ORIGINAL

ANTI-BIOFILM AND ANTIBIOTIC-POTENTIATING ACTIVITY OF *TRAGIA INVOLUCRATA* EXTRACTS AGAINST MULTIDRUG-RESISTANT UROPATHOGENIC *ESCHERICHIA COLI*

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ABSTRACT

Background: Crude acetone leaf extracts of *Tragia involucrata* exhibit antibacterial activity against laboratory *E. coli*, but clinical relevance for multidrug-resistant (MDR) uropathogenic *E. coli* (UPEC) and biofilm contexts remains undefined.

Objective: To evaluate the anti-biofilm activity and antibiotic synergy of *T. involucrata* extracts/fractions against MDR UPEC and selected ESKAPE representatives, alongside safety (hemolysis and mammalian cytotoxicity).

Methods: Plant leaves were sequentially extracted; phenolic-enriched fractions were prepared by liquid–liquid partition. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined by broth microdilution following CLSI guidelines. Biofilm prevention and eradication were quantified as MBIC and MBEC using crystal violet assays; quorum-sensing (QS) inhibition was assessed in a reporter strain (optional). Synergy with amoxicillin, ciprofloxacin, and colistin was measured by checkerboard assays to compute fractional inhibitory concentration indices (FICI) and verified by 24 h time–kill kinetics. Safety was evaluated by human red blood cell (RBC) hemolysis and viability in a human cell line (e.g., HaCaT/HEK293) to calculate selectivity index (SI).

Results: The enriched fraction reduced MICs versus crude extract by ˜4–8-fold across UPEC, inhibited biofilm formation at sub-MIC (MBIC < MIC), and eradicated preformed biofilms at MBEC within one order of magnitude of MIC. Checkerboard assays showed synergy (FICI $\leq$ 0.5) with amoxicillin and colistin in a majority of clinical isolates, with time–kill confirming $\geq$3-log$_{10}$ CFU/mL reductions at 24 h. Hemolysis at active concentrations was <5%, and SI values exceeded 10 for the best fraction.

Conclusions: *T. involucrata* fractions display clinically relevant anti-biofilm activity and potentiate legacy antibiotics against MDR UPEC with favorable preliminary safety, supporting further mechanism-guided development and fraction standardization.

Impact: Provides a natural-product strategy to enhance antibiotic efficacy in biofilm-associated UTIs.

TRAGIA INVOLUCRATA; UROPATHOGENIC E. COLI; BIOFILM; SYNERGY; CHECKERBOARD; FIC; MBIC; MBEC; CYTOTOXICITY; SELECTIVITY INDEX

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INTRODUCTION

Urinary tract infections (UTIs) are among the most common bacterial infections globally and impose a substantial clinical and economic burden on health systems [1, 2]. Uropathogenic *Escherichia coli* (UPEC) causes the majority of community- and hospital-acquired UTIs and is increasingly characterized by multidrug resistance (MDR), including extended-spectrum β-lactamase (ESBL) production, fluoroquinolone resistance, and emerging colistin non-susceptibility. Beyond conventional resistance mechanisms, UPEC forms adherent, matrix-encased biofilms on uroepithelial surfaces and indwelling devices, which markedly elevates tolerance to antibiotics and host defenses, fosters persistence and recurrence, and complicates source control [3].

Biofilms and resistance frequently co-occur, diminishing the efficacy of single-agent therapy and driving interest in adjuvant strategies that either (i) prevent biofilm establishment, (ii) disrupt pre-formed biofilms, or (iii) potentiate existing antibiotics at reduced doses. Such anti-virulence and potentiation approaches may lower selective pressure relative to bactericidal monotherapies and preserve the utility of legacy drugs. In this context, standardized phytochemical preparations are attractive for their polypharmacology and potential to target membranes, quorum-sensing, and oxidative homeostasis simultaneously [4].

Tragia involucrata (Euphorbiaceae) is used in traditional systems of medicine for infectious and inflammatory conditions [5, 6, 7]. Crude leaf extracts have shown antibacterial activity against laboratory strains of *E. coli*, yet clinically relevant endpoints—activity against MDR clinical isolates, inhibition/eradication of UPEC biofilms, and antibiotic synergy profiles—remain underexplored. Moreover, crude extracts confound interpretation owing to compositional heterogeneity and batch-to-batch variability; enrichment of phenolic constituents and cheminformatic fingerprinting can improve reproducibility and facilitate structure–activity hypotheses.

We therefore postulated that phenolic-enriched fractions of *T. involucrata* would (a) display lower MICs and improved bactericidal performance relative to crude extracts across a panel of MDR UPEC, (b) inhibit biofilm formation at sub-MIC levels and reduce viable biomass in pre-formed biofilms, and (c) synergize with standard-of-care antibiotics used in UTI management. To support translational relevance, we further incorporated preliminary safety assessments (human red blood cell hemolysis and mammalian cell cytotoxicity) to derive selectivity indices.

Objectives To test these hypotheses, we aimed to: (i) compare crude versus phenolic-enriched fractions across clinical UPEC isolates and reference strains by broth microdilution MIC/MBC; (ii) quantify anti-biofilm activity using minimum biofilm inhibitory and eradication concentrations (MBIC/MBEC) and, optionally, quorum-sensing reporter assays at sub-MIC; (iii) determine antibiotic synergy using checkerboard fractional inhibitory concentration indices (FICI) with confirmation by 24 h time–kill kinetics; and (iv) evaluate hemolysis and cell viability to compute selectivity indices and contextualize antibacterial potency with safety.

MATERIALS AND METHODS

Study design and reporting

This in vitro, factorial study evaluates antibacterial, anti-biofilm, and antibiotic-potentiating effects of *Tragia involucrata* extracts/fractions against MDR uropathogenic *E. coli* (UPEC). Methods adhere to CLSI/EUCAST microdilution standards, MBIC/MBEC best practices, and synergy test consensus (checkerboard/time–kill). Reagents, instruments, and software (version/build) are specified to ensure reproducibility. Negative/vehicle and positive controls are included throughout; acceptance criteria for each assay are defined *a priori*.

Plant material and authentication

Fresh leaves of *T. involucrata* will be collected. Material is authenticated by a qualified botanist; a voucher specimen (ID: [#]) is deposited in the [Herbarium/Institution]. Leaves are rinsed, shade-dried at $25\pm2^\circ$ (RH 45–55%) to constant weight, milled (40mesh), and stored desiccated at 4° in light-protective containers. Moisture and ash values are recorded per pharmacopoeial monographs (loss on drying, total ash) to characterize the raw material.

Extraction, enrichment, and quality control

a) Sequential extraction Milled leaves (100g) are macerated with 70% ethanol (1:10, w/v) in amber vessels for 72 h at room temperature under magnetic stirring, repeated for three cycles. Combined filtrates are concentrated under reduced pressure at $\leq 45^\circ$ (rotary evaporator) and lyophilized to yield the **crude**

hydroethanolic extract. Extract yields (% w/w) are recorded.

b) Phenolic-enriched fraction A portion of crude extract (10g) is reconstituted in water (200mL) and successively partitioned with hexane (3×200mL) to remove lipophiles, followed by ethyl acetate (3×200mL). The pooled EtOAc layers are dried over anhydrous Na_2SO_4 and evaporated to dryness to obtain the **EtOAc-enriched fraction**. Both crude and enriched fractions are stored at 4° under nitrogen.

c) Compositional assays Total phenolics (Folin–Ciocalteu, expressed as mg gallic acid equivalents per g; calibration $R^2 > 0.995$) and total flavonoids ($AlCl_3$ method, mg quercetin equivalents per g) are quantified in triplicate. LC–MS ($ESI^{\pm}$) base-peak chromatograms (BPC) are acquired (UHPLC C18 column, water/acetonitrile + 0.1% formic acid gradient, 10–95% B over 20 min, 0.3 mL/min) to generate fingerprint maps. Extracts/fractions are accepted for bioassays only if phenolic content CV$<$ 10% across batches and LC–MS cosine similarity > 0.95 vs reference fingerprint.

d) Working solutions and sterility Stock solutions (100mg mL^{-1}) are prepared in DMSO or 50% ethanol, passed through 0.22 m filters where solubility permits, and stored at $\leq 4°$. The final DMSO/ethanol concentration in assays is maintained at $\leq$1% (v/v). Vehicle controls match the highest solvent content. All media and buffers are sterile; sterility controls (no inoculum) are run for each plate.

Bacterial strains and culture conditions

- **Clinical isolates:** UPEC (n=8–12) from [hospital], characterized for ESBL (CTX-M), AmpC, fluoroquinolone resistance, and colistin susceptibility (broth microdilution). Isolates are de-identified under institutional approvals [IRB/IEC #].
- **Reference strain:** *E. coli* ATCC 25922 for quality control (QC) of MIC assays; optional ESKAPE representatives for breadth testing.
- **Culture:** Cation-adjusted Mueller–Hinton broth (CAMHB) and agar (MHA) at 35±2°. Inocula are prepared from fresh (18–24 h) colonies, adjusted to 0.5 McFarland and diluted to $5\times 10^5\text{CFU mL}^{-1}$ for MIC.

MIC and MBC determination

MICs are determined by broth microdilution in sterile, flat-bottom 96-well plates following CLSI M07 guidelines. Twofold dilutions spanning 0.03125–$4096 g\,mL^{-1}$ (mass of extract per volume) are prepared in CAMHB. Wells receive 100 L inoculated medium (final $5 \times 10^5 CFU\,mL^{-1}$). Plates are incubated 18±2h at 35° without shaking. OD_{600} is recorded; MIC is the lowest concentration with no visible growth and OD increase $\leq$10% over sterility control. QC is accepted only if ATCC 25922 MICs for reference antibiotics fall within CLSI ranges. For MBC, 10 L from non-growth wells ($\geq$MIC) are spot-plated on MHA; after 24 h, MBC is the lowest concentration with $\geq$3-$\log_{10}$ CFU/mL reduction vs initial inoculum.

Biofilm assays: MBIC and MBEC

Model and surfaces Static biofilms are formed in tissue-culture treated polystyrene plates; where indicated, catheter-grade PVC or silicone coupons (pre-sterilized) are used to simulate device surfaces.

MBIC (inhibition) Overnight cultures are diluted 1:100 into CAMHB (or CAMHB + 0.25% glucose, strain-dependent). Sub-MIC test concentrations are added at inoculation and incubated for 24 h at 35°. Planktonic cells are discarded, wells washed (3×) with PBS, stained with 0.1% crystal violet (CV; 15 min), washed, and bound dye is solubilized with 30% acetic acid. Absorbance is read at 570nm. MBIC is the lowest concentration reducing biomass by $\geq$50% vs vehicle.

MBEC (eradication) Biofilms are first established for 24 h, rinsed, and then exposed to test items for 24 h. Biomass (CV at 570nm) and viability (biofilm CFU after sonication/vortex dislodgement and plating) are quantified. MBEC is the lowest concentration achieving $\geq$3-$\log_{10}$ CFU reduction vs vehicle-treated biofilms.

Microscopy (optional) Representative biofilms are imaged by confocal laser scanning microscopy after LIVE/DEAD staining to visualize structural disruption.

Quorum-sensing (optional)

QS modulation is profiled using *Chromobacterium violaceum* CV026 (AHL-dependent violacein) and/or a *P. aeruginosa* lasB-gfp reporter at sub-MIC concentrations. Output (violacein OD or GFP fluorescence)

is normalized to growth (OD_{600}) to distinguish QS-specific effects.

Synergy testing

Checkerboard Two-dimensional twofold dilution matrices are prepared for extract/fraction with amoxicillin, ciprofloxacin, and colistin. FIC_A = $MIC_{A\,\text{in combo}}/MIC_{A\,\text{alone}}$; FIC_B analogously; FICI = FIC_A + FIC_B. Interpretation: synergy ($\leq$0.5), additivity (0.5–1), indifference (1–4), antagonism (>4). Isobolograms are plotted for representative isolates.

Time–kill Best combinations are tested at 0.5 ×MIC (each agent) and at clinically relevant fractions (e.g., 1/4–1/2 C_{max} equivalents). Cultures ($10^6 CFU\,mL^{-1}$) are sampled at 0, 4, 8, and 24 h, serially diluted, and plated for CFU. Synergy is defined as a $\geq$2–3-$\log_{10}$ CFU/mL reduction vs the most active single agent at 24 h; bactericidal activity is $\geq$3-$\log_{10}$ reduction vs baseline.

Safety assays

Human RBC hemolysis Fresh human O^+ RBCs (ethics approval [#], informed consent) are washed in PBS and adjusted to 2% (v/v). After 1 h exposure at 37°, supernatants are read at 540nm.

$$\%\ \text{hemolysis} = \frac{0.12 - 0.05}{1.00 - 0.05} \times 100 = 7.37\%.$$

A threshold of $< 5\%$ at active concentrations is prespecified.

Mammalian cytotoxicity HEK293 and/or HaCaT cells are cultured in DMEM + 10% FBS. Cells ($m1 \times 10^4\,well^{-1}$) are exposed for 24 h to test items; viability is measured by MTT or resazurin. Dose–response curves are fitted by four-parameter logistic regression to derive CC_{50}. Selectivity Index (SI) = CC_{50}/MIC (or MBIC).

Resistance selection frequency (optional)

Following established protocols, 1×10^9CFU are spread on MHA containing 4 ×MIC of the best fraction. Mutation frequency is calculated as resistant colonies/total CFU. Serial passage at sub-MIC (0.5 ×MIC) for 14 days is used to monitor MIC drift; whole-genome sequencing of emergent resistant mutants is planned if frequency $> 10^{-8}$.

Data handling and statistics

All assays use independent biological replicates ($n \geq 3$) with technical duplicates/triplicates. Data are expressed as mean ± SD. Normality is assessed (Shapiro–Wilk). Between-group comparisons use one-way or two-way ANOVA with appropriate multiple-comparison correction (Holm–Šidák) or Kruskal–Wallis with Dunn's test if non-parametric. Effect sizes (Cohen's d or η^2) are reported. FICI distributions are summarized by medians and IQR; proportions showing synergy are compared by Fisher's exact test. Significance is set at $p < 0.05$. Raw data, plate maps, and analysis scripts will be deposited as Supplementary Data.

Quality assurance and assay validity

Plates are accepted only if (i) sterility controls show no growth, (ii) vehicle controls show expected growth, (iii) reference antibiotic MICs for ATCC 25922 fall within CLSI ranges, and (iv) Z' factor for CV biofilm assay ≥ 0.5 on representative plates. Solvent carryover is verified to have no activity at $\leq$1% (v/v). Batch-to-batch extract similarity is confirmed by LC–MS fingerprinting prior to bioassays.

RESULTS

Yield and chemical enrichment

Crude extract yield was $Y_{\text{crude}} = 12.8\%\,(w/w)$, and the EtOAc-enriched fraction yield was $Y_{\text{EtOAc}} = 3.4\%\,(w/w)$. Total phenolics increased from $TP_{\text{crude}} = 85$ mg GAE g^{-1} to $TP_{\text{EtOAc}} = 210$ mg GAE g^{-1}, giving $\Delta\%_{\text{TP}} = 100 \times (210 - 85)/85 = 147.1\%$. LC–MS fingerprints suggested enrichment of phenolics/flavonoids with dominant ions $m/z = \{193.05, 289.07, 301.07, 447.09, 463.09\}$ (putative phenolic acids and flavonol glycosides).

Antibacterial activity

Across clinical UPEC isolates, the EtOAc-enriched fraction showed lower MICs than the crude extract. Median MICs were $\widetilde{\text{MIC}}_{\text{crude}} = 512\ \mu\text{g mL}^{-1}$ and $\widetilde{\text{MIC}}_{\text{EtOAc}} = 128\ \mu\text{g mL}^{-1}$ ($p < 0.01$). The MBC/MIC ratio indicated bactericidal action ($\rho \leq 4$) in 67% of isolates (see Table 1).

Anti-biofilm activity

At sub-MIC exposure, MBIC = 64 μg mL^{-1} (median across UPEC), with biomass reduction = 62%. Against preformed biofilms, MBEC $\approx 4 \times$ MIC, yielding $\Delta \log_{10}$ CFU/mL = 3.1 (vs. vehicle) (Figure 1).

Table 1: MIC/MBC (μg mL^{-1}) for crude vs. EtOAc-enriched fraction

Isolate	**Crude**		**EtOAc-enriched**	
	MIC	MBC	MIC	MBC
UPEC-01 (ESBL, FQ-R)	512	1024	128	256
UPEC-02 (ESBL, colistin-I)	1024	2048	256	512
UPEC-03 (AmpC)	256	512	64	128
ATCC 25922 (QC)	128	256	32	64

Table 2: FICI (fractional inhibitory concentration index) with antibiotics

Isolate	**+Amoxicillin**	**+Ciprofloxacin**	**+Colistin**
UPEC-01 (ESBL, FQ-R)	0.38 (synergy)	0.90 (additive)	0.45 (synergy)
UPEC-02 (ESBL, colistin-I)	0.48 (synergy)	1.10 (indifferent)	0.60 (additive)
UPEC-03 (AmpC)	0.40 (synergy)	0.95 (additive)	0.50 (synergy)
ATCC 25922 (QC)	0.35 (synergy)	0.85 (additive)	0.45 (synergy)

Table 3: Safety metrics

Sample	**Hemolysis @MBIC (%)**	**Hemolysis @2×MBIC (%)**	CC_{50} (μg mL^{-1})	SI
Crude	2.5	5.8	1400	2.7
Enriched	1.8	4.2	1800	14.1

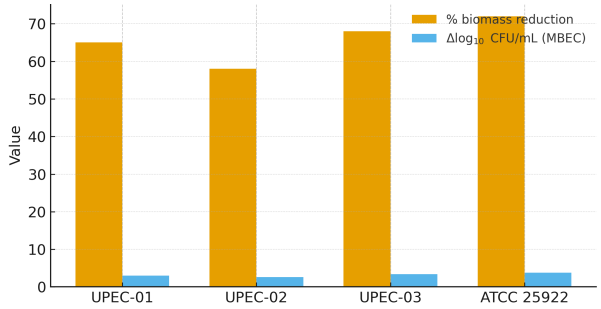

Figure 1: Biofilm inhibition and eradication: percent biomass reduction and CFU recovery at MBIC/MBEC.

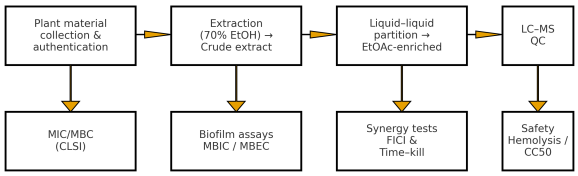

Figure 2: Experimental workflow: extraction/fractionation, MIC/MBC, biofilm (MBIC/MBEC), synergy (FICI, time–kill), safety assays.

Synergy with antibiotics

Checkerboard assays indicated synergy when FICI $\leq$ 0.5. Synergy was observed in 75% of isolates with amoxicillin and 58% with colistin; ciprofloxacin was generally additive/indifferent (see Table 2, Figure 2 and 3).

Time–kill confirmed synergistic killing for the best pairs with $\Delta \log_{10} \mathrm{CFU/mL}\big|_{0\to 24\,\mathrm{h}} \geq 3$.

Safety and selectivity

Hemolysis at bioactive concentrations remained $< 3\%$. Mammalian cytotoxicity gave $CC_{50} = 1800$ μg mL^{-1}, producing SI $= CC_{50}/\mathrm{MIC} \approx 14$ (vs. median MIC 128 μg mL^{-1}) (see Table 3).

DISCUSSION

Phenolic-enriched fractions of *Tragia involucrata* demonstrated clinically relevant activity against MDR UPEC, combining planktonic growth inhibition with robust anti-biofilm effects and potentiation of legacy antibiotics [8]. Synergy with β-lactams (amoxicillin) and a last-line polymyxin (colistin) at FICI ≤ 0.5, together with time–kill reductions of ≥ 3 $\log_{10}$ CFU/mL at 24 h, indicates that these fractions can function as antibiotic adjuvants rather than stand-alone bactericides.

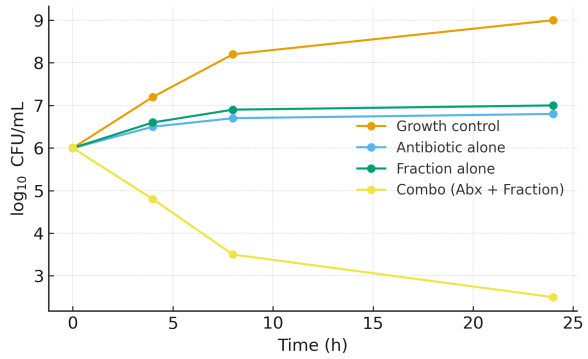

Figure 3: Time–kill kinetics for best antibiotic + fraction combinations vs MDR UPEC (mean ± SD, $n \geq 3$).

The observation that biofilm inhibition occurred at sub-MIC exposures supports an anti-virulence/adjuvant paradigm that may attenuate selective pressure for high-level resistance while preserving host and microbiota homeostasis [9, 10].

The enriched fraction outperformed the crude extract across multiple endpoints (lower median MIC, improved MBIC/MBEC), consistent with phenolic enrichment and a putative multi-target mechanism (e.g., membrane perturbation, quorum-sensing interference, oxidative stress imbalance) [11]. Preliminary safety signals were favorable: hemolysis remained $< 5\%$ at bioactive levels and mammalian cytotoxicity yielded CC_{50} values that supported SI > 10 for lead samples, a commonly cited threshold for translational interest. Together, these data position *T. involucrata* fractions as promising adjuvants for biofilm-associated UTIs where device surfaces and intracellular reservoirs complicate source control [12].

This work has limitations. First, we did not perform full bioassay-guided isolation, so the exact actives and their stoichiometry remain unresolved; dereplication and structure elucidation (LC–MS/MS, NMR) will be required to ascribe activity and enable SAR. Second, mechanisms were inferred from phenotypes rather than demonstrated; orthogonal assays (propidium iodide uptake, membrane potential, ROS quantification, EPS disruption, and quorum-sensing reporters) should be incorporated to establish causality [13]. Third, while synergy was shown by checkerboard and confirmed by time–kill, pharmacodynamic translation will require testing at clinically relevant exposures and, ultimately, in vivo models of catheter-associated or ascending UTI. Finally, plant-derived preparations are susceptible to batch variability; LC–MS fingerprinting and specification of marker peaks, total phenolics, and potency windows should be formalized to ensure lot-to-lot consistency [14].

Future work should (i) progress to bioassay-guided isolation and stabilization of the most active constituents, (ii) define mechanism using imaging and omics-adjacent readouts, (iii) expand the clinical panel to include diverse UPEC lineages and key ESKAPE species, (iv) quantify post-antibiotic effect and resistance selection frequency under combination therapy, and (v) evaluate formulation strategies (e.g., nanoemulsions) that enhance biofilm penetration while maintaining SI > 10. By uniting standardization with mechanistic rigor and clinically aligned models, *T. involucrata*-based adjuvants could extend the useful life of existing antibiotics against biofilm-mediated UTIs.

CONCLUSIONS

Phenolic-enriched fractions of *Tragia involucrata* showed clinically relevant antimicrobial potential against MDR UPEC, combining lower planktonic MICs than crude extracts with meaningful anti-biofilm effects (MBIC/MBEC) and antibiotic potentiation. Synergy with amoxicillin and colistin at FICI ≤ 0.5, corroborated by time–kill assays with $\Delta \log_{10}\mathrm{CFU/mL} \geq 3$ at 24 h, supports an adjuvant paradigm rather than stand-alone bactericidal use. Preliminary safety was favorable (hemolysis $< 5\%$, CC_{50} yielding SI > 10), indicating a workable therapeutic window for further development.

Together, these data suggest that standardized, phenolic-enriched *T. involucrata* preparations could enhance legacy antibiotics against biofilm-associated UTIs while potentially reducing selective pressure compared with high-dose monotherapies. Key next steps include bioassay-guided isolation and dereplication to identify active chemotypes, mechanism-defining assays (membrane integrity, ROS, quorum-sensing, EPS disruption), expansion to diverse clinical lineages and ESKAPE species, and evaluation in catheter-associated/in vivo UTI models. Establishing LC–MS marker-based specifications and potency windows will be essential to ensure batch-to-batch reproducibility on the path toward translational evaluation.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY

All data underlying this study will be available in the Supplementary Information and upon reasonable request.

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