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ARTICLE

Antibacterial activity of hydroethanolic leaf extracts of *Allium ursinum* before and after flowering against selected food-associated bacteria

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ABSTRACT Ramsons (*Allium ursinum* L.; wild garlic) is a seasonal edible *Allium* species traditionally consumed for health-promoting properties. Here we investigated whether the antibacterial activity of crude leaf extracts depends on the phenological stage of the plant. Fresh leaves collected before and after flowering were extracted with ethanol:water (30:70, v/v), concentrated, re-dissolved in 30% ethanol, sterile-filtered, and tested against a panel of food-associated bacteria and pathogens by broth microdilution. Minimum inhibitory concentrations (MIC) were defined by absorbance-based growth inhibition, and minimum bactericidal concentrations (MBC) were determined by viability plating (track plate method). The pre-flowering extract showed no clear inhibitory activity within the tested concentration range (MIC >20 mg/mL for all strains). In contrast, the post-flowering extract displayed measurable antibacterial activity, most notably against *Escherichia coli* (MIC >10.8 mg/mL; MBC 10.8 mg/mL) and *Staphylococcus aureus* (MIC 21.5 mg/mL). Several strains exhibited bactericidal endpoints only at the highest tested concentration, while *Serratia marcescens* remained poorly susceptible based on MIC values. In addition, *Bacillus cereus* var. *mycoides* showed an unusual concentration-dependent response with growth stimulation at subinhibitory concentrations. Our findings indicate that the antibacterial potential of crude *A. ursinum* leaf extracts is stage-dependent and highlight the need for chemical characterization and standardized preparation to support food- or health-related applications. **Acta Biol Szeged 69(2):118-123 (2025)**

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Introduction

Ramsons (*Allium ursinum* L.; wild garlic) is a widely consumed wild edible plant in Europe and parts of Asia. Beyond its culinary value, *A. ursinum* has long been used in traditional medicine, and its biological activities are commonly attributed to sulfur-containing compounds, phenolics, and other secondary metabolites typical of the genus *Allium* (Stajner et al. 2006). Phenological stage-dependent qualitative and quantitative changes in bioactive constituents are well documented in many medicinal plants, and such variability can substantially affect antimicrobial performance.

While *A. ursinum* extracts have been reported to show antimicrobial effects in vitro, comparative data on antibacterial activity in different phenophases are scarce. In the present study, we evaluated the antibacterial activity of crude hydroethanolic leaf extracts prepared from *A. ursinum* collected at two phenological stages (before and after flowering) against selected Gram-positive and Gram-negative bacteria relevant to food spoilage and food safety.

Although antimicrobial activity has often been attributed to thiosulfinate-type organosulfur compounds and phenolic constituents of *Allium* species, the abundance and profile of these metabolites may change during plant development (Voca et al. 2022). Such phenological variation can influence biological activity and may partly explain inconsistent antimicrobial effects reported for crude preparations.

Recent studies have continued to confirm antibacterial activity of *A. ursinum* leaf extracts, although reported potency varies with extraction procedure and plant origin (Krstin et al. 2018; Stupar et al. 2022; Barbu et al. 2023). Moreover, large-scale sampling across wild populations highlighted considerable natural variation in antibacterial and antifungal activity, with allicin emerging as a key metabolite linked to activity (Burton et al. 2023; Pârvu et al. 2011).

Seasonal and phenological variation in *A. ursinum* chemistry has been reported for several metabolite classes across different harvest times (Lachowicz et al. 2018). Similar stage-dependent shifts in antimicrobial and anti-biofilm activity have been demonstrated for other medicinal plants, including thyme and sage essential oils collected at different flowering phenophases (Assaggaf et al. 2022; Bakó et al. 2023), supporting the rationale for comparing pre- and post-flowering *A. ursinum* leaves.

In our earlier work, crude hydroethanolic extracts of *A. ursinum* displayed broad-spectrum anti-yeast activity, including fungicidal endpoints for several *Candida* species (Nacsa-Farkas et al. 2014). Building on this observation, we hypothesized that leaf extracts may also exhibit antibacterial effects and that the magnitude of this effect may depend on the stage of plant development.

Therefore, the aim of the present study was to compare the antibacterial activity of hydroethanolic leaf extracts of *A. ursinum* collected before and after flowering against selected Gram-positive and Gram-negative bacteria. Antibacterial activity was evaluated by broth microdilution (MIC) and a viability-based track plate assay (MBC), and a concentration-dependent growth response was documented for *Bacillus cereus* var. *mycoides*.

Materials and Methods

Plant material

Fresh leaves of *A. ursinum* were obtained from a local market in Szeged, Hungary, in April (before flowering) and in early June (after flowering). Leaves were transported to the laboratory in a cooled container and processed on the day of purchase. Leaves were rinsed with tap water followed by distilled water, gently blotted dry, and visibly damaged tissues were removed prior to extraction.

Bacterial strains

The following strains were used: *Escherichia coli* SZMC 0582, *Serratia marcescens* SZMC 0567, *Bacillus subtilis* SZMC 0209, *Bacillus cereus* var. *mycoides* SZMC 0042 (SZMC, Szeged, Hungary); *Pseudomonas putida* DSM 291 (DSMZ, Braunschweig, Germany); *Staphylococcus aureus* ATCC 25923 and MRSA (*S. aureus* ATCC 43300) (ATCC, Manassas, VA, USA).

Strains were maintained on nutrient agar slants at 4 °C and subcultured twice before testing. For assays, a single colony was inoculated into Mueller–Hinton broth (MHB) and incubated for 16-18 h with shaking. The turbidity of the overnight cultures was adjusted to 0.5 McFarland standard (≈1×10^8 CFU/mL) and further diluted in MHB to obtain a final inoculum of 1×10^5 CFU/mL in the microtiter wells.

Preparation of leaf extracts

Fresh leaves were cut into small pieces and homogenized. For each extract, 10 g of the fresh homogenate was macerated in 100 mL of ethanol:water (30:70, v/v) for 24 h at room temperature in the dark with continuous agitation. The suspension was filtered (paper filter), and the filtrate was concentrated to dryness by rotary evaporation at 50 °C. The dry residue was weighed; extraction yield was expressed as mg dry extract per g fresh plant material. For dry matter standardization, 1 mL aliquots of each stock solution were dried to constant weight at 60 °C and the total soluble solids were expressed as mg/mL. The dry residue was re-dissolved in 30% (v/v) ethanol to obtain a stock concentration of 40-45 mg/mL (mg dry extract per mL). Extracts were sterilized by membrane filtration (0.45 µm, Millipore) and stored at -20 °C until use. Immediately before testing, working solutions were prepared by twofold serial dilution in MHB from the highest test concentrations (Table 1).

Broth microdilution assay and endpoint definitions

Antibacterial activity was assessed using a broth microdilution method in sterile 96-well microtiter plates. Wells contained 100 µL of extract dilution and 100 µL of bacterial inoculum in double-strength MHB, result-

Table 1. MIC and MBC values (mg/mL) of *A. ursinum* leaf extracts before and after flowering.

Microorganism	MIC (before)	MBC (before)	MIC (after)	MBC (after)
Escherichia coli	>20	20	>10.8	10.8
Pseudomonas putida	>20	20	>21.5	21.5
Bacillus subtilis	>20	>20	>21.5	21.5
Bacillus cereus var. mycoides	>20	>20	>21.5	>21.5
Serratia marcescens	>20	>20	>21.5	21.5
Staphylococcus aureus (ATCC 25923)	>20	>20	21.5	>21.5
MRSA (S. aureus ATCC 43300)	>20	>20	21.5	>21.5

">" indicates that the endpoint was not reached at the highest tested concentration.

ing in a final volume of 200 μL per well. Final extract concentrations ranged from 0.16 to 20 mg/mL for the pre-flowering extract and from 0.17 to 21.5 mg/mL for the post-flowering extract (twofold serial dilutions). Plates were incubated for 24 h at 37 °C without shaking.

Each plate included a growth control (inoculated medium without extract), a sterility control (non-inoculated medium), and a solvent control containing the same final concentration of ethanol as in the corresponding extract dilution. Optical density was measured spectrophotometrically (OD_{620}) using a microplate reader (Spectrostar Nano, BMG Labtech, Ortenberg, Germany). MIC was defined as the lowest extract concentration at which the absorbance of the treated culture was ≤10% of the untreated growth control after 24 h.

Track plate assay for MBC determination

For MBC determination, 10 μL aliquots were taken from wells at and above the MIC and spotted onto Mueller–Hinton agar plates (track plate method). After incubation for 24 h at 37 °C, the lowest concentration showing no visible colony growth was recorded as the MBC.

Calculation of concentration-dependent growth response

To characterize the non-inhibitory and potentially growth-promoting effects observed for *Bacillus cereus* var. *mycoides*, relative growth (%) was calculated for each concentration as $(OD_{treated}/OD_{control}) \times 100$ after 24 h incubation. Values were used to generate dose-dependent growth response plots (Fig. 1).

Replication and statistical analysis

Microdilution assays were performed in three technical replicates and repeated in at least two independent experiments. Relative growth data are presented as mean ± SD. Differences from the untreated control were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test; $P < 0.05$ was considered statistically significant.

Results

The antibacterial activity of crude *A. ursinum* leaf extracts differed between the two phenological stages. The extract obtained before flowering displayed no measurable MIC within the tested range (MIC >20 mg/mL for all strains). In contrast, the after-flowering extract showed inhibitory and/or bactericidal effects for several bacteria (Table 1). The most pronounced activity was observed for *E. coli* (MBC 10.8 mg/mL), while *S. aureus* and MRSA exhibited inhibition only at the highest tested concentration (MIC 21.5 mg/mL).

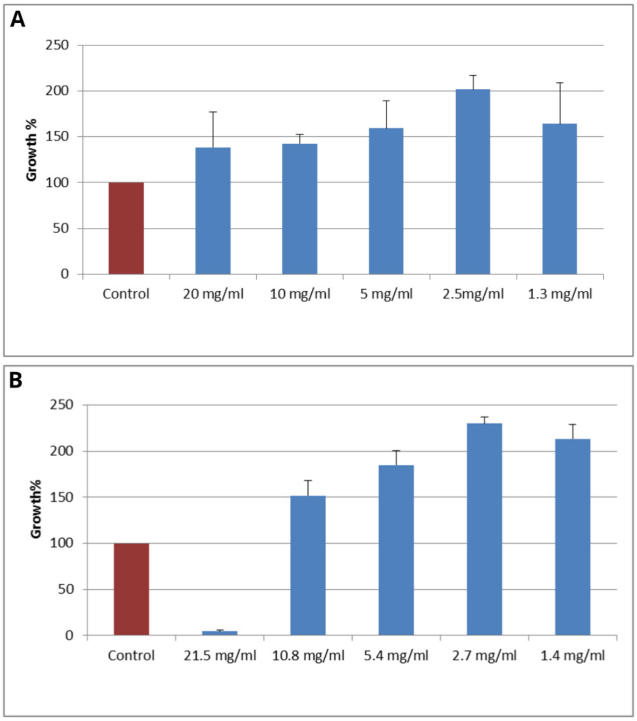

Figure 1. Concentration-dependent growth response of *B. cereus* var. *mycoides* after 24 h exposure to *A. ursinum* leaf extracts collected before flowering (A) and after flowering (B). Relative growth (%) was calculated from endpoint optical density values compared to the untreated growth control (100%).

Notably, *B. cereus* var. *mycoides* responded atypically: the highest concentration of the after-flowering extract suppressed growth, whereas lower concentrations promoted growth relative to the control (a concentration-dependent biphasic response). *S. marcescens* remained poorly susceptible based on MIC values (Table 1).

MIC and MBC values are summarized in Table 1. No inhibitory endpoints were reached for any tested strain with the pre-flowering extract (MIC >20 mg/mL), whereas the post-flowering extract exhibited measurable inhibitory and/or bactericidal activity for several bacteria.

The concentration-dependent growth response of *B. cereus* var. *mycoides* is shown on Fig. 1. While the pre-flowering extract consistently stimulated growth relative to the untreated control across the tested concentrations, the post-flowering extract caused near-complete inhibition at 21.5 mg/mL but stimulated growth at subinhibitory concentrations, indicating a biphasic (hormetic-like) response.

Representative track plate assays supporting bactericidal endpoints are presented in Fig. 2.

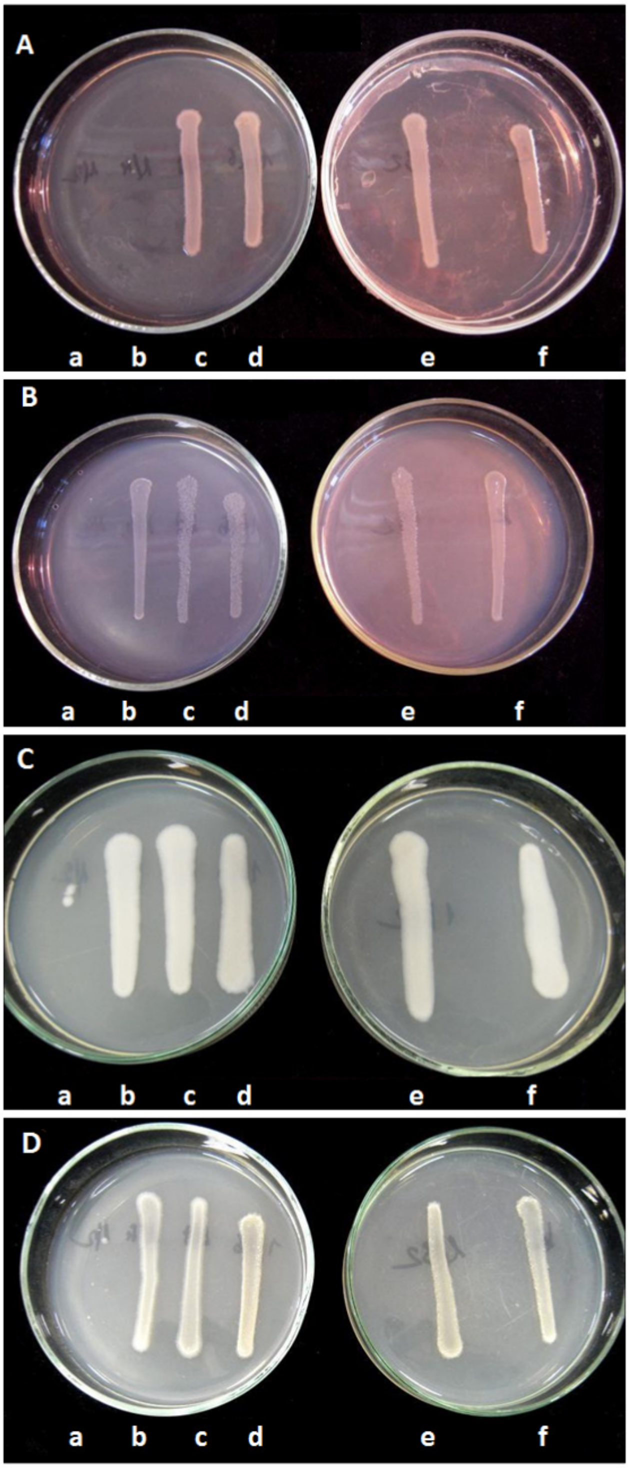

Figure 2. Representative track plate assays showing inhibition/bactericidal effects of *A. ursinum* leaf extracts (after flowering) against selected bacteria after 24 h exposure. A: *E. coli*, B: *P. putida*, C: *B. cereus* v. *mycoides*, D: *B. subtilis*, a – 21.5mg/ml, b – 10.8 mg/ml, c – 5.4 mg/ml, d – 2.7 mg/ml, e – 1.4 mg/ml, f – control.

Discussion

The bacterial panel used in this study was selected to represent both Gram-negative and Gram-positive taxa that are relevant to food safety and food spoilage, while also providing "challenge organisms" with known intrinsic tolerance traits. *E. coli* was included as a widely accepted indicator of fecal contamination and a clinically and food-relevant Gram-negative model, enabling assessment of extracts activity against bacteria protected by an outer membrane barrier. *P. putida* represents the *Pseudomonas* group frequently implicated in spoilage processes, particularly under refrigerated storage and in moist processing environments; thus, it is a practical model for evaluating the potential applicability of the extracts in food-related contexts.

In addition, *S. marcescens* was chosen as an opportunistic, environment-associated species that can occur as a contaminant and is often considered relatively tolerant to multiple stressors, thereby providing a stringent test for crude plant extracts. Among Gram-positive bacteria, *S. aureus* was examined due to its major importance in foodborne disease, including toxin-mediated intoxication, and because it serves as a clinically relevant target for natural antimicrobials. The inclusion of MRSA (ATCC 43300) extends this relevance by addressing an antibiotic-resistant *S. aureus* background, allowing evaluation of whether activity persists against a resistant phenotype.

Finally, the spore-forming *Bacillus* species were incorporated to capture a technologically important group associated with raw plant materials and food-processing environments. *B. subtilis* serves as a robust Gram-positive model frequently encountered as an environmental contaminant, while the *B. cereus* group is of particular concern in food microbiology due to its association with foodborne illness and the high stress tolerance of its spores. Testing *B. cereus* var. *mycoides* is especially justified for plant-derived matrices, as it reflects a realistic soil-/plant-surface-associated contaminant and provides insight into extract effects on a resilient, sporulating Gram-positive background.

In the present study, the antibacterial activity of crude *A. ursinum* leaf extracts was strongly dependent on the phenological stage of the plant. Under our assay conditions, the extract prepared from pre-flowering leaves did not reach inhibitory endpoints within the tested range (MIC >20 mg/mL across the panel), whereas the post-flowering extract exhibited measurable activity against several test organisms. The most pronounced effect was detected against *E. coli*, for which a bactericidal endpoint was achieved at 10.8 mg/mL, indicating that post-flowering leaves contained (or released) antibacterial constituents at levels sufficient to exert killing rather than only growth suppression. In contrast, for multiple strains the post-flowering extract produced inhibition only at the highest tested concentration (e.g., *S. aureus* and MRSA with MIC 21.5 mg/mL), suggesting a generally moderate potency of the crude preparation and/or strain-dependent tolerance. Notably, *S. marcescens* remained poorly susceptible based on MIC values, consistent with its reputation as a relatively robust Gram-negative challenge organism. Among the spore-forming Gram-positive bacteria, the atypical, concentration-dependent response of *B. cereus* var. *mycoides*—growth inhibition at the highest concentration but stimulation at lower (subinhibitory) concentrations—highlights the importance of interpreting sub-MIC exposures cautiously and supports the need for standardized extract preparation and chemical profiling when considering practical applications.

Taken together, these findings support a phenological-stage dependence of antibacterial activity and suggest that the bioactive composition of *A. ursinum* leaves changes during development. Because identical extraction and testing procedures were applied, the different biological responses most likely reflect phenological variation in extractable constituents rather than methodological factors. *Allium* leaves contain sulfur-containing precursors that can be enzymatically converted into reactive thiosulfinates upon tissue disruption (Sobolewska et al. 2015); the amount and stability of these compounds, as well as the accompanying phenolic fraction, may vary between pre- and post-flowering plants.

The post-flowering extract showed the clearest bactericidal activity against *E. coli*, while Gram-positive staphylococci required the highest concentration to reach an MIC. Such differences may relate to strain-specific permeability barriers, stress responses and detoxification pathways; however, chemical characterization of the extracts is required to identify the compounds responsible for activity and to clarify spectrum-of-action differences.

The biphasic growth response of *B. cereus* var. *mycoides* is notable from a food microbiology perspective. Growth stimulation at subinhibitory concentrations may arise from nutrient effects of plant-derived carbohydrates or other growth-supporting components, or from adaptive stress responses triggered by low-level antimicrobial exposure. Regardless of mechanism, this finding highlights the importance of assessing subinhibitory exposures when considering crude plant preparations for food-related applications.

Previous studies have reported antibacterial effects of *A. ursinum* extracts against food- and clinically relevant bacteria, although activity estimates differ substantially between studies (Krstin et al. 2018; Stupar et al. 2022; Barbu et al. 2023). Such variability is expected for crude plant preparations, where solvent polarity, extraction intensity, and handling of fresh tissue can influence both the yield and the stability of reactive organosulfur constituents.

With respect to developmental timing, stage-dependent antimicrobial performance has been demonstrated in other medicinal plants, including thyme and sage essential oils harvested at different flowering phenophases (Assaggaf et al. 2022; Bakó et al. 2023). Together with evidence for seasonal changes in *A. ursinum* chemistry across harvest times (Lachowicz et al. 2018), phenophases (Voca et al. 2022) and for natural variation in antibacterial activity within wild populations (Burton et al. 2023), our results reinforce that plant developmental stage should be controlled and explicitly reported when comparing antimicrobial properties of *A. ursinum* preparations.

A limitation of the present study is that the extracts were standardized only by dry mass concentration; no chemical fingerprinting (e.g. total phenolics or organosulfur markers) was performed. Moreover, growth was assessed mainly as an endpoint after 24 h incubation. Future work should include chemical profiling of stage-specific extracts, determination of extraction yields and batch-to-batch variability, time-resolved growth kinetics, and validation in relevant food model systems.

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