Bioactive Compounds and Antioxidant Properties of *Myrrhis odorata* Deodorized Residue Leaves Extracts from Lithuania and France Origins

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Abstract Agricultural and industrial residues obtained after isolating essential oil very often constitute more than 99% of the raw material and represent a potential natural source for antioxidants and other valuable substances; however, nowadays they are poorly exploited. For the first time, this study investigated antioxidant properties and bioactive compounds in the deodorized residue extracts of *Myrrhis odorata* leaves collected in Lithuania and South of France in order to evaluate the potential of by-products obtained after essential oil isolation as a source for antioxidants. The extracts isolated with polar solvents were strong antioxidants, mainly due to the presence of kaempferol-7-*o*-glucoside and cynarin, which were reported in *Myrrhis odorata* for the first time. Deodorized residues of mature leaves of *Myrrhis odorata* were valued as an antioxidants bioresource for the first time.

Keywords Myrrhis odorata; Deodorized extracts; Antioxidant activity; Phenols, Bioactive compounds.

Introduction

Myrrhis odorata (L.) Scop. is a perennial herb (Apiaceae family) growing up to 1 m high with soft large leaves, up to 30 cm length, cut bi-or tripinnate. The plant is native in central (Switzerland, Austria) and southern (Albania, ex-Yugoslavia, Italy, Spain) Europe and has been cultivated for its anise-flavoured leaves, seeds and stalks. *M. odorata* is called sweet cicely and has been used as a sweetener in desserts and food condiments [1]. *M. odorata* essential oils were studied previously [2-5], while, to the best of our knowledge, the solid residue remaining from plant after volatile oil removal by hydro-distillation, called "deodorized residue", was not evaluated until now. For the first time, this study aim to evaluate the antioxidant properties of total phenolic compounds and the phytochemical composition of deodorized residue extracts isolated from *M. odorata* leaves. Beside, as phytochemical composition of plant's secondary metabolites depends on cultivation area, climatic conditions, vegetation phase, and genetic modifications, this study was carried out on the same plant collected from two different geographical sites: Lithuania and south of France.

Materials and Methods

Plant Material

The leaves of sweet cicely (*M. odorata*) were collected in Saint-Cyr-en-Val region of France (47° 49′ 9.09″ N/1° 56′ 51.3″ E) during July (further referred as FRMO), and in Kaunas Botanical Garden of Vytautas Magnus University, Lithuania (54° 52′ 14″ N/ 23° 54′ 40″ E) during August, 2009 (further referred as LTMO). Climatic conditions in

both locations were favourable for plant development; however, mean temperature and rainfall amount were higher in Saint-Cyr-en-Val (10.9°C and 771 mm) than in Kaunas (7.1°C and 676 mm).

Preparation of Extracts

The volatile oil was removed by hydrodistillation and remaining deodorized residues were separated into solid and liquid fractions. The solid fraction was dried at 30°C and extracted with acetone (AE), methanol (ME) or ethanol (EE), while the liquid fraction was considered as water extract (WE) (Fig. 1). The yield of the extracts was from 8.0 to 16.1% (d.w.). All extractions were performed in triplicate.

Analysis of Total Phenolic Compounds (TPC)

The content of TPC in the extracts was determined with Folin–Ciocalteau reagent [6]. Calibration curve was prepared by using 1 ml reference gallic acid solutions in ethanol (0.025, 0.075, 0.100, 0.175 and 0.350 mg/ml), which were mixed with 5 ml of a standard Folin-Ciocalteau reagent and diluted with distilled water (1:10) and 4 ml of 7.5% sodium carbonate solution in distilled water. The absorption was read after 30 min at 765 nm and the concentration of TPC was expressed in mg of gallic acid equivalents (GAE) per g of plant extract.

DPPH• Radical Scavenging Assay

Radical scavenging capacity (RSC) of plant extracts against stable 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH•, Sigma-Aldrich, Steinheim, Germany) was determined by measuring the changes of reaction colour from deep violet to light yellow at 515 nm on a UV/Vis spectrophotometer Spectronic Genesys 8 (Rochester, USA) [7]. Dry *M. odorata* extracts were dissolved in methanol. The solution of DPPH• in methanol (6.5×10^{-5} M) was prepared daily before measurements. Two ml of this solution were mixed with 50 μl of extract solution in 1 cm path length disposable microcuvette. The samples were kept in the dark for 30 min at room temperature and then the decrease in absorption was measured. The absorption of a blank sample containing the same amount of methanol and DPPH• solution was prepared and measured daily. The measurements were carried out in triplicate and RSC was calculated by the following formula: $I = [(A_B-A_A)/A_B] \times 100$; where I is DPPH• inhibition, %; A_B is the absorption of blank sample (t=0 min); A_A is the absorption of extract solution (t=30 min). The amount of extract required to decrease the initial DPPH• concentration in the reaction mixture by 50 % is referred as an effective concentration IC_{50}.

On-line HPLC–UV-DPPH•

The extracts were analysed on a HPLC/UV system supplemented with DPPH• radical scavenging detector. Two chromatograms were recorded simultaneously, one of which representing the UV absorbance at 265 nm prior to the reaction, while the other one was obtained by recording the absorbance at 517 nm after the reaction of eluent with DPPH• in the reaction coil. The mobile phase was supplied to the column by the HPLC gradient pump, model 9012 (Varian, USA) at a flow rate 0.75 ml/min. The samples of 20 μl were injected by means of Cheminert C1 injector (Valco Instruments, USA). Reverse-phase LiChroSpher RP-18e, 5 μm 12.5×0.4 cm column and 0.5×0.4 cm precolumn (Merck, Germany) were used. The DPPH• reagent was prepared by dissolving 0.01 M DPPH• in 0.1 M sodium citrate buffer (pH=7.6), methanol and acetonitrile (50:25:25 v/v). It was continuously supplied into a fused-silica capillary reaction coil (3 m, 0.25 mm id) by the HPLC pump, model 2200 (Bischoff, Germany) at a flow rate 0.75 ml/min. The signals were acquired at 265 and 517 nm wavelengths by means of Linear 206 PHD and Linear UVIS 200 UV-Vis detectors, respectively. The solution A (bidistilled water with 0.05% TFA) and B (methanol with 0.05% TFA) were used as the mobile phase components for the gradient elution: 10% of B at 0 min, 25% of B at 5 min, 40% of B at 25 min. 95% of B at 40 min. 95% of B at 43 min and 10% of B at 44 min. Clarity chromatography software (DataApex, Czech Republic) was used for data acquisition.

HPLC and LC-MS Analysis

LC system consisted of a Thermo-Fisher Spectra System (TFSP, San Jose, CA) P1000XR pump, a TFSP 6000LP Photodiode Array Detector and a TFSP AS 3000 autosampler. Separation of compounds was performed on a Varian Pursuit XRs 5 C18 column (250 mm×4.6 mm ID, 5 μm) using a linear gradient of 0–30 min., 3–97% of acetonitrile (A) to bidistilled water with 0.1% TFA (v/v) (B), 30–35 min., 20–80% of A to B, 35-45 min., 3-97% of A to B. Flow rate was 1 ml/min and 10 μl of the sample was injected. Mass spectra were acquired using a Thermo-Fisher LCQ mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an atmospheric pressure chemical ionization source (APCI) using both positive and negative ion mode. The APCI source operating parameters were as follows: the capillary and APCI vaporizer temperatures were 250°C and 450°C, respectively, and the spray was stabilized with nitrogen sheath and auxiliary gas (80 and 25 arbitrary units, respectively). Discharge current was 5 μA and capillary voltage was +15 V and −15 V in the positive and negative ion mode, respectively. The mass spectra were

acquired in the data dependent mode with wideband activation (i.e. the most intense ion obtained for each scan in the full mass spectrum is further submitted to 38% collision energy for MS/MS).

Statistical Analysis
The values are expressed as a means of 3 replicates with standard deviations (SD). RSC and TPC values were calculated using MS Excel software (CORREL statistical function).

Results and Discussion
Antioxidant activity of *M. odorata* Extracts
There are many methods for assessing antioxidant potential of plant raw materials, however TPC and RSC in the reaction with a stable free DPPH• radical are among the most popular assays for the rapid screening of extracts. Phenolics, as primary antioxidants terminating free radical chain reactions constitute the major group of antioxidatively active compounds in many plants. The content of TPC extracted from *M. odorata* was solvent dependent and varied in the range of 10.78±0.02 to 85.25±1.32 mg GAE/g (Table 1). The content of polyphenols isolated by different solvents decreased in the following order: ME > EE > WE > AE for FRMO and EE > ME > WE > AE for Lithuanian *Myrrhis odorata* (LTMO). Ethanol and methanol are quite similar protonic solvents; however both of them were tested because ethanol is more acceptable for the isolation of food grade ingredients. The differences in the TPC content in EE and ME between Lithuanian and French origin plants were not remarkable, however, the biggest sum of TPC (ME+WE+AE = 179.47 mg GAE/g) in French *Myrrhis odorata* (FROM) was higher than in LTMO (EE+WE+AE = 126.93 mg GAE/g). It is interesting to note that lower polarity phenolics extracted with acetone were almost 4 times more abundant in FRMO than in LTMO.
The RSC of EE and WE was slightly stronger than that of ME in DPPH• assay, while AEs were approximately 6-9 times weaker radical scavengers than polar extracts (Table 1).

Table1: The amount of total phenolic compounds and DPPH radical scavenging capacity of deodorized *Myrrhis odorata* extracts from France (FR) and Lithuania origin (LT). Values are mean ± SD of triplicate measurements; IC$_{50}$ of Trolox = 0.025 mg/ml.

Plant origin	Extraction solvent			
	Methanol	Ethanol	Water	Acetone
	Total phenolic compounds, mg GAE/d			
FR	85.25±1.32	68.97±0.30	55.03±0.04	39.19±0.32
LT	62.32±0.08	80.60±0.97	35.55±0.06	10.78±0.02
	DPPH radical scavenging capacity, IC$_{50}$, mg/ml			
FR	0.14±0.002	0.12±0.01	0.13±0.00	0.83±0.08
LT	0.16±0.01	0.11±0.00	0.12±0.00	0.98±0.082

So far as polar phenolic constituents are dominant antioxidants in the majority medicinal and aromatic plants, this result is in agreement with many previously performed studies. Usually the TPC are in a strong correlation with RSC, however in our study this correlation was not so evident. For instance, the content of TPC in WE and AE of FRMO was remarkably higher that in LTMO, while the differences in the RSC between the extracts isolated from the two different origin plants with the same solvents were negligible. EE contained high amount of phenolics and showed very good RSC, while the content of phenolics in WE, which also possessed good RSC was remarkably lower than in EE and ME extracts.
Preliminary screening of antioxidant potential indicate that non-volatile fraction of *M. odorata* contain relatively high amount of phenolic compounds which might be of interest for the isolation of valuable food and or nutraceutical ingredients. It also showed that polyphenolic composition and antioxidant properties depend on the plant geopgraphical origin.

Phytochemical Composition and RSC of M. odorata Constituents
The assessment of antioxidant properties of *M. odorata* by TPC and RSC of DPPH• assays suggests that there might be remarkable differences in the extract composition between the plants of French and Lithuanian origin, particularly in case of WE and AE. It is known that the antioxidant activity of individual phenolic compounds can differ significantly depending on their molecular structure; therefore the content of TPC in plant extracts is not very informative indicator of their RSC. The structures of isolated constituents need to be elucidated and assessed in order to obtain more precise results. An on-line HPLC–DPPH• method is a convenient option for the preliminary screening of RSC of the compounds present in the extracts. Therefore, further studies were performed to evaluate RSC of chromatographically separated constituents.

Kaempferol-7-O-glucoside and cynarin were identified in *M. odorata* and they were the major antioxidatively active compounds in the analysed extracts, however the former compound was more abundant in all LTMO extracts while the latter one was dominant in WE of FROM. To the best of our knowledge these compounds were not previously reported in this plant. Peak areas of kaempferol-7-O-glucoside and cynarin in EE and WE extracts were in the range of 639.7-6038.2 and 0-849.1, while their input in the total RSC was 34.5-97.1 and 0-45.4 %, respectively (Table 2).

Table 2: Composition and radical scavenging capacity of *Myrrhis odorata* constituents evaluated by the on-line HPLC-UV-DPPH method. (ni = not identified; FR = France; LT= Lithuania).

Plant origin	Active compound	RT, min	Peak area, mV×s	% of total RSA
		Ethanol extract		
FR	K-7-O-glc	29.7	1436.6	77.2
	Ni	30.7	229.9	12.4
	Ni	32.0	58.0	3.1
	Ni	34.2	67.6	3.6
	Ni	35.0	26.5	1.4
	K-7-O-glc	29.8	6038.2	97.1
LT	Ni	35.5	55.5	0.9
	Ni	36.4	115.9	1.9
		Water extract		
FR	Ni	8.5	51.4	3.4
	Ni	13.2	201.2	10.8
	Cynarin	15.5	849.1	45.4
	Ni	26.7	26.4	1.4
	K-7-O-glc	29.9	639.7	34.5
	Ni	32.0	33.6	1.8
	Cynarin	15.5	231.2	7.8
	K-7-O-glc	29.9	2547.8	86.4
LT	Ni	35.7	22.3	0.5
	Ni	37.9	13.3	0.3
		Acetone extract		
FR	K-7-O-glc	29.9	375.1	90.4
	ni	36.5	32.6	7.9
LT	K-7-O-glc	29.9	214.4	77.2
	ni	36.5	43.4	15.6

Kaempferol-7-*O*-glucoside was found in *Smilax china*; this flavonoid induced G2/M phase arrest and apoptosis on HeLa cells in a p53-independent manner (Xu et al. 2008). Cynarin is an ester formed from quinic acid and two units of caffeic acid and was reported as biologically active chemical constituent of artichoke 58 years ago [8]; diuretic, choleretic and hepatoprotective activities of artichokes leaves extract were often related to the cynarin content [9,10]. In addition, cynarin is responsible for the sensation of sweetness that occurs after eating artichokes; it appears to suppress bitter tongue taste receptors while enhancing the sweet ones; consequently it may also be responsible for the sweet taste of *M. odorata*. Most recently it was reported that cynarin, which was abundant in sunflower (*Helianthus annuus*) sprouts possessed both antiglycative and antioxidant activities and was able to intervene against glycoxidation [11].

The chromatographic profiles of WE (Figure 1) obtained by the on-line HPLC–DPPH$^\bullet$ method clearly show that FRMO contain more radical scavenging constituents than LTMO. Strong antioxidant cynarin was present in FRMO in remarkably higher amounts than in LTMO, however, the content of kaempferol-7-O-glucoside was more abundant in LTMO (Figure 1) and (Table 2).
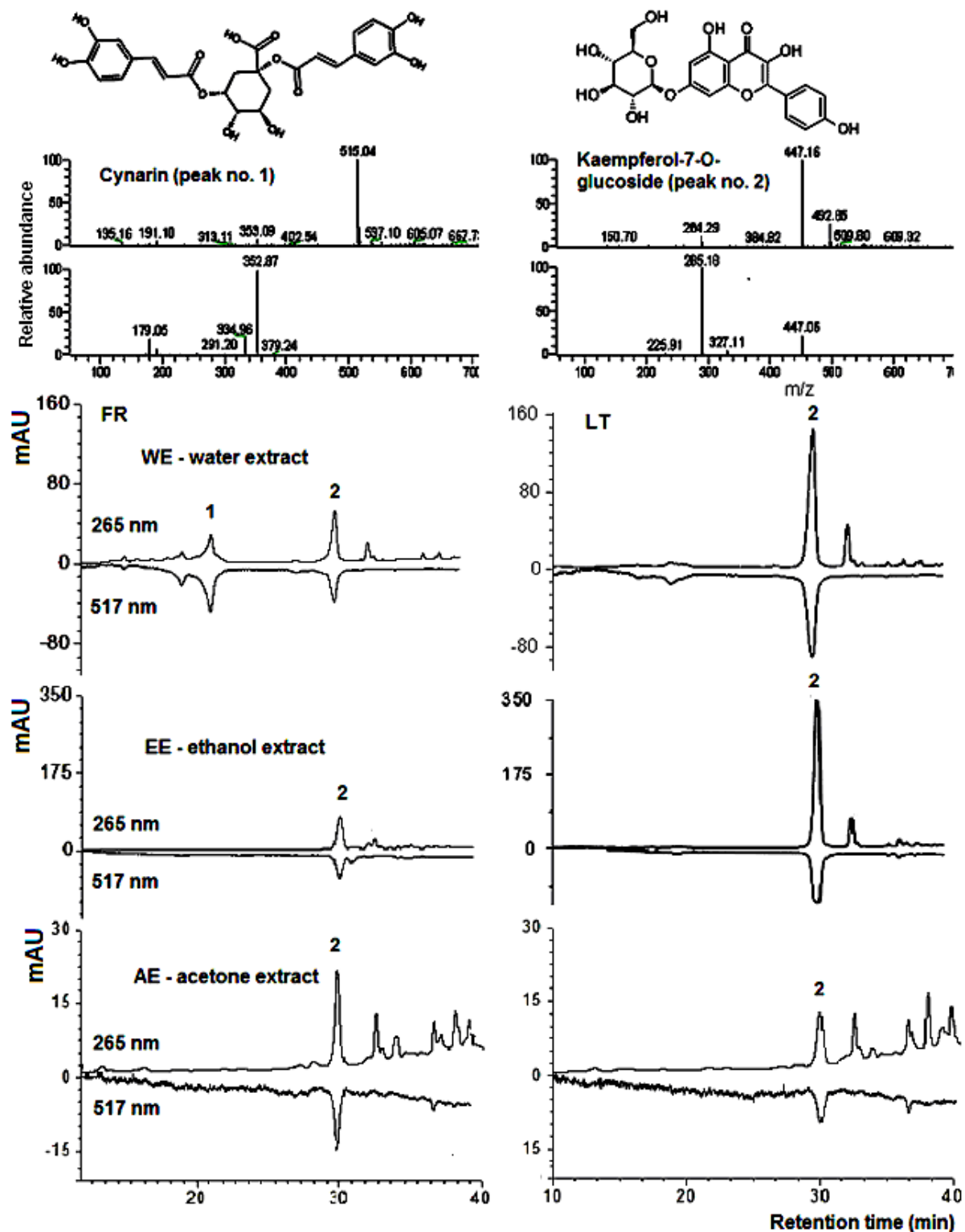
Figure 1: HPLC–DPPH˙ chromatograms of Myrrhis odorata extracts of French (FR) and Lithuanian (LT) origin and m/z and the structures of identified compounds.

Total peak area of radical scavenging peaks in WE of LTMO and FRMO was 2814.6 and 1801.4, respectively, and these values are in agreement with RSC (IC_{50}) of these extracts, which were 0.12 and 0.13 mg/l, respectively. However, in case of EE direct comparison of on-line and batch evaluation of RSC is not possible. The IC_{50} of EE of FRMO was only slightly lower than that of LTMO, while peak area of the main EE constituent kaempferol-7-O-glucoside was more than 4 times bigger in the LTMO. Possibly, EE of FRMO contains other radical scavengers which were not detected at the applied HPLC analysis conditions. As it may be observed in the chromatograms the extracts contain more antioxidatively active compounds, however, their input in the total RSC was less remarkable and constituted from 0.8 % (WE of LTMO) to 20.5 % (EE of FROM) of the total RSC (Table 2). Mass spectra and UV data was not sufficient to identify these compounds. Also the content of detected constituents in AE was remarkably lower than in EE and WE.

Conclusion

M. odorata accumulate valuable phenolic and antioxidatively active compounds, which may be isolated with polar solvents from the plant residues after essential oil distillation. Kaempferol-7-O-glucoside was dominant constituent in ethanol extracts of *M. odorata* from France and Lithuania; however cynarin was most abundant compound in water extract of French origin plants. Data obtained in this study may be considered as a first step in valorising the processing of *M. odorata* raw material in order to obtain valuable substances, present both in volatile and non-volatile fractions. From the scientific point of view further studies should be focused on purification and structure elucidation of other bioactive compounds while technological and economic feasibility should be evaluated for practical applications.

Acknowledgements

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