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Phytochemical profile, antioxidant, antimicrobial activity, antibiofilm, cytotoxicity, and HR-LCMS of *Tribulus terrestris*

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ABSTRACT

Background: Tribulus Terrestris (TT) is a Phytotherapeutic species employed in ethanopharmacological practices, stimulating spermatogenic and androgenic pathways, enhancing stamina, and promoting prophylaxis of nephrolithiasis. In this study, the focus will be on the phytochemical composition, of (TT), that contributes to antioxidant activity, antimicrobial, anti-biofilm properties.

Methods: The ethanolic extract of TT (ETT) was obtained through the Soxhlet extraction process, which was subsequently followed by rotary evaporation and lyophilization. A preliminary screening for phytochemicals was conducted, and the antioxidant activity was assessed using DPPH FRAP, and ABTS assays. Antimicrobial and antibiofilm assays were conducted. The cytotoxic effects of the ETT were evaluated utilizing L929 and HEK-293 cell lines. Scanning electron microscopy (SEM) was used to observe the morphological changes in bacteria. HR-LCMS analysis was conducted to identify its bioactive compounds.

Results: Phytochemical screening verified the existence of phenolics, alkaloids, saponins, glycosides, flavonoids, carbohydrates, proteins, amino acids, tannins, and steroids. The phenolic concentration was measured at 7.96 μg/mL, while the flavonoid concentration was found to be 23.22 μg/ml. The ethanolic extract exhibited antioxidant activity with IC_{50} values of 46.65 μg/mL (DPPH), 30.52 μg/ml (FRAP), IC50 = 41.42 μg/ml (ABTS). Cytotoxicity assays revealed that ETT was non-toxic to both L929 and HEK293 cell lines across all tested concentrations. ETT has an effect against MDR bacteria. ETT markedly decreased biofilm density in a concentration-dependent manner, showing the greatest inhibition at 200 mg/ml. This was further supported by strong correlation coefficients and statistically significant p-values ($p < 0.05$). SEM analysis indicated considerable cellular damage in both gram-positive and gram-negative bacteria. HR-LCMS analysis identified a diverse bioactive compounds, including alkaloids, flavonoids, phenolics, saponins, tetrapyrrolic compounds, triterpenoids, glycosides, steroids, fatty acids, indanes, and methoxyphenols.

Conclusion: The multifaceted bioactivity of (TT) ethanolic extract, such as antioxidant activity, antimicrobial, and anti-biofilm inhibition, stems from its diverse phytochemical metabolites. This underscores their relevance in traditional ayurvedic practices and validates their.

Abbreviations: ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid),; CRA, Congo Red Agar; DMSO, Dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ELISA, Enzyme-linked Immunosorbent Assay; ETT, Ethanolic extract of TT; FRAP, Ferric Reducing Antioxidant Potential; HEK-293, human embryonic kidney cells; HR-LCMS, High-resolution liquid chromatography-mass spectrometry; L929, mouse fibroblast cells; MAR, Multiple Antibiotic Resistance; MBC, Minimum bactericidal concentration; MIC, Minimum inhibitory concentration; MRSA, Methicillin-Resistant *Staphylococcus aureus*; MTT, Tetrazolium salt; SEM, Scanning electron microscopy; TFC, Total Flavonoid Content; TPC, Total phenolic content; TT, Tribulus Terrestris; UTI, Urinary tract infection.

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1. Introduction

Natural products derived from medicinal plants have long been recognized as a valuable source of health-promoting agents and therapeutic compounds. They play a crucial role in disease prevention and management due to their structural diversity, biological compatibility, and broad spectrum of pharmacological activities, including antioxidant, antimicrobial, anti-inflammatory, and cytoprotective effects. In recent years, growing concerns regarding antimicrobial resistance and adverse effects associated with synthetic drugs have renewed scientific interest in plant-based remedies as safer and more sustainable alternatives.[1]

Tribulus terrestris (TT), a small, leafy herbaceous plant belonging to the Zygophyllaceae family, is a renowned medicinal plant that has been utilized for centuries in Ayurveda, Traditional Chinese Medicine, and Unani medicine. Native to warm, arid regions of Asia, Africa, Europe, and Australia, TT has been traditionally valued for its diuretic, aphrodisiac, anti-inflammatory, and antimicrobial properties.[2] The pharmacological potential of TT is attributed to its diverse bioactive phytochemicals, including flavonoids, alkaloids, tannins, glycosides, and steroidal saponins. These compounds contribute to its antioxidant, antimicrobial, and anti-urolithic effects, making TT a promising candidate for managing urinary tract disorders, reproductive health issues, and metabolic diseases.[3] Notably, TT has been used to treat kidney stones, urinary infections, hypertension, and sexual dysfunction, while its role in enhancing testosterone levels and physical performance has garnered interest in sports nutrition.[4] Despite its extensive traditional use, comprehensive scientific validation of TT's mechanisms of action, safety, and therapeutic efficacy remains limited. Recent studies have explored its antibacterial, antifungal, and cytotoxic properties, yet a detailed phytochemical profiling coupled with bioactivity assessments is essential to bridge traditional knowledge with modern pharmacological applications.[5]

Recent evidence indicates that bioactive compounds derived from plants may positively influence urinary tract health and conditions associated with nephrolithiasis through their antioxidant, antimicrobial, and antibiofilm properties. Nonetheless, investigations correlating extensive phytochemical profiling with antibiofilm effectiveness and cellular safety assessment are limited.[6] This study seeks to systematically investigate the phytochemical composition, antioxidant potential, antimicrobial and antibiofilm properties, as well as the cytotoxic effects of TT extracts. Additionally, HR-LCMS will be employed to identify and quantify bioactive compounds, providing insights into their potential mechanisms and therapeutic applications. By elucidating the phytochemical profile and biological activities of TT, this research seeks to validate its ethnomedicinal uses and explore its potential as a source of novel drug leads for modern medicine.

2. Materials and methods

2.1. Plant Material

TT fruits were purchased from a commercial herbal supplier, Sree Lakshmi Trading Company, Tiruvalla, Kerala, India (Batch No: 21314159000004). Ensuring traceability and batch consistency. Two commercially packaged lots (2 kg each) belonging to the same batch were procured to minimize intra-batch variability and to reflect material available in the local herbal market. The fruit was cleaned thoroughly, shade-dried, and mechanically ground into a fine powder. For exhaustive extraction of bioactive constituents, approximately 30 g of powdered material was subjected to Soxhlet extraction using 250 ml HPLC-grade ethanol per cycle. The extraction was continued for several hours until the siphon tube solvent became colorless, indicating completion of the extraction. The obtained extract of TT was concentrated under reduced pressure using a rotary evaporator and subsequently lyophilized to obtain a dry ethanolic extract for further analysis.

2.2. Extraction yield

The yield of ETT was expressed as a percentage using the formula. $ExtractionYield = \frac{Finalextractweght}{Initialsampleweight} \times 100$ indicating the efficiency of the extraction process.[7]

2.3. Phytochemical screening test

2.3.1. Qualitative phytochemical screening

Preliminary qualitative phytochemical screening was performed using standard colorimetric methods to obtain an overview of the major classes of secondary metabolites present in the ETT. These assays were employed as preliminary phytochemical screening step to guide subsequent biological evaluations and to complement the detailed metabolite identification performed later using HR-LCMS analysis.

Alkaloids: Hager's test was conducted on 2 ml of ethanolic ETT, treated with Hager's reagent, while Wagner's test was conducted on 3 mL of ethanolic ETT, treated with Wagner's reagent.[8]

Saponins: The ETT was vigorously shaken with a few drops of sodium carbonate.[8]

Test for glycosides: The Keller-Killiani test involved combining 2 ml of ETT with 0.5 ml of ferric chloride and glacial acetic acid, followed by adding 1 ml of concentrated H_2SO_4.

Steroids: To detect steroids, combine concentrated sulfuric acid, chloroform, and ETT in a mixture and heat for five minutes.[9]

Test for phenolic and Tannins

The gelatin test detects phenolics by mixing ETT with a solution containing 10% NaCl. The lead tetra-acetate test uses ETT with a solution, while the iodine and ferric chloride tests measure tannins by adding neutral solutions.[10]

Tests for flavonoids: The alkaline reagent test involves mixing 2 ml of ETT with 2–3 drops of sodium hydroxide and a few drops of diluted hydrochloric acid.[10]

Carbohydrates: The presence of carbohydrates in the ETT is identified using qualitative tests such as Molisch's, Benedict's, and Fehling's, which include the addition of an alcoholic α-naphthol solution.[9]

Protein: Proteins in the ETT are detected using Millon's and Biuret tests. Millon's test combines the extract with a specific reagent, while the Biuret test involves adding sodium hydroxide and copper sulfate.[9]

Amino acid: The Xanthoproteic test uses two milliliters of plant sample extract and a few drops of nitric acid. The yellow hue shows the current amino acid.[10]

2.3.2. Quantitative phytochemical screening

Estimation of the total phenolic content

The total phenolic content (TPC) of the ETT was determined through the Folin–Ciocalteu reagent assay, and the results were reported as gallic acid equivalents (GAE) per gram of the dried extract. The reaction mixture was incubated at room temperature in the dark, and absorbance was measured at 765 nm using a UV–Vis spectrophotometer. Quantification was performed using a gallic acid calibration curve, and all measurements were conducted in triplicate.[8,11]

Determination of total flavonoid content

The total flavonoid content of the ETT was evaluated using the aluminum chloride colorimetric method, with quercetin used as the standard for measurement. Absorbance was recorded at 415 nm using a UV–Vis spectrophotometer, and quantification was based on a quercetin calibration curve. All experiments were performed in triplicate.[11]

2.4. Antioxidant assay

2.4.1. DPPH scavenging assay

The study involved adding 0.1 mM ethanolic DPPH solution to varying concentrations of ETT, shaking it, and incubating it in the dark for 30 min. Absorbance was recorded using a UV-spectrophotometer,

with gallic acid as the standard.[7,12] Absorbance was measured at 517 nm, and all measurements were performed in triplicate. The percentage of DPPH radical scavenging was determined using the following equation.:

$$DPPHScavengingactivity(\%) = \frac{(Acontrol - Asample)}{Acontrol} \times 100$$

The IC_{50} value, which indicates the concentration needed to attain 50% antioxidant activity, was calculated from the scavenging activity data.

2.4.2. Ferric reducing antioxidant potential (FRAP)

The ferric reducing antioxidant power (FRAP) assay was used to assess the antioxidant activity of ETT. Ascorbic acid was used as a standard, with calibration curve concentrations ranging from 6.25 to 100 μg/ml. The FRAP reagent was added to each sample or standard, and absorbance was measured at 593 nm using a UV-spectrophotometer[13,14]. All samples were incubated at room temperature, and experiments were conducted in triplicate.

2.4.3. ABTS radical scavenging assay

The neutralization of $ABTS\bullet^{+}$ radicals was monitored using the ABTS test to assess the ETT's capacity to scavenge free radicals. After preparing a stock solution of the extract (10 mg/ml), serial dilutions (6.25–100 μg/ml) were labeled T1–T5. After adding 7 mM ABTS solution to each sample, they were all left to incubate for 15 min in the dark. As the standard, 10 mM ascorbic acid was utilized. The absorbance was measured at 734 nm using a UV spectrophotometer. Each experiment was conducted three times to ensure accuracy and reliability.[15] The percentage of ABTS radical scavenging was calculated using the following equation:

$$\text{Radical scavegingctivity}\,(\%) = \frac{(AcontrolOD - AsampleOD)}{AcontrolOD} \times 100$$

2.5. Cytotoxic activity (MTT Assay)

The cytotoxic effects of ETT were evaluated using the MTT assay on L929 mouse fibroblast cells and HEK-293 human embryonic kidney cells. The cells were placed in 96-well plates and allowed to incubate overnight at 37°C. Cells were seeded at an appropriate density to ensure logarithmic growth during the assay. Various concentrations of the test compound, ranging from 0.01 to 100 μg/ml, were added, and the plates were incubated for another 24 h. Following this incubation period, MTT reagent was introduced to each well, and the plates were covered and incubated for 3 h. The resulting formazan crystals were dissolved using an appropriate solubilization solution prior to absorbance measurement. The assay included three controls: a medium control, a negative control, and a positive control (Doxorubicin). Absorbance was measured at 570 nm using an ELISA plate reader[16,17]. All experiments were performed in triplicate, and cell viability was expressed as a percentage relative to the untreated control. The selection of L929 and HEK-293 cell lines was based on their widespread use as representative normal mammalian cell models for preliminary cytotoxicity and biocompatibility assessment. L929 cells are commonly employed to evaluate general cytotoxic effects, and HEK-293cells were chosen to assess potential renal cellular toxicity, which is particularly relevant given the proposed application of ETT in urinary tract health and nephrolithiasis-related conditions. Together, these cell lines provide complementary insights into the general and organ-relevant safety profile of ETT under *in vitro* conditions[18].

2.6. Antibiotic susceptibility and Multiple Antibiotic resistance (MAR) analysis of bacterial strains

The study utilized multidrug-resistant (MDR) bacterial strains isolated from urinary tract infection (UTI) samples collected at Pushpagiri Medical College Hospital, Tiruvalla, Kerala, India. The identified isolates included *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, MRSA (Methicillin-Resistant Staphylococcus aureus),* and *Enterococcus faecalis.* The bacterial isolates were identified using standard microbiological and biochemical methods prior to susceptibility testing. The strains were grown on nutrient agar slants and incubated at 37°C for 24 h. The assessment of antibiotic resistance was conducted using the Kirby-Bauer disc diffusion technique. Bacterial inoculum density was adjusted to match the 0.5 McFarland standard before plating. The antibiotic discs (HiMedia) tested included Ampicillin, Cefazolin, Cefuroxime, Nalidixic Acid, Norfloxacin, Nitrofurantoin, Imipenem, Methicillin, Amikacin, Tetracycline, Cefepime, Levofloxacin, Fosfomycin, Erythromycin, Ofloxacin, Gentamicin, Ciprofloxacin, Penicillin, Chloramphenicol, Colistin, and Bacitracin. Bacterial cultures prepared in overnight nutrient broth were uniformly spread on Mueller-Hinton Agar plates using sterile swabs[19]. After incubation at 37°C for 18–24 h, inhibition zone diameters were measured and interpreted according to standard guidelines. The Multiple Antibiotic Resistance Percentage (MAR%) of the pathogen was determined using the following formula:

$$MAR\% = \frac{Noofantibioticstowhichtheisolateshowedresistance}{Totalnumberofantibioticstested}$$

2.7. Plant antimicrobial activity

The antibacterial effectiveness of ETT against pathogens causing urinary tract infections was evaluated using the Kirby-Bauer well diffusion method. Bacterial suspensions were standardized to 0.5 McFarland turbidity prior to inoculation. Extract solutions were prepared in dimethyl sulfoxide (DMSO) at 5%, 10%, and 20% w/v concentrations. DMSO was the negative control, while 1% w/v azithromycin was used as the positive control[19]. Aliquots of the extract were added into pre-punched wells on Mueller–Hinton agar plates, followed by incubation at 37°C for 24 h. Zones of inhibition were measured in millimeters, and all experiments were performed in triplicate.

2.8 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC): The resazurin microplate test was used to find the MIC of ETT against MDR bacteria. Following a series of dilutions of the extract (initial concentration of 200 mg/mL) in Mueller-Hinton broth, bacteria (1×10^8 CFU/ml) were introduced, and the mixture was incubated for 24 h at 37°C. Each assay was conducted in 96-well microplates, including growth and sterility controls. To identify live bacteria, resazurin dye was applied; the MIC is the concentration at which growth is inhibited. A color change from blue to pink indicated bacterial viability, while no color change indicated inhibition of growth. MBC was detected by subculturing onto Mueller-Hinton agar; the MBC with the lowest concentration did not exhibit any bacterial growth[2021]. All determinations were performed in triplicate to ensure reproducibility.

2.8. Biofilm formation in Congo red agar

Biofilm production was assessed semiquantitatively using the HiMedia Congo Red Agar (CRA) method, as outlined by Freeman et al. The CRA medium was made up of 100 ml of distilled water, ten grams of sucrose, three grams of agar, 7.4 g of brain heart infusion broth, and 0.16 g of Congo Red indicator. The Congo Red stain was autoclaved for 15 min at 121°C, after which it was combined with sucrose and autoclaved brain–heart infusion agar at 55°C. The medium was poured into sterile Petri plates and allowed to solidify under aseptic conditions. Following inoculation on CRA plates, the test organisms were cultivated at 37°C for 24 h. Biofilm formation was indicated by the appearance of black colonies[2223]. All observations were recorded visually, and experiments were performed in triplicate to ensure consistency.

Table 1
Qualitative analysis of phytochemicals in ETT.

Name component	Experiment	Results
Flavonoids	Alkaline reagent test	+++
	Shinod's test.	–-
Alkaloids	Mayer's test.	––
	Hager's test.	+++
	Wagner's test	+++
Glycosides	Keller Killiani test.	+++
Phenolic compounds and tannins	Ferric chloride test	–-
	Lead tetra acetic acid test	+++
	Glatein test	+++
	Iodine test	––
Saponins	Tests for saponins	+++
Triterpenoids	Horizon test.	+
Proteins	Biuret test.	–-
	Ninhydrin test.	–-
	Millon's test	+++
Amino acid	Xanthoprotein	+++
Carbohydrates	Molish test.	+++
	Benedict's test.	+++
	Fehling's test.	+++
Steroids	Salkowski test	+++
Starch	iodine test	–-

+++: more abundance,++:abundance,–:absence.

Table 2
IC_{50} values of ETT compared with standard antioxidants in DPPH, FRAP, and ABTS assays.

No	Sample	DPPH assay	FRAP assay	ABTS assay
1	ETT IC_{50}	46.65 ± 1.86 µg/ml	30.52 ± 2.01 µg/ml	41.42 ± 1..34 µg/mL
2	Standard IC_{50}	23.98 ± 1.97 µg/ml (Gallic acid)	14.72 ± 1.55 µg/ml (Ascorbic acid)	25.0 ± 0.96 µg/ml (Ascorbic acid)

2.9. Antibiofilm assay

The anti-biofilm activity of the ETT was assessed at concentrations between 12.5 and 200 mg/ml in test tubes that included the ETT, bacterial suspension, and sterile nutritional broth. The bacterial culture that used DMSO to promote biofilm production served as the positive control, whereas the negative control consisted of sterile nutrient broth without any extract. The initial bacterial inoculum was standardized prior to incubation. To assess biofilm production, the tubes were incubated for 72 h at 37°C, washed with phosphate-buffered saline (PBS), left to air dry, and subsequently stained with crystal violet. After washing, 1% acetic acid was used to dissolve the bound dye, and biofilm inhibition was assessed by measuring absorbance at 595 nm[24]. All assays were conducted in triplicate, and mean OD values were used for analysis. The ETT exhibited antibiofilm activity, as evidenced by a decrease in optical density (OD) values when compared to the positive control. The percentage inhibition of biofilm formation or disruption was calculated using the following formula:.$Inhibition Percentage(\%) = \frac{(ControlOD - TestOD)}{ControlOD} \times 100$

2.10. Scanning electron microscopy (SEM) analysis

The antibacterial effects of ETT on MRSA and *E. coli* were investigated through scanning electron microscopy (SEM) to examine changes in bacterial structure. Separate cultures of *MRSA* and *E. coli* were prepared in sterile Mueller-Hinton broth (MHB) and adjusted to the 0.5 McFarland standard. Each 100 ml culture was treated with 2 mL of ETT at a concentration of 200 mg/ml and incubated at 37°C for 24 h, while untreated cultures were kept as controls. All experiments were conducted under aseptic conditions. Following incubation, the cultures were centrifuged at 10,000 × g for 10 min, and the resulting pellets were rinsed twice with sterile Milli-Q water. The washing step was performed to remove residual medium and extract prior to imaging. A 10 µl sample from each suspension was placed on a sterile glass slide and allowed to air-dry. The dried samples were then coated with a thin layer of gold to improve conductivity and imaging clarity. Gold sputter coating was performed to a uniform thickness to minimize charging effects. SEM analysis was performed using a JEOL JSM-6390 microscope with a tungsten filament electron source. Imaging took place under high vacuum in both secondary electron (SE) and backscattered electron (BSE) modes at an accelerating voltage of 10 kV, with images captured at magnifications ranging from 500 × to 3,300 × to evaluate morphological changes in the bacterial cells[25].

2.11. Identification of bioactive compounds by HR-LCMS analysis

The ETT was analyzed using high-resolution liquid chromatography–mass spectrometry (HR-LCMS) at the Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay, Mumbai, India. The analysis was conducted with an Agilent 1290 Infinity UHPLC system connected to an Agilent G6550A iFunnel quadrupole time-of-flight (Q-TOF) mass spectrometer. The system could detect masses between m/z 120 and 1200, had a resolving power of about 40,000 full width at half maximum (FWHM), and was accurate to less than 1 ppm. Chromatography used a reverse-phase C18 column at 40°C to separate the samples. The mobile phase was made up of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). A gradient elution program was used, starting with 5% B and ending with 95% B after 25 min. The program stayed at that level for 30 min, and then it went back to the starting conditions for 35 min. The injection volume was 5 µl and the flow rate was 0.3 mL/min. We used a Dual AJS electrospray ionization (ESI) source to ionize the material. It could work with both positive and negative ions and had Auto MS/MS acquisition. The source settings were a capillary voltage of 3500 V, a nozzle voltage of 1000 V, a gas temperature of 250°C, a gas flow rate of 13 L/min, a nebulizer pressure of 35 psig, a sheath gas temperature of 300°C, and a sheath gas flow of 11 L/min.Using ramped collision energy, we did collision-induced dissociation. We used Agilent MassHunter software to get the data and process it after chromatography. Accurate mass measurements, matching isotopic patterns, MS/MS fragmentation patterns, and comparing with spectral databases and published literature were all used to figure out what the compounds were. Identified compounds were reported as phytochemicals that had not yet been fully characterized[2627].

2.12. Statistical analysis

We did all the experiments three times (n = 3), and the results are shown as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to do the statistical analysis. Differences were considered statistically significant at $p < 0.05$. Furthermore, Pearson's correlation analysis was conducted to assess the relationship between extract concentration and the percentage inhibition of biofilm formation, with the corresponding correlation coefficients (r) and p-values reported.

3. Result

3.1. Yield of extraction

The extraction yield of ETT was 13.3%, obtained using ethanol as the extraction solvent. The yield was calculated from an initial plant material weight of 300 g, which yielded 40 g of dried extract after lyophilization and evaporation of the sample.

3.2. Qualitative phytochemical screening

The presence of various phytochemicals in the ETT is summarized in

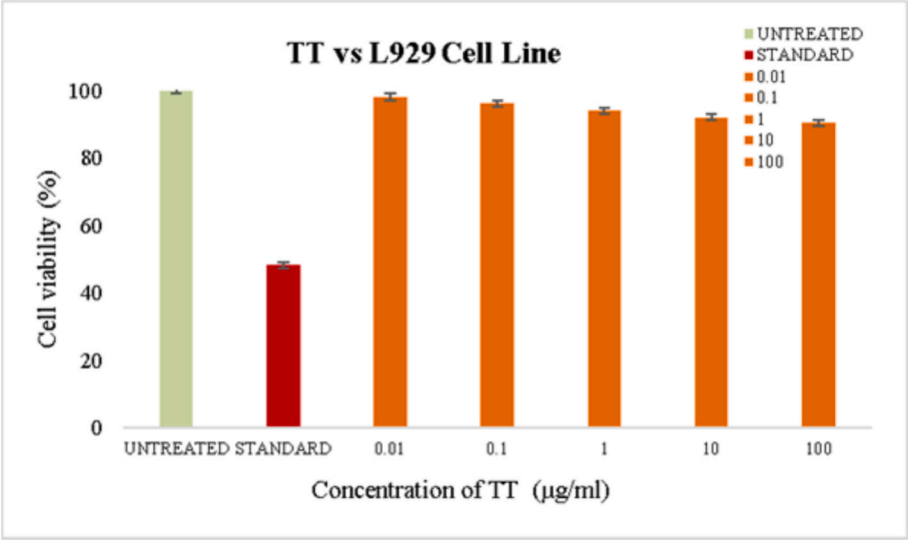

Fig. 1. Effect of ETT on L929 cell viability after 24 h of incubation. The findings reveal that ETT did not exhibit cytotoxicity and preserved high cell viability across all concentrations tested, indicating its biocompatibility and safety.

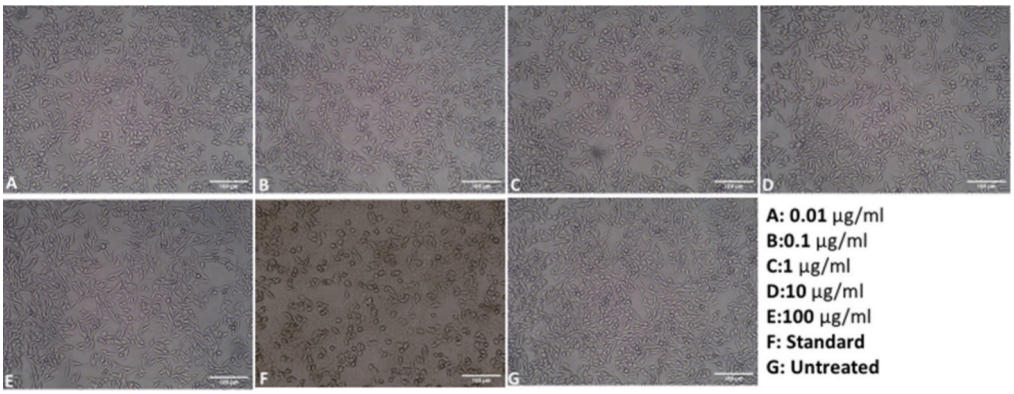

Fig. 2. Morphological assessment of L929 cells subjected to various concentrations following 24 h of incubation.

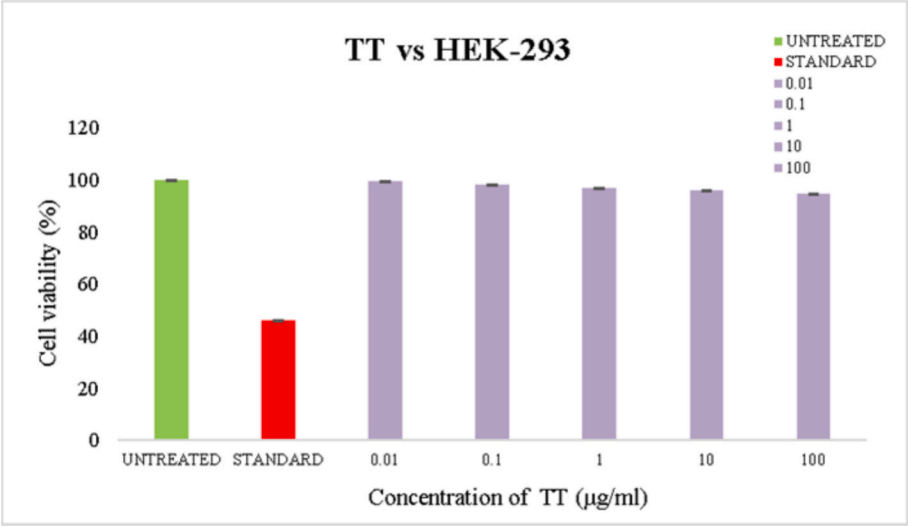

Fig. 3. Effect of ETT on HEK-293 cell viability after 24 h of incubation. The findings reveal that ETT did not exhibit cytotoxicity and preserved high cell viability across all concentrations tested, indicating its biocompatibility and safety.

Table 1.

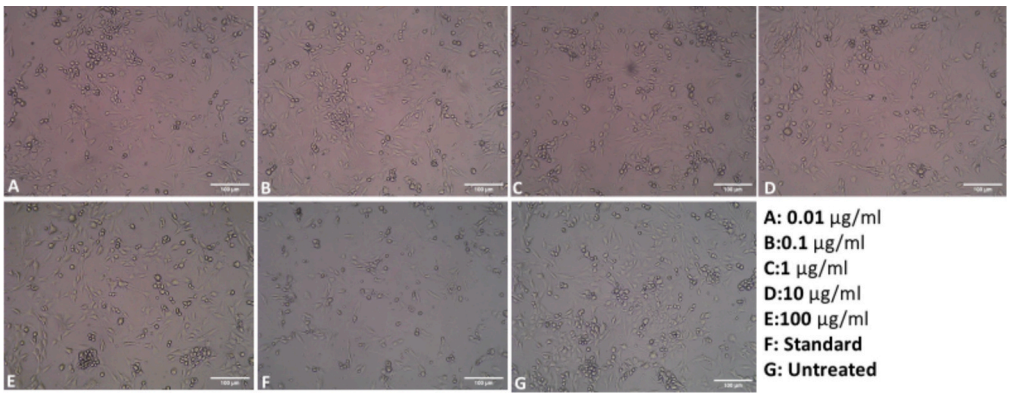

Fig. 4. Morphological assessment of HEK-293 cells treated with various concentrations after 24 h of incubation.

Table 3
Antibiotic Susceptibility and Multiple Antibiotic Resistance (MAR) Analysis of Bacterial Strains.

Si. No	Name of bacteria	Number of Antibiotics Resisted by Bacteria	Number of Antibiotics to Which Bacteria Are Sensitive	Total No. of antibiotics	MAR %
1	*Klebsiella pneumonia*	17	4	21	80.95%
2	*Pseudomonas aeruginosa*	15	1	16	93.75%
3	*E. coli*	17	4	21	80.95%
4	*Acinetobacter baumannii*	10	7	17	58.82%
5	*Enterococcus fecalis*	5	4	9	55.55%
6	MRSA	6	5	11	54.54%

3.3. Assessment of total phenolic content (TPC)

The gallic acid standard curve showed excellent linearity ($y = 0.012x + 0.045$, $R^2 = 0.998$), and the TPC of the ETT was calculated as 7.96 µg/ml. The calibration curve is provided in the Supplementary Material.

3.4. Assessment of total flavonoid content (TFC)

The quercetin calibration curve showed good linearity ($y = 0.009x + 0.031$, $R^2 = 0.997$), and the TFC of the ETT was determined to be 23.22 µg/ml. The standard curve is provided in the Supplementary Material.

3.5. Antioxidant assay

We used the DPPH, FRAP, and ABTS tests to see how well the ETT could act as an antioxidant. The results are shown in Table (2). In the DPPH radical scavenging test, ETT had a moderate antioxidant activity

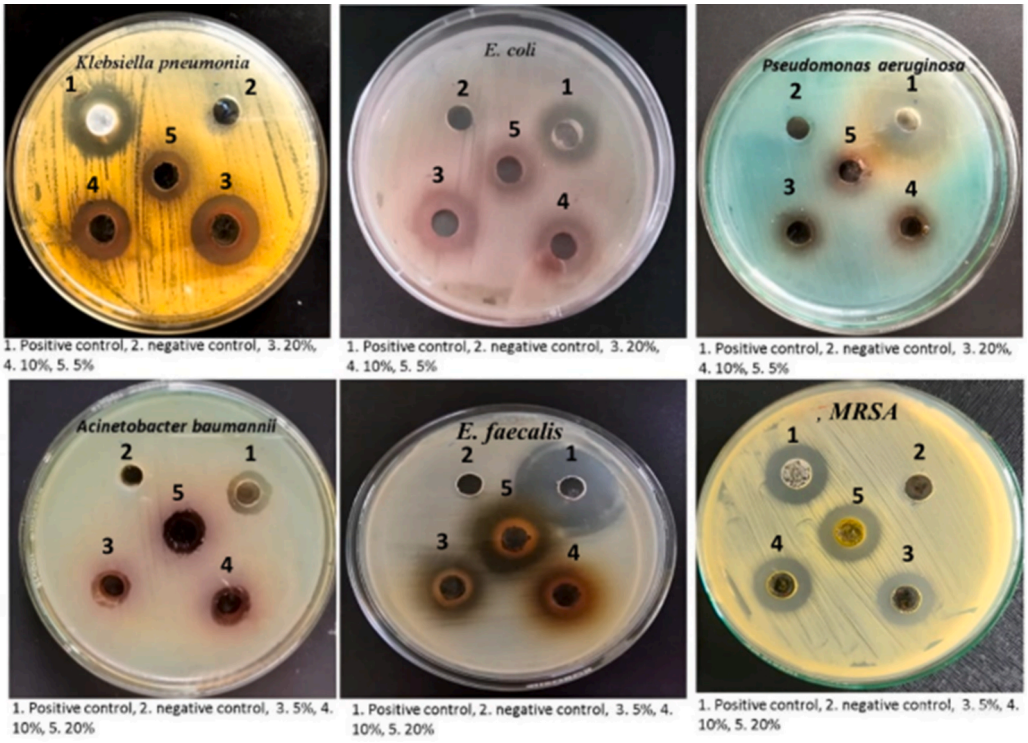

Fig. 5. Antimicrobial activity of tt against mdr bacteria.

Table 4
Results of the mean zones of inhibition of TT extract against MDR bacteria.

Name	Concentration 5% W/V (mm)	Concentration 10% W/V (mm)	Concentration 20% W/V(mm)	Positive control: Azithromycin 1%w/v (mm)	Negative control: DMSO 100% V/V
K.pneumoniae	11 ± 0.002	14 ± 0.001	17 ± 0.003	18 ± 0.001	No zone
E. Coli	15 ± 0.001	17 ± 0.005	19 ± 0.003	21 ± 0.002	No zone
P. aeruginosa	13 ± 0.006	16 ± 0.002	18 ± 0.001	28 ± 0.002	No zone
A. baumannii	12 ± 0.002	14 ± 0.003	17 ± 0.001	20 ± 0.004	No zone
E. fecalis	12 ± 0.001	16 ± 0.002	19 ± 0.005	24 ± 0.003	No zone
MRSA	14 ± 0.002	17 ± 0.004	20 ± 0.003	22 ± 0.001	No zone

with an IC_{50} value of 46.65 ± 1.86 μg/ml. In contrast, the reference standard gallic acid had a much lower IC_{50} value of 23.98 ± 1.97 μg/ml, which means it was better at getting rid of free radicals. The FRAP test also showed that ETT can reduce things, with an EC_{50} value of 30.52 ± 2.01 μg/ml, which is higher than the 14.72 ± 1.55 μg/ml value for ascorbic acid. The ABTS test showed that ETT was good at scavenging radicals, with an IC_{50} value of 41.42 ± 1.34 μg/ml, but it was not as good as the standard ascorbic acid (25.0 ± 0.96 μg/ml). These results all support the idea that ETT is an antioxidant, most likely because it contains phenolic and flavonoid compounds.

3.6. Cytotoxic activity (MTT Assay)

The results of the MTT experiment (Figs. 1-4) showed that after 24 h of incubation, the test compounds had no cytotoxic effects on the L929 and HEK-293 cell lines. All experiments were performed in triplicate (n = 3), and the results are expressed as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc test. No statistically significant reduction in cell viability was observed when compared with the untreated control ($p > 0.05$), confirming the absence of acute cytotoxic effects under the tested conditions. The morphological images (Figs. 1 - 4) qualitatively support the MTT results, showing preserved cell morphology and integrity without signs of shrinkage, detachment, or membrane damage in ETT-treated L929 and HEK-293 cells compared to controls. This visual evidence complements the quantitative viability data and supports the cellular biocompatibility of ETT.

3.7. Antibiotic susceptibility and Multiple Antibiotic resistance (MAR) analysis of bacterial strains

Table 3 displays the antibiotic resistance profiles of six distinct bacterial species, highlighting varying levels of resistance among them.

3.8. Antimicrobial activity

The antibacterial effectiveness of ETT was assessed through the agar well diffusion technique against multidrug-resistant uropathogenic isolates from six different bacterial strains. Fig. 5 illustrates a comparative analysis of the extract's efficacy alongside a negative control (DMSO) and a positive control (azithromycin at 1% w/v). Table 4 summarizes the mean inhibition zones (in mm) produced by the TT extracts against *E. coli, MRSA, K. pneumoniae, P. aeruginosa, Acinetobacter baumannii, and E. faecalis,* including the control results. The antimicrobial performance of ETT at concentrations of 5%, 10%, and 20% w/v was compared with that of the positive control.

3.9. MIC and MBC

Using a microtiter plate test based on resazurin, the MIC of the ETT against MDR bacteria isolated from urinary tract infections (UTI) was ascertained. The findings are displayed in Fig. 6 and Table 5.

3.10. Biofilm formation

The results of the biofilm formation assay, shown in Fig. 7, indicate that all tested bacterial strains were capable of forming biofilms, as evidenced by the appearance of black colonies.

3.11. Anti-biofilm activity of ethanolic TT extract against UTI Pathogens

All of the examined bacterial strains were able to generate biofilms, as seen in Fig. 8. The density of the biofilm, however, diminished in relation to the dosage of ETT given, with the highest level of inhibition observed at 200 mg/ml.

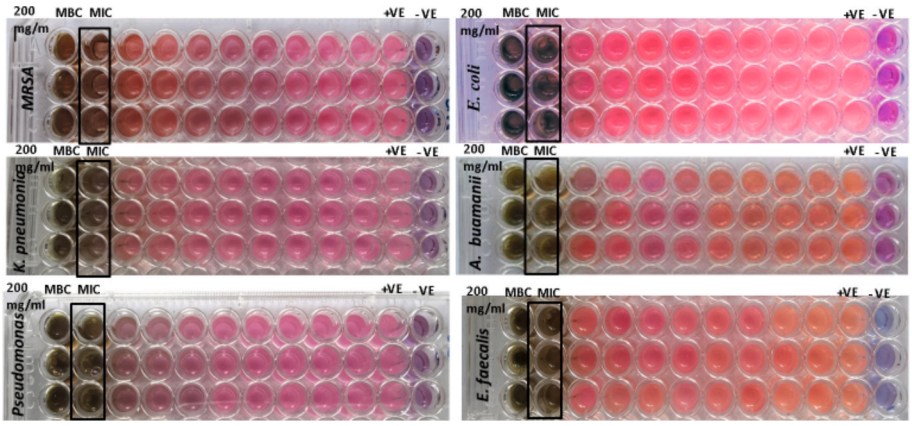

Fig. 6. MIC of TT against MDR bacteria.

Table 5
MIC and MBC values of ethanolic TT extract against UTI pathogens.

Bacterial Strain	MIC (mg/mL)	MBC (mg/mL)
MRSA	100	200
Klebsiella pneumoniae	100	200
Pseudomonas aeruginosa	100	200
Escherichia coli	100	200
Acinetobacter baumannii	100	200
Enterococcus faecalis	200	200

3.11.1. Inhibition of MDR bacterial biofilm growth by TT extract at different concentrations

Results show that the ETT reduces optical density (OD) readings, indicating a dose-dependent inhibition of biofilm formation by MDR bacteria (Fig. 9).All experiments were performed in triplicate (n = 3), and data are presented as mean ± SD. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test, revealing a significant, concentration-dependent inhibition of biofilm formation in all tested MDR bacterial strains compared with the positive control ($p < 0.001$).

3.11.2. Inhibition % of biofilm growth

An apparent dose-dependent antibacterial activity is shown by the findings in Table 6, where higher extract concentrations are accompanied with rising inhibition percentages. Strong positive relationships between concentration and bacterial inhibition are suggested by the high correlation coefficients, and the statistical significance of these results is confirmed by p-values less than 0.05.

3.12. SEM analysis

A scanning electron microscope (SEM) analysis of untreated *MRSA* and *E. coli* revealed smooth and intact cell surfaces, displaying their typical cocci and rod-shaped forms, respectively. The cells appeared densely arranged, suggesting normal integrity and healthy growth. In contrast, *MRSA* and *E. coli* cells that were treated with ETT showed significant morphological damage. The *MRSA* cells were characterized by irregular shapes, collapsed surfaces, and signs of cell lysis, pointing to a severe disruption of the cell wall. The treated *E. coli* cells had rough, wrinkled, and ruptured surfaces, along with a noticeable decrease in cell density when compared to the untreated samples. These structural changes suggest that ETT prompts the destruction of bacterial cell walls and membrane integrity, resulting in the leakage of cellular contents and subsequent cell death. The SEM results support the potent antibacterial effect of ETT against both Gram-positive (*MRSA*) and Gram-negative (*E. coli*) bacteria (Fig. 10).

3.13. HR-LCMS results

When ETT was analyzed using high-resolution LC-MS (HR-LCMS), 117 bioactive chemicals were found. Table 2 provides specifics on these compounds, including their molecular weights, retention times (RT), chemical formulae, compound classes, and known biological activity. The existence of multiple important bioactive components is further supported by the noticeable peaks seen in the chromatograms (Figs. 11 and 12).

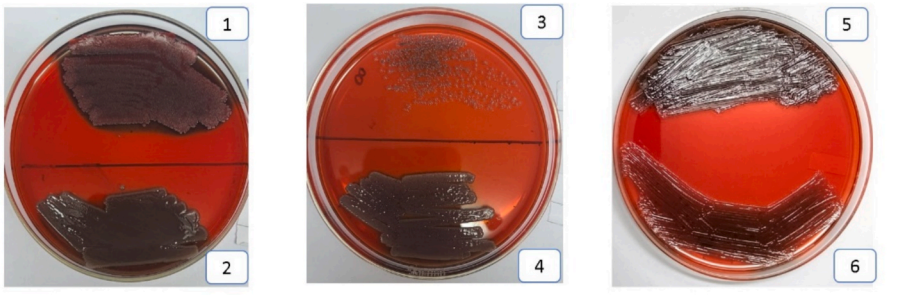

Fig. 7. Biofilm formation of MDR bacteria in Congo red agar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

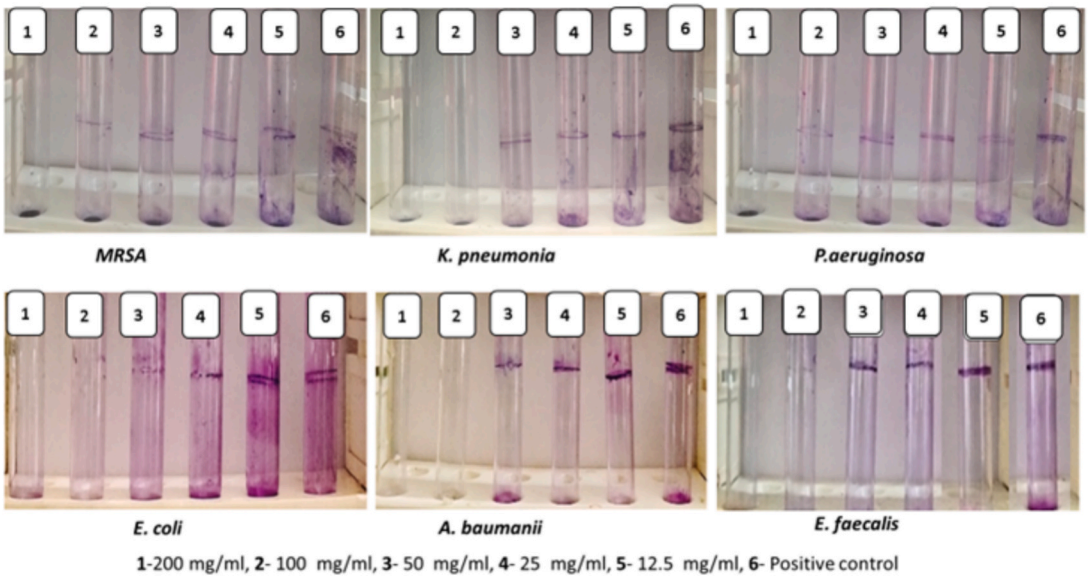

Fig. 8. Antibiofilm of TT against MDR bacteria.

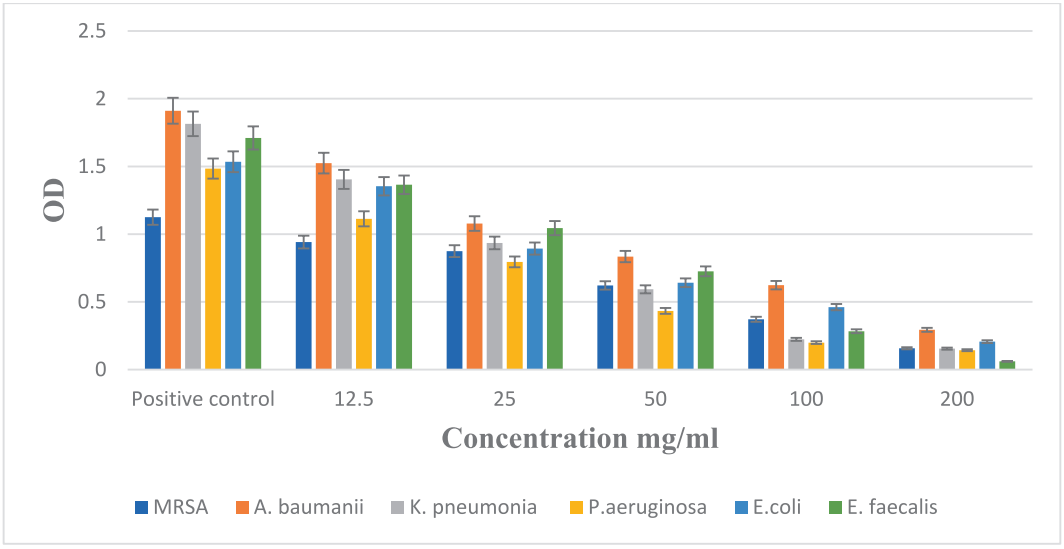

Fig. 9. Inhibition of MDR bacteria biofilm growth observed at various concentrations of extract, measured by optical density (OD).

Table 6
Detection of the inhibition % of biofilm growth.

s. no	Concentration Mg/ml	*K. pneumonia* Inhibition %	*A. baumanii*	*MRSA*	*P. aeruginosa*	E. coli	*E. faecalis*
1	Positive control	0	0	0	0	0	0
2	12.5	22.612	20.230	16.321	24.994	11.794	20.210
3	25	48.457	43.582	22.274	46.418	41.768	38.920
4	50	67.340	56.330	44.786	70.806	58.210	57.571
5	100	87.692	67.387	67.002	86.570	69.939	83.434
6	200	91.476	84.635	86.018	90.343	86.555	96.452
7	correlation coefficient	0.831	0.874	0.946	0.821	0.875	0.904
8	P value	0.040*	0.023*	0.004**	0.045*	0.023*	0.013*

* = $p < 0.05$. ** = $p < 0.01$.

4. Discussion

The therapeutic repertoire of TT spans across various traditional cultures, utilized for centuries as curative interventions for inflammation, infertility, and urinary disorders. Recent investigations have emphasized its potent antibacterial activity, particularly against

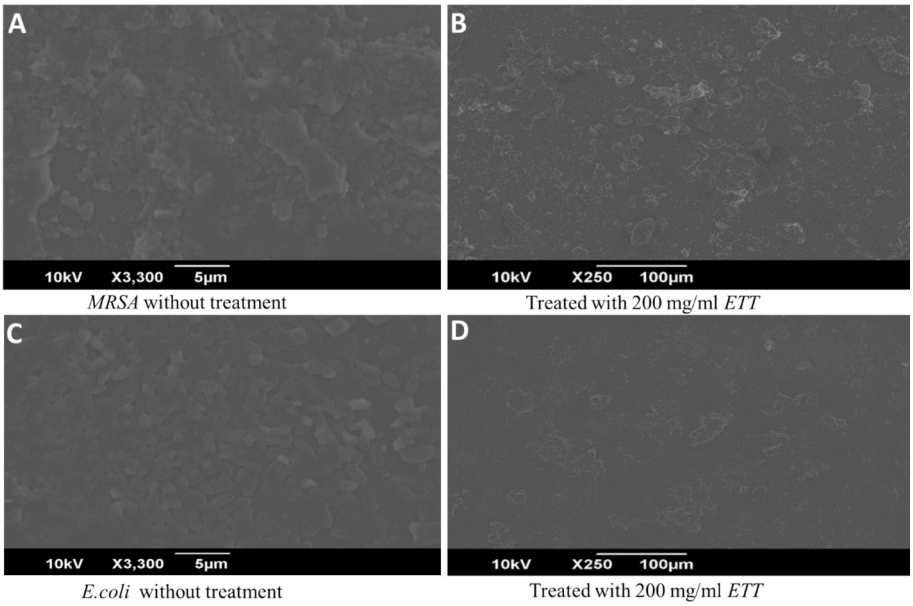

Fig. 10. Results of SEM analysis demonstrating the impact of ETT on *MRSA* and *E. coli*. (A) *MRSA* without treatment; (B) *MRSA* subjected to 200 mg/ml ETT; (C) Untreated *E. coli*; (D) *E. coli* exposed to 200 mg/ml ETT.

uropathogenic bacteria associated with urinary tract infections (UTIs). This effect is primarily attributed to its rich phytochemical profile, including steroidal saponins and flavonoids, which may disrupt bacterial membranes or inhibit quorum-sensing mechanisms. Studies from the past five years have shown promising results where TT extracts significantly inhibited the growth of *E. coli, Proteus mirabilis*, and *Klebsiella pneumoniae*, key pathogens in UTIs. Moreover, the plant has demonstrated potential in reducing bacterial adhesion and biofilm formation, critical in the persistence and recurrence of UTIs. These findings support the relevance of TT as a potential natural therapeutic agent for managing UTIs and combating antimicrobial resistance.[28,19,29].

The extraction yield of ETT was 13.3%, obtained from 300 g of raw material, yielding 40 g of dried extract. The result suggests an efficient extraction process and aligns with literature-reported yields, highlighting the potential of the extract for pharmacological use.[30,10]. However, future studies using multiple batches of TT are recommended to confirm consistency.

The results in Table 1 show that the qualitative phytochemical screening of TT revealed the presence of several bioactive constituents, including flavonoids, alkaloids, glycosides, phenolic compounds, tannins, saponins, amino acids, carbohydrates, and steroids. These findings are consistent with previous reports highlighting the plant's rich phytochemical composition and therapeutic potential.[31,32].

The ETT demonstrated a total phenolic content of 7.96 μg/ml and a total flavonoid content of 23.22 μg/mL, as depicted in Figs. 1 and 2. These values indicate a moderate presence of antioxidant phytoconstituents, which are known to contribute to the plant's therapeutic potentialPhenolic compounds play an essential role in combating free radicals and reducing oxidative stress, whereas flavonoids are well-known for their anti-inflammatory and antioxidant effects. Recent studies have reported varying levels of phenolic and flavonoid contents in TT, influenced by extraction methods, solvent polarity, and the plant part used[33,34].

The findings in Figs. 3 and 4 show that ETT displayed significant antioxidant activity, as revealed by the DPPH and FRAP assays. The extract had an IC_{50} value of 46.65 μg/mL in the DPPH assay, indicating a moderate ability to scavenge free radicals. In contrast, the FRAP assay yielded an IC_{50} of 30.52 μg/ml, reflecting its ferric-reducing antioxidant power. The ETT showed relatively lower but significant activity compared to the reference standard gallic acid (IC_{50} = 23.98 μg/ml in DPPH). These findings suggest that the antioxidant potential of the extract may be attributed to its phenolic and flavonoid constituents, supporting the plant's traditional use in oxidative stress-related conditions. The results align with recent reports highlighting ETT as a promising source of natural antioxidants[35,36]. Recent studies have highlighted that the antioxidant activity of ETT is closely associated with the presence of polyphenols and flavonoids, which play a crucial role in mitigating oxidative stress and cellular damage. The findings of the present study are in agreement with these reports and further reinforce the antioxidant relevance of ETT.

The cytotoxicity assessment of the ethanolic extract of ETT using the MTT assay demonstrated no significant reduction in cell viability in L929 and HEK-293 cell lines after 24 h of exposure (Figs. 6–9). Cell viability remained above the commonly accepted cytotoxicity threshold across all tested concentrations, indicating an absence of acute cytotoxic effects under the *in vitro* conditions employed. These results suggest preliminary biocompatibility of ETT with standard cell lines; however, they necessitate careful interpretation, as *in vitro* cytotoxicity assays fail to reflect the complexities of *in vivo* systems. Consequently, the results solely indicate transient cellular tolerance and fail to demonstrate systemic safety or human relevance. Further investigations, including *in vivo* toxicological studies and supplementary safety evaluations, are necessary to validate the pharmacological potential of ETT.[37].It is important to note that the absence of cytotoxicity in normal cell lines represents a preliminary safety indicator only Although *in vitro*. assays cannot fully predict *in vivo* toxicity, *in silico* studies will help evaluate the factors such as absorption, distribution, metabolism, and excretion (ADME), as well as long-term and organ-specific effects,[638].

Multiple Antibiotic Resistance (MAR) in Table 2 shows *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and MRSA in this research are consistent with other studies. The exact high MAR percentages were noted in *Klebsiella pneumoniae* and *E. coli* (80.95%), *Pseudomonas aeruginosa* (93.75%), and *Acinetobacter baumannii* (58.82%), which are in line with findings from[39,40,41]. Furthermore, *Enterococcus faecalis* and *MRSA* demonstrated moderate resistance, similar to the findings of Malik et al. (2022). These observations reflect the widespread antibiotic resistance among clinical pathogens and emphasize the necessity for effective antimicrobial stewardship and alternative therapy strategies[42].

The ETT demonstrated antibacterial activity in a dose-dependent

Table 7
Bioactive Compounds Annotated in the ETT through HR-LCMS Analysis: Positive (1–51) and Negative (52–109) Modes.

Sl. No	Name of Compound	Class of compound	RT	Formula	Mass
1	Heliotrine	a pyrrolizidine alkaloid	6.693	$C_{16}H_{27}NO_5$	313.1875
2	Europine	Alkaloid	6.918	$C_{16}H_{27}NO_6$	329.1824
3	Conessine	Steroidal alkaloid	7.196	$C_{24}H_{40}N_2$	356.3174
4	Praziquantel	Tetrahydrogenated isoquinoline derivatives.	7.207	$C_{19}H_{24}N_2O_2$	312.1841
5	N1,N10-Dicoumaroylspermidine	Polyamine	7.823	$C_{25}H_{31}N_3O_4$	437.2289
6	Quercetin	Flavonoid	8.243	$C_{15}H_{10}O_7$	302.041
7	(+)-Norushinsunine N-oxide	Pyrrolizidine	8.665	$C_{18}H_{17}NO_4$	311.1148
8	Lasiocarpine	Pyrrolizidine alkaloid	8.932	$C_{21}H_{33}NO_7$	411.2221
9	Methoprotryne	Triazinone	9.297	$C_{11}H_{21}N_5OS$	271.1437
10	N-*trans*-p- Coumaroyloctopamine	hydroxycinnamic acid amide	9.361	$C_{17}H_{17}NO_4$	299.1147
11	Metolachlor	Chloroacetamide	10.219	$C_{15}H_{22}ClNO_2$	283.1378
12	Piperidolate	Piperidine	10.415	$C_{21}H_{25}NO_2$	323.1881
13	(S)-Edulinine	alkaloid belonging to the aporphine	10.472	$C_{16}H_{21}NO_4$	291.1483
14	Dihydrocapsaicin	Capsaicinoid	10.677	$C_{18}H_{29}NO_3$	307.2138
15	Methyl 15- cyanopentadecanoate	Fatty acid methyl ester	10.688	$C_{17}H_{31}NO_2$	281.2363
16	Penbutolol	Beta-blocker	10.892	$C_{18}H_{29}NO_2$	291.2185
17	Dihydrocapsaicin	Alkaloid	10.956	$C_{18}H_{29}NO_3$	307.2132
18	Ajaconine	Aconitine-type diterpenoid alkaloids	11.383	$C_{22}H_{33}NO_3$	359.2427
19	Senecionine	Alkaloid	11.39	$C_{18}H_{25}NO_5$	335.1715
20	Benzoylecgonine	Cocaine	11.48	$C_{16}H_{19}NO_4$	289.1326
21	(+)-Prosopinine	Piperidine alkaloid	12.878	$C_{18}H_{35}NO_3$	313.2615
22	Aminopentol	Pentolamine	13.508	$C_{22}H_{47}NO_5$	405.3453
23	Gibberellin Ga115	c20-gibberellin 6-carboxylic acid	13.678	$C_{20}H_{26}O_6$	362.1735
24	Gibberellin A75	tetracyclic diterpenoids	14.432	$C_{19}H_{24}O_8$	380.1466
25	Secodemethylclausenamide	Alkaloid	15.31	$C_{17}H_{19}NO_3$	285.1353
26	Mitoxantrone	Anthracenedione	15.888	$C_{22}H_{28}N_4O_6$	444.2021
27	Notoginsenoside T2	Saponin	16.046	$C_{37}H_{62}O_{10}$	666.4342
28	Antibiotic CP 412065	Diterpenoids	17.141	$C_{25}H_{34}O_6$	430.2348
29	6beta,17beta- Dihydroxyandrost-4-en-3-one diacetate	Steroid	17.145	$C_{23}H_{32}O_5$	388.2248
30	Glutathionylaminopropylcadav erine	polyamine-thiol conjugates	17.162	$C_{18}H_{36}N_6O_5S$	448.2458
31	Diasarone 2	Indanes	17.219	$C_{24}H_{32}O_6$	416.2194
32	Panaxynol linoleate	panaxynol, a polyacetylene derivative	17.454	$C_{35}H_{54}O_2$	506.4188
33	Hulupone	vinylogous acids	17.654	$C_{20}H_{28}O_4$	332.1992
34	Dihydrocapsaicin	Methoxyphenols	17.655	$C_{18}H_{29}NO_3$	307.2132
35	Limonoate	Terpenoids	17.903	$C_{26}H_{34}O_{10}$	506.2171
36	PE(20:2(11Z,14Z)/15:0)	Phospholipid	18.851	$C_{40}H_{76}NO_8P$	729.5218
37	Spirolide D	Spirolides	18.901	$C_{43}H_{65}NO_7$	707.4772
38	Convallasaponin A	Saponin	19.067	$C_{32}H_{52}O_9$	580.3543
39	(ent-2b,4S,9a)-2,4,9-Trihydroxy-10(14)-oplopen-3- one 2-(2-methylbutanoate) 9- (3-methyl-2E-pentenoate)	Terpenoid	19.244	$C_{26}H_{40}O_6$	448.2767
40	Convallasaponin A	Steroidal saponin	19.44	$C_{32}H_{52}O_9$	580.3545
41	Stenocereol	Triterpenoids	20.675	$C_{28}H_{46}O_2$	414.349
42	4Z,15E-Bilirubin IXa	Tetrapyrrolic	21.996	$C_{33}H_{36}N_4O_6$	584.2621
43	Irinotecan	Pyranoindolizino quinoline	23.05	$C_{33}H_{38}N_4O_6$	586.2779
44	Oxidized dinoflagellate luciferin	Bilenes	23.267	$C_{33}H_{38}N_4O_7$	602.273
45	Endomorphin-2	opioid peptides	24.016	$C_{32}H_{37}N_5O_5$	570.2835
46	Ganoderic acid F	Triterpenoid	24.252	$C_{32}H_{42}O_9$	570.2832
47	Irinotecan	Alkaloid	24.311	$C_{33}H_{38}N_4O_6$	586.2772
48	Armillatin	protoilludane norsesquiterpenoid esters	24.469	$C_{38}H_{58}O_6$	608.4148
49	Ganoderic acid F	Triterpenoid	24.555	$C_{32}H_{42}O_9$	570.2832
50	Euphornin	Saponins	24.712	$C_{33}H_{44}O_9$	584.2981
51	Ganosporelactone A	esquiterpenoids and saponins	24.785	$C_{30}H_{40}O_7$	512.2779
52	Quinoline-3-carboxamides	Quinoline	1.442	$C_{21}H_{14}FN_3O_2$	359.1091
53	Galactinol dihydrate	Glycosylated cyclitols.	1.473	$C_{12}H_{22}O_{11}$	342.1187
54	Xanthosine	Nucleosides	3.977	$C_{10}H_{12}N_4O_6$	284.0775
55	Calendoflavobioside	Flavonoid glycosides	7.985	$C_{27}H_{30}O_{16}$	610.158
56	Myricitrin	Flavonoid glycosides	8.28	$C_{21}H_{20}O_{12}$	464.0989
57	Quercetin 3,7-dirhamnoside	Flavonoid glycosides	8.527	$C_{27}H_{30}O_{15}$	594.1633
58	Scoparin 2″-glucoside	Flavonoid diglycosides	8.651	$C_{28}H_{32}O_{16}$	624.1742
59	N-*cis*-Caffeoyltyramine	Phenylpropanoid amides.	9.125	$C_{17}H_{17}NO_4$	299.1179
60	Nonate	Fatty acid salts	9.257	$C_9H_{16}O_4$	188.106
61	Finaconitine	Diterpene alkaloids	9.295	$C_{33}H_{46}N_2O_{10}$	630.3129
62	Salicylic acid	Phenolic acid	9.547	$C_7H_6O_3$	138.0324
63	Muricinine	Alkaloids	10.241	$C_{18}H_{19}NO_4$	313.1337
64	Auxin a	Indole	11.655	$C_{18}H_{32}O_5$	328.2274
65	9S,12S,13S-trihydroxy-10E-octadecenoic acid	Fatty acids	12.279	$C_{18}H_{34}O_5$	330.2429
66	Etofenprox	Synthetic pyrethroid	14.097	$C_{25}H_{28}O_3$	376.2034
67	(9Z,11R,12S,13S,15Z)-12,13-Epoxy-11-hydroxy-9,15-octadecadienoic acid	Fatty acids	14.767	$C_{18}H_{30}O_4$	310.2157

(*continued on next page*)

Table 7 (*continued*)

Sl. No	Name of Compound	Class of compound	RT	Formula	Mass
68	11S-HpODE	Fatty acids	14.993	$C_{18}H_{32}O_4$	312.2314
69	9,10-DiHOME	Fatty acids	15.902	$C_{18}H_{34}O_4$	314.2469
70	1-Naphthylacetylspermine	Polyamine	17.114	$C_{22}H_{34}N_4O$	370.274
71	MG(0:0/15:0/0:0)	Glycerolipid	17.246	$C_{18}H_{36}O_4$	316.2628
72	10-Oxo-11-octadecen-13-olide	Macrolides	17.287	$C_{18}H_{30}O_3$	294.2208
73	Geranylfarnesyl diphosphate	Terpenoid	17.885	$C_{25}H_{44}O_7P_2$	518.258
74	Hexazinone	Triazine herbicide	18.337	$C_{12}H_{20}N_4O_2$	252.1562
75	12-Hydroxy-8,10-octadecadienoic acid	Fatty acids	18.359	$C_{18}H_{32}O_3$	296.2365
76	α-Linolenic Acid	Omega-3 fatty acid	18.389	$C_{18}H_{30}O_2$	278.2261
77	Lauryl hydrogen sulfate	Alkyl sulfates	18.406	$C_{12}H_{26}O_4S$	266.1564
78	9-HOTE	Hydroxy fatty acids	18.89	$C_{18}H_{30}O_3$	294.2209
79	Loxtidine	H2 receptor antagonists	19.042	$C_{19}H_{29}N_5O_2$	359.2328
80	3-keto stearic acid	Oxidized fatty acids	19.116	$C_{18}H_{34}O_3$	298.2522
81	Neamine (Neomycin A)	Aminoglycoside	19.295	$C_{12}H_{26}N_4O_6$	322.1815
82	N-Undecylbenzenesulfonic acid	Aromatic sulfonic acids	19.311	$C_{17}H_{28}O_3S$	312.1773
83	12-Hydroxy-8,10-octadecadienoic acid	Hydroxy fatty acids	19.311	$C_{18}H_{32}O_3$	296.2367
84	Salannin	Triterpenoid limonoids	19.545	$C_{34}H_{44}O_9$	596.3
85	LysoPE(20:2(11Z,14Z)/0:0)	Lysophosphatidylethanolamines (LysoPEs)	20.097	$C_{25}H_{48}NO_7P$	505.3202
86	Pateamine	Alkaloids	20.104	$C_{31}H_{45}N_3O_4S$	555.312
87	Azukisapogenol	Triterpenoid sapogenins	20.605	$C_{30}H_{48}O_4$	472.358
88	8S-HODE	Hydroxy fatty acids	21.027	$C_{18}H_{32}O_3$	296.2366
89	G-418	Aminoglycoside	21.224	$C_{20}H_{40}N_4O_{10}$	496.2739
90	makisterone B	Steroids	21.974	$C_{28}H_{46}O_7$	494.3276
91	Elaidolinoleic acid	Unsaturated fatty acids	21.998	$C_{18}H_{30}O_2$	278.226
92	Linalyl caprylate	Ester	22.001	$C_{18}H_{32}O_2$	280.241
93	Sodium Tetradecyl Sulfate	Alkyl sulfate surfactants	22.105	$C_{14}H_{30}O_4S$	294.1883
94	Macrocarpal B	Phloroglucinol	22.214	$C_{28}H_{40}O_6$	472.2855
95	4-Dodecylbenzenesulfonic acid	Aromatic sulfonic acids	22.243	$C_{18}H_{30}O_3S$	326.1933
96	DG(18:3(6Z,9Z,12Z)/14:0/0:0)	Diacylglycerols	22.309	$C_{35}H_{62}O_5$	562.4638
97	(Z)-15-Oxo-11-eicosenoic acid	Oxidized fatty acids	22.372	$C_{20}H_{36}O_3$	324.2682
98	2,4,12-Octadecatrienoic acid isobutylamide	Fatty acid amides	23.19	$C_{22}H_{39}NO$	333.3056
99	Dioctyl hexanedioate	Diesters	23.204	$C_{22}H_{42}O_4$	370.3099
100	Hydrocortisone cypionate	Corticosteroid esters	23.208	$C_{29}H_{42}O_6$	486.293
101	Ethyl 2E,4Z-hexadecadienoate	Fatty acid esters	23.282	$C_{18}H_{32}O_2$	280.2421
102	10,20-Dihydroxyeicosanoic acid	Hydroxy fatty acids	23.423	$C_{20}H_{40}O_4$	344.2948
103	2R-hydroxy-stearic acid	Hydroxy fatty acids	24.299	$C_{18}H_{36}O_3$	300.2682
104	Imperialine	Steroidal alkaloids	24.461	$C_{27}H_{43}NO_3$	429.3261
105	Pipercitine	Amide alkaloids	24.502	$C_{23}H_{43}NO$	349.3372
106	Diepomuricanin A	Diterpenoids	24.543	$C_{35}H_{62}O_4$	546.469
107	Surfactin	Lipopeptides	24.545	$C_{53}H_{93}N_7O_{13}$	1035.6915
108	Oleic Acid	Monounsaturated fatty acids	24.688	$C_{18}H_{34}O_2$	282.2576
109	1,2,10-Trihydroxydihydro-*trans*-linalyl oxide 7-O-beta-D-glucopyranoside	Monoterpene glycosides	24.698	$C_{16}H_{30}O_{10}$	382.1835

manner against all tested pathogens, with inhibition zones measuring between 17 and 20 mm at a concentration of 20% w/v. Notably, the extract demonstrated significant efficacy against *Escherichia coli*, *Enterococcus faecalis*, and *MRSA*, with inhibition zones comparable to those produced by azithromycin (1% w/v). Comparable antimicrobial effects of ETT against both Gram-positive and Gram-negative bacteria have been previously reported, supporting the broad-spectrum activity observed in this study. These findings suggest that the detected phytochemicals may interfere with bacterial cell integrity and metabolic functions. No inhibitory effect was observed with the DMSO negative control, confirming the extract's antimicrobial properties. These results align with earlier research that has highlighted the antimicrobial potential of ETT. For instance,[44] demonstrated that ethanol extracts from TT fruits exhibited significant antibacterial activity against various Gram-positive and Gram-negative bacteria, including *E. coli* and *Klebsiella pneumoniae*. Similarly,[45] reported that ethanolic extracts of TT fruits showed notable antibacterial effects against common uropathogens such as *E. coli*,*Pseudomonas aeruginosa*, *K. pneumoniae*, and *E. faecalis*.

These findings align with recent studies.[45,46] reported that ETT exhibited notable antibacterial activity against common uropathogens, including *E.coli*, *Pseudomonas aeruginosa*, *K. pneumoniae*, and *E. faecalis*. The study emphasized that higher concentrations of the extract resulted in larger zones of inhibition, demonstrating a dose-dependent antibacterial effect. The slightly higher MIC observed for *E. faecalis* may be due to its intrinsic resistance mechanisms, suggesting that higher concentrations or combination therapies might be necessary for effective treatment. The present study demonstrated that the ETT effectively inhibits biofilm formation in MDR uropathogens in a dose-dependent manner, with the highest inhibition observed at 200 mg/ml. All tested strains exhibited strong biofilm-forming capacity, underscoring their role in persistent UTI. The extract's significant anti-biofilm activity suggests potential therapeutic value in managing biofilm-associated infections.

These findings are in agreement with recent reports indicating the anti-biofilm efficacy of plant-derived phytochemicals, particularly saponins and flavonoids, which disrupt quorum sensing and hinder extracellular polymeric substance (EPS) production. The anti-biofilm activity of ETT may therefore be attributed to its rich content of secondary metabolites that interfere with bacterial adhesion and biofilm maturation[47,48].

The biofilm inhibition assay demonstrated that the ETT exhibited a significant, dose-dependent reduction in biofilm biomass across all tested uropathogens. As shown in Table 6, biofilm inhibition increased with rising extract concentrations, with maximum inhibition observed at 200 mg/ml, particularly notable in *E. faecalis* (96.45%), *K. pneumoniae* (91.47%), and *P. aeruginosa* (90.34%). Statistical analysis further supported the biological relevance of these findings. The strong positive correlation coefficients (ranging from 0.821 to 0.946) indicate a robust linear relationship between concentration and inhibition

percentage. Additionally, p-values below 0.05 for all strains (and below 0.01 for *MRSA*) confirm the statistical significance of the dose–response trend.

The extract's efficacy against both Gram-positive and Gram-negative biofilm producers indicates that its phytochemical components, especially saponins, flavonoids, and tannins, may disrupt essential phases of biofilm formation, including adhesion, EPS production, and quorum sensing. This aligns with previous reports on the anti-biofilm potential of phytocompounds from ETT[48]. Biofilm inhibition by ETT has received limited attention despite its clinical relevance, particularly in persistent and recurrent infections. Previous investigations have suggested that TT-derived metabolites can disrupt biofilm architecture and bacterial adhesion mechanisms[49], which is consistent with the antibiofilm effects observed in the present work.

The SEM analysis provided morphological evidence supporting the antibacterial activity of the ETT. Untreated *MRSA* and *E. coli* cells showed intact surfaces, whereas ETT-treated cells exhibited marked structural damage, including deformation and cell wall disruption, indicating loss of membrane integrity and bacterial cell death. These findings support a bactericidal mode of action and are consistent with the observed antimicrobial and antibiofilm effects. Comparable SEM-documented morphological disruptions have been reported for plant-derived antimicrobials[25], further supporting the mechanistic basis of ETT antibacterial activity.

The use of preliminary colorimetric screening prior to HRLC-MS analysis allowed an initial classification of phytochemical groups, which facilitated the interpretation of biological activities and SEM analysis observations. The HRLC-MS analysis of ETT identified a broad spectrum of bioactive compounds, including alkaloids, flavonoids, phenolics, saponins, tetrapyrrolic compounds, triterpenoids, glycosides, steroids, fatty acids, indanes, and methoxyphenols (Table 7). Earlier studies have demonstrated that steroidal saponins, phenolic acids, and flavonoids identified in TT extracts contribute significantly to its antimicrobial and antioxidant properties[6,43]. The qualitative HR-LCMS profiling performed in this study provides further support for these associations. These phytochemicals play a role in the pharmacological effects of TT, which include antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory activities. The presence of these components reinforces the traditional use of TT for treating urinary tract infections, as well as addressing inflammation and oxidative stress. The findings are consistent with recent studies, reinforcing TT's relevance in herbal medicine and its potential for therapeutic development[50,51].

5. Conclusion

The multifaceted bioactivities of ETT, such as antioxidant, antimicrobial and antibiofilm inhibition stems from their diverse phytochemical metabolites. This underscores their relevance in traditional ayurvedic practices, and this investigation supports their potential as a promising translational medicine to treat nephrolithiasis.

CRediT authorship contribution statement

Faheem Q. AL-Mojahid: Writing – original draft, Methodology, Investigation. **Tijo Cherian:** Writing – review & editing, Formal analysis. **Afaf A. Alsosowaa:** Writing – review & editing. **N. Sharmila Devi:** Writing – review & editing. **Ardra A.P.:** Writing – review & editing, Formal analysis. **Namitha Vijay:** Formal analysis. **Sijo Asokan:** Formal analysis. **Teena Merlin:** Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Methodology, Data curation, Conceptualization. **Willie J.G.M. Peijnenburg:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgeb.2026.100683.

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