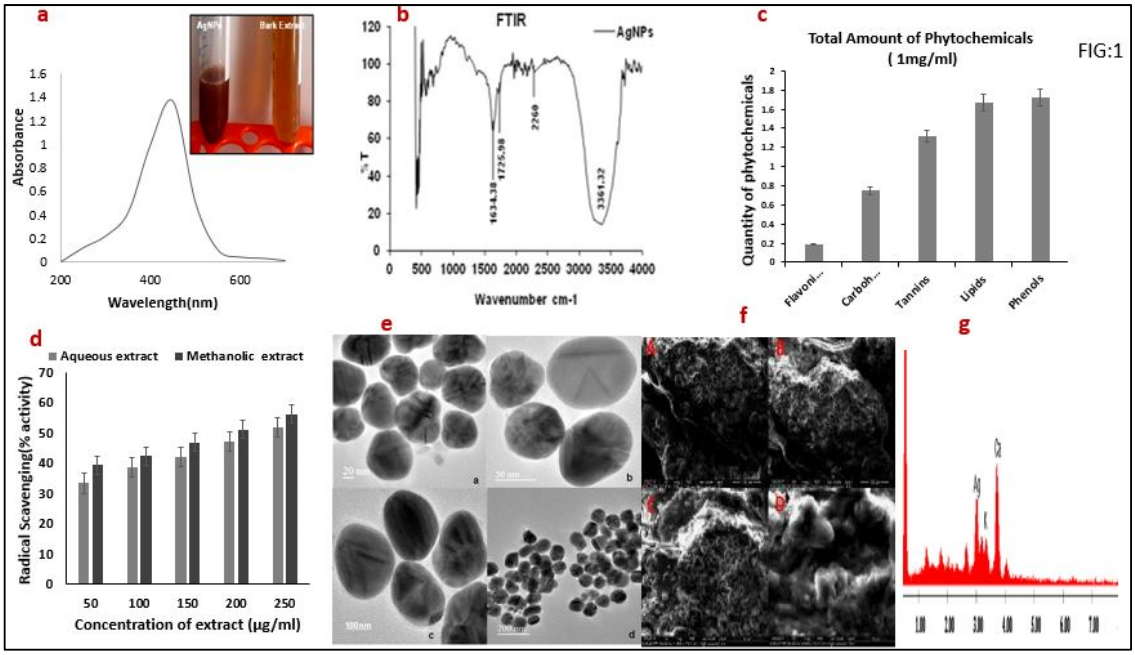
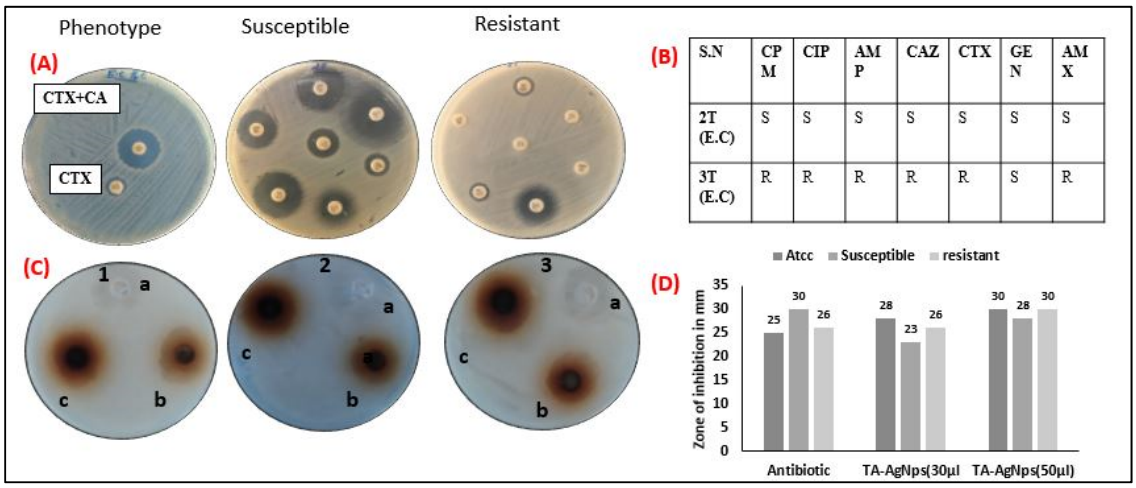


Fig. 1:(a) Synthesis of silver nanoparticles from bark extract of *T. arjuna* inside showing color change from yellowish to dark brown and UV-VIS spectra of AgNPs showing maximum absorbance at 450 nm. (b) The FTIR analysis of Ta-AgNPs synthesized from bark extract of *T. arjuna* showing the presence of different functional groups.(C) Quantitative phytochemical analysis of bark extract of *T.arjuna* and (d) Free radical scavenging activity of Aqueous and methanolic extract. Error bars indicate the SE of triplicates.(e) High Resolution Transmission Electronic Micrograph (HR-TEM) of silver nanoparticles with particle size of [(a) 20 nm (b) 50nm (c) 100nm and, (d) 200nm].(f)Scanning Electronic Micrograph (SEM) images of silver nanoparticles at different magnifications.(g) EDAX image of silver nanoparticles synthesized from bark extract.

Silver nanoparticles are bound with many different types of biomolecules, Hence, to identify and characterize these biomolecules FTIR spectroscopic analysis was carried out. The spectrum of AgNPs of T. arjuna bark extract exhibited enumerate peaks at 1634.38, 1725.98, 2260.0 and 3343.32 which might be stretches of (N–H) primary amines, (C=O) Aldehyde, saturated aliphatic, (C≡N) Nitriles, (O–H) H–bonded alcohols, phenols respectively and confirmed the presence of phytochemical constituents are incorporated inside Ta-AgNps(Fig. 2b).21

The external morphology such as size, shape and composition of AgNPs biosynthesized from bark extract of *T.arjuna* was determined by HR-TEM, SEM and EDAX analysis. The SEM analysis revealed lattice interference fringes and confirmed the aggregation of smaller nanoparticles (Fig. 1f). Most of the nanoparticles are spherical in shape with the size range from 20-100 nm (Fig. 1f). Results of the study have in agreement with earlier reports [22, 23]. Some of the nanoparticles have



S.N	CPM	CIP	AMP	CAZ	CTX	GEN	AMX
2T (E.C)	S	S	S	S	S	S	S
3T (E.C)	R	R	R	R	R	S	R

Fig. 2: (A and B) Phenotypic confirmatory test for ESBL production using combined disk diffusion test against clinical isolate *E. coli* was showing zone diameter around the disk containing, cefotaxime + clavulanic acid (CTX+CA) ≥ 5 mm than the zone diameter nearby the disk containing CTX alone(A and B) CPM: Cefepime, CIP: Ciprofloxacin, AMP: Ampicillin, CAZ: Ceftazidime, CTX: Cefotaxime, GEN: Gentamicin, AMX: Amoxicillin, S: Sensitive, R: Resistance and Antibiotic screening according to CLSI recommendation. (C) Antibacterial activity of Ta- AgNPs against human bacterial pathogens 1(*E. coli* -ATCC) ,2(*E.coli Susceptible),3(E.coli -Resistant*) (a(Antibiotic),b(Ta-AgNPs 30µl),c(Ta-AgNPs 50µl)) by well diffusion method (D) showing Zone of inhibition in graphical representation.

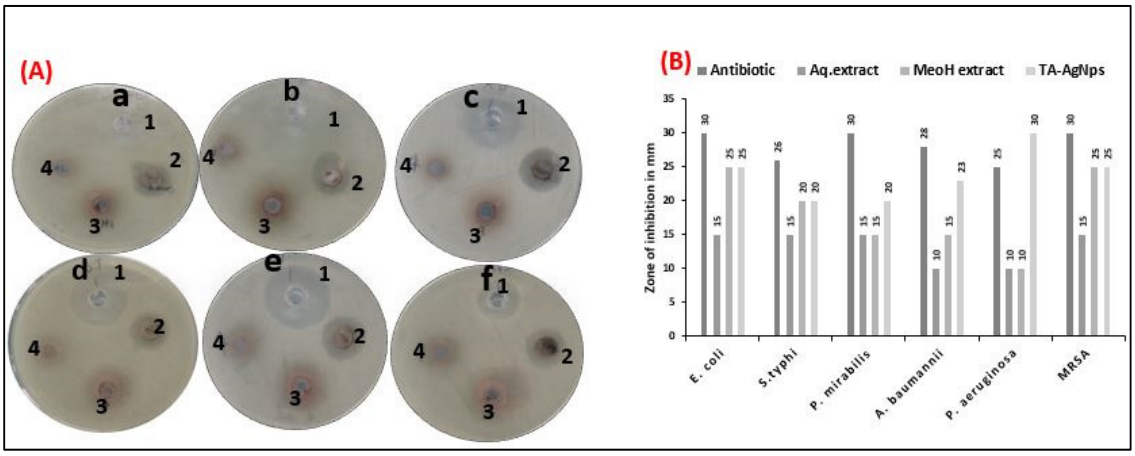


Fig. 3: (A and B) Antibacterial activity of aqueous and methanolic extract of *T. arjuna* bark and TA- AgNPs against human bacterial pathogens (a) *E. coli*(b) *P.aeruginosa* (c) *A. baumannii* (d)*S. typhimurium*(e) *P. mirabilis* (f)*Methicillin Resistant Staphylococcus Aureus* (MRSA) by well diffusion method andGraphicalrepresentation

Table 1:CTXM-15 interaction with different compounds present in *Terminalia arjuna*

S.No	Compound Name	Pubchem Id	Total_Score	Crash	Polar	D_SCORE	PMF_SCORE	G_SCORE	CHEMSCORE	CSCORE	GLOBAL_ CSCORE
1	TANNIC ACID	16129778	11.7327	-5.2799	9.7837	-388.9514	-84.989	-330.0013	-0.2323	5	4
2	Arjunone	14034821	5.6757	-0.7264	1.0994	-122.5695	3.9637	-167.7988	-14.6418	2	2
3	Arjunolic acid	73641	5.2248	-2.027	3.8681	-96.0919	-18.9453	-181.2937	-16.3431	3	2
4	NONANOIC ACID	8158	5.1006	-0.5205	2.7827	-77.6567	39.548	-165.0527	-8.6791	4	2
5	Gallic acid	370	4.4472	-0.4776	5.1984	-69.6761	-6.5237	-65.1551	-10.8947	3	2
6	Ellagic acid	5281855	3.9736	-0.4336	3.8403	-89.978	-47.3349	-85.282	-13.7738	3	2
7	Arjunic acid	15385516	3.7668	-2.1032	0.9694	-103.1865	-17.8627	-204.8596	-17.3694	3	1
8	Erythrodiol	101761	3.4439	-3.2248	1.6708	-97.2322	-9.7697	-209.1991	-15.9271	5	1
9	Apigenin	5280443	3.2338	-2.5057	2.0405	-95.3183	-12.0356	-159.0619	-18.1288	5	1

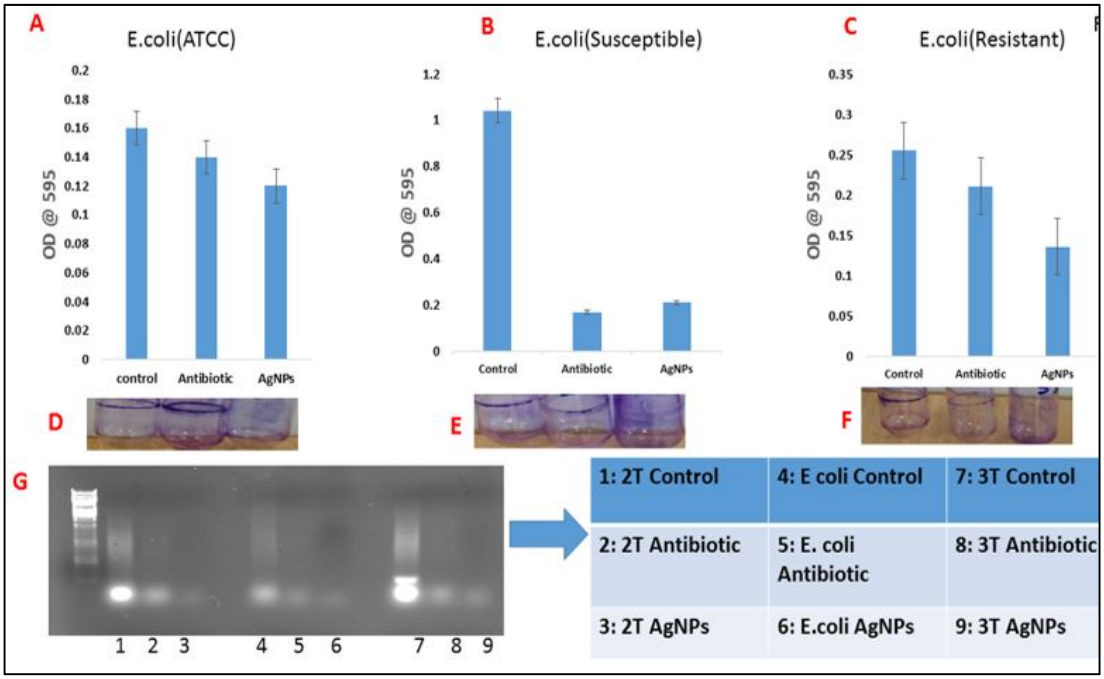


Fig. 4: (A-F) The quantification of ATCC, Susceptible and resistant biofilm formation was evaluated by crystal violet staining after 24 h of challenge with Antibiotic, TA-AgNPs and without treatment. A gradual decrease in biofilm formation was observed in TA-AgNPs treated strains as compared to Control (Untreated strains).While (G) represents the CTXM-15 gene expression through PCR. The band intensity are clearly visible in the diagram which shows the downregulation of CTXM-15 gene when treated with the antibiotic and TA-AgNPs

clustered together and showed some variation in the size of nanoparticles. (Fig. 1g) confirmed the presence of silver.

Antibacterial activity

Antibacterial activity against human bacterial pathogens displayed varying degree of zone of inhibition with Ta-AgNPs when compared to methanol and aqueous extract of *T.arjuna* bark. The maximum zone of inhibition (25 mm) was observed in *E. coli*, *P. aeruginosa* followed by *A.baumannii* (23mm), *S.typhimurium, P.mirabilis,* and *MRSA* (20mm) with AgNPs. While the zone of inhibition in aqueous and methanol extracts were between (10-25mm) (Fig. 3 a, b].

In-silico studies

A total of 9 compounds from *T.arjuna* were utilized for nano informatics studies (Table 1). The three-dimensional structures of all these bioactive compounds were retrieved from PubChem and the final optimized ligands were used for molecular docking. Docking analysis of CTX-M-15 with the 9 bioactive compounds along with the residues and the bond length of each compound is shown in (Fig. 5).

Detection of phenotypic ESBL

Out of 83 clinical isolates, 82% was found to be resistant to at least one of the third generation and fourth generation antibiotics including

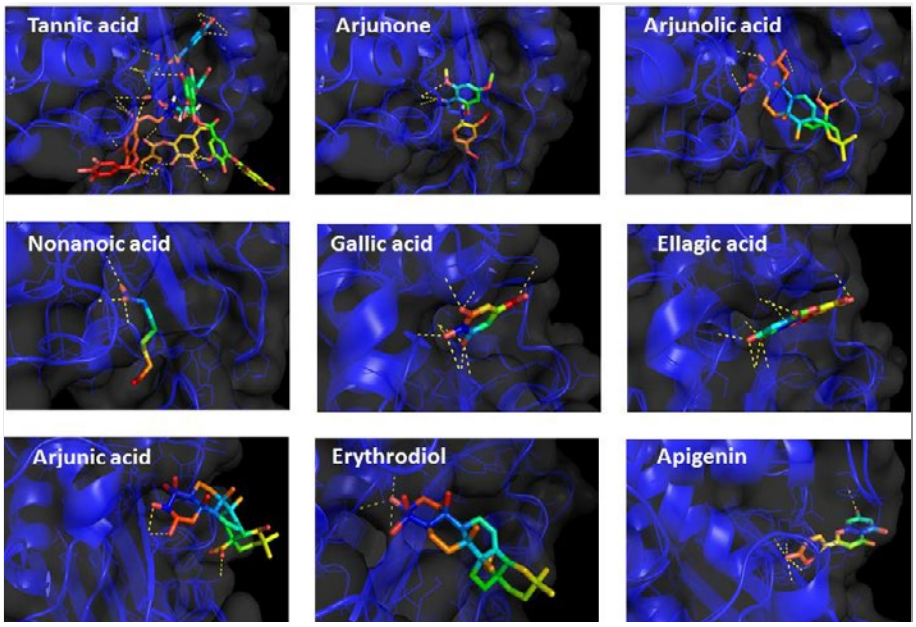


Fig. 5:Molecular docking of CTXM-15 with 9 different compounds from *T. arjuna*

cephalosporin or ampicillin that are used, by the disk diffusion test. In the CLSI phenotypic confirmatory test using 3rd & 4th generation cephalosporin/clavulanic acid combination antibiotic disc, all seventy-eight strains were analysed highly susceptible to ceftazidime, cefotaxime, cefepime and amoxicillin in the presence of clavulanic acid, thereby confirmed the production of ESBL by these clinical isolates of *E.coli.* The isolates showed positive for at least one of the confirmatory tests and by utilizing CAZ/CLA, CTX/CLA, CPM/CLA, and AMX/AMC disk.

Antibiotic Susceptibility Testing

The general resistance of ESBL producers to a variety of antibiotics was as follows: meropenem (31%), imipenem (5%), aztreonam (88%), cefpodoxime (88%), cefotetan (42%), gentamicin (41%), ciprofloxacin (65%) and levofloxacin (61%), respectively (Fig. 3), therefore revealing multidrug resistance. MIC determination of ESBL positive isolates to cefotaxime showed that all of them were in range of 128 to >256 µg/ml, ceftazidime were in the range 128 to >256 µg/ml, cefepime were in the range 16 to >256 µg/ml and amoxicillin were in the range of 32 to >256 µg/ml [Fig. 2 a, b] therefore exhibiting a higher range of resistance against the 3rd and 4th generation cephalosporin, particularly ceftazidime.

Antibacterial activity through well plate method

Ta-AgNps weretested against susceptible and resistant ESBL producing pathogenic isolated of *E.coli* and ATCC strain of *E. coli.* Antibiotic streptomycin was used as a positive control and Ethyl Acetate was used as a negative control. Two different concentrations of Ta-AgNps were used to check the antibacterial effect ranging from 30µg/ml-50µg/ml of extract showed better zones of inhibition against ESBL producing strains of *E. coli.* (Fig. 2 c, d).

Static biofilm assay

The biofilm formation was inhibited by Ta-AgNps in ATCC and pathogenic strains of *E.coli*. In both susceptible and resistant strains of *E.coli* the Ta-AgNPs inhibited biofilm formation when compared to control and antibiotic treatment (Fig. 4 a, b, c).

DNA isolation and gene expression analysis by PCR

The genomic DNA of ESBL producing strains of *E. coli* was isolated and amplified for $bla_{CTX\text{-}M\text{-}15}$ encoding gene to analyse the gene expression after treatment with Ta-AgNps. In susceptible strain, there is no expression when treated with antibiotics and Ta-AgNps. However in resistant strain the expression was observed in control and not in the treatment. Furthermore, ATCC strain of *E. coli* which was used as a negative control showed no expression at all (Fig. 4 g).

CONCLUSION

The green synthesis of silver nanoparticles from bark extract of *T.arjuna* and characterization of synthesized nanoparticles was executed and verified through UV-VIS spectrophotometer, FTIR and TEM approaches. The nanoparticles gives spherical appearance with smooth edges and their size conglomerate from 20 to 200 nm but the most of the nanoparticles ranged from 20 to 50 nm. The antibacterial potential of AgNPs reveals maximum efficacy with 20µg/ml to counter the both gram positive and gram negative pathogenic strains. Furthermore, the bark of *T.arjuna* provides a strong foundation of green synthesis of silver nanoparticles with an alternative potential drug for the inhibiting broad spectrum human pathogenic strains.

ACKNOWLEDGEMENT

We are thankful to B. S. Abdur Rahman University, Chennai for providing the fellowship to Saroj Kumar Sah, Mohd Shahanbaj Khan and research facility to conduct the research. We also acknowledge the NCCR, and Chemistry department IIT Madras, Chennai, for HRTEM, SEM with EDAX analysis.

CONFLICTS OF INTEREST

Authors declare they have no actual or potential conflict of interest.

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