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Direct microwave treatment enhances antioxidant and antibacterial properties of the seed extracts of Kékfrankos grapes

Tamás Hofmann [a,*], Eszter Visi-Rajczi [a], Silvia Vaculciakova [b], Roman Guran [b], Stanislava Voberkova [b], Martina Vrsanska [b], Ondrej Zitka [b], Levente Albert [a]

[a] University of Sopron, Faculty of Forestry, Institute of Environmental Protection and Nature Conservation, Bajcsy-Zsilinszky út 4, Sopron, 9400, Hungary

[b] Department of Chemistry and Biochemistry, Faculty of AgriSciences, Mendel University in Brno, CZ-61300, Brno, Czech Republic

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ABSTRACT

The Kékfrankos is the most frequently cultivated wine grape in Hungary, with a significant national and regional impact, resulting in considerable amounts of byproducts (e.g. pomace, seeds). To the best of our knowledge no research has been conducted on the antioxidant and antibacterial properties of its seed extracts (GSE). A novel apporach of applying direct microwave treatment on grape seeds was implemented for the first time to enhance antioxidant and antimicrobial properties of GSE. Antioxidant properties were assayed using the DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power) and TPC (Folin-Ciocâlteu's Total Polyphenol Content) methods. Profile and content of polyphenols was studied using high-performance liquid chromatography/tandem mass spectrometry and matrix-assisted laser desorption/ionization mass spectrometry. Antibacterial properties were evaluated using Gram-positive *Staphylococcus aureus* (SA), methicillin-resistant *Staphylococcus aureus* (ST239) (MRSA) and Gram-negative *Escherichia coli* (EC) bacteria strains. Results proved that the mild direct microwave treatment of grape seeds significantly increased total polyphenol, (+)-catechin, (−)-epicatechin as well as antioxidant capacity levels by 20–30 % compared to untreated samples and resulted the best antibacterial properties based on bacterial growth curves (SA and MRSA: 0.015625 mg/mL, EC: 0.25 mg/mL). Results justify the importance of further pharmacological investigations on Kékfrankos grape seed extracts and that the direct microwave treatment of grape seeds is an innovative approach for the fast and cost efficient improvement of the antibacterial properties of grape seed extracts.

1. Introduction

The grape (*Vitis vinifera* L.) is known as the 'queen' of horticultural crops. It is widely grown in the world and is popular among customers due to its attractive sweet flavor and high nutritional value [1–3]. According to the data of the Food and Agriculture Organization of the United Nations worldwide grape production exceeded 73.5 M tonnes in 2020 [4]. One of the byproducts of grape berries is seeds, which comprises about 8–20 % of their weight, depending on variety [5] which represents a vast amount (6–15 M

* Corresponding author.
E-mail addresses: hofmann.tamas@uni-sopron.hu (T. Hofmann), visine.rajczi.eszter@uni-sopron.hu (E. Visi-Rajczi), xvaculci@mendelu.cz (S. Vaculciakova), roman.guran@mendelu.cz (R. Guran), voberkovas@gmail.com (S. Voberkova), martina.vrsanska88@gmail.com (M. Vrsanska), ondrej.zitka@mendelu.cz (O. Zitka), albert.levente@uni-sopron.hu (L. Albert).

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tonnes) of waste biomass without planned utilization, despite the fact that numerous studies have dealt with the topic so far [5,6]. Grape seed is composed of fiber (35–40 %), proteins (11 %) and extractives, latter of which mostly comprises of polyphenols (4–10 %, e.g. tannins, phenolic acids, stilbenes) and lipophilic substances or oils (7–20 %, e.g. tocopherols, tocotrienols, sterols, fatty acid esters, triglycerides) [6–11]. One of the potential uses of grape seeds is the preparation of grape seed extracts (GSE) for pharmaceutical, food and cosmeceutical industries, based on their antioxidant, anti-diabetic, anti-platelet, anti-cholesterol, anti-inflammation, anti-aging, anti-microbial and anti-tumor effects [5,6] and for the production of silver nanoparticles [12–14]. The use of grape seed as a source of bioactive compounds is also remarkable in the perspective of circular economy [15], local economy and potential contribution to territorial capital [16].

For the extraction of GSE, various techniques have been used among which microwave-assisted extraction (MAE) is especially effective and thoroughly researched [9,17–19]. The efficiency of MAE lies in the fact that microwave energy destroys the cell wall, which together with rapid inner heating causes instant elevated temperatures that enhance diffusion, and extraction yields in short times. However, MAE can mostly be applied at a laboratory scale for small amounts of plant material and not on an industrial scale.

Kékfrankos is a red grape variety, originating from north-eastern Slovenia [20]. Its distribution and popularity is highlighted by the fact that there are about 120 synonyms of the variety (e.g. Blue Franc, Blaufränkisch, Lemberger, Borgona, Frankovka Modra, Burgund Mare, etc.) depending on the country of cultivation [21]. It is the most frequently cultivated wine grape in Hungary, grown on 8000 ha which corresponds to about 12 % of the total wine grape producing area (62,000 ha) of the country [22]. The Sopron Wine Region, located in the western part of Hungary, is especially important in this regard, as the rate of Kékfrankos-growing area here is the overall highest (49 %) in the whole of Hungary, earning the city of Sopron the title of the „Capitol of the Kékfrankos". Despite its local, national and worldwide significance, to the best of our knowledge, no data has been published yet on the chemical composition and antibacterial properties of the GSE of Hungarian-grown Kékfrankos grape variety.

The present study focused on the assessment of the polypehnolic composition, as well as on the antioxidant and antibacterial properties of the Kékfranos GSE. To take advantage of these beneficial effects of microwave energy, the present study used direct microwave treatment of grape seeds for the first time for the efficient extraction of GSEs and for the improvement of antibacterial properties. According to the literature, so far a similar approach has been used with success only for the improvement of seed oil quality [23] and wine polyphenol content [24] but not for improvement of antioxidant and antibacterial properties of the GSE.

Antioxidant properties were assayed using the DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power) and TPC (Folin-Ciocâlteu's Total Polyphenol Content) methods, while HPLC-PDA-ESI-MS/MS (high-performance liquid chromatography/photodiode array detection/tandem electrospray mass spectrometry) and MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) analysis was done for the characterization of phenolic compounds. Antibacterial properties were tested on Gram-positive *Staphylococcus aureus* (SA), methicillin-resistant *Staphylococcus aureus* (MRSA) (ST239) and Gram-negative *Escherichia coli* (EC) using the Disc diffusion test (DDT), Minimal inhibitory concentration (MIC) and by the evaluation of growth curves. These strains were selected, as they are significant water and food contaminants worldwide [25]. They cause foodborne diseases as well as food poisoning and they are capable of expressing a number of extracellular toxins and enzymes [26–28]. Moreover, MRSA is the cause of *Staphylococcus* infection that is difficult to treat because of its resistance to methicilin antibiotics, making MRSA-related infections a very challenging health issue worldwide [29]. It is clear that new approaches are needed to treat bacteria

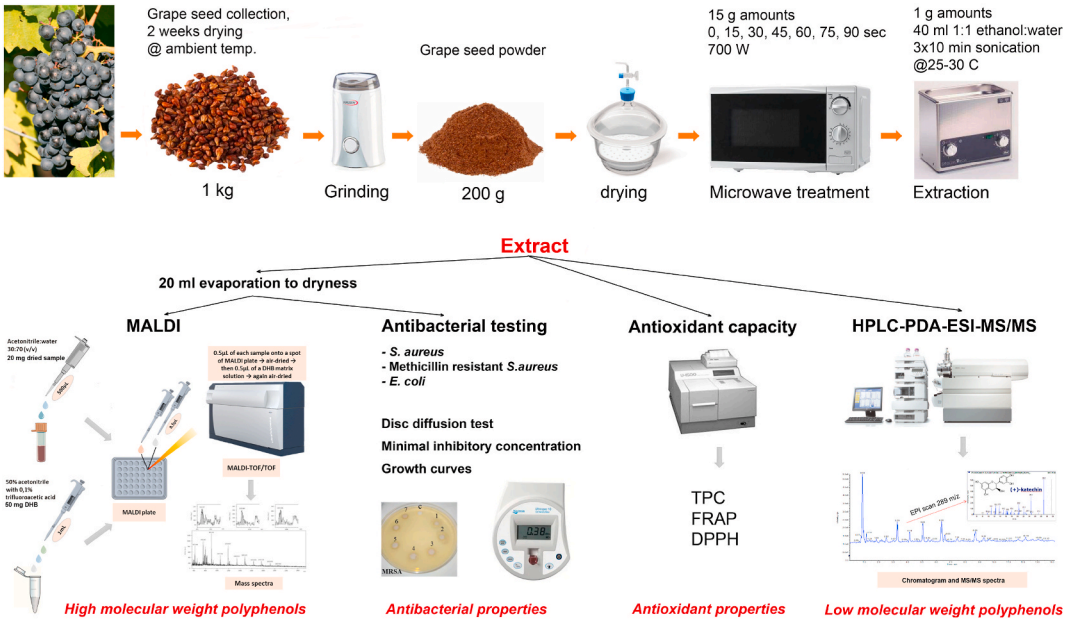

Fig. 1. The graphical scheme of study approach.

without using antibiotics and disinfectants and plant extracts are specially important in this regard. The graphical scheme of the study approach is presented in Fig. 1.Besides the first-time characterisation of the Kékfrankos GSE polyphenols the present research implements a novel sample pretreatment method for the improvement of the antioxidant and antibacterial properties of Kékfrankos GSE which can be also applied in the future on the seeds of other grape varieties and other plants.

2. Materials and methods

2.1. Chemicals and reagents

Double distilled water was prepared for the extractions and chromatographic separation, using conventional distillation equipment. Ethanol, methanol, (analytical grade) and acetonitrile (LC-MS grade) were obtained from VWR International (Budapest, Hungary). Gallic acid, (+)-catechin, (−)-epicatechin, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), DPPH, 2,4,6-tripyridyl-S-triazine (TPTZ), iron(III)-chloride, acetic acid, sodium acetate, hydrochloric acid, sulfuric acid, sodium carbonate, potassium hydrogen phosphate and potassium dihydrogen phosphate were obtained from Sigma-Aldrich (Budapest, Hungary). Folin-Ciocâlteu reagent was purchased from Merck (Darmstadt, Germany). For MALDI analysis 2,5-dihydroxybenzoic acid (DHB) and trifluoracetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA), water and acetonitrile (LC-MS grade) were purchased from VWR-International (Budapest, Hungary). Columbia blood agar, amoxicillin, penicillin, Mueller Hinton agar and Mueller Hinton broth were obtained from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Sample collection and processing

Sample was collected from the Sopron wineyards of the Sopron Wine Region during October 2021 at a local wine producer (Lajos Salamon). About 5 kg of Kékfrankos grape pomace was collected and the seeds were separated by hand from grape skin and other foreign material to result 1 kg of pure grape seed. Seeds were dried in a cool dry place for 2 weeks. Seeds were then homogenized by hand and ground using a conventional household coffee grinder (Hauser grinder G-731) and stored in freezer (−20 °C) until further processing.

2.3. Microwave treatment

About 200 g of ground seed sample was dried in an desiccator for 2 days prior to extraction. Precisely weighed 15 g amounts of the dried sample were subjected to microwave treatment for 15, 30, 45, 60, 75 and 90 s using a 700 W power output household microwave oven (Vision MMO700). Non-microwave-treated dried seed material served as a control sample.

2.4. Extraction

For the determination of the total extractive content, antioxidant capacity as well for HPLC-PDA-ESI-MS/MS analyses, the extraction was carried out as follows: 1 g grape seed was homogenized with 40 mL ethanol:water 50:50 v/v solution in a 50 mL volume centrifuge tube and sonicated for 3 × 10 min in an ultrasonic bath (Elma Transsonic T570 ultrasonic bath, Elma Schmidbauer GmbH, Singen, Germany) maintaining the bath temperature between 25 and 30 °C. For extractive and antioxidant content analysis, extracts were centrifuged at 4000/min for 10 min; for HPLC analysis, 1 mL extract was centrifuged at 18.000/min for 10 min (Hettich EBA 21, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany).

For MALDI-TOF analysis, and for the determination of antibacterial activity, 20 mL of the extracts centrifuged at 4000/min for 10 min, were evaporated to dryness under a gentle stream of N_2 gas at 45 °C using an MD200-2 type sample concentrator (Hangzhou Allsheng Instruments, Hangzhou, China). These dried extracts were collected and stored in a freezer at −20 °C until use.

2.5. Total extractive content

The centrifuged extracts (2 mL) were evaporated to dryness at 70 °C in a laboratory oven and the remaining solids were dried in an exsiccator and weighed. Experiments were carried out in triplicate. The total extractive content (TEX) was expressed as % related on dry weight.

2.6. Antioxidant capacity assays

All antioxidant assays were done in triplicate using a U-1500 type spectrophotometer (Hitachi Ltd., Tokyo, Japan). The TPC was determined by the Folin-Ciocâlteu assay [30] using gallic acid standard as follows: 0.5 ml extract solution was mixed with 2.5 ml 10-fold diluted Folin-Ciocâlteu reagent. After 1 min, 2 ml 0.7 M Na_2CO_3 solution was added and the mixture was heated for 5 min in a 50 °C water bath. The reaction was stopped by cooling to room temperature in a cold water bath. The absorbance of the solution was measured at 760 nm. The results were expressed as mg equivalents of gallic acid/g dry weight (mg GAE/g d.w.). The FRAP assay was performed using trolox as the standard [31]. Results were expressed as mg equivalents of trolox/g dry weight (mg TE/g d.w.).

The DPPH radical scavenging activity of the extracts was determined as follows: 2800 μl methanolic DPPH solution (80 μM) and 200 μl extract were mixed and incubated in the dark at room temperature for 30 min. The decrease in absorbance was measured at 515

nm and the results were expressed as mg equivalents of trolox/g dry weight (mg TE/g d.w.)

2.7. The HPLC-PDA-ESI-MS/MS analysis of polyphenols

For the separation, identification and quantitative determination of the polyphenolic compounds, a Shimadzu LC-20 type liquid chromatograph coupled with a Shimadzu SPD-M20A type diode array detector (PDA) (Shimadzu Corporation, Kyoto, Japan) and an AB Sciex 3200 QTrap triple quadrupole/linear ion trap LC/MS/MS detector (AB Sciex, Framingham, USA) was used. A Phenomenex Synergy Fusion-RP 80A, 250 mm × 4.6 mm, 5 μm column was used at 40 °C for the separation with a Phenomenex SecurityGuard ULTRA LC type guard column (Phenomenex Inc., Torrance, USA). The injection volume was 15 μL. A gradient of the mobile phases A (H_2O + 0.1 % HCOOH) and B (CH_3CN + 0.1 % HCOOH) was run with 1.2 mL/min flow-rate as follows: 2 % B (0–1 min), 12 % B (25 min), 40 % B (60 min), 100 % B (70 min), 100 % B (70–80 min), 2 % B (81–92 min). Separation was carried out in triplicates from each sample.

Chromatograms were obtained from the PDA detector signal from the 250–300 nm range. The quantitative determination of (+)-catechin, (−)-epicatechin and gallic acid was done using the PDA chromatogram at the wavelength ranges of 270–285 nm for (+)-catechin and (−)-epicatechin, and 267–277 nm for gallic acid.

Identification of the polypehnolic compounds was done by mass spectrometric detection. Electrospray ionization was used for the MS detector in a negative mode. The ion source parameters were as follows: ion spray voltage: −4500 V, curtain gas (N_2) pressure: 40 psi, spray gas (N_2) pressure: 30 psi, drying gas (N_2) pressure: 30 psi, ion source temperature: 500 °C. Because of the relatively high flow rate of the mobile phase, flow-splitting was applied using a split valve, which allowed 0.6 mL/min flow to enter the MS ion source. Mass spectrometric fragmentation data was acquired using the Information Dependent Analysis (IDA) scanning function of the mass spectrometer by performing automatic on-line MS/MS experiments; survey (Q1) scans were performed between 150 and 1700 *m/z*. After the selection of a particular *m/z* ion and Q2 fragmentation, the dependent (Q3) product ion scans were performed between 80 and 1700 *m/z*. The recorded MS/MS spectra were evaluated using scientific data found in the literature. Data were acquired and evaluated using the Analyst 1.6.1 software.

2.8. The MALDI-TOF analysis of the samples

Measurements were performed on a Bruker UltrafleXtreme mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) according to the modified method of the authors [32], as follows: a 2,5-dihydrohybenzoic acid (DHB) was prepared at a concentration of 50 mg/mL in acetonitrile:water:trifluoroacetic acid 50:50:0.1 v/v/v. Positive reflector mode in the 0–5 kDa range was used. The final spectra were averaged from 5000 laser shots/spectra for each sample spot. The laser power was set 5–10 % above the threshold. Before the MALDI-TOF analysis, the dried extracts (20 mg) were reconstituted in a solution of acetonitrile:water 30:70 v/v (500 μL). Samples were mixed on the Multi-Rotator (Multi Bio RS-24, Biosan, Riga, Latvia) for 1 h, then 0.5 μL of each sample was spotted onto a MALDI plate, air-dried, and then 0.5 μL of a DHB solution was applied and again air-dried.

2.9. Antibacterial assays

2.9.1. Cultivation of bacterial strains

The tested bacterial cultures causing infections (Gram-positive *Staphylococcus aureus* (CCM 4223), methicillin-resistant *Staphylococcus aureus* (ST239) and Gram-negative *Escherichia coli* (CCM 3954)) were obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic. The bacterial strains were stored as a frozen stock solution in 20 % (*v/v*) glycerol at −80 C. Before use, the strains were thawed, and the glycerol was washed with sterile water. The strains were cultivated over night at 37 °C on a shaker at 600 rpm using 5 % Columbia blood agar.

2.9.2. Measurement of antibacterial activity

About 50–200 mg of the dried extracts (prepared according to section 2.4) were dissolved in 1 % ethanol to get a final concentration of 1 mg/mL. Pure 1 % ethanol solution was used as a blank probe.

2.9.3. Disc diffusion test

The qualitative antimicrobial effect of extracts in a concentration of 2 mg/mL was tested by DDT [33]. The bacterial inoculum was prepared from a suspension of 0.5 Mc Farland density in Mili-Q water. As a positive control, amoxicillin was used for EC, whereas penicillin was used for SA and MRSA. 6 mm sterile paper discs were used and 20 μL of each extract was transported onto each disc. These discs were placed in Mueller Hinton Agar and incubated at 37 °C for 24 h. After incubation, the inhibition zones were determined. The DDT was run in duplicates.

2.9.4. Minimal inhibitory concentration

To measure the MIC [34], 96-well microtiter plates were applied, using a two-fold Mueller Hinton broth to obtain inoculum suspension of 0.5 Mc Farland density followed by a 100-times dilution to cell density 1-2-times 10^6 CFU/mL. The extracts were diluted in sterile Mili-Q water to obtain selected concentrations (0.016–1.0 mg/mL) with 100 μL of bacterial inoculum pipetted into each well of the microplate. Distilled water and bacterium was used as a control. Absorbance (620 nm) was determined at the beginning of the reaction and after 24 h. Inoculum with extracts was cultivated at 37 °C on a plate shaker at 120 rpm. Besides minimal inhibitory

concentration, the results were also evaluated as IC_{50} value (mg/mL), which is the extract concentration that causes 50 % growth inhibition of tested bacterial strains. The MIC was performed in triplicates.

2.9.5. Growth curve of bacterial strains after exposure to extracts

The growth rate of pathogenic bacteria after exposure of extracts was determined using the broth dilution method with 100-wells microtiter plates. The same extract preparation procedure was carried out as for the MIC assay with concentrations between 0.004 and 1.0 mg/mL. The growth rate of strains was measured by a Bioscreen C MBR device (Dynex, Czech Republic). Absorbance (600 nm) was monitored at 30 min intervals for 24 h at 37 °C. The growth curve was performed in duplicates.

2.10. Statistical evaluation

For the comparison of respective results, ANOVA analysis was run using Statistica 11 (StatSoft Inc., Tulsa, USA) software with the Tukey HSD calculation method for the post-hoc test. Values of the measurements were first checked for normal distribution, and then the variables were checked for the homogeneity of variances using Bartlett's Chi-square test.

3. Results and discussion

3.1. Evaluation of extractive content and antioxidant capacity

The beneficial effects of many natural extracts are closely related to their content of antioxidant compounds. For this reason, the total extractive content as well as the antioxidant activity of the samples were assessed and compared. Table 1 summarizes the total extractive contents as well as the TPC, FRAP and DPPH antioxidant capacities of the samples treated with different durations of microwave irradiation at 700 W power. Sample 6 was slightly toasted, while sample 7 was slightly burnt (charred), yet for the sake of comparison they were not excluded from evaluation. No visual changes were observed on other samples.

The TEX content of the control (1) sample was 13.5 ± 0.7 %, which increased significantly as an effect of the microwave treatment, with the highest values determined in sample 2 (18.2 ± 1.0 %) and sample 5 (18.4 ± 0.9 %). The lowest TEX content was found in sample 7 (12.0 ± 1.4 %). The values are in accordance with literature data on grape seed extractive contents [6]. The TPC as well as DPPH and FRAP antioxidant capacities also increased significantly with microwave treatment. The overall highest values were also found in samples 2 and 5. Compared to sample 1 (control), the TEX increased by 34 % and 36 %, the TPC increased by 25 and 28 % the FRAP increased by 19 % and 20 %, and the DPPH increased by 23 % and 38 % in samples 2 and 5 respectively. The mild decease of the values between samples 2 → 4 was not found to be significant, except for FRAP, which was not explained. There was a significant decrease for all measured parameters in samples 5 → 7, indicating the degradation processes.

According to literature, the polyphenolic content of grape seeds ranges between 4 and 10 % depending on the grape variety [10], which were in accordance with the values (4.9–6.3 %) calculated using the TPC values in Table 1. The TPC results are also comparable with results for other grape varieties [9,18,19,35,36] ranging between 5.66 and 125.52 mg GAE/g dw. However, it must be noted that TPC results do not only depend on the variety, but are also influenced by the solvent composition as well as extraction method and circumstances. The FRAP and DPPH antioxidant capacity results are difficult to compare with other researchers' data [18,37,38], as these assays are often run and evaluated differently (e.g. using different extraction method, standards, units) which should encourage the standardization of these methods. Our results indicate that direct microwave treatment leads to the change of the chemical composition of the GSE, with mild treatment increases antioxidant capacity and extractive content while strong treatment triggers adverse effects, which indicates that not only the concentration but also the composition of the GSE is changed, requiring the analysis of the molecular constituents of the extracts.

3.2. Liquid chromatographic/mass spectrometric analysis

Extractives contributing to the antioxidant properties of grape seeds can be either hydrophylic (polyphenols) or lipophilic (tocopherols, tocotrienols) [5,6,8,23]. The aqueous ethanol solution primarily faciliates the extraction of hydrophilic compounds. Thus the separation, identification and quantification of polyphenolic compounds is best accomplished using reverse phase (C18) high-performance liquid chromatography, coupled with photodiode array- and tandem electrospray mass spectrometry detection [39,

Table 1
The total extractive contents (TEX) as well as the TPC, FRAP and DPPH antixodant capacities of the samples. Results are indicated as mean ± standard deviation. Different superscript letters indicate significant differences at $p < 0.05$ level except for TPC ($p < 0.04$).

Sample	MW treatment (sec)	Remark	TEX (%)	TPC (mg GAE/g dw)	FRAP (mg TE/g dw)	DPPH (mg TE/g dw)
1	0	Control	13.5 ± 0.7^{ab}	48.9 ± 1.3^{a}	55.1 ± 2.27^{a}	26.4 ± 1.2^{a}
2	15	n/a	18.2 ± 1.0^{c}	61.1 ± 4.1^{c}	65.7 ± 1.2^{b}	32.6 ± 1.8^{bc}
3	30	n/a	16.5 ± 0.8^{c}	55.1 ± 2.3^{abc}	51.3 ± 4.7^{a}	31.0 ± 1.2^{bc}
4	45	n/a	16.0 ± 0.6^{bc}	56.8 ± 3.0^{abc}	51.9 ± 1.2^{a}	30.8 ± 1.4^{bc}
5	60	n/a	18.4 ± 0.9^{c}	62.9 ± 1.1^{c}	66.2 ± 2.26^{b}	36.6 ± 1.0^{d}
6	75	slightly toasted	17.0 ± 0.7^{c}	58.6 ± 1.6^{bc}	64.0 ± 3.08^{b}	33.4 ± 0.6^{c}
7	90	slightly burnt	12.0 ± 1.4^{a}	50.6 ± 5.1^{ab}	52.9 ± 1.3^{a}	29.8 ± 1.3^{b}

40].

According to the literature data, the most frequent polyphenols in GSEs are phenolic acids (gallic-, coumaric-, protocatechuic-, hydroxbenzoic-, chlorogenic-, syringic-, caffeic-, ferulic acid), flavonoids and their glycosides (quercetin, rutin, kaempferol derivatives), catechins ((+)-catechin, (−)-epicatechin, (epi)catechin-gallate, gallocatechin), procyanidins (dimers to undecamers) and stilbenes (*trans*-resveratrol) with catechins and procyanidins usually being the most frequent components in aqueous extracts [5,7,9,11,18,41,42].

The list of the identified compounds is included in Table 2, Fig. 2 depicts the HPLC-PDA (250–300 nm) chromatograms of the GSE samples. Altogether 50 compounds have been tentatively identified and described by using the literature data [11,43,44] including gallic acid, monogalloyl-glucose, (+)-catechin, (−)-epicatechin, quercetin, kaempferol, and most abundantly a great variety of procyanidin compounds. *Trans*-resveratrol or other stilbenes were not found in the samples. Peaks 2, 3, 4 and 7 appear only in the chromatogram of sample 7, and are presumably polar degradation products with a poor ionization, yielding no mass spectrometric data.

By comparing the chromatograms of different samples in Fig. 2, it was observed that chromatographic profiles differ significantly, primarily in the intensities of corresponding peaks which can be attributed to the effect of the microwave treatment. Generally, peaks

Table 2
Chromatographic/mass spectrometric identification of Kékfrankos grape seed polyphenols.

Peak	t_r (min)	Compound	$[M-H]^-$ m/z	MS/MS *m/z*
1	4.72	Gallic acid	169	125
2	5.7	Unknown	n/a	
3	6.2	Unknown	n/a	
4	6.8	Unknown	n/a	
5	7.2	Monogalloyl-glucose	331	313, 295, 271, 241, 211, 169, 125
6	10.9	Procyanidin dimer	577	451, 425, 407, 289, 245, 125
7	12.5	Procyanidin dimer	577	451, 425, 407, 289, 245, 125
8	13.1	(+)-Catechin	289	245, 203, 125, 123, 109
9	13.3	Procyanidin dimer	577	451, 425, 407, 289, 245, 125
10	13.8	Procyanidin trimer	865	739, 695, 755, 407, 289, 125
11	14.25	Procyanidin dimer	577	451, 425, 407, 289, 245, 125
12	15.5	Procyanidin trimer	865	739, 695, 755, 407, 289, 125
13	15.9	Procyanidin tetramer	1153	865, 695, 577, 451, 407, 289, 245, 125
14	16.1	Procyanidin dimer	577	451, 425, 407, 289, 245, 125
15	16.6	Procyanidin dimer	577	451, 425, 407, 289, 245, 125
16	17.8	Procyanidin trimer	865	739, 695, 755, 407, 289, 125
17	18	Procyanidin dimer	577	451, 425, 407, 289, 245, 125
18	18.44	(−)-epicatechin	289	245, 203, 125, 123, 109
19	19	Procyanidin pentamer	1441	$[M-2H]^{2-}$: 720; 865, 577, 451, 425, 289, 125
20	20.5	Procyanidin trimer monogallate	1017	865, 729, 695, 577, 451, 407, 289, 169, 125
21	20.7	Procyanidin dimer monogallate	729	451, 407, 289, 245, 169, 125
22	21.4	Procyanidin trimer monogallate	1017	865, 729, 695, 577, 451, 407, 289, 169, 125
23	21.8	Procyanidin dimer monogallate	729	451, 407, 289, 245, 169, 125
24	23.4	Procyanidin dimer monogallate	729	451, 407, 289, 245, 169, 125
25	23.6	Procyanidin dimer monogallate	729	451, 407, 289, 245, 169, 125
26	24.2	Procyanidin tetramer	1153	865, 695, 577, 451, 407, 289, 245, 125
27	24.6	Procyanidin trimer	865	739, 695, 755, 407, 289, 125
28	24.7	Procyanidin dimer monogallate	729	451, 407, 289, 245, 169, 125
29	25.2	Procyanidin pentamer	1441	$[M-2H]^{2-}$: 720; 865, 577, 451, 425, 289, 125
30	25.3	Procyanidin dimer	577	451, 425, 407, 289, 245, 125
31	25.5	Procyanidin tetramer	1153	865, 695, 577, 451, 407, 289, 245, 125
32	26.1	Procyanidin trimer monogallate	1017	865, 729, 695, 577, 451, 407, 289, 169, 125
33	26.7	Procyanidin pentamer	1441	$[M-2H]^{2-}$: 720; 865, 577, 451, 425, 289, 125
34	26.9	Procyanidin trimer monogallate	1017	865, 729, 695, 577, 451, 407, 289, 169, 125
35	27.7	(epi)Catechin monogallate	441	289, 169, 125
36	30.6	Procyanidin tetramer	1153	865, 695, 577, 451, 407, 289, 245, 125
37	30.8	Procyanidin pentamer	1441	$[M-2H]^{2-}$: 720; 865, 577, 451, 425, 289, 125
38	31.5	Procyanidin dimer digallate	881	729, 577, 451, 407, 289, 169, 125
39	31.6	(epi)Catechin monogallate	441	289, 169, 125
40	31.7	Procyanidin trimer monogallate	1017	865, 729, 695, 577, 451, 407, 289, 169, 125
41	32.8	Procyanidin trimer digallate	1169	1017, 881, 729, 577, 289, 169
42	33	Procyanidin trimer monogallate	1017	865, 729, 695, 577, 451, 407, 289, 169, 125
43	33.2	Procyanidin tetramer monogallate	1305	1153, 865, 729, 451, 407, 169
44	35	Procyanidin trimer monogallate	1017	865, 729, 695, 577, 451, 407, 289, 169, 125
45	35.1	Procyanidin trimer digallate	1169	1017, 881, 729, 577, 289, 169
46	37	Procyanidin trimer monogallate	1017	865, 729, 695, 577, 451, 407, 289, 169, 125
47	37.7	Procyanidin dimer monogallate	729	451, 407, 289, 245, 169, 125
48	39.1	Procyanidin dimer digallate	881	729, 577, 451, 407, 289, 169, 125
49	41.5	Quercetin	301	273, 255, 233, 179, 151
50	46	Kaempferol	285	267, 229, 211, 159

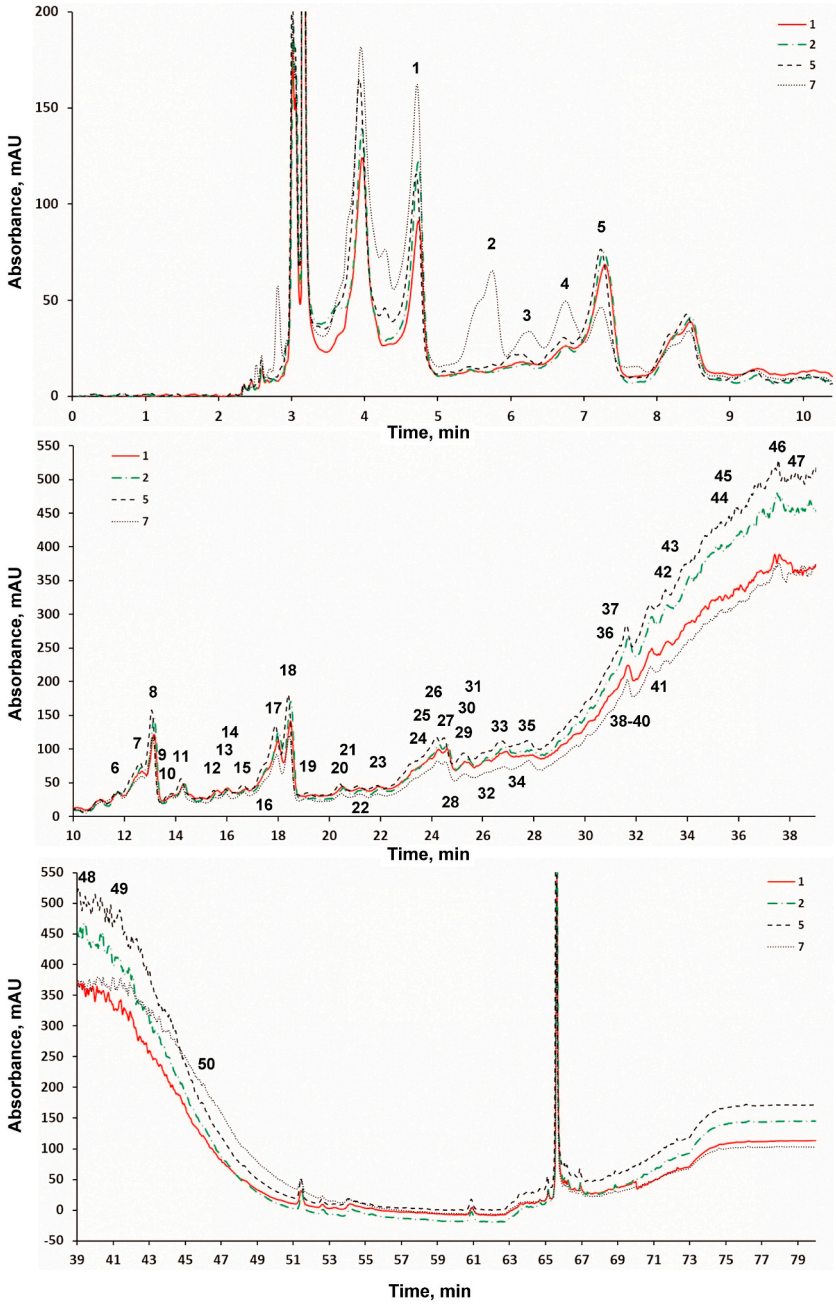

Fig. 2. The HPLC PDA (250–300 nm) chromatogram of the GSE extracts 1, 2, 5 and 7.

are higher in samples 2 and 5 which was attributed to better extraction efficiency due to microwave treatment, whereas in sample 7 the decrease of peaks was explained by degradation.

According to literature, the various types of pretreatments on grape seed have a significant effect on the composition of extractives. As shown earlier, heat treatment significantly increased the concentrations of caffeine and gallocatechin gallate in GSE, suggesting that heating could be used as a method to improve the antioxidant activity of grape seed extracts, however too intense treatments may cause degradation effects [45,46]. Ultrasonic treatment (particularly in an ultrasonic bath) produced a considerable increase of the peaks corresponding to catechin, oligomeric- and polymeric procyanidin up to 49, 41 and 35 %, respectively, in GSE [42]. The direct microwave treatment of Cabernet Sauvignon grapes improved the wine's chromatic characteristics, significantly increasing the content of anthocyanins, tannins, and stable pigments [24]. According to the results of Oomah et al. microwave conditioning of grape seeds produced changes in the quality of their oil, with such positive effects, as a decrease in chlorophyll level and an increase in α-tocopherol and α- and γ-tocotrienols [23].

Table 3 includes the quantitative evaluation of the most prominent catechins ((+)-catechin and (−)-epicatechin) and phenolic acids (gallic acid) in the Kékfrankos GSE. The literature values of (+)-catechin, (−)-epicatechin and gallic acid content of grape seeds range between 30 and 156 mg/100g dw, 11–62 mg/100 g dw and 25–80 mg/100 g dw respectively, depending on the variety and extraction method [5,9,18]. In the Kékfrankos sample, (+)-catechin content was found to be in this range, while (−)-epicatechin leveles were higher, and gallic acid content was significantly lower compared to the respective literature values.

According to Table 3, microwave treatment significantly increases (+)-catechin and (−)-epicatechin concentrations is samples 2 and 5 compared to the untreated sample. Between samples 2 → 4 a mild significant decreasing tendency was observed that could not be interpreted. In samples 6 and 7 there was also a decrease of the concentrations which justify the results presented in the previous chapter on degradation processes accompanied by the decrease of TPC, FRAP and DPPH levels. For gallic, acid a reverse tendency was observed, concentrations significantly increased in samples 6 and 7 which are also apparent in the samples' chromatograms in Fig. 2 This was explained by the formation of gallic acid from the thermal degradation of galloylated procyanidins.

The literature and present results also confirmed that the most abundant groups of polyphenols in the GSE were the procyanidins. As the mass range of the triple quadrupole/linear ion trap mass spectrometer used in the HPLC-PDA-ESI-MS/MS analyses limits the detection of compounds only up to 1700 *m/z*, the GSE procyanidins with a higher degree of polymerization (up to undecane) and with parent ion masses up to 3600 *m/z* cannot be detected [47]. The MALDI-TOF technique was used to study the structure, degree of polymerization, degree of galloylation and mass distribution of these compounds as well as the effect of microwave treatment on these properties.

3.3. Results of the MALDI-TOF analyses

The MALDI-TOF technique has been widely used for the analysis of high molecular mass GSE procyanidins [11,41,42,48]. In general it can be concluded that results depend mostly on the grape variety, with a highest degree of polymerization up to 11. In many varieties the gallic acid esters of procyanidins are also present with a degree of galloylation up to 7 [47].

The MALDI-TOF analysis of GSE procyanidins is done in the positive ionization mode and spectra include most commonly K^+ and Na^+ adducts of the compounds. Fig. 3b shows the spectrum of sample 1 with Fig. 3a presenting high molecular mass ranges with minor signals, while Appenedix contains the respective spectra for samples 2–7 (see Supplementary Material Figs. S1–S6). According ot Fig. 3 peaks were detected up to *m/z* 2100, which corresponds to a degree of polymerization of 7, while the highest degree of galloylation was 2. Identification of procyanidins was shown by their 288 Da mass differences, while the presence of galloylation was verified by the 152 Da mass increase compared to the mass of the respective procyanidin compound.

Table 4 includes the comparative evaluation of the presence of the major identified procyanidin compounds in the seed extracts of Kékfrankos grapes. Due to the way of ionization, the MALDI-TOF technique provides qualitative information (molecular mass distribution and verification of presence of compounds) rather than quantitative data on the sample. For this reason the data in Table 4 provides only a semi-quantitative evaluation, indicating the occurrence and relative abundance of compounds represented by the signal-to-noise ratio (S/N) of the peaks. By increasing the intensity of the microwave treatment, the presence and abundance of higher molecluar mass procyanidins gradually decreases, and in sample 7 only traces can be detected. The degradation of these compounds is also verified by the formation and increasing concentration of gallic acid as detailed in previous chapter.

The presence and abundance of polymeric procyanidins is also important as they can have a significant impact on the antioxidant and antibacterial properties of the GSEs [48–51].

3.4. Antibacterial properties

According to Baydar et al. GSE showed antibacterial effects against fourteen pathogenic and spoilage bacteria [25]. GSE is known to be more effective against Gram-positive than Gram-negative bacteria [52–56], but there are only a limited number of studies on the inhibitory effects of GSE on foodborne bacteria [12,54]. Their inhibitory effects are mostly attributed to the presence of the various types of phenolic compounds [25] but other compounds (e.g. tocopherols, tocotrienols) have been known to contribute to GSE antioxidant properties [8], thus potentially contributing to antibacterial effects, too.

3.4.1. MIC values

The grape seed extracts 1, 2, 3 and 5 were shown to be effective as they caused 50 % inhibition of SA and MRSA and 2, 3 and 5

Table 3
The (+)-catechin, (−)-epicatechin and gallic acid content (mg/100g dw) of the samples. Results are indicated as mean ± standard deviation. Different superscript letters indicate significant differences at $p < 0.05$ level.

Sample	(+)-catechin	(−)-epicatechin	gallic acid
1	42.4 ± 2.3^{a}	60.7 ± 2.1^{b}	3.6 ± 0.6^{a}
2	55.2 ± 2.3^{bd}	74.2 ± 2.1^{de}	4.3 ± 0.4^{ab}
3	52.2 ± 1.9^{b}	68.3 ± 1.6^{cd}	3.4 ± 0.3^{a}
4	46.0 ± 1.6^{ac}	66.7 ± 3.2^{bc}	3.4 ± 0.3^{a}
5	57.3 ± 0.8^{d}	77.8 ± 1.4^{e}	3.8 ± 0.3^{ab}
6	50.7 ± 1.7^{bc}	68.0 ± 1.9^{c}	4.6 ± 0.4^{b}
7	44.2 ± 1.1^{a}	46.5 ± 2.3^{a}	6.3 ± 0.3^{c}

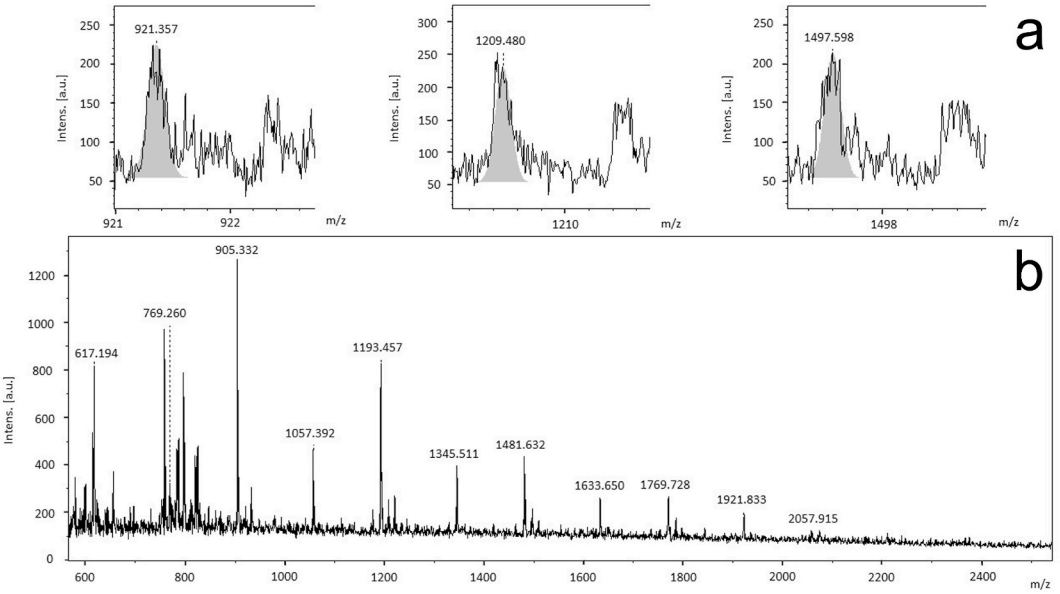

Fig. 3. Identification of minor peaks in the high molecular mass range of the MALDI-TOF spectrum (a) and the spectrum of sample 1 (b). The respective MALDI-TOF spectra of samples 2–7 are presented in Figs. S1–S6 in the Supplementary matarial.

Table 4
The MALDI-TOF analysis of the Kékfrankos GSE samples. xxx: S/N > 10, x: S/N: 3–10, t: traces (S/N:2–3), -: not detected. S/N: signal-to-noise ratio of respective peaks.

		Sample						
Compound	$[M+K]^+$	1	2	3	4	5	6	7
Procyanidin dimer	617	xxx	xxx	x	x	x	x	t/-
Procyanidin dimer monogallate	769	x	x	t	–	t	t/-	–
Unidentified	796	x	x	x	x	x	–	–
Procyanidin trimer	905	xxx	xxx	x	x	x	x	–
Procyanidin dimer digallate	921	t	t	t	t	–	–	–
Procyanidin trimer monogallate	1057	x	x	x	x	x	x	–
Procyanidin tetramer	1193	xxx	xxx	x	x	x	x	–
Procyanidin trimer digallate	1209	x	t	t	–	–	t	–
Procyanidin tetramer monogallate	1345	x	x	x	x	t	x	–
Procyanidin pentamer	1481	x	x	x	x	t	t	t/-
Procyanidin tetramer digallate	1497	x	–	t	–	–	t	–
Procyanidin pentamer monogallate	1633	x	x	x	t	–	t	–
Procyanidin hexamer	1769	x	t	t	t	–	t	–
Procyanidin hexamer monogallate	1921	x	–	t	–	–	–	–
Procyanidin heptamer	2057	t	–	–	–	–	–	–
Procyanidin hexamer digallate	2073	t	–	–	–	–	–	–

caused a 50 % inhibition of EC (Table 5). There was no observable inhibition effect for samples 4, 6 and 7.

Overall, the best MIC values were observed for sample 2 (MIC: 0.25 mg/mL for EC and MRSA and 0.125 mg/mL for SA) and sample 5 (MIC: 0.25 mg/mL for EC and SA and 0.5 mg/mL for MRSA). According to Tables 1 and 2, these two samples also showed significantly increased levels of the TPC, FRAP and DPPH antioxidant capacities as well as (+)-catechin and (−)-epicatechin levels compared to the untreated sample. The HPLC chromatograms presented in Fig. 2 also showed that the concentration of condensed tannins increased as an effect of microwave treatment in samples 2 and 5 which may also contribute to better antibacterial effects. The results

Table 5
MIC (mg/mL) values of the samples with the highest inhibition effects. Samples 4, 6 and 7 did not show any inhibiton.

	Sample			
	1	2	3	5
SA	0.25	0.125	0.5	0.25
EC	not observed	0.25	0.25	0.25
MRSA	0.25	0.25	0.5	0.5

of the MALDI-TOF analysis revealed that too strong microwave irradiation will result in the loss of condensed tannins, (especially with higher condensation degree), and according to Tables 5 and in the loss of antibacterial activity. In fact, the influence of procyanidin oligomers on antibacterial properties seems to be complex. Yoshida et al. reported that procyanidin dimers and a trimer tested against the pathogenic *Helicobacter pylori* strain showed weak antibacterial activity (MIC = 50–100 μg/mL), and a procyanidin polymer (average dodecamer) showed no antibacterial activity at 100 μg/mL [57]. However, according to other researchers, procyanidins do possess evaluable antibacterial properties against several pathogenic bacteria strains [49,50,58]. Oligomeric procyanidins showed a significant antimicrobial effect against *S. aureus*, but a lower inhibitory effect was reported against *Escherichia coli* [51].

According to our results, shorter microwave exposure times facilitated the extraction of (+)-catechin, (−)-epicatechin and procyanidin oligomers from grape seed, also increasing TPC, FRAP and DPPH levels, due to the destruction of the cell wall membranes and increasing dissolution and diffusion of compounds in the extraction solvent [24]. However, as shown in Table 3, too high microwave power will significantly destroy procyanidin compounds (samples 3 → 7) and increase the concentration of degradation products, (e.g. gallic acid).

The study of Silva et al. on the antibacterial effects of the GSE of Portuguese red wine grapes concluded that the high activity of the GSE against Gram-positive strains may be attributed to the high concentrations of catechin, epicatechin and *trans*-resveratrol [59].

Shrestha et al. reported antibacterial effects with a MIC at 0.625 mg/mL on both tested strains of *S. aureus* [60]. Sheng et al. observed a MIC value of 2 mg/mL against *E. coli* O26:H11 and 4 mg/mL against the other non-O157 *E. coli* [61]. Similar results were observed in an-other study revealing high MIC values against *P. gingivalis* (4 mg/mL) and *F. nucleatum* (2 mg/mL) [62].

The MIC determined in the present study against EC (0.25 mg/mL) was lower compared to the results of Jayaprakasha et al. [54], who reported GSE MIC values for *E. coli* O157:H7 of approximately 1 mg/mL, but was higher (0.0474 mg/mL) compared to the results of Levy et al. [55]. MIC <0.2 mg/mL and 4.0 mg/mL prevented the growth of *E. coli* O157:H7 inoculated at 3.25 and 4.43 log CFU/plate, respectively [63]. It must be noted, however that the MICs of the same bacterial species could also differ due to an intrinsic difference [64].

Comparing these values to the results of the present study, it was concluded that the MIC values of the Kékfrankos GSE were lower than the literature values which justify the potent antibacterial effects of the studied samples and the benefice of direct mild microwave treatment on antibacterial activity. Nevertheless, the use of the microplate method and the discrepancies in methodology or using different extraction methods may also cause differences in the MIC values and also contribute to high values (1–4 mg/mL) [65]. Further studies must be performed to investigate the effect of GSE on the reduction of growth of human pathogens.

3.4.2. Disc diffusion test

The agar disc diffusion method is considered as a standard screening test to check the antimicrobial activity of GSE [60]. The disk diffusion test revealed that samples 2, 3 (eventually 4) showed moderate activity against SA with inhibition zones of 8 mm–7 mm and the extracts 1, 2 also showed an inhibition zone of 9 mm–7 mm against MRSA. Against EC, minimal or no reactivity (no zones) was found (Fig. 4). The results of DDT are in accordance with the results of MIC: both tests revealed that sample 2 was effective against SA and MRSA strains.

3.4.3. Bacterial growth curves

According to MIC and DDT tests sample 2 showed the most promising results; thus bacterial growth curves are presented only for this sample. Fig. 5 depicts the growth curves of bacteria after treatment with various concentrations of the extract solution of sample 2. The increasing level of the seed extract concentrations generally shows an increase in their inhibition effects and a reduction in the rate of growth of the tested bacteria [25]. EC growth was inhibited from 0.25 mg/mL and higher concentrations, while SA and MRSA growth was inhibited from 0.015625 mg/mL and higher concentrations.

Low concentrations (0.5 mg/mL) of GSE could reduce AI-2 production in all tested non-O157 Shiga toxin-encoding *E. coli* strains [61]. It was shown that grape seed phenolic extract had a bactericidal effect on the Gram-positive *B. linens* bacterium and reduced the microbial growth of *E. coli* after 48 h incubation on a solid medium [66]. The studies of Zillich et al. showed the beneficial effects of

Fig. 4. DDT of the 7 tested grape seed extracts using three pathogenic strains. (c is control sample without extract). SA: *Staphylococcus aureus*, MRSA: *Methicillin-Resistant Staphylococcus aureus,* EC*: Escherichia coli.*

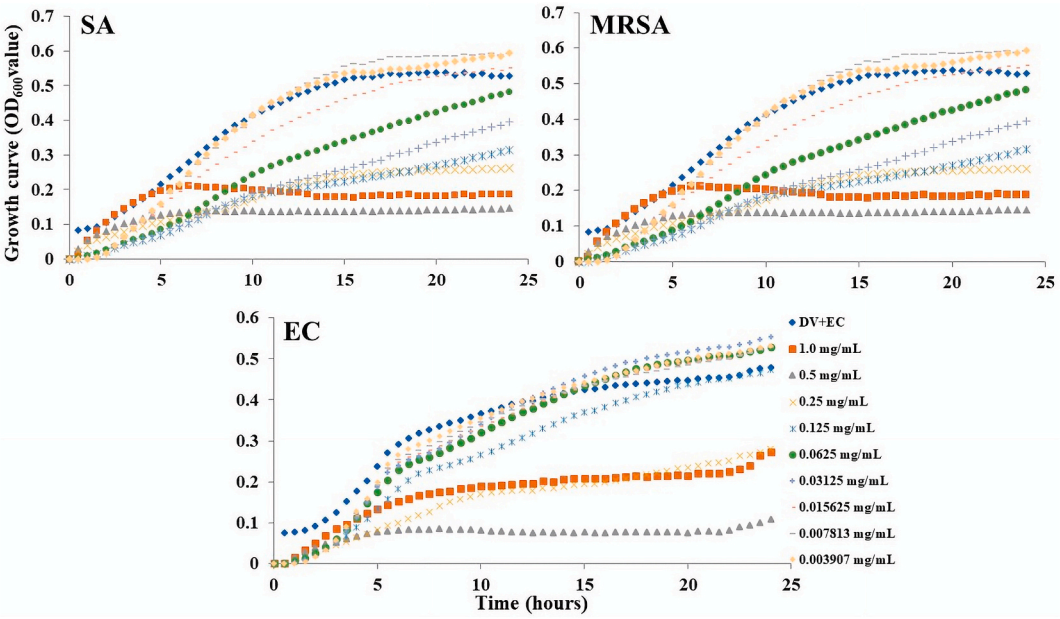

Fig. 5. Growth curves of three tested bacteria with sample 2. Concentration range: 0.004–1.0 mg/mL. SA: *Staphylococcus aureus*, MRSA: *Methicillin-Resistant Staphylococcus aureus,* EC: *Escherichia coli*.

polyphenols in protection against human skin pathogens and have resulted the wide application of polyphenol-rich grape extracts in numerous skin care products [67,68]. Other researchers concluded that GSE inhibits the growth of oxacillin-resistant *Staphylococcus aureus* and it exerts an abhesive effect against it [69]. Al-Mousawi et al. indicated that *Staphylococcus aureus* and *Staphylococcus haemolyticus* bacterial isolates were able to produce a biofilm which was prevented by the methanolic extracts of the crude seeds of *Vitis vinifera* rich in galloylated catechin esters of gallic acid [29].

Results on the growth curves of sample 2 show that extracts of mild microwave-treated seeds of Kékfrankos grapes inhibit the growth of the tested SA and MRSA strains even at low concentrations which also justify the importance of further pharmacological investigations.

4. Conclusion

The present study investigated for the first time the polyphenolic composition, antioxidant and antibacterial properties of the extracts of the Kékfrankos grape variety, which is the most frequently grown wine-grape variety in Hungary. Using liquid-chromatography/mass spectrometry analysis 50 compounds have been tentatively identified and described, including gallic acid, (+)-catechin, (−)-epicatechin, quercetin, kaempferol and procyanidins. MALDI-TOF analysis of the samples revealed that the highest degree of polymerization of procyanidins is 7 while the galloylation degree of procyanidins is 0–2. In order to increase antioxidant and antibacterial properties of the extracts, the seeds were directly treated with microwave irradiation – an approach used for the first time in the literature to the best of our knowledge. It was found that mild microwave treatment significantly increased the total polyphenol, FRAP and DPPH levels as well as (+)-catechin, (−)-epicatechin concentrations by 20–30 % and resulted in significantly better antibacterial properties against the tested *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* strains compared to the untreated sample's extract. Too long treatment times caused these values to decrease and also the degradation of procyanidins. By optimization of the applied microwave energy, treatment time and amount of the treated seed material further improvement of antibacterial and antioxidant could be achieved in the future and treatment method can also be adapted to the seed material of other grape varieties. The prepared extracts can be utilized in food industry, medicine, pharmacology and cosmetics for example as bio-preservatives and functional ingredients in active and intelligent packaging systems, in medical preparations against human pathogens, for the production of nanoparticles and in the research of new drugs with antioxidant properties. However, potential degradation products of microwave treatment need also to be analyzed in the future. The utilization of grape seed byproduct fortifies circular economy and could also represent added value for local wine producers. Results also justify the importance of further pharmacological investigations on Kékfrankos grape seed extracts and the use of direct microwave energy on grape seeds to enhance the antibacterial properties of the extracts.

5. Data availability statement

Data will be made available on request.

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CRediT authorship contribution statement

Tamás Hofmann: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Eszter Visi-Rajczi:** Formal analysis, Investigation. **Silvia Vaculciakova:** Data curation, Formal analysis, Methodology, Writing – original draft. **Roman Guran:** Conceptualization, Writing – original draft, Writing – review & editing. **Stanislava Voberkova:** Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Martina Vrsanska:** Data curation, Investigation, Writing – original draft, Writing – review & editing. **Ondrej Zitka:** Conceptualization, Funding acquisition, Methodology, Writing – original draft. **Levente Albert:** Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:T. Hofmann reports financial support was provided by National Research Development and Innovation Office. S. Vaculciakova reports financial support was provided by Mendel University in Brno, Czechia. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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