Pharmaceutical Biology
2003, Vol. 41, No. 5, pp. 379–383

1388-0209/03/4105-379$16.00

Antioxidant Activity of Supercritical CO_2 Extracts of *Helichrysum italicum*

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Abstract

The antioxidant activity of supercritical CO_2 extracts of *H. italicum* dried flower heads derived from the commercial drug and from plants grown in different areas of north-east Italy with different culturing conditions was determined. In particular, the characterization of the antioxidant activity was made by the DPPH and β-carotene bleaching test methods. The four kind of *H. italicum* extracts were also tested for their ability to scavenger superoxide radicals. All extracts showed, although with different importance, an antioxidant activity with all the methods performed. The supercritical extracts obtained from commercial dried *H. italicum* flower heads and from dried flower heads belonging to wild plants exhibited the highest activity. These results established *H. italicum* supercritical extracts as important antioxidant solvent-free matrices in alimentary (i.e., dietary, nutraceutical, flavouring) and cosmetic fields, as well as the value of coastal Mediterranean areas to serve as an exploitable source of important plant matrices.

Keywords: Antioxidant activity, β-carotene bleaching test, DPPH method, *Helichrysum italicum*, radical scavenger activity, SFE.

Introduction

Helichrysum italicum (Roth) Don (*Asteraceae)* is a shrub widely distributed in dry and sandy-rocky areas of the Mediterranean region. The main medicinal properties ascribed to this plant are anti-inflammatory and anti-allergic, reported in the folk medicines of the Mediterranean area as typical remedies of the decoctions of its dried flower heads (Bruni et al., 1997). In cosmetics, various kinds of *H. italicum* matrices obtained by different extraction methods – flower heads collected immediately after blooming and subsequently dried – are most often employed in fragrances and in the formulation of phyto-derived products to protect delicate, irritated skin. Such properties of the *Helichrysum*-derived cosmetic products are most likely due to the presence of significant amounts of polyphenols – as flavonoids – which are known to protect against oxidants (Maffei Faccino et al., 1990; De la Puerta et al., 1999). The increasing interest in alimentary applications (i.e., dietary, nutraceutical, flavouring) of plant matrices rich in antioxidants is due to the possible correlation between the oxidant action of free radicals and the onset of some important pathologies like ischemia-reperfusion injury, atherosclerosis, thrombotic and cancer diseases (Mak et al., 1992). This kind of correlation has led the health nutrition industry to seek antioxidants from plant matrices that can be used in the formulation of preventive phytocomplexes. Strict legislation on the use of synthetic food additives and consumer preferences have also shifted the attention of manufacturers from synthetic to natural antioxidants (Dapkevicius et al., 1998). Noteworthy is that natural matrices rich in antioxidants have proved to be less toxic than synthetic molecules such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which not only are highly volatile and unstable at high temperatures – making their industrial scale application difficult – but might possibly induce carcinogenesis despite the fact that they inhibit lipid peroxidation (Thabrew et al., 1998; Amarowicz et al., 2000; Idaomar et al., 2002). Therefore, the

Accepted: October 10, 2002

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purpose of the present work was to evaluate the antioxidant activity of supercritical CO_2 extracts of *H. italicum* dried flower heads derived from the commercial drug and from plants grown in different areas of North-East Italy with different culturing conditions. The reasons which led us to employ supercritical fluids as extracting tools instead of classical methods are linked to numerous advantages. These can be mainly summarized in preventing contact with atmospheric oxygen – thus minimizing degradation of the labile components – and even more important, in the production of solvent-free extracts which can then be directly processed in industrial applications in the alimentary and cosmetic areas (Bartle, 1990; Hawthorne, 1990).

Materials and methods

Plant material

In the present study the following plant material was used: flower heads of wild *Helichrysum italicum* growing in the sandy dunes along the North East Italian coast (Rosolina, Rovigo, Italy) (sample A); flower heads of *H. italicum* plants obtained through vegetative reproduction from the wild plants and grown in the experimental open fields of the Veneto Agricoltura Centro Sperimentale Ortofloristico "Po di Tramontana" (Rosolina, Rovigo, Italy) (sample B); flower heads of *H. italicum* from nursery (sample C); selected flower heads of *H. italicum* from packages purchased from the market (sample D). All the fresh samples of *H. italicum* flower heads (samples A, B, C) were dried at room temperature, in the dark for 15 days with 10% constant humidity.

The wild *H. italicum* plants were collected and identified by Prof. F. Piccoli, University of Ferrara. A voucher specimen of the wild species was deposited in the Herbarium Universitatis Ferrariensis of the Dipartimento delle Risorse Naturali e Culturali of the University of Ferrara (code no. FER 002553 2A5).

Supercritical Fluids Extraction (SFE): apparatus and conditions

The same amount of each *Helichrysum* sample (7 kg) was subjected to supercritical CO_2 extraction using an SF extractor pilot plant (Model Fedegari Autoclavi Spa, Italy). The extraction was processed for 3 h at a constant pressure of 260 bar. The CO_2 flow rate was 50 kg/h and the oven temperature was 50 °C. The extracts were kept in the dark at –20 °C until they were used for the determination of the antioxidant capacity.

Chemicals

All the chemicals employed for the antioxidant evaluation were purchased from Sigma Chemical Co., St. Louis, MO, USA).

Determination of the antioxidant capacity

DPPH method

Free radical scavenging activity of the crude extracts was determined using a method based on the reduction of an ethanolic solution of the colored free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) (Wang et al., 1998). An ethanolic DPPH solution (1×10^{-4} M) was mixed with ethanolic solutions of extract concentration (5, 15, 25, 50, 100, 200 µg/ml). Then the solutions were shaken vigorously and kept in the dark for 30 min at room temperature. Sample absorbancy was measured at 517 nm with a Perkin Elmer 554 spectrophotometer. An ethanolic solution of butylated hydroxyanisole (BHA) was used as reference at all concentrations used for the extracts; an ethanolic solution of DPPH (1×10^{-4} M) was employed as blank. Antioxidant activity of each extract was determined as follows, according to the percentage of DPPH decoloration:

$$\text{decolouration percentage} = [1 - (\text{absorbance with compound/absorbance of the blank})] \times 100.$$

All tests were run in triplicate and averaged.

β-Carotene bleaching test

The test procedure used was similar to the one described by Taga et al. (1984). The technique consisted of measuring β-carotene bleaching as a result of oxidation by linoleic acid degradation products. The ethanolic solution concentration of the extracts used in this test was 2 mg/ml. An ethanolic butylated hydroxyanisole solution (BHA) (2 mg/ml) was used as a reference. β-Carotene (0.5 mg) was dissolved in 1 ml of chloroform. Linoleic acid (25 µl) and 200 µl of Tween 40 were added to the β-carotene solution. Chloroform was removed using a rotavapor at 50 °C. Fifty ml of oxygenated distilled water were added and the mixture was vigorously shaken to form an emulsion. Five ml of the emulsion was added to 0.2 ml of the antioxidant solution and the samples were set in an agitating water bath at 50 °C. The absorbance at 470 nm was measured with a spectrophotometer after 28 and 56 h of incubation. In order to avoid the interference of the colour of the extract with the spectrophotometric detection, the method reported in Gazzani et al. (2000) was adopted. The antioxidant activity of the extracts was calculated using an RAA (Relative Antioxidant Activities) coefficient, obtained using the following equation (Dapkevicius et al., 1998):

$$\text{RAA} = \text{Sample absorbance/BHA absorbance}$$

All determinations were performed in triplicate and averaged.

Assessment of superoxide radical scavenger activity

To investigate the ability of the extracts to scavenger superoxide anions, experiments were also carried out to determine

if these extracts inhibited the cytochrome c reduction caused by such oxygen radicals.

Superoxide radicals were generated by preparing a mixture of xanthine and xanthine oxidase (EC 1.1.3.22) from buttermilk. One ml of the reaction mixture contained the following: 50 mM KH_2PO_4-KOH, pH 7.4, 1 mM EDTA, 100 µM xanthine and 100 µM cytochrome c. The reaction was started by adding 0.066 units of xanthine oxidase (freshly diluted in 100 µl of the above phosphate buffer), and the rate of cytochrome c reduction was measured at 550 nm with a spectrophotometer at 25 °C (De las Heras et al., 1998). The ethanolic solutions of the extracts were tested at the reaction mixture concentration of 25, 50, 75, 100 and 200 µg/ml. The results were expressed as percentage of inhibition of cytochrome c reduction. Control experiments were carried out to determine if the extracts themselves could reduce cytochrome c directly. Ethanolic solutions of the extracts were added to a solution containing 100 µM cytochrome c and the absorbance was measured at 550 nm (De las Heras et al., 1998). At the concentrations evaluated, the extracts did not reduce cutochrome c by themselves. Superoxide dismutase (SOD) (EC 1.15.1.1) from bovine erythrocytes was used as a reference at the concentration of 500 units/ml. All determinations were performed in triplicate and averaged.

Results and discussion

The supercritical fluid extractions were performed on dried flower heads collected from wild *Helichrysum italicum* plants grown along the Italian north-east Mediterranean coast (sample A), on dried flower heads from plants cultivated in experimental fields (sample B), on dried flower heads from plants grown in the nursery (sample C) and on flower heads derived from the *Helichrysum italicum* commercial drug. The four kind of extracts were all greenish-yellow pastes and all had an intense aromatic smell. In terms of extraction yields, those obtained from the commercial drug were slightly lower than those of the other dried flower heads, which therefore were substantially similar (Table 1).

The antioxidant activity of *H. italicum* extracts from dried flower heads is only reported with regard to matrices obtained through polar solvent extraction, and the biological activity is often associated with isolated polyphenols (Maffei et al., 1990). With particular reference to this class of chemicals, the SF extraction is known to provide good qualitative and quantitative performance. The main reason is because it is particularly selective towards the non-polar extractable fractions of the plant matrices, often associated with antioxidant polyphenols which protect unsaturated substances (i.e., polyunsaturated fatty acids) (Taga et al., 1984).

The antioxidant activity of all extracts was evaluated with the DPPH method and the β-carotene bleaching test. Both these techniques are particularly fit for the evaluation of the antioxidant activity of crude extracts (Amarowicz et al., 2000). Moreover, these methods are rapid, simple, sensitive reproducible and require conventional laboratory equipment. Application of these methods to the *H. italicum* supercritical extracts provided interesting and homogeneous results. In particular, the DPPH assay showed that all extracts showed minimal antioxidant activity even at the lowest test concentration of 5 µg/ml (Table 2). On the whole, at a concentration of 100 µg/ml, the sample extracts A, B and D exhibited an antioxidant activity similar to that obtained for the same concentration of synthetic antioxidant (BHA). At a higher concentration (200 µg/ml), these extracts did not show any significant increase in scavenging activity. On the other hand, the sample extract C showed equivalent activity to BHA only

Table 1. Extraction yield percentage obtained from *H. italicum* dried flower heads by SFE.

Extracts	Extraction Yield (%)
A	4.5 ± 0.1
B	4.5 ± 0.1
C	4.9 ± 0.15
D	3.9 ± 0.09

Extracts: A = dried flower heads from wild *H. italicum*; B = dried flower heads from *H. italicum* grown in experimental open fields; C = dried flower heads from *H. italicum* grown in the nursery; D = dried flower heads from *H. italicum* commercial drug. Each value is the average of three extractions performed for each sample ± standard deviation.

Table 2. Scavenging effect of SFE extracts of *H. italicum* on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical.

Extracts	5 µg/ml	15 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
A	17.91 ± 0.2	51.45 ± 1.2	68.9 ± 1.3	77.1 ± 1.3	94 ± 1.5	95.2 ± 1.5
B	23.77 ± 0.5	58.9 ± 1.3	77.2 ± 1.3	83.1 ± 1.4	94.8 ± 1.6	95.2 ± 1.5
C	10.79 ± 0.3	34.57 ± 0.7	44.11 ± 1.1	52.3 ± 1.2	73.9 ± 1.3	90.1 ± 1.4
D	21.24 ± 0.6	54.1 ± 1.1	73.3 ± 1.3	79.4 ± 1.3	94.2 ± 1.4	95.1 ± 1.4
BHA	80.2 ± 1.3	92.14 ± 1.4	93.3 ± 1.4	94.41 ± 0.6	94.9 ± 1.6	95.3 ± 1.7

Extracts: A = dried flower heads from wild *H. italicum*; B = dried flower heads from *H. italicum* grown in experimental open fields; C = dried flower heads from *H. italicum* grown in the nursery; D = dried flower heads from *H. italicum* commercial drug. Reference compound: butylated hydroxyanisole (BHA). Each value in the table was obtained by calculating the average of three experiments ± the standard deviation.

at 200 µg/ml, while at the lower dose of 100 µg/ml, its activity was slightly lower than that observed for the other extracts at the same concentration.

In regard to the variation in antioxidant activity after 28 and 56 h, the β-carotene bleaching test showed that the RAA (Relative Antioxidant Activities) values for the samples extracts A, B and D were quite similar at the first step (28 h) (Table 3). In particular, a comparison with the reference compound (BHA) evidenced that the antioxidant activity of these *Helichrysum* extracts was only approximately 19% lower than that of BHA at this concentration. On the other hand, the sample extract C showed lower activity than the other samples. In fact, the RAA value of the C sample was 43% lower than BHA at the same concentration. After 56 h (second step) only the sample extract B maintained relatively constant RAA values, with a 6% drop from the first step (28 h). After 56 h, instead samples A and D presented approximately a 20% drop in antioxidant activity, reaching values comparable with those of the C sample.

Table 3. Relative antioxidant activity (RAA) of SFE extracts of *H. italicum* determined by the β-carotene bleaching test.

Extracts	28 h	56 h
A	0.71 ± 0.035	0.57 ± 0.027
B	0.857 ± 0.04	0.8 ± 0.039
C	0.57 ± 0.024	0.5 ± 0.026
D	0.78 ± 0.036	0.64 ± 0.031
BHA	1	1

Extracts: A = dried flower heads from wild *H. italicum*; B = dried flower heads from *H. italicum* grown in experimental open fields; C = dried flower heads from *H. italicum* grown in the nursery; D = dried flower heads from *H. italicum* commercial drug. Reference compound: butylated hydroxyanisole (BHA). Each value in the table was obtained by calculating the average of three experiments ± standard deviation.

With the object of determining the scavenging capacity of all the *Helichrysum* extracts versus superoxide radical (O_2) – one of the oxygen radicals possibly involved in the onset of human pathologies as inflammation, allergies, heart ischemia-reperfusion, atherosclerosis, diabetes emphysema and cancer (Halliwell & Gutteridge, 1999) – the percentage inhibition of cytochrome c reduction was determined (Table 4). On the whole, the results of this test were analogous to those obtained in the previous evaluations. In fact, samples A, B and D showed similar scavenger capacity at all the tested concentrations. In particular, the production of superoxide radicals was inhibited more than 50%, at a concentration of 50 µg/ml. Among these extracts, the sample D proved to be the most active since, at 75 µg/ml, it showed a protective action against the superoxide radical similar to the reference compound (SOD). At all the concentrations tested, the sample C showed lower scavenger activity.

In conclusion, the antioxidant activity found for SF extracts derived from the dried flower heads of wild *H. italicum* (sample A), cultivated in the field (sample B) and commercial (sample D) were all similar. The fact that the dried flower heads from wild plants showed similar results as the commercial drug suggests the potential exploitation of the sandy coastal areas of north-east Italy as a source of these important plant matrices. On the other hand, the variability found in plants derived from nursery cultures (sample C) may be linked not only to genetic variance (chemotypes) but also to a different qualitative and quantitative characterization of the polyphenol fraction, which is well documented for *H. italicum* and considered responsible for its scavenger activity (Maffei et al., 1990). In fact, the extracts derived from plant matrices with potential antioxidant properties are characterized by a complex mixture of phenols with a wide qualitative and quantitative variability even within the same species, in particular related to the different numbers of hydroxyl and methoxy groups substituted on the phenols and to their different arrangements and degrees of polymerization (Amarowicz et al., 2000).

Table 4. Percentage of inhibition of SFE extracts of *H. italicum* on the production of the superoxide radical (O_2^-), generated by the xanthine-xanthine oxidase enzyme system.

Extracts	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	200 µg/ml
A	24.3 ± 0.3	65.2 ± 1.8	83 ± 2.8	100 ± 2.9	nd
B	21 ± 0.2	62.3 ± 1.7	81 ± 2.8	100 ± 3.1	nd
C	0.0	8.2 ± 0.1	16.5 ± 0.2	40.3 ± 1.6	63.6 ± 1.8
D	25 ± 0.4	73.4 ± 2.8	100 ± 3.2	nd	nd

Extracts: A = dried flower heads from wild *H. italicum*; B = dried flower heads from *H. italicum* grown in experimental open fields; C = dried flower heads from *H. italicum* grown in the nursery; D = dried flower heads from *H. italicum* commercial drug. Each value in the table was obtained by calculating the average of three experiments ± the standard deviation. nd: could not be determined.

Reference compound: SOD (500 units/ml). The percentage of inibition of O^-_2 production was 100% at this concentration.

Moreover, the interesting antioxidant capacity of examined *Helichrysum* SF extracts, and the non-toxicity of *Helichrysum* lipophilic fractions evidenced by Idaomar et al. (2002), stressed the importance of possibly employing these plant matrices as a source of natural antioxidants. This suggestion could be supported by the increased interest of manufactures for natural additives (Dapkevicius et al., 1998) to the detriment of synthetic antioxidant – usually industrially used – as BHA and BHT, suspected of being involved in inducting severe pathologies (Thabrew et al., 1998). In addition, the extraction method employed, the supercritical fluid extraction, presents not only the advantage of a particularly selective technique to extract low polarity compounds as lipids and lipid related polyphenols, but also allows the production of solvent free extracts. It also reduces the time of extraction and oxidation due to the atmospheric oxygen. In particular, the possibility of obtaining solvent-free extracts represents an important additional value for the nutraceutical, food and cosmetic industry, because of the potential to directly employ these natural matrices in industrial processing.

Acknowledgements

Investigation supported by a grant of VENETO AGRICOLTURA, intervento n° 36 – U.E. program Leader II – GAL DELTA PO

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