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THE PHYTOCHEMICAL AND ANTIMICROBIAL EXAMINATION OF PHENOLIC ACIDS CONTAINED IN EXTRACTS FROM FOUR *MARRUBIUM* SPECIES

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Abstract: The aim of the work done was the qualitative and quantitative assessment of a polyphenolic fraction contained in methanolic extracts obtained from the flowering herbs of *Marrubium incanum* Desr., *M. peregrinum* L., *M. thessalum* Boiss. & Heldr., and *M. candidissimum* L. The samples containing phenolic acids, having undergone acid and alkaline hydrolysis, were investigated by TLC, RP-HPLC, and Q-TOF-MS. In all methanolic extracts of *Marrubium* species, neochlorogenic, chlorogenic, protocatechuic, p-hydroxybenzoic, caffeic, *p*-coumaric, gentisic, ferulic acids and flavonoids: rutin, kaempferol rhamnohexoside, kaempferol hexoside, isorhamnetin hexoside, apigenin were identified. This is the first study dedicated to the qualitative and quantitative analysis of polyphenolic compounds in flowering herbs of selected *Marrubium* sp. Both the total phenolic content (TPC) and the antioxidant activity of water extracts obtained from aerial parts were assessed. TPC was estimated with the spectrophotometric method, whereas the antioxidant activity was evaluated by DPPH assay using Trolox, rutin, caffeic, and gallic acid as reference substances. We also presented the evaluation of antioxidant activity of 70% (v/v) methanolic extracts, that was expressed both as a percentage of DPPH radicals inhibition and IC_{50} values (mg/mL). The same *Marrubium* extracts showed the highest concentration of phenolic acids. Ethanolic (70%, v/v), methanolic, acetone and water extracts of *Marrubium* sp. were screened for their antibacterial and antifungal activity using the agar microdilution method against a panel of reference strains of 20 microorganisms, including Gram-positive and Gram-negative bacteria and fungi belonging to yeasts.

Keywords: *Marrubium* sp., HPLC, phenolic acids**,** antimicrobial activity, antioxidant activity

The *Marrubium* L. genus (Eng. horehound) belongs to the family Lamiacae and comprises about 97 species reported for their medicinal properties. Selected *Marrubium* taxa are widely known in traditional and modern medicine as they are used as choleretic, digestive, anti-inflammatory, antihypertensive, antispasmodic, analgetic, antimicrobial, insecticidal, even antileukemic agents and cytotoxic/cytostatic effects against four human cancer cell lines, specifically HeLa, MCF-7, FM3 and HCT-116. (1-5). In the group of active substances identified in various horehound species, diterpenoids, sterols, and phenylpropanoids (including caffeic acid derivatives and flavonoids) have been reported (4, 5).

The aim of the research was to determine the total phenolic content (TPC) and provide qualitative and quantitative analysis of phenolic acids (FAs) in ethanolic 70% (v/v), methanolic, acetone and water extracts obtained from the flowering herbs of *M. incanum*, *M. peregrinum*, *M. thessalum* and *M. candidissimum*. For this purpose, spectrophotometric, TLC and RP-HPLC/PDA methods were used. Simultaneously, the antimicrobial and antioxidant assays of the aforementioned extracts have been realized.

MATERIALS AND METHODS

Plant material

According to the seed exchange policy, the seeds of four *Marrubium* species: *M. incanum* Desr. (Botanical Garden University of Tartu, Estonia), *M. peregrinum* L. (Core Facility Botanischer Garten der

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Universtät Wien, Austria), *M. thessalum* Boiss. & Heldr. and *M. candidissimum* L. (Botanischer Garten und Botanisches Museum Berlin-Dahle, Berlin, Germany) were obtained. They were cultivated for the planned phytochemical and biological experiments in Medicinal Plant Garden (a special unit of the Department of Pharmacognosy, Medical University of Lublin, Poland). Flowering herbs were collected in July. Plant material was authenticated by a specialist on plant taxonomy – Prof. K. Głowniak (Medical University of Lublin), and deposited in the herbarium of the Department of Pharmacognosy as voucher specimens *Marrubium* 01-04/2016. Plant material was dried and powdered according to the guidelines of the Polish Pharmacopoeia the 6th.

Reagents

All chemicals used for extraction, TPC and radical scavenging assays, including methanol, ethanol, acetone, acetonitrile, benzene, formic acid, sodium formate, Folin-Ciocalteu reagent were purchased from Avantor Performance Materials (Gliwice, Poland). The solvents for HPLC analysis (methanol, acetic acid) and for ESI-QTOF-MS analysis (acetonitrile, ammonium formate) were purchased from J.T. Baker (Deventer, Holland). The standards of phenolic acids, flavonoids, DPPH, sulphanilic acid, sodium carbonate were obtained from Sigma Aldrich (St. Louis, MO, USA).

Extraction techniques used for the isolation of phenolic acids from horehound species

Accelerated Solvent Extraction (ASE)

ASE was performed using a previously elaborated method published by Kozyra and Skalicka-Woźniak (6). Dried, powdered flowering herbs (1.0 g) of the selected horehound species was subjected to accelerated solvent extraction in ASE 100 apparatus (Dionex, Sunnyvale, CA, USA). The following extraction conditions were used: 70% methanol as an extraction solvent, temperature of 85^{o}C, flush volume: 60%, purge time: 150 s, number of cycles: 3, duration of a cycle: 10 min. The obtained extracts were concentrated under reduced pressure at 50^{o}C, dissolved in methanol and filtered (PTFE Cronus Syringe Filter, 25mm, 0.45 μm) into 10 mL calibrated vials. Then they were analyzed by RP-HPLC, followed by the investigation of their antioxidant and antimicrobial activities.

Preparation of plant extracts using other extraction methods

Milled plant samples (7.0 g) of horehound species were soaked in 50 mL of 70% (v/v) methanol in a round-bottomed flasks, then extracted under reflux (at a boiling extractant temperature) for 30 min. Each extract was carefully filtered through a wad of cotton wool and the plant material was re-extracted twice with a fresh portion (50 mL) of solvent. The obtained methanolic extracts were evaporated to dryness and dissolved in 50 mL of hot water and left in a refrigerator for 24 h. The precipitated ballast substances were filtered off through a paper filter and the filtrates were extracted with diethyl ether (5 x 40 mL). Diethyl ether and aqueous fractions were obtained for each horehound species, that were subjected to phenolic acids (FAs) analysis with the method described by Schmidtlein & Hermann (7) and Ibrahim and Towers (8). As a result of these procedures, three polyphenolic fractions: FA (free FAs), FB (FAs after acidic hydrolysis) and FC (FAs after alkaline hydrolysis) were obtained. They were further evaporated to dryness, dissolved in 1 mL of methanol, and analyzed by two-dimensional thin-layer chromatography (2D-TLC) on cellulose plates, followed by RP-HPLC.

Preparation of extracts for microbiological studies

Milled samples (5.0 g) of four horehound species were soaked in 50 mL of 70% (v/v) methanol, methanol, 96% ethanol, acetone, water in a 250 mL round-bottomed flasks, then extracted under reflux in solvent boiling points for 30 min. Each extract was re-extracted twice with the same portion of extractant. Each final extract obtained was evaporated to dryness and, together with FA, FB, FC fractions, dissolved in 1 mL of dimethyl sulfoxide (DMSO), and screened *in vitro* for antibacterial and antifungal activities using the broth microdilution method.

Qualitative and quantitative analysis of FAs

Qualitative two-dimensional (2D) thin-layer chromatographic (TLC) analysis

Qualitative identification of FAs was performed using 2D-TLC on 100 x 100 x 0.1 mm cellulose plates (E. Merck, Darmstadt, Germany). Different available standards of FAs, i.e. ferulic, vanillic, protocatechuic, *p*-hydroxybenzoic, *p*-coumaric, caffeic, gallic, chlorogenic, syringic and gentisic were used for identification. Each fraction containing FAs and standards was spotted on 2D TLC plates and the plates were developed in horizontal DS chambers (Chromdes, Lublin, Poland) using the following mobile phases: benzene-methanol-acetonitrile-acetic acid (80 : 10 : 5 : 5, v/v/v/v) – first direction; water-formic acid-sodium formate (200 : 1 : 10, v/v/w) – second direction. (9, 10) Before the

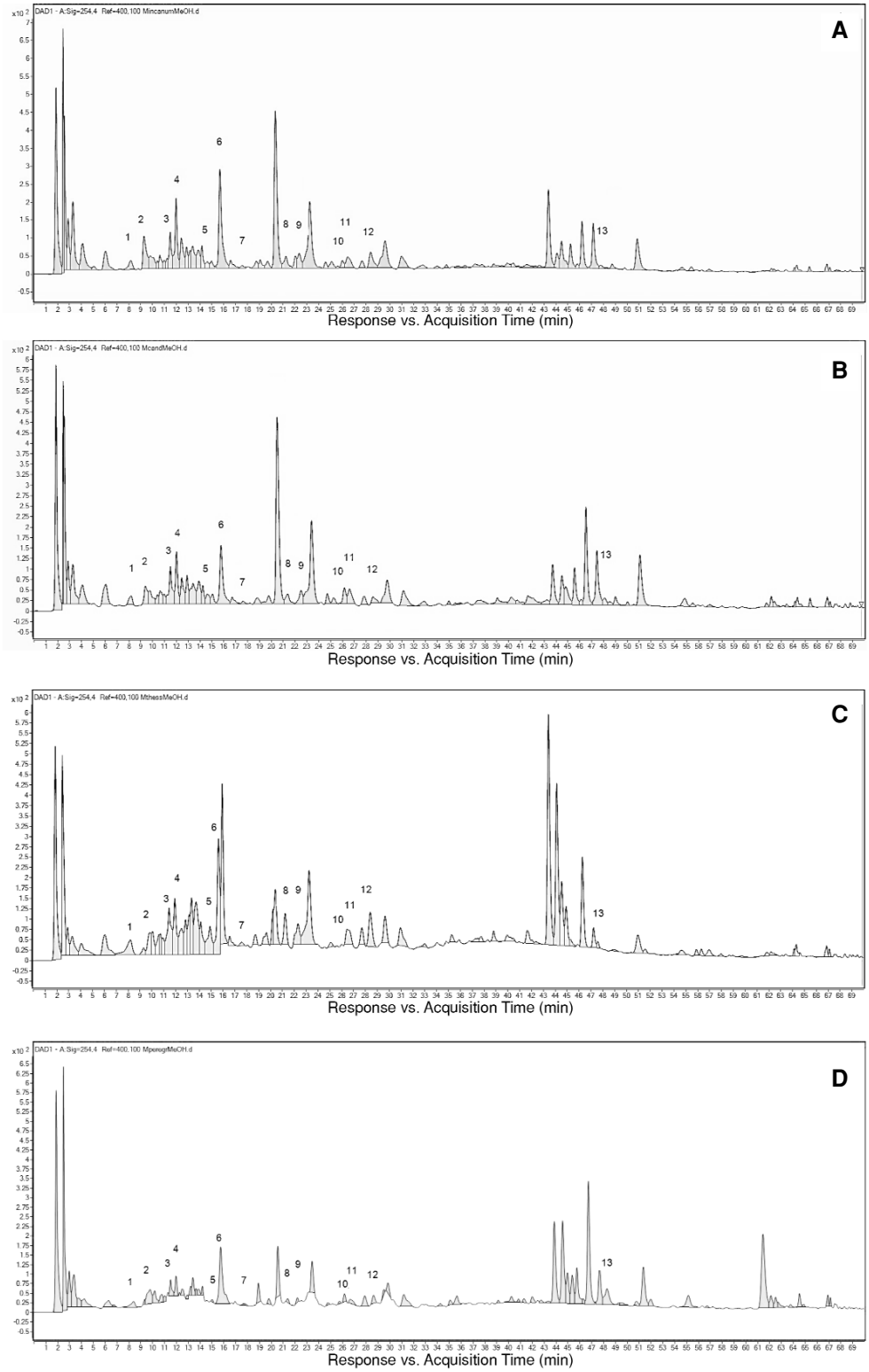

Figure. 1. HPLC–DAD chromatograms of 70% (v/v) methanolic extracts from: A) *M. incanum,* B) *M. candidissimum*, C) *M. thessalum,* D) *M. peregrinum* obtained using ASE.

Peaks: 1- protocatechuic acid 2- neochlorogenic acid, 3- p-hydroxybenzoic, 4- chlorogenic acid, 5- gentisic acid, 6- caffeic acid, 7- p-coumaric acid, 8- rutin, 9- ferulic acid, 10- kaempferol rhamnohexoside, 11- isorhamnetin hexoside, 12- kaempferol hexoside, 13- apigenin

development, the plates were conditioned in the chamber for about 5 min in the vapors of solvent mixture benzene-methanol-acetic acid (94 : 1 : 5, v/v/v). All chromatograms were observed under UV light (λ = 254 and 366 nm). Derivatization (after 2D-TLC) was performed by spraying the plates with diazotized sulphanilic acid followed by 20% sodium carbonate aqueous solution (1 : 1, v/v). Digital photos of the sprayed plates were taken in visible light with a TLC visualizer (Camag, Muttenz, Switzerland). The compounds were identified according to their R_f values compared with those of the FAs standards.

Quantitative spectrophotometric determination of polyphenolic constituents

For the spectrophotometric (Vis) quantitation of FAs in the extracts obtained from flowering herbs of four horehound species, we used a method with the Arnov's reagent as described in the Polish Pharmacopoeia the 6th (FP VI). The absorbance measurement was done at λ = 490 nm using Helios Beta (Unicam, UK) spectrophotometer.

Additionally, total phenolic content (TPC) in aqueous extracts of *Marrubium* spp. was determined using the Folin–Ciocalteu (FC) reagent according to the method described by Singleton and Rosi (11) and Atanassova et al. (12). In order to prepare the reaction mixture, 100 µL of each extract (c = 3,52 mg/mL) was mixed with 100 µL of FC and 800 µL of Na_2CO_3 aqueous solution (0.075 g/mL). The mixture was shaken immediately, and 20 min later the absorbance was measured at 760 nm using spectrophotometric method. The TPC concentration was calculated as gallic acid equivalent (GAE) per 1 mg of each extract using the following equation obtained for GA calibration curve (R^2 = 0.9864; y = 13.838 x; mean value for n = 3). To prepare the curve, the absorbance of different concentrations of GA (0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.10 mg/mL) was measured following the procedure described above.

RP-HPLC/PDA qualitative and quantitative analysis

HPLC analysis was performed using Agilent 1100 (Agilent Technologies, USA) chromatographic system equipped with an autosampler, a column thermostat, a diode–array detector (PDA), and a Zorbax Eclipse XDB-C18 column (250 × 4.6 mm I.D., d_p= 5 µm). In order to achieve sufficient resolution of the peaks, a stepwise mobile phase gradi-

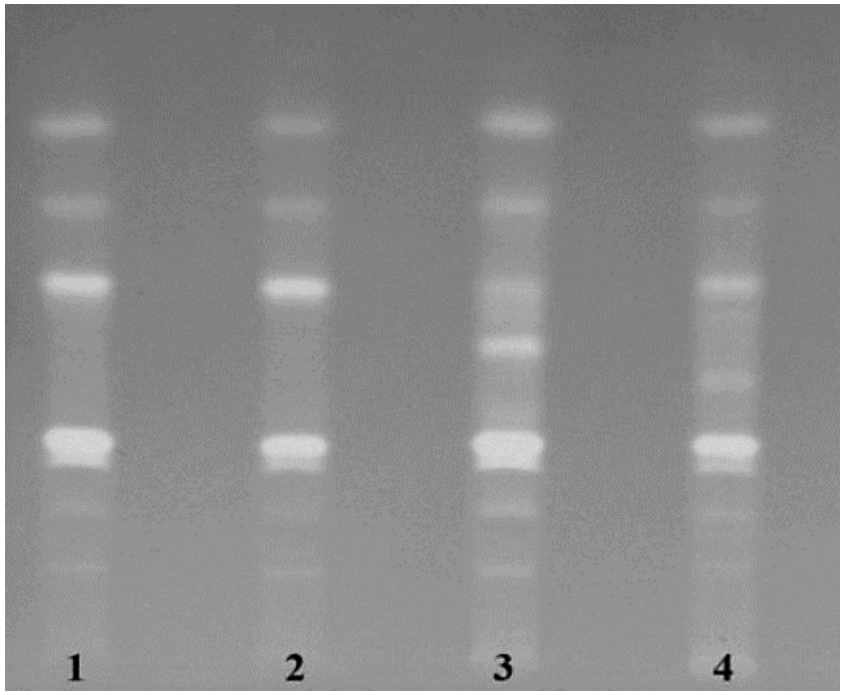

Figure. 2. TLC chromatographic separation of polyphenolic constituents occurring in 70% (v/v) methanolic extracts of four *Marrubium* species after DPPH derivatization.
1 – *M. incanum*; 2 – *M. candidissimum*; 3 – *M. thessalum*; 4 – *M. peregrinum*

Table 1. PAs identified by RP-HPLC in fractions (FA, FB and FC) obtained from *Marrubium* L. species.

PAs	*M. incanum*			*M. candidiss.*			*M. thessalum*			*M. peregrinum*		
	FA	FB	FC	FA	FB	FC	FA	FB	FC	FA	FB	FC
Ferulic	+	+	+	+	+	+	+	+	+	+	+	+
p-Cumaric	+	+	+	+	+	+	+	+	+	+	+	+
Syringic	-	+	+	-	-	-	-	+	-	-	-	-
Caffeic	+	+	+	+	+	+	-	+	+	+	+	+
Gentisic	-	+	-	-	+	-	-	+	-	-	+	-

ent prepared from 1% (v/v) aqueous acetic acid solution (component A) and methanol (component B) (v/v) was used. The concentration of component B in mobile phase changed as stated: 5-20% from 0 to 5 min, 20-35% from 5 to 20 min and 35-55% from 20 to 35 min. The flow-rate of 1 mL min^{-1}, a sample injection volume of 10 µL, and a temperature of 25°C were used. The compounds were identified by comparing the retention times and UV spectra with those of appropriate standard substances analyzed under the same conditions. The acquisition of spectroscopic data was realized at 320 nm (maximum absorption of cinnamic acid derivatives) and 254 nm (for benzoic acid derivatives).

The quantification of FAs was performed by comparing the peak areas of the compounds present in the extracts with those of the external standards. For this procedure, the same standards of FAs as in 2D-TLC analysis were used. The precision of the analytical method was evaluated using intra-day assays that were performed by replication of three aliquots of the same sample on the same day. Peak area of each of the extract components was measured and RSD (relative standard deviation, %) values were calculated.

HPLC–DAD-Electrospray Ionization Q-TOF-MS

The purified samples were analyzed quantitatively by an HPLC/ESI-QTOF- MS system in negative ion mode using a 6530B Accurate-mass-QTOF-MS (Agilent Technologies, Inc., Santa Clara, CA, USA) mass spectrometer with an ESI-Jet stream ion source. The chromatograph was equipped with DAD, autosampler, binary gradient pump, and column oven. A Eclipse XDB C18 Stable Bond chromatographic column (150 × 4.6 mm, 3.5 µm was used in the analyses. Gradient of solvents: acetonitrile (1%) with 10 mM ammonium formate (0.1%) and acetic acid 0.1% (solvent A) and acetonitrile (95%) with 10mM ammonium formate (0.1%) and acetic acid 0.1% (solvent B) were used as the mobile phases. The following gradient procedure was adopted: 0–70 min, 0-5 min, 5–15% B; 5–25 min, 15–25% B; 25–55 min, 20–45% B; 55–70 min, 45–95% B; post time 12 min. Total time of analysis was 70 min, with a stable flow rate at 0.200 mL/min. Injection volume for sample was 10 µL. ESI-QTOF-MS analysis was performed according to the following parameters of the ion Source: Dual spray jet stream ESI, positive and negative ion mode, gas (N2) flow rate: 12 L/min., nebulizer pressure: 35 psig, vaporizer temp.: 300°C; m/z range 100–1000 mass units, with acquisition Mode Auto MS/MS, collision-induced dissociation (CID): 15 and 20 eV with MS scan rate 1 spectrum per s, 2 spectra per cycle, V Cap 4000V, skimmer: 65 V, fragmentor: 145 V and octopole RF Peak: 750 V. Qualitative analysis of the extract from TF was made additionally in auto MS/MS with excluded: m/z at 966.0007 and 112.9855 for negative ion mode corresponding to the m/z of reference ions. The MassHunter software was used to record and analyze spectra. The identification of compounds present in samples was performed by comparison of MS/MS spectra with literature data and records from the METLIN database. The tentative identification of some derivatives was based on the fragmentation patterns of known compounds.

Antioxidant assays

TLC-DPHH antioxidant assay

Methanolic (70%, v/v) plant extracts (5 µL) and FAs standards (each at volume of 1 µL) were applied on TLC Kieselgel $60F_{254}$ plates, 100 × 200 mm (Merck, Germany) using an automatic sampler (CAMAG TLC Sampler III). The plates were developed on the distance of 8 cm using the mobile phase: ethyl acetate-acetic acid-formic acid-water (100 : 11 : 11 : 26, v/v/v/v) as it was described in a paper. (13) After development and drying, the plate underwent

derivatization. For this purpose, the plate was sprayed with 0.2% methanolic solution of DPHH and then placed in the dark for 30 min. Visualization and documentation of TLC chromatograms were carried out using a TLC visualizer (Camag, Muttenz, Switzerland). Zones of separated compounds showing antioxidant activities were observed as yellow spots on a purple background (Fig. 2).

Evaluation of antioxidant activity using spectrophotometric (Vis) assay

Antioxidant activity of extracts was also determined spectrophotometrically according to a slightly modified method of Brand-Williams et al. (14) using the methanolic solution of DPPH (0.0085 mg per 1 mL). Dilutions of horehound extracts were made to obtain the concentrations of 0.125, 0.188, 0.250, 0.375, 0.500, 0.625, 0.750, 0.875, 1.000, 1.125 and 1.258 mg of referred plant substance per 1 mL. The diluted extracts were mixed with 3.9 mL of DPPH methanolic solution. The mixture was shaken vigorously and left to stand for 30 min in the dark at room temperature. Next, the absorbance was measured at 517 nm against a blank in a spectrophotometer. The inhibitory concentration at which 50% DPPH radicals were scavenged (EC_{50}) was obtained by data interpolation based on linear regression analysis done in triplicate. Radical scavenging activity was calculated as the percentage of

Table 2. QTOF-MS/MS data of identified and characterized of PAs and flavonoids in 70% (v/v) methanol extracts of *Marrubium* sp.

	t_r (min)	Polyphenols	UV max	Empirical Formula	$[M-H]^-$ (m/z)	Fragment ions (m/z)	Diff (ppm)
1	8.099	Protocatechuic acid	225 260 295	$C_7H_5O_4^-$	153.0183	109.0295	6.7
2	9.404	Neochlorogenic acid	220 245 325	$C_{16}H_{17}O_9^-$	353.0891	191.0569 179.0353	-0.83
3	11.078	*p*-Hydroxybenzoic	220 260 220	$C_7H_5O_3^-$	137.0250	93.0359	-4.22
4	12.251	Chlorogenic acid	245 325	$C_{16}H_{17}O_9^-$	353.0906	179.0353	-7.89
5	14.965	Gentisic acid	235 330	$C_7H_6O_4^-$	153.0203	109.0291	-6.28
6	15.868	Caffeic acid	220 240 295 325	$C_9H_7O_4^-$	179.0351	135.0457	-0.65
7	17.711	*p*-Coumaric acid	230 315	$C_9H_7O_3^-$	163.0412	119.0504	-6.9
8	21.294	Rutin	220 260 360	$C_{27}H_{29}O_{16}^-$	609,1462	301.0339	-0,15
9	22.332	Ferulic acid	235 325	$C_9H_{10}O_4^-$	193.0508	178.0272 149.0570	-0.86
10	26.468	Kaempferol rhamnohexoside	220 265 355	$C_{27}H_{29}O_{15}^-$	593.1510	285.0405	0.33
11	26.619	Isorhamnetin hexoside	210 255 275	$C_{22}H_{21}O_{12}^-$	477.1054	315.0728	-3.24
12	28.428	Kaempferol hexoside	220 265 350	$C_{21}H_{19}O_{11}^-$	447.0956	285.0401 284.0337	-5.17
13	48.155	Apigenin	215 270 340	$C_{15}H_9O_5^-$	269.0462	159.0456	-2,42

Table 3. The mean (n = 3) content of FAs (ĕg/g, dry wt) in *Marrubium* extracts obtained using various extraction solvents.

Extracts	Species	FAs				
		Neochlorogenic	Chlorogenic	Caffeic	*p*-Cumaric	Ferulic
Methanol	*M. incanum*	223	201	55	61	3
	M. candidissimum	154	127	24	41	2
	M. thessalum	179	88	41	30	ND
	M. peregrinum	274	84	56	30	4
Methanol (70%)v/v	*M. incanum*	1096	201	67	99	3
	M. candidissimum	642	136	48	82	2
	M. thessalum	1068	200	72	77	9
	M. peregrinum	1801	151	111	128	100
Ethanol	*M. incanum*	34	109	34	31	2
	M. candidissimum	21	55	32	14	8
	M. thessalum	55	58	41	29	11
	M. peregrinum	25	74	42	19	2
Acetone	*M. incanum*	292	32	28	56	1
	M. candidissimum	517	13	37	47	1
	M. thessalum	338	25	27	39	ND
	M. peregrinum	655	33	63	80	22
Water	*M. incanum*	24	8	10	15	0.60
	M. candidissimum	21	ND	ND	ND	ND
	M. thessalum	ND	ND	ND	ND	ND
	M. peregrinum	24	ND	5	ND	0.31

ND not detected

DPPH that was scavenged using the following formula: % inhibition = [(Ab – Aa)/Ab] × 100, where: Ab-absorption of a blank sample; Aa-absorption of extract solution. To assess more precisely the scavenging properties of the investigated extracts, the EC_{50} value was calculated also for reference substances including Trolox, rutin, caffeic and gallic acid (Table 3). The results were presented as average values ± standard deviation (SD).

Evaluation of antimicrobial activity

The extracts obtained from aerial parts of *M. incanum*, *M. candidissimum*, *M. peregrinum* and *M. thessalum* were screened *in vitro* for antibacterial and antifungal activities using the broth microdilution method according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (15) and Clinical and Laboratory Standards Institute (16) guidelines. In this study, reference microbial strains from American Type Culture Collection (ATCC) were used, including Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pyogenes* ATCC 19615, *Streptococcus pneumoniae* ATCC 49619, *Streptococcus mutans* ATCC 25175, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, *Micrococcus luteus* ATCC 10240), Gram-negative bacteria (*Escherichia coli* ATCC 3521, *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 12453, *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027, *Pseudomonas aeruginosa* ATCC 27853, *Bordetella bronchiseptica* ATCC 4617) and fungi belonging to yeasts (*Candida albicans* ATCC 2091, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019).

Microbiological assay

The microbial cultures were subcultured on the nutrient agar or Sabouraud agar at 35°C for 18-24 h or 30°C for 24-48 h for bacteria and fungi, respectively. The surface of Mueller-Hinton agar or Mueller-Hinton agar with sheep blood (for bacteria) and RPMI 1640 with MOPS (for fungi) were inoculated with the suspensions of bacterial or fungal species. Microbial suspensions were prepared in sterile 0.85% NaCl with an optical density of McFarland standard scale 0.5 – approximately 1.5×10^8 CFU (Colony Forming Units)/ml for bacteria and 0.5 McFarland standard scale – approximately 5×10^5 CFU/mL for fungi.

Samples (10 mg) of 70% (v/v) methanol, methanol, ethanol, acetone, and aqueous horehound dried extracts, related to 5 g of herbal substance,

were dissolved in 1 mL dimethyl sulfoxide (DMSO). Next, 50 µL of these extracts were dropped into the wells (d = 9 mm) on the agar media. The agar plates were preincubated at room temperature for 1 h, followed by the incubation at 37°C for 24 h and 30°C for 48 h for bacteria and fungi, respectively. After the incubation period, the zones of growth inhibition were measured and average values were calculated. The wells containing DMSO without the tested compound were used as controls. Ciprofloxacin and fluconazole (Sigma) were used as reference antibacterial or antifungal compounds, respectively. In our study we also used the following reference phenolic acids: ferulic, caffeic, *p*-coumaric and chlorogenic (Sigma, ROTH) to determine the antimicrobial activity.

Subsequently, MIC (Minimal Inhibitory Concentration) of the extracts or fractions was examined by the microdilution broth method prepared in 96-well polystyrene plates. In this method, two-fold dilutions of the extracts or fractions in a suitable broth from 10 000 to 78.1 µg/mL or 1000 to 0.488 µg/mL were used, respectively. Next, 2 µL of each bacterial or fungal suspension with the optical density of 0.5 McFarland standard, was added per each well containing 200 µL broth with various concentrations of the examined compounds. The microplates were incubated in suitable conditions (37°C or 30°C, 24 h for bacteria or fungi, respectively), and next MIC was assessed spectrophotometrically. Appropriate DMSO, growth and sterile controls were carried out. The medium with no tested substances was used as a control.

The MBC (Minimal Bactericidal Concentration) or MFC (Minimal Fungicidal Concentration) are described as the lowest concentration of the compounds that are required to kill a particular bacterial or fungal species. MBC/MFC was determined by removing 20 µL of the culture using for MIC determinations from each well and spotting it onto an appropriate agar medium. After incubation (37°C or 30°C, 24 h for bacteria or fungi, respectively), the compounds of lowest concentrations with no visible growth observed were assessed as a bactericidal/fungicidal concentration. All the experiments were performed three times similarly to the previously described procedures. (17-19).

In this study, no bioactivity was defined as a MIC > 1000 µg/mL, mild bioactivity as a MIC in the range 501 – 1000 µg/mL, moderate bioactivity with MIC from 126 to 500 µg/mL, good bioactivity as a MIC in the range 26 – 125 µg/mL, strong bioactivity with MIC between 10 and 25 µg/mL and very strong bioactivity as a MIC < 10 µg/mL. The MBC/MIC or MFC/MIC ratios were calculated in order to determine bactericidal/fungicidal (MBC/MIC = 4, MFC/MIC = 4) or bacteriostatic/fungistatic (MBC/MIC > 4, MFC/MIC > 4) effect of the tested compounds. (20)

RESULTS AND DISCUSSION

In that work FAs for the first time were investigated in four *Marrubium* species. Flavonoids were also identified by HPLC–DAD-Electrospray Ionization Q-TOF-MS.

Table 4. The mean (n = 3) content of FAs (µg/g, dry wt) in 70% v/v methanolic extracts obtained from *Marrubium* L. species.

Species	Parameter	FAs				
		Neochlorogenic	Chlorogenic	Caffeic	*p*-Cumaric	Ferulic
M. incanum	mean	1096	201	67	99	3
	SD	61	32	9	12	0.3
	RSD	6	16	13	12	10
M. candidissimum	mean	642	136	48	82	2
	SD	16	29	7	12	0.2
	RSD	2.4	21	14	15	8
M. thessalum	mean	1068	200	72	77	9
	SD	74	28	15	19	0.6
	RSD	7	14	22	25	6
M. peregrinum	mean	1801	151	111	128	100
	SD	156	5.3	20	11	10
	RSD	8.7	3.5	18	8.6	1.8

SD - standard deviation, RSD - relative standard deviation (%) ND - not detected

Table 5. The growth inhibition (mm) in the presence of the tested extracts from *Marrubium* sp. at concentration 10 mg/mL for the reference strains of microorganisms.

Extracts	Species	The diameter of growth inhibition (mm) for the reference strains of microorganisms			
		S. epidermidis ATCC 12228	*M. luteus* ATCC 10240	*B. subtilis* ATCC 6633	*B. cereus* ATCC 10876
Methanol	*M. incanum*	9	7	12	9
	M. candidissimum	10	10	13	9
	M. peregrinum	9	7	12	8
	M. thessalum	7	6	11	10
Methanol (70%)v/v	*M. incanum*	6	6	12	10
	M. candidissimum	7	7	11	10
	M. peregrinum	6	6	12	8
	M. thessalum	6	7	12	7
Ethanol	*M. incanum*	6	6	10	7
	M. candidissimum	6	7	13	10
	M. peregrinum	8	7	13	10
	M. thessalum	7	7	11	8
Acetone	*M. incanum*	7	6	12	10
	M. candidissimum	8	6	13	10
	M. peregrinum	8	7	12	10
	M. thessalum	9	7	12	9
Water	*M. incanum*	-	-	-	-
	M. candidissimum	-	-	-	-
	M. peregrinum	-	-	-	-
	M. thessalum	-	-	-	-

Table 6. Free radical (DPPH) scavenging properties of methanolic extracts obtained from Marrubium sp. (n = 3) and reference substances expressed as EC_{50} value (± SD).

Extract/Reference substance	EC_{50} (mg/mL)
M. incanum	0.59 ± 0.014
M. candidissimum	0.72 ± 0.012
M. thessalum	0.59 ± 0.013
M. peregrinum	0.59 ± 0.006
Caffeic acid	0.00455 ± 0.00013
Gallic acid	0.00395 ± 0.00011
Rutin	0.01450 ± 0.00041
Trolox	0.01050 ± 0.00022

The total percentage of FAs in aerial parts of *Marrubium* spp. was calculated and expressed as caffeic acid. Aerial parts of *M. thessalum* and *M. incanum* were especially rich in FAs (0.66% and 0.43%, respectively), whereas *M. candidissimum* and *M. peregrinum* showed lower contents of the compounds (0.30% and 0.33%). FAs from the flowering herbs of *Marrubium sp*. were analyzed by chromatographic methods (2D TLC, RP-HPLC, and HPLC–DAD-ESI-Q-TOF-MS analysis). Figure 1 shows the results of HPLC–DAD separation of 70% (v/v) methanolic extracts obtained from flowering herbs of four *Marrubium* species. The HPLC method combined with PDA detection confirmed the presence of the following FAs: ferulic, *p*-coumaric, caffeic, gentisic, neochlorogenic, chlorogenic, acids in all samples examined. Similar qualitative results were obtained for HPLC analysis of FA, FB and FC fractions (Table 1). The analysis on ESI-QTOF- MS system in negative ion mode confirmed the presence of earlier mentioned FAs and made it possible to identify additionally protocatechuic, p-hydroxybenzoic acids and flavonoids: rutin, kaempferol rhamnohexoside, kaempferol hexoside, isorhamnetin hexoside apigenin (Table 2).

Qualitative and quantitative analysis of free FAs in flowering herbs of *Marrubium* spp. was carried out using different eluents. The greatest amount of FAs (neochlorogenic, chlorogenic, coffee, *p*-coumaric, ferulic acid) was achieved using 70% v/v methanol as an extractant than methanolic, ethanolic, acetone and aqueous. The lowest content of active compounds was observed in aqueous extracts. The amounts (μg/g) of these compounds detected in the analyzed samples are shown in (Tables 3, 4). Neochlorogenic and chlorogenic acids

were found in all extracts in different proportions, depending on species. Neochlorogenic acid was predominant in fraction FA in all studied taxa. The highest content of this compound was observed in 70% (v/v) methanolic extracts of *M. peregrinum* (1801 µg/g), whereas the lowest in *M. candidissimum* (642 µg/g). The highest concentration of chlorogenic acid was reported for *M. incanum* and *M. thessalum* (201 µg/g and 200 µg/g), whereas caffeic acid for *M. peregrinum* (111 µg/g). The amounts of ferulic acid reached the lowest levels in all investigated extracts. The biological potential of extracts strongly depends on the type of extraction solvent used for the isolation of polyphenolic fraction. The results of the HPLC studies prove that FAs may be involved in the pharmacological activity of *Marrubium* spp. because of their high variability and content in extracts examined. The abovementioned observation clearly reflects in the results of the antimicrobial activity (Table 5). 70% v/v methanol

Table 7. The activity data expressed as MIC (MBC) (µg/mL) against the reference strains of bacteria established for *Marrubium* extracts.

The obtained extracts from *Marrubium* sp.		MIC (MBC) [µg/mL] of the extracts			
		S. epidermidis ATCC 12228	*M. luteus* ATCC 10240	*B. subtilis* ATCC 6633	*B. cereus* ATCC 10876
Methanol	*M. incanum*	2500 (10000)	1250 (5000)	625 (625)	1250 (2500)
	M. candidissimum	5000 (10000)	1250 (5000)	312.5 (312.5)	1250 (2500)
	M. peregrinum	2500 (5000)	1250 (2500)	1250 (1250)	625 (1250)
	M. thessalum	2500 (10000)	2500 (5000)	1250 (1250)	2500 (2500)
Methanol (70%)v/v	*M. incanum*	2500 (5000)	1250 (2500)	1250 (2500)	1250 (1250)
	M. candidissimum	5000 (10000)	1250 (5000)	625 (1250)	1250 (1250)
	M. peregrinum	5000 (10000)	1250 (5000)	625 (625)	1250 (1250)
	M. thessalum	2500 (5000)	2500 (10000)	1250 (1250)	2500 (2500)
Ethanol	*M. incanum*	2500 (10000)	1250 (10000)	2500 (5000)	2500 (2500)
	M. candidissimum	5000 (10000)	1250 (5000)	625 (1250)	1250 (1250)
	M. peregrinum	5000 (> 10000)	625 (2500)	625 (2500)	625 (1250)
	M. thessalum	2500 (5000)	2500 (5000)	5000 (5000)	2500 (2500)
Acetone	*M. incanum*	5000 (> 10000)	1250 (10000)	625 (1250)	1250 (1250)
	M. candidissimum	10000 (> 10000)	1250 (5000)	1250 (5000)	2500 (5000)
	M. peregrinum	10000 (10000)	1250 (5000)	1250 (2500)	1250 (2500)
	M. thessalum	5000 (10000)	2500 (10000)	1250 (5000)	2500 (5000)
Water	*M. incanum*	-	-	-	-
	M. candidissimum	-	-	-	-
	M. peregrinum	-	-	-	-
	M. thessalum	-	-	-	-

Table 8. The activity data expressed as MIC and MBC (µg/mL) against the reference strains of bacteria for fractions from *Marrubium* sp. and standard phenolic acids.
The standard antibiotic – ciprofloxacin (CIP) for bacteria used as positive control.

The obtained fractions from *Marrubium* sp.		MIC (MBC) [µg/mL] of the extracts					
		S. aureus ATCC 6538	*S. aureus* ATCC 25923	*S. epidermidis* ATCC 12228	*M. luteus* ATCC 10240	*B. cereus* ATCC 10876	*B. subtilis* ATCC 6633
M. incanum	FA	500 (1000)	500 (500)	500 (1000)	125 (1000)	125 (500)	62.5 (125)
	FB	1000 (1000)	1000 (1000)	1000 (> 1000)	500 (1000)	250 (1000)	125 (250)
	FC	1000 (> 1000)	1000 (1000)	1000 (10000	1000 (> 1000)	500 (1000)	250 (500)
M. candidissimum	FA	500 (1000)	500 (1000)	500 (1000)	125 (1000)	125 (500)	125 (125)
	FB	-	1000 (> 1000)	1000 (1000)	1000 (> 1000)	1000 (1000)	250 (500)
	FC	1000 (> 1000)	1000 (> 1000)	1000 (> 1000)	1000 (> 1000)	500 (500)	500 (500)
M. thessalum	FA	1000 (> 1000)	-	1000 (> 1000)	1000 (> 1000)	250 (1000)	250 (250)
	FB	-	-	1000 (> 1000)	1000 (> 1000)	1000 (1000)	1000 (> 1000)
	FC	1000 (1000)	1000 (1000)	1000 (1000)	500 (1000)	500 (> 1000)	250 (500)
M. peregrinum	FA	250 (1000)	250 (500)	500 (> 1000)	125 (1000)	250 (> 1000)	125 (250)
	FB	1000 (> 1000)	1000 (> 1000)	1000 (> 1000)	1000 (> 1000)	1000 (> 1000)	1000 (> 1000)
	FC	1000 (> 1000)	1000 (> 1000)	1000 (> 1000)	1000 (> 1000)	1000 (> 1000)	500 (500)
Standards	Chlorogenic acid	-	-	250 (500)	1000 (> 1000)	1000 (> 1000)	1000 (> 1000)
	p-coumaric acid	-	-	1000 (> 1000	500 (> 1000	1000 (> 1000	1000 (> 1000)
	Ferulic acid	-	-	500 -	1000 (> 1000)	1000 (> 1000)	1000 (> 1000)
	Caffeic acid	-	-	500 (> 1000)	1000 (> 1000)	1000 (> 1000)	1000 (> 1000)
	CIP	0.244	0.488	0.122	0.976	0.061	0.030

MIC – Minimal Inhibitory Concentration; MBC – Minimal Bactericidal Concentration; – no activity

extracts showed the highest antibacterial activity (MIC, the lowest values of MBC), whereas aqueous extracts showed no activity at all.

Using Folin-Ciocalteu method, total phenolic content (TPC) in water extracts of the studied species was determined, having the following values: 20.82 mg GAE/g for *M. incanum;* 17.07 mg GAE/g for *M. candidissimum;* 20.79 mg GAE/g for *M. thessalum;* 16.76 mg GAE/g for *M. peregrinum*. TLC–DPPH• assay enabled a rapid detection of antioxidant activity of polyphenolics in methanolic (70%) extracts obtained by ASE. The TLC chromatogram revealed the presence of several zones with compounds having the ability to scavenge free radicals – Figure 2.

Because the highest amounts of FAs were shown in 70% (v/v) methanolic extracts, they were further studied for their antioxidant activity using a spectrophotometric analysis. EC_{50} value for the extracts of *M. candidissimum* was 0.72 mg/mL, whereas *M. incanum*, *M. thessalum, M. peregrinum* exhibited similar activity (EC_{50} = 0.59 mg/mL). For

comparison, EC_{50} was also calculated for reference antioxidants: gallic and caffeic acids, rutin and Trolox. Mean values obtained were 0.00395, 0.00455, 0.01450 and 0.01050 mg/mL, respectively (Table 6). The antioxidant activity is attributed not only to the FAs but also to the other phenolic compounds identified and described in the literature (5). The results suggest that the *Marrubium sp*. may serve as an economical and effective source of natural antioxidants. The present study focuses on the antimicrobial activity of extracts and fractions obtained from flowering herbs of different species of *Marrubium* spp. The results indicate that the examined organic extracts: methanol (100% and 70%), ethanol and acetone from these plants had no activity against Gram-negative bacteria and yeasts belonging to *Candida* spp. However, extracts showed some antibacterial activity against Gram-positive bacteria, especially opportunistic *S. epidermidis, M. luteus* and *Bacillus* spp. with MIC = 312.5–10 000 µg/mL.

According to the data presented in the literature (1), the most active antioxidant for *M. peregrinum* the methanol extract (EC_{50} = 187.4 mg/mL). Aqueous extracts (EC_{50} = 481 mg/mL) and ethyl acetate (EC_{50} = 551 mg/mL) show a less potent activity. Chlorogenic acid (EC_{50} = 11.65 mg/mL) and BHA (EC_{50} = 5.39 mg/mL) were used as standards.

Recently, the development of bacterial resistance to presently available antibiotics, a high cost of synthetic compounds, as well as undesirable side effects of certain drugs, have necessitated the search for new antibacterial agents from alternative sources including medicinal plants. It has been observed that many plants contain different substances with antimicrobial activities, which can serve as a source and template for the synthesis of new antimicrobial drugs. (19, 21)

The knowledge about the therapeutic possibility of using both extracts and isolated phytochemicals from *Marrubium* spp. has become more popular as well. The present study focuses on the antimicrobial activity of extracts and fractions obtained from whole ground parts of different species of *Marrubium* spp., i.e. *M. peregrinum M. incanum*, *M. candidissimum* and *M. thessalum*. The results indicate that the examined organic extracts: methanol (100% and 70%), ethanol and acetone from these plants had no activity against Gram-negative bacteria and yeasts belonging to *Candida* spp. However, extracts showed some antibacterial activity against Gram-positive bacteria, especially opportunistic *S. epidermidis, M. luteus* and *Bacillus* spp. with MIC = 312.5 – 10 000 µg/mL. The activity of all organic extracts obtained was similar towards these bacteria. The aqueous extracts had no effect against reference microorganisms. According to the data delivered by Sen A. (22), all types of extracts obtained using organic solvents show antibacterial activity. Therefore, the organic extracts showed more potent bacteriostatic and bactericidal properties when compared to aqueous extracts which display no such activities.

The antimicrobial activity of extracts obtained from whole ground parts of plants was studied for *M. vulgare* (23), *M. peregrinum* (24), *M. alysson* (25) and *M. globosum* (2). The results of these studies indicated that organic extracts (methanol, ethanol, acetone) of *Marrubium* spp., show some antimicrobial activity on certain bacterial strains, which proved similar in our analysis.

According to the data found in the literature (24), methanol extracts of *M. peregrinum* indicated similar antibacterial activity against *Bacillus cereus*, *Bacillus pumilus* and *Sarcina lutea* with MIC = 321.5 µg/mL, against *Bacillus subtilis* – MIC = 625 µg/mL and against *Staphylococcus aureus* (MIC = 1250 µg/mL). Our results confirm these reports, because minimal concentrations of methanol (100% and 70%), ethanol and acetone extracts of *M. peregrinum,* which inhibited the growth of *Bacillus subtilis* and *Bacillus cereus*, ranged from 625 µg/mL to 1250 µg/mL. The same authors indicate a wide spectrum of activity in essential oils obtained from *Marrubium peregrinum L.* towards bacteria *(Staphylococcus aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *Micrococcus flavus* ATCC 10240, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* NCIMB 9111 and *Pseudomonas aeruginosa* ATCC 27853), and two strains the yeast *Candida albicans* (ATCC 10259 and ATCC 24433).

The data found in Masoodi M. (23) proved that methanol extracts from *M. vulgare* also show antimicrobial activity towards *Staphylococcus epidermidis* (MIC = 200 mg/mL), *Staphylococcus aureus* (MIC = 100 mg/mL) and *Bacillus subtilis* (MIC = 100 mg/mL). Moreover, the literature on the antimicrobial activity of the species the *Marrubium* genus L. also highlights good antibacterial and antifungal properties of essential oils of *M. vulgare* (26) and *M. incanum* (27).

Our data indicate that the obtained extracts of *Marrubium* spp. contain some FAs, i.e. chlorogenic, neochlorogenic, *p*-coumaric, ferulic and caffeic acids. Moreover, these acids show the activity against staphylococci, micrococci and *Bacillus* spp.

The largest amount of these acids was achieved using 70% methanol as an extractant. The smallest content of the active compounds was observed in aqueous extracts. It was also showed that fractions with FAs of *Marrubium* spp. had a higher antibacterial activity than extracts. Moreover, these fractions additionally influenced the growth of pathogenic *S. aureus*.

Comprehensive literature review gives no information about the antibacterial and antifungal activities of extracts and fractions obtained from whole ground parts of *M. incanum*, *M. candidissimum* and *M. thessalum*. These studies were probably evaluated for the first time.

Our results indicate that the tested extracts at a concentration 10.0 mg/mL, obtained from the flowering herbs of *Marrubium* spp., had different activity against the reference strains of microorganisms. Using agar plate method, it was shown that all these extracts had no bioactivity against the reference strains of Gram-negative bacteria and yeasts belonging to *Candida* spp., whereas aqueous extracts had no influence on the growth of all tested microorganisms. Using this method, various diameters of inhibition growth methanol (100% and 70%), ethanol and acetone extracts were indicated for the reference strains of some Gram-positive bacteria, i.e. staphylococci, micrococci and *Bacillus* spp. belonging to opportunistic bacteria. Moreover, the antibacterial activity against *S. epidermidis* ATCC 12228, *M. luteus* ATCC 10240 and *B. cereus* ATCC 10876 with the zones of growth inhibition 7 – 10 mm. was also shown. In turn, the diameters of growth inhibition of *B. subtilis* ATCC 6633 were 10 – 13 mm for these extracts (Table 7).

According to the data presented in Table 6, on the basis of minimal inhibitory concentration values obtained by the broth microdilution method, it was shown that the examined extracts: methanol (100% and 70%), ethanol and acetone of the four species of *Marrubium* had a similar influence on the growth of staphylococci, micrococci and *Bacillus* spp.

These extracts showed the highest activity against *B. subtilis* ATCC 6633 with moderate effect of 100% methanol extract of *M. candidissimum* L. (MIC = 312.5 µg/mL) and a mild effect of several extracts (MIC = 625 µg/mL). Minimum concentrations, which inhibited the growth of these bacteria, ranged from 312.5 to 5000 µg/mL (MBC = 625 – 5000 µg/mL). The activity towards *B. cereus* ATCC 10876 was similar with MIC = 625 – 5000 µg/mL and MBC = 1250 – 5000 µg/mL and towards *M. luteus* ATCC 10240 (MIC = 625 – 2500 µg/mL and MBC = 2500 – 10000 µg/mL. The activity against *S. epidermidis* ATCC 12228 was lower (MIC = 2500 – 1000 µg/mL and MBC = 5000 – > 10000 µg/mL).

It was also indicated that fractions of *Marrubium* spp. had a higher activity against the reference strains of Gram-positive bacteria than extracts. The fractions had an influence on the growth both pathogenic *S. aureus* ATCC and opportunistic staphylococci, micrococci and *Bacillus* spp. Among them, fraction FA showed a good activity towards *M. luteus* ATCC 10240 and *Bacillus* spp. ATCC with MIC = 125 – 250 µg/mL (MBC = 125 – >1000 µg/mL) and had a moderate effect on staphylococci with MIC = 250 – 500 µg/mL (MBC = 500 – > 1000 µg/mL). The remaining fractions indicated a lower activity against these bacteria. Our data also indicate that the standard acids: ferulic, caffeic, p-coumaric and chlorogenic show some activity against opportunistic staphylococci, micrococci and *Bacillus* spp. (see Table 8).

CONCLUSIONS

For the first time, the qualitative and quantitative analysis of FAs in the flowering herbs of *M. incanum*, *M. candidissimum*, *M. thessalum* and *M. peregrinum* was carried out. By the 2D TLC, RP-HPLC and HPLC/ESI-QTOF- MS analysis eight FAs, namely protocatechuic, p-hydroxybenzoic, ferulic *p*-coumaric, caffeic, gentisic, neochlorogenic, chlorogenic and five flavonoids namely rutin, kaempferol rhamnohexoside, kaempferol hexoside, isorhamnetin hexoside, apigenin were identified in aerial parts of all *Marrubium* species examined. The highest amount of FAs was achieved using 70% (v/v) methanol as an extractant. The lowest concentration was observed for aqueous extracts. This clearly reflects the results of antimicrobial activity, where 70% (v/v) methanolic extracts showed the highest antimicrobial activity (the lowest value of MIC and MBC), whereas aqueous extracts showed no activity at all. The ability to scavenge free radicals by a methanol-water extracts from the aerial parts of *M. incanum, M. candidissimum, M. thessalum* was put forward for the first time. Our data also indicated that the examined extracts: methanol (100% and 70%), ethanol and acetone obtained from *Marrubium* spp. had a similar influence on the growth of opportunistic *S. epidermidis, M. luteus* and *Bacillus* spp. The fraction showed additionally the activity against pathogenic *S. aureus*. The aqueous extracts had no effect towards reference microorganisms. For the first time, a significant antimicrobial activity of methanol (70% v/v and 100%), ethanol, acetone and water extracts obtained from the aerial

parts of the *M. incanum*, *M. candidissimum*, and *M. thessalum* was presented.

In conclusion, our studies confirmed that biological (antioxidant and antimicrobial) of *Marrubium* extracts were strongly correlated with the high amounts of TPC particular FAs and flavonoids.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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