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Phenolic and Triterpenoid Antioxidants from *Origanum majorana* L. Herb and Extracts Obtained with Different Solvents

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Antioxidant properties of marjoram (*Origanum majorana* L.) herb and extracts obtained with ethanol, *n*-hexane, and supercritical CO_2 extraction are presented. Individual antioxidants, ursolic acid, carnosic acid, and carnosol, were quantified with high-performance liquid chromatography. The effects of different parameters (temperature and pressure) of high-pressure extraction on the yield of carnosol were studied. Furthermore, two marjoram herbs from Hungary and Egypt were compared measuring hydrogen-donating abilities with 1,1-diphenyl-2-picrylhydrazyl by spectrophotometric and the total scavenger capacities by chemiluminometric methods from the aqueous extracts of the herbs. The antioxidant activities of the solvent extracts were performed using the Rancimat method. The Egyptian herb and its extracts possessed better antioxidant activities than Hungarian ones. Applying supercritical CO_2 extraction, the highest value of carnosol was obtained at 400 bar and 60 °C.

KEYWORDS: Marjoram (*Origanum majorana* L.); antioxidant properties; DPPH method; H_2O_2/OH•-microperoxidase-luminol system; Rancimat method; ursolic acid; carnosol and carnosic acid

INTRODUCTION

Although oxygen is the most important element for aerobic life, it has also been shown, however, to participate in a number of toxic chemical reactions. In particular, lipid peroxidation is a toxic reaction that commonly occurs in food via organoleptic deterioration during processing, distribution, and later storage stages. Therefore, the prevention of such adverse reactions is obvious with potential financial and nutritional gains to be attained for the food industry. The use of antioxidants, preferably being obtained from natural sources, has been shown to be invaluable in the prevention of such oxidative deterioration.

A well-known antioxidant is carnosic acid, a diterpene phenol, which has a structure similar to rosmarinic acid and has been thoroughly investigated in the literature (*1*−*9*). The oxidative hydroxylation of carnosic acid leads to the formation of carnosol, a derivative with increased stability, while still possessing antioxidant properties (*1*−*3*, *6*, *7*). A more novel antioxidant is ursolic acid, a pentacyclic triterpenoid compound, which can be found in numerous plants. Similar to steroids, triterpenoids have been shown to induce dramatic biological effects, subsequently resulting in a growing interest in their properties. The study of carnosic acid and carnosol has shown them to have both anti-inflammatory (*6*, *10*−*12*) and antitumor (*13*−*15*) effects, while ursolic acid has been reported to possess hepatoprotective (*16*, *17*) and lipid lowering (*18*, *19*) properties. Lamiaceae plants have been widely studied as natural antioxidant sources because of their high concentration of phenolic compounds (*1*, *3*, *7*−*9*, *20*, *21*). *Origanum majorana* L. is an herb that commonly grows in Mediterranean regions and is widely used in traditional medicine as well as the food and cosmetic industries. This herb has carminative, antispasmodic, diaphoretic, and diuretic properties (*22*, *23*). In particular, sweet marjoram herb contains up to 3% volatile oil, flavonoid glycosides, tannins, steroids (e.g., β-sitosterol), and triterpenoids (oleanolic acid and ursolic acid) (*24*, *25*). These different extracts of marjoram possess antioxidant, antimicrobial, and anti-inflammatory effects (*15*, *26*−*28*). To obtain these biologically active natural products, it is desirable to use an environmentally friendly separation process. The use of supercritical fluid extraction (SFE) using supercritical CO_2 is particularly advantageous as low temperatures can be used to extract thermally labile compounds with the additional benefit of solvent residue free extracts (*29*).

The aim of this study was to investigate the antioxidant activities of different products obtained by both conventional solvent extraction and SFE from two species of *Origanum majorana* L. herbs. The carnosic acid, carnosol, and ursolic acid

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content of marjoram was quantified, and the effect of different parameters (pressure and temperature) of supercritical CO_2 extraction on the yield of carnosol was studied. The use of supercritical CO_2 (due to its low dielectric constant) is well known to predominantly extract compounds possessing a low polarity. For comparison, the conventional solvent extractions were performed using *n*-hexane and ethanol to compare the carnosol and ursolic acid composition and antioxidant properties. Furthermore, two marjoram herbs from Hungary and from Egypt were compared.

EXPERIMENTAL PROCEDURES

Materials. The dried, finely ground marjoram sample was obtained from Kalocsa, Hungary (sample 1). The raw material was composed of grayish-brown leaves and tops with a characteristic scent. Sample 2 was purchased from an herb supplier with official control originating from Egypt, which had lighter green color, containing the leaves and the flowering tops also. Moisture content of sample 1 was 12.07 ± 0.62% (w/w), and of sample 2 was 10.31 ± 0.69% (w/w). The CO_2 used was 99.5% (w/w) pure and was 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-phthalazinedion (luminol) were purchased from Sigma Chem. Corp. (St. Louis, MO). For determination of the antioxidant activities of extracts with the Rancimat apparatus, sunflower oil (Floriol, Hungary) and butylated hydroxytoluene (BHT) (Merck, Hungary) control were 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 96.5%) was purchased from Sigma (Germany).

Methods. Standard methods were applied for the determination of the oleoresin (by ethanol and *n*-hexane Soxhlet extraction), and the moisture contents of marjoram samples (*30*). Aqueous extracts were obtained as each raw material was infused with of 90−100 °C double distilled water and kept for 30 min in a water-bath. The solvent extraction was carried out in laboratory Soxhlet apparatus using *n*-hexane and ethanol. Pilot plant extraction (in 5 L volume extractor) using ethanol was carried out too. The extractor was packed with 1000 g of marjoram. The extraction temperature was set for 40 °C, and the flow rate of ethanol was 6−7 kg/h. The extraction was carried out for 320−360 min, until the concentration of extract was below 0.1% of the raw material. Supercritical CO_2 extraction 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 (*31*). The extraction vessel was filled with about 800−1000 g of ground raw material. The desired temperature (50 °C) and pressure (450 bar) were adjusted, and the CO_2 feed was started. The carbon dioxide flow rate was 7 kg/(h × kg raw material). The accumulated product samples were collected in a separator (pressure operated at 40 bar and the temperature was 20 °C) and weighed at certain time intervals. The extraction was carried on until the amount of the product sample collected for 1 h decreased under 0.1% of the raw material.

Analytical Methods. *Determination of Polyphenol Content.* The polyphenol content of the herb was measured by spectrophotometric method at 750 nm, using pyrogallol as reference standard (*30*). This method is based on the formation of blue-colored products by redox reaction with Folin-reagent. The absorbance of colored solutions is proportional to polyphenol concentrations.

Flavonoid Content Determination. The flavonoid content of the samples was determined by spectrophotometric method measuring the flavonoids in $AlCl_3$-complex form of the purified ethyl acetate phase obtained after acid hydrolysis (*32*). Glycosides and aglycones were determined together in aglycone form.

Determination of Tannins. Tannin was determined quantitatively by its adsorption on standard hide powder (*30*). This method is an indirect determination. The tannin content is equivalent to the difference between the total polyphenol content and the polyphenol content that remained after the tannins were adsorbed by hide powder.

Determination of Oxidative Stability and Radical Scavenger Activity of Herb and Extracts. *The hydrogen-donating ability (HDA)* 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. (*33*). DPPH solution was freshly prepared as a free radical source. From aqueous extracts, methanolic solutions in four concentrations (0.008, 0.018, 0.028, and 0.04% w/w) were prepared by adding 500 μL of DPPH to each and incubating at room temperature (25 ± 2 °C) for 30 min. As a control, 20% (v/v) of DPPH methanolic solution was prepared and treated as the samples. The absorbance (Abs) of the control and samples was measured, and the DPPH scavenging activity in percentage is presented below:

$$\text{DPPH scavenging activity (\%)} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] \times 100$$

The total scavenging capacity (TSC) of the aqueous extracts was detected in the H_2O_2/OH•-luminol-microperoxidase system with a Lumat LB 9051 luminometer by the chemiluminometric method (*34*). Unstable free radicals (OH•) originating from H_2O_2 via the Fenton reaction result in the chemical reaction of luminol into amino-phthalic acid, when monochromatic light is emitted. 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:

$$\text{RLU (\%)} = \left[\frac{\text{RLU}_{\text{sample}}}{\text{RLU}_{\text{standard}}}\right] \times 100$$

$$\text{TSC (\%)} = 100 - \text{RLU (\%)}$$

The Rancimat method is an automated version of the 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 concentrations (0.1, 0.5, 1, 1.5, and 2% w/w) were measured with 4−4 g refined sunflower oil, which was the control. For comparison, BHT synthetic antioxidant was added to the oil in different concentrations (0.01 and 0.1% w/w). Three parallel samples were filled into the reaction vessels and put in the measuring block. It was kept at stable temperature (100 °C), and the air was pumped with a 20 L/h flow rate. The induction time was detected by conductivity measurements and recorded by computer. The protection factor (PF) was calculated by dividing 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. To determine the content of antioxidants in the raw plant materials, 100 mg of marjoram herbs was mixed with 100 mL aliquots of ethanol:methanol:2-propanol (90:5:5 v/v) and extracted in an ultrasonic bath for 1 h. Afterward, the samples were filtered and analyzed by HPLC.

Determination of Carnosic Acid and Carnosol. The 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 (250 × 4 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.

Determination of Ursolic Acid. The apparatus was the same as for the determination of carnosic acid and carnosol. A Waters Spherisorb ODS2 (250 × 4 mm, 5 μm) column (Waters) was used. The mobile

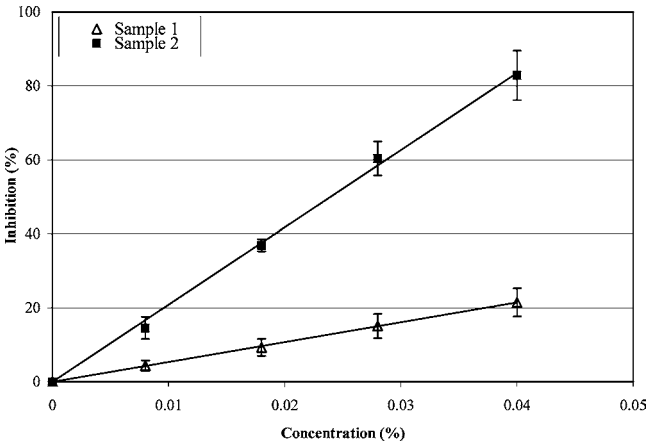

Figure 1. Hydrogen-donating ability of aqueous extracts of marjoram sample 1 ($y = 503.2x$, $R^2 = 0.9929$) and sample 2 ($y = 2053.8x$, $R^2 = 0.997$). Results are mean ± standard deviation ($n = 3$).

Table 1. Phytochemical Composition of the Marjoram Herb

	concentration[a] (w/w %)	
	sample 1	sample 2
flavonoids	0.538 ± 0.0007	0.410 ± 0.0012
tannins	6.454 ± 0.0004	6.357 ± 0.0008
polyphenols	11.665 ± 0.0008	13.813 ± 0.0006

[a] Mean ± standard deviation ($n = 5$).

Table 2. Total Scavenger Capacity of Marjoram Herbs

	chemiluminescence intensity[a] (RLU%)	
sample concentration (w/w, %)	sample 1	sample 2
0.008	100	100
0.018	100	100
0.028	100	0.57 ± 0.10
0.04	53.58 ± 20.08	0.17 ± 0.11

[a] Mean ± standard deviation ($n = 3$).

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 the 95% confidence range results had no statistical differences.

RESULTS AND DISCUSSION

Antioxidant Activity in Aqueous Systems. Due to the phytochemical compositions of samples 1 and 2, no significant differences could be found between the concentrations of tannins and flavonoids, although the content of polyphenols in sample 2 was higher (**Table 1**). The aqueous solutions of Hungarian (sample 1) and Egyptian (sample 2) marjoram in the applied concentrations showed hydrogen-donating ability in the presence of DPPH stable radical, as can be seen in **Figure 1**. The results are well described with fitted straight lines. The inhibition of the samples showed dependence on the concentration. Sample 2 exhibited significantly stronger oxidative stability. The scavenging activities were also concentration dependent. The results obtained by the chemiluminescence method in the H_2O_2/OH•-luminol-microperoxidase system are summarized in **Table 2**. Better antioxidant activity of sample 2 was stated in this test system, although significantly strong scavenging capacities of both samples were measured in higher concentrations. The total scavenging capacity of Hungarian marjoram (sample 1) was less effective as examined by the chemiluminescence method.

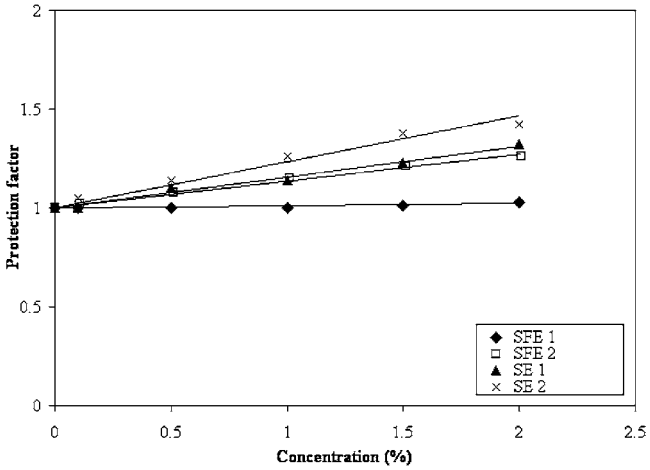

Figure 2. Comparison of protection factors of the ethanol (SE) and supercritical CO_2 extracts (SFE) of samples 1 and 2 obtained by the Rancimat method. Supercritical CO_2 extraction was carried out at 450 bar and 50 °C in both cases. Results of linear regression are: SE1 – $y = 0.1565x + 1$, $R^2 = 0.9869$; SE2 – $y = 0.2324x + 1$, $R^2 = 0.9664$; SFE1 – $y = 0.01x + 1$, $R^2 = 0.9377$; SFE2 – $y = 0.1359x + 1$, $R^2 = 0.9909$.

Antioxidant Activity in Lipid System As Measured by the Rancimat Method. The antioxidant activities of oleoresins of samples 1 and 2 extracted with different solvents (ethanol and supercritical CO_2) were determined in lipid oxidation assay and compared to BHT, a synthetic antioxidant in different concentration ranges. The protection factors versus concentrations of the samples are summarized in **Figure 2** fitted with well-characterized straight lines. 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 herb. The ethanolic extract of Hungarian sample and the supercritical CO_2 extract of Egyptian sample have almost similar antioxidant activities in the lipid system. The protection factors of BHT at the concentrations of 0.01% and 0.1% were 1.14 and 1.42, respectively. The ethanolic extracts exhibited antioxidant power comparable to that of BHT at the concentration of 0.1%. BHT is carcinogenic in rats; it has a dosage limit (0.01%) in foodstuffs (*35*). Regarding this, the same antioxidant activity can be achieved only using BHT in higher and health damaging concentrations as the same antioxidant activity can be proved with natural ethanolic extracts from marjoram.

Qualification and Quantification of Antioxidant Compounds in Marjoram Extracts. The contents of ursolic acid (UA), carnosic acid (CA), and carnosol (C) antioxidant compounds were determined by the HPLC method in the two marjoram herbs, in the conventional solvent extracts and in the extracts obtained by SFE. The quantification of ursolic acid and carnosol can be found in **Table 3**. UA was found in the highest amount among the identified antioxidant compounds in the plant materials as well as in the extracts. This triterpenoid was obtained with ethanol in the highest concentration, with recovery of 59−95% to the raw materials of samples 1 and 2. The amount of carnosol was relatively high in the samples and possessed better soluble properties in apolar solvents. The recovery of C in the extracts obtained with *n*-hexane and $scCO_2$ was between 24% and 37% to the raw materials. The highly sensitive CA was under the detection level (<0.001%) in all extracts that might have occurred due to the low level of CA of herbs and the quick degradation of this sensitive compound.

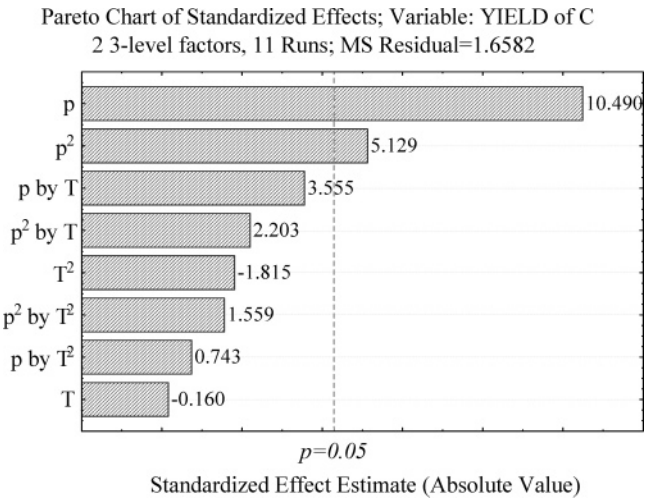

Figure 3. Pareto chart of the effects of the process parameters of supercritical CO_2 extraction on the yield of carnosol (mg/100 g dry material).

Table 3. Extraction Yields and Concentrations of Antioxidant Compounds in Marjoram Herb and Extracts

	extraction yield[b] (%, g/100 g dm)	ursolic acid (%, w/w)	carnosol (%, w/w)
plant material 1		0.971	0.073
SE 1 – ethanol	13.35 ± 0.94	4.304	0.080
SE 1 – hexane	5.00 ± 0.12	2.108	0.535
SFE 1[a]	3.76 ± 0.17	0.103	0.492
plant material 2		0.708	0.056
SE 2 – ethanol	28.99 ± 0.63	2.315	0.032
SE 2 – hexane	7.03 ± 0.33	4.043	0.193
SFE 2[a]	5.39 ± 0.20	0.191	0.250

[a] Supercritical CO_2 extraction was carried out at 450 bar and 50 °C. [b] Mean ± standard deviation ($n = 3$).

Effect of Process Parameters of Supercritical CO_2 Extraction on the Yield of Carnosol. The effects of the temperature (T) and pressure (p) of the extraction on the yield of C were examined by applying a 3^2 full factorial design with three repeated measurements in the center. 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, 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 estimated effects of the terms are demonstrated in the form of a Pareto chart (**Figure 3**). The vertical dashed line, titled $P = 0.05$, gives the critical limit on 95% significance level. Effects that have larger absolute values than this limit were qualified as significant. It is apparent that pressure had a very strong effect on carnosol extraction, as the linear pressure term [p] and the quadratic pressure term [p^2] were significant. The effects of other terms were not significant, because the values in the rows of these terms were under the critical limit of significance level.

The three-dimensional response−surface plot fitted to the experimental results is shown in **Figure 4**. It is seen that both the pressure and the 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. This curved surface in the [p] variable reflected the quadratic pressure dependence, which gave an optimal pressure, but probably a less optimal temperature in the experimental region. At higher pressure ($p \geq 300$ bar) and at higher temperature, a higher yield of C was produced, while at lower pressures ($p \approx$ 100 bar) carnosol was not extracted. The perfect range of extraction temperature for C was higher than 55 °C.

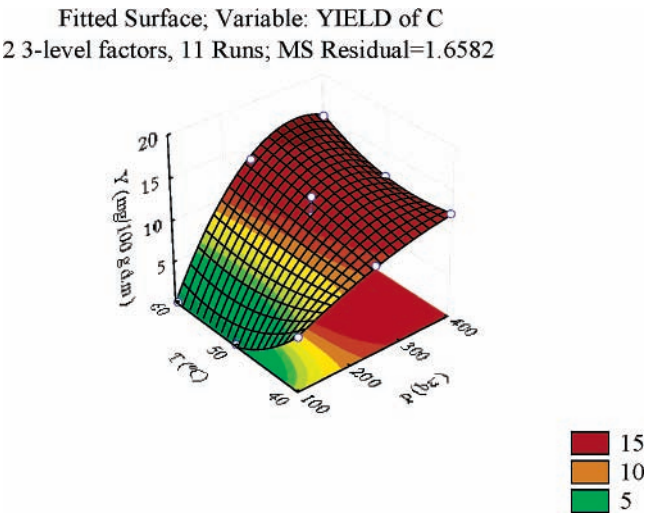

Figure 4. Three-dimensional fitted surface of carnosol (mg/100 g dry material) in supercritical CO_2 extracts of marjoram.

In conclusion, the *Origanum majorana* L. herbs and their extracts originating from Hungary or Egypt possess relatively strong antioxidant activities, which can promptly be measured by several methods. The phytochemical analysis showed slight differences between the examined herbs. Concerning the hydrogen-donating abilities and the total scavenging capacities of aqueous solutions, the Egyptian herb showed stronger antioxidant properties. It can be explained with the geographic differences. These secondary metabolites, like flavonoids, tannins, and polyphenols, are produced as self-defense compounds against radiation (e.g., UVA, UVB), microbes, viruses, and other harming beings. The observed differences in the amounts of phenolic compounds of the plants might have been caused by the different climate features (e.g., higher numbers of sunny days in Egypt); therefore, the different antioxidant properties of the selected herbs were revealed. The products obtained by conventional solvent extraction and supercritical CO_2 extraction still exhibited strong antioxidant activities; the activities were significantly higher using polar solvent (ethanol) for the extraction. Natural antioxidant compounds were quantified from the herbs and extracts; marjoram contained a high amount of ursolic acid and carnosol, while the highly labile carnosic acid was absent from the extracts. The Hungarian herb and its extracts contained ursolic acid and carnosol in higher concentrations than those of the Egyptian sample; meanwhile, the Egyptian extracts showed better antioxidant properties. It points out the importance of recovery of the antioxidant activity of the complex extracts rather than individual compounds; however, numerous unidentified biological active compounds have important roles in the antioxidant activities expressed. 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 is highly reasonable within food, cosmetic, and pharmaceutical industries.

ABBREVIATIONS USED

SFE, supercritical fluid extraction; SE, Soxhlet extraction; T, extraction temperature (°C); p, extraction pressure (bar); P, value belongs to the F-test statistic during ANOVA; d.m., dry material; HDA, hydrogen-donating ability; DPPH, 1,1-diphenyl-2-picrylhydrazyl; TSC, total scavenging capacity; BHT, buthyl-

ated hydroxytoluene; UA, ursolic acid; CA, carnosic acid; UVA, ultraviolet A ray; UVB, ultraviolet B ray.

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