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Radical scavenging and antibacterial properties of the extracts from different *Thymus pulegioides* L. chemotypes

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Abstract

Radical scavenging and antibacterial properties of large thyme extracts isolated from five chemotypes of *Thymus pulegioides* L. growing wild in Lithuania were studied. The chemotypes were defined according to the main essential oil components: linalool (L), geranial/geraniol/neral (G/G/N), thymol (T), carvacrol/γ-terpinene/*p*-cymene (C/γT/*p*C) and thymol/carvacrol/γ-terpinene/*p*-cymene (T/C/γT/*p*C). The contents of phenolic compounds, flavonoids and flavonols were determined. It was found that the extracts of phenolic chemotypes containing remarkable concentrations of thymol and/or carvacrol were stronger DPPH and ABTS free radical scavengers in the model systems. The antibacterial activity of the extracts depended on the plant chemotype, extract preparation, solvent used and finally the sensitivity of bacteria. *Bacillus cereus*, *Micrococcus luteus*, *Staphylococcus epidermidis* and *Staphylococcus aureus* were the most sensitive to the all extracts applied, whereas *Escherichia coli*, *Salmonella typhimurium* and *Enterobacter aerogenes* remained resistant.

Keywords: *Thymus pulegioides* L.; Chemotypes; Antioxidant activity; DPPH; ABTS; Antibacterial activity

1. Introduction

Plant kingdom is a good source of natural preparations containing effective bioactive compounds which can be used for different applications, particularly as food additives and health promoting ingredients in the formulations of functional foods and nutraceuticals (Gibson & Williams, 2000; Meskin, Bidlack, Davies, & Omaye, 2002; Shahidi, 1997). Fresh and dried aromatic plants as well as their processed products have been widely used as flavourings since ancient times, however, during last few decades they also have become a subject for a search of natural antioxidants and antibacterial agents (Evans & Reyhout, 1992; Pokorny, 1991; Madsen & Bertelsen, 1995; Venskutonis, 2004).

The genus *Thymus* comprises 215 species with *Thymus vulgaris* being one of the most important and thoroughly investigated aromatic plant. Chemistry, processing and application of *Thymus* species were previously investigated. *Thymus* species as well as many other aromatic plants biosynthesize remarkable amount of volatile compounds referred as the essential oil, therefore chemical classification of such plants is based on the main essential oil components. Chemical polymorphism is characteristic to the species of *Thymus*; numerous chemotypes have been defined, such as carvacrol and thymol, α-terpineol, thujone, geraniol, linalool and others (Thompson, Manicacci, & Tarayre, 1998). Essential oils containing high amount of thymol and carvacrol were reported to possess the highest antioxidant activity (Aeschbach et al., 1994; Dapkevičius, Venskutonis, Van Beek, & Linssen, 1998; Deighton, Glidewell, Deans, & Goodman, 1994; Farag, Badei, & ElBaroty, 1989). In addition, these compounds exhibit other bioactivities, e.g. thymol is an antiseptic, while carvacrol possesses antifungal properties (Menphini, Pagiotti, & Capuccella, 1993). Non-volatile antioxidants, such as flavonoids and vitamin E were also found in the extracts of *T. vulgaris*

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(Dapkevicius et al., 2002; Guillén & Manzanos, 1998). Therefore essential oils of thyme can be used as natural preservative ingredients in the food industry (Banias, Oreopoulou, & Thomopoulos, 1992; Conner & Beuchat, 1984; Curtis, Shetty, Cassagnol, & Peleg, 1996; Economou, Oreopoulou, & Thomopoulos, 1991; Karapmar & Aktug, 1987; Shapiro, Meier, & King, 1994).

Thymus pulegioides L. is less important commercially species comparing to *T. zygis* and *T. vulgaris* (Lawrence, 1992). However, the essential oil of *T. pulegioides* was studied quite intensively (Stahl-Biskup & Sáez, 2002). For instance, *T. pulegioides* growing wild in Lithuania was characterised as a plant with a remarkable chemical polymorphism; as a consequence six chemotypes were defined for the species according to the main essential oil constituents, namely linalool (L), geranial/geraniol/neral (G/G/N), thymol (T), carvacrol/γ-terpinene/*p*-cymene (C/γT/*p*C), thymol/carvacrol/γ-terpinene/*p*-cymene (T/C/γT/*p*C) and α-terpenyl acetate (Ložienė, Venskutonis, & Vaičiūnienė, 2002, 2003; Mockutė & Bernotienė, 1999, 2001). However, the reports on antioxidant and antibacterial properties of *T. pulegioides* as well as composition of non-volatile fraction of its extracts are rather scarce and fragmentic (Vila, 2002).

The main aim of this study was to examine the antioxidant and antibacterial properties of *T. pulegioides* extracts isolated from different plant chemotypes by using various solvents and extraction procedures.

2. Materials and methods

2.1. Plant material and chemicals

Thymus pulegioides plants were collected from different natural habitats and replanted in the experimental field collection of the Institute of Botany, Lithuania. The plants according to the main components in their essential oil previously were attributed to five chemotypes, namely linalool (L), geranial/geraniol/neral (G/G/N), thymol (T), carvacrol/γ-terpinene/*p*-cymene (C/γT/*p*C), thymol/carvacrol/γ-terpinene/*p*-cymene (T/C/γT/*p*C) (Ložienė & Venskutonis, 2005). Individual plants were selected from each predefined chemotype described in the above cited work. These five plants of a different chemotype were cultivated vegetatively in separate experimental plots in 2003. They were harvested at the flowering phase, air-dried at room temperature and ground before analysis.

Food grade ethanol was obtained from the local distillery (AB Stumbras, Kaunas Lithuania). Technical grade methanol (MeOH, Lachema, Neratovice, Czech Republic) was distilled prior to use for preparative chromatography. Analytical grade MeOH and glacial acetic acid (99.8%) for HPLC were from Lachema (Neratovice, Czech Republic).

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95%) anhydrous sodium carbonate, gallic acid were from Sigma–Aldrich Chemie (Steinheim, Germany), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), standard Folin–Ciocalteu's phenol reagent, KCl, NaCl, Na_2HPO_4, $K_2S_2O_8$ were from Merck (Darmstadt, Germany); KH_2PO_4 from Jansen Chimica (Beerse, Belgium). Ultra pure water was used for determination of total phenolic compounds.

2.2. Essential oil analysis

Twenty grams of plant material (three replicates for every chemotype) were submitted, for 2 h, to hydrodistillation using a European Pharmacopoeia apparatus. The essential oils obtained were diluted in diethyl ether (20 μl of oil in 1 ml of solvent) and analysed on a Fisons 8261 gas chromatograph with a flame ionisation detector (FID) on a fused silica capillary column DB-5, 25 m, i.d. 0.32 mm, film thickness 0.5 μm. Helium was used as a carrier gas with a flow rate of 1.6 ml/min; detector temperature was 260 °C, oven temperature was programmed from 40 to 250 °C at the rate of 4 °C/min. Split injector was heated at 250 °C, split ratio was 15:1. Data were processed on a DP 800 integrator. For the identification essential oils were also analysed on a HP 5890 (II) instrument equipped with a 5971 series mass selective detector operating in the electron impact ionisation mode at 70 eV, and the following GC parameters: split inlet 1:10; helium as a carrier gas at a flow rate of 2 ml/min; fused silica HP5 MS column (Hewlett Packard, crosslinked 5% phenyl methyl silicone) 30 m length, 0.25 mm i.d., 0.25 μm film thickness, temperature program from 40 to 250 °C increasing at 4 °C/min. Identification was based mainly on the comparison of retention indices (RI) (Adams, 2001; Davies, 1990) and mass spectra (NIST/EPA/NIH Mass Spectral Database NBS75K).

2.3. Preparation of extracts

Three different polarity solvents, *n*-hexane, acetone and ethanol, were used for the isolation of active components from the whole and deodorised *T. pulegioides* herb. Deodorisation was performed by hydrodistilling volatile oil from the ground material in a European Pharmacopoeia apparatus for 2 h. All extractions were carried out at room temperature for 2 h; solvent and plant material ratio was 1–17.5 (m/v). The solids after deodorisation and each extraction were separated from the liquid by filtration and dried at 50–60 °C temperature in a drying oven. The extracts were concentrated in a rotary vaccuum evaporator Büchi (Flavil, Switzerland) at temperature not higher than 50 °C; dry extracts were stored in a refrigerator before further analysis. Extraction is summarised in Fig. 1.

2.4. DPPH radical scavenging assay

Radical scavenging activity (RSA) of thyme extracts against stable DPPH$^{\cdot}$ was determined by a slightly modified DPPH$^{\cdot}$ radical scavenging assay (Brand-Williams, Culivier, & Berset, 1995). It is widely used reaction based

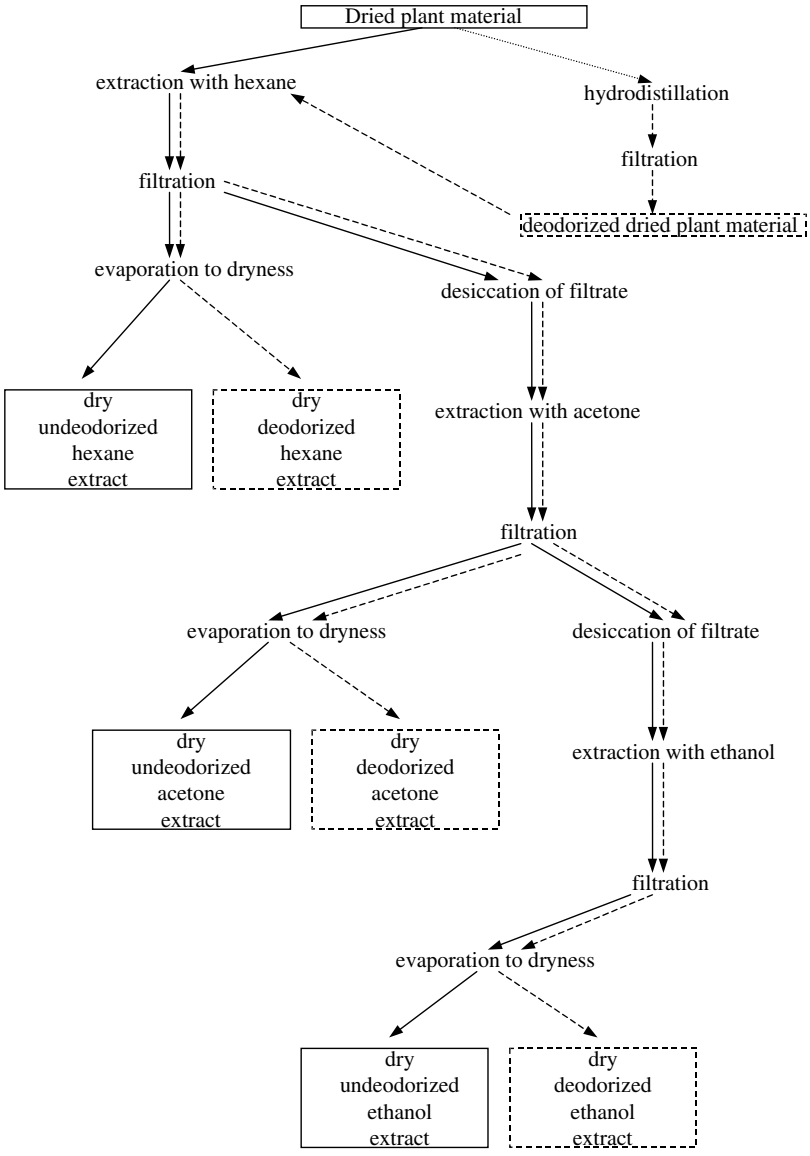

Fig. 1. Extraction scheme for the preparation of *Thymus pulegioides* extracts.

on the ability of antioxidant molecule to donate hydrogen to DPPH$^{\cdot}$, which consequently turns into an inactive form. The solution of DPPH$^{\cdot}$ (6×10^{-5} M) was prepared daily, before measurements on a UV/visible light spectrophotometer (Spectronic Genesys 8, Rochester, USA) at 515 nm. Three milliliters of this solution were mixed with 77 μl extract solution in 1 cm path length disposable microcuvette (Greiner Labortech, Alpher a/d Rijn, The Netherlands). The decreasing absorbance was read during 15 min reaction time at 10 s intervals until the absorbance stabilized. Simultaneously the absorption of a blank sample containing the same amount of methanol and DPPH$^{\cdot}$ solution was prepared and measured daily. The measurements were performed in triplicate. The RSA was calculated by a formula $I = [(A_B - A_A)/A_B] \times 100$, where I is the DPPH$^{\cdot}$ inhibition, %; A_B is the absorption of blank sample ($t = 0$ min); A_A is the absorption of extract solution ($t = 16$ min).

2.5. ABTS radical cation decolourisation assay

ABTS$^{\cdot+}$ radical cation was produced by reacting ABTS with potassium persulfate ($K_2S_2O_8$) (Re et al., 1999). Stock solution of ABTS (2 mM) was prepared by dissolving in 50 ml of phosphate buffered saline (PBS) obtained by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of ultra pure water. If the pH was lower than 7.4, it was adjusted with NaOH. Ultra pure water was used to prepare 70 mM solution of $K_2S_2O_8$. ABTS$^{\cdot+}$ radical cation was produced by reacting 50 ml of ABTS stock solution with 200 μl of $K_2S_2O_8$ solution and allowing the mixture to stand in the dark at room temperature for 15–16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the assessment of extracts, the ABTS$^{\cdot+}$ solution was diluted with PBS to obtain the absorbance of 0.800 ± 0.030 at 734 nm. Three milliliters of ABTS$^{\cdot+}$ solu-

tion were mixed with 30 μl ethanol solution of leaf extract in 1 cm path length microcuvette. The absorbance was read at ambient temperature exactly after 1, 4, 6 and 10 min. PBS solution was used as a blank sample. All determinations were performed in triplicate. The percentage decrease of the absorbance at 734 nm was calculated by formula $I = [(A_B - A_A)/A_B] \times 100$, where I is the $ABTS^{\cdot+}$ inhibition, %; A_B is the absorption of blank sample ($t = 0$ min); A_A is the absorption of extract solution ($t = 10$ min).

2.6. Assessment of antibacterial effect

Seven food spoilage bacteria, including Gram-positive, namely *Bacillus cereus* (ATCC 10876), *Micrococcus luteus*, *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* and Gram-negative pathogens, namely *Salmonella typhimurium* (ATCC 14028), *Esherichia coli* (ATCC 25922) and *Enterobacter aerogenes* (ATCC 13048) were used in this study. The antimicrobial properties were evaluated by the agar well diffusion method. For the preparation of cell suspension bacteria were grown 18 h at 37 °C on the slant agar (Oxoid, CM325). After washing the cell suspensions were adjusted according to McFarland No 0.5 standard (Andrews, 1992) and mixed in a Vortex mixer. For the determination of the total bacteria count the suspension of bacteria cells was poured into the dissolved and cooled to 47 °C agar, and mixed one more time to obtain even distribution of the cells. Ten milliliters of each bacteria culture were pipetted into 90 mm diameter Petri plates. After the setting 6 wells (8 mm diameter each) were punched in the agar and filled with 50 and 10 μl of 15% ethanolic solutions of *T. pulegioides* extracts. The plates were incubated at 37 °C during 24 h and the antimicrobial effect was assessed by the diameter of clear zones developed around wells. When such zones were not observed it was accepted that the extracts do not possess antimicrobial effect.

2.7. Total amount of phenolic compounds

The concentration of phenolic compounds in ethanol extracts of *T. pulegioides* leaves was determined by Folin–Ciocalteu method (Folin & Ciocalteu, 1927). Calibration curve was prepared by using 1 ml reference gallic acid solutions in ethanol (aliquots of 0.025, 0.075, 0.100, 0.175 and 0.350 mg/ml) which were mixed with 5 ml of a standard Folin–Ciocaleu reagent and diluted with distilled water (1:10) and 4 ml of 7.5% sodium carbonate solution in water. The absorption was read after 20 min at 765 nm on a Spectronic Genesys 8 spectrophotometer and the calibration curve ($y = 9.6994x + 0.104$, $R^2 = 0.9973$) was drawn. Plant phenolics were determined in 1 ml of extract solution in ethanol by the same analysis procedure; blank sample was prepared by using pure water instead of plant extract. All determinations were performed in triplicate. Total content of phenolic compounds (C) in extracts in gallic acid equivalents (mg/g GAE) was calculated by formula $C = c \times V/m$, where c is the concentration of gallic acid determined from the calibration curve (mg/ml); V is the volume of extract (ml); m is the weight of pure *T. pulegioides* ethanolic extract (g).

2.8. Determination of flavonoids

The percentage of flavonoids was measured using rutin as a reference (State Pharmacopoeia, 1989). One milliliter of plant extract solution in 95% ethanol (10 g/L) and 1 ml of aluminium trichloride solution in 95% ethanol (20 g/L) were pipetted into a 25 ml volumetric flask and made up with 95% ethanol. The absorbance was read at 415 nm after 40 min at 20 °C. Blank samples were prepared from the mixture of 1 ml of plant extract and 1 drop of diluted acetic acid. The absorbance of a reference solution, which was prepared by using 1 ml of rutin solution instead of plant extract was read simultaneously. Rutin solution was prepared from 0.05 g of dried at 130–150 °C for 3 h rutin, which was diluted in 100 ml of 95% ethanol. All determinations were performed in triplicate. The percentage of flavonoids in plant AO extracts was calculated by formula $X = (A \times m_o \times 100 \times 10)/(A_o \times m \times 100)$; where A is the absorbance of extract; A_o is the absorbance of rutin; m is the weight of pure extract (g); m_o is the weight of rutin (g).

2.9. Determination of flavonols

Series of reference rutin solutions containing 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 mg/ml of rutin were prepared. Two milliliters of such reference were mixed with 2 ml of aluminium trichloride solution (20 g/L) in 95% ethanol and 6 ml of sodium acetate solution in ethanol (50 g/L) were added. The absorbance was read at 440 nm after 2.5 h at 20 °C and the calibration curve on the dependance of the absorbency on the concentration of rutin was drawn. Plant extract samples were prepared under the same conditions by using 2 ml of extract (10 g/l) in 95% ethanol instead of rutin. All determinations were performed in triplicate. The percentage of flavonols was calculated by the formula $X = (C \times V \times 100)/(m \times 100)$, where C is the concentration of rutin, determined form the calibration curve (mg/ml); V is the volume of plant extract (ml); m is the weight of pure plant extract (g).

2.10. High pressure liquid chromatography and mass spectrometry (HPLC/UV/MS)

The extracts were analysed by Waters Macromass system consisting of 1525 binary pump and ZQ 2000 mass detector (Millipore, Waters Chromatography, Milford, USA). Additionaly the system was equipped with Lachrom UV detector L-7400 (Merck, Germany). Extract components were separated on a Synergi MAX–RP analytical column, 250 × 4.60 mm i.d. (Phenomenex, Torrance, USA) packed with Luna C_{18} stationary phase, particle size 4 μm. The

linear binary gradient was used at a flow rate of 0.8 ml/min. The time of HPLC run was over 40 min. Binary mobile phase consisted of a solvent A (ultra pure water with 10% methanol and 1% of glacial acetic acid) and solvent B (100% methanol). Elution from the column was achieved with the following linear gradient: 0–30 min B increased from 30% to 100% and kept constant till 33 min; 33–36 min B decreased back to 30% and kept constant till 40 min. UV detector was operating at 254 nm wavelength. Mass detector parameters were as follows: polarity ES+ and ES−; capillary voltage 3.00 and 3.08 kV; ionisation 40.0 V; temperature of ion source 120 °C; evaporation temperature 250 °C; ion source gas (nitrogen) flow 80 L/h; evaporation gas (nitrogen) flow 250 L/h; mass range 40–600 amu. During the scanning of MS spectra the flow rate was 0.5 ml/min. The injection volume was 10 μl.

For chromatographic analysis 0.5% extract solutions in methanol were used, which were filtered before analysis. The identification of extract components was based on MS and available literature data.

3. Results and discussion

3.1. Essential oil composition of T. pulegioides chemotypes

The content of essential oil in different plant chemotypes was as follows: thymol chemotype $-0.7 \pm 0.03\%$; geranial/geraniol/neral chemotype $-0.5 \pm 0.03\%$; linalool chemotype $-0.5 \pm 0.06\%$; carvacrol/γ-terpinene/*p*-cymene chemotype $-0.5 \pm 0.03\%$; thymol/carvacrol/γ-terpinene/*p*-cymene chemotype $-0.4 \pm 0.03\%$. The composition of essential oils of the studied *T. pulegioides* chemotypes is presented in Table 1. Volatile oil components from the same chemotypes of *T. pulegioides* were monitored in 1998–2002 (Ložienė & Venskutonis, 2005). However, it is known that the composition of secondary metabolites, such as essential oil constituents depend on various factors, particularly on climatic and environmental conditions. Therefore further monitoring of essential oil composition of different chemotype plants was of some interest. It can be observed that the composition of the essential oil of *T. pulegioides* harvested in 2003 was similar to the composition of relevant chemotypes harvested earlier, e.g., in 2002 (Ložienė & Venskutonis, 2005). The content of linalool in L chemotype was 80.3%, geranial, geraniol and neral in G/G/N chemotype 64.4%, thymol, carvacrol, γ-terpinene and *p*-cymene in T/C/γT/*p*C chemotype 65.1%, carvacrol, γ-terpinene and *p*-cymene in C/γT/*p*C chemotype 36.0%, thymol and thymol methyl ether in T chemotype 37.9%.

3.2. Radical scavenging activity (RSA) of T. pulegioides extracts

Two most widely used model reaction systems containing stable radical DPPH$^{\cdot}$ and cation radical ABTS$^{\cdot+}$ were used to assess radical scavenging activity (RSA) of *T. pulegioides* extracts. The results obtained demonstrate the effect of three factors on the RSA, namely the polarity of solvent, the type of free radical used in reaction and plant material treatment before extraction (whole plant or deodorised part). Therefore, all these effects will be briefly discussed in this section.

First of all it can be clearly observed that the extracts isolated with polar solvent ethanol were considerably stronger radical scavengers as compared with acetone in both reaction systems (Figs. 2 and 3). For instance, the decrease in absorbance during DPPH$^{\cdot}$ reaction with radical scavengers present in ethanolic extracts from the whole plant material was higher than 80%, while in case of acetone extracts the same reaction index varied from 20% (L chemotype) to 60% (C/γT/*p*C chemotype). It indicates that in the former case almost all radicals have been scavenged by the active extract components present in ethanolic extracts. The RSA of acetone extracts from the so-called phenolic chemotype plant extracts containing remarkable amounts of thymol and/or carvacrol was higher comparing to L and G/G/N chemotypes. It is also interesting to note that the differences in the RSA of ethanolic extracts from *T. pulegioides* chemotypes were more remarkable in the ABTS$^{\cdot+}$ reaction than in DPPH$^{\cdot}$ reaction (Figs. 2a and 3a). Thus, the RSA of the all non-deodorised extracts in DPPH$^{\cdot}$ reaction was in the range of 80–90%, while in ABTS$^{\cdot+}$ reaction it varied from 12% (L) to 33% (C/γT/*p*C). The differences in RSA in the two reaction systems were also observed in the previously published reports (Dvaranauskaite, Venskutonis, & Labokas, 2006; Miliauskas, Venskutonis, & van Beek, 2004). Most likely, reaction kinetics between various radical scavengers occurring in crude plant extracts and DPPH$^{\cdot}$/ABTS$^{\cdot+}$ free radicals proceed in a different way.

The results obtained revealed remarkable differences in the RSA between the extracts from the whole and from the deodorised plant material, however these differences again depend on the solvent, plant chemotype and reaction system. The RSA of ethanolic and acetone extracts from deodorised plant materials was lower than that from non-deodorised one except for G/G/N chemotype. In the latter case the RSA of ethanolic extract from deodorised G/G/N thyme was almost similar in DPPH$^{\cdot}$ reaction and slightly higher in ABTS$^{\cdot+}$ system. Antioxidant activity of deodorised water extracts of some Labiatae herbs was reported earlier, however it was not compared with the activity of non-deodorised extract (Dorman, Peltoketo, Hiltunen, & Tikkanen, 2003). The increase of antioxidant activity after deodorisation of savory extract was reported in rapesed oil (Bandonienė, Venskutonis, Gruzdienė, & Murkovic, 2002). It is worth noting that some extracts are losing almost all radical scavengers after plant deodorisation; the RSA of deodorised extracts of C/γT/*p*C chemotype in DPPH$^{\cdot}$ reaction, C/γT/*p*C, L and T/C/*p*C/γT chemotypes in ABTS$^{\cdot+}$ reaction was close to zero. On the contrary, ethanolic extracts from the deodorised T and L chemotype plants contained effective concentrations of strong radical scavengers which were sufficient to neutralise

Table 1
Composition of essential oil of different *Thymus pulegioides* L. chemotypes, GC area % (T/C/γT/*p*C – thymol/carvacrol/γ-terpinene/*p*-cymene, G/G/N – geranial/geraniol/neral, T – thymol, L – linalool, C/γT/*p*C – carvacrol/γ-terpinene/*p*-cymene chemotypes)

Components	*Thymus pulegioides* L. chemotypes				
	T/C/γT/*p*C	G/G/N	T	L	C/γT/*p*C
α-Thujene	0.9	–	1.6	–	1.1
α-Pinene	0.4	–	0.6	–	0.5
Camphene	0.1	–	–	–	0.4
Sabinene	tr	–	–	–	0.1
β-Pinene	0.1	–	–	–	0.2
1-Octen-3-ol	0.5	2.4	5.3	0.8	0.5
3-Octanone	0.3	–	0.5	–	0.2
Myrcene	1.3	–	1.5	0.2	1.4
3-Octanol	1.0	0.4	0.8	0.2	tr
α-Phelandrene	0.4	–	0.3	–	0.2
δ-3-Carene	0.1	–	–	–	0.1
α-Terpinene	2.3	–	2.0	–	2.4
p-Cymene	14.3	–	10.2	0.1	16.7
Limonene	1.1	–	0.3	0.1	1.2
(*Z*)-β-Ocimene	0.4	–	0.2	tr	0.2
(*E*)-β-Ocimene	tr	–	1.0	0.1	tr
γ-Terpinene	16.3	–	9.9	tr	21.4
cis-Sabinene hydrate	0.6	–	0.4	–	0.3
p-Mentha-3,8-diene	–	–	–	tr	–
Terpinolene	0.1	–	–	0.1	tr
trans-Sabinene hydrate	–	–	–	–	tr
Linalool	0.4	0.5	–	80.3	0.2
allo-Ocimene	tr	–	–	–	tr
Camphor	tr	–	–	–	–
iso-Borneol	–	0.3	–	–	–
p-Mentha-1,5-dien-8-ol	–	0.3	–	–	–
Borneol	0.2	0.6	–	–	0.7
Terpinen-4-ol	0.1	–	0.4	–	0.1
α-Terpineol	tr	–	–	0.4	0.1
Nerol	–	4.9	–	–	–
Thymol methyl ether	2.5	–	11.8	–	0.4
Neral	–	9.2	–	–	–
Carvacrol methyl ether	4.3	–	4.3	0.1	7.1
trans-Sabinene hydrate acetate	–	–	–	4.7	–
Geraniol	–	43.8	0.3	–	–
Geranial	–	11.4	–	–	–
Thymol	11.9	–	26.1	–	0.2
Carvacrol ethyl ether	–	–	–	–	0.6
Geranyl formate	–	0.4	–	–	–
Carvacrol	22.6	–	1.5	–	24.9
α-Elemene	0.3	–	–	–	0.6
4-Terpineol acetate	–	–	–	–	0.4
α-Terpinyl acetate	tr	–	–	–	0.2
α-Cubebene	0.1	–	–	–	0.5
Carvacrol acetate	tr	–	–	–	0.1
α-Copaene	0.1	–	–	–	0.1
Geranyl acetate	tr	2.0	–	–	–
β-Bourbonene	0.1	0.7	0.2	0.4	0.1
β-Elemene	–	0.2	–	0.1	tr
iso-Caryophyllene	–	–	–	–	tr
β-Caryophyllene	5.1	4.3	10.1	5.7	8.3
β-Gurjunene	0.2	0.1	–	0.1	0.1
γ-Elemene	tr	–	–	–	tr
(*Z*)-β-Farnesene	0.1	–	–	–	tr
(*E*)-*iso*-Eugenol	tr	–	–	tr	tr
α-Humulene	0.2	0.2	0.5	0.2	0.3
allo-Aromadendrene	tr	–	–	–	–
γ-Muurolene	0.4	–	0.6	–	0.2
Germacrene D	1.2	3.4	2.2	2.7	1.2
cis-β-Guaiene	–	–	–	–	tr

(*continued on next page*)

Table 1 (*continued*)

Components	*Thymus pulegioides* L. chemotypes				
	T/C/γT/*p*C	G/G/N	T	L	C/γT/*p*C
Bicyclogermacrene	0.6	–	–	0.6	–
α-Muurolene	0.6	–	–	–	0.1
trans-β-Guaiene	–	–	0.3	–	0.1
(*Z*)-α-Bisabolene	–	–	0.6	–	–
β-Bisabolene	4.7	9.2	3.3	1.4	5.1
γ-Cadinene	0.5	–	0.5	–	0.1
δ-Cadinene	0.9	0.2	–	tr	0.4
Cadina-1,4-diene	0.1	–	0.9	–	tr
α-Cadinene	0.1	0.1	–	–	0.1
Spathulenol	–	0.2	–	–	–
Caryophyllene oxide	0.3	1.4	1.2	0.5	0.6
epi-α-Cadinol	0.1	–	–	–	0.1
α-Cadinol	–	0.3	–	–	–
Total	97.9	96.5	99.4	98.8	99.9

tr = trace (⩽0.05%).

DPPH· radicals in the used model system. Acetone extracts from deodorised plants were not effective except for G/G/N chemotype.

Three important aspects should be considered in attempting to explain the above presented findings on the RSA of *T. pulegioides* extracts: the volatility of active com-

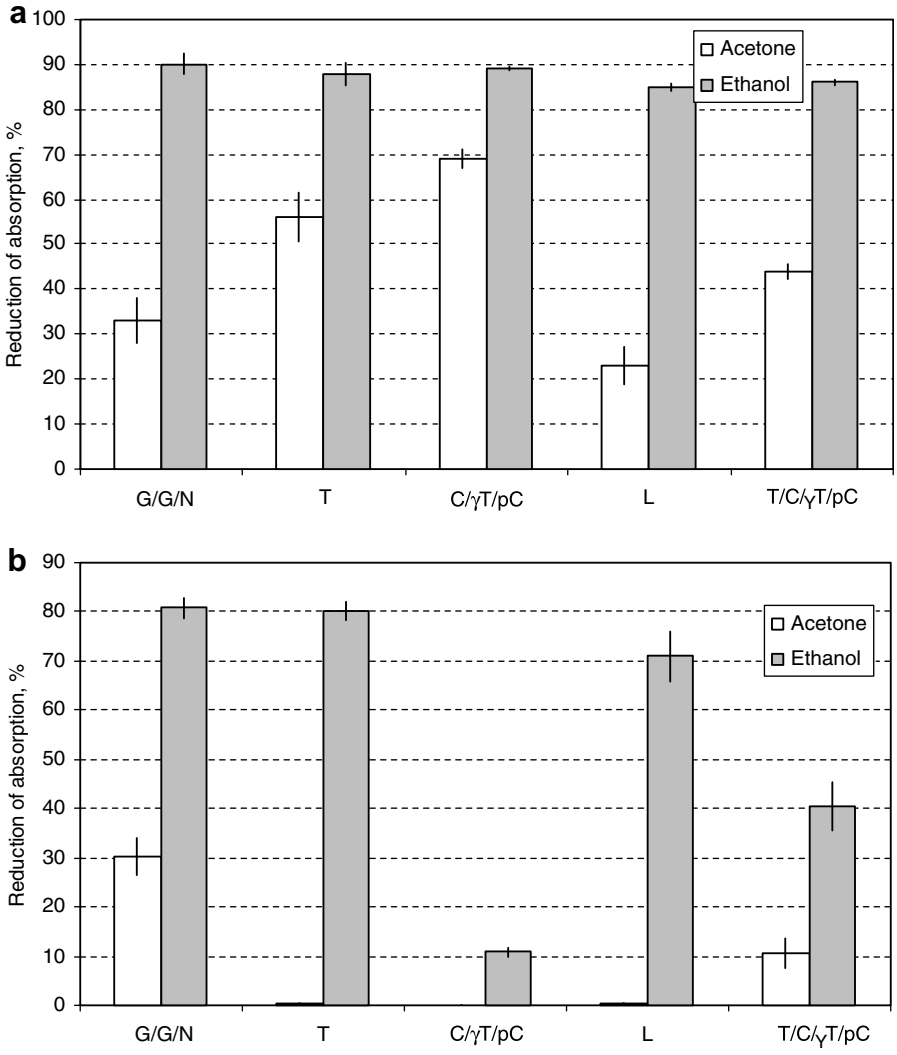

Fig. 2. Radical scavenging activity of *Thymus pulegioides* extracts against DPPH·: (a) whole plant material; (b) deodorised plant material.

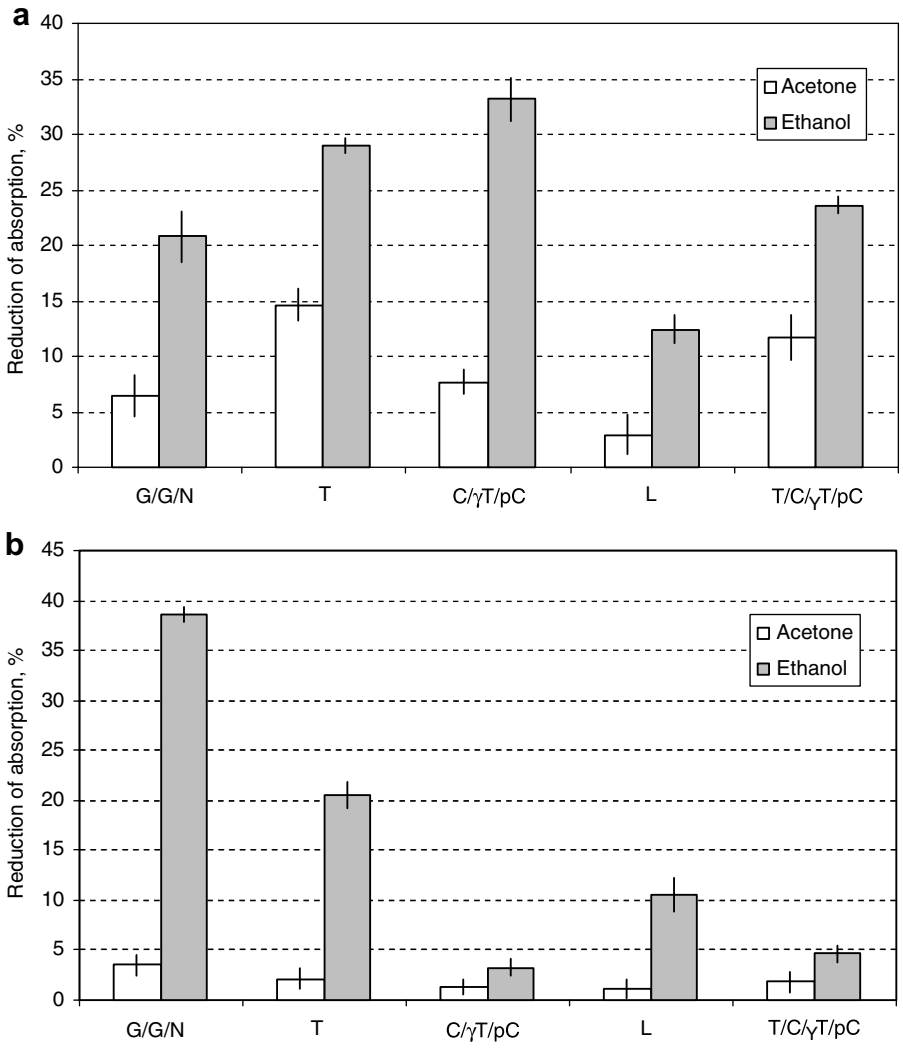

Fig. 3. Radical scavenging activity of *Thymus pulegioides* extracts against $ABTS^{\bullet+}$: (a) whole plant material; (b) deodorised plant material.

pounds, their polarity and possible changes of these compounds during deodorisation. So far as the procedure of deodorisation was performed in this study, possible radical scavengers should be separated into the volatile and non-volatile groups of compounds. The volatile compounds are removed from the plant material during distillation; the extracts from deodorised residue will not contain these compounds and consequently such extracts will loose part of the RSA of the initial non-deodorised material (Dorman, Deans, Noble, & Surai, 1995). It is important to note that from the practical point of view deodorisation should be an important step in the preparation of natural antioxidant formulation when the flavour of a particular aromatic and/or spicy plant is not acceptable for the intended food product. The main radical scavenging compounds in phenolic chemotypes of *T. pulegioides* are two isomers, thymol and carvacrol (Yanishlieva, Marinova, Gordon, & Raneva, 1999), therefore one of the reasons for the reduction in the RSA of the deodorised extracts from such chemotypes is most likely due to the loss of volatile phenolic compounds. In one of the previously published reports it was demonstrated that among several tested plants volatile oil from *T. vulgaris* was the only essential oil retarding bleaching of β-carotene in the reaction of its co-oxidation with linoleic acid; the main component in this oil was thymol (Dapkevičius et al., 1998).

The polarity of plant radical scavenging components is another important factor defining extract RSA. The results of this study clearly indicate that *T. pulegioides* contains high polarity antioxidants; the RSA of ethanolic extracts was remarkably higher than that of acetone extracts. Antioxidatively active compounds and radical scavengers were widely investigated in the other *Thymus* species *T. vulgaris*; for instance, in one of the studies most of the identified components were high polarity phenolic components, simple phenolic compounds and flavonoids (Dapkevicius et al., 2002).

And finally, active compounds of *T. pulegioides* can undergo some changes during deodorisation, i.e., boiling in water during 2 h. Most likely, chemical degradation and hydrolysis are the most important reactions in the redistribution of antioxidants during deodorisation. For

instance, glycosidically bound phenolic antioxidants which almost always are present in the raw plant material, can be released due to their hydrolysis. In this case the total RSA of the extract will change depending on the RSA of glycoside and its aglycone. Some of active components depending on their structure can loose antioxidant activity due to degradation and/or interactions with other plant compounds. Thus, it can be suggested that in case of ethanolic extract of T chemotype the loss of DPPH$^{\cdot}$ scavengers during deodorisation (e.g., thymol) was compensated by the formation, release or easier extraction of new active compounds. Except for G/G/N chemotype, these compounds were not soluble in acetone. These findings clearly indicate that the differences in the composition of non-volatile antioxidants in different *T. pulegioides* chemotype plants should be quite remarkable and this fact encourages further investigations, preferably focussed on the identification of these compounds in the extracts.

3.3. Amount of phenolic compounds in T. pulegioides extracts

In general, antioxidant and radical scavenging properties of plant extracts is associated with the presence of phenolic compounds possessing the ability to donate hydrogen to the radical. Numerous reports indicated good correla-

Table 2
Antibacterial activity of *Thymus pulegioides* L. extracts (A, acetone; E, ethanol; U, non-deodorised; D, deodorised) assessed by the diameter of inhibition zone (mm)

Chemotype	Solvent	Material	Amount (μl)	*B. cereus*	*M. luteus*	*E. coli*	*S. typhimurium*	*S. epidermidis*	*S. aureus*	*E. aerogenes*
T/C/γT/*p*C	A	U	50	4.0 ± 0.0	4.0 ± 0.0	1.3 ± 0.1	0.0	4.0 ± 0.0	4.0 ± 0.0	0.0
			10	4.0 ± 0.0	4.0 ± 0.0	1.2 ± 0.0	0.0	4.0 ± 0.0	4.0 ± 0.0	0.0
T/C/γT/*p*C	E	U	50	2.9 ± 0.2	2.6 ± 0.6	0.0	0.0	3.5 ± 0.7	3.4 ± 0.6	0.0
			10	2.2 ± 0.1	1.3 ± 0.1	0.0	0.0	1.8 ± 0.0	2.0 ± 0.0	0.0
T/C/γT/*p*C	A	D	50	2.3 ± 0.1	0.0	0.0	0.0	2.5 ± 0.0	2.3 ± 0.1	0.0
			10	–	0.0	0.0	0.0	1.2 ± 0.0	1.4 ± 0.1	0.0
T	A	N	50	2.2 ± 0.2	2.8 ± 0.1	0.0	0.0	2.2 ± 0.0	3.9 ± 0.1	0.0
			10	1.7 ± 0.1	1.9 ± 0.1	0.0	0.0	1.7 ± 0.0	2.7 ± 0.2	0.0
T	E	N	50	2.1 ± 0.1	2.8 ± 0.3	1.3 ± 0.0	1.5 ± 0.1	2.2 ± 0.0	2.5 ± 0.1	0.0
			10	1.7 ± 0.0	1.8 ± 0.1	1.2 ± 0.0	0.0	1.5 ± 0.1	1.4 ± 0.1	0.0
T	A	D	50	2.0 ± 0.0	0.0	0.0	0.0	1.8 ± 0.0	1.7 ± 0.1	0.0
			10	1.2 ± 0.0	0.0	0.0	0.0	0.0	1.4 ± 0.0	0.0
G/G/N	A	U	50	2.5 ± 0.2	2.8 ± 0.3	0.0	0.0	2.5 ± 0.0	2.4 ± 0.1	0.0
			10	1.6 ± 0.1	0.0	0.0	0.0	1.6 ± 0.1	1.2 ± 0.0	0.0
G/G/N	E	U	50	3.0 ± 0.2	1.4 ± 0.1	0.0	0.0	2.5 ± 0.0	2.0 ± 0.0	0.0
			10	1.6 ± 0.0	1.2 ± 0.0	0.0	0.0	1.8 ± 0.3	0.0	0.0
G/G/N	A	D	50	1.9 ± 0.1	1.2 ± 0.0	0.0	0.0	1.5 ± 0.1	1.9 ± 0.1	0.0
			10	1.7 ± 0.1	0.0	0.0	0.0	1.3 ± 0.0	0.0	0.0
G/G/N	E	D	50	2.2 ± 0.2	1.8 ± 0.1	0.0	1.3 ± 0.2	1.9 ± 0.0	2.0 ± 0.0	1.0 ± 0.0
			10	1.7 ± 0.1	1.1 ± 0.0	0.0	0.0	1.9 ± 0.1	1.5 ± 0.0	0.0
L	A	U	50	1.5 ± 0.1	1.9 ± 0.1	0.0	0.0	1.8 ± 0.0	2.3 ± 0.1	0.0
			10	1.8 ± 0.1	0.0	0.0	0.0	1.5 ± 0.1	1.4 ± 0.1	0.0
L	E	U	50	2.0 ± 0.0	2.0 ± 0.0	1.3 ± 0.0	1.5 ± 0.1	2.0 ± 0.1	2.0 ± 0.0	0.0
			10	1.4 ± 0.0	0.0	0.0	0.0	1.3 ± 0.1	1.5 ± 0.0	0.0
L	A	D	50	1.7 ± 0.0	0.0	0.0	0.0	1.5 ± 0.0	1.5 ± 0.1	0.0
			10	1.4 ± 0.1	0.0	0.0	0.0	1.1 ± 0.1	0.0	0.0
L	E	D	50	2.1 ± 0.1	2.1 ± 0.1	0.0	0.0	2.2 ± 0.1	1.8 ± 0.1	0.0
			10	1.7 ± 0.0	1.8 ± 0.1	0.0	0.0	1.5 ± 0.0	1.7 ± 0.0	0.0
C/γT/*p*C	A	U	50	2.0 ± 0.0	2.2 ± 0.1	1.2 ± 0.0	0.0	2.3 ± 0.1	2.4 ± 0.2	0.0
			10	1.5 ± 0.1	1.2 ± 0.1	0.0	0.0	1.3 ± 0.1	1.3 ± 0.1	0.0
C/γT/*p*C	E	U	50	1.8 ± 0.3	1.2 ± 0.0	1.0 ± 0.0	0.0	1.5 ± 0.0	1.5 ± 0.0	0.0
			10	1.2 ± 0.0	0.0	0.0	0.0	0.0	0.0	0.0
C/γT/*p*C	A	D	50	2.0 ± 0.0	1.0 ± 0.0	0.0	0.0	0.0	0.0	0.0
			10	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C/γT/*p*C	A	D	50	1.8 ± 0.3	0.0	0.0	0.0	0.0	0.0	0.0
			10	0.0	0.0	0.0	0.0	0.0	0.0	0.0

tion between the RSA and the concentration of phenolic compounds measured by Folin–Ciocalteu method. A great number of simple phenolic compounds as well as flavonoids can act as antioxidants, however, their antioxidant power depends on some important structural prerequisites, particularly on the number and the arrangement of hydroxyl groups, the extent of structural conjugation and the presence of electron-donating and electron-accepting substituents on the ring structure (Miliauskas, van Beek, de Waard, Venskutonis, & Sudholter, 2005; Miliauskas, van Beek, Venskutonis, Linsses, & de Waard, 2004a; Miliauskas et al., 2004b).

The total amounts of phenolic compounds, flavonoids and flavonols in *T. pulegioides* extracts are presented in Table 3. As a rule, the concentration of phenolic compounds was higher in the extracts isolated from the plants attributed to the 'phenolic' chemotypes, i.e., from those whose essential oils contain remarkable concentration of thymol and carvacrol: T, C/γT/*p*C and T/C/γT/*p*C. For instance, the concentration of phenolic compounds in the non-deodorised extracts from linalool (L) chemotype was approximately 2–3 times lower comparing to the above mentioned chemotypes. G/G/N chemotype plants occupy middle position; they accumulate higher amounts of non-volatile phenolics than the plants belonging to L chemotype and lower amounts comparing to T, C/γT/*p*C and T/C/γT/*p*C chemotype plants. The concentration of phenolics in T, C/γT/*p*C and T/C/γT/*p*C chemotype extracts were quite similar (Table 3). It is interesting to note that after deodorisation total amount of phenolics in T, C/γT/*p*C and T/C/γT/*p*C chemotype samples decreased more than two times, while the concentration of flavonoids and flavonols did not change so remarkably. It can be explained by the removal of important phenolic compounds, thymol and carvacrol during hydrodistillation.

Preliminary screening of extract composition was performed by HPLC-MS/UV. Typical chromatographic profile of ethanolic extract isolated from the phenolic chemotype of *T. pulegioides* is presented in Fig. 4. Chromatographic analysis showed that some components were present in the extracts isolated by the all solvents, however, their concentrations as assessed by the peak area were dependant on the solvent polarity. For instance, the compounds with retention time 26.40 and 27.44 min were most efficiently extracted by the most polar solvent ethanol; the peaks of these components in acetone extracts were considerably lower, while in hexane extracts they were not detected. On the contrary, another unidentified component (RT = 39 min) was present at comparatively high concentrations both in acetone and hexane extracts, whereas it was minor constituent in ethanol extract. Detection method was also important; some compounds gave large peaks with UV detector (RT = 26.40, 39.00) and were not detected by MS (no ionisation), while some other components, which did not absorb at the selected UV wavelength gave clear mass spectra (RT = 45.60, 47.70).

Chromatographic analysis also revealed that the composition of the extracts undergoes remarkable changes during deodorisation. For instance, HPLC analysis of deodorised ethanol extracts (Fig. 4) resulted in a considerably higher number of peaks comparing to non-deodorised ones. The concentration of some components after deodorisation considerably increased, particularly that of RT = 39.11. Most likely, similar compound with the same RT was present in hexane and acetone extracts. It can be assumed that during deodorisation at 100 °C part of this compound dissolves in hot water. The content of this component in acetone and hexane extracts after deodorisation severely reduced; most likely the compound is relatively volatile and evaporated during hydrodistillation.

Used detection methods were not sufficient for the positive identification of some compounds present in *T. pulegioides* extracts. However, two components were identified based on their RT and mass spectra: rosmarinic acid (RT ≅ 26 min) and luteolin (RT ≅ 37 min). Mas spectra also suggests that the compound with RT = 46 min is a caffeic acid derivative. Rosmarinic acid as a very strong antioxidant was found in many Labiatae plants, however, to our knowledge this compound was not previously reported in *T. pulegioides*. Rosmarinic acid was dominating in the extracts obtained by polar solvents, ethanol and water (Fig. 5); its concentration in deodorised extracts was considerably lower than in full plant extracts. Most likely, rosmarinic acid dissolves in the water which is used for hydrodistillation.

The concentration of luteolin, as a compound of lower polarity as compared to rosmarinic acid was almost similar in the ethanol and acetone extracts (Fig. 6). It is also interesting to note that the concentration of luteolin was higher in deodorised extracts. Most likely the main reason for this finding is that during deodorisation some compounds are released from plant material and the remaining components are to some extent preconcentrated. Another possible explanation for this increase could be a hydrolysis of

Table 3
The amounts of phenolic compounds, flavonoids and flavonols in the extracts of different *Thymus pulegioides* L. chemotypes (GAE, gallic acid equivalents; RE, rutin equivalents)

Chemotype, extract	Total amount of phenolic compounds mg/g GAE	Amount of flavonoids, mg/g RE	Amount of flavonols, mg/g RE
C/γT/*p*C, non-deodorised	18.82	2.50	0.85
C/γT/*p*C, deodorised	8.58	2.63	1.02
T, non-deodorised	19.77	2.97	1.03
T, deodorised	7.02	2.27	0.91
T/C/γT/*p*C, non-deodorised	16.64	3.04	1.13
T/C/γT/*p*C/, deodorised	7.77	2.11	1.06
L, non-deodorised	7.42	1.66	0.43
L, deodorised	6.74	1.22	0.28
G/G/N, non-deodorised	12.11	2.04	0.64
G/G/N, deodorised	11.49	2.13	0.71

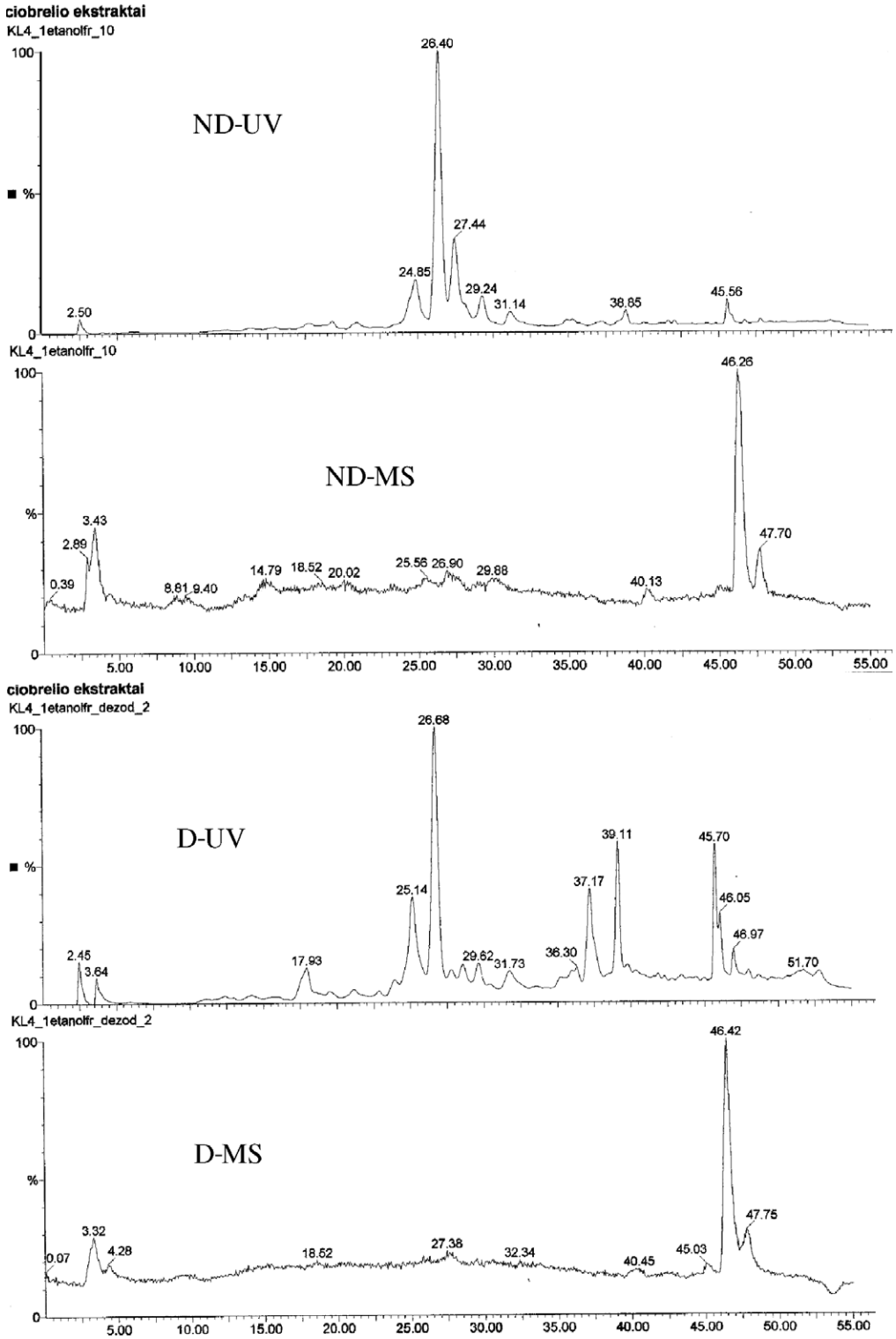

Fig. 4. UV and MS chromatograms of deodorised (D) and non-deodorised (ND) ethanol extracts isolated from the phenolic chemotype of *Thymus pulegioides*.

luteolin glycosides during hydrodistillation at 100 °C during two hours. The identification of other components in *T. pulegioides* should require further purification of extract components and their examination by the other spectroscopic methods, such as NMR and IR.

3.4. Antibacterial activity of T. pulegioides extracts

Antibacterial properties of plant essential oils and extracts were reported in numerous studies. Essential oils of various *Thymus* species exhibit distinctive antibacterial

activity mainly due to the presence of phenolic compounds, thymol and carvacrol. The effect of non-volatile components of thyme is less thoroughly documented. Food pathogens were selected as target microorganisms for the preliminary antibacterial screening of thyme extracts in the present study (Table 2). The antibacterial activity of the extracts depended on the plant chemotype, extract preparation, solvent used and finally the sensitivity of bacteria. *B. cereus*, *M. luteus*, *S. epidermidis* and *S. aureus* were the most sensitive to the all extracts applied, while *E. coli*, *S. typhimurium* and *E. aerogenes* were resistant to the *T. pulegioides* components extracted by ethanol and/or acetone.

It can be observed that the extracts of T/C/*p*C/γT chemotype were slightly more effective and L chemotype less effective antibacterial agents comparing to the other chemotypes. As it can be expected the dose of 50 μl was more efficient, however, in some cases, e.g., in case of T/C/*p*C/γT non-deodorised acetone extract, 10 μl was sufficient dose for effective inhibition of *B. cereus*, *M. luteus*, *S. epidermis* and *S. aureus*.

The extracts isolated from the whole plant material were more effective comparing to the deodorised ones with few exceptions, however the differences were not remarkable. The reduction in antibacterial activity after deodorisation of phenolic chemotypes can be easily explained by the loss of strong bactericides, thymol and/or carvacrol. Comparatively small differences in antibacterial properties between non-deodorised and deodorised extracts can be explained by the small concentration of essential oil components in the extracts. Most likely volatile components in most cases constitute smaller part in the total antibacterial activity of extracts. It is known that non-volatile phenolic compounds, including flavonoids, also demonstrate antibacterial activity against various bacteria (Cushnie, Hamilton, & Lamb, 2003).

There is no clear evidence about the preference of the solvent in terms of antibacterial power; in some cases

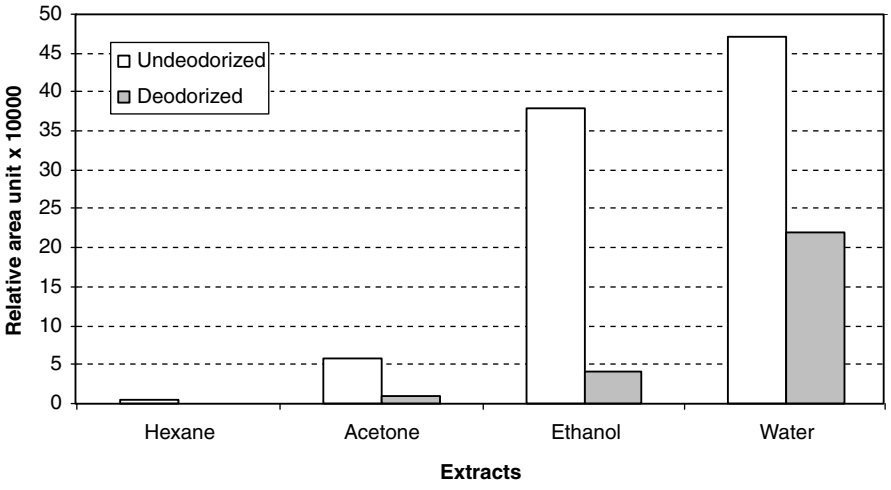

Fig. 5. The amounts of rosemarinic acid in non-deodorised and deodorised extracts of thymol/carvacrol/γ-terpinene/*p*-cymene chemotype of *Thymus pulegioides* L.

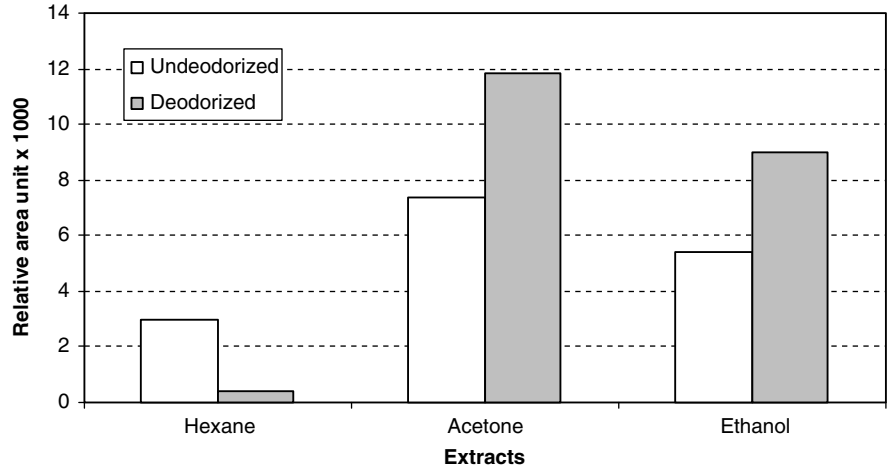

Fig. 6. The amounts of luteolin in non-deodorised and deodorised extracts of thymol/carvacrol/γ-terpinene/*p*-cymene chemotype of *Thymus pulegioides* L.

acetone extracts were more effective, while in the other cases better inhibition was obtained by using ethanol extracts. The complexity in extract composition of different plant chemotypes and possible changes of plant components during deodorisation which was discussed in the previous section can be also applied to explain data obtained during testing antibacterial properties.

4. Conclusions

The extracts obtained from the phenolic chemotypes of *Thymus pulegioides* were effective scavengers of free radicals. The use of polar solvents, such as ethanol resulted in the isolation of more effective fractions, which contained higher concentration of phenolic components as compared with the fractions isolated with lower polarity solvents (acetone and hexane). Rosmarinic acid, which is reported in *T. pulegioides* for the first time, was the main phenolic antioxidant in ethanolic extract. Taking into account that chain-radical reactions are involved in the oxidation of lipids and other biomolecules (e.g., DNA) both *in vitro* and *in vivo* the selected breeds of *T. pulegioides* can be considered as a promising plants in the development of bioactive ingredients for functional foods, nutraceuticals, medicinal preparations and other applications. The concentration of antioxidants in the non-phenolic chemotypes was remarkably lower, however these chemotypes biosynthesize high amounts of valuable essential oil components, such as geranial, neral, geraniol and linalool. The results of this study also show that some chemotypes of *T. pulegioides* are promising plants for obtaining two valuable products, antioxidatively active deodorised fraction and essential oil for flavouring purposes. Antimicrobial effects of the extracts, although being selective in terms of pathogenic bacteria and comparatively weak bactericides can also contain some potential for practical applications as a complementary property, e.g., in designing hurdle food preservation technologies.

Acknowledgements

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