Original Article

TANp – a Potent Antibiotic Resistant Breaker

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Abstract

Background: Nanoresearch is currently an area of intense scientific research, due to its potential applications in biomedical, optical, and electronic fields.

Objectives: *Trachyspermumammi* which is commonly known as 'Ajwain' is used to treat many medical ailments.Ajwain extract was prepared from fresh Ajwain powder and utilized for green synthesis of silver nanoparticles.

Materials and Methods: The synthesis of *Trachyspermumammi* silver nanoparticle (TANp) was confirmed through various biophysical characterizations, including UV-VIS spectroscopy, FTIR, FESEM and EDAX. The antimicrobial activity of the TANp was tested against *E.coli* ATCC (25922) and pathogenic Ampicillin resistant clinical isolate of *E.coli* strain.

Results: The expression of genes encoding AmpC was analysed using polymerase chain reaction (PCR), to check the effect of TANp on these genes involved in antibiotic resistance in *E.coli*.

Conclusions: The current study suggested that TANp acted as a potential antibiotic resistance breaker by controlling the growth and biofilm formationin pathogenic E.coli by modulating the expression of AmpC gene.

Key words : Green Nanoparticle, Trachyspermumammi,Antimicrobial activity, AmpC

Introduction

Nanoparticles exhibit novel properties which depend on their size, shape and surface charge. Nanoparticles and Nanomaterials are frequently evolving in various new fronts such as food, biomedical, health care, drug-gene delivery, mechanics, chemical industries, optics, catalysis, electronics, single electron transistors, light emitters, space industry, energy science and photochemical applications. Silver nanoparticles have shown excellent bactericidal properties against a wide range of micro-organisms. Many approaches are used for the synthesis of nanoparticles such as chemical, biological and green synthesis methods.[1]

The chemical and physical approaches are relatively costly and conceivably dangerous to the environment, because of use of harmful materials for nanoparticle synthesis which leads to various biological disorders. Green synthesis uses bacteria, fungi and plants-based compounds, has gained a great interest in modern years, because it is eco-friendly, easily scaled up and economical. Naturally derived compounds are used as capping agent for the stabilization of silver nanoparticles (AgNPs). AgNPs have capability to kill bacteria by penetrating into the cell membranes. AgNPs have been reported to be more effective antibacterial agent towards different bacteria and fungi even at very low concentration.[2]

Gram-negative bacteria such as Escherichia coli, are accountable for many of the infections which are hospital acquired. Drug resistant E.coli producing AmpC enzymes are responsible for major infections such as nosocomial infections, urinary tract infection and other life threatening diseases which gradually lead to death. AmpC-type β-lactamase are the enzymes produced by the organism which confers resistance to cephalosporins.[3] Hence, there is an immediate need to formulate alternative to existing antibiotics. Herbal based AgNPs can play a crucial role in controlling the multidrug resistant pathogens, and it could be potent alternative to antibiotics. Herbal based nanoparticles can be formulated in different forms for the public health application. There are reports supporting the antimicrobial properties of green nanoparticles against Gram-negative pathogens and Gram-positive pathogens. In addition, nanoparticles are more effective, because of high-surface-area-to-volume ratio which can combat numerous bacterial cells when compare to pure ionic silver.[4]

Ajwain (*Trachyspermumammi*) is well known for its characteristic aroma and pungent taste. Ajwain has several health benefits, including digestive stimulant, toothache, common cold, lessen greying of hair, Arthritis, constipation, kidney disorders, asthma, excessive flow and uneven periods, and to reduce body weight. Ajwain seed is reported to contain fibers, carbohydrates, tannins, saponins, glycosides, flavones, proteins, fats and minerals such as nicotinic acid, phosphorous, calcium and iron. Ajwain contains essential oil such as thymol, carvone, limonene and dillapiole. Ajwain have many pharmacological activities such as antihypertensive, broncho-dilator, antilithiasis, antiplasmodic, antidiuretic, abortifacient, to prevent platelet aggregation, anti- inflammatory, antitussive, antifilarial, detoxification of toxins including aflatoxins, ameliorative, antimicrobial, hypolipedomic, digestive stimulant and antinematicidal activity.[5]

The present research work focused on synthesis of green nanoparticles using the aqueous extract of *Trachyspermumammi* (Ajwain) and physiochemical characterization to confirm the synthesis of nanoparticle (TANp). The antimicrobial properties of TANp was validated through various assays such as Minimum Inhibition Concentration (MIC), Minimum Bactericidal Concentration (MBC), Biofilm inhibition assay and AmpC gene expression assay.

Materials and Methods

E.colistrains such as ATCC (25922) and clinical isolate of biofilm forming, pathogenic, Multi Drug Resistant (MDR) strain of *E.coli* was obtained from Tagore Medical College and Hospital, Chennai after proper ethical approval from BSACIST (Ref. no. BSAU: REG-OFF: 2016/02SLS).

Extraction of Trachyspermumammiseed

Trachyspermumammi (Ta) extract was prepared by soaking 5g of fresh seed (Trachyspermumammi) in 100mL of sterile distilled water. The soaked seeds were kept overnight at 37°C shaker. Next day the mixture was boiled at 80°C for 30 min. Then the solution was allowed to cool at room temperature. Finally, the Ta-extract was filtered through Whatman No 1 filter paper. The filtrate was stored at 4°C for further use.[6]

Qualitative phytochemical analysis of *Trachyspermumammi* extract

The phyto chemical compounds present in the *Trachyspermumammi* extract were qualitatively screened.[7]

Synthesis of *Trachyspermumammi* mediated Silver Nanoparticles (TANp) and physiochemical characterization

Trachyspermumammi nanoparticle synthesis (TANp) was achieved by mixing 1 volume of the extract with 4 volumes of 1 mM silver nitrate solution. The solution was incubated in dark at 37°C for overnight under static condition.The bio-reduction of silver nitrate solution into *Trachyspermumammi* nanoparticle (TANp) was periodically monitored for visual colour change. Once the strong colour change was observed the synthesis of nanoparticle was confirmed using Ultraviolet-Visible (UV) spectrophotometer by taking the absorption spectrum in the range 100 -800 nm (Jasco V-730 spectrophotometer). Followed the confirmation of SPR peak of TANp, the mixture was centrifuged for 20 min at 15,000 rpm in a cooling centrifuge. The resulting TANp was washed with sterile distilled water to remove the impurities. The resulting pellet was dissolved in 30% DMSO and subjected to mild sonication. The resulting TANp was used for further experiments.[8]

Fourier Transform Infrared spectroscopy (FT-IR) was carried out to observe the functional groups present in the *Trachyspermumammi* extract and (TANp) which would have acted as capping and reducing agent during the synthesis of TANp. The extract and TANp was dried and mixed with KBr pellets individually and analysed for FTIR spectrum by scanning in the range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1}.[9]

Size, shape and morphology of TANp was obtained through Field Emission Scanning Electron Microscopy (FESEM). Small amount of the sample was placed on a carbon coated copper grid and prior to visualization grids were allowed to dry at room temperature. The elemental analysis of TANp was

qualitatively detected through EDAX equipped with FESEM (SIGMA HV—Carl Zeiss with Brukern Quantax 200—Z10EDS Detector).[8]

Antibacterial assay

Antibacterial activity of TANp was validated through determining the growth rate, minimum inhibitory concentration, minimum bactericidal concentration, biofilm inhibition and AmpC gene expression assaysby screening E.coli ATCC (25922) and antibiotic resistant clinical isolate. Before carrying all the assays bacterial strains were revived in fresh LB medium at 37°C incubator shaker (Scigenics India Pvt ltd) at 110 rpm until the culture reaches optical density (OD) of 0.1 (correspond to ~1X10^8 CFU/ml) at 600 nm.[9]

Determination of Growth rate, Minimum Inhibitory Concentration, Minimum Bactericidal Concentration

TANps were examined for their inhibitory activity against two bacterial strains such as Escherichia coli (ATCC 25922), AmpC producing pathogenic clinical strain of E.coli. The Minimum Inhibitory Concentration (MIC) was examined using broth dilution assay.[10] TANp was serially diluted in microtiter plate with the initial concentration of 1 mg/mL (100, 50, 25, 12.5, 6.25, 3.125, 1.5, 0.8 µg/ml). Bacterial culture without TANp was kept as control and strains treated with ampicillin was considered as positive control. Microtiter plate was incubated at 37°Cshaker for overnight. The concentration at which it completely inhibits the visible growth of organism was calculated as MIC. After 24 h of incubation the growth rate of organism was observed by measuring the microtiter plate OD at 600 nm. From the absorbance the influence of TANp on growth rate can be plotted. Minimum Bactericidal Concentration (MBC) was determined by drop plate method. The wells with invisible growth of organism was chosen, 2 µl of culture from selected well were inoculated in LB agar plates. The plates were inoculated at 37°C for overnight. After 24 hours of incubation, the concentration at which the organisms are completely killed are noted for MBC.[11]

Antibiofilm Assay

To evaluate the efficacy of TANp against biofilm formation, the experiment was performed in 96 well microtiter plate. 100 µl of LB broth was filled in wells and serially diluted with 100 µL of TANp with initial concentration of 1 mg/ml. 2 µl of grown culturewas inoculated in all wells. The experiment was performed along with control and Ampicillin treatment. Then the plates were incubated for 24 hours at 37°C. After the period medium was discarded and washed with autoclaved water to remove planktonic bacteria. Biofilm formation was observed by staining the biofilm with crystal violet dye (0.1%, w/v) and incubated for 15 minutes. Unbound stain was removed by washing thrice, with mild flow of water. Then the plates should be dried completely. After drying, 200 µl of 30% glacial acetic acid was added to the wells. The absorbance at 570 nm was measured using multimode reader and absorbance obtained was considered as index for the biofilm formation.[12, 13]

Effect of TANp on controlling the expression of antibiotic resistant gene AmpC

Template DNA for Polymerase Chain Reaction (PCR) was prepared by Heat lysis method. 150 µl of overnight Culture was taken from control, Ampicillin treated and TANp treated cultures of *E.coli* ATCC (25922) and clinical isolate. Culture was centrifuged at 13000 rpm for 10 minutes. After discarding the supernatant, the pellet was washed in 100 µl autoclaved water by spinning at 3000 rpm for 10 minutes. The pellet was suspended in 100 µl of autoclaved distilled water. The cells were mixed well and incubated at 100°C for 15 minutes to lyse the cells and were centrifuged at 3000rpm for 5 minutes and supernatant was transferred to fresh tubes and used for amplification.

PCR was carried out using AmpC primers. For amplification of AmpC gene, Forward primer, 5' –ATT CCG GGT ATG GCC CGT- 3' and reverse primer 5' –GGG TTT ACC TCA ACG GGC -3' were used. PCR Master mix comprises of primers-1 µl each, master mix 5 µl and 3 µl of template DNA. The PCR reactions were performed in a thermocycler according to the following cycles: Initial denaturation of (94 °C, 5 min), 35 thermal cycles, (95 °C, 1min; 55°C, 44s; 72°C, 1 minute and 50 s) and final extension (72°C, 5 minutes). The PCR products were electrophoresed on 1.8% agarose gels (0.24 g agarose, 30 ml 10X, 0.5 µL ethidium bromide).[6, 8]

Results and discussion

Extraction and Phytochemical screening of *Trachyspermumammiextract*

Trachyspermumammi extract was prepared as per the procedure described in materials and methods and screened for phytochemical constituents. From the test it was observed the *Trachyspermumammi* contains phytocompounds such as phenols, saponins, tannins, steroids, flavonoids. These phytocompounds plays a crucial role in nanoparticles synthesis (Table 1).

Extract	Phenol	Tannins	Flavonoids	Saponins	Anthocyanins	Terpenoids	Alkaloids	Steroids	Carotenoids
Trachyspermumammi	+	+	+	+	-	-	-	+	-

Table 1: Phytochemical analysis of Trachyspermumammiextract

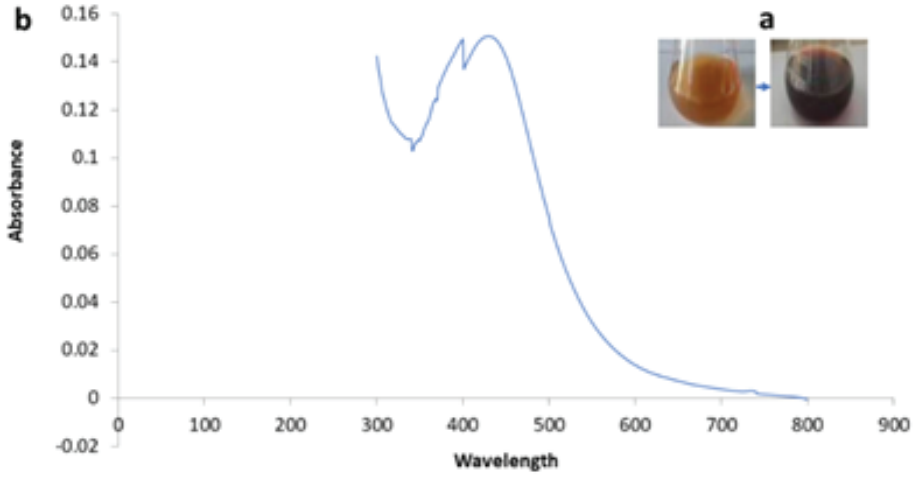


Figure 1a: Inlet figure represent the colour change during Trachyspermumamminanoparticle (TANp) synthesis.
Figure 1b: UV Vis absorption spectrum of TANp, SPR peak at 435nm confirms TANp synthesis

Biosynthesis and Physiochemical characterisation of TANp

Trachyspermumammi extract was mixed with 1 mM AgNO3 solution in the ratio of 1:4. After 24hours of incubation. The colour change was observed from yellowish to dark brown (Inlet Figure 1a), which confirmed the reduction of ionic silver. The colour change is the preliminary signature for the reduction of ionic silver into *Trachyspermumammi* nanoparticle (TANp). Phytochemicals such as phenol, saponins, steroids and flavonoids help in the bioreduction of ionic silver to form TANp. 14UV- Vis spectrum is the preliminary characterisation technique to confirm the synthesis of TANp. The surface plasmon resonance peak at 435 nm confirmed the synthesis of TANp as shown in Figure 1b.

The functional group responsible for the TANp synthesis was confirmed by FTIR spectrum. FTIR spectrum of both the extract and TANp were taken and depicted in figure 2a & 2b.

These are the groups which increase the activity of nanoparticles and act as reducing, capping and stabilising agent during the synthesis of TANp.[6] The IR- spectra of *Trachyspermumammi* extract and TANp revealed the existence of different stretches and various functional groups including alkynes, alcohol, nitro compound, aromatic, phenol, flouro, benzene and carboxyl groups (Table2 & 3). These

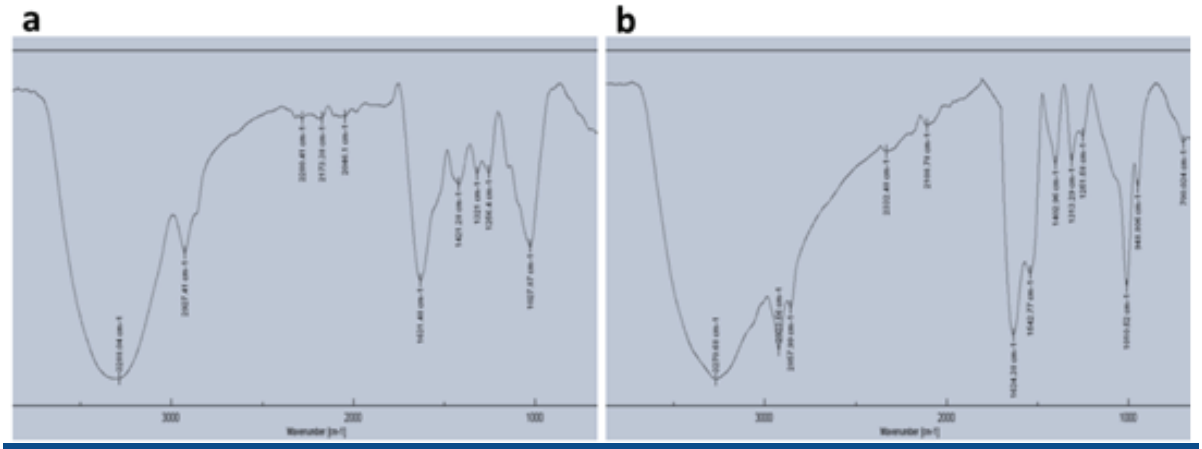


Figure 2a: FTIR spectrum of Trachyspermumammiextract
Figure 2b: FTIR spectrum of Trachyspermumamminanoparticles (TANp)

Frequency (/cm)	Bond	Functional group
3288.04	OH stretch	Alcohol
2927.41	=C-H stretch	C-H stretch
1631.48	C=O	Amide
1421.28	C=C	Aromatic compound
1027.87	C-F	Flouro compounds

Table 2: Different peaks of FTIR spectra of Trachyspermumammiextract showing the different bonds and functional groups

Frequency (/cm)	Bond	Functional group
3270.68	O-H stretching	Alcohol
2923.56	O-H stretching	Carboxylic acid
2857.99	O-H stretching	Alcohol
2332.48	O=C=O stretching	Carbon dioxide
2108.78	C≡C stretching	Alkynes
1634.38	C-H bending	Aromatic compounds
1542.77	N-O stretching	Nitro compound
1402.96	S=O stretching	Sulfonyl chloride
1313.29	O-H bending	Phenol
1251.68	C-O stretching	Alkyl aryl ether
1010.52	C-F stretching	Fluoro compound
700.34		Benzene derivative

Table 3: Different peaks of FT-IR spectra of *Trachyspermumammi* nanoparticle (TANp) showing the different bonds and functional groups

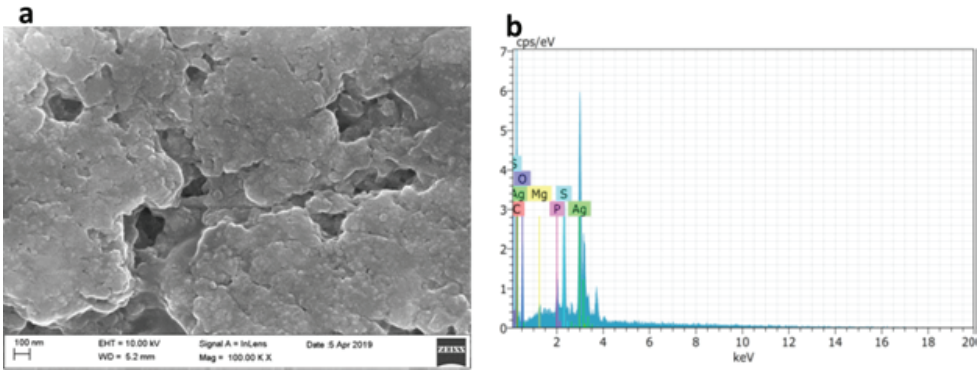


Figure 3a: FESEM microscopic image of TANP at 100.00 KX magnification, shows the spherical shaped TANp.

Figure 3b: EDAX analysis confirms the Ag as one of the element in TANp along with other element C, O, Mg, P, S from phytochemical components.

functional groups may be derived from the phyto-constituents, proteins and enzymes that are present in the aqueous extract of *Trachyspermumammi*.

The structure, shape and size of the TANp was detected using field emission scanning electron microscopy. At 100.00 KX magnification, the size was found to be less than 100nm, and it was spheri cal in shape. The size and shape of TANp would be the major factor for its potent antibacterial activity (Figure 3a). EDAX results reveals elemental composition of TPNp contains Ag, Mg, P, S, C, O. The strong signal at 3 Kev confirms the presence of silver. The percentage weight of C, Ag, O, S, P, Mg are 37.39%, 32.50%, 21.90%, 6.16%, 1.56%, 0.49% respectively.

The percentage atoms of C, Ag, O, S, P, Mg are 61.70%, 5.97%, 27.13%, 3.81%, 1.00%, 0.04% respectively (Figure 3b). There were literatures supporting that *Trachyspermumammi* extract contains minerals such as phosphorous. Along with Silver, other elements such as Carbon, Magnesium, Sulphur and phosphorous act synergistically to improve its potent antibacterial activity.[5]

Antibacterial Activity of TANp

Assessment of MIC, MBC and Growth curve: The bacteriostatic and bactericidal concentration of TANp was calculated by measuring the minimum concentration of TANp which was required to inhibit and kill the growth of ATCC and clinical isolate. The MIC concentration of *E.coli* ATCC (25922) and clinical isolate was found to be 6.25 µg/ml and 12.5 µg/ml respectively (Figure 4a). MBC concentration-of E.coli ATCC (25922) and clinical isolate was found to be 12.5 µg/ml. The MIC concentration was higher for clinical isolate, when compared to ATCC, but the MBC concentration was same (Table 4). This assay shows that TANp has potent antibacterial activity by showing potent bacteriostatic and bactericidal activity even at low concentration, when compared to Ampicillin treated strains. The clinical isolate, which is multidrug resistant strain, was very susceptible to TANp even at the concentration of 12.5 µg/ml.

There are several reports supporting the fact that green nanoparticles could be potential alternative to antibiotic resistant strains. There are several mechanisms through which nanoparticles can easily target the cell. One such mechanism is nanoparticles provide large surface to volume ratio for cells to come in contact with the nanoparticles. So that nanoparticles can easily produce pits on the surface of cell wall, so the cell wall will lose its membrane permeability, which in turn increases ROS of the cell

STRAIN	Concentration(µg/ml)	
	MIC	MBC
E. coli ATCC(25922)	6.25	12.5

Table 4: MIC and MBC of TANp required to inhibit and kill the growth of E.coliATCC (25922) and clinical isolate.

E.coli ATCC (25922) and clinical pathogen were grown in medium supplemented with 12.5 µg/ml of TANp. After 24 hours, the growth of the organism was followed by measuring absorbance at 600nm (Figure 4a). From this assay it was found that the growth rate of organism was decreased by 96% in both *E.coli* ATCC (25922) and clinical pathogen. There are reports supporting our result that TANp enter into the cell and intercalates with DNA, so the replication, transcription and translation of the cells will be stopped, hence multiplication of bacteria in the TANp supplemented media will be inhibited.[16]

Assessment of Antibiofilm activity

The biofilm inhibition potential of TANp in E.coli ATCC (25922) and clinical pathogen was assessed by growing bacteria in a medium supplemented with TANp. The percentage of biofilm formation wascalculated by measuring the absorbance at 570nm. From this assay it was concluded that TANp has very good biofilm inhibition activity. TANp can reduce the biofilm formation of *E.coli* ATCC (25922) and clinical pathogen by 81% and 92% respectively (Figure 4b). Biofilm formation is the major cause for several infection. From this result, it was observed that even at the concentration of 12.5 µg/ml, TANp can reduce the biofilm formation in multi drug resistant organism. TANp may act as a quorum quencher, which block the quorum sensing signals produced by the pathogenic organism during biofilm formation.[17] The

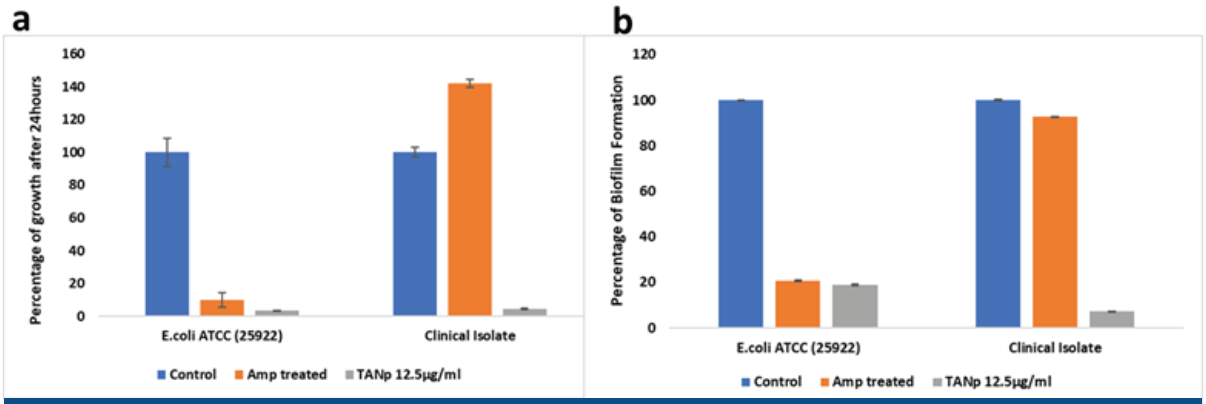


Figure 4a: Percentage growth of *E.coli* ATCC (25922) and clinical isolate after 24 ours of TANp treatment.

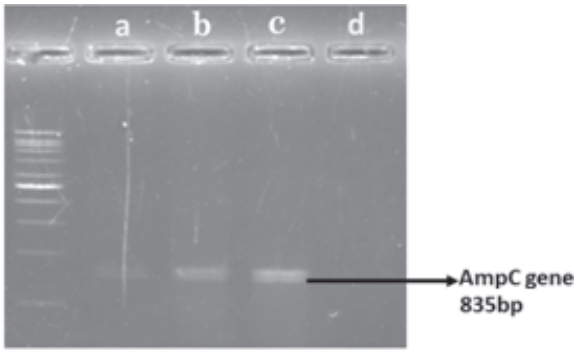
Figure 4b: Percentage of Biofilm formation of E.coli ATCC (25922) and clinical isolate after 24 hours of TANp treatment

phytocompound loaded TANp acts synergistically with all metals to combat the biofilm formation and it may reduce the severity of the disease. TANp showed distinct differences between ATCC and clinical pathogens, because the nature of TANp interacting with the cell varies from one organism to other. There are several physical, chemical and biological properties which may be involved during the interaction. Physical property of penetration, Chemical property like interaction between the biofilm and the nanoparticles, biological property of absorption into the cell, may vary between ATCC and clinical isolate.[18] These nanoparticles act in such a way that it decreases the secretion of extra polysaccharide, which is responsible for biofilm formation. The results suggest that TANpcan be used as a potent biofilm disruptor.

Effect of TANp on antibiotic resistant gene AmpC

AmpC gene amplification was carried to validate the effect of TANp treated E.coli ATCC (25922) and clinical pathogen. ATCC E.colidoes not express AmpC gene, but clinical isolate is multidrug resistant strain, which expresses AmpC gene. From the amplification result, it was observed that when the clinical pathogen was treated with sub MIC concentration 5µg/ml of TANp, the expression of gene was reduced when compared to the control and Ampicillin treated strain (Figure 5).

Well - a: showed down regulation in the expression of AmpC gene upon treatment with TANP when compared to its control (well - c) and ampicillin treated (well - b). Whereas in well – d: E.coli ATCC (25922) non-pathogenic strain does not express AmpC gene.



a) Clinical isolate treated with TANp
b) Clinical isolate treated with Ampicillin
c) Clinical isolate without any treatment
***d) E.coli* ATCC (25922) control**

Figure 5: Expression of gene encoding AmpC; a) Clinical isolate treated with TANp, b) Clinical isolate treated with Ampicillin, c) Clinical isolate without any treatment, d) *E.coli* ATCC (25922) control

This shows that TANp can effectively target the gene encoding AmpC, which is responsible for producing the enzyme which degrades the cephalosporin before its action. Thus,TANp could be an effective alternative in controlling the antibiotic resistant pathogens by playing a role as bacteriostatic, bactericidal, antibiofilm agent and suppressing the expression of antibiotic resistant gene encoding AmpC.

Conclusion

In this study, the TANp was synthesised by using simple, cost effective, rapid method for mass production. From this study, the antibacterial and antibiofilm activity of TANp was explored through various assays and found as very effective and cost-effective alternative to antibiotics. Further research on exploring and formulating different topical formulation could be validated for commercialisation of TANpfor multiple purposes.

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Conflict of interest

Authors have no conflict of interest

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