



Antibacterial activity of herbal extracts towards uropathogenic *Enterococcus* isolates as a natural approach in control of urinary tract infections

Ivica Dimkić^{a,1}, Ivana Gobin^{b,1}, Gabrijela Begić^b, Davorka Repac Antić^{b,c}, Petar Ristivojević^d, Karlo Jurica^e, Tanja Berić^a, Jelena Lozo^a, Maja Abram^{b,c}, Slaviša Stanković^{a,*}

^a Faculty of Biology, University of Belgrade, Belgrade, Serbia

^b Department of Microbiology, Faculty of Medicine, University of Rijeka, Rijeka, Croatia

^c Department of Clinical Microbiology, Clinical Hospital Centre Rijeka, Rijeka, Croatia

^d Department of Analytical Chemistry, Faculty of Chemistry, University of Belgrade, Belgrade, Serbia

^e Special Security Operations Directorate, Ministry of Interior, Zagreb, Croatia

ARTICLE INFO

Keywords:

Antibacterial effect
Arbutus unedo
Biofilm inhibition
Enterococcus spp.
Herbal extracts
Synergistic effect

ABSTRACT

Antibacterial and antibiofilm efficacy through a synergistic effect of herbal leaf extracts of bearberry (BE), bearberry tea (BTE), parsley (PE), and the strawberry tree (STE) was evaluated to discover an alternative approach to control urinary tract infections caused by *Enterococci*. UHPLC–DAD MS/MS analysis indicated that herbal extracts were rich in ellagic and gallic acids, catechin, quercetin, and arbutin, which are strong antioxidants. Mostly, *Enterococcus faecalis* isolates showed resistance only to gentamicin and/or norfloxacin (26.3 %) and moderate biofilm production (31.5 %), while 12 isolates of *Enterococcus faecium* showed multiple antibiotic resistance. The minimum inhibitory concentration for the majority of isolates was 1.25 mg ml^{−1} for STE, but 2.5 mg ml^{−1} for BE and BTE. Although individual extracts gave higher MIC values, for most isolates, a synergistic effect at lower concentrations was achieved, when BE or BTE (0.625 mg ml^{−1}) was combined with STE (0.31 mg ml^{−1}). The BTE extract was shown to have the strongest individual effect on initial adhesiveness and on biofilm formation to all selected isolates, while the synergism of BTE and STE caused significant biofilm inhibition at all concentrations tested. The synergistic effect achieved of the tested extract combinations might be a starting point in the development of alternative products, effective against antibiotic resistant *Enterococcus* isolates.

1. Introduction

Uropathogenic bacteria have the capability of adhering, forming a biofilm, and/or resisting the immune system of the host, which can result in colonization and infection of the urinary tract. Urinary tract infections (UTIs) are among the most common bacterial infections and are important clinical problems. Various studies showed that most microorganisms found in nature are organized as biofilms and that 80 % of infections in humans are a consequence of their formation (Zijngje et al., 2010). Microorganisms that have the capability of biofilm formation are often up to 1000 times more resistant to different antimicrobial agents than planktonic cells (Verma and Patil, 2016). Traditionally regarded, species of the genus *Enterococcus* are low grade pathogens. However, nowadays they have been causing a number of various nosocomial infections (Sood et al. 2008). The genus *Enterococcus* includes several

species, but two of them (*Enterococcus faecalis* and *Enterococcus faecium*) are most commonly isolated from humans. These species are often resistant to one or more antibiotics, including cephalosporins, vancomycin, clindamycin, ampicillin, etc. (Hollenbeck and Rice, 2012; Miller et al., 2014). Although appropriate antibiotics can cure many patients, the infection is recurrent in 25 % of the infected (Khan et al., 2009). This has prompted a search for new sources of antimicrobials. According to the World Health Organization (WHO), medicinal herbs will be a valuable source of new medication in the future (Savoia, 2012). Medicinal and aromatic herbs are rich in secondary metabolites that protect them from herbivores and pathogenic microorganisms. Phenolic compounds are widespread in the natural flora and are characterized by numerous pharmacological and biological activities. The most common polyphenols are phenolic acids, flavonoids, and tannins. Phenolic acids such as gallic, protocatechuic, ellagic, ferulic, and caffeic acids, have an

* Corresponding author at: Full professor, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000, Belgrade, Serbia.

E-mail address: slavisas@bio.bg.ac.rs (S. Stanković).

¹ These authors contributed equally to this work.

important role in antimicrobial protection (Borges et al., 2013). One of the plant species used in traditional and folk medicine so far for the treatment of UTIs is *Arbutus unedo* L. (known as the strawberry tree) of the family Ericaceae (Oliveria et al., 2011), which is widespread in the Mediterranean region, as well as the region of Asia Minor and Western Europe (Mariotto et al., 2008). The leaves of *A. unedo* showed a wide range of biological effects (Oliveria et al., 2011), and they are rich in tannins, flavonoids, phenols, and glycosides (Jurica et al., 2017). The content of polyphenols in *A. unedo* leaves often depend on the climate and the geolocation of their origin. In the family Ericaceae, catechin, myricetin, and kaempferol are most often isolated as flavonoids (Pallauf et al., 2008). Bearberry or uva (*Arctostaphylos uva-ursi* L.), another traditionally used plant, grows in Central and Western Europe. Bearberry is also an official medicine in many pharmacopoeias. Parsley (*Petroselinum crispum* (Mill.) Fuss), from the family Apiaceae also plays an important role in the treatment of UTIs as an alternative medicine (Simin and Quinin, 1988). Herbal medicines against UTIs can often be found on the market, the majority of them being relatively heterogeneous mixtures of various herbs. One of the most widespread preparations on the market is a plant mixture called bearberry tea, for which the combination of herbal types is chosen by the manufacturer and has previously been described in detail by the German Regulatory Commission E (Schulz et al., 2001).

Antibiotic resistance of clinical as well as all other bacterial isolates in combination with biofilm formation ability, motility, adhesion and the production of other virulence factors are raising the demand for the search for new antimicrobials. In that sense, use of various combinations of extracts and/or different antimicrobials is a new approach to increase the efficacy of individual extracts by taking advantage of their synergistic and additive effects. A collection of bacterial isolates from the urine of patients under treatment at the Clinical-Hospital Centre in Rijeka was identified in this paper. Whether the isolates formed a biofilm was checked, and the detected biofilms were categorized. In addition, the antibacterial effects of individual extracts of bearberry (BE), bearberry tea (BTE), parsley (PE), and the strawberry tree (STE) on all isolates were determined, as well as their potential synergistic influence in certain combinations on the inhibition of biofilm formation on selected isolates.

2. Methods

2.1. Plant material and extract preparation

Herbal extracts analyzed in this study were obtained from strawberry tree, bearberry, and parsley leaves and from the mixture of plant species called bearberry tea (Table 1). The leaves of strawberry tree (STE) were collected from a number of trees on the island of Koločep in Croatia. Herbal material was identified by Dr. Dario Kremer and deposited in the Fran Kušan Pharmaceutical Botanical Garden at the Faculty of Pharmacy

Table 1
Plant samples and species used for extraction of plant secondary compounds.

Sample	Plant species/parts	Origin
Strawberry tree leaf	<i>Arbutus unedo</i> L. folium	Koločep, Croatia, 42.6759 °N, 18.0095 °E
Bearberry leaf	<i>Arctostaphylos uva-ursi</i> L. folium	Institute for Medicinal Plant Research "Dr Josif Pančić" from Belgrade, Serbia.
Bearberry tea	<i>Uvae ursi</i> folium 30%	Institute for Medicinal Plant Research "Dr Josif Pančić" from Belgrade, Serbia.
	<i>Herniariae</i> herba 20%	
	<i>Equiseti</i> herba 10%	
	<i>Petroselini</i> folium 5%	
	<i>Betulae</i> folium 15%	
Parsley leaf	<i>Ononidis</i> radix 20%	Institute for Medicinal Plant Research "Dr Josif Pančić" from Belgrade, Serbia.
	<i>Petroselinum crispum</i> (Mill.) Fuss folium	

and Biochemistry, University of Zagreb, Croatia. The dried leaves were ground in a laboratory mill and stored in plastic containers at room temperature until analysis. Other herbal materials were commercially purchased from the Dr. Josif Pančić Institute for Medicinal Plant Research in Belgrade. Approximately 10 g of each sample was cut and extracted with 50 mL of a mixture of ethanol and water (4:1, v/v) in an ultrasound bath (MRC Lab Equipment, London, UK) for 45 min (Dimkić et al., 2016). Subsequently, the extracts were filtered, evaporated to dryness (Büchi Rotavapor R-215, Flawil, Switzerland), and diluted with methanol up to a final concentration of 100 mg mL⁻¹ for each sample. The methanolic solutions were refrigerated prior to analysis.

2.2. UHPLC–DAD MS/MS analysis of polyphenolic compounds

Separation and quantification of phenolics in the four herbal extracts were performed according to Gašić et al. (2015). A Dionex Ultimate 3000 UHPLC system equipped with a diode array detector (DAD) connected to a TSQ Quantum Access Max triple-quadrupole mass spectrometer (Thermo Fisher Scientific, Basel, Switzerland) was used here. Elution was performed at 40 °C on a Syncronis C18 column. The mobile phase consisted of water + 0.1 % formic acid (A) and acetonitrile (B), which were applied in the following gradient of elution: 5 % B for the first 2.0 min, 5–95 % B from 2.0 to 12.0 min, 95–5 % B from 12.0 to 12.2 min, and 5 % B until the 15th min. The flow rate was set to 0.3 mL min⁻¹ and the detection wavelengths to 254 and 280 nm. The injection volume was 5 µL. A TSQ Quantum Access Max triple-quadrupole mass spectrometer equipped with heated electrospray ionization (HESI) source was used (Gašić et al., 2015). Mass spectrometry data were acquired in the negative ion mode, in the *m/z* range of from 100 to 1000. Time-selected reaction monitoring (tSRM) experiments for quantitative analysis were performed using two MS² fragments for each previously defined dominant compound. Xcalibur 2.2 software (Thermo Fisher, Bremen, Germany) was used for instrument control. Phenolics were identified by direct comparison with commercial standards. The total amounts of each compound were evaluated by calculation of the peak areas and expressed as mg l⁻¹.

2.3. Bacterial isolates and antibiotic sensitivity tests

Ninety-six isolates of the genus *Enterococcus* originating from the urine of patients collected during 2016 at the Clinical Hospital Centre in Rijeka, Croatia were used. Bacteriological analyses were performed by inoculating urine samples on chromogenic UTI agar (HiCrome UTI Modified, HiMEDIA, India). The bacteria were stored at –80 °C in 10 % glycerol broth (Biolife, Italy). For experiments, bacteria were cultivated in Mueller-Hinton agar and broth (MHB) (Difco, MD, USA). All bacterial strains were cultured overnight at 37 °C in the MHB medium. Suspensions were adjusted to 0.5 McFarland standard turbidity (BioMérieux, Marcy-l'Étoile, France), which corresponds to 10⁸ CFU mL⁻¹.

The Kirby–Bauer disk diffusion method (Hudzicki, 2009) according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) protocol was used to evaluate the antimicrobial susceptibility profiles of isolates using a standardized single disk (BBL, Sensu Disc, Becton, Dickinson and Co. (BD), USA) at the following concentrations (µg disk⁻¹): ampicillin (2), gentamicin (30), norfloxacin (30), vancomycin (5), teicoplanin (30), imipenem (10), and nitrofurantoin (100). The antibiotic sensitivity was read after incubation of 24 h at 37 °C and expressed in mm, and the isolates were classified as susceptible (S) or resistant (R).

2.3.1. Ethical approval

The ethical committee of the Clinical Hospital Centre Rijeka (decision No. 2170-29-02/15–17-2) obtained the clinical strains after approval. Clinical uroisolates of *Enterococcus* spp. were collected in Department of Clinical Microbiology in Rijeka and belong to the collection of the Department of Microbiology, Faculty of Medicine

University of Rijeka, Croatia. The clinical isolates were randomly selected from urine samples through routine clinical work during 2016. Patient identifiable information was anonymized, so no informed consent was necessary for this study. Patient demographics anonymization was performed in two steps; first, personal data was coded by the head of the clinical microbiology laboratory at the Clinical Hospital Centre Rijeka where the isolates were obtained from, and secondly by assigning a different code by the principal investigator at the Department of Microbiology, Faculty of Medicine University of Rijeka. The authors had no contact or interaction with the patients. None of the clinical data obtained previously were associated with the isolates of this study.

2.4. DNA extraction and PCR amplification

Genomic DNA from *Enterococcus* spp. was isolated according to the method described by Dimkić et al. (2013). A 16S rRNA gene sequence from the isolates was determined from PCR-amplified fragments using universal primers UN1_{16S}F (GAGAGTTTGATCCTGGC) and UN1_{16S}R (AGGAGGTGATCCAGCG). The PCR amplification was performed with KAPA Taq polymerase (KAPA Biosystems, USA) at annealing temperature of 50 °C. The PCR products were purified using a QIAquick PCR Purification KIT/250 (QIAGEN GmbH, Hilden, Germany) and sent for sequencing to the MacroGen Sequencing Service (The Netherlands). The obtained sequences were searched for homology with sequenced genes in the GenBank database using the National Center for Biotechnology Information's blast search program for nucleotides.

The sequences were submitted to the NCBI database under the GenBank submission number SUB6279502 and GenBank accession numbers were received for *Enterococcus faecalis* strains (MN422521 – MN422596). Plus, *Enterococcus faecium* (MN422597 – MN422616) strains.

2.5. Minimum inhibitory concentration (MIC) assay

A broth microdilution method described previously (Dimkić et al., 2016) was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the herbal extract samples against all *Enterococcus* isolates. Two-fold serial dilutions of the samples with MHB medium in 96-well microtiter plates were performed in the concentration range of from 0.009 to 5 mg ml⁻¹. The final concentration of methanol as a solvent was 10 %. Besides a negative control, a sterility control, and a control for the solvent (methanol), the antibiotic vancomycin (Sigma-Aldrich, USA) in the concentration range of from 0.0003 to 0.256 mg ml⁻¹ was also tested as a positive control. All dilutions were carried out in triplicate. Each well, except for the sterility control, was inoculated with 20 µl of bacterial culture (1 × 10⁸ CFU ml⁻¹), reaching a final volume of 200 µl. Lastly, 22 µl of resazurin was added to each well. The plates were incubated for 24 h at 37 °C. All tests were performed in a lit environment, but the plates were incubated in the dark. Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth. It is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin and finally to colorless dihydroresorufin by oxidoreductases within viable cells (Karuppusamy and Rajasekaran, 2009). The lowest concentration, which showed no change in color, was defined as the MIC. The MBC was determined by sub-culturing the test dilutions from each well without color change on MHA plates and incubating for 24 h. The lowest concentration that showed no bacterial growth was defined as the MBC value. The results are expressed in mg ml⁻¹.

2.6. Determination of effects of herbal extracts in different combinations

Determination of the type of interaction between STE extract and individual extracts (BE, PE, and BTE) was carried out by using the checkerboard method (Schwalbe et al., 2007). Two-fold serial dilutions of each extract were made individually, on two separate plates, within

the concentration range of from 0.009 to 5 mg ml⁻¹. The well contents were mixed on a single plate and all 60 possible combinations of concentrations were tested. All steps after that were carried out as described in MIC assay. After 24 h of incubation at 37 °C, the lowest concentration which showed no change in color was defined as the MIC value for certain combinations of extracts. The synergism, additivism, indifference, or antagonism of all the combinations of herbal extracts was determined. The MICs of individual extracts in different combinations were transformed into fractional inhibitory concentrations (FIC) in the following manner:

$FIC_{(A)} = (\text{MIC of compound A in the presence of compound B}) / (\text{MIC of compound A})$;

and

$FIC_{(B)} = (\text{MIC of compound B in the presence of compound A}) / (\text{MIC of compound B})$.

The fractional inhibitory concentration index (FICI) was calculated from FIC values for each herbal extract as follows: $FICI = FIC_{(A)} + FIC_{(B)}$, where A represents the first extract and B the second one in combinations. The FICI was interpreted as follows: synergistic effect when $FICI \leq 0.5$; additive effect when FICI was 0.5–1; indifferent effect when FICI was 1–2; and antagonistic effect when $FICI \geq 2$ (EUCAST, 2000).

2.7. Determination of biofilm formation

Quantification of biofilm formation was performed by a modified version of the method of Stepanović et al. (2003). Each strain was tested in triplicate, and all tests were carried out in three repetitions. The wells of a 96-well flat-bottomed microplate were filled with a final volume of 200 µL [180 µL of MHB and 20 µL of bacteria (1 × 10⁸ CFU ml⁻¹)]. The negative control wells contained MHB only. After incubation, the plates were decanted and the wells washed three times with 300 µL of phosphate buffer (1 × PBS, pH 7.2, 25 °C). The remaining attached bacteria were fixed with 200 µL of methanol per well, and after 20 min the plates were emptied and left to air dry. The final step was staining for 15 min with 200 µL per well of 2% Crystal Violet (Lach-Ner, Czech Republic). Excess stain was rinsed off with running tap water. After air drying, the remaining stain was solubilized with 200 µl of 96 % ethanol per well. The optical density of initial adhesiveness and biofilm formation were measured after 24 and 48 h at 570 nm using an automated Multiskan FC reader (Thermo Fisher Scientific, China). Based on the optical densities (OD) for each strain, the cut-off OD value (OD_c) was defined (three standard deviations above the average mean OD of the negative control) according to Stepanović et al. (2003). Each strain was classified into one of the following categories: non-adherent or not a biofilm producer ($OD \leq OD_c$, category 0 or -); weakly adherent or a weak biofilm producer ($OD \leq OD_c \leq 2 \times OD_c$, category 1 or +); moderately adherent or a moderate biofilm producer ($2 \times OD_c \leq OD \leq 4 \times OD_c$, category 2 or ++); and strongly adherent or a strong biofilm producer ($4 \times OD_c \leq OD$, category 3 or +++).

2.8. Influence of individual extracts and their combinations on biofilm formation

The effects of three individual extracts and their combinations on the formation of biofilm were tested against four selected isolates (E43, E61, E89, and E94). Two-fold serial dilutions of the samples with MHB medium in 96-well microtiter plates were performed as described in the MIC assay. Briefly, according to previously determined MIC values of individual extracts and their combinations against each selected isolate, supra- and sub-MICs (4 - ¼ MIC) were used. The final concentration in the first wells of individual extracts STE, BE, and BTE was 5, 10, and 10 mg ml⁻¹, respectively. The PE extract was excluded from further testing due to the lowest antibacterial activity scored previously. The effects of

two different combinations of individual extracts (STE + BE and STE + BTE) on biofilm formation were also examined. Based on the FIC_i values and interactions achieved, an average MIC concentration of the appropriate extract combinations was determined for each selected isolate. For the STE + BTE combination, the average MIC values were 0.019 and 0.30 mg ml⁻¹ (isolates E43, E61, and E89), as well as 0.15 and 0.30 mg ml⁻¹ (isolate E94). For the STE + BE combination, the average MIC values were 0.074 and 0.30 mg ml⁻¹ (E43 and E89); 0.038 and 0.30 mg ml⁻¹ (E61); and 0.074 and 0.30 mg ml⁻¹ (E94). As a positive control, the antibiotic vancomycin was used at a final concentration in the first wells of 0.004 mg ml⁻¹ (E43, E61, and E89) and 0.008 mg ml⁻¹ (E94). The individual extracts and their combinations, only diluted with medium (without bacteria) in the same concentration range, were used as blanks. Each strain was tested in triplicate, and all tests were carried out in three repetitions. Initial adhesiveness, biofilm formation, and biofilm categories were determined according to the scheme described above. As the optical density is proportional to thickness of the produced biofilm, the values of initial adhesiveness and biofilm inhibition were also calculated and expressed as a percentage of inhibition according to the following formula (Balaji et al., 2013):

$$\text{Percentage of inhibition} = ([\text{Control OD570 nm} - \text{Test OD570 nm}]/\text{Control OD570 nm}) \times 100.$$

2.9. Statistical analysis

The analysis of variance was supported by the Kolmogorov–Smirnov test for the normality of residuals and Levene's test for homogeneity of variance. The obtained data were subjected to analysis of variance (ANOVA), and separation of the mean percentages of initial adhesiveness and biofilm inhibition for treatment with individual extracts and their combinations was accomplished by Tukey's HSD (honest significant difference) test. The results were deemed significant at $P < 0.05$. Statistical analyses were conducted by the general procedures of STATISTICA v. 7 (StatSoft, Inc.) and IBM SPSS Statistics v. 20 (SPSS, Inc.).

3. Results

3.1. UHPLC–DAD MS/MS analysis of herbal extracts

Mass spectrometry was applied in order to determine the concentration of polyphenolic compounds in the tested herbal extracts. The presence of 37 phenolic compounds was confirmed, and ellagic acid was present in the highest concentrations in the STE, BE, and BTE extracts (Table 2). Of 27 phenolic compounds, catechin, arbutin, and gallic acid were the most abundant in the STE extract. Apart from the largest number of identified phenolic compounds (32) and ellagic and gallic acids present in the highest concentrations, BE is also characterized by considerably higher concentrations of catechin and arbutin in comparison with other extracts. The highest identified concentration of ferulic acid was found in BTE in addition to ellagic and gallic acids, catechin, and arbutin. The PE extract showed the presence of higher concentrations of apigenin 7-*O*-apioglucoside, rutin, and chlorogenic acid in comparison with the other extracts, as well as the absence of arbutin and gallic acid.

3.2. Molecular identification of *Enterococcus* isolates

Sequencing and BLASTn analysis of the obtained amplicons showed existence of two species of the genus *Enterococcus*. The reconstruction based on manually analyzed nucleotide sequences clustered as many as 76 isolates (79.2 %) as *E. faecalis*, while the rest of the isolates (20.8 %) were related to the species of *E. faecium* (Table 3). Accordingly, BLASTn analysis based on partial sequence of 16S rRNA showed that the all

Table 2

UHPLC–DAD MS/MS analysis of phenolic composition in herbal extracts (mg ml⁻¹).

Quantified compounds	STE	BE	BTE	PE
Gallic acid	55.13	255.24	151.23	NF
Protocatechuic acid	7.57	9.29	6.80	4.13
Aesculin	2.42	1.11	NF	2.91
Chlorogenic acid	0.09	2.40	18.22	30.40
Catechin	226.31	461.27	250.55	0.98
Gentisic acid	0.64	0.75	1.57	0.11
<i>p</i> -Hydroxyphenylacetic acid	NF	10.98	NF	NF
Caffeic acid	0.11	0.51	2.93	1.41
Galocatechin gallate	1.62	1.44	NF	NF
Epigallocatechin gallate	NF	1.90	NF	NF
Rutin	7.19	4.85	12.99	29.65
<i>p</i> -Coumaric acid	NF	1.55	4.35	2.58
Quercetin 3- <i>O</i> -galactoside (Hyperoside)	51.13	36.53	47.83	4.22
Ellagic acid	586.33	769.07	411.96	6.19
Apigenin 7- <i>O</i> -apioglucoside (Apiin)	0.25	0.06	22.31	57.66
Ferulic acid	NF	6.21	93.51	13.19
Naringin	0.57	NF	1.19	NF
Sinapic acid	3.96	NF	NF	NF
Kaempferol 3- <i>O</i> -glucoside (Astragalin)	2.16	0.51	0.77	0.13
Luteolin 7- <i>O</i> -glucoside (Cynaroside)	10.36	4.69	4.21	7.42
Apigenin 7- <i>O</i> -glucoside (Apigetrin)	0.13	0.09	0.29	0.70
Phlorizin	NF	4.51	1.75	0.24
Myricetin	3.52	1.69	2.74	0.23
Coniferyl aldehyde	0.27	0.35	0.64	0.21
Quercetin	NF	1.90	0.50	NF
Daidzein	NF	NF	0.36	NF
Hesperetin	3.77	8.38	5.52	0.07
Luteolin	0.05	3.08	5.11	1.34
Phloretin	0.03	NF	NF	NF
Naringenin	0.22	0.43	0.90	0.13
Apigenin	0.16	0.48	7.15	2.46
Genistein	NF	0.24	7.01	2.73
Kaempferol	3.72	2.00	1.56	0.14
Chrysin	NF	0.03	0.23	0.09
Pinocembrin	0.02	0.06	0.06	0.03
Galangin	0.32	NF	NF	0.04
Arbutin	190.7	465.40	258.90	NF

*NF - not found; STE - strawberry tree extract; BE - bearberry extract; BTE - bearberry tea extract; PE - parsley extract.

strains of *E. faecalis* were the most related to *Enterococcus faecalis* ATCC 19,433 NR_115765 (Query cover: 100 %; E value: 0.0; Ident.: 99.21–100 %), while others shown similarity with *Enterococcus faecium* ATCC 19,434 NR_115764 (Query cover: 100 %; E value: 0.0; Ident.: 98.6–99.9 %).

3.3. Antibiotic resistance profile

The antibiotics susceptibility was examined using the Kirby-Bauer disk diffusion method. All isolates of the species *E. faecalis* showed sensitivity to vancomycin, teicoplanin, and nitrofurantoin, while only one isolate (E95) was resistant to the effect of ampicillin and imipenem, while E93 was resistant toward ampicillin (Table 3). The highest percentage of resistant isolates of species *E. faecalis* (26.3 %) was obtained in the tests towards gentamicin and norfloxacin, with gentamicin resistance being the more pronounced. A large number of *E. faecium* isolates 12 out of 20 isolates (E20–E31), for the most part demonstrated multiple resistances to all of the tested antibiotics and to vancomycin (and thus were VRE - vancomycin-resistant enterococci). It is necessary to note that eight isolates of the same species (E40, E62, E64, E66, E88, E92, E94, and E96) showed a completely opposite response and were sensitive to vancomycin, teicoplanin, and nitrofurantoin.

3.4. Antimicrobial activity of herbal extracts

The microdilution method was used to examine antimicrobial activity of the herbal extracts STE, BE, BTE, and PE through determination

Table 3Antibiotic susceptibility testing of *Enterococcus* isolates and determination of MIC and MBC values of the herbal extracts.

No.	Isolate/species according to 16S rRNA	Protocol No.	Antibiogram test (µg disc ⁻¹)							Vancomycin (µg ml ⁻¹)		STE (mg ml ⁻¹)		BE (mg ml ⁻¹)		BTE (mg ml ⁻¹)	
			Amp	Gen	Nor	Imi	Van	Tec	Nif	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E1	<i>Enterococcus faecalis</i>	52491	S	R	R	S	S	S	S	0.5	0.5	1.25	2.5	2.5	5	2.5	5
E2		52056	S	S	S	S	S	S	S	0.5	1.6	1.25	2.5	2.5	5	2.5	5
E3		52014	S	S	S	S	S	S	S	0.25	0.5	1.25	2.5	2.5	5	5	-
E4		52836	S	S	S	S	S	S	S	0.25	0.5	1.25	2.5	2.5	5	5	-
E5		52567	S	S	S	S	S	S	S	0.25	0.5	1.25	2.5	2.5	5	2.5	5
E6		52748	S	R	S	S	S	S	S	0.25	4	1.25	2.5	2.5	5	5	-
E7		52203	S	R	R	S	S	S	S	0.75	8	1.25	2.5	2.5	5	5	-
E8		52096	S	S	S	S	S	S	S	0.25	4	1.25	2.5	2.5	5	5	-
E9		51462	S	S	S	S	S	S	S	1	16	1.25	2.5	2.5	5	2.5	5
E10		53012	S	R	R	S	S	S	S	0.5	8	1.25	2.5	2.5	5	5	-
E11		53236	S	R	R	S	S	S	S	0.5	8	1.25	2.5	2.5	5	5	-
E12		53108	S	S	S	S	S	S	S	6.3	8	1.25	2.5	2.5	5	2.5	5
E13		53292	S	S	S	S	S	S	S	0.5	8	1.25	2.5	2.5	5	5	-
E14		53041	S	S	S	S	S	S	S	0.75	8	1.25	2.5	2.5	5	5	-
E15		53373	S	S	S	S	S	S	S	1	16	1.25	2.5	2.5	5	2.5	5
E16		53379	S	S	S	S	S	S	S	0.5	16	1.25	2.5	2.5	5	2.5	5
E17		53463	S	S	S	S	S	S	S	0.5	8	1.25	2.5	2.5	5	2.5	5
E18		53491	S	S	S	S	S	S	S	1	32	1.25	2.5	2.5	5	2.5	5
E19		53484	S	S	S	S	S	S	S	0.5	32	1.25	2.5	2.5	5	2.5	5
E32		39997	S	S	S	S	S	S	S	0.5	8	1.25	2.5	2.5	5	5	-
E33		40080	S	S	S	S	S	S	S	0.5	32	1.25	2.5	2.5	5	5	-
E34		54674	S	S	S	S	S	S	S	1.5	128	1.25	2.5	2.5	5	5	-
E35		54604	S	S	S	S	S	S	S	1	8	1.25	2.5	2.5	5	2.5	5
E36		54540	S	S	S	S	S	S	S	0.5	16	1.25	2.5	2.5	5	2.5	5
E37		54537	S	R	R	S	S	S	S	0.5	16	1.25	2.5	2.5	5	2.5	5
E38		54685	S	S	S	S	S	S	S	1	16	1.25	2.5	2.5	5	2.5	5
E39		54672	S	S	S	S	S	S	S	0.5	16	1.25	2.5	2.5	5	2.5	5
E41		54998	S	S	S	S	S	S	S	0.75	16	1.25	2.5	2.5	5	2.5	5
E42		55009	S	S	S	S	S	S	S	2	4	1.25	2.5	2.5	5	2.5	5
E43		54946	S	S	S	S	S	S	S	1	4	1.25	2.5	2.5	5	2.5	5
E44		55985	S	S	S	S	S	S	S	0.5	4	1.25	2.5	2.5	5	2.5	5
E45		54510	S	S	S	S	S	S	S	1	4	1.25	2.5	2.5	5	2.5	5
E46		56115	S	S	R	S	S	S	S	0.5	4	1.25	2.5	2.5	5	2.5	5
E47		55947	S	S	S	S	S	S	S	2	32	1.25	2.5	2.5	5	2.5	5
E48		55933	S	S	S	S	S	S	S	0.5	8	1.25	2.5	2.5	5	2.5	5
E49		56711	S	S	S	S	S	S	S	1	64	1.25	2.5	2.5	5	2.5	5
E50		56707	S	S	S	S	S	S	S	0.5	16	1.25	2.5	2.5	5	2.5	5
E51		56411	S	S	S	S	S	S	S	1	64	1.25	2.5	2.5	5	2.5	5
E52		56818	S	R	R	S	S	S	S	5	32	1.25	2.5	2.5	5	2.5	5
E53		56590	S	S	S	S	S	S	S	1	32	1.25	2.5	2.5	5	2.5	5
E54		56813	S	S	S	S	S	S	S	1	32	1.25	2.5	2.5	5	2.5	5
E55		56803	S	S	R	S	S	S	S	5	32	1.25	2.5	2.5	5	5	-
E56		56540	S	S	S	S	S	S	S	1	4	1.25	2.5	2.5	5	2.5	5
E57		56306	S	R	S	S	S	S	S	1	4	1.25	2.5	2.5	5	2.5	5
E58		57439	S	R	R	S	S	S	S	1	8	1.25	2.5	2.5	5	5	-
E59		57427	S	S	S	S	S	S	S	0.75	8	1.25	2.5	2.5	5	2.5	5
E60		57433	S	S	S	S	S	S	S	2	8	1.25	2.5	2.5	5	2.5	5
E61		57470	S	S	S	S	S	S	S	1	2	1.25	2.5	2.5	5	2.5	5

(continued on next page)

Table 3 (continued)

E63	55917	S	S	S	S	S	S	S	0.25	2	1.25	2.5	2.5	5	5	-	
E65	57670	S	S	S	S	S	S	S	1	2	1.25	2.5	2.5	5	2.5	5	
E67	57513	S	S	S	S	S	S	S	0.75	4	1.25	2.5	2.5	5	2.5	5	
E68	58016	S	S	S	S	S	S	S	0.5	40	2.5	5	2.5	5	5	-	
E69	57758	S	R	R	S	S	S	S	0.75	64	1.25	2.5	2.5	5	2.5	5	
E70	57913	S	S	S	S	S	S	S	2	4	1.25	2.5	2.5	5	5	-	
E71	58319	S	R	R	S	S	S	S	1	-	1.25	2.5	2.5	5	2.5	5	
E72	58570	S	R	R	S	S	S	S	1	32	1.25	2.5	2.5	5	2.5	5	
E73	58151	S	S	S	S	S	S	S	2	64	1.25	2.5	2.5	5	2.5	5	
E74	58497	S	S	S	S	S	S	S	1	48	1.25	2.5	2.5	5	2.5	5	
E75	58705	S	R	R	S	S	S	S	1	32	1.25	2.5	2.5	5	2.5	5	
E76	58268	S	S	S	S	S	S	S	1	32	1.25	2.5	2.5	5	2.5	5	
E77	58214	S	S	S	S	S	S	S	2	32	1.25	2.5	2.5	5	2.5	5	
E78	58848	S	S	S	S	S	S	S	2	32	2.5	5	2.5	5	2.5	5	
E79	58022	S	S	S	S	S	S	S	2	32	1.25	2.5	2.5	5	2.5	5	
E80	58326	S	S	S	S	S	S	S	2	32	1.25	2.5	2.5	5	2.5	5	
E81	59764	S	S	S	S	S	S	S	1.5	32	1.25	2.5	2.5	5	2.5	5	
E82	59801	S	S	S	S	S	S	S	1	32	1.25	2.5	2.5	5	2.5	5	
E83	59739	S	S	S	S	S	S	S	1	32	1.25	2.5	2.5	5	2.5	5	
E84	59144	S	S	S	S	S	S	S	1	32	1.25	2.5	2.5	5	2.5	5	
E85	59271	S	R	R	S	S	S	S	1	16	1.25	2.5	2.5	5	2.5	5	
E86	59530	S	R	S	S	S	S	S	1	32	1.25	2.5	2.5	5	5	-	
E87	59170	S	S	S	S	S	S	S	0.5	32	1.25	2.5	2.5	5	2.5	5	
E89	60306	S	S	S	S	S	S	S	1	16	1.25	2.5	2.5	5	2.5	5	
E90	60637	S	S	S	S	S	S	S	1.5	16	1.25	2.5	2.5	5	2.5	5	
E91	61036	S	R	S	S	S	S	S	1	32	1.25	2.5	2.5	5	2.5	5	
E93	62798	R	S	R	R	S	S	S	1	24	2.5	5	5	-	5	-	
E95	64562	R	R	R	S	S	S	S	3	32	1.25	2.5	2.5	5	5	-	
E20	3687	R	R	R	R	R	R	R	256	-	1.25	2.5	2.5	5	2.5	5	
E21	4406	R	S	R	R	R	R	S	256	-	2.5	5	2.5	5	2.5	5	
E22	5271	R	S	R	R	R	R	S	256	-	1.25	2.5	2.5	5	2.5	5	
E23	46952	R	S	R	R	R	R	S	256	-	2.5	5	1.25	2.5	5	-	
E24	46928	R	R	R	R	R	R	R	256	-	1.25	2.5	2.5	5	2.5	5	
E25	9804	R	S	R	R	R	R	S	256	-	1.25	2.5	2.5	5	2.5	5	
E26	10745	R	R	R	R	R	R	R	256	-	1.25	2.5	2.5	5	2.5	5	
E27	15338	R	R	R	R	R	R	R	256	-	1.25	2.5	2.5	5	2.5	5	
E28	18978	R	S	R	R	R	R	S	256	-	1.25	2.5	2.5	5	5	-	
E29	29512	R	R	R	R	R	R	R	256	-	1.25	2.5	2.5	5	5	-	
E30	38343	R	R	R	R	R	R	S	256	-	1.25	2.5	2.5	5	5	-	
E31	38412	R	S	R	R	R	R	S	256	-	2.5	5	2.5	5	5	-	
E40	54631	R	R	R	R	S	S	S	0.5	4	1.25	2.5	2.5	5	2.5	5	
E62	57143	R	R	R	R	S	S	S	0.25	1	2.5	5	2.5	5	5	-	
E64	57578	R	S	R	R	S	S	S	0.5	0.5	1.25	2.5	2.5	5	2.5	5	
E66	57612	R	R	R	R	S	S	S	1	64	1.25	2.5	2.5	5	2.5	5	
E88	60336	R	R	R	R	S	S	S	1	24	1.25	2.5	2.5	5	2.5	5	
E92	62014	R	S	R	R	S	S	S	1	16	2.5	5	2.5	5	5	-	
E94	62269	S	R	R	S	S	S	S	2	16	1.25	2.5	2.5	5	2.5	5	
E96	67423	R	R	R	R	S	S	S	1.5	24	1.25	2.5	2.5	5	2.5	5	
No.	Isolate/species according to 16S rRNA	Protocol No.	Amp	Gen	Nor	Imi	Van	Tec	Nif	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Antibiogram test (µg disc ⁻¹)									Vancomycin (µg ml ⁻¹)		STE (mg ml ⁻¹)		BE (mg ml ⁻¹)		BTE (mg ml ⁻¹)		

Amp – Ampicillin (2 $\mu\text{g disc}^{-1}$); Gen – Gentamicin (30 $\mu\text{g disc}^{-1}$); Nor – Norfloxacin (30 $\mu\text{g disc}^{-1}$); Imi – Imipenem (10 $\mu\text{g disc}^{-1}$); Van – Vancomycin (5 $\mu\text{g disc}^{-1}$); Tec – Teicoplanin (30 $\mu\text{g disc}^{-1}$); Nif – Nitrofurantoin (100 $\mu\text{g disc}^{-1}$). STE - strawberry tree extract; BE - bearberry extract; BTE - bearberry tea extract. S - Sensitive; R - Resistant. According to EUCAST Clinical Breakpoint v.7.0 vancomycin susceptibility in MIC assay was expressed as $S \leq 4$ and $R > 4 \mu\text{g ml}^{-1}$.

of their minimum inhibitory (MIC) and bactericidal (MBC) concentrations. Of all the tested extracts, STE had the lowest MIC value (1.25 mg ml^{-1}) for almost all of the isolates (Table 3). Several isolates showed a higher MIC value (2.5 mg ml^{-1}), five of them belonging to the species *E. faecium* (E21, E23, E31, E62, and E92). Such a discordance was also noticed in three isolates of the species *E. faecalis* (E68, E78, and E93), whose MIC values were likewise higher in comparison with other isolates. In the case of BE, MIC values were 2.5 mg ml^{-1} , except for E23 and

E93. The lowest MIC value for BTE also was 2.5 mg ml^{-1} . Additionally, 27 isolates were inhibited with 5 mg ml^{-1} of BTE, as well. The PE extract showed the lowest antibacterial activity towards all of the tested isolates and the MIC value was not determined because it was higher than the highest tested concentration ($>5 \text{ mg ml}^{-1}$). According to achieved MIC values of individual extracts towards each isolate simultaneously, six different susceptibility groups of isolates were obtained. Vancomycin was used as a positive control and the results, measured according to the

Table 4The synergistic effect of selected plant extract combinations against *Enterococcus* isolates.

Combination of strawberry tree + bearberry tea extracts (STE + BTE)							
E23				E68			
MIC_{STE} 2.5 MIC_{BTE} 5.0	1.25 BTE + 0.625 STE	0.625 BTE + 0.625 STE	1.25 BTE + 0.31 STE	1.25 BTE + 0.16 STE	1.25 BTE + 0.08 STE	1.25 BTE + 0.625 STE	1.25 BTE + 0.31 STE
FIC _{BTE}	0.25	0.125	0.25	0.25	0.25	0.25	0.25
FIC _{STE}	0.25	0.25	0.124	0.064	0.032	0.25	0.124
FICI	0.5	0.375	0.374	0.314	0.282	0.5	0.374
Interaction	S	S	S	S	S	S	S
	E3, E58, E63 and E95	E63	E3				
MIC_{STE} 1.25 MIC_{BTE} 5.0	1.25 BTE + 0.31 STE	0.625 BTE + 0.31 STE	1.25 BTE + 0.16 STE				
FIC _{BTE}	0.25	0.125	0.25				
FIC _{STE}	0.248	0.248	0.128				
FICI	0.498	0.373	0.378				
Interaction	S	S	S				
	E50, E65, E73, E75, E79 and E90	E90					
MIC_{STE} 1.25 MIC_{BTE} 2.5	0.625 BTE + 0.31 STE	0.625 BTE + 0.16 STE	0.625 BTE + 0.08 STE				
FIC _{BTE}	0.25	0.25	0.25				
FIC _{STE}	0.248	0.128	0.064				
FICI	0.498	0.378	0.314				
Interaction	S	S	S				
Combination of strawberry tree + bearberry extracts (STE + BE)							
E93							
MIC_{STE} 2.5 MIC_{BE} 5.0	1.25 BE + 0.625 STE		0.625 BE + 0.625 STE	0.31 BE + 0.625 STE			
FIC _{BE}	0.25		0.125	0.062			
FIC _{STE}	0.25		0.25	0.25			
FICI	0.5		0.375	0.312			
Interaction	S		S	S			
	E21						
MIC_{STE} 2.5 MIC_{BE} 2.5	0.625 BE + 0.625 STE						
FIC _{BE}	0.25						
FIC _{STE}	0.25						
FICI	0.5						
Interaction	S						
	E25, E35, E46, E47, E49, E50, [E52-E54], [E56-E58], [E64-E66], E69, [E71-E76], E79, E81, E82, E84, E90, E91, E94 and E95		E52, E53, E56, E57 and E90	E50, E65, E75 and E90		E90	
MIC_{STE} 1.25 MIC_{BE} 2.5	0.625 BE + 0.31STE		0.31 BE + 0.31STE	0.625 BE + 0.16 STE		0.625 BE + 0.08 STE	
FIC _{BE}	0.25		0.124	0.25		0.25	
FIC _{STE}	0.248		0.248	0.128		0.064	
FICI	0.498		0.372	0.378		0.314	
Interaction	S		S	S		S	

EUCAST Clinical Breakpoint v. 7.0 ($R > 4 \mu\text{g ml}^{-1}$), confirmed antibiogram test for 12 VRE isolates, as well as susceptibility of almost all *E. faecalis* isolates, except three isolates (E12, E52, and E55) which were resistant to the tested antibiotic. The MBC values of all isolates were twice as high as the determined MIC values or were not determined for some isolates.

3.5. Interactions of herbal extracts

Types of interaction among the chosen combinations were examined with the aim of determining the contribution of individual herbal extracts and their relationships in manifestation of antimicrobial activity. The values of FIC and FICI were calculated on the basis of MIC values in order to express the antimicrobial effect of extracts in different combinations of individual extracts. The results of testing the types of interaction between the extracts STE, BE, and BTE are shown in Supplementary Tables S1-S3. The extract PE combined with STE extract showed a strictly antagonistic or indifferent effect on all isolates in the highest tested concentrations, while lower concentrations always caused the growth of isolates. These were therefore excluded from further analyses (data not shown). In analysis of interaction based on the achieved MIC values of individual extracts towards each isolate simultaneously,

six different susceptibility groups of isolates were obtained and the same groups of combinations were tested according to the following scheme:

- The combination of STE (MIC 2.5 mg ml⁻¹) and BTE (MIC 5.0 mg ml⁻¹);
- The combination of STE (MIC 1.25 mg ml⁻¹) and BTE (MIC 5.0 mg ml⁻¹);
- The combination of STE (MIC 1.25 mg ml⁻¹) and BTE (MIC 2.5 mg ml⁻¹);
- The combination of STE (MIC 2.5 mg ml⁻¹) and BE (MIC 5.0 mg ml⁻¹);
- The combination of STE (MIC 2.5 mg ml⁻¹) and BE (MIC 2.5 mg ml⁻¹);
- The combination of STE (MIC 1.25 mg ml⁻¹) and BE (MIC 2.5 mg ml⁻¹).

A synergistic effect was recorded for as many as 36 isolates out of their total number in all tested checkerboard schemes (Table 4, Supplementary Tables S1-S3). A synergistic effect of STE and BE was observed in 32 isolates, while the combination of STE and BTE showed a synergistic effect against 12 isolates. It is interesting to note that a synergistic effect on eight isolates of the species *E. faecalis* (E50, E58,

E65, E73, E75, E79, E90, and E95) was confirmed in both combinations used. From the total number of isolates, the synergistic effect towards *E. faecalis* was slightly more pronounced (39.5 %) than the one towards *E. faecium* isolates (30 %).

The synergistic effect of STE and BE extracts was noticed in only one isolate of *E. faecium* (E23), while this combination was more effective in the case of *E. faecalis* isolates. The combination of STE and BTE, as well as the combination of STE and BE, showed a synergistic effect on the majority of isolates when the concentration of BTE or BE was 0.625 mg ml⁻¹ and that of STE was 0.31 mg ml⁻¹ (Table 4). For two other groups with isolates E21 and E78 (MIC of STE and BTE, 2.5 mg ml⁻¹ each) and isolate E23 (MIC of STE, 2.5 mg ml⁻¹; and MIC of BE, 1.25 mg ml⁻¹) all types of interaction with the exception of a synergistic effect were scored. Additivism in the lowest concentrations for isolates E21 and E78 was achieved in the combination of 0.04 mg ml⁻¹ BTE and 1.25 mg ml⁻¹ STE, as well as in the combination of 1.25 mg ml⁻¹ BTE and 0.31 mg ml⁻¹ STE. The strongest additivism for isolate E23 was achieved in the combination of 0.625 mg ml⁻¹ STE and 0.625 mg ml⁻¹ BE.

3.6. Determination of biofilm formation

The capacity of all isolates for biofilm formation was marked as previously described way, i.e., isolates were assigned to categories of ones showing weak, moderate, or strong biofilm formation (Supplementary Table S4). The results of determining initial adhesiveness and biofilm formation of identified isolates of the genus *Enterococcus* are

shown in Fig. 1. The graph shows that almost all isolates, apart from six isolates (E27, E28, E31, E40, E66, and E75) mostly of the species *E. faecium* (Supplementary Table S4), which showed no cut-off OD values in their initial adhesiveness, have the ability to adhere on microtiter plate wells. Less than 48 h later, the E66 and E75 isolates showed no adherence ability. Weak formation of biofilm (category 1) was displayed by the majority of isolates of both species, while only three isolates (E43, E93, and E94) had very strong biofilm formation (category 3), one of them (E94) belonging to the species *E. faecium*. Twenty-four isolates of the species *E. faecalis* had moderate biofilm formation (category 2). Most of the isolates showed no changes of biofilm formation after the initial adhesiveness, i.e., they remained in the same category even after 48 h. Biofilm increase was noticed after initial adhesiveness for a fairly small number of isolates (Supplementary Table S4).

3.7. Effect of individual herbal extracts and their combinations on biofilm formation

After determining the categories of initial adhesiveness and biofilm formation of all tested isolates, we chose four isolates that showed moderate (E61 and E89) or strong (E43 and E94) biofilm formation. The effect of individual extracts and their combinations on biofilm formation was followed on the chosen isolates (Supplementary Tables S5 and S6). A concentration range from 1/4 to 4 MIC for both individual extracts and their combinations, which gave the best results in terms of additive

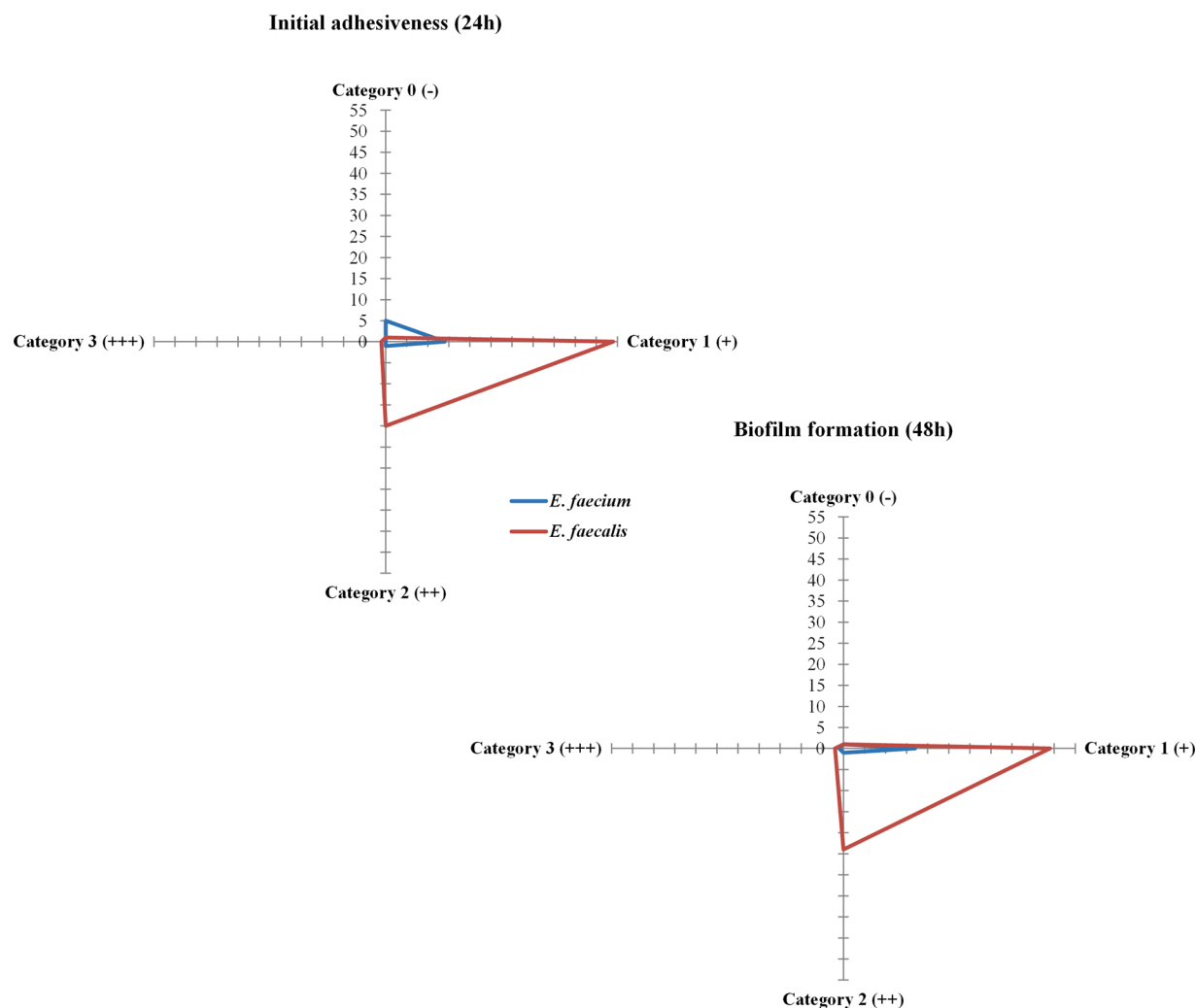


Fig. 1. Initial adhesiveness and biofilm formation of the *Enterococcus* isolates.

Table 5

The percentage of inhibition of the initial adhesiveness and biofilm formation of selected isolates treated with the plant extracts.

24 h		STE	BTE	BE	Van	STE + BTE	STE + BE
E43	4 MIC	99.5 ^a ± 1.4	100.9 ^a ± 1.0	92.1 ^a ± 4.3	88.7 ^a ± 0.2	94.4 ^a ± 6.6	99.1 ^a ± 1.7
	2 MIC	51.5 ^b ± 0.9	97.5 ^a ± 4.0	86.2 ^a ± 2.5	88.8 ^a ± 1.0	70.1 ^{ab} ± 4.6	91.8 ^a ± 1.3
	1 MIC	26.5 ^c ± 1.7	100.0 ^a ± 0.2	85.1 ^{ab} ± 0.8	88.6 ^a ± 0.3	55.4 ^{bc} ± 4.6	34.2 ^b ± 0.7
	1/2 MIC	18.5 ^d ± 1.0	72.1 ^b ± 4.1	64.2 ^b ± 8.7	52.7 ^b ± 0.1	53.5 ^{bc} ± 6.9	36.7 ^b ± 3.5
	1/4 MIC	12.2 ^e ± 0.3	50.3 ^c ± 4.0	36.5 ^c ± 3.2	48.3 ^c ± 1.2	36.1 ^c ± 3.3	31.2 ^b ± 3.2
E61	4 MIC	97.5 ^a ± 1.2	100.8 ^a ± 1.6	84.6 ^a ± 2.3	98.3 ^a ± 0.4	78.1 ^a ± 5.3	95.8 ^a ± 2.4
	2 MIC	92.4 ^a ± 1.3	95.1 ^{ab} ± 1.7	85.9 ^a ± 1.9	98.2 ^a ± 0.32	60.3 ^{ab} ± 1.5	96.6 ^a ± 1.1
	1 MIC	82.2 ^b ± 0.9	91.7 ^b ± 1.4	85.9 ^a ± 3.2	95.9 ^a ± 0.9	44.7 ^b ± 7.5	45.9 ^b ± 2.6
	1/2 MIC	74.4 ^{bc} ± 2.8	76.5 ^c ± 0.7	53.5 ^b ± 2.1	96.0 ^a ± 1.4	44.4 ^b ± 3.9	43.2 ^b ± 6.0
	1/4 MIC	72.8 ^c ± 2.3	42.2 ^d ± 2.7	49.5 ^b ± 3.0	90.8 ^b ± 0.2	9.7 ^c ± 5.1	37.3 ^b ± 2.0
E89	4 MIC	100.9 ^a ± 0.6	102.8 ^a ± 4.7	100.1 ^a ± 1.9	97.3 ^a ± 0.6	98.8 ^a ± 5.9	99.6 ^a ± 2.8
	2 MIC	72.6 ^b ± 5.4	97.1 ^a ± 4.6	90.6 ^b ± 2.9	97.6 ^a ± 0.1	98.8 ^a ± 0.8	99.5 ^a ± 0.5
	1 MIC	44.5 ^c ± 2.4	99.9 ^a ± 0.6	91.4 ^b ± 0.8	96.3 ^a ± 0.6	92.3 ^a ± 1.4	72.1 ^b ± 3.0
	1/2 MIC	34.6 ^c ± 1.0	99.0 ^a ± 0.5	66.6 ^c ± 1.8	95.5 ^a ± 1.2	73.1 ^b ± 2.9	25.4 ^c ± 3.2
	1/4 MIC	30.7 ^c ± 4.3	82.4 ^b ± 1.0	51.9 ^d ± 1.3	26.5 ^b ± 0.8	33.9 ^c ± 4.9	0.3 ^d ± 0.03
E94	4 MIC	100.8 ^a ± 0.7	100.7 ^a ± 0.01	92.8 ^a ± 2.5	92.8 ^a ± 0.5	100.5 ^a ± 8.5	89.8 ^a ± 1.1
	2 MIC	98.4 ^a ± 3.03	98.2 ^a ± 2.3	92.8 ^a ± 3.8	94.4 ^a ± 0.4	99.8 ^a ± 0.8	86.8 ^a ± 1.6
	1 MIC	60.7 ^b ± 1.3	99.7 ^a ± 2.1	49.0 ^b ± 6.1	83.1 ^a ± 4.9	90.8 ^a ± 2.9	87.3 ^a ± 2.3
	1/2 MIC	62.0 ^b ± 1.8	93.1 ^{ab} ± 1.2	25.0 ^c ± 4.2	81.8 ^a ± 2.5	87.5 ^a ± 2.1	87.5 ^a ± 5.6
	1/4 MIC	58.0 ^b ± 6.4	86.9 ^b ± 3.2	20.1 ^c ± 0.7	33.4 ^b ± 9.5	83.7 ^a ± 3.03	77.5 ^a ± 7.6
48 h		STE	BTE	BE	Van	STE + BTE	STE + BE
E43	4 MIC	85.0 ^a ± 1.1	100.3 ^a ± 0.7	100.1 ^a ± 4.4	90.0 ^a ± 2.2	91.0 ^a ± 5.5	94.6 ^a ± 7.2
	2 MIC	84.1 ^a ± 0.8	100.4 ^a ± 0.0	99.2 ^a ± 5.67	88.4 ^a ± 1.6	64.8 ^b ± 1.6	86.1 ^a ± 3.0
	1 MIC	59.3 ^b ± 2.3	94.5 ^a ± 1.5	86.2 ^a ± 1.39	88.8 ^a ± 1.5	63.8 ^b ± 3.5	43.3 ^b ± 14.7
	1/2 MIC	41.3 ^c ± 2.5	72.9 ^b ± 9.5	43.5 ^b ± 0.7	13.1 ^b ± 0.2	60.1 ^b ± 5.4	25.3 ^{bc} ± 2.2
	1/4 MIC	26.3 ^d ± 0.5	54.7 ^b ± 2.1	29.4 ^b ± 1.4	10.8 ^b ± 3.6	51.4 ^b ± 4.4	0.0 ^c ± 0.0
E61	4 MIC	77.5 ^a ± 3.4	98.9 ^a ± 0.2	102.7 ^a ± 3.8	91.2 ^a ± 0.6	84.3 ^a ± 5.0	91.2 ^a ± 5.5
	2 MIC	61.9 ^{ab} ± 5.0	95.7 ^a ± 2.8	74.4 ^b ± 3.2	90.8 ^a ± 0.5	68.3 ^a ± 3.8	85.6 ^a ± 2.4
	1 MIC	63.6 ^b ± 0.8	80.5 ^b ± 4.9	76.0 ^b ± 0.9	91.4 ^a ± 0.9	45.8 ^b ± 4.8	72.9 ^{ab} ± 6.8
	1/2 MIC	61.9 ^b ± 0.0	67.3 ^c ± 0.4	74.2 ^b ± 1.1	53.1 ^b ± 4.6	48.5 ^b ± 3.9	51.0 ^{bc} ± 3.2
	1/4 MIC	50.4 ^b ± 3.2	63.7 ^c ± 1.8	74.3 ^b ± 1.9	51.5 ^b ± 7.2	0.0 ^c ± 0.0	44.2 ^c ± 9.6
E89	4 MIC	94.9 ^a ± 2.6	101.2 ^a ± 2.5	102.0 ^a ± 0.5	91.5 ^a ± 0.5	101.4 ^a ± 2.4	101.1 ^a ± 0.3
	2 MIC	96.3 ^a ± 0.4	97.6 ^a ± 1.7	70.2 ^b ± 4.7	89.3 ^a ± 1.4	99.5 ^a ± 0.7	98.8 ^a ± 3.4
	1 MIC	54.5 ^b ± 0.7	78.1 ^b ± 1.1	62.2 ^b ± 2.8	90.3 ^a ± 1.6	90.3 ^a ± 1.7	86.0 ^b ± 1.7
	1/2 MIC	25.4 ^c ± 5.8	65.9 ^c ± 0.0	28.4 ^c ± 2.7	20.8 ^b ± 6.9	63.5 ^b ± 3.4	51.2 ^c ± 1.8
	1/4 MIC	24.2 ^c ± 4.7	55.3 ^d ± 0.6	2.4 ^d ± 1.7	0.0 ^c ± 0.0	31.0 ^c ± 4.6	5.9 ^d ± 2.3
E94	4 MIC	85.7 ^a ± 1.9	97.8 ^a ± 2.1	97.7 ^a ± 3.8	80.4 ^a ± 1.4	97.3 ^a ± 13.3	99.4 ^a ± 4.0
	2 MIC	87.7 ^a ± 1.2	97.2 ^a ± 2.1	75.1 ^a ± 11.2	65.0 ^a ± 2.6	92.6 ^a ± 8.7	95.6 ^a ± 1.8
	1 MIC	81.2 ^{ab} ± 0.5	95.0 ^a ± 2.6	32.4 ^b ± 3.8	67.8 ^a ± 6.8	92.9 ^a ± 0.7	95.1 ^a ± 2.9
	1/2 MIC	70.6 ^b ± 1.9	74.6 ^b ± 0.7	25.9 ^b ± 4.1	13.6 ^b ± 2.4	95.1 ^a ± 5.0	91.1 ^{ab} ± 5.5
	1/4 MIC	57.5 ^c ± 5.5	74.7 ^b ± 1.3	22.7 ^b ± 1.3	17.0 ^b ± 3.5	88.1 ^a ± 6.4	76.0 ^b ± 1.5

*Van - Vancomycin; STE - strawberry tree extract; BE - bearberry extract; BTE - bearberry tea extract.

or synergistic effects during the previous phase of research, was used for this purpose. Table 5 shows the results obtained in testing the influence of individual herbal extracts and their combinations on decrease of initial adhesiveness and biofilm formation after 24 and 48 h. For the E43 isolate (with strong initial adhesiveness and biofilm formation) the absence of any statistically significant difference in pre-treatment with individual extracts in the higher concentrations tested was confirmed. A concentration of 1 MIC was for the most part high enough to achieve 80 % of biofilm inhibition, particularly in the case of BTE and BE treatment. However, in some cases the use of supra-MIC concentration of BE or STE extracts towards biofilm formation of E94 and E89, respectively lead to better inhibition of biofilm, than MIC itself. The sub-MIC concentrations tested (1/4 and 1/2 MIC) caused a considerably lower biofilm inhibition, i.e., the effect of these two concentrations was mostly statistically insignificant. Supplementary Table S5 shows that there was an inhibition, i.e., alteration, of the cut-off OD values (biofilm categories) at the lower concentrations tested, especially after 48 h. Similar results were noticed for the *E. faecalis* E61 and E89 isolates (Supplementary Table S6). Both isolates had moderate biofilm formation, and it is interesting to note that the higher tested concentrations of individual extracts showed an inhibition of over 50 %. A statistically significant inhibition of biofilm formation was noticed at lower concentrations for

the E61 isolate as well. The applied concentrations of extracts (especially BTE and BE) caused weakening of initial adhesiveness and consequent biofilm formation of the last tested *E. faecium* E94 isolate. These results mostly match those obtained with E43, but stronger inhibition of initial adhesiveness at lower concentrations for the E94 isolate was observed. The BTE extract had the strongest effect on initial adhesiveness and biofilm formation towards all isolates, although a strong effect of the BE extract also, was observed at higher concentrations. The combination of STE with BE or with BTE caused statistically significant differences of biofilm formation between the higher and the lower concentrations for all isolates. Both combinations of extracts caused a significant weakening of initial adhesiveness of E94 isolate at all concentrations. This is in accordance with the synergistic antimicrobial effect of the STE and BE, as well as with the additive antimicrobial effect of the STE and BTE on the E94 isolate. In regard to the other isolates, both combinations showed equal inhibition at the highest concentrations, while a more pronounced effect of biofilm inhibition was also noticed at lower concentrations of the STE and BTE combination. An equal inhibition of biofilm was noted for all isolates when vancomycin was used at the highest concentrations tested (Table 5) but, statistically speaking, the ability to inhibit biofilm formation decreased considerably (four times or more) at lower concentrations.

4. Discussion

Today there are increasing numbers of urinary tract infections (UTIs) with microbes that are multidrug resistant. As a solution to this problem, there has been a renewed interest in alternative antimicrobials. The individual and synergistic effects of bearberry (BE), bearberry tea (BTE), parsley (PE), and the strawberry tree (STE) herbal extracts for the treatment of urinary infections caused by *Enterococcus* spp. was examined. In earlier research, various polyphenolic compounds were identified in strawberry tree leaves (Dib et al., 2013). These leaves are characterized by high contents of phenolic acids, terpenes, and flavonoids, which are present in different percentages, depending on the plant growth region (Celikel et al., 2008). The most abundant phenolic compounds found in strawberry tree leaves collected in Croatia included catechin, rutin, myricetin, gallic acid, and arbutin, which was present at the highest concentration, 627 mg l^{-1} (Oliveira et al., 2009). Twelve phenolic compounds were identified in leaves collected in Central Italy, arbutin being the most common one, while the presence of gallic and ellagic acids and eight flavonoids was also noticed (Fiorentino et al., 2007). The phenolic glycoside arbutin (4-hydroxyphenyl- β -D-glucopyranoside) was identified as one of the major compounds in the leaves of strawberry tree and bearberry. Arbutin is decomposed into hydroquinone and glucoses under the influence of β -glucosidase, with hydroquinone having an antiseptic effect on the urinary tract (Blaut et al., 2006). According to the European Pharmacopeia, the content of arbutin in dried bearberry leaves must be above 7 % (Panusa et al., 2015). The arbutin content in leaves is of utmost importance for antimicrobial activity and it varies depending on the climate, the soil in which the plant grows, and age of the plant itself (Jurica et al., 2015). Previous research showed that, apart from arbutin, bearberry contains large amounts of gallic and ellagic acids, quercetin, myricetin, kaempferol, and catechin (García de Arriba et al., 2013). Apigenin and kaempferol glucosides were confirmed as the main components of parsley (Sakakibara et al., 2003), which coincides with the results of the authors research, at least in regard to apigenin glucoside. Shan et al. (2005) showed that the main phenolic components of parsley were apigenin, caffeic, *p*-coumaric, and ferulic acid, which were noted in the study extract as well, although at lower concentrations.

Dominance of two species in comparison with other uropathogenic *Enterococcus* representatives was noticed earlier (Brigante et al., 2006). *E. faecalis* is found as a cause of 80–90 % of UTIs in contrast to *E. faecium* which is a cause of 10–20 % (Oli et al., 2012) which is in correlation with results obtained in this study. Resistant isolates of the genus *Enterococcus* were first noticed in France and Italy in 1980. Their number increased in time, the reason for that being the wide use of antibiotics (Schouten et al., 2000). In previous research in the Balkans (in the republics of Bosnia and Herzegovina and Croatia), 13.8 % (*E. faecalis*) and 80 % (*E. faecium*) out of a total of 64 isolates were characterized as VRE (Jakovac et al., 2017). The same study also confirmed that all of the isolates of *E. faecium* showed ampicillin and gentamicin resistance, but also sensitivity to the effect of teicoplanin (74 % of the isolates). The fairly large number of VRE isolates is concerning, due to the fact that they often cause infections which are not susceptible to conventional antibiotic therapy.

As an alternative approach, the present study involved the examination of herbal extracts traditionally applied in the treatment of urinary infections. Previous research showed a strong antimicrobial effect of methanol leaf extracts of the strawberry tree against some specific clinical and reference strains of the genus *Enterococcus* (Jurica et al., 2017). A similar effect was obtained using a petroleum leaf extract of the strawberry tree (Diba et al., 2010). However, the strong antimicrobial potential of STE is attributable to increased β -glucosidase production by the affected isolates, which convert arbutin into free hydroquinone, a substance that exhibits a strong antibacterial activity (Jurica et al., 2017). According to Schindler et al. (2002), an antibacterial effect of bearberry was attributable to the effect of arbutin and its metabolite,

hydroquinone. Besides arbutin, other polyphenolic compounds can also have a distinct antimicrobial potential. It has been confirmed that caffeic, gallic, and ellagic acids can inhibit bacterial growth and cause severe damage to the cell membrane (Borges et al., 2013). The same study also established that ferulic acid has a good antimicrobial potential towards *E. faecalis* isolates, causing an imbalance in structure of the cell membrane, its breakdown, and intracellular content leakage. Catechin, one of the major components of the strawberry tree and bearberry extracts tested in this study also showed distinct antimicrobial activity towards Gram-positive bacteria and slightly lower activity towards Gram-negative bacteria (Diba et al., 2010). However, parsley extract showed no distinct antibacterial activity, either in this study or in *E. faecalis* research in which a 5 mg ml^{-1} MIC value was measured (Manderfeld et al., 1997). Ojala et al. (2000) also showed low antimicrobial activity of parsley towards *Bacillus subtilis*, *E. faecalis*, and *Staphylococcus aureus* isolates, with an MIC value in the range of $2\text{--}3 \text{ mg ml}^{-1}$. Although the antimicrobial effect is mostly attributable to those compounds with the highest share, the influence of other traceable components should not be disregarded due to possible interactions that can affect antimicrobial activity.

Although reference data on STE and BE and/or BTE and their combinations are missing, previous research showed that the phenolic acids dominant in this study extracts are precisely the ones responsible for the more considerable synergistic effect towards Gram-positive bacteria (Daglia, 2012). Various general mechanisms of antimicrobial interaction producing synergism have been described and accepted up to this point (Bassolé and Juliani, 2012). These can include sequential inhibition of the shared biochemical route, the inhibition of protective enzymes, and the use of active cell wall components for better penetration of other antimicrobial agents (Santesteban-Lopez et al., 2007). Whatever the case, the discovery of appropriate alternative combinations of herbal extracts could contribute to the constant fight against antimicrobial resistance.

Biofilm formation makes possible a much higher rate of bacterial survival than under individual conditions (Lee and Tan, 2015). Members of the genus *Enterococcus* vary in their ability to produce a biofilm. Research carried out in Italy on clinical isolates of urine showed that 80 % of *E. faecalis* isolates and 48 % of *E. faecium* isolates had the ability to produce a biofilm (Baldassarri et al., 2001). Also, 59 % of clinical isolates of *E. faecalis* collected in Poland formed a biofilm (Dworniczek et al., 2005). In the study of Mohamed and Huang (2007) it was concluded that *E. faecalis* forms a stronger biofilm in medium with glucose due to the fact that glucose stimulates the synthesis of superficial proteins that facilitate simpler adhesion. There is also biofilm formation in *E. faecium*, although considerably weaker.

In regard to the considerable effect of 1 MIC, 2 MIC, and 4 MIC concentrations, it can be stated that the inhibition of initial adhesiveness and biofilm formation resulted from inhibition of cell growth, as well as from cell death. The application of lower concentrations of herbal extracts caused greater semi-permeability of the cell membrane and changed its structure, but did not affect its viability, while the application of higher concentrations caused severe membrane damage, homeostatic breakdown, and cell death (Lima et al., 2001). Cai et al. (2016) showed that the arbutin extract caused significant lessening of bacterial colonization and development of a biofilm on the surface of urinary catheters. Blaut et al. (2006) proved that arbutin and its metabolite hydroquinone prevent biofilm formation by species of the genus *Enterococcus*, as well as other genera. Earlier research showed that a significant part in inhibition of biofilm formation is played by gallic and ellagic acids (Slobodnková et al., 2013), which they were also among the main components of our extracts. It has been shown that ellagic acid can limit biofilm formation by *E. faecalis* up to a degree which can correlate with its high sensitivity to antibiotics (Schindler et al., 2002). Tannins also have antimicrobial activity that prevents biofilm formation, especially catechin, which can interact with the lipid bilayer and cause leakage of cell content (Ikigai et al., 1993).

5. Conclusions

Species of the genus *Enterococcus* are among the leading causes of UTIs, and they often show resistance to one or more antibiotics. Herbal leaf extracts rich in phenolics, could be used more often in combination with antibiotics to simultaneously enhance their efficacy, lessen their dosage, and reduce negative reactions to them. In the present study, treatment with individual extracts (particularly BTE extract) and selected combinations resulted in a statistically significant inhibition of biofilm formation and demonstrated a considerable antimicrobial potential. The synergistic effect of the tested extract combinations achieved, when BE or BTE was combined with STE, makes their use an alternative worth investigating further in future research, especially relying on the synergism of BTE and STE extracts.

Funding

This work was supported by the Ministry of Education, Science and Technological Development of Serbia and Ministry of Science and Education of Croatia through Croatia-Serbia bilateral cooperation program "Knowledge for progress and stability" (2016–2018). Also, this work was supported by the Ministry of Education, Science and Technological Development of Serbia (Grant No. 451-03-9/2021-14/ 200178) and University of Rijeka, Croatia (Grants No. 13.06.2.2.60 and 13.06.1.1.07). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CRedit authorship contribution statement

Ivica Dimkić, Ivana Gobin, Maja Abram, Slaviša Stanković: Conceived and designed the experiments. **Ivica Dimkić, Ivana Gobin, Gabrijela Begić, Davorka Repac Antić, Petar Ristivojević:** Performed the experiments. **Ivica Dimkić, Ivana Gobin, Gabrijela Begić, Petar Ristivojević:** Analyzed the data. **Ivica Dimkić, Petar Ristivojević, Karlo Jurica, Slaviša Stanković, Maja Abram:** Contributed reagents/materials/analysis tools. **Ivica Dimkić, Ivana Gobin:** Writing - Original Draft. **Ivica Dimkić, Ivana Gobin, Petar Ristivojević, Tanja Berić, Jelena Lozo, Karlo Jurica, Maja Abram, Slaviša Stanković:** Writing - Review & Editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.hermed.2021.100445>.

References

- Balaji, K., Thenmozhi, R., Pandian, S.K., 2013. Effect of subinhibitory concentrations of fluoroquinolones on biofilm production by clinical isolates of *Streptococcus pyogenes*. *Indian J. Med. Res.* 137, 963–971.
- Baldassarri, L., Cecchini, R., Bertuccini, L., Ammendolia, M.G., Iosi, F., Arciola, C.R., Montanaro, L., Di Rosa, R., Gherardi, G., Dicuonzo, G., Orefici, G., 2001. *Enterococcus* spp. produces slime and survives in rat peritoneal macrophages. *Med. Microbiol. Immunol.* 190, 113–120.
- Bassolé, I.H.N., Juliani, H.R., 2012. Essential oils in combination and their antimicrobial properties. *Mol.* 17, 3989–4006.
- Blaut, M., Braune, A., Wunderlich, S., Sauer, P., Schneider, H., Glatt, H., 2006. Mutagenicity of arbutin in mammalian cells after activation by human intestinal bacteria. *Food Chem. Toxicol.* 44, 1940–1947.
- Borges, A., Ferreira, C., Saavedra, M.J., Simões, M., 2013. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. *Microb. Drug Resist.* 19, 256–265.
- Brigante, G., Luzzaro, F., Bettaccini, A., Lombardi, G., Meacci, F., Pini, B., Stefani, S., Toniolo, A., 2006. Use of the phoenix automated system for identification of *Streptococcus* and *Enterococcus* spp. *J. Clin. Microbiol.* 44, 3263–3267.

- Cai, T., Gallelli, L., Meacci, F., Brugnolli, A., Proserpi, L., Roberta, S., Eccher, C., Mazzoli, S., Lanzafame, P., Caciagli, P., Malossini, G., 2016. The efficacy of umbelliferone, arbutin, and N-acetylcysteine to prevent microbial colonization and biofilm development on urinary catheter surface: results from a preliminary study. *J. Pathog.*, 1590952.
- Celikel, G., Demirsoy, L., Demirsoy, H., 2008. The strawberry tree (*Arbutus unedo* L.) selection in Turkey. *Sci. Hort.* 118, 115–119.
- Daglia, M., 2012. Polyphenols as antimicrobial agents. *Curr. Opin. Biotechnol.* 23, 174–181.
- Dib, M.E.A., Allali, H., Bendiabdellah, A., Meliani, N., Tabti, B., 2013. Antimicrobial activity and phytochemical screening of *Arbutus unedo*. *J. Saudi Chem. Soc.* 17, 381–385.
- Diba, M.A., Paolini, J., Bendahou, M., Varesi, L., Allali, H., Desjobert, J.M., Tabti, B., Costa, J., 2010. Chemical composition of fatty acid and unsaponifiable fractions of leaves, stems and roots of *Arbutus unedo* and *in vitro* antimicrobial activity of unsaponifiable extracts. *Nat. Prod. Commun.* 5, 1085–1090.
- Dimkić, I., Živković, S., Berić, T., Ivanović, Ž., Gavrilović, V., Stanković, S., Fira, D., 2013. Characterization and evaluation of two *Bacillus* strains, SS-12.6 and SS-13.1, as potential agents for the control of phytopathogenic bacteria and fungi. *Biol. Control* 65, 312–321.
- Dimkić, I., Ristivojević, P., Janakiev, T., Berić, T., Trifković, J., Milojković-Opsenica, D., Stanković, S., 2016. Phenolic profiles and antimicrobial activity of various plant resins as potential botanical sources of Serbian propolis. *Ind. Crops Prod.* 94, 856–871.
- Dworniczek, E., Wojciech, L., Sobieszczanska, B., Seniuk, A., 2005. Virulence of *Enterococcus* isolates collected in Lower Silesia (Poland). *Scand. J. Infect. Dis.* 37, 630–636.
- European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), 2000. Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. EUCAST Definitive Document E. Def 1.2. *Clin. Microbiol. Infect.* 6, 503–508.
- Fiorantino, A., Castaldi, S., D'Abrascia, B., Natale, A., Carfora, A., Messere, A., Monaco, P., 2007. Polyphenols from the hydroalcoholic extract of *Arbutus unedo* living in a monospecific Mediterranean woodland. *Biochem. Syst. Ecol.* 35, 809–811.
- García de Arriba, S., Naser, B., Nolte, K.U., 2013. Risk assessment of free hydroquinone derived from *Arctostaphylos uva-ursi* folium herbal preparations. *Int. J. Toxicol.* 32, 442–453.
- Gasić, U.M., Natić, M.M., Mišić, D.M., Lušić, D.V., Milojković-Opsenica, D.M., Tešić, Ž.L., Lušić, D., 2015. Chemical markers for the authentication of unifloral *Salvia officinalis* L. Honey. *J. Food Compos. Anal.* 44, 128–138.
- Hollenbeck, B.L., Rice, L.B., 2012. Intrinsic and acquired resistance mechanisms in *Enterococcus*. *Virulence* 3, 421–569.
- Hudzicki, J., 2009. Kirby-Bauer disk diffusion susceptibility test protocol. *Am. Soc. Microbiol* 1–23. <http://www.asmscience.org/content/education/protocol/protocol1.3189>.
- Ikigai, H., Nakae, T., Hara, Y., Shimamura, T., 1993. Bactericidal catechins damage the lipid bilayer. *Biochim. Biophys. Acta.* 1147, 132–136.
- Jakovac, S., Bojić, E.F., Ibrisić, M.A., Tutiš, B., Ostojić, M., Hukić, M., 2017. Characteristics of Vancomycin-resistant *Enterococcus* strains in the West Balkans: a first report. *Microb. Drug Resist.* 23, 122–126.
- Jurica, K., Karačonić, I.B., Šegan, S., Opsenica, D.M., Kremer, D., 2015. Quantitative analysis of arbutin and hydroquinone in strawberry tree (*Arbutus unedo* L., Ericaceae) leaves by gas chromatography-mass spectrometry. *Arh. Hig. Rada Toksikol.* 66, 197–202.
- Jurica, K., Gobin, I., Kremer, D., Čepo, D.V., Grubešić, R.J., Karačonić, I.B., Kosalec, I., 2017. Arbutin and its metabolite hydroquinone as the main factors in the antimicrobial effect of strawberry tree (*Arbutus unedo* L.) leaves. *J. Herb. Med.* 8, 17–23.
- Karuppusamy, S., Rajasekaran, K.M., 2009. High throughput antibacterial screening of plant extracts by resazurin redox with special reference to medicinal plants of Western Ghats. *Glo. J. Pharm.* 3, 63–68.
- Khan, R., Islam, B., Akram, M., Shakil, S., Ahmad, A.A., Ali, S.M., Siddiqui, M., Khan, A. U., 2009. Antimicrobial activity of five herbal extracts against multi drug resistant (MDR) strains of bacteria and fungus of clinical origin. *Mol.* 14, 586–597.
- Lee, P., Tan, S.K., 2015. Effects of epigallocatechin gallate against *Enterococcus faecalis* biofilm and virulence. *Arch. Oral Biol.* 60, 393–399.
- Lima, K.C., Fava, L.R., Siqueira Jr, J.F., 2001. Susceptibilities of *Enterococcus faecalis* biofilms to some antimicrobial medications. *J. Endod.* 10, 616–619.
- Manderfeld, M.M., Schafer, H.W., Davidson, P.M., Zottola, E.A., 1997. Isolation and identification of antimicrobial furocoumarins from parsley. *J. Food Prot.* 60, 72–77.
- Mariotto, S., Esposito, E., Di Paola, R., Ciampa, A., Mazzon, E., de Prati, A.C., Darra, E., Vincenzi, S., Cucinotta, G., Caminiti, R., Suzuki, H., 2008. Protective effect of *Arbutus unedo* aqueous extract in carrageen an-induced lung inflammation in mice. *Pharmacol. Res.* 57, 110–124.
- Miller, W.R., Munita, J.M., Arias, C.A., 2014. Mechanisms of antibiotic resistance in enterococci. *Expert Rev. Anti-infect. Ther.* 12, 1221–1236.
- Mohamed, A.J., Huang, D.B., 2007. Biofilm formation by enterococci. *J. Med. Microbiol.* 56, 1581–1588.
- Ojala, T., Remes, S., Haansuu, P., Vuorela, H., Hiltunen, R., Haahtela, K., Vuorela, P., 2000. Antimicrobial activity of some coumarin containing herbal plants growing in Finland. *J. Ethnopharmacol.* 73, 299–305.
- Oli, A.K., Raju, S., Rajeshwari, S., Nagaveni, S., Kelmani Chandrakanth, R., 2012. Biofilm formation by multidrug resistant *Enterococcus faecalis* (MDEF) originated from clinical samples. *J. Microbiol. Biotechnol.* 2, 284–288.

- Oliveira, I., Coelho, V., Baltasar, R., Pereira, J.A., Baptista, P., 2009. Food chem. Toxicol. 47, 1507–1511.
- Oliveria, I., Baptista, P., Bento, A., Pereira, J.S., 2011. *Arbutus unedo* L. and its benefits on human health. J. Food Nutr. Res. 2, 73–85.
- Pallauf, K., Rivas-Gonzalo, J.C., Del Castillo, M.D., Cano, M.P., de Pascual-Teresa, S., 2008. Characterization of the antioxidant composition of strawberry tree (*Arbutus unedo* L.) fruits. J. Food Compos. Anal. 21, 273–281.
- Panusa, A., Petrucci, R., Marrosu, G., Multari, G., Gallo, R.F., 2015. UHPLC–PDA–ESI–TOF/MS metabolic profiling of *Arctostaphylos pungens* and *Arctostaphylos uva-ursi*. A comparative study of phenolic compounds from leaf methanolic extracts. Phytochemistry 115, 79–88.
- Sakakibara, H., Honda, Y., Nakagawa, S., Ashida, H., Kanazawa, K., 2003. Simultaneous determination of all polyphenols in vegetables, fruits, and teas. J. Agric. Food Chem. 51, 571–581.
- Santisteban-Lopez, A., Palou, E., López-Malo, A., 2007. Susceptibility of food-borne bacteria to binary combinations of antimicrobials at selected a(w) and pH. J. Appl. Microbiol. 102, 486–497.
- Savoia, D., 2012. Plant-derived antimicrobial compounds: alternatives to antibiotics. Future Microbiol. 7, 979–990.
- Schindler, G., Patzak, U., Brinkhaus, B., Nieciecki, A., Wittig, J., Krähmer, N., Glöckl, I., Veit, M., 2002. Urinary excretion and metabolism of arbutin after oral administration of *Arctostaphylos uva-ursi* extract as film-coated tablets. J. Clin. Pharmacol. 42, 920–927.
- Schouten, M.A., Hoogkamp-Korstanje, J.A.A., Meis, J.F.G., Voss, A., 2000. European VRE study group. Prevalence of vancomycin-resistant enterococci in Europe. Eur. J. Clin. Microbiol. Infect. Dis. 11, 816–822.
- Schulz, V., Hänsel, R., Tyler, V.E., 2001. Rational Phytotherapy: a Physician's Guide to Herbal Medicine. Psychology Press.
- Schwalbe, R., Steele-Moore, L., Goodwin, A.C., 2007. Antimicrobial Susceptibility Testing Protocols. CRC Press.
- Shan, B., Cai, Z.Y., Sun, M., Corke, H., 2005. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. J. Agric. Food Chem. 53, 7749–7759.
- Simin, J.E., Quinin, J., 1988. Characterization of essential oil of parsley. J. Agric. Food Chem. 36, 467–472.
- Slobodníková, L., Fialová, S., Hupková, H., Grančai, D., 2013. Rosmarinic acid interaction with planktonic and biofilm *Staphylococcus aureus*. Nat. Prod. Commun. 8, 1747–1750.
- Sood, S., Malhotra, M., Das, B.K., Kapil, A., 2008. Enterococcal infections & antimicrobial resistance. Indian J. Med. Res. 128, 111–121.
- Stepanović, S., Ćirković, I., Mijač, V., Švabić-Vlahović, M., 2003. Influence of the incubation temperature, atmosphere and dynamic conditions on biofilm formation by *Salmonella* spp. Food Microbiol. 20, 339–343.
- Verma, S., Patil, S., 2016. *In vitro* biofilm formation by uropathogenic bacteria and their antibiotic susceptibility pattern. JKMSU 5, 61–67.
- Zijne, V., Van Leeuwen, M.B.M., Degener, J.E., Abbas, F., Thurnheer, T., Gmür, R., Harmsen, H.J., 2010. Oral biofilm architecture on natural teeth. PLoS One 5, 1–9.