ANTIOXIDANT ACTIVITY OF *ORIGANUM MAJORANA* L. HERB AND EXTRACTS OBTAINED BY SUPERCRITICAL CO_2 EXTRACTION

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As the usage of synthetic antioxidants (e.g. butylated hydroxytoluene) is not approved in several developed countries, thus natural antioxidants have gained popularity in recent years. Among Lamiaceae plants many contain phenolic compounds which may lead the strong antioxidant activity. In this study, the antioxidant properties of marjoram (*Origanum majorana* L.) herb and extracts obtained by ethanol, *n*-hexane and supercritical CO_2 extraction are presented. Individual antioxidants (ursolic acid, carnosic acid and carnosol) were identified with high performance liquid chromatography. The effects of the parameters (temperature and pressure) of high pressure extraction on the yields of carnosol and ursolic acid were analysed. The effects of fractionation of extracts were followed at different extraction pressure. For screening the antioxidant activities of the herb aqueous and solvent extracts widely used analytical methods were applied. The free radical scavenging activities with 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the total scavenger activity by chemiluminometric method were compared. From solvent extracts the antioxidant activity was performed using Rancimat method.

INTRODUCTION

Marjoram (*Origanum majorana* L.) is widely known and used Mediterrian spice, which contains up to 3% of volatile oil. Other compounds like flavonoid glycosides (luteolin-7-diglucoside, apigenin-7-glucoside), arbutin, tannins, caffeic acid, labiatic acid, rosmarinic acid, steroids (e.g., β-sitosterol), triterpenoids (ursolic acid, etc.), paraffins and others can be found in the herb [1-4]. Sweet marjoram is considered to have carminative, antispasmodic, diaphoretic, and diuretic properties [3]. Marjoram used locally to relieve the symptoms of the common cold, such as nasal congestion and in mouthwashes for oral hygiene [1-3]. Besides its antimicrobial activity [4-5], lately further examination has been carried out to map all the possible effects of marjoram extracts. One purified compound from methanolic extract of *O. majorana* was shown to possess oxigene anion scavenging activity in vitro tests [6]. Ursolic acid isolated from marjoram herb seemed to be efficient acetylcholinesterase inhibitor (key compound in Alzheimer's disease) and this compound also showed strong antioxidant activity on Abeta-induced neurotoxicity [7-8].

The aim of this study is to map the antioxidant activity of different products obtained by several extraction methods from two species of *Origamum majorana* L. herbs (originated from Hungary and Egypt). The ursolic acid and carnosol contents of marjoram were revealed and the effects of the parameters (pressure and temperature) of supercritical CO_2 extraction on the yields of them were determined. Compounds with low polarity can be extracted with supercritical CO_2 therefore conventional solvent extractions (with n-hexane and ethanol) were carried out for comparison of their antioxidant properties.

I - MATERIALS AND METHODS

Materials

The dried, finely ground marjoram sample was obtained from Kalocsa, Hungary (sample 1). Sample 2 was purchased from a herb supplier with officially controlled originating from Egypt. Moisture content of sample 1 was 12.07 ± 0.62 % (w/w), of sample 2 was 10.31 ± 0.69 % (w/w). The CO_2 used was 99.5 % (w/w) pure and supplied by Messer Griesheim Hungaria. Nitrogen gas used was 99.999% (w/w) pure and supplied by Linde (Hungary). Reagent-grade ethyl alcohol and n-hexane were used for conventional Soxhlet extractions. Analytical grade reagents (Reanal Ltd., Budapest, Hungary) were used for phytochemical analysis. 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable radical, microperoxidase and 5-amino-2,3-dihydro-1,4-phtalazinedion (luminol) were purchased from Sigma Chem. Corp. (St. Louis, USA). For determination of the antioxidant activities of extracts with Rancimat apparatus sunflower oil (Floriol, Hungary) was used. All chemicals used for HPLC analysis were purchased from Merck (Darmstadt, Germany). Carnosic acid standard (purity 95.29%, HPLC) and carnosol standard (purity 96.40%, HPLC) were supplied by Cromadex (USA). Ursolic acid standard (purity 96.50%) was purchased from Sigma (Germany).

Methods

Standard methods described in the Hungarian Pharmacopoea Ed. VII. were applied for the determination of the oleoresin (by ethanol and n-hexane Soxhlet extraction) of marjoram samples. Supercritical CO_2 extraction (SFE) was carried out in a high-pressure apparatus equipped with a 5 L volume extractor vessel. A more detailed description of the apparatus and extraction is given extensively elsewhere [9]. Samples of 800-1000 g of the plant material were weighed accurately and put into the extraction vessel. The total extracts of the plants were recovered.

To determine the content of antioxidants in the raw material, 100 mg of herbs were mixed with 100 ml aliquots of ethanol:methanol:2-propanol (90:5:5 v/v) and extracted in an ultrasonic bath for 1 h. Afterwards, the samples were filtered and analysed by HPLC.

Analytical methods

Hydrogen donating ability of aqueous extracts was examined in the presence of 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable radical at 517 nm according to the method of Hatano et al. [10]. DPPH solution was freshly prepared as a free radical source. From aqueous extracts in four concentrations (0.008, 0.018, 0.028 and 0.04 % w/w) methanolic solutions were prepared adding 500 µl DPPH to each and incubated at room temperature (25 ± 2°C) for 30 min. As control 20% (v/v) of DPPH methanolic solution was prepared and treated as the samples. For characterization of the activity, the inhibition in percentage is presented.

Inhibition (%) = [(control absorbance – sample absorbance)/ control absorbance] × 100.

Total scavenging capacity (TSC) of the aqueous extracts was detected by chemiluminometric method [11] with Lumat LB 9051 luminometer in the H_2O_2 / OH?-luminol-microperoxidase system. In the presence of radical scavenging molecules the emitted light is reduced, expressed as the percentage of the standard light of the H_2O_2 / OH?–luminol-microperoxidase system (RLU%=Relative Light Unit %). From the two marjoram herbs aqueous solutions in four concentrations (0.008, 0.018, 0.028 and 0.04 % w/w) were prepared. RLU % and the total scavenging capacity (TSC) can be expressed as:

RLU (%) = [sample light (mV)/ control light (mV)] × 100; TSC (%) = 100 – RLU (%).

Rancimat method is an automated version of active oxygen method for the determination of induction time the so-called stability time of fatty or oily extracts. In this method the highly volatile organic acids produced by autoxidation are absorbed in water and used to indicate the induction time. Metrohm 743 Rancimat apparatus (Metrohm, Switzerland) was used for the measurements. The supercritical CO_2- and alcoholic extracts in different concentration (0.1, 0.5, 1, 1.5 and 2%; w/w) were measured with 4 – 4 gram refined sunflower oil (control oil). It was kept at stable temperature (100°C) and the air was pumped with 20 L/h flow rate. The induction time was detected by conductivity measurements and recorded by computer. Protection factor (PF) was calculated by diving the induction time of the sample by the induction time of control sunflower oil. When the materials do not have antioxidant activity, the induction time of their dispersion is equal to the induction time of control sample and PF is equal to 1.

High-performance liquid chromatography

For the determination of carnosic acid and carnosol HPLC system consisted of a Spectra SERIES P100 pump, a Spectra SYSTEM UV1000 UV-VIS detector and a Rheodyne injector (Cotati, CA). Data were monitored with OS2 software, A KROMASIL 100 C18 (250x4 mm, 5 µm) column (BIA separations, Slovenia) was used. The mobile phase was a mixture of acetonitrile and water (70:30 (v/v)) and contained 0.5% phosphoric acid. The flow rate was 1.5 ml/min and the detection wavelength was 230 nm.

To determine the ursolic acid the same apparatus was applied as for the determination of carnosic acid and carnosol. A Waters Spherisorb ODS2 (250x4 mm, 5 µm) column (Waters) was used. The mobile phase was a mixture of methanol and water (91:9 (v/v)). The flow rate was 0.70 ml/min and the detection wavelength was 210 nm. Both methods were validated and on 95% confidence range results had no statistical differences.

II - RESULTS AND DISCUSSION

Antioxidant activity in aqueous systems

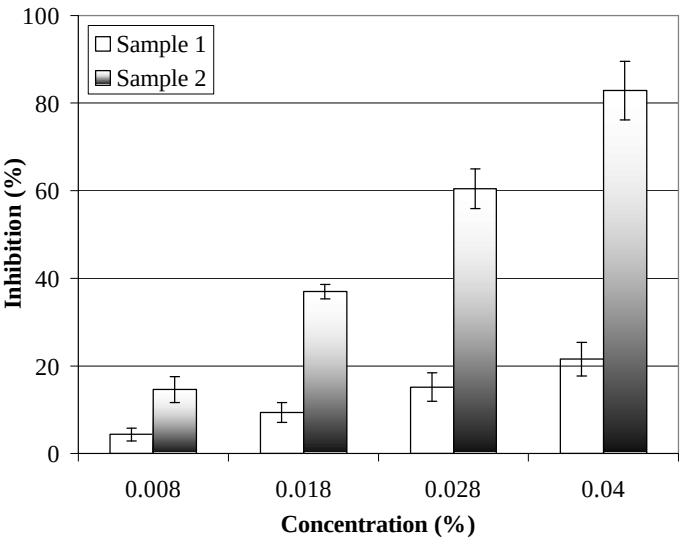

Figure 1: Hydrogen donating ability of aqueous extracts of marjoram.

The results obtained by chemiluminescence method in the H_2O_2/OH?-luminol-microperoxidase system revealed differences between sample 1 and 2. In 0.028 % concentration sample 2 showed significantly strong scavenger capacity, while sample 1 had no scavenger activity. At the highest applied concantration (0.04%, w/w) sample 1 possessed 53.58 % of RLU value, while sample 2 had 0.17 % RLU value. The results well represent the stronger scavenging activities of sample 2, while the other showed significantly lower activities. In Figure 1 the antioxidant activities of sample 1 and 2 are summarized. Both samples

showed hydrogen-donating activities in the presence of free radical DPPH. The scavenging activities were strongly concentration dependent. It can be seen that sample 2 exhibited significantly higher antioxidant activities in aqueous test systems.

Antioxidant activity in lipid systems: Rancimat method

The antioxidant activities of oleoresins of samples 1 and 2 extracted with different solvent powers (ethanol, *n*-hexane and supercritical CO_2) were determined in lipid oxidation assay. The protection factors versus concentrations of the samples are summarized in Table 1. It can be concluded that ethanolic extracts of both samples showed stronger antioxidant activities than the SFE extracts. The Egyptian (sample 2) marjoram possessed stronger antioxidant activity than the Hungarian one (sample 1). The ethanolic extracts of Hungarian sample and the supercritical CO_2 extracts of Egyptian sample have almost similar antioxidant activities in lipid system.

Table 1 : Comparison of protection factors of the ethanolic Soxhlet extraction (SE) and supercritical CO_2 extracts (SFE) of samples 1 and 2 obtained by Rancimat method

Concentration	Sample 1		Sample 2	
(%)	**SFE**	**SE**	**SFE**	**SE**
0.1	1±0.09	1±0.45	1.02±0.02	1.05±0.04
0.5	1±0.06	1.1±0.22	1.08±0.01	1.14±0.11
1	1±0.17	1.14±0.56	1.15±0.12	1.26±0.27
1.5	1.01±0.06	1.23±0.22	1.21±0.24	1.38±0.55
2	1.03±0.08	1.32±0.45	1.26±0.09	1.42±0.30

Qualification and quantification of antioxidant compounds in marjoram extracts

Ursolic acid (UA), carnosic acid (CA) and carnosol (C) compounds were determined by HPLC in the two marjoram herbs (samples 1 and 2), in the conventional solvent extracts and in the extracts obtained by SFE. The concentrations of UA and C in the raw materials are the followings : 0.97% of UA and 0.07% of C in sample 1 and 0.71% of UA and 0.06% of C in sample 2. In highest amount UA was found in the herb as well as in the extracts among the identified antioxidant compounds. The highest concentration of this triterpenoid can be obtained with ethanol, 4.30 and 2.32% in sample 1 and 2, respectively. Although the concentration of C is low in the raw material, in the extracts the amount of C is relatively high. The carnosol is more soluble in apolar solvents (*n*-hexane and $scCO_2$), however only 24 - 37 % of C of raw material was extracted. With ethanol only ~15 % of C could be extracted, while with *n*-hexane or $scCO_2$ twice amount of carnosol was determined. The highest amount of C was obtained at 450 bar and 50°C, 18.53 mg/100 g d.m., respectively.

The highly sensitive CA was under the detection level (< 0.001 %) in all extracts that might have been caused by the low level of CA of herbs and also the possible quick degradation of the molecule after extraction.

In the case of sample 1 the quantification of UA, C and CA compounds was investigated in $scCO_2$ extracts obtained between different ranges of extraction temperatures and pressures. Ursolic acid was relatively low soluble in $scCO_2$ solvent, the highest amount of UA was found in extract obtained at 400 bar and 40°C, 81.58 mg/100 g d.m., which is 8.4% of the UA of the raw material. The amounts of UA in the extracts increased with pressure. With extraction at 250 bar and 50°C 71.42 mg/ 100 g d.m. UA was found in the extract. In all extracts obtained at 60°C the amounts of UA were lower than that of obtained at lower temperatures.

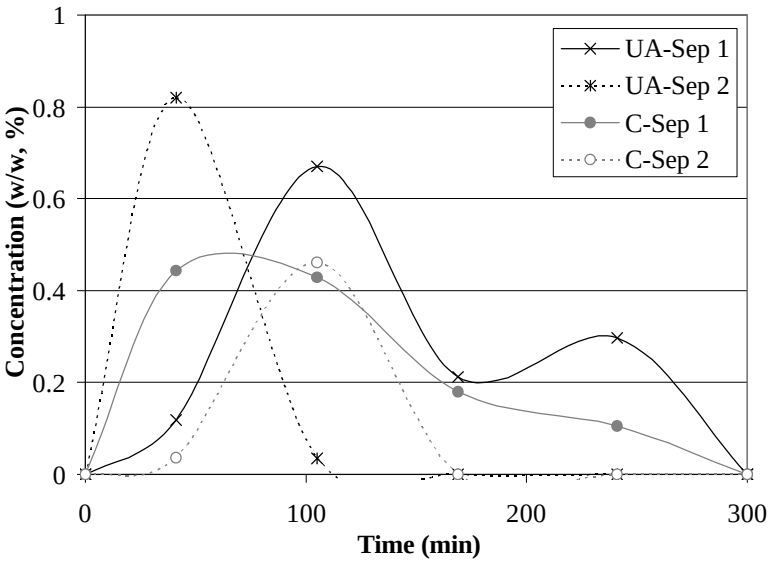

Figure 2: Concentrations changes of UA and C during SFE of marjoram

The changes of the extracted amount of triterpenoid and diterpene phenol compounds in the extracts in the term of time / or consumed $scCO_2$ solvent were investigated. The changes in concentrations in 1st and 2nd separators were followed as it can be seen in Figure 2. Fractionated extraction was carried out at 450 bar and 50°C with two separators in series. The pressure of 1st separator was kept at 80 bar, while that of 2nd separator was 20 bar. Ursolic acid was found in bigger concentration in 2nd separator than in 1st separator (0.82 and 0.12%), while carnosol was concentrated in 1st separator (0.33%). At the beginning of extraction high amount of UA was collected from the 2nd separator, after this compound was concentrated in 1st separator in decreasing concentration. Carnosol was mainly found in the 1st separator, although after 100 min high amount of C was measured in 2nd separator. This phenomenom can be explained with that, the polar, non-volatile free triterpene, ursolic acid is located in leaf cuticular, where the semivolatile organic compounds (SOCs) and linear alkanes are accumulated. Therefore, at the beginning of the extraction this triterpene is extracted with the volatile oils, SOCs and linear alkanes and could be collected from the 2nd separator.

Effects of process parameters of supercritical CO_2 extraction on the yields of carnosol and ursolic acid

The effects of the temperature (T) and pressure (p) of the extractor on the yield of C and UA were examined by applying a 3^2 full factorial design with three repeated measurements in the centre. The three levels of temperature were 40, 50, and 60 °C, while those of the pressure were 100, 250 and 400 bar. The dependent variable was the yield of C and UA, expressed in mass ratio of C to the dry material (mg C / 100 g d.m.). The effects of independent variables were calculated by Statistica for Windows software.

The contour plot fitted to the experimental results is shown in Figure 3. It is seen that both the

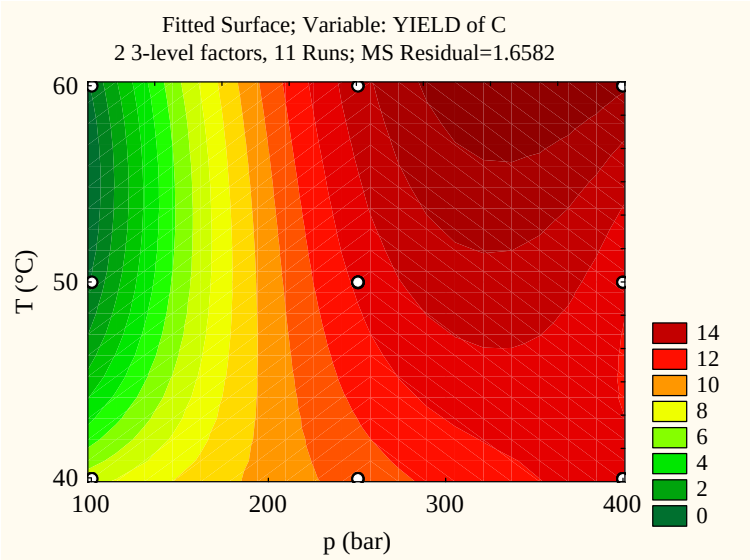

Figure 3 : Contour plot of carnosol in SFE extracts of marjoram

pressure and temperature of the extractor affected the yield of carnosol, although the effect of pressure was obviously stronger due to the more curving surface in the terms of pressure. At higher pressure ($p \geq 300$ bar) and at higher temperature, higher yield of C is produced, while at lower pressures ($p \sim 100$ bar) carnosol cannot be extracted. The perfect range of extraction temperature for C is higher than 55°C.

CONCLUSION

In conclusion the *Origanum majorana* L. herbs and their extracts whether are originating from Hungary (sample 1) or Egypt (sample 2) possess relatively strong antioxidant activities which can adequately be measured by several methods. Concerning the hydrogen donating abilities and the total scavenging capacities of aqueous solutions the Egyptian herb showed stronger antioxidant properties. The products obtained by conventional solvent extraction and supercritical CO_2 extraction still exhibited strong antioxidant activities, that were significantly higher using polar solvent (ethanol) for extraction. Natural antioxidant compounds were quantified from the herbs and extracts, marjoram contained high amount of ursolic acid and carnosol, while the highly labile carnosic acid was absent from the extracts. The amount of carnosol can be enhanced with the optimization of the extraction conditions (pressure and temperature). The usage of certain extractable compounds and / or the whole herb and its extracts are highly reasonable within food-, cosmetic or pharmaceutical industries.

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