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In vitro antibacterial activity of Leaf Extract of *Rubus fruticosus* toward pathogens isolated from Urinary Tract Infection

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Abstract :

Urinary tract infectious illnesses (UTIs) are globally amongst the greatest predominant microbial infectious illnesses, representing an ongoing public concern of health because of raising antimicrobial resistances (AMRs) and the restricted effectiveness of presenting antimicrobials. The search for modern alternatives of antimicrobials from environmental origins has consequently gained significance. Leaves of blackberry, scientific name called *Rubus fruticosus* are flavonoid and phenolic compounds-rich with stated features of antioxidants and antimicrobials, yet their specific activity toward uropathogenic microorganisms remains inadequately explored. The work aimed to estimate the *in-vitro* activities of the antifungals and antibacterials of ethanolic extract of *R. fruticosus* leaf toward UTI patients-isolated clinical pathogens, including *Klebsiella* spp., *Proteus mirabilis*, *Candida albicans*, *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli (E. coli)*. With clotrimazole as a positive control, the method of agar well diffusion was employed using concentrations of extract of 10–40 mg/mL. At 40 mg/mL of extract, the mean ± SD of maximal activities was noted toward *C. albicans* (32.0 ± 1.0 mm) and *Klebsiella* spp. (31.0 ± 1.0 mm), followed by *P. mirabilis* (25.3 ± 1.52 mm) and *S. aureus* (22.0 ± 0.0 mm), whereas *E. coli* and *S. typhi* revealed moderate inhibitory impact (15.3 ± 0.57 and 15.0 ± 1.0 mm, respectively). Outcomes represented noteworthy dose-dependent inhibitory impact ($p<0.01$) through entire examined UTI pathogenic microbes. These outcomes prove the probable of leaf extract of *R. fruticosus* as a natural antimicrobial agent toward UTI pathogenic microbes, providing its additional improvement as a complementary treatment for management of AMR.

Keywords: Phenolic compounds; Ethanolic extract; Urinary tract infection; Antimicrobial activity; *R. fruticosus*.

النشاط المضاد للبكتيريا في المختبر لمستخلص أوراق العليق (Rubus fruticosus) ضد الممرضات المعزولة من التهاب المسالك البولية

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مستخلص:

هدفت هذه الدراسة إلى تقدير النشاط المضاد للبكتيريا والفطريات في المختبر لمستخلص أوراق العليق الإيثانولي ضد العزلات السريرية المأخوذة من مرضى التهاب المسالك البولية، والتي شملت (*Klebsiella spp.*) و(*Proteus mirabilis*) و(*Candida albicans*) و(*Staphylococcus aureus*) و(*Salmonella typhi*) و(*Escherichia coli*). باستخدام الكلوتريمازول كعنصر ضبط إيجابي، تم تطبيق طريقة الانتشار في الأجار (Agar Well Diffusion) بتركيزات من المستخلص تراوحت بين 10–40 ملغم/ مل. عند تركيز 40 ملغم/ مل، بلغت أعلى القيم المتوسطة ± الانحراف المعياري للنشاط المثبط تجاه (*C. albicans*) هي (32.0 ± 1.0 مم) و(*Klebsiella spp.*) هي (31.0 ± 1.0 مم)، تلتها (*P. mirabilis*) وهي (25.3 ± 1.52 مم) و(*S. aureus*) هي (22.0 ± 0.0 مم)، في حين أظهرت كل من (*E. coli*) و(*S. typhi*) تأثيرًا مثبطًا متوسطًا بلغ (15.3 ± 0.57 مم) و(15.0 ± 1.0 مم) على التوالي. أظهرت النتائج تأثيرًا مثبطًا معنويًا يعتمد على الجرعة ($p<0.01$) ضد جميع الممرضات المدروسة المسببة لعدوى المسالك البولية. وتُثبت هذه النتائج القدرة المحتملة لمستخلص أوراق العليق كمضاد ميكروبي طبيعي ضد ممرضات المسالك البولية، مما يؤهله ليكون علاجًا تكميليًا واعدًا في مواجهة مقاومة الميكروبات للمضادات الحيوية (*AMR*).

الكلمات المفتاحية: المركبات الفينولية؛ المستخلص الإيثانولي؛ اصابات المسالك البولية؛ النشاط المضاد للميكروبات؛ نبات العليق.

Introduction

Urinary tract infectious illnesses (UTIs) are globally amongst the greatest common infectious illnesses and represent an important burden of public-health through hospital settings and community. Their administration is complex because of raising the AMR, which increases the risks of therapeutic failures and recurrences and narrows effective empiric options. Consistently, regional and global surveillances recognize Gram-negative Enterobacterales as important uropathogenic microbes, with uropathogenic *E. coli* (UPEC) classically dominant and go together with *P. mirabilis* and *Klebsiella* spp. Also, *S. aureus* enhances particularly in some works. Current surveying investigates additional display a non-negligible component of fungi, including species of *Candida*, in cultures of urine. These trends reinforce the requirement for alternative strategies of antimicrobials and locally applicable information that can relieve or complement pressure on presenting antimicrobials [1], [2].

Complicating this landscape, candidal UTIs and catheter-related candiduria happen principally in instrumented or hospitalized individuals and may resolve with exchange or removal of device alone, highlighting the significance of stewardship and accurate diagnosis. While *C. albicans* is classically implicated, non-albicans *Candida* are increasingly reported. On other hand, urinary infectious illnesses that caused via *Salmonella*—whether typhoidal or non-typhoidal—are infrequent but recognized, characteristically rising in patients with chronic carriage, immunocompromise, or urological abnormalities. Recognitions of these fewer common etiologies matters when designing panels of antimicrobials for *in-vitro* examining to mirror real-world isolates from patients with UTI [3], [4], [5].

Escalating resistances among uropathogenic microbes, such as prevalence of carbapenem-resistant strains and ESBL-producing Enterobacterales, has been emphasized in global prioritization exercises and contemporary clinical guidance. Guidance of IDSA emphasizes the challenges of therapy posed by CRE, ESBL-E and other dif-

ficult-to-treat Gram-negatives, while the Bacterial Priority Pathogenic List of 2024 WHO ranks third-generation cephalosporin-resistant *E. coli* and carbapenem-resistant *Klebsiella* pneumoniae through life-threatening cases. Therefore, exploring adjunctive or novel antimicrobial agents obtained from natural sources is not simply of academic attention; however, a practical requirement to diversify the anti-infective collection [6], [7], [8].

Medicinal plants propose a bioactive compounds-rich source—specifically polyphenols—with established activities of antifungals and antibacterial agents via mechanisms that include modulation of oxidative stress, chelation of metal, interactions of protein and nucleic-acid, and perturbation of membrane. Contemporary reviews detail how phenolics can disturb envelopes of microbes, prevent key enzymes of metabolites and formation of biofilm, and sometimes act synergistically with other ions or phytochemicals. Such multi-target actions could, in principle, reduce the likelihood of rapid resistance emergence and make plant extracts attractive candidates for screening toward priority uropathogens [9], [10], [11].

Within this space, *R. fruticosus* (blackberry) is notable for its leaves' abundant phenolics (e.g., flavonols, ellagitannins, phenolic acids) and related bioactivities. Systematic phytochemical analyses of blackberry leaves report high total phenolic content, with rutin frequently dominant, and document antioxidant, enzyme-inhibitory, anti-inflammatory and—critically—antimicrobial activities in leaf extracts. Parallel work on blackberry fruits and by-products corroborates antimicrobial effects toward bacteria including *E. coli*, *S. typhi*, and S. aureus, while antifungal effects appear more variable. Together, these data position blackberry leaf extract as a biologically plausible candidate to test toward clinically relevant UTI isolates [12], [13].

However, despite the growing phytochemical and bioactivity evidence base, there is a specific gap regarding targeted in-vitro evaluation of *R. fruticosus* **leaf** extract toward a panel representative of pathogens isolated from UTIs—namely *E. coli*, *S. typhi*, *S. aureus*, *C. albicans*, *P. mirabilis* and

Klebsiella spp. Addressing this gap could clarify the breadth of antibacterial (and antifungal) action, benchmark potency toward reference strains and recent clinical isolates, and provide foundational data for future fractionation, mechanism-of-action studies, and potential adjuvant applications. The present study therefore investigates the in-vitro antibacterial activity of R. fruticosus leaf extract toward this clinically relevant set of uropathogens, aiming to inform subsequent translational work and help diversify strategies toward AMR in UTIs.

Materials and Methods

Plant Material Collection

Fresh leaves of *R. fruticosus* (blackberry) were collected from the agricultural region of Al-Tarmiyah City, Iraq, in January 2024. The leaves were methodically washed with distilled water to eliminate debris and dust and then air-dried at temperature of ambient laboratory with intermittent turning to avoid contamination of fungi. Then, the dried leaves were preserved in fresh, airtight paper, clean, bags till extractions.

Preparation of the Ethanolic Extract

The dried material of plant was crushed utilizing a dry mechanical grinder for obtaining of the fine powders. Forty grams (40 g) of powdered leaves of *R. fruticosus* were softened in 160 mL of ethanol (70%) and mixed utilizing a shaker of laboratory at 4 °C for 24 hours to improve efficiency of extraction. The extract was first sieved utilizing many layers of sterile muslin cloth. Then, it re-filtered utilizing filter paper of Whatman No. 1 to eliminate residual particulates and fibers of the plant. In an oven at 40°C, the filtrate was evaporated till whole removal of ethanol, leaving a concentrated extract at the bottom of the beaker, as described by Al-Joboory and Al-Rawi [14]. At −20°C, the dried extract was then preserved in compactly closed glass vials till usage.

Preparation of Stock Solution and Dilutions

A stock solution of the leaf extract was prepared by dissolving 1 g of the dried extract in 10 mL of sterile distilled water, resulting in a concentration of 100 mg/mL. The solution was sterilized using Whatman No. 1 filter

paper to eliminate microbial contaminants. Serial dilutions were prepared from the stock to obtain concentrations of 10, 20, 30, and 40 mg/mL. These concentrations were used to evaluate antibacterial and antifungal activities. Clotrimazole (480 µg/mL) was used as the positive control.

Microbial Strains

The 6 clinically significant isolates of microbes related with urinary tract infectious illnesses (UTIs) were examined: *Klebsiella* spp., *P. mirabilis*, *C. albicans*, *S. aureus*, *S. typhi* and *E. coli*. These microorganisms were obtained from 52 clinical urine samples of UTI-diagnosed patients and established through microscopic identification and standard biochemical methods, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2023).

Antimicrobial Assay

The antibacterial activity of *R. fruticosus* leaf extract was evaluated using the agar well diffusion method according to Perez et al. [15]. Briefly, 25 mL of sterile nutrient agar was poured into each Petri dish and allowed to solidify. The agar surface was inoculated by evenly spreading 0.1 mL of a standardized microbial suspension (1.5×10^8 CFU/mL), equivalent to 0.5 McFarland standard, using a sterile spreader. After air-drying at room temperature, wells of 5 mm diameter were made in the agar using a sterile cork borer. Each well was filled with 0.2 mL of the prepared extract concentrations (10–40 mg/mL) using a sterile micropipette. Control wells were filled with clotrimazole solution (positive control) and sterile distilled water (negative control). The plates were incubated at 37 °C for 48 hours. Following incubation, antibacterial and antifungal activities were determined by measuring the diameter (in millimeters) of the inhibitory impact zones surrounding each well using a digital caliper. Each treatment was performed in triplicate, and mean inhibitory impact zone diameters were calculated. The absence of microbial growth in the inhibitory impact zone indicated susceptibility of the tested organism to the plant extract. Recent reports recommend the use of agar diffusion for screening plant extracts due to its reproducibility and correlation with MIC assays.

Statistical analyses

Statistical analyses were carried out utilizing SPSS software, version 20.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics, including standard deviation, mean, were utilized to data. P-value= < 0.01 (**) were regarded as extremely significant, and a p-value < 0.05(*) as statistically significant.

Results and discussion

Distribution of Uropathogenic Isolates

A total of 40 isolates were obtained from patients with urinary tract infectious illnesses (UTIs). The distribution of pathogens revealed that *E. coli* represented the most frequent isolate, accounting for 26.9% (n=14) of all samples (Table 1).The second most common isolate was *Klebsiella* spp., comprising 17.3% (n=9) of cases. *P. mirabilis* and *C. albicans* each accounted for 9.6% (n=5) of isolates. *S. aureus* constituted 7.7% (n=4) of isolates. The lowest prevalence was observed for *S. typhi* at 5.8% (n=3).

Table (1): Distribution of Uropathogenic Isolates.

UTI pathogens		No.	Percentages (%)
1	*E. coli*	14	35%
2	*Klebsiella* spp.	9	23%
3	*P. mirabilis*	5	13%
4	*C. albicans*	5	13%
5	*S. aureus*	4	10%
6	*S. typhi*	3	8%
Total		**40**	**100%**

Across 52 culture-positive UTI isolates, *E. coli* was the leading pathogen (26.9%, 14/52), followed by *Klebsiella* spp. (17.3%, 9/52). *S. typhi* in 5.8% (3/52), *S. aureus* in 7.7% (4/52) as well as *C. albicans* and *P. mirabilis* were each recovered in 9.6% (5/52). This manner—Gram-negative bacilli enteric dominating, with *E. coli* foremost—is consistent with contemporary evidence from multiple regions. Recent cross-sectional and meta-analytic re-

ports continue to identify *E. coli* as the principal uropathogen in both community and hospital settings, typically contributing ~30–60% of isolates, with *Klebsiella* spp., *P. mirabilis*, and *Enterococcus* spp. comprising most of the remainder. In this work, *E. coli* share (26.9%) sits at the lower end of this expected range but remains directionally concordant with the previous studies [16], [17], [18].

Comparisons with recent work from Iraq and the broader Middle East show close alignment. A study from Thi-Qar reported *E. coli* (45%) and *Klebsiella pneumoniae* (30%) as the top uropathogens, while a 2023 Iraqi overview similarly highlighted *E. coli* and *Klebsiella* as the predominant agents. Although this study's *E. coli* proportion is lower, the rank order matches, and *Klebsiella* as the second most common isolate mirrors those datasets. Differences in percentages likely reflect case-mix (inpatient vs outpatient), prior antibiotic exposure, and sampling periods [19], [20].

The recovery of *P. mirabilis* at 9.6% is compatible with recent summaries that place *Proteus* among the common Gram-negative contributors to UTIs, particularly in catheterized or complicated cases. In this work, *S. aureus* frequency (7.7%) is also within ranges reported in recent series where *S. aureus* features as the leading Gram-positive uropathogen after enteric Gram-negatives. A 2025 PLOS One study, for example, found *E. coli* (~30%) and *S. aureus* (~15%) most prevalent among Gram-negative and Gram-positive isolates, respectively—again broadly consistent with the distribution in this work, acknowledging setting differences [18], [21].

C. albicans accounted for 9.6% of isolates in this cohort. Contemporary literature indicates candiduria is uncommon in community UTIs but can reach notable proportions in hospitalized or device-associated infectious illnesses; recent reports estimate *Candida* involvement in ~5–10% of healthcare-associated UTIs, with higher rates where urinary catheters, diabetes, or broad-spectrum antibiotics are prevalent. In this work, candiduric proportion therefore suggests either a mixed inpatient/outpatient sample or enrichment for patients with typical

candiduria risk factors [22], [23].

Isolation of *S. typhi* from urine (5.8%) is unusual and exceeds the very low rates generally reported. Contemporary reviews emphasize that *Salmonella* UTIs—especially typhoidal serovars—are rare, often representing 0.01–0.1% of UTIs, and are typically linked to structural urinary tract abnormalities, nephrolithiasis, or chronic carriers. The relatively higher share in the sample or this work may reflect small denominator effects (n=52), local epidemiology, or specific patient-level risk factors (e.g., stones or recent systemic salmonellosis). Targeted chart review to assess such factors would help contextualize this finding [24], [25].

Antibacterial and Antifungal Activity of *R. fruticosus* Leaf Extract

The *R. fruticosus* ethanolic leaf extract displayed comprehensive spectra of activity of antimicrobe against entire examined microbes, with the degree of inhibitory influence raising proportionately with concentration of extract (10–40 mg/mL). Statistical significance of variations ($p<0.01$) were detected through concentrations for entire microbes (Table 2) (Figure 1), representing a strong relationship of doses–responses. At 10 mg/mL, inhibitory impact zones were minimal, ranging from 0 mm in *S. Typhi* to 11 mm in *P. mirabilis*. When the concentration was increased to 20 mg/mL, inhibitory impact zones expanded substantially—for example, S. aureus increased from 7 mm to 13 mm (an 85.7% rise), and C. albicans increased from 9 mm to 17.3 mm (92.2% rise). At 30 mg/mL, the average inhibitory impact zone for all pathogens increased by approximately 55–110% relative to 20 mg/mL, confirming enhanced bioactivity with higher extract concentration. At the maximum concentration (40 mg/mL), the extract displayed the highest inhibitory effect, particularly toward *C. albicans* (32 mm) and *Klebsiella* spp. (31 mm)—equivalent to inhibitory impact levels exceeding 290% and 244%, respectively, compared to their 10 mg/mL values. *P. mirabilis* and *S. aureus* followed with inhibitory impact zones of 25.3 mm and 22 mm, corresponding to increases of approximately 130% and 214%, respectively, from their initial readings. In contrast, *E. coli* and S.

Typhi represented smaller inhibitory impact zones of 15.3 mm and 15 mm, yet still increased by over 200% relative to the 10 mg/mL treatment. These data indicate that *R. fruticosus* leaf extract exhibits dose-dependent antimicrobial efficacy across both Gram-positive and Gram-negative bacteria, as well as fungal isolates.

Table (2): Antibacterial and Antifungal Activity of *R. fruticosus* Leaf Extract.

Uropathogenic isolates (n=52)	Concentration of extract				*p*-value
	Mean ± SD (mg/ml)				
	10	20	30	40	
E.coli	5 ± 0.01	7.0 ± 0.1	12.1 ± 1.25	15.3 ± 0.57	**0.007****
S. typhi	0.0 ± 0.0	9.0 ± 1.0	12.0 ± 1.0	15.0 ± 1.0	**0.009****
S. aureus	7.0 ± 1.0	13.0 ± 1.0	18.1 ± 1.0	22.0 ± 0.0	**0.01****
C. albicans	9.0 ± 1.0	17.3 ± 0.57	22.6 ± 0.57	32.0 ± 1.0	**0.007****
P. mirabilis	11.0 ± 0.01	12.3 ± 0.57	23.6 ± 1.52	25.3 ± 1.52	**0.012****
***Klebsiella* spp.**	9.0 ± 1.0	14.0 ± 2.0	23.0 ± 1.0	31.0 ± 1.0	**0.001****

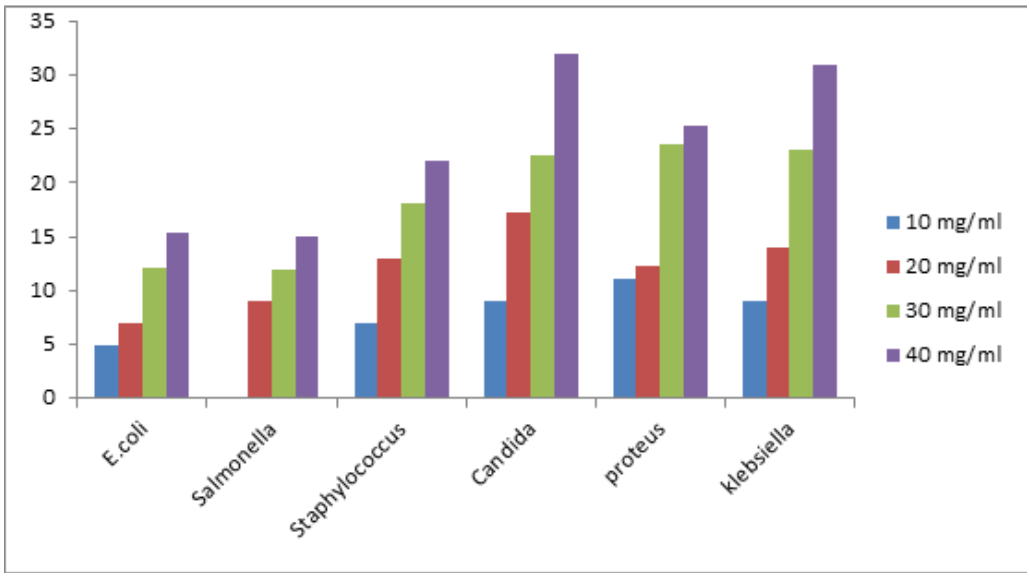

Figure 1: Antibacterial and Antifungal Activity of R. fruticosus Leaf Extract.

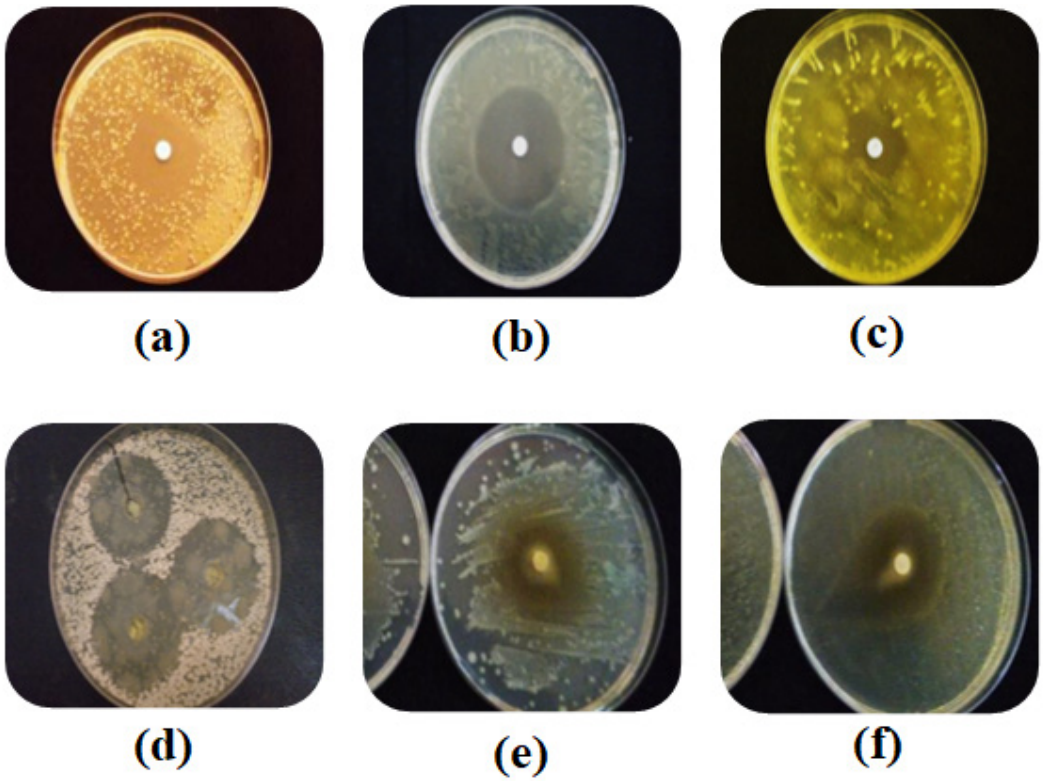

Figure 2: The effect of the ethanolic extract of blackberry leaves toward (a) *E. coli*; (b) *S. typhi*; (c) *S. aureus*; (d) *C. albicans*; (e) *P. mirabilis*; (f) *Klebsiella* spp.

Across all pathogens, inhibitory impact zones increased monotonically with extract concentration (10–40 mg/mL) and the between-dose differences were statistically significant ($p < 0.01$), indicating a strong dose–response. The magnitude of change was large: for example, S. aureus rose from 7 mm at 10 mg/mL to 13 mm at 20 mg/mL (+85.7%), and *C. albicans* from 9 mm to 17.3 mm (+92.2%). Pooled across organisms, the 30 mg/mL dose expanded mean zones by roughly 55–110% versus 20 mg/mL, and the 40 mg/mL dose produced the largest effects, consistent with greater availability of phenolics and other bioactives at higher concentrations. These patterns align with contemporary reports that antimicrobial outputs of botanical extracts scale with total polyphenol dose and extract enrichment, reflecting membrane-active and redox-active mechanisms that intensify with concentration [26].

At 40 mg/mL, *C. albicans* (32 mm) and *Klebsiella* spp. (31 mm) were the

most susceptible, equivalent to >290% and ~244% gains versus their 10 mg/mL baselines. This strong antifungal response agrees with recent evidence that Rubus leaf preparations (and closely related species) exhibit pronounced activity toward *Candida* through polyphenol-driven membrane destabilization, ROS induction, and interference with ergosterol-associated functions. Comparable inhibitory impact of *Candida* by *Rubus* leaf extracts has been reported, including for *R. ulmifolius* leaves, supporting a genus-level antifungal potential. Mechanistically, complex tannins (e.g., ellagitannins), flavonols, and terpenoid fractions abundant in *Rubus* leaves can permeabilize fungal membranes and impair biofilm development, which rationalizes the large zones we observed for *C. albicans* [26], [27], [28].

Gram-positive S. aureus represented robust inhibitory impact (22 mm at 40 mg/mL; +214% from baseline), consistent with literature noting higher susceptibility of Gram-positives to polyphenols due to the absence of an outer membrane barrier. Contemporary studies on blackberry extracts report meaningful anti-staphylococcal effects and link activity to phenolic richness and extract polarity—features compatible with our ethanolic preparation. Our dose-responsive increases mirror these findings and support the role of ethanol in extracting membrane-active aglycones and mid-polarity phenolics that efficiently diffuse through peptidoglycan layers [27], [29].

Among Gram-negatives, *Klebsiella* spp. and P. mirabilis were comparatively sensitive (31 mm and 25.3 mm at 40 mg/mL), whereas *E. coli* and S. Typhi exhibited smaller but still meaningful zones (15.3 mm and 15 mm; both >200% above 10 mg/mL). This heterogeneity is consistent with strain-specific outer-membrane permeability and efflux phenotypes. Recent work using *R. fruticosus* leaf phenolic extracts demonstrated measurable growth inhibitory impact and sub-lethal growth perturbations in Gram-negatives (e.g., *Listeria* models and foodborne surrogates), with membrane integrity loss and altered cultivability at increasing sub-MIC levels—mechanisms that plausibly underlie our graded responses and the need for higher doses to sur-

mount Gram-negative barriers [30], [31].

The overall spectrum observed here—activity toward S. aureus, *E. coli*, *Klebsiella* spp., P. mirabilis, S. Typhi, and C. albicans—fits with recent characterizations of the *Rubus* phytochemical space. Current profiling detect rich pools of phenolic acids, flavonols, and ellagitannins, in matrices of blackberry, with antimicrobial actions spanning inhibitory impact of protein synthesis, inactivation of enzyme, and disruption of membrane. The significant p-values across doses in our Table 2 are therefore pharmacologically credible: as concentration rises, multiple targets are engaged synergistically, translating to larger inhibitory impact zones across diverse taxa [27], [32].

Finally, these findings compare favorably with post-evaluations of *Rubus* extracts that highlight (i) stronger antifungal than antibacterial effects in several models, (ii) notable anti-staphylococcal activity, and (iii) clear concentration dependence. While direct head-to-head organisms differ across studies, the directions and relative magnitudes we report are concordant with these contemporary datasets, strengthening confidence that *R. fruticosus* leaves are a credible source of broad-spectrum antimicrobial agents, particularly at higher extract loads [29], [30].

Given the pronounced dose–response and the strong effects on *Candida* and *Klebsiella*, bioassay-guided fractionation focused on ellagitannin-rich and flavonol-rich subfractions from blackberry leaves is warranted, alongside MIC/MBC/MFC determinations and synergy testing with standard antimicrobials to translate inhibitory impact-zone gains into clinically interpretable potency metrics [27], [33].

Conclusions

The ethanolic leaf extract of *R. fruticosus* exhibited significant and concentration-dependent antimicrobial activity toward all tested uropathogens. The extract was most effective toward *C. albicans* and *Klebsiella* spp., followed by *P. mirabilis* and *S. aureus*, confirming a broad-spectrum efficacy encompassing both Gram-positive, Gram-negative, and fungal species. Statistical analysis ($p < 0.01$) validated

the dose–response relationship, highlighting increased inhibitory impact with higher extract concentrations. These outcomes substantiate that the bioactive phytochemicals—especially phenolics and flavonoids—present in *R. fruticosus* contribute to its potent antimicrobial action. The study underscores the plant's therapeutic promise as an adjunct or alternative to conventional antibiotics, especially toward resistant uropathogens. Future investigations should focus on isolating active constituents, defining their mechanisms of action, determining minimum inhibitory concentrations, and exploring synergistic potential with existing antimicrobial agents.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Data availability

All data supporting the findings of this study, including raw inhibitory impact zone measurements and statistical analyses, are provided in the supplementary materials submitted with this article.

AI Disclosure

The authors declare that no artificial intelligence (AI) tools were used in the generation of data, analysis, or writing of this manuscript.

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