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Chemical analysis and antibacterial activity of optimized *Satureja montana* L. extracts

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Summary

The study aimed at the phytochemical characterization of the optimized ethanol and acetone extracts of *Satureja montana* L., and the evaluation of their antibacterial and synergistic activity with an antibiotic, along with their potential mechanisms of action. Response surface methodology was employed to optimize the extraction conditions. The antibacterial activity was evaluated using microdilution, checkerboard, time-kill kinetics, and cell membrane permeability methods. *S. montana* extracts were effective against different bacterial species, including the Gram-positive *Staphylococcus aureus*, *Enterococcus* sp., *Bacillus cereus*, and the Gram-negative *Acinetobacter* sp. and *Proteus mirabilis*. Notably, the acetone extract enhanced the efficacy of gentamicin up to 16-fold against a gentamicin-resistant strain of *P. mirabilis*. Additionally, the extracts exhibited bactericidal activity against certain strains within a 3-hour and 6-hour time frame and increased cell membrane permeability, thereby disrupting normal cellular functions. The ethanol and acetone extracts reached a total phenolic content of 79.44 ± 0.5 mg GAE/g and 53.88 ± 0.2 mg GAE/g, and a total flavonoid content of 27.93 ± 0.3 mg RUE/g and 16.35 ± 0.3 mg RUE/g, respectively. Rosmarinic acid, chlorogenic acid, luteolin, quercetin, and naringenin were detected in the highest quantities in the extracts. *S. montana* extracts may serve as potential natural antibacterial agents.

Keywords: Medicinal plant; phytochemical analysis; antibacterial activity; mechanism of action; synergism

Introduction

Medicinal plants, known for their diverse array of secondary metabolites, not only offer numerous health benefits for humans but also present a potential solution to the global challenge of antimicrobial resistance (Abdallah et al., 2023). The concerning fact is that the occurrence of acquired resistance to antibiotics in pathogenic bacteria is increasing (Gauba and Rahman, 2023). Among the most notable multidrug-resistant pathogens are Gram-positive bacteria such as *Staphylococcus aureus*, the *Streptococcus* species, and the *Enterococcus* species, while prominent Gram-negative pathogenic bacteria include members of the Enterobacteriaceae family, *Pseudomonas aeruginosa*, and the *Acinetobacter* species (Kaapu et al., 2023). Consequently, significant efforts are underway to discover alternatives that could fully replace antibiotics or enhance their effectiveness.

Recently, there has been a resurgence of interest in investigating plant extracts as potential remedies for antimicrobial resistance, due to their established antimicrobial properties. Plant extracts contain bioactive compounds such as coumarins, flavonoids, phenolics, tannins, terpenoids, and alkaloids which serve as foundations for antibiotic development (Abdallah et al., 2023). They exert various effects on bacterial cells, including interference with intermediary metabolism, the disruption of DNA/RNA synthesis and functionality, and the modulation of critical events within the pathogenic progression (Abdallah et al., 2023).

Satureja montana L., known as winter savory, is a perennial shrub or sub-shrub plant that belongs to the Lamiaceae family (Kremer et al., 2015). Its natural distribution spans the Mediterranean region and the Balkans, and it is also a successfully cultivated plant (Hudz et al., 2020). Winter savory is known for its distinct aroma and belongs to the aromatic plant species, traditionally used as a spice and a natural food preservative (Hudz et al., 2020). The aerial parts of this plant are recognized in traditional medicine for their effectiveness in treating various ailments. They are particularly beneficial for conditions related to the inflammation of lymphatic and respiratory systems, as well as for gastrointestinal problems and coughs (Matejić et al., 2020). Secondary metabolites of *S. montana*, including phenolic compounds, tocopherols, alkaloids, tannins, carotenoids, and terpenoids, contribute to its diverse biological activities. Notably, these include anti-cancer, anti-inflammatory, antiviral, anti-allergic, anti-proliferative, antioxidant, and antibacterial activity (Tepe et al., 2016; Vladić et al., 2020; Abdelshafeek et al., 2023).

The antibacterial activity of *S. montana* extracts has been reported in previous studies (Serrano et al., 2011; Gomes et al., 2020; Aćimović et al., 2022), the mechanisms of action have not been studied in detail. Extensive work on the species *S. montana* has revealed its chemical complexity, varied bioactive molecules, and diverse biological activities but *S. montana* is still unexplored even though it has versatile ethnomedical uses. Despite the common association of antimicrobial activity with the essential oil of *S. montana* (Santos et al., 2019); phenolic compounds have also been identified as key contributors to its biological activity (Tepe et al., 2016). Given the chemical complexity of plant extracts, focusing on phenolic compounds provides a broader understanding of the antimicrobial potential of *S. montana*. Therefore, this study emphasizes the analysis of phenolic constituents to investigate their contribution to the antibacterial effect, complementing the existing knowledge on essential oils.

Additionally, there is limited information on the optimization of extraction processes for secondary metabolites from *S. montana*, as the yield and quality of the extracts strongly depend on the extraction process used, among others. The current study investigated the antibacterial activity of *S. montana* extracts against both antibiotic-resistant and antibiotic-sensitive bacterial strains by observing changes in cell membrane integrity and permeability. By broadening our understanding of their mechanisms of action, we may uncover new therapeutic strategies that can help overcome the challenges posed by antimicrobial resistance.

Recognizing that the content of secondary metabolites in extracts is

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significantly influenced by the extraction process, the first goal of this study was to optimize an extraction process to achieve a higher content of phenolic compounds, specifically the total phenolic content (TPC) and total flavonoid content (TFC), and besides that to optimize the antibacterial activity of *S. montana*. Further, the study aimed to conduct a detailed analysis of phenolic compounds using liquid chromatography - mass spectrometry (LC-MS) and high-performance thin-layer chromatography (HPTLC), along with an *Escherichia coli*/*Staphylococcus aureus* bioautography assay. The final objective was to investigate the antibacterial activity of the optimized extracts and explore potential mechanisms of action at the cellular membrane level, as well as to examine the synergistic effects of the extracts in combination with the antibiotic gentamicin.

Materials and methods

Plant material

The plant material (*S. montana* L.) was purchased in the form of herbal tea from the Institute for Medicinal Plant Research "Dr. Josif Pančić" (Belgrade, Serbia). It consisted of aerial parts, including stem, leaves, and flowers. Detailed information regarding the geographical origin, developmental stage at the time of harvest, drying method, and storage conditions was not available.

Extraction optimization

Experimental design

Extraction optimization was conducted following the approach used in our previous research (Stanković et al., 2024). The design was based on the Box-Behnken method, which included 18 randomized runs with 6 center points. The independent variables were extraction temperature (A, ranging from 25 to 60 °C), extraction time (B, ranging from 10 to 30 minutes), and plant material to solvent ratio (C, ranging from 1:10 to 1:40 g/mL). Tab. S1 presents the high (+1), medium (0), and low (-1) levels of these variables. The goal of the optimization was to maximize the TPC, TFC, and enhance the antibacterial activity of the extracts. A second-order polynomial equation was employed to model the relationship between the dependent and independent variables, with the goal of determining the optimal extraction conditions:

$$Y=\beta_0+\beta_1 A+\beta_2 B+\beta_3 C+\beta_{12} AB+\beta_{13} AC+\beta_{23} BC+\beta_{11} A^2+\beta_{22} B^2+\beta_{33} C^2 \quad (1)$$

where Y is the dependent variable; A, B, and C represent the independent variables (temperature, time, and solid-solvent ratio), respectively; β_0 is the model intercept coefficient; and β_i, β_{ii}, and β_{ij} represent linear, quadratic, and regression coefficients of interaction, respectively (Ayyildiz et al., 2018).

Extraction procedure

To optimize the extraction process, one gram of plant material per sample was used, while the solvent volume varied according to the design conditions (Tab. S2). The plant material and solvent were mixed in glass bottles and subjected to UAE (ultrasonic bath, Vabsonic, Niš, Serbia, operating at an ultrasound frequency of 40 kHz) under the specified temperature and time outlined in the design (Tab. S2). Following extraction, the samples were centrifuged at 1110 × g for 5 minutes. TPC was measured using the method previously described by Wootton-Beard et al., 2011 and was expressed as micrograms of gallic acid equivalents (GAE) per gram of dry plant weight (μg GAE/g DW). TFC was determined according to the method of Quettier-Deleu et al., 2000 and was expressed as micrograms of rutin equivalents (RUE) per gram of dry plant weight (μg RUE/g DW). All measurements were performed in triplicate, and the mean values were calculated.

After TPC and TFC determination, the supernatants were evaporated using a rotary evaporator (IKA-Werke, Breisgau, Germany) at 40 °C. The resulting dried extracts were then utilized for evaluation of antibacterial activity. Antibacterial testing was conducted using the agar-well diffusion method, following CLSI guidelines 2015, on two test bacteria: *E. coli* ATCC 25922 (G^-) and *S. aureus* ATCC 25923 (G^+). Briefly, the extracts were dissolved in 1 mL of 10% aqueous solution of DMSO. Next, 100 μl of each extract solution was introduced into wells (ø10 mm) on inoculated Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India) plates. The diameters of the zones of inhibition were measured after 18 hours of incubation at 37 °C. All measurements were conducted in triplicate, and mean values were calculated. The final concentration of 10% DMSO has been demonstrated to exert no inhibitory effect on the tested bacterial strains.

Preparation of plant extracts under optimal extraction conditions

Extracts for further research were prepared under the optimized extraction conditions (Tab. 1). For this process, 20 g of plant material was used per solvent. After extraction, the resulting extracts were filtered and concentrated under reduced pressure at 40 °C using a rotary evaporator to obtain dry extracts. The final weights of the ethanol and acetone extracts were 2.24 g and 2.11 g, corresponding to extraction yields of 11.2% and 10.6% (w/w), respectively. These extracts were stored at -20 °C until needed. For *in vitro* bacterial tests, stock solu-

Tab. 1: Predicted and experimental values of TPC, TFC, and DIZ under the optimal extraction conditions.

	Optimal extraction conditions			Maximum value		Desirability
	A (°C)	B (min)	C (g/mL)	Predicted*	Experimental value**	
Responses for ethanol extract						
TPC	60	30	1:40	11424.28-13833.15	13171.76 ± 9.41	1
TFC				2688.12 - 3311.80	3107.92 ± 37.92	0.971
DIZEC				12.8 – 14.6	12.5 ± 0.7	1
DIZSA				46.4 – 58.1	52 ± 0	1
Responses for acetone extract						
TPC	60	30	1:22	5155.47 - 6017.77	5802.82 ± 20.71	0.724
TFC				1859.79 - 2159.3	2064.10 ± 3.90	0.950
DIZEC				13.5 – 15.5	13.5 ± 0.7	1
DIZSA				48.5 – 49.5	49 ± 0	0.897

A – temperature (°C); B – time (min); C - solid-solvent ratio (g/mL); TPC - total phenolic content (μg GAE/g DW); TFC - total flavonoid content (μg RUE/g DW); DIZEC – diameter of growth inhibition zone of *E. coli* ATCC 25922 (mm); DIZSA – diameter of growth inhibition zone of *S. aureus* ATCC 25923 (mm); *95% Confidence interval; ** Data are presented as mean ± SD, n = 3.

tions of the extracts were prepared in dimethyl sulfoxide (DMSO, >99.8%) and then diluted with nutrient broth to achieve a final concentration of 10% DMSO. A solvent control was included to confirm that 10% DMSO did not exhibit any toxic effects.

Phytochemical analysis of optimized extracts

Determination of TPC, TFC, and total proanthocyanidin content (TPAC)

The extracts' concentrations of 1 mg/mL were prepared in methanol (> 99.8%). TPC was determined using Folin-Ciocalteu's reagent, as previously described by Wootton-Beard et al., 2011, and results were reported as milligrams of gallic acid equivalents (GAE) per gram of extract (mg GAE/g extract). TFC was measured using the aluminum chloride method, following the procedure outlined by Quettier-Deleu et al., 2000, and was expressed as milligrams of rutin equivalents (RUE) per gram of extract (mg RUE/g extract). TPAC was assessed using the butanol-HCl method with ferric ammonium sulfate as a catalyst, as previously described by Hagerman et al., 2000, and results were reported as milligrams of cyanidin chloride equivalents (CChE) per gram of extract (mg CChE/g extract). All measurements were conducted in triplicate.

High-performance thin-layer chromatography analysis (HPTLC)

Five µL of the initial extract (20 mg/mL) was applied as 8 mm bands on a 20 × 10 cm HPTLC plate coated with silica gel 60 utilizing Linomat 5 (CAMAG, Muttenz, Switzerland). Chromatographic separation was carried out in a Twin Trough Chamber measuring 20 × 10 cm using a mobile phase consisting of ethyl acetate, toluene, formic acid, and water (16:4:3:2 *v/v/v/v*). The mobile phase migrated at a distance of 70 mm from the lower plate edge. Before separation, the chamber was equilibrated for 5 minutes with 5 mL of the mobile phase in the second trough. Subsequently, the HPTLC chromatogram was briefly immersed in natural product (NP) reagent for 3 seconds (a solution of 250 mg of diphenylborinic acid 2-aminoethyl ester in 200 mL of ethanol), and after 2 minutes drying, immersed in 5% (w/v) methanolic polyethylene glycol (PEG 400) solution using a Chromatogram Immersion Device 3 at a speed of 3.5 cm/s (Ristivojević et al., 2019). The resulting HPTLC chromatograms were scanned and saved in TIFF format.

Escherichia coli/Staphylococcus aureus bioautography assay

The prepared HPTLC chromatograms were immersed in *E. coli* ATCC 35218 and *S. aureus* ATCC 6538 suspensions of 4.50 McFarland units density, separately, for 10 seconds using a Chromatogram Immersion Device 3 (CAMAG, Muttenz, Switzerland) after air-drying for 15 minutes using cold airflow. Then they were placed in an incubator at 37 °C for 1.30 hours. To visualize bioactive bands, the plates were further immersed in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye solution (1 mg/mL). The addition of one drop of Triton X-100 per 10 mL of aqueous MTT solution was observed to enhance the color intensity. After derivatization with MTT, the TLC plates were incubated for an additional 1 hour at 37 °C (Ristivojević et al., 2019). The resulting HPTLC chromatograms were scanned and saved in TIFF format.

LC-MS qualitative and quantitative analysis

The chemical analysis of *S. montana* extracts was conducted using LC/MS (Thermo Scientific™ Vanquish™ Core UHPLC system) coupled to the Orbitrap Exploris 120 mass spectrometer (San Jose, CA, USA). All LC/MS parameters are detailed in Stojković et al., 2024. The obtained MS data were processed and analyzed using R Studio software (version 2023.09.1, build 494). Identification of bioactive compounds was conducted based on their chromatographic behavior and HRMS/MS^2 data, with comparisons made to standard compounds, when available, and literature data for tentative identification. Data acquisition was carried out using Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA). Phenolics were identified and quantified according to the corresponding spectral characteristics: mass spectra, accurate mass, characteristic fragmentation, and characteristic retention time. The results are expressed as milligrams of phenolic compound per kilogram of dry plant weight (mg/kg DW). All chemicals used in this study had an HPLC purity of 99.9%.

Determination of antibacterial activity of the optimized extracts

Bacterial strains

A total of 24 laboratory-confirmed strains of medically important and food-borne pathogenic bacteria were selected (Fig. 3). The antibiotic susceptibility profiles and isolates' sources were described in our previous paper (Stanković et al., 2024). The strains were sourced from the Microbiology Laboratory of the Hospital in Paraćin, Serbia, as well as from the Institute of Public Health in Kragujevac, Serbia. Reference strains from the American Type Culture Collection (ATCC) were acquired from Sigma Aldrich, St. Louis, USA (Fig. 3). The strains were preserved in a medium containing 20% glycerol at -80 °C. For experimental purposes, the strains were subcultured twice on Nutrient agar (Torlak, Belgrade, Serbia) at 37 °C for 18 hours.

Microdilution method

The minimum inhibitory concentration (MIC) was determined using the microdilution method (CLSI, 2018). Extract stocks were subsequently diluted with Mueller–Hinton broth (MHB) (HiMedia Laboratories, Mumbai, India) using a two-fold dilution to achieve the required concentrations (0.156 - 20 mg/mL). Bacterial suspensions were prepared from overnight cultures and subsequently adjusted to a 0.5 McFarland turbidity standard using a McFarland densitometer (DEN-1, BIOSAN, Riga, Latvia). The final density of the bacterial suspension was approximately 5×10^5 colony-forming units (CFU)/mL per well. The inoculated plates were incubated at 37 °C for 20 hours. Following this incubation period, 5 µl of a 1.6 mg/mL aqueous solution of resazurin (w/v) was added to each well. The plates were then incubated at 37 °C for an additional 2 hours. The MIC was visually determined as the lowest concentration of the extract that showed no color change of the indicator from blue to pink. The minimum bactericidal concentration (MBC) was determined by subculturing 10 µl aliquots from wells that exhibited no color change onto Nutrient agar (NA) plates (Torlak, Belgrade, Serbia). The MBC was defined as the lowest concentration of the extract at which no bacterial growth was observed on NA plates following overnight incubation. Each experiment included a growth control (broth + bacterium) and a sterility control (broth + extract). Tetracycline (Sigma–Aldrich, St. Louis, USA), dissolved in MHB, was utilized as a positive control.

Time-kill assay

The time-kill assay was performed according to the standard protocol established by the Clinical and Laboratory Standards Institute (CLSI 1999). Overnight bacterial cultures were diluted to a concentration of 1×10^6 CFU/mL in Mueller–Hinton broth and subsequently exposed to the tested extracts at concentrations corresponding to the MBC for various time intervals, specifically at 0, 3, 6, 9, and 24 hours at 37 °C. At each designated time point, 100 µl aliquots were withdrawn from the treatment tubes, serially diluted in a sterile 0.85% NaCl solution and plated onto Nutrient agar plates. After overnight incubation at 37 °C, colony counts were recorded to determine the CFU/mL, with results expressed as $\log_{10}$ CFU/mL. A reduction of ≥3 $\log_{10}$ in CFU from the initial count was interpreted as indicative of a bactericidal effect. The detection limit (LOD) was established at 1.5 $\log_{10}$ CFU/mL, equivalent to 30 CFU/mL. Additionally, parallel growth control samples were prepared under the same conditions.

Protein leakage assay and crystal violet uptake assay

Bacterial suspensions, prepared from overnight cultures and adjusted to a 1 McFarland turbidity standard, were suspended in a phosphate buffer solution (PBS, pH 7.4). These suspensions were then treated with plant extracts at concentrations corresponding to the MIC and incubated for 18 hours at 37 °C. After incubation, the samples were centrifuged at 9391 × g for 10 minutes to separate the supernatants from the bacterial pellets. For the protein leakage assay, 0.5 mL of the supernatant was combined with 1.5 mL of Bradford reagent (Sigma Aldrich, St. Louis, USA) and incubated for 5 minutes at room temperature. Absorbance was measured at 595 nm, with appropriate blanks used for correction (extract + Bradford reagent for treated samples and PBS + Bradford reagent for untreated samples). All measurements were performed in triplicate. In the crystal violet uptake assay, bacterial pellets from both treated and untreated samples were resuspended in crystal violet solution (10 µg/mL) and analyzed following the previously described method (Khan et al., 2017). The percentage of crystal violet uptake was calculated using the following formula:

$$\text{Dye uptake (\%)} = 100 - [(\text{ODSample}/\text{ODcrystal violet solution}) \times 100] \quad (2)$$

Checkerboard method

The bacterial strains under investigation, *E. coli* LM3, *P. mirabilis* LM1, and *Staphylococcus* sp. LM1, exhibited resistance to gentamicin. In the experimental setup, gentamicin was diluted two-fold horizontally across a 96-well microtiter plate, while pre-diluted extracts were added vertically to each well. Each well contained a final volume of 100 µl, with concentrations ranging from 1/32 MIC to MIC. For strains where MICs were not determined, the highest tested concentrations were utilized. The inoculum density was set at 5×10^5 CFU/mL per well. Control wells included a growth control (broth + bacterium) and a sterility control (broth + tested agents). The microtiter plates were incubated at 37 °C for 20 hours. The percentage of inhibition was assessed by measuring the optical density (OD) at 600 nm using an ELISA microplate reader (RT-2100C, Rayto, Shenzhen, China). SynergyFinder was used to calculate drug combination responses based on the Zero interaction potency (ZIP) reference model. The interaction between the two drugs was classified as antagonistic if the synergy score was < -10, additive if the score ranged from -10 to 10, and synergistic if the score was > 10 (Ianevski et al., 2022). In addition, the MIC values of the extract/antibiotic combinations were recorded by resazurin color change. The FIC index was calculated according to the formula below:

$$\text{FICI} = \frac{\text{MIC A/B}}{\text{MIC A}} + \frac{\text{MIC B/A}}{\text{MIC B}} \quad (3)$$

where MIC A - MIC of extract alone and MIC B - MIC of antibiotic alone; MIC A/B - MIC of extract in combination with antibiotic and MIC B/A - MIC of antibiotic in combination with an extract. The results were interpreted using the following criteria: synergism (FICI ≤ 0.5); additivity (FICI > 0.5–1); indifference (FICI > 1–4) and antagonism (FICI > 4) (Odds, 2003).

Statistical analysis

Design Expert software (Trial version 11.0, STAT-EASE Inc., Minneapolis, MN, USA) was employed to analyze the experimental data for optimizing extraction conditions. This involved constructing 3D response surface models and evaluating the significance of the independent variables and their interactions through analysis of variance (ANOVA), with statistical significance determined at $p < 0.05$. Derringer's desirability function approach was used to identify the optimal extraction conditions.

For analyzing the phytochemical and biological assay results, SPSS software (version 21, IBM SPSS Statistics, Chicago, IL, USA) was employed. Data normality was evaluated using the Kolmogorov-Smirnov test and the Shapiro-Wilk test. Significant differences between groups were identified using the independent t-test, Mann-Whitney test, and Kruskal-Wallis test, with a significance threshold established at $p < 0.05$.

Results

Optimization of UAE

The TPC, TFC and DIZ values are presented in Tab. S2. The significance of the model was confirmed in all six analyzed responses (Tab. S3, S4). Regression equations (Tab. S5) were established to analyze the impact of the independent variables on the responses.

Linear factors of temperature and time had a positive influence on the TPC in the ethanol extract. Increasing the temperature from 53 °C to 60 °C and extending the extraction time from 25 to 30 min led to a better yield of TPC in the ethanol extract (Fig. 1A). The interaction of time and solid-solvent ratio had the greatest impact on the TPC in the acetone extract. Specifically, extending the extraction time from 25 to 30 minutes and increasing the solvent amount from 1:34 to 1:40 mL resulted in a higher TPC (Fig. 2A). All three linear factors of temperature, time, and solid-solvent ratio had a positive influence on the TFC in the ethanol extract (Fig. 1B). However, the TFC in acetone extract was positively affected by the linear factor of temperature and time, while the linear factor of solid-solvent ratio has a negative impact (Fig. 2B). In both cases raising the temperature to 60 °C and extending the extraction time from 25 to 30 minutes led to a better TFC. The efficiency of ethanol extract against *E. coli* ATCC 25922 was predominantly enhanced by the interaction between temperature and extraction time and by the interaction between temperature and solid-solvent ratio (Fig. 1C). Antibacterial efficiency of acetone extract against *E. coli* ATCC 25922 was positively enhanced by all three linear factors of temperature, time, and solid-solvent ratio (Fig. 2C). The interaction between temperature and extraction time (AB) had the best effect on *S. aureus* ATCC 25923 in the case of the ethanol extract (Fig. 1D). Neither the interactions between factors nor their quadratic terms positively influenced the antibacterial effect of the acetone extract; however, the linear terms had a positive impact (Fig. 2D).

Using Derringer's desirability function method, the optimal extraction parameters were established to maximize the responses of TPC, TFC, and DIZ for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 for each solvent independently. The independent variables were maintained within specified ranges, while the responses were optimized to achieve their maximum values. The optimal extraction conditions are presented in Tab. 1. Upon implementing these optimized conditions, the experimental results closely aligned with the predicted outcomes, with a confidence interval of 95% (Tab. 1).

Phytochemical characterization of optimized extracts

Determination of TPC, TFC, and TPAC

The TPC and TFC were higher in the ethanol extract compared to the acetone extract ($p < 0.05$). Specifically, the ethanol extract contained 79.44 ± 0.5 mg GAE/g of TPC and a TFC of 27.93 ± 0.3 mg RUE/g, suggesting that ethanol is a more effective solvent for extracting these phytochemicals. Conversely, while the acetone extract showed lower values for both TPC (53.88 ± 0.2 mg GAE/g) and TFC (16.35 ± 0.3 mg RUE/g), it still provided measurable amounts of these compounds. Additionally, the TPAC was relatively low in both extracts, 0.56 ± 0.1 mg CChE/g in ethanol extract and 0.59 ± 0.2 mg CChE/g in acetone extract ($p = 0.065$).

HPTLC analysis and E. coli/S. aureus bioautography

Visual inspection of the HPTLC chromatograms (Fig. S1; 1 and 2) revealed the presence of six phenolic compounds in both extracts: rutin

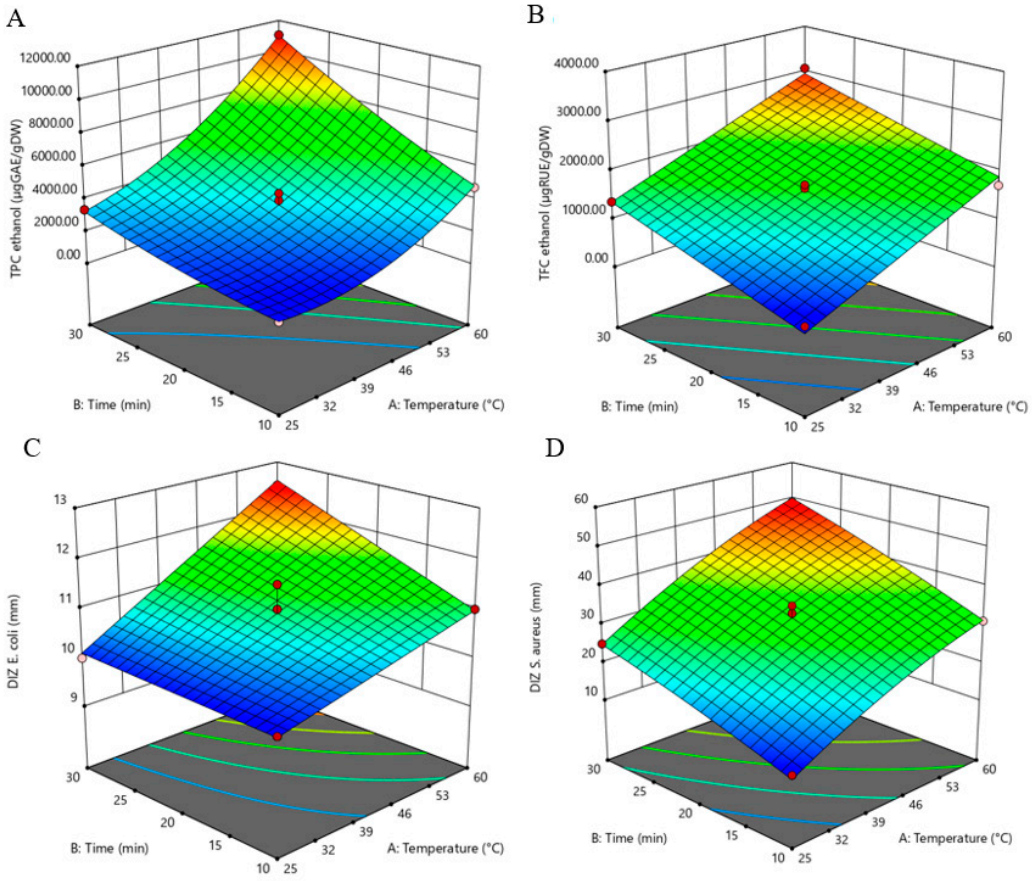

Fig. 1: Response surface plots for the effect of temperature, time, and solid-solvent ratio on TPC (A), TFC (B), DIZ of *E. coli* (C), and DIZ of *S. aureus* (D) of the ethanol extract.

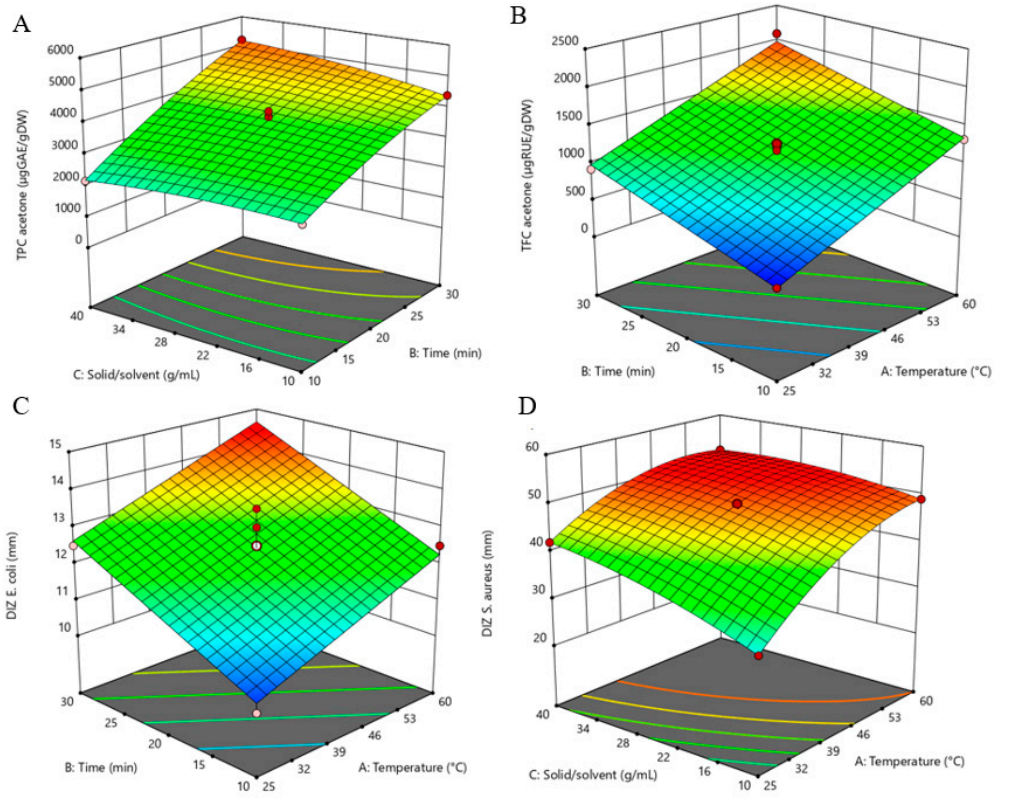

Fig. 2: Response surface plots for the effect of temperature, time, and solid-solvent ratio on TPC (A), TFC (B), DIZ of *E. coli* (C), and DIZ of *S. aureus* (D) of the acetone extract.

(RU, R_F 0.09), chlorogenic acid (CHA, R_F 0.24), quercetin-3-*O*-glucoside (Q-3-O-G, R_F 0.36), quercetin (Q, R_F 0.53), caffeic acid (CA, R_F 0.80), and luteolin (LU, R_F 0.84). The profile of the ethanol extract (Fig. S1; 1) was more abundant compared to the acetone extract (Fig. S1; 2), although both exhibited similar phenolic zones. Quercetin was predominantly present in both extracts. Analysis of the corresponding bioautographic zones (Fig. S1; 1A; 1B, 2A; 1B) revealed the presence of inhibitory zones against tested bacteria within both extracts, which included compounds at R_F 0.39, R_F 0.86, R_F 0.91, and R_F 0.94.

LC-MS analysis

LC-MS qualitative analysis of *S. montana* ethanol and acetone extracts revealed 106 identified metabolites (Tab. S6). Metabolite identification was achieved by studying the exact mass of the compound in a full scan MS, as well as MS^2 fragmentation in high resolution. The tentative identification of the compounds was proposed based on a comprehensive review of the literature on *Satureja* metabolites and the SciFinder database was used as a reference search tool (CAS SciFinder-n database 2024). Tab. S6 lists the LC-MS data for identified compounds (hydroxybenzoic acid derivatives (13 compounds), hydroxycinnamic acids (34 compounds), flavonoid *C*-glycosides (6 compounds), flavonoid *O*-glycosides (30 compounds), and flavonoid aglycones (23 compounds)) together with the representation of the given compounds in the two tested samples, i.e. two extraction agents, in the form of peak areas. Fig. S2 shows the base peak chromatograms of these two investigated *S. montana* extracts.

LC-MS quantitative analysis showed the presence of 14 phenolic compounds, including 8 phenolic acids and different types of flavonoids, mainly flavones, flavonols, and one flavonoid glycoside (Tab. 2). The compound quantified in the highest amount was rosmarinic acid (9795.27 mg/kg), which was extracted three times more in the ethanol extract than in the acetone extract (2681.80 mg/kg). The extracts contained protocatechuic acid, chlorogenic acid and ferulic acid in significant amounts. In addition, luteolin, quercetin, and naringenin were the dominant flavonoids in both extracts.

Tab. 2: Quantitative analysis of phenolics in *S. monatana* ethanol and acetone extracts.

No.	mg/kg DW	Ethanol extract	Acetone extract
1	Gallic acid	15.61	7.52
2	Protocatechuic acid	380.67	152.40
3	Chlorogenic acid	745.25	113.24
4	Caffeic acid	250.99	93.25
5	Syringic acid	434.94	82.43
6	p - Coumaric acid	70.10	44.60
7	Ferulic acid	206.42	133.90
8	Isorhamnetin 3-O-glucoside	35.27	54.74
9	Rosmarinic acid	9795.27	2681.80
10	Luteolin	840.44	1142.52
11	Quercetin	712.91	943.60
12	Naringenin	1228.74	2060.22
13	Kaempferol	148.69	223.81
14	Isorhamnetin	75.72	87.86

Antibacterial activity of optimized extracts

Microdilution method

The results regarding the antibacterial effects of the ethanol and acetone extracts of *S. montana* are presented in Fig. 3. Ethanol and acetone extracts demonstrated strain-dependent antibacterial activity, with MIC values ranging from 0.31 to 20 mg/mL for both extracts. The MBC values were slightly higher, ranging from 0.63 to 20 mg/mL for the ethanol extract and from 1.25 to 20 mg/mL for the acetone extract.

There was a statistically significant difference in antibacterial activity between ethanol and acetone extract ($p < 0.05$). The ethanol extract demonstrated the highest activity against the following strains:

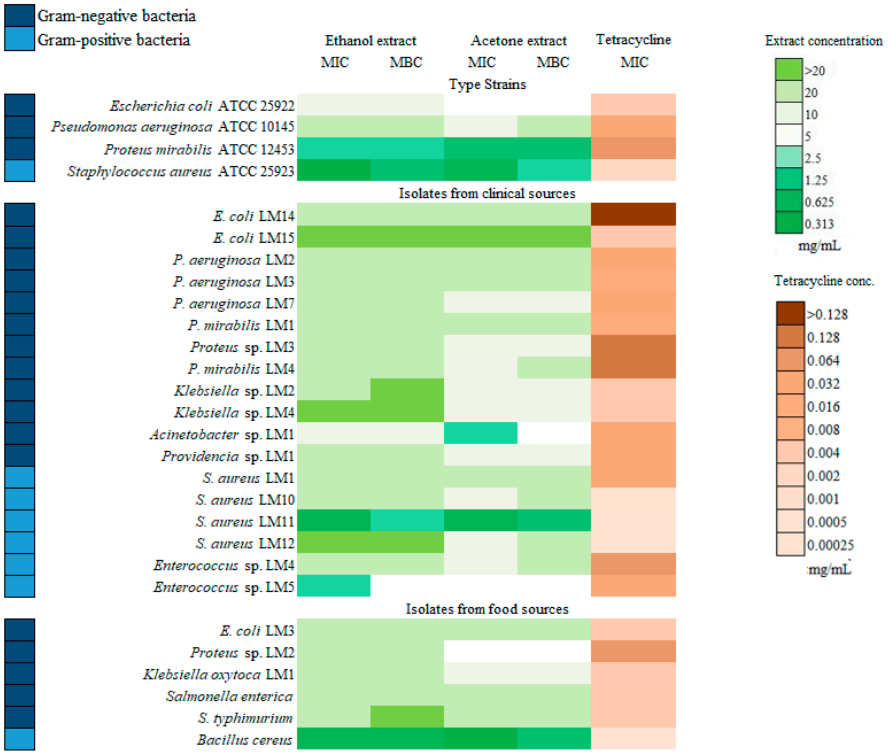

Fig. 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *S. monatana* ethanol and acetone extracts and tetracycline

S. aureus ATCC 25923 at a concentration of 0.31 mg/mL, *S. aureus* LM11 and *Bacillus cereus* at a concentration of 0.63 mg/mL, and *Proteus mirabilis* ATCC 12453 and *Enterococcus* sp. LM5 at a concentration of 2.5 mg/mL. The most susceptable strains to the acetone extract were *B. cereus* at a concentration of 0.31 mg/mL, *S. aureus* ATCC 25923 and *S. aureus* LM11 at a concentration of 0.63 mg/mL, *P. mirabilis* ATCC 12453 and *Enterococcus* sp. LM5 at a concentration of 1.25 mg/mL, and *Acinetobacter* sp. LM1 at a concentration of 2.5 mg/mL. The growth of other strains was inhibited at extracts' concentrations ranging from 5 to 20 mg/mL. The strain that showed complete resistance to both extracts was *E. coli* LM15.

The extracts' effectiveness against strains of Gram-positive and Gram-negative bacteria was significant ($p = 0.001$). This finding supports the established notion that Gram-positive bacteria are generally more susceptible to plant extracts than Gram-negative bacteria, which can be attributed to the distinct structural differences in their cell walls. Additionally, a statistically significant difference was observed based on the origin of the strains, specifically, ATCC strains were more susceptible than those derived from clinical sources and food samples ($p = 0.011$).

Time-kill assay

The time-kill assay is advantageous over the MIC/MBC test as it provides insight into the rate of bactericidal activity of the extracts. The results are illustrated in Fig. 4, with data presented as changes in $\log_{10}$ CFU/mL. Both extracts showed a bactericidal effect on *E. coli* ATCC 25922 and *P. mirabilis* ATCC 12453 after 3 hours and 6 hours of exposure, respectively. The ethanol extract inhibited the growth of *Acinetobacter* sp. LM1 after 9 h, and *S. aureus* ATCC 25923 and *Enterococcus* sp. LM5 growth after 24 hours. Notably, the growth of *S. aureus* ATCC 25923 was inhibited after 6 hours of exposure to the acetone extract. Additionally, the acetone extract significantly reduced the number of viable cells of *Acinetobacter* sp. LM1 and *Enterococcus* sp. LM5 after 9 hours of exposure.

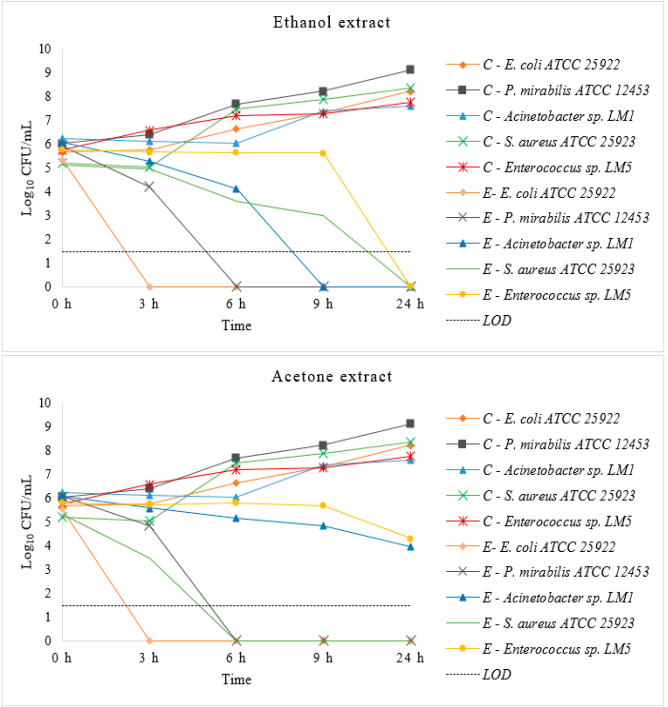

Fig. 4: Time-kill curves for bacteria treated with *S. montana* extracts; (C) control sample; (E) sample treated with the extract; LOD – limit of detection.

Mechanism of action

In this study, the mechanisms of action of *S. montana* extracts were investigated with a focus on their effects on the cell membranes of selected bacterial strains. The results indicated that the ethanol ex-

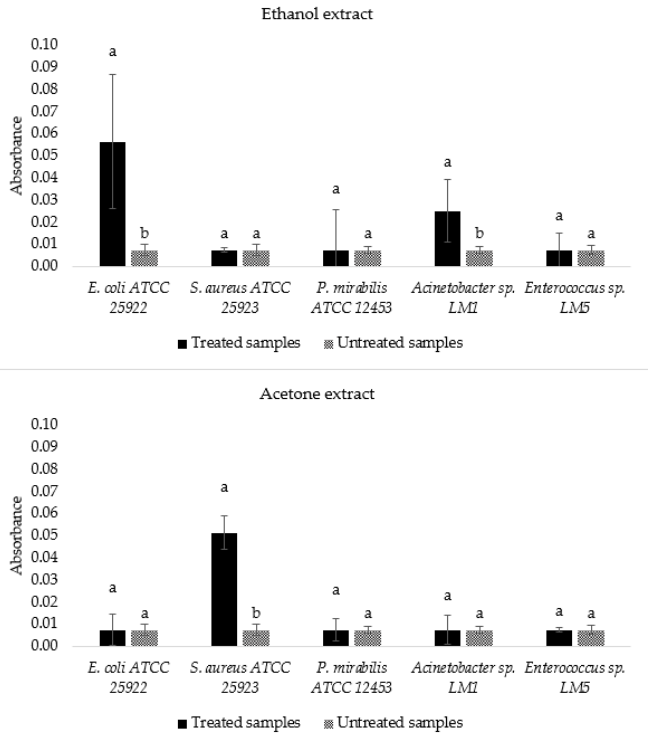

Fig. 5: Protein leakage determination. Data are presented as mean ± SD, n = 3. [ab] Statistically significant differences between treated and untreated samples ($p < 0.05$).

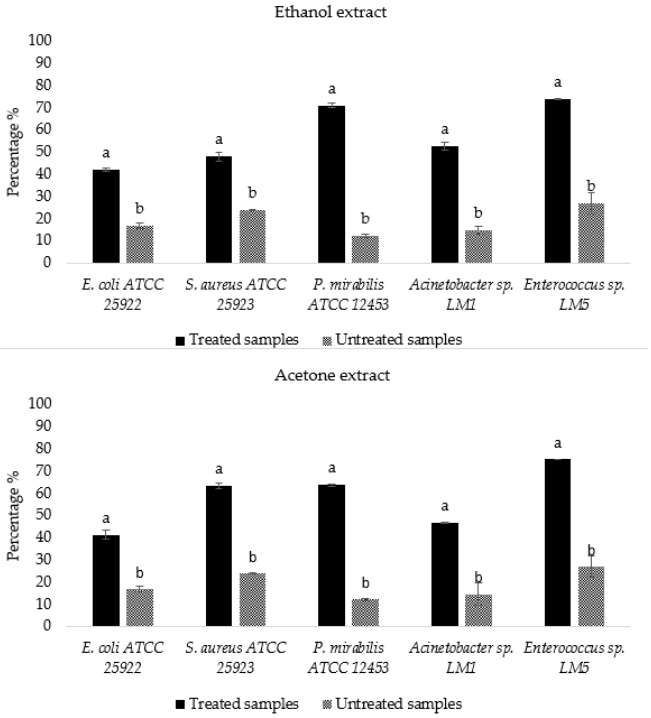

Fig. 6: Percentage of crystal violet uptake. Data are presented as mean ± SD, n = 3. [ab] Statistically significant differences between treated and untreated samples ($p < 0.05$).

tract increased cell membrane permeability in *E. coli* ATCC 25922 and *Acinetobacter* sp. LM1, as evidenced by elevated protein release (Fig. 5A). Furthermore, the acetone extract disrupted cell membrane function, with increased permeability observed in *S. aureus* ATCC 25923 (Fig. 5B). The crystal violet assay, which measured the percentage of dye binding by bacterial cells, corroborated these findings by showing damage to the cell membrane induced by both extracts across all tested strains (Fig. 6). Statistical analysis revealed a significant difference ($p < 0.05$) between treated and untreated samples, highlighting increased cell membrane permeability in the treated strains.

Checkerboard method

The results of the potential synergistic effects of *S. montana* extracts in combination with the antibiotic gentamicin on three bacterial strains are presented in Tab. 3. The acetone extract exhibited a synergistic effect with gentamicin against resistant *P. mirabilis* LM1, leading to a significant 16-fold reduction in the MIC of gentamicin (FICI = 0.31). The ethanol extract with gentamicin showed an additive effect (FICI = 0.53) on *P. mirabilis* LM1. However, the combination of extracts and gentamicin against *S. aureus* LM1 and *E coli* LM3 demonstrated indifference. The results obtained using the SynergyFinder software tool (Fig. S3) align with those derived from the FIC index. This indicated the synergistic effect of *S. montana* acetone extract and gentamicin against *P. mirabilis* LM1 (Fig. S3).

Discussion

The yield and composition of phenolic and non-phenolic compounds in plant extracts can be significantly affected by several factors, including the extraction technique, solvent type, liquid-solid ratio, extraction temperature and time, and particle size. To investigate the impact of these factors on the extraction process, it is advisable to optimize the process using a statistical technique such as response surface methodology (RSM). RSM seeks to establish models describing the relationships between independent and dependent variables. The UAE optimization aimed to maximize the TPC and TFC in the ethanol and acetone extracts of *S. montana*, while also enhancing the antibacterial activity of the extracts. The findings of this study suggested that by raising the temperature to 60°C, the TPC in ethanol extract and TFC in both ethanol and acetone extracts were enhanced. Additionally, the extraction temperature also positively affected the antibacterial efficacy of the obtained extracts. Extending the extraction time positively influenced all measured responses. The solid-solvent ratio had the least effect on TPC and TFC in the extracts of *S. montana*, as well as on the antibacterial efficiency of the obtained extracts. Our findings align with previous studies focused on optimizing polyphenol extraction from plant materials (Reungoat et al., 2020; Šavikin et al., 2021).

The phytochemical analysis of *S. montana* optimized extracts indicated that the extraction of phenolic compounds was dependent on the solvent used. Notably, TPC and TFC in the ethanol extract were significantly higher. The more successful extraction of phenolic compounds in ethanol can be attributed to the solvent's polarity. Ethanol has a higher polarity index than acetone, making it a better solvent for extracting polar secondary metabolites. Additionally, the hydroxyl groups (–OH) present in ethanol can form hydrogen bonds with the oxygen atoms in phenolic compounds. This interaction explains the stronger affinity of polar protic solvents like ethanol for these compounds, in contrast to polar aprotic solvents such as acetone (Galanakis et al., 2013). Čutović et al., 2023 performed phytochemical analysis of ethanol extracts obtained through ultrasonic extraction at varying durations. The findings revealed that the highest TPC and TFC values were observed at 30 minutes of extraction, which aligns with our results. The measured TPC was 21.1 ± 0.6 mg GAE/g, while the TFC was 8.4 ± 0.1 mg QE/g. The TPC and TFC in *S. montana* from seven different regions in Croatia were investigated. Extraction was performed using ultrasound under the following parameters: 20 °C for 60 minutes, with 80% ethanol as solvent. The TPC ranged from 155.36 to 271.35 mg/g, depending on the locality. In contrast, the concentration of flavonoids was significantly lower, ranging from 10.26 to 22.08 mg/g (Kremer et al., 2015).

The LC-MS analysis of *S. montana* extracts revealed the presence of 106 metabolites. The compounds quantified in the highest amounts were rosmarinic acid, chlorogenic acid, luteolin, quercetin, and naringenin. A total of 44 phenolic compounds were identified in *S. montanta* subsp. *kitaibelii*. Two main families of phenolic compounds were found: phenolic acids (hydroxybenzoic and hydroxycinnamic acids) and flavonoids (flavones and flavonols) (López-Cobo et al., 2015). Our results are in agreement with those of Pavlović et al., 2021, who reported a high content of rosmarinic acid and significant levels of flavonoids (quercetin, rutin, naringin, and luteolin-7-O-glucoside) in *S. montana* extracts.

The antibacterial activity of *S. montana* extracts was observed against several medically important pathogens. Notable sensitivity was found among strains of Gram-positive bacteria, including *S. aureus* ATCC 25923, *S. aureus* LM11, *Enterococcus* sp. LM5, and *B. cereus*, with MICs ranging from 0.31 to 0.63 mg/mL. *S. aureus* is a human pathogenic bacterium responsible for a range of infections that occur in both community and hospital settings (Kluytmans-VandenBergh and Kluytmans, 2006). The *Enterococcus* species are considered opportunistic pathogens, particularly concerning in healthcare environments. Their ability to translocate from the gastrointestinal tract to other tissues, combined with their virulence factors and antibiotic re-

Tab. 3: Results of the checkerboard method.

Bacterial strains	MIC of gentamicin (mg/mL)	MIC Combination	FIC A	FIC B	FIC Index
E. coli LM3	0.016	1/2 MIC of ethanol extract 1/2 MIC of gentamicin	0.5	0.5	1 (Indifference)
		MIC of acetone extract 1/32 MIC of gentamicin	1	0.031	1.03 (Indifference)
S. aureus LM1	0.128	MIC of acetone extract 1/32 MIC of gentamicin	1	0.031	1.03 (Indifference)
		MIC of acetone extract 1/32 MIC of gentamicin	1	0.031	1.03 (Indifference)
P. mirabilis LM1	> 0.128	1/2 MIC of acetone extract 1/32 MIC of gentamicin	0.5	0.031	0.53 (Additivity)
		1/4 MIC of acetone extract 1/16 MIC of gentamicin	0.25	0.063	0.31 (Synergism)

sistance, complicates efforts to suppress these infections (Krawczyk et al., 2021). *B. cereus* is a pathogenic bacterium primarily associated with food poisoning, causing infections that are mainly of the diarrheal or emetic type (Kotiranta et al., 2000). The strains of Gram-negative bacteria that showed greater sensitivity to the action of the tested extracts were *P. mirabilis* ATCC 12453 and *Acinetobacter* sp. LM1 with MIC values at ≤ 2.5 mg/mL. The mentioned bacteria can be the causative agents of urinary tract infections, pneumonia, sepsis, and other severe nosocomial infections (Exner et al., 2017). Earlier, the efficacy of *S. montana* ethanol extract was confirmed against *Brochothrix thermosphacta* (MIC = 3.80 mg/mL), *Listeria innocua* (MIC = 3 mg/mL), *L. monocytogenes* (MIC = 3 mg/mL), *E. coli* (MIC = 15.10 mg/mL), *Pseudomonas putida* (MIC = 0.04 mg/mL), *Salmonella typhimurium* (MIC = 30.30 mg/mL), and *Shewanella putrefaciens* (MIC = 3 mg/mL), while the aqueous extract did not exhibit antibacterial activity (Serrano et al., 2011). Aćimović et al. (2022) noticed that *S. montana* extracts were inactive. Oppositely, the decoction of *S. montana* demonstrated inhibitory effect on the growth of *S. aureus* and *Klebsiella pneumoniae* (Gomes et al., 2020).

Time-kill kinetics of the ethanol and acetone extracts of *S. montana* varied among the tested bacterial strains. This variation is likely attributed to the differing compositions of secondary metabolites in the extracts and the specific bacterial species involved. The findings indicate that the ethanol extract exhibits more potent activity than the acetone extract. The bactericidal effects against *E. coli* ATCC 25922, *P. mirabilis* ATCC 12453, and *S. aureus* ATCC 25923 were noticeable. By investigating the potential mechanisms of action, we found that the acetone and ethanol extracts of *S. montana* increased the permeability of the cell membranes in the tested strains, thereby disrupting their function. Rosmarinic acid was identified as the main component of the extracts. Previous studies have established that its antibacterial mechanism involves cell membrane damage (Wang et al., 2024). This phenolic acid may also inhibit bacterial cell proteins or Na+/K+-ATPase (Zhang et al., 2022). Luteolin, quercetin, and naringenin were the most abundant flavonoids in both extracts. Luteolin and quercetin have shown cell membrane disruption as a mechanism of action, which can be related to the activity of the tested extracts (Nguyen and Bhattacharya, 2022). While the antibacterial mechanism of naringenin is not fully understood, evidence suggests that high concentration increases membrane permeability and alter the cell morphology of *S. aureus* (Cai et al., 2023). Our findings align with the research conducted by Gomes et al., 2020, which also demonstrated that decoction of *S. montana* can disrupt the normal functioning of the cell membrane in *S. aureus*, leading to its damage, especially after 3 hours upon exposure. *In vitro* research examining effect of *S. montana* against food pathogen *Campylobacter jejuni*, revealed that both the ethanol extract and essential oil impair the bacterial cell membrane and inhibit efflux pump activity (Šimunović et al., 2020). Although the concentrations of key phenolic compounds in *S. montana* extracts were not among the highest reported, the results of this study demonstrate that they exhibit notable antibacterial activity (MIC ≤ 2.5 mg/mL) against clinically relevant pathogens. This indicates that the observed bioactivity is not solely determined by the quantitative presence of individual phenolics. Rather, the pronounced effect may be attributed to the specific overall chemical composition of the extract, potential synergistic interactions among constituents, or the role of minor yet biologically active compounds. These findings suggest that *S. montana* extracts may represent a promising natural alternative to conventional antibacterial agents.

Moreover, the tested acetone extract of *S. montana* demonstrated a synergistic effect with gentamicin against the resistant *P. mirabilis* LM1 strain. These results align with previous studies that confirmed the synergistic interactions between plant extracts and antibiotics (Álvarez-Martínez et al., 2021; Saquib et al., 2021). Plant active compounds could modify the mechanisms of acquired resistance and thus exhibit a synergistic effect with antibiotics. The mechanism of synergistic action could be explained by: i) modification of active sites on bacterial cells, ii) inhibition of enzymes that catalyze degradation or modification of antibiotics, iii) increase of membrane permeability, and iv) inhibition of efflux pumps (Álvarez-Martínez et al., 2021). It is hypothesized that *S. montana* extracts may enhance the penetration of the antibiotic into bacterial cells by disrupting membrane permeability. This assumption suggests that such an effect could help to overcome the resistance of certain pathogenic bacteria and improve antibiotic effectiveness, potentially explaining the observed synergism. A precise understanding of the mechanisms underlying the observed synergistic effect could provide a basis for the development of new antibacterial agents.

Conclusions

The optimized extracts obtained using UAE exhibited broad-spectrum antibacterial activity, particularly against Gram-positive strains. A promising outcome was the synergistic effect between the acetone extract and gentamicin against a resistant strain of *P. mirabilis* LM1. The antibacterial mechanism was confirmed to involve disruption of membrane integrity. The results of LC-MS analysis indicated that the aerial parts of *S. montana* are a rich source of phenolic compounds, primarily phenolic acids and flavonoids. The compounds quantified in the highest amounts were rosmarinic acid, chlorogenic acid, luteolin, quercetin, and naringenin. These findings suggest that *S. montana* extracts could be considered as natural antimicrobial agents, either as a stand-alone treatment or in combination with conventional antibiotics. Further studies are warranted to evaluate their efficacy *in vivo* and to explore their potential for applications in the pharmaceutical or food industry.

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Conflict of interest

No potential conflict of interest was reported by the authors.

Data Availability Statement

The data that support the findings of this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

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