ACTIVITY OF NATURAL ANTIOXIDANTS EXTRACTED FROM GREATER CALAMINT, SWEET CICELY AND COLTSFOOT CULTIVATED IN LITHUANIA AND IN FRANCE

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Abstract
The residues obtained after hydrodistillation of three aromatic plants (*Calamintha grandiflora*, *Myrrhis odorata* and *Tussilago farfara*) were separated into solid and liquid fractions. The liquid fraction was dried while the solid fraction was extracted with acetone, methanol or ethanol. Two main types of antioxidant activity tests were employed: i) assays to evaluate oxidation of fats, oils and other fat containing foods (Oxipress); ii) assays to evaluate radical scavenging activity in model systems (DPPH, FRAP). The antioxidant activities were expressed as gallic acid equivalents (GAE) with the aim of standardizing these methods and that allow data comparisons. The three antioxidant assay methods give different antioxidant activity trends. An initial screening of ethanol extracts from the three aromatic plants for antioxidant activity in oil reported them as the best. Despite hydrodistillation residues presented strong antioxidant activities, the total phenolics content in acetone, methanol, ethanol leaves extracts for *Tussilago farfara* was about twice lower than others plants extracts.
Key words: Aromatic plants, antioxidant activity, by-products valorization

Introduction

Agricultural and industrial residues/by-products obtained during processing of raw plants represent potential natural sources for antioxidants that are rarely exploited (wastes could be represent up to 99.5% of the raw material). Taking into account this situation our aims are analysing and evaluating such by-products as possible sources for food antioxidants. Chemical and biological diversity of aromatic and medicinal plants depending on such factors, as cultivation area, climatic conditions, genetic modifications and others is an important impetus to study flora present in different growing sites, countries and geographical zones. Aromatic and medicinal plants are used since ancient times for different purposes, e.g. for flavouring of foods, for preservation and for disguising unpleasant odours. Mostly this is linked with the discovery of synthetic drugs, which usually are more effective. Nevertheless a big part of the world population still widely relies on traditional herbal medicine. Many medicinal plants contain large amounts of antioxidants such as polyphenols, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases (Anderson et al. 2001). Herbs are most often defined as any part of a plant that is used in the diet for its aromatic properties (Hacskaylo, 1996; Davidson and Jaine, 2006; Smith and Winder, 1996). Recently, however, herbs have also been identified as sources of various phytochemicals, many of which possess important antioxidant activity (Velioglu et al., 1998; Larson, 1988; Kähkönen et al., 1999). Two main types of antioxidant activity tests were employed assays to evaluate oxidation of fats and assays to evaluate radical scavenging activity in model systems. This work is aimed at a preliminary screening for radical scavenging activity of extracts from aromatic plants namely *Calamintha grandiflora, Myrhis odorata* and T*ussilago farfara* has been performed using DPPH, FRAP radical scavenging assays as well as the total phenolic content estimated by the Folin-Ciocalteu assay. Residue extracts from these plants were added to sunflower oil, and oxidative deterioration was measured by the Oxipres method.

Materials and Methods

Plant Material. The following plants greater calamint, sweet cicely and coltsfoot (*Calamintha grandiflora, Myrrhis odorata and Tussilago farfara)* were harvested from the collection of

medicinal plants at Kaunas Botanical Garden (Vytautas Magnus University, Lithuania) and in Midi- Pyrénées (south west of France), during April-August, 2009.

Sample Preparation. Preparation of Extracts. The residues (by-product) of hydrodistillation were separated into solid and liquid fractions. The solid residue was dried at 30 °C and extracted with acetone, methanol and ethanol while the liquid fraction (water extract) was divided in two parts, one of which was spray-dried and the other freeze-dried. The yield of extracts varied from 8.0 to 17.1%. The extractions were performed in triplicate.

Evaluation of Antioxidant Activity. DPPH Radical Scavenging Assay. The RSC of aromatic plants extracts against stable 2,2-diphenyl-2-picrylhydrazyl hydrate radical DPPH (Sigma-Aldrich, Germany) was determined by a slightly modified spectrophotometric method of (Brand-Williams et al. 1995). A 2 ml aliquot of $DPPH^{\bullet}$ solution was mixed with a 50 µl of extract solution in 1 cm path length disposable microcuvette. The decreasing absorbance was read during 30 min reaction time at 1 min intervals until the absorbance curve reached the plateau phase. The amount of extract required to decrease the initial $DPPH^{\bullet}$ concentration in the reaction mixture by 50% is referred as an effective concentration, IC_{50}. *FRAP assay*. The ferric reducing power (FRAP) assay was performed in a Biotek EL808 microplate reader (Vermont, USA). The working FRAP reagent was prepared by mixing the acetate buffer (300 mM pH 3.6): 10 mM TPTZ (2.4.6-tripyridyl-s-triazine) (Sigma-Aldrich, Germany) in 40 mM HCl: 20 mM $FeCl_3 6H_2O$ in the ratio 10:1:1 (Benzie and Strain, 1996). Firstly, 300 µl of freshly prepared FRAP reagent was heated to 37 °C and an absorbance (A0) of a blank reagent was read at 593 nm. Then 10 µl of 0.1% sample solution in water and 30 µl H_2O were added (final dilution of samples in the reaction mixture was 1:34) and the absorbance (A) were recorded every 1 min during the whole monitoring period which lasted up to 30 min The change in the absorbance (ΔA593nm) between the final reading and A_0 was calculated for each sample and related to the ΔA593nm of a FeII reference solution which was measured simultaneously.

Total Amount of Phenolic Compounds. The concentration of phenolic compounds in extracts was determined by Folin–Ciocalteu method (Folin & Ciocalteu 1927). For the preparation of a calibration curve 1 ml reference gallic acid solutions in ethanol (aliquots of 0.025, 0.075, 0.100, 0.175, and 0.350 mg ml^{-1}) were mixed with 5 ml of standard Folin-Ciocalteu 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. The concentration of phenolics compounds (*C*) was expressed in milligrams of gallic acid equivalents (GAE) per gram of plant extract.

Method of OXIPRES. Infused oil were prepared by mixing sunflower oil with different concentrations of extracts (0.05%, 0.1%, 0.2%) 5 g of infused oil was weighted (nearest 0.001 g) into a reactor tube which then placed thermostated (110 °C) and under oxygen atmosphere (5 bars). Pressure changes were recorded by using OXIPRESS apparatus (Mikrolab Aarhus). The protection factor (*PF*) value of sunflower oil in case of using plants extracts and their antioxidant activities (*AA*) were calculated by the following formulas:

$$PF = \frac{IP_X}{IP_K}; \quad (1)$$

$$AA = \frac{IP_X - IP_K}{IP_{BHT} - IP_K}; \quad (2)$$

where: IP_X – induction period of sample with additive, h;
IP_K - induction period of sample without additive, h;
IP_{BHT} – induction period of sample with BHT, h.

Results and Discussion

The antioxidant activity of the extracts was determined using a DPPH and FRAP scavenging assay. DPPH assay is known to give reliable information concerning the antioxidant ability of the tested compounds (Huang et al., 2005). The principle of the assay is based on the color change of the DPPH solution from purple to yellow as the radical is quenched by the antioxidant (Parthasarathy et al., 2009). The results of DPPH scavenging assay by different plant extracts are summarized in the Table 1. The acetone extracts were the most effective DPPH radical scavengers for *Calamintha grandiflora*. IC_{50} values 0.112 mg ml^{-1}. The IC_{50} values of *Myrhis odorata* ranged between 0.828 and 0.982 mg ml^{-1}. The lowest IC_{50} value (highest antioxidant activity) of 0.049 mg ml^{-1} was obtained for *Tussilago farfara* Leaves-AE (France). The free radical scavenging activity of spray dried water extract (*Calamintha grandiflora*-SD) exhibited slightly lower IC_{50} value against DPPH radicals (0.158 mg ml^{-1}). The DPPH radical seems to be more reactive with leaves acetone extract for *Tussilago farfara* (France). The roots extracts for *Tussilago farfara* had very low antiradical activity comparing with other extracts of blossom and leaves. Methanol and ethanol extracts were more active against the DPPH radical than acetone for *Myrhis odorata*.

Table 1

DPPH scavenging assay of *Calamintha gradiflora, Myrhis odorata* and *Tussilago farfara*

Plant extracts	DPPH	
	IC_{50} mg·ml^{-1}	
	Lithuania	France
***Calamintha grandiflora*-AE**	–	0.112±0.004
***Calamintha grandiflora*-MeOH**	–	0.227±0.008
***Calamintha grandiflora*-EtOH**	–	0.221±0.004
***Calamintha grandiflora*-FD**	–	0.217±0.001
***Calamintha grandiflora*-SD**	–	0.158±0.006
***Myrrhis odorata* -AE**	0.982±0.075	0.828±0.067
***Myrrhis odorata* -MeOH**	0.160±0.003	0.140±0.002
***Myrrhis odorata* -EtOH**	0.107±0.001	0.123±0.005
***Myrrhis odorata* -FD**	0.117±0.002	0.115±0.001
***Myrrhis odorata* -SD**	0.241±0.004	0.226±0.003
***Tussilago farfara* Blossom-AE**	0.270±0.001	0.387±0.007
***Tussilago farfara* Blossom-MeOH**	0.201±0.012	0.352±0.039
***Tussilago farfara* Blossom-EtOH**	0.154±0.017	0.219±0.011
***Tussilago farfara* Leaves-AE**	0.103±0.005	0.049±0.003
***Tussilago farfara* Leaves-MeOH**	0.091±0.005	0.234±0.019
***Tussilago farfara* Leaves-EtOH**	0.315±0.006	0.175±0.024
***Tussilago farfara* Roots-AE**	0.877±0.041	0.877±0.021
***Tussilago farfara* Roots-MeOH**	0.184±0.052	0.461±0.029
***Tussilago farfara* Roots-EtOH**	0.231±0.066	0.415±0.031

Based on the result obtained from the analyses DPPH it is important to notice that, there is not significant similitude between the same plants of different climates and country (Lithuania and France), the only important difference is obtained by the use of different solvent extraction. On the other hand, the results obtained from the FRAP analyses showed in the figure 1 above, we can find that the plants located in Lithuania has more antioxidant activity that the plants located in the Midi- Pyrénées zone. The FRAP is versatileand can be readily applied to both aqueous, alcohol and acetone extracts of different plants. In this assay, the antioxidant activity is determined on the basis of the ability to reduce ferric (III) iron to ferrous (II) iron. The results

were expressed as mg ferrous iron equivalents per ml of sample The ferric reducing power (FRAP) assay of the alcohol extracts was in the range of 0.147–0.986 mg Fe (II)/ml (Figure 1).

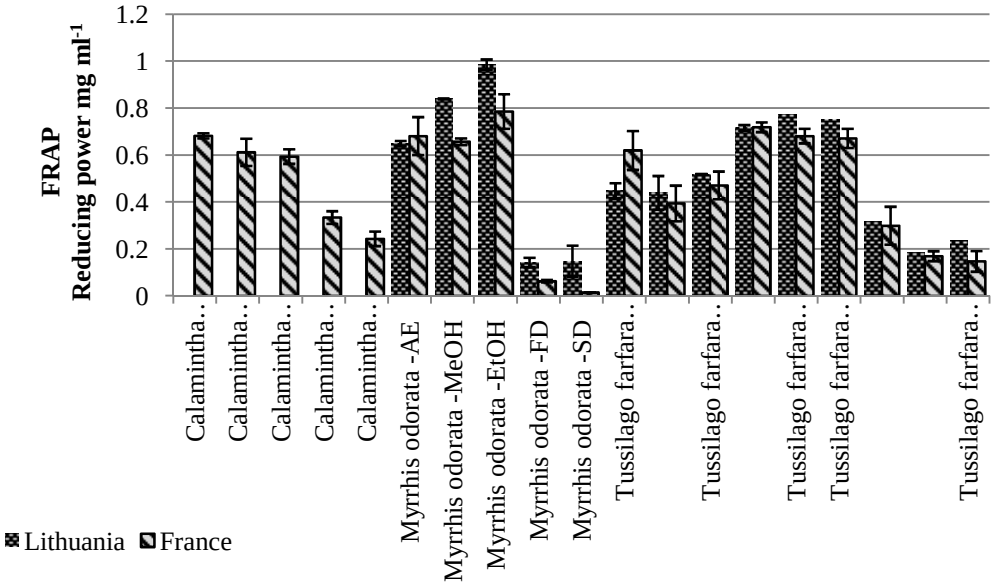

Figure 1. Ferric Reducing Capacity assay of *Calamintha gradiflora, Myrhis odorata* and *Tussilago farfara*

For the aqueous extracts, the antioxidant activity ranged from 0.014 to 0.334 mg Fe (II) ml^{-1}. Ethanol, methanol, acetone extracts for plants exhibited highest antioxidant capacity, than the spray dried and freeze dried water extracts.

Table 2

Total phenolic content of *Calamintha gradiflora, Myrhis odorata* and *Tussilago farfara*

Plant	Total phenolic compounds, mg g^{-1} plant extract (in GAE)				
	Acetone extract	Methanol extract	Ethanol extract	Freeze-dried extract	Spray-dried extract
Calamintha grandiflora	27.99±0.12	64.79±0.22	82.76±1.02	58.89±0.72	72.78±1.11
***Myrrhis odorata* (France)**	39.19±0.32	85.25±1.32	68.97±0.30	27.25±0.02	55.03±0.04
***Myrrhis odorata* (Lithuania)**	10.78±0.02	62.32±0.08	80.60±0.97	25.89±0.03	35.55±0.06
***Tussilago farfara* Blossom (France)**	123.16±1.25	48.79±0.17	66.23±1.01	-	-
***Tussilago farfara* Leaves (France)**	151.36±1.87	204.57±1.54	131.88±0.99	-	-
***Tussilago farfara* Roots (France)**	13.58±0.01	41.64±0.03	11.30±0.06	-	-
***Tussilago farfara* Blossom (Lithuania)**	94.79±0.98	48.79±0.03	157.25±0.02	-	-
***Tussilago farfara* Leaves (Lithuania)**	102.00±0.01	144.33±1.20	157.25±0.08	-	-
***Tussilago farfara* Roots (Lithuania)**	6.05±0.01	41.08±0.78	38.87±0.65	-	-

The amounts of total phenolic compounds in the herbs are presented in Table 2. The leaves extracts of *Tussilago farfara* possessed approximately twice-higher amounts of phenolics (102–204.57 GAE) than the other plant extracts. However, the total phenolics content in *Calamintha grandiflrora* acetone extract was lower than in methanol, ethanol extracts, and lower than in some other herbs having lower antioxidant activities.

Table 3

Antioxidant activity (*AA*) the extracts of *Myrhis odorata* and *Calamintha grandiflora* as compared with the effect of BHT and their effect on the stability of sunflower oil, expressed in protection factor values (*PF*)

Extracts	Conc %	IP		PF		AA	
		Lithuania	France	Lithuania	France	Lithuania	France
***Myrrhis odorata* -AE**	0.20	2.17	2.19	1.09	1.10	0.08	0.09
	0.10	2.09	2.11	1.05	1.06	0.04	0.05
	0.05	2.05	2.07	1.03	1.04	0.02	0.03
***Myrrhis odorata* -MeOH**	0.20	2.19	2.10	1.10	1.05	0.09	0.05
	0.10	2.07	2.02	1.04	1.01	0.03	0.01
	0.05	2.01	2.00	1.01	1.00	0.00	0.00
***Myrrhis odorata* -EtOH**	0.20	2.30	2.30	1.15	1.15	0.14	0.14
	0.10	2.10	2.20	1.05	1.10	0.05	0.10
	0.05	2.02	2.07	1.01	1.04	0.01	0.03
***Myrrhis odorata* -FD**	0.20	2.36	2.42	1.18	1.21	0.17	0.2
	0.10	2.21	2.34	1.11	1.17	0.10	0.16
	0.05	2.19	2.29	1.10	1.15	0.09	0.14
***Myrrhis odorata* -SD**	0.20	2.51	2.21	1.26	1.11	0.25	0.10
	0.10	2.34	2.02	1.17	1.01	0.16	0.01
	0.05	2.26	2.01	1.13	1.01	0.13	0.00
***Calamintha grandiflora*-AE**	0.20	–	2.48	–	1.24	–	0.35
	0.10	–	2.72	–	1.36	–	0.33
	0.05	–	2.68	–	1.34	–	0.23
***Calamintha grandiflora*-MeOH**	0.20	–	2.81	–	1.41	–	0.39
	0.10	–	2.71	–	1.36	–	0.34
	0.05	–	2.58	–	1.29	–	0.28
***Calamintha grandiflora*-EtOH**	0.20	–	2.98	–	1.49	–	0.47
	0.10	–	2.93	–	1.47	–	0.45
	0.05	–	2.78	–	1.39	–	0.38
***Calamintha grandiflora*-FD**	0.20	–	2.61	–	1.31	–	0.29
	0.10	–	2.54	–	1.27	–	0.26
	0.05	–	2.30	–	1.15	–	0.14
***Calamintha grandiflora*-SD**	0.20	–	2.78	–	1.39	–	0.38
	0.10	–	2.51	–	1.26	–	0.25
	0.05	–	2.37	–	1.19	–	0.18
BHT	0.02	4.07		2.04		1.00	
Sunflower oil	0.00	2.00		1.00		–	

This finding showed that the content of total phenolics in herbs is not a reliable indicator of their antioxidant activity. The structures of the individual constituents need to be elucidated and assessed in order to obtain more precise results and information. The oxipres method, which was used to evaluate the antioxidant activity (AA) of extracts, is a very convenient procedure performed without using any chemicals. The change of pressure at the end of the induction

period can be rather precisely measured. In case of adding plants extracts to sunflower oil, the pressure drop was delayed for some period of time, indicating the antioxidative effect of the added substance. The effects of the applied extracts at 0.05, 0.1 and 0.2% concentrations are summarized in Table 3. The extracts obtained from *Calamintha gradiflora* and *Myrhis odorata* were found to be the most effective natural antioxidants. The effect of extracts on the stability of Sunflower oil during accelerated oxidation was comparable with the effect of butylated hydroxytoluene (BHT) at the same concentration. The extracts of *Calamintha grandiflora* was the most effective natural antioxidant as compared with extracts of *Myrhis odorata*. It has also should be emphasized that no positive correlation was found between the total amount of phenolic compounds and AA. For instance, methanol extract of *Myrhis odorata* which were rich in the total phenolics possessed the lowest AA.

Conclusions

The test result in the DPPH analyses obtained in preliminary investigations show that the type of compounds that have a different antioxidant activity differ depending on the solvent used and do not depend in the zone of origin. This study suggests that the *Myrhis odorata, Calamintha gradiflora* and *Tussilago farfara* extracts contain valuable antioxidant active components, which might be helpful in preventing or slowing the progress of various oxidative stresses. We can find a practical application of these plants in different areas, particularly in formulation and production of food additives. It also can be concluded that methanol and ethanol extraction are a suitable way to prepare extracts with antioxidant activity in oil, this conclusion was based in the observations obtained by the analyses. Continues with this investigation a further analyses on the isolation and identification of antioxidants components of the plants mentioned previously will be realised.

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