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Iron-Responsive Inhibition of Growth and Post-Antibiotic Regrowth of Uropathogenic *Escherichia coli* by Aqueous Plant Extracts

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Abstract

Recurrent urinary tract infections caused by uropathogenic *Escherichia coli* (UPEC) are associated with bacterial persistence and survival following antibiotic treatment, motivating interest in non-antibiotic adjuncts. We investigated aqueous extracts of six medicinal plants against UPEC 536, assessing their chemical profiles, growth-inhibitory activity, effects on biofilm formation, and post-antibiotic regrowth. *Arctostaphylos uva-ursi* and *Geum urbanum* exhibited the strongest antibacterial activity, with minimum inhibitory concentrations (MICs) of 5 and 10 mg/mL, respectively. Both extracts contained chromatographic signals consistent with hydrolysable tannins. *Betula pendula* and *Juniperus communis* delayed bacterial growth, whereas *Arnica montana* and *Solidago virgaurea* showed minimal activity. Supplementation with 100 µM $FeCl_3$ counteracted growth inhibition by both active extracts, whereas $MgCl_2$ did not. At 10 mg/mL, *G. urbanum* and *A. uva-ursi* reduced biofilm biomass to approximately 8% and 14% of untreated controls, respectively. Following ampicillin exposure (100× MIC), surviving bacteria regrew to approximately 10^9 CFU/mL in extract-free medium, whereas subinhibitory concentrations of either extract maintained viable counts near the post-antibiotic baseline. These findings identify iron-responsive growth inhibition and suppression of post-antibiotic regrowth as distinct activities of tannin-containing aqueous plant extracts against UPEC.

Keywords: uropathogenic *Escherichia coli*; urinary tract infection; *Arctostaphylos uva-ursi*; antibiotic tolerance; biofilm; natural products; herbal drugs; post-antibiotic regrowth

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1. Introduction

Urinary tract infections (UTIs) are among the most common bacterial infections [1]. They affect about half of all women at least once in their lifetime, and a large proportion of patients experience recurrence within months of the first episode [1,2]. Uropathogenic *Escherichia coli* (UPEC) causes most uncomplicated community-acquired UTIs [3]. Its success as a uropathogen relies on adhesion to the uroepithelium, invasion of bladder epithelial cells and the formation of intracellular bacterial communities [4,5]. These intracellular reservoirs, together with antibiotic-tolerant persister cells, survive standard therapy and seed recurrent infection [6,7].

Antibiotics remain the first-line treatment, but repeated courses drive antibiotic resistance and disturb the gut and urogenital microbiota [4]. There is therefore a clear need for non-antibiotic approaches that prevent or support the treatment of UTIs, particularly in recurrent disease [8].

Herbal drugs have a long tradition in the management of UTIs and remain widely used worldwide, both as self-medication and as adjuvants to antibiotic therapy. The mechanisms of action of herbal preparations used for urinary tract disorders include antimicrobial, anti-adhesive, and diuretic effects [8]. Urinary antiseptics and anti-adhesion agents, such as bearberry leaf (*Arctostaphylos uva-ursi*), lingonberry leaf (*Vaccinium vitis-idaea*), and cranberry fruit (*Vaccinium macrocarpon*), reduce bacterial presence in the urinary tract: bearberry and lingonberry release antibacterial hydroquinone conjugates in the urine [9], while cranberry inhibits bacterial adhesion to the uroepithelium through A-type proanthocyanidins [10]. Diuretic drugs, such as birch leaf (*Betula pendula*), goldenrod herb (*Solidago virgaurea*), and juniper berry (*Juniperus communis*), increase urine flow and flush bacteria from the urinary tract [11–13]; several have EU herbal monographs for this use [14–17].

To investigate whether antibacterial activity against UPEC is associated with traditional urological use or extends to medicinal plants with other therapeutic indications, we compared four herbal drugs traditionally used for urinary tract disorders with two non-urological comparators, Arnica flower heads (*Arnica montana*) and wood avens rhizome (*Geum urbanum*). Although traditionally used for different therapeutic purposes, both comparators contain constituents with reported antibacterial activity, with *G. urbanum* being particularly rich in ellagitannins [12].

The antibacterial potential of these drugs has commonly been attributed to their polyphenols, such as hydrolysable tannins, proanthocyanidins, and flavonoids [18,19]. Among the mechanisms proposed for tannins, interference with microbial iron acquisition is directly relevant to the urinary tract. Iron acquisition is essential for UPEC fitness in the iron-limited urinary tract, where bacteria employ multiple systems for acquiring iron, including siderophore-mediated uptake and heme utilization [20–22]. Plant polyphenols, particularly tannins, can interact with metal ions, and iron deprivation has been proposed as one mechanism contributing to their antibacterial activity [19,23]. Previous work on herbal drugs has largely reported MIC values or anti-adhesive activity; however their effects on bacterial recovery following antibiotic exposure remain less well characterized [24]. The present study asks whether iron availability influences growth inhibition, and it examines the regrowth of antibiotic-surviving UPEC following antibiotic withdrawal.

The aim of this study was to evaluate aqueous extracts of herbal drugs traditionally used in urinary tract disorders for their activity against uropathogenic *E. coli* and to compare them with drugs without a urological indication. We characterized the phytochemical composition of extracts by LC-MS and assessed their effects on UPEC growth, biofilm formation, and post-antibiotic regrowth. The results identify bearberry leaf and wood avens rhizome as the most active materials and highlight compound classes potentially contributing to their antibacterial activity.

2. Results

2.1. Phytochemical Characterization

HPLC-DAD-MS^n analysis revealed distinct chromatographic fingerprints for the six aqueous extracts (Figures S1–S6). Tentative compound identifications based on retention times, UV absorption characteristics and mass spectral fragmentation patterns are presented in Tables S1–S6.

The *Geum urbanum* rhizome extract contained signals characteristic of hydrolysable tannins and ellagic acid derivatives [12]. A later-eluting signal, with m/z 301 and UV absorption maxima at 252 and 366 nm, was consistent with ellagic acid (Table S1). These findings support the presence of ellagitannin-related constituents, although the identities of individual tannin isomers could not be established.

The *Betula pendula* and *Solidago virgaurea* extracts exhibited signals characteristic of caffeoylquinic acid derivatives and flavonoid glycosides (Tables S2 and S3) [13,25]. In *S. virgaurea*, ions at m/z 609 and 463, both yielding fragments at m/z 301, were consistent with quercetin glycosides. The *Arnica montana* extract contained caffeoylquinic acid derivatives, including compounds exhibiting precursor ions at m/z 353 and 515, together with flavonoid glycosides [26] (Table S4). The *Juniperus communis* extract contained flavonoid glycosides and proanthocyanidins, including a signal at m/z 577 with characteristic fragmentation consistent with a B-type procyanidin dimer [27,28] (Table S5).

The *Arctostaphylos uva-ursi* extract displayed a chemically distinct profile characterized by hydroquinone glycosides and galloylated phenolic compounds (Table S6) [29]. Arbutin was tentatively identified at RT 3.6 min, with ions at m/z 271 ($[M - H]^-$) and 317 ($[M + HCOO]^-$). Additional signals at m/z 483, 635, and 939 were consistent with galloylglucose and higher galloylglucose derivatives, while ions at m/z 423 and 575 supported the presence of galloylarbutin derivatives. The profile also contained signals assigned to other hydrolysable tannins, including ellagitannin-related compounds (Table S6). Free hydroquinone was not specifically identified in the available chromatographic data. Among the six extracts, signals assigned to hydrolysable tannins were identified in *G. urbanum* and *A. uva-ursi*.

2.2. Growth-Inhibition Activity

The antibacterial activity of six aqueous plant extracts against UPEC 536 was evaluated by monitoring bacterial growth (Figure 1). The extracts exhibited different degrees of growth inhibition, with *A. uva-ursi* and *G. urbanum* showing the strongest antibacterial activity.

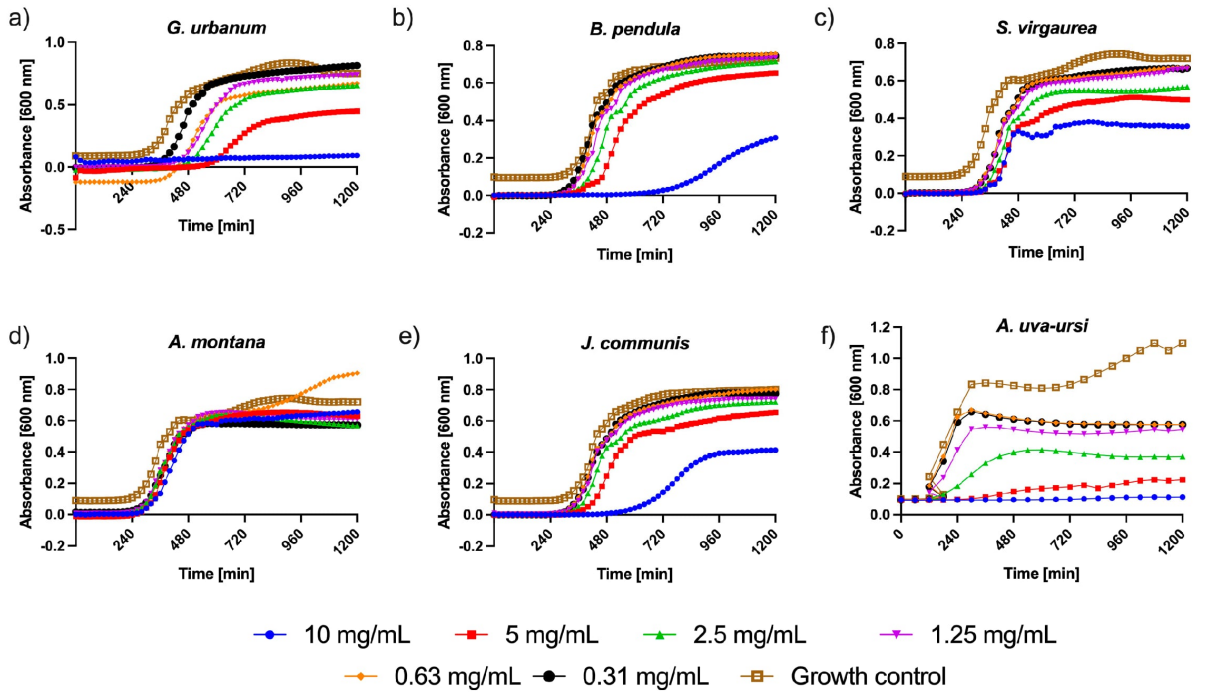


Figure 1. Effects of aqueous plant extracts on the growth of UPEC 536. Bacterial growth was monitored by measuring optical density at 600 nm (OD_{600}) in the presence of increasing concentrations of six aqueous plant extracts. Extract-free cultures served as growth controls. Curves represent mean values from three independent biological experiments ($n = 3$). Error bars were omitted for clarity.

The *A. uva-ursi* extract exhibited the lowest MIC among the tested extracts, at 5 mg/mL. The *G. urbanum* extract completely prevented a detectable increase in optical density at 10 mg/mL, corresponding to its MIC under the assay conditions. At 5 mg/mL, bacterial growth was delayed, with the lag phase increasing from 338 ± 12 min in the untreated control to 515 ± 5 min (Table 1).

Table 1. Growth inhibition and lag-phase duration of UPEC 536. Values are presented as mean ± SD from three independent biological experiments ($n = 3$). Lag-phase duration was calculated using the maximum-slope extrapolation method implemented in BioTek Gen5 software (version 3.12). MIC values represent the lowest concentrations preventing detectable growth under the assay conditions.

Extract	MIC (mg/mL)	Control Lag (min)	Lag at 5 mg/mL (min)	Lag at 10 mg/mL (min)
A. uva-ursi	5	175 ± 7	No detectable growth	No detectable growth
G. urbanum	10	338 ± 12	515 ± 5	No detectable growth
B. pendula	>10	368 ± 5	440 ± 7	806 ± 15
J. communis	>10	316 ± 9	393 ± 12	421 ± 9
A. montana	>10	291 ± 4	298 ± 4	312 ± 8
S. virgaurea	>10	290 ± 2	266 ± 16	281 ± 18

The *B. pendula* extract produced the greatest prolongation of the lag phase among extracts that did not completely inhibit growth. At 10 mg/mL, the lag phase increased from 368 ± 5 min in the control to 806 ± 15 min, whereas at 5 mg/mL it reached 440 ± 7 min. The *J. communis* extract exhibited a weaker effect, increasing the lag phase from 316 ± 9 min in the control to 393 ± 12 and 421 ± 9 min at 5 and 10 mg/mL, respectively.

In contrast, *A. montana* and *S. virgaurea* exhibited limited effects on bacterial growth, with no complete inhibition observed at concentrations up to 10 mg/mL. Their lag times remained close to those of the corresponding untreated controls (Table 1). Ciprofloxacin and ampicillin, used as positive antibacterial controls, prevented detectable growth at minimum concentrations of 0.016 µg/mL and 6 µg/mL, respectively.

Overall, *A. uva-ursi* exhibited the lowest MIC, followed by *G. urbanum*, whereas *B. pendula* and *J. communis* primarily delayed bacterial growth without complete inhibition at the concentrations tested (Table 1). To independently validate the OD-based MIC determinations, viable bacterial counts were assessed following 20 h of exposure to *A. uva-ursi* (5 mg/mL) and *G. urbanum* (10 mg/mL). In extract-free controls, bacterial counts increased from $(7.1 \pm 0.6) \times 10^4$ to $(8.2 \pm 1.0) \times 10^8$ CFU/mL. In contrast, viable counts after 20 h remained at $(7.1 \pm 6.3) \times 10^4$ CFU/mL for *A. uva-ursi* and $(4.0 \pm 3.0) \times 10^4$ CFU/mL for *G. urbanum* (mean ± SD, $n = 3$). These results confirm that both extracts prevented net bacterial population expansion at their respective OD-based MICs (Figure S8).

2.3. Iron-Supplemented Growth

To investigate whether iron availability influences the growth-inhibitory activity of the two most effective extracts, bacterial growth was monitored in the presence of *G. urbanum* and *A. uva-ursi*, with or without $FeCl_3$ supplementation (100 µM) (Figure 2).

The addition of $FeCl_3$ reversed the growth inhibition observed with both extracts. In cultures treated with *G. urbanum* at its MIC (10 mg/mL), absorbance remained unchanged throughout the 20 h incubation period. In contrast, $FeCl_3$ supplementation restored a typical bacterial growth curve.

A similar effect was observed with *A. uva-ursi*. At 5 mg/mL and 10 mg/mL, supplementation with $FeCl_3$ restored bacterial growth, reaching a final absorbance of approximately 0.88.

This effect was specific to $FeCl_3$. Supplementation with $MgCl_2$ at the same molar concentration (100 µM) did not restore UPEC growth in the presence of either extract at its MIC. These findings demonstrate that $FeCl_3$ counteracts the growth-inhibitory effects of both extracts under the tested conditions, indicating an iron-responsive effect.

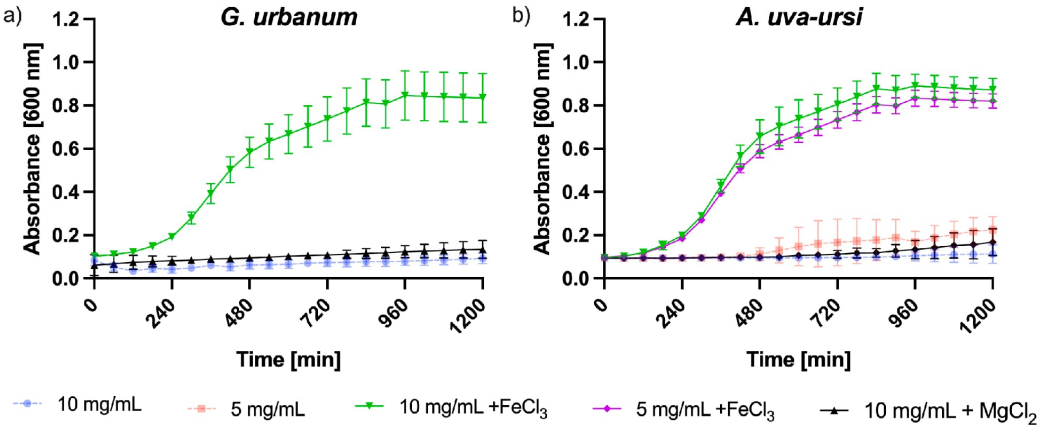


Figure 2. Effect of $FeCl_3$ and $MgCl_2$ supplementation on the growth-inhibitory activity of *G. urbanum* and *A. uva-ursi* extracts against UPEC 536. Growth was monitored by OD_{600} in the presence of $FeCl_3$ (100 µM) or $MgCl_2$ (100 µM). Semi-transparent dashed lines represent the corresponding growth curves obtained in the MIC assay without supplementation, whereas solid lines represent cultures supplemented with $FeCl_3$ or $MgCl_2$. Data represent mean ± SD from three independent biological experiments ($n = 3$).

2.4. Effects of Plant Extracts on Biofilm Formation

Based on the initial growth inhibition screening, *A. uva-ursi*, *G. urbanum*, *B. pendula*, and *J. communis* were selected for further evaluation of their effects on biofilm formation. These extracts exhibited concentration-dependent reductions in attached biofilm biomass following 48 h of incubation (Figure 3). *G. urbanum* showed the strongest reduction in biofilm biomass, reaching approximately 8% of the untreated control at 10 mg/mL. Notably, reductions were also observed at 2.5 and 5 mg/mL, corresponding to approximately 68% and 47% of control biomass, respectively. These concentrations were below the MIC determined in planktonic growth assays. Similarly, *A. uva-ursi* markedly reduced biofilm formation, with relative biomass decreasing to approximately 17% and 14% of the control at 5 and 10 mg/mL, respectively.

The effects of *B. pendula* and *J. communis* were less pronounced, with biofilm biomass reaching approximately 58% and 77% of control values at 10 mg/mL, respectively (Figure 3). At lower concentrations, both extracts produced only minor changes, with a slight biofilm-promoting tendency.

Overall, these results demonstrate that *G. urbanum* and *A. uva-ursi* exhibited the strongest effect on biofilm development by UPEC 536, with *G. urbanum* showing reduced biofilm accumulation even at concentrations below its planktonic MIC.

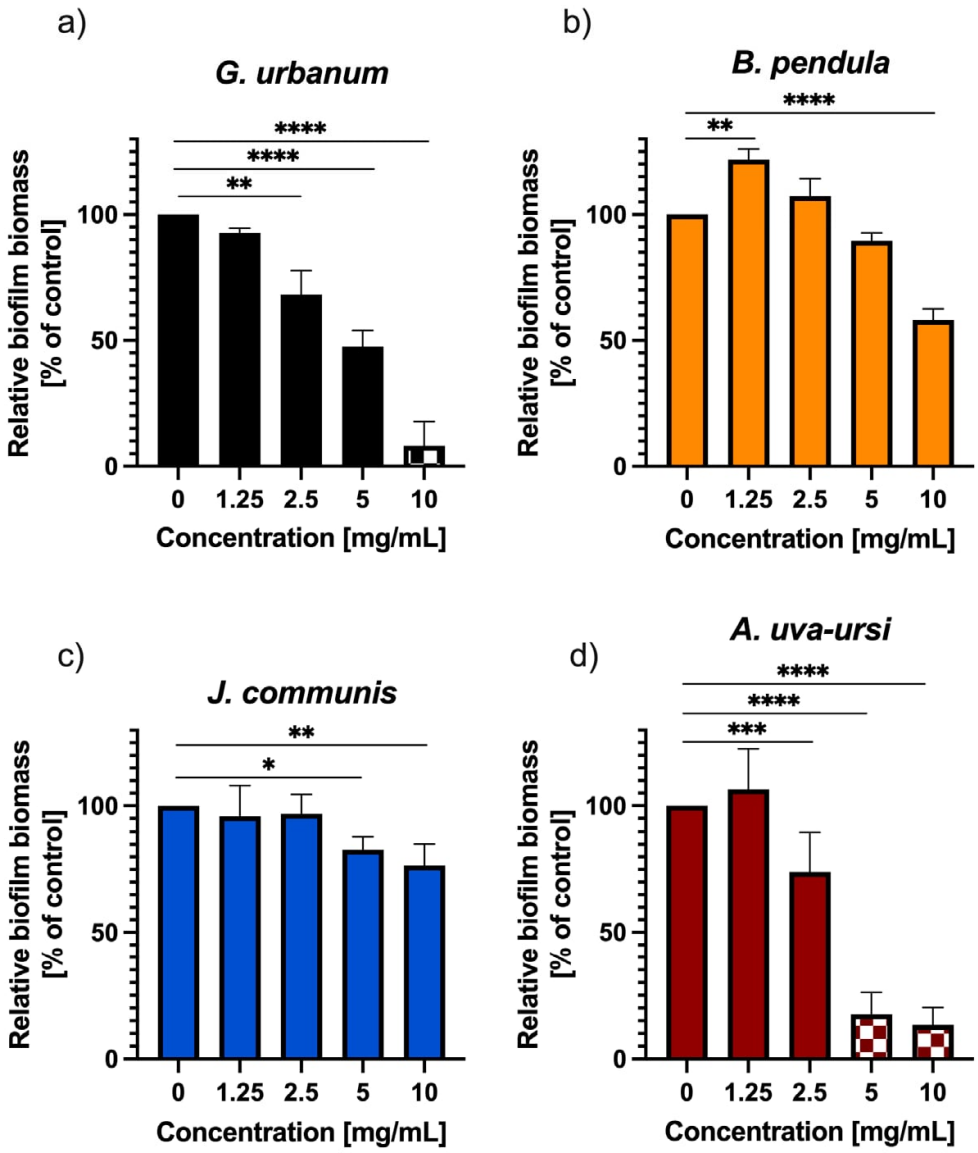


Figure 3. Effects of aqueous plant extracts on biofilm formation by UPEC 536. Biofilm biomass was quantified by crystal violet staining after 48 h of incubation in the presence of extracts and expressed as a percentage of the untreated control (100%). Checkered bars indicate inhibitory concentrations determined in planktonic growth assays. Data represent mean ± SD from three independent biological experiments ($n = 3$). Statistical significance was assessed using two-way ANOVA followed by Dunnett's multiple-comparison test. Only statistically significant differences are indicated: adjusted $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

2.5. Plant Extracts Restrict Post-Antibiotic Regrowth of UPEC

To investigate bacterial recovery following antibiotic exposure, UPEC was treated with ampicillin at 600 µg/mL (100× MIC) for 48 h. Following antibiotic removal, surviving bacteria were transferred to fresh BHI medium containing β-lactamase, either without extracts or supplemented with subinhibitory concentrations of *G. urbanum* (5 mg/mL) or *A. uva-ursi* (2.5 mg/mL). Bacterial recovery was monitored over an additional 48 h by determining colony-forming units (CFU/mL) (Figure 4) and optical density (OD_{600}) (Figure S7).

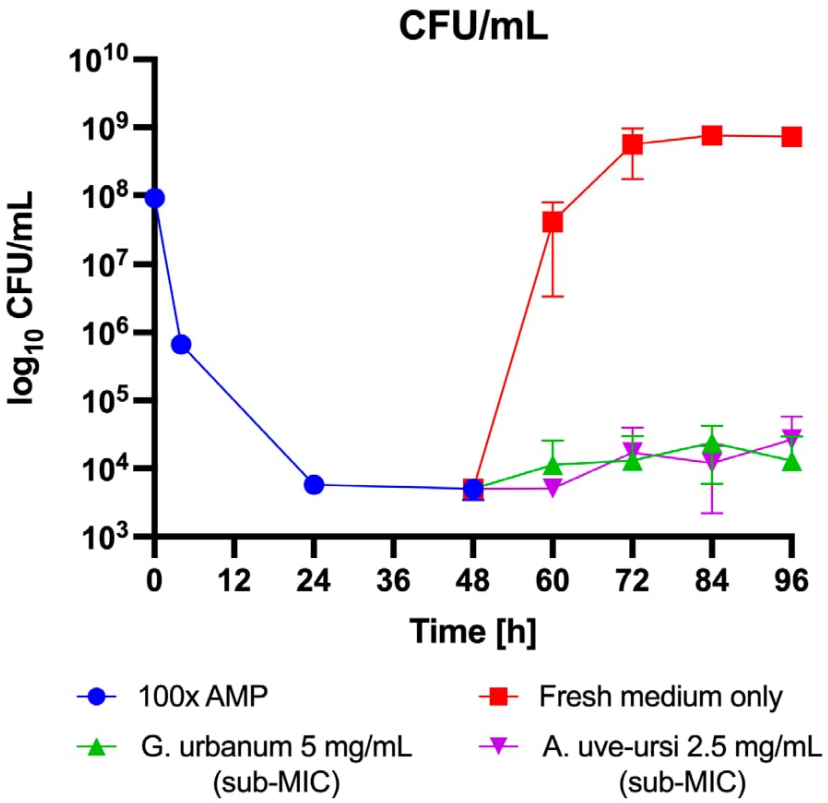


Figure 4. Effects of aqueous plant extracts on post-antibiotic regrowth of UPEC. Bacteria were exposed to ampicillin (600 µg/mL; 100× MIC) for 48 h, followed by recovery in fresh medium without extracts or supplemented with *G. urbanum* (5 mg/mL) or *A. uva-ursi* (2.5 mg/mL). Bacterial viability was determined by CFU enumeration. Data represent mean ± SD from three independent biological experiments (n = 3) and are presented on a logarithmic scale.

Ampicillin exposure reduced viable bacterial counts from approximately 10^8 to ~10^3 CFU/mL, with a surviving population remaining detectable after 48 h. The biphasic killing curve suggests the presence of a surviving antibiotic-tolerant subpopulation [7]. Following antibiotic withdrawal, surviving bacteria resumed growth in extract-free medium containing β-lactamase, reaching approximately 10^9 CFU/mL. In contrast, both plant extracts markedly restricted bacterial recovery, with viable counts remaining around 10^4 CFU/mL throughout the recovery period. No detectable regrowth was observed in control cultures maintained in the presence of ampicillin or when β-lactamase was omitted from the recovery medium. Furthermore, the ampicillin MIC of regrowing bacteria remained unchanged compared with the parental strain.

These findings demonstrate that subinhibitory concentrations of *G. urbanum* and *A. uva-ursi* substantially restrict post-antibiotic regrowth of the antibiotic-tolerant UPEC population without eliminating the surviving bacteria.

3. Discussion

The present study demonstrates that aqueous plant extracts differ in their effects on UPEC growth, biofilm formation, and recovery following antibiotic exposure. Among the six investigated preparations, *A. uva-ursi* and *G. urbanum* exhibited the strongest antibacterial activity, with MICs of 5 and 10 mg/mL, respectively. Notably, *G. urbanum* showed strong antibacterial activity despite having no established traditional urological indication, highlighting that activity against UPEC is not restricted to medicinal plants traditionally used for urinary tract disorders. Both active extracts contained hydrolysable tannin-related

constituents, which may contribute to their antibacterial effects. They also reduced biofilm biomass and markedly restricted bacterial regrowth following ampicillin withdrawal at subinhibitory concentrations. Furthermore, supplementation with $FeCl_3$ counteracted their inhibitory effects in planktonic growth assays, suggesting that iron availability influences their antibacterial activity. Together, these findings highlight the antibacterial potential of chemically distinct plant preparations and suggest that traditional therapeutic indications alone may not predict their activity against UPEC.

Phytochemical analysis provided qualitative profiles without absolute quantification of individual constituents and revealed that both active extracts contained constituents compatible with hydrolysable tannins, although their chemical profiles differed considerably. The *G. urbanum* extract was characterized by ellagitannin-related compounds and ellagic acid derivatives, whereas *A. uva-ursi* contained arbutin, galloylarbutin derivatives, galloylglucoses, and other hydrolysable tannin-related constituents. Tannins exhibit antimicrobial activity through several mechanisms, including interactions with bacterial proteins and membranes and sequestration of essential metal ions [19,23]. The observation that the most active extracts contained hydrolysable tannin-related constituents is consistent with these mechanisms [18].

The reversal of growth inhibition following $FeCl_3$ supplementation represents an important observation. Previous studies have demonstrated that iron supplementation can counteract the antibacterial effects of tannic acid and certain plant polyphenols [19,30]. Similarly, the inhibitory effects of *G. urbanum* and *A. uva-ursi* were markedly reduced following the addition of 100 µM $FeCl_3$. This response is compatible with reduced iron availability contributing to growth inhibition. Such a mechanism is biologically relevant because iron acquisition is essential for UPEC fitness, and uropathogenic strains possess multiple systems for acquiring iron under iron-limited conditions [20–22]. However, Fe(III) may also interact directly with extract constituents, modifying their solubility, chemical properties, or biological availability. Furthermore, the response was examined at a single iron concentration and primarily assessed through optical density measurements. Consequently, these findings support iron-responsive growth inhibition but do not establish direct iron chelation or iron deprivation as the principal antibacterial mechanism.

Tannins readily interact with proteins and other medium constituents, and their apparent antibacterial activity can depend on nutrient availability [31,32]. Štumpf et al. [32] demonstrated that increasing the concentration of growth medium increased the MICs of several tannins and tannin-containing extracts against *E. coli*. Such interactions may influence both the availability of inhibitory compounds and the nutrients accessible to bacteria. The absence of a comparable effect with $MgCl_2$ indicates that the reversal is not a general consequence of adding a cation to the medium. The obtained results, however, should be interpreted within the tested experimental conditions and not extrapolated directly to iron-limited environments encountered during infection.

The effects on biofilm formation further distinguished the investigated extracts. *G. urbanum* and *A. uva-ursi* substantially reduced biofilm formation, particularly at concentrations corresponding to their respective MICs. Because the extracts were present throughout biofilm formation, reduced biomass may partly reflect inhibition of bacterial proliferation rather than a biofilm-specific mechanism. This interpretation is supported by the pronounced biomass reductions at growth-inhibitory concentrations [33]. The present findings therefore demonstrate reduced biofilm accumulation under extract exposure but do not establish activity against preformed biofilms or a biofilm-specific mechanism.

A particularly relevant finding was the inhibition of bacterial recovery following ampicillin exposure. Treatment with ampicillin at 100× MIC substantially reduced the viable bacterial population, leaving a small surviving fraction after 48 h. Upon antibiotic with-

drawal, these survivors rapidly regrew in fresh medium. In contrast, supplementation with sub-MICs of *G. urbanum* and *A. uva-ursi* restricted the regrowth of the culturable population, maintaining viable counts close to the post-antibiotic baseline. The unchanged ampicillin MIC of recovered bacteria argues against the selection of stable resistant mutants, while the reduced killing rate during antibiotic exposure is consistent with phenotypic tolerance [7]. The extracts did not demonstrate eradication of surviving bacteria but prevented the regrowth observed in untreated recovery cultures. Previous work has shown that cranberry-derived pectic oligosaccharides can reduce UPEC persistence during ampicillin exposure [24]. The present experiments examined extract activity after antibiotic removal, addressing a distinct stage of bacterial recovery. These findings demonstrate the ability of the tested extracts to restrict the regrowth of antibiotic-surviving UPEC under the tested conditions.

The mechanisms underlying growth inhibition and restricted post-antibiotic recovery may not be identical. Although iron supplementation reversed growth inhibition in the planktonic assay, whether iron availability similarly influences the recovery of antibiotic survivors remains to be determined.

Differences between optical density and viable counts during post-antibiotic recovery also require consideration. Cultures supplemented with the extracts showed increased turbidity despite minimal expansion of the culturable population. Given the presence of tannin-related constituents, interactions with components of the nutrient-rich BHI medium may have contributed to light scattering through aggregation or precipitation. Optical density therefore cannot be assumed to reflect bacterial biomass accurately under these conditions. CFU enumeration provides the more direct measure of culturable bacterial recovery [34].

It is important to distinguish the antibacterial effects observed in this study from biofilm-associated antibiotic tolerance and genetically acquired resistance. While the growth inhibition assays measured the direct effects of plant extracts on bacterial proliferation, the biofilm experiments assessed biomass accumulation rather than antibiotic susceptibility of biofilm-associated cells. Biofilm-associated tolerance may arise from physiological heterogeneity, reduced metabolic activity, and limited antibiotic penetration, whereas antibiotic resistance involves mechanisms that permit bacterial growth at otherwise inhibitory antibiotic concentrations [7,35,36]. Our post-antibiotic experiments demonstrated restricted regrowth of surviving UPEC without a change in ampicillin susceptibility but did not establish reversal of antibiotic resistance or a specific mechanism targeting bacterial tolerance.

Several limitations should be considered when interpreting these findings. All experiments were performed using UPEC 536 and plant materials obtained from a single commercial batch for each species. Consequently, the reproducibility of the observed effects across clinical isolates and independent plant batches remains to be established. Moreover, the extracts were investigated as chemically complex aqueous preparations without quantitative standardization of individual constituents. The relatively high concentrations required for growth inhibition (5–10 mg/mL) raise questions about their achievable levels in urine following oral administration. In addition, the stability of extract constituents under the experimental conditions and their potential toxicity were not evaluated. For *A. uva-ursi*, safety assessment is particularly relevant because arbutin-derived hydroquinone exposure is an established consideration in the use of bearberry leaf preparations [17]. Challenges related to phytochemical variability, stability, and bioavailability have also been highlighted in broader investigations of plant-derived therapeutics [37]. These considerations limit direct extrapolation to clinical applications and highlight the need for further studies addressing chemical standardization, pharmacokinetics, stability, and safety.

In conclusion, *G. urbanum* and *A. uva-ursi* aqueous extracts inhibited UPEC growth, reduced biofilm development, and substantially restricted bacterial regrowth following antibiotic exposure. The reversal of growth inhibition following $FeCl_3$ supplementation suggests that iron availability may influence the antibacterial activity of both extracts. Together, these findings highlight the potential of tannin-containing plant preparations to restrict UPEC growth and post-antibiotic recovery, providing a basis for further investigation of their underlying mechanisms.

4. Materials and Methods

4.1. Plant Materials and Preparation of Aqueous Extracts

Plant materials were selected to represent different traditional therapeutic uses. *Arctostaphylos uva-ursi*, *Betula pendula*, *Solidago virgaurea*, and *Juniperus communis* were included because of their traditional use in urinary tract disorders. *Arnica montana*, traditionally used for its topical anti-inflammatory properties, and *Geum urbanum*, known for its astringent properties and tannin-rich composition [12], were selected as non-urological comparators. The materials were commercially available leaves of *Arctostaphylos uva-ursi* (L.) Spreng. (*Uvae ursi folium*) (NANGA Przemysław Figura, Złotów, Poland), rhizome of *Geum urbanum* L. (Dary Natury Sp. z o. o., Grodzisk, Poland), leaves of *Betula pendula* Roth (Zakład Zielarski „KAWON—HURT" Nowak sp.j., Gostyń, Poland), fruit of *Juniperus communis* L. (Herbapol Lublin S.A.—Oddział w Białystoku, Białystok, Poland), flowers of *Arnica montana* L. (Herbapol Lublin S.A.—Oddział w Białystoku, Poland) and herb of *Solidago virgaurea* L. (NANGA Przemysław Figura, Złotów, Poland). Dried plant material was ground before extraction. 50 g of each material were extracted three times with 500 mL of distilled water in an ultrasonic bath at room temperature for 30 min per extraction. The combined extracts were filtered through filter paper, frozen at −20 °C and lyophilized. Extraction yields were calculated as the mass of lyophilized extract relative to the initial mass of dried plant material and expressed as percentage weight/weight (%w/w). The yields were 5.18% for *A. uva-ursi*, 16.1% for *G. urbanum*, 21.9% for *B. pendula*, 8.0% for *J. communis*, 20.2% for *S. virgaurea*, and 8.4% for *A. montana*. For each plant species, all extracts were prepared from plant material obtained from the same commercial source and batch to ensure consistency of the starting material. Representative voucher specimens were deposited in the Department of Pharmaceutical Biology, Medical University of Warsaw, Poland.

4.2. Chromatographic Profiling

Chromatographic and mass spectrometric analyses of extracts were performed using an ultra-high-performance liquid chromatography system (Ultimate-3000 RS, Dionex, Leipzig, Germany) equipped with a diode array detector and coupled to an amaZon SL ion trap mass spectrometer (HPLC-DAD-MS^n) via an electrospray ionization source (Bruker Daltonik GmbH, Bremen, Germany). UV-Vis spectra were recorded over a wavelength range of 190–450 nm. The mass spectrometer was operated with a nebulizer pressure of 40 psi, a drying gas flow rate of 9 L/min, a nitrogen gas temperature of 134 °C, and a capillary voltage of 4.5 kV, and mass spectra were acquired over an m/z range of 70–2200 m/z. Mobile phase A consisted of water/formic acid (100:0.1, v/v), and mobile phase B consisted of acetonitrile/formic acid (100:0.1, v/v). Chromatographic separation was performed on a reversed-phase Kinetex XB-C18 column (150 mm × 3.0 mm, 2.6 µm; Phenomenex, Torrance, CA, USA) maintained at 25 °C. Separation was carried out at a constant flow rate of 0.3 mL/min using the following linear gradient: 0–1 min, 1% B; 1–60 min, 1–26% B; and 60–120 min, 26–95% B. The bearberry extract was analyzed using the following gradient: 0–1 min, 3% B; 1–60 min, 3–26% B; and 60–80 min, 26–90% B. The injection volume was 5 µL. Data were analyzed in Bruker Compass DataAnalysis 4.4 software. Compound annotations

were based on UV-Vis absorption spectra, MS^n fragmentation patterns, retention behavior, and comparison with published phytochemical data and information available in the Reaxys database (Elsevier, Amsterdam, The Netherlands). As authentic reference standards were not used, all compound identifications were considered tentative.

4.3. Growth Inhibition Assays

UPEC 536 (O6:K15:H31) [22] was used throughout. Bacteria were recovered from frozen stocks on BHI agar, and a single colony was used to inoculate broth cultures. To prepare the inoculum, UPEC 536 was grown overnight in BHI broth (Roth, Karlsruhe, Germany) at 37 °C with shaking, diluted 1:99 into fresh BHI, and incubated at 37 °C with shaking to the late exponential phase. Plant extracts were filter-sterilized using a 0.22 µm filter before use. Antibacterial activity was assessed using a broth microdilution assay [38] with twofold serial dilutions of extracts ranging from 0.02 to 10 mg/mL. Assays were performed in sterile 96-well plates with a final volume of 200 µL per well, comprising 70 µL of extract dilution, 100 µL of double-strength BHI, and 30 µL of bacterial inoculum, yielding a final density of 10^4 CFU/mL. Growth controls, sterility controls, and cell-free extract blanks at the corresponding concentrations were included. Plates were sealed with adhesive film and incubated at 37 °C in a microplate reader (Agilent BioTek Synergy H4, Agilent Technologies, Santa Clara, CA, USA), with bacterial growth monitored kinetically by measuring optical density at 600 nm (OD_{600}) overnight. Absorbance values were corrected using the corresponding cell-free extract blanks. The minimum inhibitory concentration (MIC) was defined as the lowest extract concentration at which no detectable growth occurred during the incubation period. Lag-phase duration (λ, min) was calculated from individual growth curves using the maximum-slope extrapolation method implemented in BioTek Gen5 software. Ciprofloxacin (Polpharma, Starogard Gdański, Poland; 0.002–1 µg/mL) and ampicillin (Sigma-Aldrich, Taufkirchen, Germany; 0.19–96 µg/mL) were included as antibacterial controls. To validate the MIC values obtained from optical density measurements, viable bacterial counts were determined for *A. uva-ursi* and *G. urbanum* at their respective MICs. Bacterial viability was assessed by CFU enumeration at the start of the experiment and after overnight incubation, using extract-free cultures as growth controls. Each experiment was performed in three independent biological replicates.

4.4. Iron and Magnesium Supplementation

To assess the influence of iron supplementation on growth inhibition, the growth assay was repeated with *G. urbanum* (10 mg/mL) and *A. uva-ursi* (5 and 10 mg/mL) in BHI supplemented with 100 µM $FeCl_3$ or without supplementation. $MgCl_2$ (100 µM) was included as a control for supplementation with another metal cation and was tested at the respective MICs. Additional controls included supplemented medium without extracts and extract-containing medium without added salts. $FeCl_3$ was examined at a single concentration of 100 µM as an exploratory condition to determine whether iron supplementation could counteract growth inhibition. Experiments were performed in three independent biological replicates.

4.5. Biofilm Formation Assay

Four extracts (*G. urbanum*, *B. pendula*, *J. communis*, and *A. uva-ursi*) were examined at 1.25, 2.5, 5 and 10 mg/mL. Cultures were prepared in LB supplemented with 2% glucose, grown overnight at 37 °C with shaking at 180 rpm, diluted 1:100 into fresh medium and grown for approximately 4 h. Wells received 100 µL of medium, 70 µL of the appropriate extract dilution and 30 µL of inoculum, for a final volume of 200 µL. Growth and sterility controls were included. Plates were incubated at 37 °C for 48 h without shaking. Plant

extracts were added at the beginning of incubation to assess their effects on biofilm formation. After incubation, non-adherent material was removed, and wells were washed three times with distilled water. Attached biomass was stained with 200 µL of 0.1% crystal violet for 15 min at room temperature. The stain was removed, the wells were washed three times and the plates were dried. The retained stain was solubilized in 125 µL of 30% acetic acid for 15 min and absorbance was measured at 595 nm [39]. Relative biomass was expressed as a percentage of the corresponding no-extract control after background correction. Experiments were performed in three independent biological replicates.

4.6. Antibiotic Exposure and Post-Antibiotic Regrowth Assay

UPEC 536 was exposed to ampicillin at 100× MIC (600 µg/mL) for 48 h. Viable bacterial counts were determined before exposure and at 0, 4, 24 and 48 h. Following antibiotic treatment, bacterial cells were collected by centrifugation and transferred to fresh BHI recovery medium. β-Lactamase (10 U/mL, Sigma-Aldrich, Taufkirchen, Germany) was added to neutralize residual ampicillin, followed by *G. urbanum* (5 mg/mL), *A. uva-ursi* (2.5 mg/mL), or no extract. Control conditions included continued ampicillin exposure and recovery without β-lactamase. Bacterial recovery was monitored over 48 h by CFU enumeration and OD_{600} measurements. The ampicillin MIC of bacteria recovered following antibiotic exposure was determined and compared with that of the parental strain. Experiments were performed in three independent biological replicates.

4.7. Statistical Analysis

Unless otherwise stated, all experiments were performed in at least three independent biological replicates, each with at least two technical replicates. Results are presented as mean ± standard deviation (SD), with the number of independent biological replicates (n) indicated in the corresponding figure legends. Statistical analyses were performed using GraphPad Prism 10.1.1. Where applicable, statistical significance was assessed using two-way analysis of variance (ANOVA), followed by Dunnett's multiple-comparisons test to compare each extract concentration with its corresponding untreated control. Adjusted p-values < 0.05 were considered statistically significant.

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