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Modulation of the bacterial virulence and resistance by well-known European medicinal herbs

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ABSTRACT

Ethnopharmacological relevance: *Salvia officinalis* L., *Sambucus nigra* L., *Matricaria chamomilla* L., *Agrimonia eupatoria* L., *Fragaria vesca* L. and *Malva sylvestris* L. are plants that have a long tradition in European folk medicine. To this day, they are part of medicinal teas or creams that help with the healing of skin wounds and the treatment of respiratory or intestinal infections. However, so far these plants have not been investigated more deeply than in their direct antibacterial effect.

Aim of the study: Our research is focused on adjuvants that inhibit the mechanism of antibiotic resistance or modulate bacterial virulence. Based on a preliminary screening of 52 European herbs, which commonly appear as part of tea blends or poultice. Six of them were selected for their ability to revert the resistant phenotype of nosocomial bacterial strains.

Methods: Herbs selected for this study were obtained from commercially available sources. For the extraction of active compounds ethanol was used. Modulation of virulence was observed as an ability to inhibit bacterial cell-to-cell communication using two mutant sensor strains of *Vibrio campbellii*. Biofilm formation, and planktonic cell adhesion was measured using a static antibiofilm test. Ethidium bromide assay was used to checked the potential of inhibition bacterial efflux pumps. The antibacterial activities of the herbs were evaluated against resistant bacterial strains using macro dilution methods.

Results: Alcohol extracts had antibacterial properties mainly against Gram-positive bacteria. Of all of them, the highest antimicrobial activity demonstrated *Malva sylvestris*, killing both antibiotic resistant bacteria; *Staphylococcus aureus* with MIC of 0.8 g/L and *Pseudomonas aeruginosa* 0.7 g/L, respectively. *Fragaria vesca* extract (0.08 g/L) demonstrated strong synergism with colistin (4 mg/L) in modulating the resistant phenotype to colistin of *Pseudomonas aeruginosa*. Similarly, the extract of *S. officinalis* (0.21 g/L) reverted resistance to gentamicin (1 mg/L) in *S. aureus*. However, *Sambucus nigra* and *Matricaria chamomilla* seem to be a very promising source of bacterial efflux pump inhibitors.

Conclusion: The extract of *F. vesca* was the most active. It was able to reduce biofilm formation probably due to the ability to decrease bacterial quorum sensing. On the other hand, the activity of *S. nigra* or *M. chamomilla* in reducing bacterial virulence may be explained by the ability to inhibit bacterial efflux systems. All these plants have potential as an adjuvant for the antibiotic treatment.

1. Introduction

Multidrug resistance (MDR) is defined as a decrease in the effectiveness of three or more classes of antibiotics to achieve therapeutic doses at the target site. Infections caused by MDR strains are life-threatening and financially demanding. Treatment with traditional antibiotics is failing, mainly because very few antibiotics have been approved in the last two decades with only a few innovative structures

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(Murugaiyan et al., 2022). Therefore, research in the field of MDR infection treatment is currently focused on modulation of the antibiotic-resistant phenotype. If the mechanism used by the bacterium to eliminate the antibiotic is specifically inhibited, the previously ineffective antibiotic resumes its function and kills the bacterium. This approach is known as a combination or adjuvant therapy and has been used for decades since Augmentin was approved in 1981. Augmentin is a combination of amoxicillin (penicillins) and β-lactamase inhibitor, which inhibits the enzyme that cleaves the β-lactam structure of the penicillin ring (White et al., 2004). β-Lactamase inhibitors are the most widely used and clinically proven group of adjuvant molecules. These molecules are characterized by very low antimicrobial activity, but can inhibit specific resistance mechanisms in bacteria (Mulani et al., 2019). Due to the dual objectives of adjuvant therapy, the amount of antibiotic is reduced and the resistance evolution is slow (Liu et al., 2019).

The basic mechanisms by which bacteria obtain the resistant phenotype include drug inactivation by destructases, drug-target modification, and drug efflux (Chambers et al., 2019). Destructases are enzymes that modify the antibiotic structure by transferring different groups or cleaving important structural bonds (*e.g.*, β-lactamase) (Forsberg et al., 2015). One such destructase is encoded by the *aac (6′)-le-aph(2″)-la* gene and provides resistance to aminoglycosides (including gentamicin) in Gram-positive cocci such as methicillin-resistant *Staphylococcus aureus* (MRSA). Gentamicin binds to a small subunit of the ribosome; however, destructase disposes of acetyl or phosphotransferase activity and modifies the structure of gentamicin, which is then unable to bind to the ribosome. The common goal of all efflux pumps is to reduce the intracellular concentration of antibiotics, thus eliminating their effect. Since these pumps can be nonspecific for the substrate, they can reduce the concentration of various antibiotics within the bacterium and therefore render them ineffective (Du et al., 2018). Such an active efflux system may be MexXY/OprM, which contributes to polymyxin resistance in Gram-negative bacteria (*e.g.*, *Pseudomonas aeruginosa*) (Puja et al., 2020). At the same time, bacteria can use the efflux system as an intercellular communication system between the same or different bacterial strains (Kaur et al., 2021). Bacterial communication, known as a quorum sensing system (QS), increases pathogenicity, regulates the production of virulence factors (toxins), and group behavior (biofilm formation) (Bouyahya et al., 2022).

Traditional medicine in Europe is based on a wide selection of medicinal herbs available. One of the most popular is *Matricaria chamomilla* L., also called *Matricaria recutita* L. from the *Asteraceae* family. In various cultures, the so-called chamomile was reported to treat colic and diarrhoea as well as mouth, throat, and ear infections and infections in general (Dos Santos et al., 2019a; EMA, 2015b; Ginko et al., 2023). *Agrimonia eupatoria* L. (agrimony) of the *Rosaceae* family is traditionally used for beneficial effects in the case of chronic wounds, infections of the urinary and respiratory tract (Al-Snafi, 2015; EMA, 2015a; Malheiros et al., 2022). Similar conditions are traditionally treated using *Fragaria vesca* L. (wild strawberry) from the *Rosaceae* family (EMA, 2018a; Kirsch et al., 2020). *Salvia officinalis* L. (sage) from the family *Lamiaceae* has a long history of medicinal use, in folk medicine it has been used as a remedy for many problems including inflammations and gastric disturbances (EMA, 2016; Ginko et al., 2023; Hubbert et al., 2006). *Sambucus nigra* L. (elder, family *Viburnaceae*) is for centuries used to treat fever, coughing, and upper respiratory tract problems, or urinary tract infections (Mehmood et al., 2019). *Malva sylvestris* L. (common mallow, family *Malvaceae*) has been often reported to be used as an anti-inflammatory agent, but has also been used to treat gastric ulcers and the common cold or atopic dermatitis (EMA, 2018b; Ginko et al., 2023; Meysami et al., 2021). All of these medicinal herbs are a rich source of various biologically active compounds, as was reported many times. This chemical variety is caused mainly by the production of secondary metabolites often connected with the defense of the plant (such as glycoalkaloids, phenolic compounds, *etc.*) (Krizkovska et al., 2022; Kumar et al., 2021). Although many herbs have been used for centuries to treat various bacterial infections, the molecular mechanism of their activity for many of them has not been described and the biologically active substances contained in them have not been determined. As a result of the development of analytical methods in recent decades, this possibility is now achievable.

Although there are many publications devoted to the direct antibacterial activity of the mentioned plants, only a few have reached *in vivo* or clinical tests. Especially *M. chamomilla*, which is a prominent plant in traditional medicine, appears in several cream patents (dos Santos et al., 2019b) with proven healing and antibacterial effects or as part of a cream for atopic eczema (Boroujeni et al., 2017). A mouthwash containing 1% chamomile extract was also successful in a clinical test in patients with gum inflammation (gingivitis). This reduced biofilm accumulation and bleeding, which were comparable results to 0.12% chlorhexidine (positive control). The advantage of mouthwash with chamomile was the absence of side effects (allergic reaction, burning, *etc.*), which are commonly observed in the positive control (Goes et al., 2016). Another clinical study monitored the effect of the combined Berdi Sachet product (extract from *S. nigra* and cranberry) on patients with urinary tract irritation. This study was evaluated only on the basis of questionnaires filled out by patients, which does not provide more detailed information about the activities of the product. In addition, there is no information on the control or placebo group. *S. nigra* was also included in supplements combined with immune-stimulating molecules that served as supportive therapy in children with upper respiratory tract inflammation. The use of this combination therapy resulted in improved effusion-induced otitis media outcomes, as well as an improved immune response (Della Volpe et al., 2019). In an *in vivo* test, a cream containing 5 and 10% *M. sylvestris* was successful, showing better burn healing in rats. In addition, without detection of bacterial infection, which in these cases tends to be a major complication during healing (Nasiri et al., 2015). The aqueous extract of *A. eupathoria* has also been shown to improve open wound healing in rats. Direct antibacterial effects were not monitored, but a possible bacterial infection would greatly complicate healing (Vasilenko et al., 2022).

Here, we report a chemical composition of six well-known European herbs and correlate it with their ability to modulate bacterial virulence. In addition, we demonstrate their synergistic effect in combination with commonly used antibiotics against clinical MDR bacterial strains.

2. Materials and methods

2.1. Chemicals

BHI broth (Brain heart infusion; MERCK; Germany); Casamino acids (MERCK; Germany); CCCP (Carbonyl cyanide 3-chlorophenylhydrazone; MERCK; Germany); colistin sulphate (MERCK; Germany); DMSO (Penta; Czech Republic); ethidium bromide (MERCK; Germany); gentamicin sulphate (Duchefa; Netherlands); KH_2PO_4 (Lach-Ner; Czech Republic); $MgSO_4 \times 7\ H_2O$ (Penta; Czech Republic); MH broth (Mueller Hinton Broth; MERCK; Germany); NaCl (Penta; Czech Republic); resazurin sodium salt (MERCK; Germany) and reserpine (MERCK; Germany). For UHPLC-HRMS/MS analysis: ethanol, p. a. (Merck, Germany), methanol, purity ≥99.9% (Honeywell, USA), ammonium formate, purity >99,0% (Merck, Germany), formic acid, purity ≥99,9% (VWR Chemicals, USA) and deionized water obtained from an internal Milli-Q system (Merck, Germany).

2.2. Plant material and extraction

The plant names have been checked with http://www.worldfloraonline.org/(17.02.2023). Dried plant material of selected herbs was obtained from commercially available sources. The extracts were prepared by macerating in 96% ethanol of commercially available dried plants. The extraction was carried out at room temperature (25 °C) without stirring for two weeks. The mass extraction ratio (shown in

Supplementary Table 2) was mostly 1:10. In some cases, the ratio was increased because the herbs significantly absorbed the solvent. After maceration vacuum filtration through Macherey-Nagel MN 615 filter paper, 90 mm, REF 431 009 was used, to separate herb biomass and extracts. Subsequently, the extracts were evaporated and the biomass was resuspended in DMSO to the stock concentration (100 g/L).

2.3. Determination and identification of secondary metabolites

For the analysis of secondary metabolites in the plant extracts, the instrumental technique of ultra-high-performance liquid chromatography coupled with high-resolution tandem mass spectrometry (UHPLC-HRMS/MS) was used. Microfiltration (0.22 μm) of the samples before the analysis was performed and due to the different extraction ratios of the samples, the extracts were also diluted with ethanol in the same ratio to improve the comparability of the results. The analysis was then performed on a DionexUltiMate 3000 chromatograph (Thermo Fisher Scientific, USA) coupled with a TripleTOF™ 6600 mass spectrometer (SCIEX, Canada) with quadrupole time-of-flight (Q-TOF) mass analyzer. Separation was carried out on an analytical column HSS T3 (2.1 × 100 mm, 1.8 μm) analytical column at 45 °C and the mobile phase consisted of A: deionized water with 5 mM ammonium formate and 0.1% formic acid and B: methanol with 5 mM ammonium formate and 0.1% formic acid. The gradient was used as follows: 0–1 min (5% B), 1–8 min (5–100% B), 8–16 min (100% B). The injection volume was 2 μl and the flow rate was 0.4 ml/min. Mass spectra were obtained in both positive and negative ionization modes with electrospray ionization. The acquisition mode was programmed to obtain spectra in full MS mode and to obtain MS/MS spectra. Electrospray ionization was used with these parameters: the source temperature was 480 °C for both polarities, the capillary voltage was +5000V/-4000 V, the collision energy was 35 eV (±15 eV).

Subsequently, a targeted screening of polyphenolic compounds was performed and, for that purpose, a database of 296 phenolic compounds was created based on the available literature. The database contained these data: i) name, ii) molecular formula, iii) occurrence in plants, and iv) availability of mass spectra in online libraries. For data processing, PeakView software (version 2.2, SCIEX, Canada) was used. The criteria for compound identification were: exact mass, mass error (<5 ppm), isotope profile, and conformity of the mass fragmentation spectra with the spectra in online mass spectra libraries (mzcloud.com, pubchem.com, metlin.com).

2.4. Antibacterial assay: minimal inhibitory concentration determination

The minimal inhibitory concentration (MIC) for each extract was determined using the broth microdilution method in a 96-well plate as the concentration inhibiting exactly 70% of the population (Holasová et al., 2022; Viktorová et al., 2020a, 2020b). The medium used was MH broth. The concentrations tested ranged from 0.002 to 1 g/L and were prepared from a fresh stock solution of 100 g/L. The MIC was determined by absorbance (590 nm) using the online calculator (https://www.aatbio.com/tools/ic50-calculator/)after 24 h of incubation (37 °C, 120 rpm/min). The following bacterial strains were used: *S. aureus* ATCC 25923, *P. aeruginosa* CCM 3955, *S. aureus* NEM 449 and *P. aeruginosa* NEM 986.

2.5. Sensitization of multidrug-resistant bacterial strains

The overnight culture of previously characterized *S. aureus* NEM 449 and *P. aeruginosa* NEM 986 was diluted in MH broth to a final concentration of 5×10^5 CFU/ml (Holasová et al., 2022). Gentamicin was added to *S. aureus* culture at the breakpoint concentration (0.001 g/L) according to the recommendation of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Testing, 2022). Colistin was added to the culture of *P. aeruginosa* at the breakpoint concentration (0.004 g/L). The extracts were tested by a binary dilution in the concentration range of 0.01–1 g/L. After 20 h of incubation (37 °C, 120 rpm/min), the absorbance was measured at 600 nm (Sunrise™, Tecan, Switzerland). All extracts were tested in triplicate and DMSO was used as a negative control to replace the volume of extracts. Sensitization was evaluated by MIC of extracts in the presence of a breakpoint concentration of antibiotics (killing more than 70% of the population; using the Web calculator https://www.aatbio.com/tools/ic50-calculator/).

2.6. Inhibition of quorum sensing

Antiquorum sensing activity (QS) was observed using two strains of *Vibrio campbellii* that can respond to autoinducers (AI-1 and AI-2) with a bioluminescence signal. Bacterial strains were tested separately according to Holasová et al. in 96-well white plates (Holasová et al., 2022). Extracts were added to cultures in triplicate at a final concentration of 1 g/L, binary diluted, and then incubated for 8 h at room temperature. The luminescence was measured every 20 min (integration time 10 s; shaking for 60 s before measurement) for 16 h in a microplate reader at 30 °C. The viability of the culture was verified by the standard resazurin assay (Riss et al., 2016). Samples that reduced culture viability below 80% were excluded from further analysis. Based on the sum of the luminescence, the effective concentration of compounds that halves the luminescence signal (EC_{50}) was calculated using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

To verify antiquorum sensing activity, the overnight cultures of clinical isolate *S. aureus* NEM 449 and *P. aeruginosa* NEM 986 were diluted in BHI broth to a final concentration of 5×10^5 CFU/mL. The plant extracts were tested in the concentration range of 0.1–1 g/L. After 4 h of incubation (37 °C, 120 rpm/min), 1 mL of each culture was centrifuged (7 min, 14 000×*g*). The resulting supernatant containing signal molecules was filtered through a membrane filter (0,2 μm, Millipore, Merck, Germany). The prepared samples were stored at −80 °C. The amount of communication molecules was evaluated by a reporter assay using strains of *V. campbelli* as described above. The overnight culture of *V. campbelli* was diluted to a final concentration of 7.5×10^5 CFU/mL and dispensed into the 96-well plate (97.5 μl to each well). Subsequently, 2 μl of the samples were added. All samples were tested in triplicate. Measurement and evaluation of the activity was as described above.

2.7. Real-time ethidium bromide accumulation assay

The ethidium bromide accumulation assay was used with minor modifications according to the previously described method (Nové et al., 2020; Szemerédi et al., 2020). Briefly, the overnight culture of *S. aureus* NEM 449 or colistin-induced (0.004 g/L) *P. aeruginosa* NEM 986 was diluted in MH broth to an $OD_{600} = 0.6$. The bacterial suspension was centrifuged at 13 000×*g* for 3 min and the pellet was washed and resuspended with phosphate-buffered saline (PBS, pH 7.4). 50 μL of samples (final concentration 0.06–0.25 g/L) in ethidium bromide (0.002 g/L or 0.001 g/L) was dispensed into the wells of a 96-well black microtiter plate. The reaction was started by adding 50 μl of the bacterial suspension. Fluorescence (530/600 nm) was monitored every minute for 1 h. The relative accumulation of ethidium bromide (RA) of plant extracts was calculated relative to the accumulation of ethidium bromide in the presence of reserpine (40 μM) for *S. aureus* or CCCP (40 μM) for *P. aeruginosa* (defined as 1) and the same volume of DMSO (1%, v/v; defined as 0).

2.8. Inhibition of cell adhesion and biofilm disruption

The prevention and disruption effect of plant extracts on bacterial biofilms was tested on *S. aureus* (ATCC, 25 923), *P. aeruginosa* (CCM, 3955) and *E. coli* (AG100) using a static anti-biofilm assay as described in our previous study (Hoang et al., 2020; Viktorová et al., 2020a). First,

the bacterial culture was incubated with extracts (0.015–1 g/L) and after 24 h of static incubation, adherent cells were evaluated using the resazurin assay. Second, the mature biofilm was incubated with the extracts (0.015–1 g/L) for 24 h after which the viability of the remaining biofilm (after incubation with the extract) in the plate was analyzed using the resazurin assay and expressed as relative metabolic activity. In both cases, cell viability was calculated relative to cell viability in the absence of extracts (defined as 100%) and pure media (defined as 0%). More than a 50% reduction in cell viability compared to control was considered significant and the concentration of extracts that halved viability (IC_{50}) was calculated using GraphPad Prism 7 software.

2.9. Statistical analysis and correlation

All experiments were performed with the appropriate number (n) of repetitions that are mentioned in each method. Results are presented as the averages of the repetitions with the standard error of the mean (SEM). Analysis of variance (ANOVA) was performed using Statistica software version 13 (Tibco Software Inc., Palo Alto, CA, USA), followed by Tukey's *post-hoc* test ($p < 0.05$) to show the differences between the groups.

The correlation of analytical and biological results was performed according to (Hoang et al., 2020; Křížkovská et al., 2020); basically, the areas of the peaks belonging to the identified metabolites served as matrix I. The results of the biological activity of six herbal extracts obtained from all the investigated assays (antimicrobial, inhibition of biofilm formation, destruction of mature biofilm, inhibition of QS) were utilized as matrix II. From these matrices, Pearson's coefficient was determined using the "CORREL" function in Microsoft Office Excel. The critical values ($\alpha = 0.05$) were determined using degrees of freedom ($df = n-2$). These values (Pearson's coefficient and critical values) were compared, and subsequently the significance of the correlation coefficient was determined.

3. Results

3.1. Chemical composition of plant extracts

In total, 53 different compounds were identified in all extracts tested (Supplementary Table 1) using UHPLC-HRMS/MS. The largest number of compounds identified was in the extract of *Agrimonia eupatoria* (49 compounds). In contrast, in the *Malva sylvestris* extract, the lowest number of compounds was identified from the samples tested. Approximately 62 compounds were identified in all the extracts tested. Two compounds (astringin and piceol) were detected only in *Salvia officinalis*.

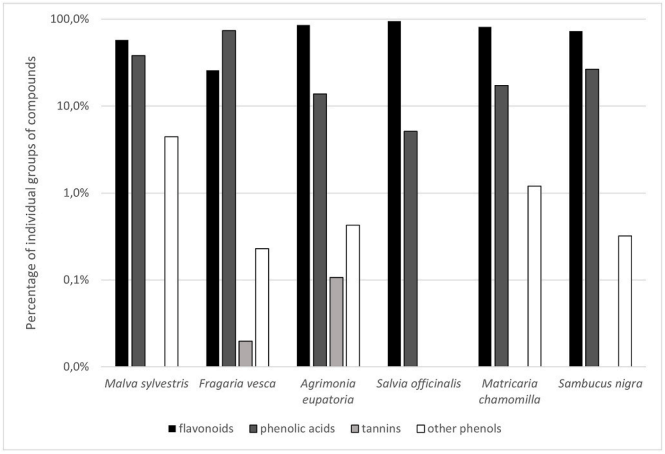

Fig. 1. Percentage representation of individual groups of substances in extracts of selected plants detected by UHPLC-HRMS/MS.

As seen in Fig. 1, the most dominant group of compounds was flavonoids followed by phenolic acids. Tannins and other phenols were detected in minimum amounts. Furthermore, tannins were detected only in two extracts (*Fragaria vesca* and *A. eupatoria*).

3.2. Antimicrobial activity

Direct antimicrobial activity was determined as the minimal inhibition concentration (MIC, g/L) of the extracts that reduces growth of bacteria by more than 70%. Most of the extracts had no or very low toxicity against all bacterial strains (Table 1). The highest antimicrobial activity against *Staphylococcus* strains demonstrated extracts made from *S. officinalis* and *A. eupatoria*. On the other hand, the only active extract against MDR *P. aeruginosa* was made from *M. sylvestris*.

3.3. Sensitization of multidrug-resistant bacterial strains

Clinical MDR bacterial strains were used to test the ability of the ethanolic extracts to revert the antibiotic-resistant phenotype. The synergistic effect of the breakpoint concentration of antibiotics and the nontoxic dose of the extract was monitored. The viability of the MDR strains was affected neither by antibiotics nor by extract, when cultivated alone; however, the combination of antibiotics and extract decreased the viability of the MDR strains under visible growth (Table 2). We achieved the best results by combining gentamicin (1 mg/L) with an extract of *S. officinalis* (21 mg/L) or *S. nigra* (36 mg/L) to MDR *S. aureus* sensitization. Extracts of *M. sylvestris, F. vesca,* and *A. eupatoria* were unable to inhibit the growth of MDR *S. aureus* in combination with gentamicin. On the contrary, the growth of Gram-negative MDR *P. aeruginosa* was inhibited in combination with colistin by all extracts tested. With the exception of the *M. sylvestris* extract, which at the highest concentration tested (1 g/L) reduced bacterial viability to 45%, all extracts reverted the colistin-resistant phenotype to the sensitive one. The combination of *F. vesca* (0.08 g/L) or *S. officinalis* (0.15 g/L) extracts with colistin (4 mg/L) was evaluated as the most effective, increasing the sensitivity of the clinical isolate of *P. aeruginosa* to colistin.

Table 1
Minimum inhibitory concentration (MIC, g/L) of crude plant extracts inhibiting the visible growth of different collection bacterial strains.

Plant	*S. aureus* ATCC 25923	*P. aeruginosa*CCM 3955	*S. aureus* NEM 449	*P. aeruginosa* NEM 986
Malva sylvestris	-	* 1 (61%)	0.8 ± 0.023^{c}	0.7 ± 0.010
Fragaria vesca	* 1 (58%)	* 1 (51%)	0.2 ± 0.015^{d}	-
Agrimonia eupatoria	* 1 (48%)	0.5 ± 0.06^{b}	0.4 ± 0.003^{b}	-
Salvia officinalis	* 1 (38%)	0.1 ± 0.02^{a}	0.4 ± 0.007^{b}	-
Matricaria chamomilla	-	* 1 (57%)	0.8 ± 0.009^{c}	-
Sambucus nigra	-	* 1 (75%)	0.5 ± 0.034^{e}	-
Imipenem	0.0001 ± 0.00001	0.008 ± 0.0005^{a}	0.02 ± 0.0003^{a}	
Tetracycline	0.0015 ± 0.00004	-	0.047 ± 0.0027^{a}	

The crude extracts were applied in the concentration range of 0.004–1 g/L. Data are presented as the average of three replicates. "-" indicates that no effect was observed up to the highest crude extract concentration tested (>1 g/L). * The highest concentration tested (1 g/L) reduced the viability of the bacterium to the value in parentheses. Data were analyzed by one-way analysis of variance (ANOVA) ($p \leq 0.05$) with the Tukey's *post-hoc* test. Statistically significant values are denoted by different letters.

Table 2
Minimum inhibitory concentration (MIC, g/L) of plant extracts inhibiting the visible growth of multidrug resistant *S. aureus* and *P. aeruginosa* strains in the presence of antibiotic breakpoint concentration. These concentrations (MIC) reverse the resistant phenotype back to a sensitive one.

Plant	*S. aureus* NEM 449	*P. aeruginosa* NEM 986
	Gentamicin [1 mg/L]	Colistin [4 mg/L]
Malva sylvestris	-	* 1 (45%)
Fragaria vesca	-	0.08 ± 0.01[a]
Agrimonia eupatoria	-	0.57 ± 0.02[b]
Salvia officinalis	0.021 ± 0.001[a]	0.15 ± 0.01[a]
Matricaria chamomilla	0.052 ± 0.002[c]	0.93 ± 0.03[d]
Sambucus nigra	0.036 ± 0.001[b]	0.73 ± 0.03[c]

The crude extracts were applied in the concentration range of 0.01–1 g/L. Data are presented as the average of three replicates. "-" indicates that no effect was observed up to the highest crude extract concentration tested (>1 g/L). * The highest concentration tested (1 g/L) reduced viability by 45%. Data were analyzed by one-way analysis of variance (ANOVA) ($p \leq 0.05$) with the Duncan's *post-hoc* test. Statistically significant values are denoted by different letters.

3.4. Inhibition of QS

Investigating inhibition of cell-to-cell communication by selected extracts was detected by production of luminescence in two mutant sensor strains of *Vibrio campbellii* responding to (a) AI-1 autoinducer (BAA1118) or (b) AI-2 autoinducer (BAA1119). In general, all extracts showed inhibition of communication based on at least one type of autoinducers that were tested (Fig. 2). In case of homoserine lactones-mediated luminescence production in *V. campbellii* BAA1118 (AI-1), the statistically highest inhibition potential was found in the extract of *M. chamomilla*. Only *S. officinalis* extract did not inhibit this type of QS up to the highest concentration tested (0.25 g/L). The second model of *V. campbellii* BAA1119 (using AI-2) was statistically best inhibited by extract of *F. vesca*. In contrast, the extract of *A. eupatoria* did not show any activity against QS caused by AI-2 up to the highest concentration tested (0.025 g/L).

The inhibition of intercellular communication was also tested using clinical isolates of antibiotic-resistant bacteria. Compared to the results obtained with the model bacterium *Vibrio*, the communication based on homeserine lactones (AI-1) was not reduced in clinical isolates by the presence of the extracts tested. However, communication based on furanosyl borates (AI-2) was significantly inhibited by all extracts. For both clinical isolates tested, the extracts of *F. vesca* and *M. chamomilla* were the most effective (Fig. 3), which is consistent with the results obtained on the model bacterium *Vibrio campbellii*.

3.5. Inhibition of cell adhesion and biofilm disruption

Inhibition of cell-to-cell communication usually decreases the ability of cells to adhere to the surface and reduces the endurance of the mature biofilm. Based on our results, all extracts reduced the adhesion of Gram-positive as well as Gram-negative bacteria (Table 3). Lower concentrations of the extracts (mainly of *F. vesca, A. eupatoria, S. officinalis,* and *M. chamomilla*) inhibited the adhesion of *S. aureus*. The most active extracts against the adhesion of *P. aeruginosa* were *F. vesca* and *A. eupatoria*. The highest doses of extract were needed to reduce the adhesion of *E. coli.* The extracts with high efficiency in this case were again *F. vesca* and *A. eupatoria.*

All the extracts tested were able to destroy a mature biofilm of *S. aureus*, in contrast to the destruction of biofilms prepared from Gram-negative bacteria, where *F. vesca* and *A. eupatoria* were mainly active (Table 4). The most active extracts against the mature biofilm of *S. aureus* were *F. vesca*, *A. eupatoria,* and *S. officinalis*.

3.6. Inhibition of efflux pump

The efflux pump system is one of the universal mechanisms responsible for the MDR phenotype, but it can also increase bacterial virulence in both Gram-positive and Gram-negative bacteria. Two of the extracts tested were able to increase the accumulation of the efflux pump substrate – ethidium bromide - inside the bacterial cells in nontoxic concentrations (1/4 MIC). Additionally, the extract of *S. nigra* was at the highest concentration (0.25 g/L) more effective inhibitor than reserpine (0.024 g/L) – a known inhibitor of efflux pumps (Fig. 4).

In Gram-negative *P. aeruginosa*, overproduction of efflux pumps was induced by colistin. A total of three extracts were active, namely *S. officinalis, M. chamomilla* and *S. nigra* (Fig. 5). The extract of *S. nigra* appears to be the most active again, showing a statistically significant accumulation of ethidium bromide compared to the positive control CCCP (0.01 g/L) at the highest concentration tested (0.25 g/L).

3.7. Correlation of biological activities and extract composition

For the determination of a relation between the biological activities measured and the compounds presented in the extracts, the Pearson's correlation coefficient was determined and evaluated. The ability of the extracts to inhibit the growth of MDR *S. aureus* was correlated with the amount of quercetin 3-O-glucoronide (Table 5). Three different detected compounds: astragalin/trifolin, ellagic acid, and tiliroside showed a significant correlation with inhibition of adhesion of *P. aeruginosa*. Furthermore, glycosidic flavonoid tiliroside correlated with both inhibition of *E. coli* AG100 adhesion and removal of mature biofilm of *P. aeruginosa*.

4. Discussion

The treatment of bacterial infections is currently a great challenge for the medicine. Antibiotics that have been used for decades are not as effective due to MDR, and new ones rarely appear. Therefore, research focuses on adjuvant substances that can support the activity of

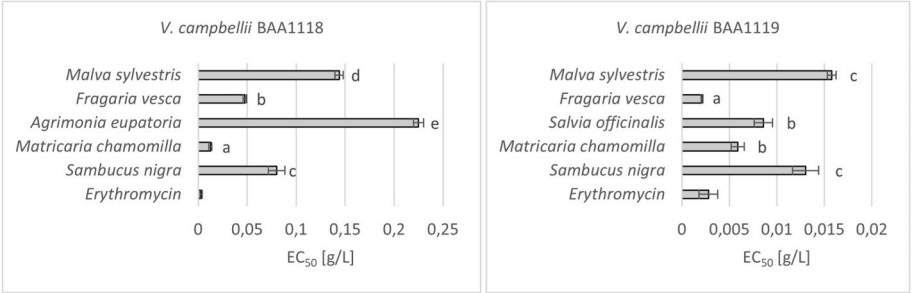

Fig. 2. Concentration of extracts [g/L] halving the bacterial quorum sensing (EC_{50}) in *Vibrio campbellii* strains. Erythromycin was used as a positive control. Data are presented as the average of three replicates ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) ($p \leq 0.05$) with the Tukey's *post-hoc* test. Statistically significant values are denoted by different letters.

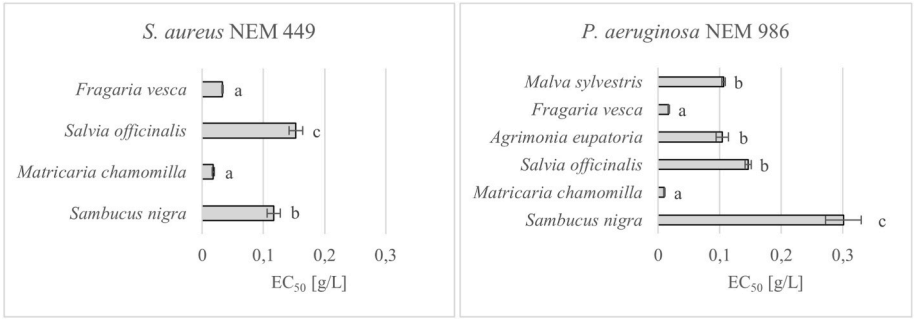

Fig. 3. Concentration of extracts [g/L] halving the AI-2 type communication (EC_{50}) of antibiotic resistant bacterial strains. Erythromycin was used as a positive control. Data are presented as the average of three replicates ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) ($p \leq 0.05$) with the Duncan's *post-hoc*. Statistically significant values are denoted by different letters.

Table 3
Concentration of extracts [g/L] halving the cell adhesion to the surface (IC_{50}).

Anti-adhesion activity			
Plant	*S. aureus* ATCC 25923	*P. aeruginosa* CCM 3955	*E. coli* AG100
Malva sylvestris	0.056 ± 0.002^{b}	0.086 ± 0.007^{c}	0.119 ± 0.009^{d}
Fragaria vesca	0.022 ± 0.002^{a}	0.024 ± 0.001^{a}	0.040 ± 0.004^{a}
Agrimonia eupatoria	0.029 ± 0.001^{a}	0.037 ± 0.001^{a}	$0.061 \pm 0.003^{a,b}$
Salvia officinalis	0.024 ± 0.002^{a}	0.068 ± 0.004^{b}	0.091 ± 0.001^{c}
Matricaria chamomilla	0.033 ± 0.004^{a}	0.059 ± 0.002^{b}	0.062 ± 0.002^{b}
Sambucus nigra	0.051 ± 0.004^{b}	0.064 ± 0.001^{b}	$0.098 \pm 0.003^{c,d}$

Data are presented as the average of three replicates ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) ($p \leq 0.05$) with the Tukey's *post-hoc* test. Statistically significant values are denoted by different letters.

Table 4
Concentration of extracts [g/L] halving the bacterial mature biofilms (IC_{50}).

Anti-biofilm activity			
Plant	*S. aureus* ATCC 25923	*P. aeruginosa* CCM 3955	*E. coli* AG100
Malva sylvestris	0.637 ± 0.040^{b}	>1	>1
Fragaria vesca	0.275 ± 0.007^{a}	0.237 ± 0.007^{a}	0.514 ± 0.002^{a}
Agrimonia eupatoria	0.298 ± 0.004^{a}	0.577 ± 0.017^{b}	0.532 ± 0.041^{a}
Salvia officinalis	0.231 ± 0.019^{a}	>1	>1
Matricaria chamomilla	0.517 ± 0.013^{c}	0.655 ± 0.030^{b}	>1
Sambucus nigra	0.688 ± 0.037^{b}	0.890 ± 0.025^{c}	>1

Data are presented as the average of three replicates ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) ($p \leq 0.05$) with the Tukey's *post-hoc* test. Statistically significant values are denoted by different letters. "> 1" indicates that no effect was observed up to the highest concentration tested (>1 g/L).

antibiotics without the risk of developing secondary resistance. New adjuvants can directly inhibit the resistance mechanism in nontoxic concentrations or modulate virulence factors, and thus reduce the danger of bacterial strains. Plant extracts are a promising source because they contain a wide range of substances. This work focuses on the modulation activities of bacterial virulence and antibiotic resistance of six well-known traditional European herbs.

All of the extracts tested were able to inhibit the bacterial growth of Gram-positive bacterial strains,. The lowest MIC values were detected in

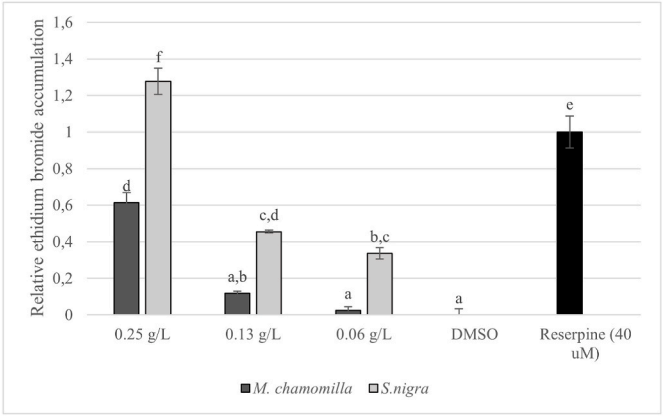

Fig. 4. Accumulation of ethidium bromide in MDR *S. aureus* caused by different concentrations of ethanolic extracts of *Matricaria chamomilla* and *Sambucus nigra.* Data are presented as the average of three replicates ± standard error of the mean (SEM). Reserpine was used as a positive control. Data were analyzed by one-way analysis of variance (ANOVA) ($p \leq 0.05$) with the Tukey's *post-hoc* test. Statistically significant values are denoted by different letters.

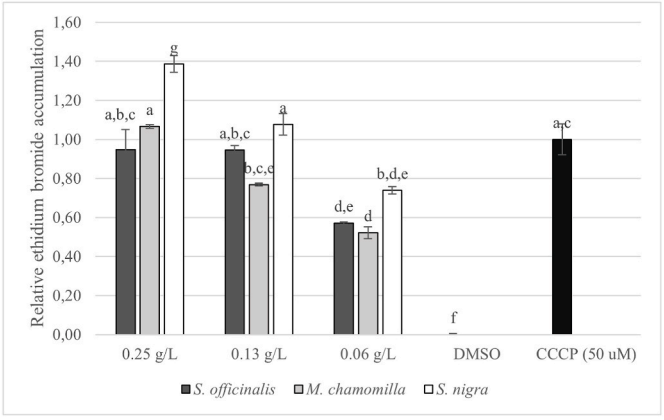

Fig. 5. Accumulation of ethidium bromide in colistin-induced MDR *P. aeruginosa* caused by different concentrations of ethanolic extracts of *Salvia officinalis*, *Matricaria chamomilla,* and *Sambucus nigra.* Data are presented as the average of three replicates ± standard error of the mean (SEM). CCCP was used as a positive control. Data were analyzed by one-way analysis of variance (ANOVA) ($p \leq 0.05$) with the Tukey's *post-hoc* test. Statistically significant values are denoted by different letters.

Table 5
Pearson's correlation coefficient demonstrating relationship between chemical composition and corresponding biological activity of extracts.

Compound	inhibition of MDR *S. aureus* viability			Anti-adhesion *P. aeruginosa*			Anti-adhesion *E. coli* AG100			Anti-biofilm *P. aeruginosa*		
	df	$R_{(crit)}$	R^2	df	$R_{(crit)}$	R^2	df	$R_{(crit)}$	R^2	df	$R_{(crit)}$	R^2
Quercetin 3-O-glucoronide	1	0.997	0.99998									
Astragalin/Trifolin				2	0.95	0.967						
Ellagic acid				4	0.811	0.816						
Tiliroside				3	0.878	0.884	3	0.878	0.902	2	0.95	0.992

R^2 is the value of the Pearson's correlation coefficient, df is the degree of freedom calculated as n−2, $R_{(crit.)}$ is the value stated in the table of the Pearson's correlation coefficient at selected significance level (α).

the cases of extracts of *F. vesca* and *S. officinalis*. On the other hand, none of the tested extracts inhibited the growth of MDR Gram-negative bacterial strain. Only *M. sylvestris* showed an inhibition potential at the highest concentration tested against *P. aeruginosa*. For each plant tested, there is at least one publication that confirms its antibacterial effects (Dias et al., 2016; Fathi et al., 2022; Gasparetto et al., 2012; Razavi et al., 2011; Shadid et al., 2021). The published MIC values differ in most cases mainly due to different extraction methods/extraction agents (Đurović et al., 2022; Muruzović et al., 2016; Shadid et al., 2021). The presence of active compounds in the extract is also influenced by the conditions under which the plant grows (soil conditions, amount of rain, sunshine) and in which generation period the plant is at the time of collection (germination, full bloom) (Milenković et al., 2015). However, all extracts tend to be more active against Gram-positive than Gram-negative bacteria at lower concentrations, which may be caused by poor permeation of active substances through the complicated membrane of Gram-negative bacteria (Fathi et al., 2022; Generalić Mekinić et al., 2019). In the literature, the MIC values of the hydroxylmethanolic extract of *F. vesca* against MRSA are similar to our measurement (0.25 g/L) (Dias et al., 2016). The published activities of *M. sylvestris* are mainly against anaerobes (Vahabi et al., 2019). The methanol extract was active against Gram-negative bacteria at higher concentrations than we tested (43 and 22 g/L). The use of different bacterial strains can also lead to different MIC values (Milenković et al., 2015).

In our case, inhibition of MRSA growth could be attributed to quercetin-3-o-glucuronide, which was identified in all extracts, especially *F. vesca* and *S. nigra*. This is in agreement with the literature, where this metabolite frequently appears in extracts that show antibacterial activity (Kim et al., 2010; Salamatullah, 2022; Yang et al., 2019).

In this work, we focus on the potential of various plant extracts to cooperate with antibiotics. The MIC values of gentamicin were decreased in the presence of extracts of *S. officinalis*, *M. chamomilla,* and *S. nigra* in the clinical isolate of MDR *S. aureus*. These findings are supported by the literature. The synergistic effect of *S. officinalis* with different antibiotics (*e.g.*, clindamycin) was previously confirmed (Mayyas et al., 2021). Horiuchi et al. extracted the active compound carnosol from *S. officinalis* that exhibits supporting activity to aminoglycosides not only against MRSA, but also against various vancomycin-resistant enterococci (Horiuchi et al., 2007). Essential oils from this herb also have a significant synergistic effect with ceftriaxone or ciprofloxacin against MRSA bacterial strains isolated from the wound. These findings suggest potential future applications for topical use (Milenković et al., 2015). In our work, six extracts tested in combination with colistin were able to decrease at least the viability of *P. aeruginosa*. In this case, the extract of *S. officinalis* was also the most active. On the other hand, the essential oil of this plant in combination with cefotaxime rather showed antagonism in *E. coli*, which may be caused by a higher concentration of volatile substances (Adrar et al., 2016). The synergistic effect of *S. officinalis* or *M. chamomilla* with antibiotics is also supported by published combinations with bactericidal substances (such as nisin or silver nanoparticles) (Dogru et al., 2017; Mehdizadeh et al., 2016). To the best of our knowledge, we are the first to show the synergistic effect of *F. vesca, A. eupatoria, M. chamomilla,* and *S. nigra* with antibiotics against resistant bacterial strains. In contrast, *M. chamomilla* and *S. officinalis* in combination with levofloxacin at nontoxic concentrations do not reveal any effect against *E. coli* strains (Tayseer et al., 2020).

The sensitivity of bacteria to antibiotics is weakened not only by the expression of resistance genes but also by the formation of biofilms. The bacteria in biofilms can be up to 100 times resistant to antibiotics (Alav et al., 2018). In addition, the production of extracellular polysaccharide mass occurs in the biofilm, which protects the bacteria from adverse external conditions. Biofilm inhibition can take the form of preventing planktonic cells from adhesion to the surface or directly removing the mature biofilm. Anti-adhesion activity was detected in all of the extracts. Higher concentrations were necessary to inhibit the biofilm formation of Gram-negative *E. coli* AG100. Our findings are supported by the literature, where the methanol extract of *M. sylvestris* inhibits the formation of biofilms in a dose-dependent manner against *S. aureus, E. faecalis,* and *K. pneumoniae* with the biofilm inhibitory concentration of 40 g/L (Fathi et al., 2022). Rinsing with *F. vesca* tea extract significantly reduces oral communities (Kirsch et al., 2020). The essential oil of *S. officinalis* was also able to inhibit the formation of biofilms by oral bacteria (Fathi et al., 2022; Mendes et al., 2020; Popa et al., 2020). The hydromethanolic extract of *F. vesca* was able to inhibit 85% of the production of MRSA biofilms at 0.25 g/L and 47–49% of the biofilm of *E. coli* strains in the concentration range 0.25–1 g/L (Dias et al., 2016). Inhibition of biofilm formation was also observed in the essential oil of *S. officinalis* against *Salmonella enterica* (Selim et al., 2022). In the case of resistant strains of *P. aeruginosa,* essential oil had antibiofilm effects without apparent concentration dependence in the tested range of 5–20 g/L (Pejčić et al., 2020). Our measurements confirm this result with an IC50 of 0.068 g/L on the collection strain of *P. aeruginosa*. It has already been published that the extract of this plant can prevent the formation of biofilms even on stainless steel (Lekbach et al., 2019). Previous work has already revealed the modulatory activity of tiliroside, especially in combination with antibiotics against MDR *S. aureus*. At the same time, the results of antibacterial activity tend to be different for an unknown reason (Kuok et al., 2017). Another secondary metabolite that was correlated with the inhibition of biofilm formation in our work was ellagic acid. Our conclusion agrees with the literature, where this substance is active especially against Gram-negative bacteria (de Souza Tavares et al., 2021; Shakeri et al., 2018).

According to previous findings, our measurements suggest that extracts/essential oils made from *S. officinalis* appear to be very efficient in the disintegration of mature biofilms of *S. aureus* (Vetas et al., 2017). On the other hand, our findings are in contrast to the results of Lekbach et al. who published the activity of the ethanolic extract against the biofilm made of *P. aeruginosa* which we did not observe (Lekbach et al., 2019; Pejčić et al., 2020). The difference could be caused mainly by the concentration tested or the difference between the bacterial strains used.

The formation of a bacterial biofilm is closely related to the QS ability of bacteria. QS is a system that regulates the expression of bacterial genes according to the density of a bacterial population. Bacterial cells are using special signaling molecules called autoinducers that are capable of binding to receptors. Autoinducer-receptor complex initiates

gene expression. This process is responsible for some specific bacterial behavior, such as biofilm formation, and relies on virulence factors. Plants, which must be able to defend themselves against bacteria, often produce molecules that can effectively interfere with these QS mechanisms (Koh et al., 2013). In our study, we measured two types of autoinducers. Autoinducer-1 (acylated homoserine lactones) is typical for Gram-negative bacteria. These types of molecules can easily diffuse through the bacterial membrane. Autoinducer-2 (*e.g.*, tetrahydroxyfuran borate) is used by both Gram-positive and Gram-negative bacteria. *Enterobacteriaceae* use the ABC transporter (Lsr) to import these molecules into the bacterial cytoplasm (Rezzonico et al., 2012). The essential oil of *S. officinalis* was able to inhibit QS at a nontoxic concentration in the case of *Salmonella typhimurium* (AI-2) (Gart et al., 2016). In the case of *P. aeruginosa,* essential oil was able to inhibit pyocyanin production by up to 58.8% (Pejčić et al., 2020). The ethanolic extract of *M. silvestris* (0.064 g/L) was able to reduce δ-toxin concentration in the supernatant of staphylococcal cultures (AI-2) (Quave et al., 2011). Inhibition of the AI-1 system was published on *S. officinalis*, where inhibition was dose dependent in the case of *Chromobacterium violaceum* (Pellegrini et al., 2014). *S. nigra* previously showed inhibition of QS as a methanolic extract against *Chromobacterium violaceum* (AI-1) and *Agrobacterium tumefaciens* (AI-1) (Tosun et al., 2021). The extract of *F. vesca* was one of the active extracts that was able to inhibit QS in all measured cases and at the same time reduced the ability of bacteria to adhere and form biofilm. This may indicate its mechanism of action. The reduction of bacterial QS may be related to the inhibition of efflux systems, which the bacterium can actively use to transport signaling molecules.

The overexpression of bacterial efflux systems may be related to some of the measured activities. Inhibition of these systems can cause an increase in the concentration of antibiotics within the bacterial cell. Furthermore, it can complicate intercellular communication and thus reduce the production of virulence factors. Our measurements revealed that extracts of *S. nigra* and *M. chamomilla* at nontoxic concentrations can inhibit MRSA and colistin-induced MDR *P. aeruginosa* efflux systems. The inhibition of efflux systems explains the mechanism of inhibition of QS by AI-2. In this case, the extracts are active at concentrations of 0.006 and 0.013 g/L respectively. Furthermore, at concentrations lower than 1/2 MIC, extracts are capable of inhibiting the formation of bacterial biofilms. They even managed to eliminate the mature biofilm of *S. aureus.* This suggests that the possible use as an adjuvant would not induce selection pressure in the bacterium and thus the creation of a secondary resistance mechanism.

5. Conclusions

In this work, the antimicrobial activity of six commonly used medicinal plants was investigated in more detail. All tested extracts have a symbiotic effect with at least one antibiotic tested (colistin or gentamicin) against MDR bacterial strains. Additionally, *S. nigra* and *M. chamomilla* showed potential to inhibit bacterial efflux pumps in nontoxic concentrations, suggesting a mechanism of action. Furthermore, we observed the ability to reduce/inhibit QS in the presence of extracts of *M. chamomila*, *F. vesca*, and *A. eupatoria*, which further reduced the biofilm formation potential of the bacteria. Two of the most active extracts (*F. vesca* and *A. eupatoria*) against bacterial biofilm formation and disruption also have a higher tannins content. Our results indicate that the extract of *M. chamomila* is able to reduce the virulence of both gram-positive and gram-negative bacteria by inhibiting efflux pumps and is therefore a suitable candidate for further investigation. Another interesting plant for us is *F. vesca*, because it reduced QS in non-toxic concentrations, thereby also the potential for biofilm formation, which it also managed to disrupt. In the future, these plants should be thoroughly investigated from the point of view of explaining the mechanism of action, but also from the point of view of an analysis of the chemical composition.

List of abbbreviations

AI: Autoinducer; ANOVA: Analysis of variance; BHI: Brain heart infusion; CCCP: Carbonyl cyanide 3-chlorophenylhydrazone; DMSO: Dimethylsulfoxide; EC_{50}: Half maximal effective concentration; EUCAST: European Committee on Antimicrobial Susceptibility Testing; MDR: Multidrug resistance; MH: Mueller Hinton; MIC: Minimal inhibitory concentration; MRSA: methicillin-resistant *Staphylococcus aureus*; MS: Mass spectrometry; IC_{50}: Half-maximal inhibitory concentration; OD: Optical density; Q-TOF: quadrupole time-of-flight; QS: Quorum sensing; RA: Relative accumulation; SEM: Standard error of the mean; UHPLC-HRMS/MS: Ultra-high-performance liquid chromatography-high resolution mass spectrometry.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Authors' contributions

BK collected the data, did the experiments connected with antibacterial activity and efflux pump inhibition, analyzed all the data, and wrote the first draft of the manuscript. LH did the experiments connected with the biofilm. DB did the experiments connected with the bacterial sensitization. KK did the experiments connected with the quorum sensing measurement. NS helped with the methodological procedure of the experiments. AL analyzed and interpreted the data of tested extracts. OK got plant material and supervised the extraction process. GS participated in the design of the study, supervision and review of manuscript. JH supervised the whole process of extracts analysis, reviewed the draft of the manuscript. PK participated in the design of the study, supervised the whole process of the extraction, and reviewed the draft of the manuscript. JV designed the study, supervised the whole process, reviewed and modified the draft of the manuscript. JL supervised the whole process, reviewed and modified the draft of the manuscript. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Bára Křížkovská: Writing – original draft, Investigation, Formal analysis. **Lan Hoang:** Investigation. **Daniela Brdová:** Investigation. **Kristýna Klementová:** Investigation. **Nikoletta Szemerédi:** Investigation, Methodology. **Anna Loučková:** Investigation, Formal analysis. **Olga Kronusová:** Investigation, Resources. **Gabriella Spengler:**

Supervision, Resources, Writing – review & editing. **Petr Kaštánek:** Conceptualization, Supervision, Funding acquisition. **Jana Hajšlová:** Supervision, Conceptualization. **Jitka Viktorová:** Writing – original draft, Funding acquisition, Conceptualization, Validation. **Jan Lipov:** Supervision, Methodology, Project administration, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2023.116484.

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