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Chemical composition and antimicrobial activity of fruit essential oils of *Myrica gale*, a neglected non-wood forest product

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Abstract

The study aimed to establish the chemical composition of fruit essential oils of *M. gale* and test their activities against the selected pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter baumannii*), yeasts (*Candida albicans*, *C. parapsilosis*), fungi (*Aspergillus fumigatus*, *A. flavus*) and dermatophytes (*Trichophyton rubrum*, *T. mentagrophytes*). Fruit samples from natural (Western Lithuania) and anthropogenic (Eastern Lithuania) *M. gale* populations were studied separately. Essential oils were isolated from dried fruits by hydrodistillation and analysed by GC/FID and GC/MS methods; enantiomeric composition of α-pinene was established by chiral-phase capillary GC. Minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of essential oils were determined using the broth microdilution method. Plants from the natural population with a humid marine climate accumulated significantly higher amounts of fruit essential oils (3.34±0.05%) than those from the anthropogenic population with a more continental climate (2.71±0.22%). In total, 39 volatiles including α-pinene (23.52–27.17%), 1,8-cineole (17.19–18.84%) and α-phellandrene (9.47–10.03%) as main compounds were identified. Chiral analysis demonstrated that (1S)-(–)-α-pinene prevailed over (1R)-(+)-α-pinene and amounted to 94.09–95.28% of all fraction of this monoterpene. The antimicrobial study *in vitro* indicated that *C. parapsilosis*, dermatophytes and *Aspergillus* fungi were more susceptible to fruit essential oils of *M. gale*, whereas *E. coli* and *C. albicans* were weakly inhibited even at the highest essential oil concentration. The strongest growth-inhibitory and bactericidal effect of sweet gale essential oil was established on *S. aureus*. This could be attributed to the major essential oil compounds with known antimicrobial activity, such as α-pinene, 1,8-cineole and α-phellandrene.

Keywords: *Myrica gale,* essential oil, chemical compounds, terpenes, enantiomers, antimicrobial

Introduction

Sweet gale, or bog myrtle (*Myrica gale* L., Myricaceae Rich. ex Kunth), a deciduous small shrub, is native species to high latitudes of the northern hemisphere including Northern and Western Europe, North America and East Asia. It is a dioecious plant with monoecious individuals or subpopulations occurring occasionally (Lloyd 1981, Skene et al. 2000, Stępień and Ciaciura 2009). As observed by Katzer (2003), the etymology of common names of *M. gale* in different languages is often linked to myrtle for the morphological similarity to *myrtle* and its strong smell. Meanwhile, common names of the plant in Slavic languages are related to the wax-bearing property of fruits of *M. gale*. Although it is not a true wax, but just "plant tallow" extracted from the surface of fruits (Williams 1958), a wide distribution of the plant's name linked to its wax-bearing property reflects, at a certain degree, its traditional use and general knowledge of the species in the region.

The species, however, is best known for its traditional uses based on aromatic plant properties. In Northern and Western Europe, it was used for flavouring beer, Scandinavian snaps and other specialty beverages (Simpson et al.

1996, Katzer 2003) as well as repellent (Chevallier 2000). Moreover, numerous literature sources (Bown 2002, Crellin 1994, Simpson et al. 1996, Skene et al. 2000, Kalle and Sõukand 2012, 2016) refer to the uses related to diuretic, styptic, anticathartic, antihelmintic properties of *M. gale* leaves and branches as well as for relieving itching with dried bark preparations (Sturluson 2018).

Plants of *M. gale* synthesize condensed tannins, phenols, flavonoids from the class of dihydrochalcones, resins, and essential oils, too (Anthonsen et al. 1971, Mathiesen 1997, Santos et al. 2002). The essential oils are accumulated in leaves, flowers (catkins) and fruits of *M. gale*; however, both quantitative and qualitative composition of essential oils of leaves and/or the whole aerial parts have been subject to studies more often than those of fruits separately. For example, essential oils of *M. gale* leaves and/or aerial parts were investigated in Spain (Negueruela et al. 1982), the Netherlands (Tattje and Bos 1974), Switzerland (Von Schantz and Kapetanidis 1971), Scotland and Finland (Carlton et al. 1992, Chang and Martin 2014, Svoboda et al. 1998), the USA (Halim and Collins 1973), Japan (Nakata et al. 2013) and Canada (Collin and Gagnon 2016, Sylvestre et al. 2005, 2006). Meanwhile, fruit essential oils of *M. gale*, taken separately, were investigated in France (Popovici et al. 2008) and Estonia (Sokolova et al. 2005).

Krogsbøll et al. (2016) showed that antibacterial activity of essential oils from leaves of M. gale was of lesser extent on *Escherichia coli* in the liquid culture than on *Bacillus subtilis*. Nakata et al. (2013) reported that essential oils from leaves of *M. gale* var. *tomentosa* indicated antimicrobial activity against Gram-positive bacteria *Bacillus subtilis* and yeast *Candida albicans*. Further, Federspil et al. (1997) reported that the leaf gland volatile oil of *M. gale* inhibited growth of fungal species of broad spectrum (*Alternaria alternata*, *Trichoderma harzianum*, *Aspergillus niger*, *Epicoccum nigrum*, *Penicillium spinulosum*, *P. citrinum*, *Apiospora montagnei* and *Fusarium sporotrichioides*) to a greater or lesser extent. Meanwhile, Stuart (1998) demonstrated antifungal activity of *M. gale* leaf essential oil against a human skin pathogen, *Trichophyton interdigitale.*

These studies reveal a rich potential of biologically active substances of the plant, although they pertain mostly to the other parts than fruits. Meanwhile, the studies on biological activity of essential oils isolated from fruits of *M. gale* are scarce, and the effects of fruit essential oils on human pathogenic microorganisms have rarely been studied. From a physiological point of view, it is known that generative organs of plants (flower, fruit, seed) usually produce concentrated amounts of biologically active compounds. Therefore, the aim of this study is to investigate the chemical composition of fruit essential oils of *M. gale* from two different populations and test their activity against the selected pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter baumannii*), yeasts (*Candida albicans*, *C. parapsilosis*), fungi (*Aspergillus fumigatus*, *A. flavus*) and dermatophytes (*Trichophyton rubrum*, *T. mentagrophytes*).

Materials and methods

Plant material and collecting site characteristics

The twigs with fruits of *M. gale* were collected from five plants in each of two habitats separately and made as sample 1 and sample 2, respectively. The sample 1 represented Habitat 1 in a natural population in Western Lithuania (Kliošiai Landscape Reserve, Klaipėda County; WGS coordinates 55.547105 N, 21.224248 E) and sample 2 represented Habitat 2 in an anthropogenic population in Eastern Lithuania (Verkiai Regional Park, Vilnius County; WGS coordinates 54.766438 N, 25.310994 E); the voucher specimens of *M. gale* have been deposited in the Herbarium of Nature Research Centre (BILAS, Vilnius, Lithuania), the numbers of the specimens are 92609 and 92610, respectively. The latter population was established by planting several shrubs in 1962 and has developed into a population of about 300 individuals (Matulevičiūtė 2007). The anthropogenic population is located about 274 km east-southeast from the natural one (see map, Figure 1). The twigs were collected in August, dried at an ambient temperature in a room protected from direct sunlight. Dried plant raw material was kept in bags. The fruits were separated from twigs before essential oil isolation.

The topsoil samples were collected from each habitat separately and dried at room temperature. Each sample was prepared in the following way: 5 subsamples (each subsample ~100 g) per habitat were taken from the depth of 10–15 cm and homogenised. Soil analysis was carried out at the Agrochemical Research Laboratory of the Lithuanian Research Centre for Agriculture and Forestry.

Figure 1. Natural distribution area of *Myrica gale* L. in Europe as provided by the Euro+Med plantbase (Uotila, 2009)
1 – Habitat 1 (natural population); 2 – Habitat 2 (anthropogenic population).

Climate data was provided by the Lithuanian Hydrometeorological Service for the respective climatic subregions, Pajūrio žemuma [Seaside Lowland] (for Habitat 1) and Aukštaičiai area (for Habitat 2), of Lithuanian climate regions. All major environmental characteristics of the collecting sites are provided in the Table 1.

Myrica gale has been included into the national list of protected species since 1962 and currently is under the category 3 (R) of the Red Data Book of Lithuania (Matulevičiūtė 2007). The permission for the sampling of *M. gale* plant material has been given by the Environmental Protection Agency (2017-08-08 No. 51).

Isolation and analysis of essential oils

The essential oils were isolated from sample 1 and sample 2 of dried fruits of *M. gale* separately by hydrodistillation in a Clevenger type apparatus for two hours (European Pharmacopoeia Commission 2008); non-ground fruits were used for the isolation of essential oils. The distillation of essential oils from each sample was carried out in three repetitions, dried with anhydrous sodium sulphate and stored in freezer until further analysis.

Essential oil solutions of 1% were prepared in the mixture of diethyl ether and n-pentane (1:1) for further investigations. The identification of compounds of essential oils was carried out by employing a Shimadzu GC-2010 coupled with Shimadzu GCMS-QP 2010 Plus mass selective detector (Shimadzu, Japan). Separation of compounds was performed on fused silica (100% dimethyl polysiloxane) column (30 m × 0.25 mm ID × 0.25 µm film thickness, Restek, USA), splitless injection; helium as carrier gas at a flow rate of 1.6 ml/min, injector and detector temperatures 250°C. The GC oven temperature was programmed as follows: initially temperature 50°C (isothermal for 7 min) increased to 250°C at the rate 4°C/min to (isothermal for 5 min) and further increased at the rate of 30°C/min to 300°C, the final temperature kept for 2 min. Mass spectra in electron mode were generated at 70 eV. The identification of the compounds was based on the comparison of computer mass spectra library (NBS75K), analytical standards (Sigma-Aldrich) and retention indices (RIs) (Adams 2007). The retention indices were determined relative to the retention times of a series of n-alkanes (C_7–C_{30}) with linear interpolation. The quantitative analyses of the main compounds were carried out using a FOCUS GC (Thermo Scientific) gas chromatograph with a flame ionisation detector (FID) on the silica capillary column TR-5MS (30 m × 0.25 mm ID × 0.25 µm film thickness; 5% diphenyl/95% dimethyl siloxane) (Thermo Electron Corporation, USA) under the same chromatographic conditions. The percentages of the investigated compounds were recalculated according to the areas of the FID chromatographic peaks assuming that all constituents of the essential oil comprise 100%.

The enantiomer separations of α-pinene were performed on a Shimadzu GC-2010 Plus equipped with aRt-bDEXsm chiral stationary phase column (30 m × 0.25 mm i.d., 0.25 µm film thickness, Restek, USA) and a flame ionization detector. The following GC parameters were used for the analysis of the α-pinene enantiomers: helium flow rate of 1.6 ml/min; temperature programme from 85°C (12 min hold) to 160°C (1 min hold) increasing at 5°C/min; detector temperature 260°C; split injector was heated at 250°C. The identification of (1R)-(+)-α-pinene and (1S)-(–)-α-pinene was carried out by the comparison of the retention time (RT) of its GC peak in investigated chromatograms with the RT of (1R)-(+)-α-pinene and (1S)-(–)-α-pinene analytical terpene standard (Sigma-Aldrich; purity (GC area %) ≥98.5% and ≥99.0%, respectively) under the same GC parameters and column. The percentages of α-pinene enantiomers were recalculated according to the areas of investigated chromatographic peaks assuming that monoterpene α-pinene fraction is 100%.

Microorganisms

The following bacteria were used as test organisms in this study: *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 43300 (MRSA), *Acinetobacter baumannii* ATCC BAA 744 and *Escherichia coli* ATCC 25922. The fungi used as test organisms: *Aspergillus fumigatus* SC 6359, *A. flavus* CBS 120264, *Trichophyton rubrum* ATCC 28188 and *T. mentagrophytes* SC 91427. The yeasts *Candida albicans* CBS 2730 and *C. parapsilosis* CBS 8836 were used in this study. The strains of bacteria were maintained on nutrient agar (Liofilchem, Italy) and the strains of fungi and yeasts – on Sabouraud dextrose agar (Liofilchem, Italy) slants and stored at 4ºC.

Bacterial inocula were obtained from bacterial cultures incubated for 24 h at 37°C on nutrient agar and brought up by dilution according to the 0.5 McFarland standard to approximately 10^8 CFU/ml. Suspensions of fungal

Table 1. Environmental characteristics of *M. gale* collecting sites in Western Lithuania (Habitat 1) and Eastern Lithuania (Habitat 2)

Environmental characteristic	Habitat 1	Habitat 2	Method / notes
Soil:			
Type	Peat	Peat	
pH	4.7	4.9	Electrometry
Total nitrogen, %	1.75	0.76	Photoelectrocolorimetry
Total phosphorus, %	0.07	0.05	Photoelectrocolorimetry
Total potassium, %	0.07	0.46	Flame photometry
Climate[1]:			
Mean annual temperature, °C	7.4	6.4	n/a
Mean temperature of the warmest month, °C	17.8 (July)	17.9 (July)	n/a
Mean temperature of the coldest month, °C	-1.9 (January)	-4.3 (January)	n/a
Annual precipitation, mm	800	650	n/a
Duration of snow cover, days	68	98	n/a
Duration of sunshine, hours	1950	1730	n/a

[1] According to the characteristics of Lithuanian climate regions (based on 1981–2010 data) provided by the Lithuanian Hydrometeorological Service. Available at http://www.meteo.lt/en/climate-regions-of-lithuania (accessed 18 October 2018).

spores were prepared from fresh mature (3- to 7-day-old) cultures that grew at 30°C on a Sabouraud dextrose agar. Spores were rinsed with sterile water with 0.05% Tween 80 to determine turbidity spectrophotometrically at 530 nm, according to the conidial size of the species (0.09 to 0.3 optical densities) (Espinel-Ingroff et al. 2009).

Antimicrobial properties of essential oil of Myrica gale

The essential oil sample from the natural population was examined as the qualitative composition of the both samples (from natural and anthropogenic populations) was very similar, while the amount of essential oils was significantly higher in the natural population. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of *M. gale* essential oil were determined using a broth microdilution method. For the antimicrobial activity tests, *M. gale* essential oil was dissolved in 96% (v/v) ethanol. Final concentration of *M. gale* essential oils was 10% (v/v). The 96-well plates were prepared by dispensing into each well 100 µL RPMI 1640 medium with L-glutamine without sodium bicarbonate (Sigma Aldrich, Germany) for fungi and yeasts, Mueller-Hinton broth (Oxoid, England) – bacteria. Final inoculum concentrations for bacteria was $1x10^7$ cfu/ml, fungi and yeast $2x10^5$ cfu/ml. A 100 µL prepared sample of oil was added into the first wells. Then 100 µL from their serial dilutions were transferred into eight consecutive wells. Thereafter, each well was inoculated with 100 µL of suspension of the culture and incubated at 37 ºC for all tested microorganisms, 24 h for bacteria and yeasts, 48 h for fungi, in order to get a reliable microbial growth. A suspension of microorganisms in the medium without *M. gale* essential oils served as growth control. A 10% (v/v) ethanol was used as a negative control for the influence of the solvents. The MIC values of gentamycin (*E. coli*), vancomycin (*S. aureus* ATCC 29213), colistin (*A. baumannii*), trimethoprim-sulfamethoxazole (*S. aureus* ATCC 43300) and itraconazole for yeasts, fungi and dermatophytes were individually determined in parallel experiments in order to control the sensitivity of the test organisms. The MIC of gentamycin, vancomycin, colistin, trimethoprim-sulfamethoxazole and itraconazole were determined using Liofilchem® MIC test strip (Liofilchem, Italy) and Interpretative Criteria and Quality Control – Rev.27/27.01.2017. After incubation, the growth of microorganisms was indicated by the presence of turbidity and 'pellets' on the well bottom. MICs were determined presumptively as the first well, in ascending order, which did not produce a pellet. To determine minimum bactericidal concentration (MBC), a 10 µL broth was taken from each well and incubated in Mueller-Hinton agar for bacteria. To determine minimum fungicidal concentration (MFB) a 10 µL broth was taken from each well and incubated in Sabouraud dextrose agar for the yeasts, fungi and dermatophytes. No visible colony growth after subsequent 24–48 h incubation was accepted as MBC/MFC. All tests were performed in duplicate (Wiegand et al. 2008). No epidemiological cutoff values (ECV) of the antibiotics were referred to as their obtained MIC values were far beyond comparison with those of *M. gale* essential oils (Espinel-Ingroff and Turnidge 2016, Lockhart et al. 2017).

Statistical analysis

Descriptive statistics, including means and standard errors, were calculated using MS Excel. To establish differences between *M. gale* populations in the major constituents of essential oils and their total amounts the t-test was used with STATISTICA® 7 software.

Results

Composition of fruit essential oils of Myrica gale

The study showed that fruits of *Myrica gale* accumulated 3.34 ±0.05% of essential oils in the natural population (Habitat 1) and 2.71 ±0.22% in the anthropogenic population (Habitat 2). The t-test showed that populations differed significantly by the percentage of essential oils ($t = 4.84$, $p < 0.01$). Thirty-nine compounds were identified, which represented 95.93% (Habitat 1) and 93.82% (Habitat 2) of the total essential oil amounts of *M. gale* fruits. Monoterpene hydrocarbons made up about a half of the whole amount of essential oils (Table 2).

The main compound of fruit essential oils was α-pinene: the mean percentage of this bicyclic monoterpene was 25.35 ±2.58% (Figure 2a). Although the percentage of α-pinene in the essential oil from natural population (Habitat 1) was nearly 4% higher in comparison to the anthropogenic population (Habitat 2), the difference between the habitats in percentage of this compound was not statistically significant. The chirality is characteristic of this monoterpene with the dextrorotatory (1R)-(+) and laevorotatory (1S)-(–) forms (enantiomers) occurring commonly. The results of our study showed that (1S)-(–)-α-pinene amounted to 95.28% and 94.09% of all α-pinene fraction in fruit essential oils of *M. gale* from Habitat 1 and Habitat 2, respectively (Figure 2b).

The second most abundant chemical compound was 1,8-cineole; its percentages were very similar in both investigated samples of the essential oils and made 18.02 ±1.17% on average (Table 2, Figure 2a). α-Phellandrene was the third most abundant chemical compound amounting to 9.75 ±0.40% on average.

Activity of essential oils of Myrica gale on different microorganisms

To analyse the antimicrobial activity of fruit essential oils of *M. gale*, the broth microdilution method was employed to determine the minimum inhibitory concentration (MIC) against the selected microorganisms. The antimicrobial activity of the studied essential oils is summarized in Table 3. The results of our experiments revealed that the essential oils of *M. gale* exhibited vari-

Table 2. Compound percentage of fruit essential oils of *Myrica gale* (GC area %) from natural population in Western Lithuania (Habitat 1) and anthropogenic population in Eastern Lithuania (Habitat 2)

Compound	RI	Habitat 1	Habitat 2	Type	Identification method[1]
α-Thujene	924	0.21	0.18	MH	MS, RI
α-Pinene	933	27.17	23.52	MH	MS, RI, Std
Camphene	946	0.88	0.82	MH	MS, RI, Std
Octen-3-ol-1	982	2.67	2.44	OT	MS, RI
Myrcene	993	2.85	2.46	MH	MS, RI, Std
α-Phellandrene	1002	10.03	9.47	MH	MS, RI
α-Terpinene	1016	1.45	1.51	MH	MS, RI, Std
p-Cymene	1024	0.80	0.86	MH	MS, RI, Std
Limonene	1030	6.69	6.45	MH	MS, RI, Std
1,8-Cineole	1028	17.19	18.84	OM	MS, RI, Std
E-β-Ocimene	1048	0.33	0.39	MH	MS, RI
γ-Terpinene	1056	2.46	2.70	MH	MS, RI, Std
Linalool	1094	0.30	0.31	OM	MS, RI, Std
Tetrahydrolavandulol	1156	0.20	0.21	OM	MS, RI
Borneol	1164	0.20	0.19	OM	MS, RI, Std
Terpinen-4-ol	1178	1.60	1.82	OM	MS, RI, Std
α-Terpineol	1198	1.54	1.66	OM	MS, RI
Geranial	1265	–	0.08	OM	MS, RI
Bornyl acetate	1285	0.59	0.53	OM	MS, RI
Menthyl acetate	1293	0.51	0.40	OT	MS, RI
α-Terpinyl acetate	1345	1.44	1.69	OM	MS, RI, Std
Citronellyl formate	1270	0.07	0.05	OM	MS, RI
Neryl acetate	1358	0.60	0.42	OM	MS, RI
α-Cubebene	1375	0.54	0.64	SH	MS, RI
β-Caryophyllene	1426	1.12	0.82	SH	MS, RI, Std
γ-Gurjunene	1474	–	0.03	SH	MS, RI
E-β-Ionone	1486	0.09	–	OT	MS, RI
Bicyclogermacrene	1499	0.12	0.14	SH	MS, RI
α-Muurolene	1501	0.07	0.15	SH	MS, RI
δ-Cadinene	1525	3.28	3.77	SH	MS, RI
cis-Calamenene	1526	1.06	1.29	SH	MS, RI
γ-Cadinene	1516	1.04	1.09	SH	MS, RI
Selina 3,7 (11)-diene	1545	1.25	2.19	SH	MS, RI
E Nerolidol	1561	4.89	4.72	OS	MS, RI
β-Elemenone	1591	0.35	0.19	OS	MS, RI
α-Cadinol	1650	–	0.02	OS	MS, RI
δ-Cadinol	1661	0.85	0.81	OS	MS, RI
Germacrone	1691	1.22	0.67	OS	MS, RI
Eudesm 7(11)-en-4-ol	1699	0.27	0.29	OS	MS, RI
Monoterpene hydrocarbons (MH)		52.96	48.36		
Oxygenated monoterpenes (OM)		23.73	25.80		
Sesquiterpene hydrocarbons (SH)		8.48	10.12		
Oxygenated sesquiterpenes (OS)		7.58	6.70		
Other compounds (OT)		3.18	2.84		
Total identified, %		95.93	93.82		

[1] Identification method: RI – retention index, MS – mass spectrometry, Std – analytical standard.

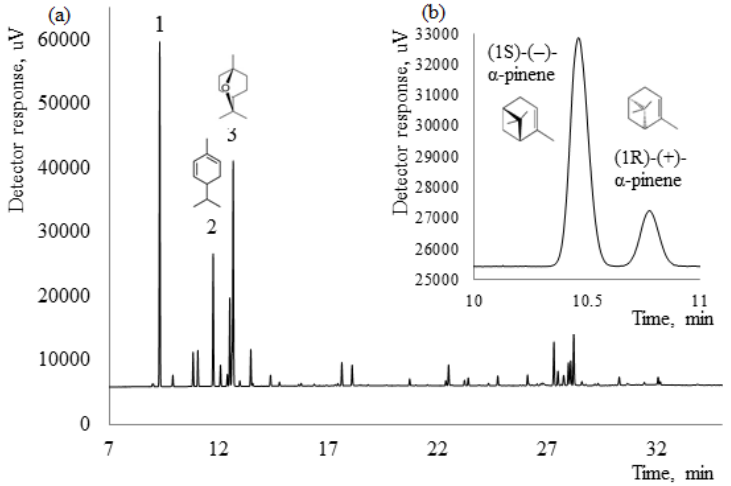

Figure 2. (a) Capillary GC analysis of *Myrica gale* essential oil (1 – α-pinene, 2 – α-phellandrene, 3 – 1,8-cineole); (b) Chiral-phase capillary GC analysis of α-pinene enantiomers in *Myrica gale* essential oil

able levels of antimicrobial activity against all microorganism strains. The MIC values of the essential oils were 316 µg/ml and 4977 µg/ml against the test bacteria and 1264 µg/ml and 4977 µg/ml against the test yeasts. The tested antibiotics exhibited variable levels of antimicrobial activity against all microorganism strains, concentrations ranging from 0.19 to 32.0 µg/ml (Table 3.).

The growth of Gram-negative *E. coli* and *A. baumannii* bacteria were weakly inhibited (MIC 4977 µg/ml and 1264 µg/ml, respectively) even at the highest concentration of the essential oil, whereas Gram-positive *S. aureus* was more susceptible (MIC 316 µg/ml). The efficiency of essential oil of *M. gale* on *C. parapsilosis* was stronger than on *C. albicans* (Table 3).

The essential oils of *M. gale* fruits showed the strongest antifungal activities against the investigated *Aspergillus* and *Trichophyton* strains with MIC values of 316 µg/ml and 1264 µg/ml, respectively (Table 3). Mean-

Table 3. Minimum inhibitory concentrations (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC) of *Myrica gale* fruit essential oil against pathogenic microorganisms and MIC values of antibiotics (all from Liofilchem, Italy)

Microorganisms	*Myrica gale* essential oils		Antibiotics
	MIC, µg/ml	MBC/MFC, µg/ml	MIC, µg/ml
Escherichia coli ATCC 25922	4977	19750	0.50[1]
Staphylococcus aureus ATCC 29213	316	4977	0.75[2]
Acinetobacter baumannii ATCC BAA-747	1264	4977	6.00[3]
Staphylococcus aureus ATCC 43300 (MRSA)	316	3160	0.19[4]
Aspergillus flavus CBS 120264	316	19750	1.50[5]
Aspergillus fumigatus 167000006359	316	19750	3.00[5]
Candida albicans CBS 2730	4977	19750	0.25[5]
Candida parapsilosis CBS 8836	1264	19750	32.00[5]
Trichophyton interdigitale Rinaldi 91427	1264	19750	32.00[5]
Trichophyton rubrum ATCC 28188	1264	19750	3.00[5]

[1] – gentamycin, [2] – vancomycin, [3] – colistin, [4] – trimethoprim-sulfamethoxazole, [e] – itraconazole.

while, it exhibited low MFC value (19750 µg/ml) against *Aspergillus* fungi, dermatophytes of the genus *Trichophyton* and *Candida* yeasts (Table 3).

Discussion

We have found surprisingly high amounts of essential oils in fruits of *M. gale* in Lithuania, averaging to 3.03 ±0.20%. According to literature, leaves and flowers of *M. gale* accumulate much lower amounts of essential oils: leaves – 0.20–0.65%, flowers – 0.97% (Jaskonis 1989, Svoboda et al. 1998, Sylvestre et al. 2005). In our study, fruits from the natural *M. gale* population (Habitat 1) accumulated more essential oils than those from the anthropogenic population (Habitat 2) by 1.2 times, and the difference was statistically significant ($p < 0.05$). The two populations of *M. gale* are quite remote from each other (ca 274 km straight line) and differ much in environmental conditions (Table 1). Although both habitats present itself as wet and acid peat soils (pH 4.7–4.9), the natural one (Habitat 1) is 2.3 times richer in total nitrogen and 6.6 times poorer in total potassium. Meanwhile, the climatic conditions differ even more: Habitat 1 is in the western part of Lithuania with a relatively soft and humid marine climate if compared to the harsher one of the Habitat 2 in the eastern part of Lithuania. The individuals of *M. gale* were better developed in the natural population presenting itself significantly larger shrubs with larger leaves than those in the anthropogenic population, and consequently accumulated higher amounts of essential oils. This is in agreement with Popovici et al. (2008) who noted that essential oil content is markedly dependent on the environment.

α-Pinene was the main compound of fruit essential oils of *M. gale* in both habitats. This implies that *M. gale*, being a rare species in Lithuania, could mainly be represented by an α-pinene chemotype. α-Pinene possesses a wide spectrum of properties such as fungicidal, antibacterial, anticarcinogenic, antioxidant, anti-inflammatory, diuretic, immunostimulant, anticonvulsive, sedative, antistressor, hypoglycaemic, anticholinesterase activity. This bicyclic monoterpene is the most volatile component of the turpentine oil which was used in phytotherapy both externally (as antiparasitic, analgesic, revulsive, disinfectant, antispasmodic, antirheumatic) and internally (active on bronchial secretion, dissolving gallstones, genitourinary tract infections, as diuretic) since ancient times (Mercier et al. 2009). Many drugs, used for hepatic and renal diseases, like those produced in Germany (*Rowachol* and *Rowatinex*), Slovenia (*Uroterp*) and Poland (*Terpichol* and *Terpinex*), contain α-pinene (Sybilska et al. 1994). Chirality is characteristic for this monoterpene: (1R)-(+) and (1S)-(–) enantiomers of α-pinene are found in plants. This phenomenon is important not only for the production of fragrances and flavours (different enantiomeric forms of the same compound can condition quite different odours) (Zawirska-Wojtasiak 2006), but it can also predetermine different biological activity of the same compound and/or diverse potential of biological activity due to different specific interactions with other chemical compounds (Rivas da Silva 2012, Ložienė et al. 2018). It has been reported that a light mint flavour is typical for (+), while pine flavour associates with (–) enantiomer of α-pinene, both differing in biological and repellent activities (Hull et al. 2004, Zawirska-Wojtasiak 2006). The present study demonstrated that (1S)-(–)-α-pinene prevailed over (1R)-(+)-α-pinene and amounted to very similar percentages of α-pinene fraction in both samples of fruits of *M. gale* collected from the natural and anthropogenic populations.

The second most abundant compound of the essential oils was 1.8-cineole. A very similar percentage of 1,8-cineole (18.9%) was found in fruit essential oil of *M. gale* growing in France, where this monoterpene was the second most abundant compound of the essential oil too (Popovici et al. 2008); while 1,8-cineole was the main compound of fruit essential oil of *M. gale* growing in Estonia and amounted to 25.7% (Sokolova et al. 2005). 1,8-Cineole is a broadly distributed monoterpene in plants with a eucalyptus-like smell, which is why it has another common name, *eucalyptol*. This monoterpene is an interesting substance with several pharmaceutically useful properties important for human treatment: it exhibits mucolytic, bronchodilating and anti-inflammatory effects. Therefore, it is used in phytotherapy of such diseases as asthma, sinusitis and bronchitis (Juergens et al. 1998, Juergens et al. 2003).

Literature data suggest that germacrone is an abundant compound in essential oils of *M. gale*. It was reported as one of the major compounds of fruit essential oils of *M. gale* collected in France (Popovici et al. 2008) and the essential oils isolated from aerial parts of *M. gale* collected in Canada (Collin and Gagnon 2016), amounting to 14.2% and 13.5%, respectively. But the present study showed that germacrone amounted to only minor percen-

tages of essential oils of fruits from both *M. gale* habitats in Lithuania. Meanwhile, α-phellandrene was the third most abundant compound (Table 2).

According to the literature above, chemical polymorphism is characteristic of *M. gale* essential oils. The present study showed that chemical composition of fruit essential oils of *M. gale* growing in two different habitats in Lithuania was quite similar. Only the quantitative composition of essential oils differed significantly (with the percentages of α-pinene differing most, although statistically not significantly) reflecting more favourable and probably optimal growth conditions of the species in its natural habitat in the western part of Lithuania.

The latest papers on chemical analysis and antimicrobial properties of *M. gale* essential oils have reported that they possess growth inhibitory effects of a varying degree against some bacteria, yeasts and fungi (Popovici et al. 2008, Nakata et al. 2013, Krogsbøll et al. 2016). But most of them dealt with the oils extracted from the other parts than fruits. In our study, fruit essential oils of *M. gale* showed similar activities against microorganisms, disease agents of skin (bacterium *S. aureus*, yeasts *C. albicans* and *C. parapsilosis*, fungi *T. rubrum* and *T. mentagrophytes*) and internal organs (bacteria *E. coli* and *A. baumannii*, fungi *A. fumigatus* and *A. flavus*), thus validating the external and internal ethnopharmacological uses of this plant. The strongest growth-inhibitory and bactericidal effects of sweet gale essential oils were established on both examined strains of bacterium *S. aureus*, which can cause severe wound infections. Therefore, from the old times *M. gale* has been used in folk medicine as wound antiseptic and as a remedy to speed up wound-healing (Isokas 2001, Gudžinskas 2012). The antimicrobial activity of *M. gale* essential oils could be attributed to the high contents of the compounds with known antimicrobial activity, such as α-pinene (Zawirska-Wojtasiak 2006, Popovici et al. 2008), 1,8-cineole (Juergens et al. 1998, Popovici et al. 2008, Labokas and Ložienė 2013) and α-phellandrene (Juergens et al. 2003). According to the literature (Bishop and MacDonald 1951, Nakata et al. 2013, Krogsbøll et al. 2016), Gram-positive bacteria seemed to be more sensitive to the essential oil than Gram-negative bacteria. Although these studies also dealt with other plant parts than fruits, an agreement could be observed with our results on the activity tests of fruit essential oils with *E. coli*, *A. baumannii* and *S. aureus* bacteria. Popovici et al. (2008) showed that the growth of *A. flavus* was weakly inhibited at the 1000 ppm (0.1%) concentration of fruit essential oils of *M. gale*, which is close to our data.

As mentioned above, enantiomeric analysis of α-pinene, the main compound of *M. gale* essential oils, showed that (1S)-(–)-α-pinene prevailed over (1R)-(+)-α-pinene, i.e., enantiomeric composition of α-pinene fraction was S>R. A recent study has demonstrated different activities of pure α-pinene of *Juniperus communis* origin with different enantiomeric composition on the same bacteria, yeasts and fungi: α-pinene with the enantiomeric composition S<R inhibited growth of bacteria *S. aureus* and E. coli and *Candida* yeasts more strongly; meanwhile, α-pinene with the enantiomeric composition S≈R affected growth of *Trichophyton* and *Aspergillus* (Rivas da Silva et al. 2012). Thus, the comparison of the results of the present study and the referred one shows that the tested strains of *Trichophyton* and *Aspergillus* are more sensitive to (1S)-(–)-α-pinene; whereas *E. coli* and *C. albicans* were weakly inhibited even at the highest *M. gale* essential oil concentration.

Conclusions

Fruits of *M. gale* accumulate high total contents of biologically active essential oils that should make their traditional use attractive. The percentage of fruit essential oils is markedly dependent on environmental factors with the typical humid marine climate providing best conditions for the species. The study showed that both populations represented the same α-pinene chemotype with the (1S)-(–)-α-pinene prevailing over the (1R)-(+)-α-pinene. The antimicrobial study *in vitro* indicated that the pathogenic yeast *C. parapsilosis*, dermatophytes *T. rubrum* and *T. mentagrophytes* and *Aspergillus* fungi were found to be more susceptible to fruit essential oils of *M. gale*, whereas the pathogenic bacterium *E. coli* and fungus *C. albicans* were weakly inhibited even at the highest essential oil concentration. The strongest growth-inhibitory and bactericidal effects of sweet gale essential oils were established on the bacterium *S. aureus*, which can cause severe wound infections. The findings of the present study indicate that fruit essential oils of *M. gale* have definite potential activity against pathogenic bacteria, yeasts and fungi including dermatophytes. This validates the traditional use of *M. gale* fruits, e.g., for treatment of dermal diseases as well as for other antimicrobial applications.

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