Antiradical Activity of Hydrolyzed and Non-hydrolyzed Extracts from *Helichrysi inflorescentia* and its Phenolic Contents

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A methanol extract was obtained from defatted (petroleum ether) inflorescence of *Helichrysum arenarium* (L.) Moench (perennial herb native to Middle and Southeast Europe). The extract was evaporated under reduced pressure and the dry residue was dissolved in hot water. The aqueous solution was stored for 6 d at 4 °C and the precipitate discarded. The remaining solution was divided into three aliquots a, b and c. Part a was extracted with ethyl acetate to obtain extract (A), part b was extracted with diethyl ether to obtain extract (B) and part c was subjected to alkaline hydrolysis and then extracted with diethyl ether to obtain extract (C). Extracts (A), (B) and (C) were evaporated under reduced pressure to obtain the dry residues A, B and C which were further investigated for phenolic compound content by TLC and HPLC and for antiradical activity with 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) as a substrate.
Residue C exhibited stronger antiradical properties than non-hydrolysed residues A and B. HPLC analysis showed a great increase of caffeic acid in residue C.
We concluded that the hydrolysis process led to a significant increase of free caffeic acid (strong antioxidant) concentration resulting in increased antiradical activity of residue C.

Key words: Antiradical Activity, Plant Extracts, Plant Phenolics

Introduction

The inflorescence of *Helichrysum arenarium* (L.) Moench, Asteraceae, is recognized by some European pharmacopoeias. Basic pharmacological properties of this plant are improving flow of bile in bile-ways (cholagogue), and increasing of bile production in liver cells (choleretic) activities, which are closely related to the presence of a phenolic fraction (Czinner *et al.*, 1999).

The therapeutic application of the everlasting was first recognized in folk medicine. Its choleretic, cholagogue, hepatoprotective (Skakun and Stepanov, 1988) and antimicrobial (Smirnov *et al.*, 1989) activities were confirmed by recent phytochemical research.

Also the antioxidant activity of the extracts isolated from *H. arenarium* was the subject of various research (Czinner *et al.*, 2000, 2001).

Phenolic compounds, among others flavonoids and phenolic acids, show antioxidant (Braca *et al.*, 2002; Lekse *et al.*, 2001) and antiradical (Alvarez *et al.*, 2002; Mojzisova and Kuchta, 2001) activities *in vitro*. There is evidence that phenolic compounds can also act as antioxidants and scavengers of free radicals *in vivo* (Mojzisova and Kuchta, 2001). The antiradical and antioxidant effects of phenolic compounds *in vivo* may slow down the ageing processes (Halliwell and Gutteridge, 1999) as well as protect the human body against diseases such as atherosclerosis (Miura *et al.*, 2001), coronary heart disease (Mojzisova and Kuchta, 2001) and cancer (Hirvonen *et al.*, 2001).

Some of the phenolic compounds identified in *H. arenarium* possess antiradical and antioxidant properties. Among them quercetin (flavonoid) and caffeic acid (phenolic acid) are known as strong antioxidants (Satyanarayana *et al.*, 2001; Erden *et al.*, 2001; Raneva *et al.*, 2001; Simonetti *et al.*, 2001).

Experimental

Preparation of the extracts

The inflorescence of *Helichrysum arenarium* (L.) Moench, dried below 35 °C, was obtained from Kawon (Herb Factory, Gostyń, Poland). The raw material was harvested in the region of Konin,

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central Poland. A voucher specimen (Herbarium No. ZS-1) was deposited in the Department of Pharmacognosy, Wrocław University of Medicine, Wrocław, Poland.

The raw material (38.5 g) was defatted with 1000 ml of petroleum ether in a Soxhlet apparatus for 2 d. After defatting the raw material the petroleum ether was removed. Then the same raw material was put again into the Soxhlet apparatus and exhaustively extracted with 750 ml of methanol at 70 °C (water bath) for 2 d. The methanol extract was evaporated to dryness under reduced pressure. The dry residue was dissolved in 600 ml of hot water, cooled and stored at 4 °C for 6 d. The precipitate obtained was filtered off and discarded. Then the water solution was divided into three aliquots a, b, c (200 ml each).

Part a was extracted with 400 ml (8 × 50 ml) of ethyl acetate. The ethyl acetate extract was evaporated under reduced pressure to obtain the dry residue A (395 mg).

Part b was extracted with 250 ml of diethyl ether (10 × 25 ml). After evaporation residue B was obtained (133 mg).

Part c was subjected to alkaline hydrolysis (pH 12) in boiling water bath for 15 min in the presence of 1 g $NaBH_4$. After acidifying (1 M HCl, pH 2) the hydrolysate was extracted with 250 ml diethyl ether (10 × 25 ml). After evaporation the dry residue C (146 mg) was obtained.

Thin layer chromatography analysis of phenolic compounds in the extracts

Residues A, B and C were dissolved in methanol at 10 mg/ml and examined by one-dimensional thin layer chromatography on silica gel plates (Kieselgel 60 F254, DC-Fertigplatten, layer thickness 0.25 mm; Merck, Darmstadt, Germany). Phenolic acids were analysed using the following developing systems: toluene/ethyl formate/formic acid (5:4:1 v/v/v) (Harborne, 1993), isopropyl ether/cyclohexane/formic acid (4:1:0.1 v/v/v), toluene/dioxane/formic acid (8:1:1 v/v/v) (Grayer, 1993), chloroform/ethyl acetate/formic acid (5:4:1 v/v/v) (Schönsiegel and Egger, 1969), toluene/ethyl formate/formic acid/water (5:100:10:12 v/v/v/v), toluene/isopropyl ether/formic acid (8:12:1 v/v/v). The best resolution of phenolic acids was obtained when the plates were developed with toluene/ethyl formate/formic acid/water (5:100:10:12 v/v/v/v) at a distance of 3 cm and next (after drying) with toluene/dioxane/formic acid (8:1:1 v/v/v) at 10–15 cm. Flavonoids were analysed with acetone/chloroform/water (4:2:0.48 v/v/v), ethyl acetate/ethyl methyl ketone/formic acid/water (5:3:1:1 v/v/v/v) (Markham, 1993), chloroform/methanol (4:1 v/v) (Markham, 1993), chloroform/methanol (9:1 v/v) (Markham, 1993), chloroform/acetone/toluene (5:7:8 v/v/v) (Markham, 1993), ethyl acetate/formic acid/acetic acid/water (100:11:11:26 v/v/v/v) (Van Sumere *et al.*, 1965). The best resolution of flavonoids was obtained for the ethyl acetate/ethyl methyl ketone/formic acid/water (5:3:1:1 v/v/v/v) system for glycosides and chloroform/methanol (4:1 v/v) for aglycones. Plates were visualised by spraying with 1% $FeCl_3$ in methanol, diazotised sulfanilic acid amide (Wong and Taylor, 1962), 0.1% $AlCl_3$ in methanol and under UV light at 360 nm. Phenolic acids as well as flavonoids were identified by TLC analysis against standards.

HPLC analysis of phenolic compounds in the extracts

Residues A, B and C were dissolved in methanol at the concentration of 1 mg/ml.

A high performance liquid chromatograph (Knauer, Berlin, Germany) was equipped with HPLC pumps 64 and an UV-VIS detector (254 nm). A LiChrospher 100, RP-18 (5 μm) column (250 × 4 mm) (Merck) was used. The flow rate was 1 ml/min and the injection volume 20 μl. The analyses of the phenolic compounds were carried out at room temperature using two linear gradients of acetonitrile (A) in 2% acetic acid in water (B). Phenolic acids and flavonoid glycosides were analysed with the gradient of Oszmiański *et al.* (1988). Flavonoid aglycones were analysed with the following elution profile: from 0 to 10 min: 3 to 10% (A) in (B); 10 to 25 min: from 10 to 45% (A) in (B); 25 to 35 min: from 45 to 100% (A) in (B). Identification and quantitative analysis were done by comparison with standards.

Evaluation of the antiradical activity

The antiradical activity of the extracts was measured in triplicate according to the method of Brand-Williams *et al.* (1995). Solution (I) was freshly prepared by dissolving 2 mg of $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) in 54 ml of methanol. For solution (II) residues A, B and C were dissolved in methanol to obtain a solution at the concentration of 0.73 mg/ml. 38 μl of (II) were

added to 1460 μl of (I) at room temperature. The absorbance at 515 nm was measured in a 1-cm glass cuvette after 0 (start of reaction), 3, 5, 7, 10, 15, 60 and 180 min and after 24 h. The control sample was prepared by adding 38 μl of methanol to 1460 μl of (I) and the absorbance was measured (515 nm) after 0, 3, 5, 7, 10, 15, 60, 180 min and after 24 h. The antiradical activity of the residues was expressed as the decrease of DPPH$^{\bullet}$ radical concentration (see caption of Table II).

Results and Discussion

Qualitative analysis by thin layer chromatography (TLC) performed for residue A showed the presence of phenolic acids such as syringic, *m*-coumaric and *p*-hydroxybenzoic acid, while apigenin, quercetin, kaempferol and glycosides such as rutin, apigenin-7-glucoside, isorhamnetin-3,7-diglucoside, luteolin-7-glucoside, kaempferol-3-glucoside were identified among flavonoids. Investigation of residue B revealed the presence of syringic, protocatechuic, caffeic, *m*-coumaric and *p*-hydroxybenzoic acid. Among flavonoids we identified the aglycones apigenin, kaempferol, quercetin, luteolin, and among glycosides isorhamnetin-3-glucoside, kaempferol-3-glucoside and apigenin-7-glucoside. Residue C revealed the presence of caffeic, *p*-coumaric, syringic, protocatechuic and *p*-hydroxybenzoic acid. Among flavonoid aglycones the presence of apigenin, kaempferol and luteolin was observed and among glycosides rutin, isorhamnetin-3-glucoside and apigenin-7-glucoside were identified.

HPLC analysis (Table I) of the phenolic compounds in residue A demonstrated the greatest amount of kaempferol-3-glucoside (about 14% w/w; calculated per dry mass of residue A). The quantity of apigenin-7-glucoside and kaempferol was found equal to 8.5 and 5.3%. Quercetin was determined as 0.9%, caffeic and syringic acids as 0.25 and 0.2%, respectively. The amount of apigenin and *p*-hydroxybenzoic acid was 0.08 and 0.03%. Other compounds were measured below 0.003%. Residue B contains 13.5% apigenin-7-glucoside and 4.4% kaempferol-3-glucoside. The amount of apigenin and kaempferol was 2.1 and 1.3%, respectively. Caffeic, syringic, protocatechuic and *p*-hydroxybenzoic acid were found in the amounts of 1.3, 0.3, 0.1 and 0.2%, respectively. Other compounds occurred in trace amounts. A high amount (12%) of caffeic acid was observed in residue C. *p*-Coumaric and *p*-hydroxybenzoic acid were found in quantities of 1%. Apigenin, kaempferol and apigenin-7-glucoside were 3.2, 2.2 and 4.5%, respectively. The amount of syringic acid was 0.1%.

Table I. The quantity of some phenolic compounds determined in residues A, B and C by HPLC. The amount of each compound is demonstrated as percentage calculated per weight of dry residue. Phenolic compounds not shown in the table were present in trace amount (below 0.003% per weight of dry residue).

Compound	Residue A	Residue B	Residue C
Caffeic acid	0.25	1.3	12.0
Syringic acid	0.2	0.3	0.1
p-Hydroxybenzoic acid	0.025	0.2	1.0
Protocatechuic acid		0.1	
p-Coumaric acid			1.0
Apigenin	0.1	2.1	3.2
Kaempferol	5.3	1.3	2.2
Quercetin	0.9		
Apigenin-7-glucoside	8.5	13.5	4.4
Kaempferol-3-glucoside	14.0	4.4	
Sum	*29.2*	*23.2*	*23.9*

The antiradical activity of residues A, B and C is demonstrated in Fig. 1 and Table II. Residue C exhibited a much stronger antiradical activity than residues A and B. The weakest antiradical activity was observed in residue B.

Our experiments showed that the hydrolysis process in everlasting residue C led to a considerable increase in the concentration of caffeic acid which is strong antioxidant (Table I). The higher concentration of caffeic acid is closely related to a stronger antiradical activity of this residue (Fig. 1, Table II).

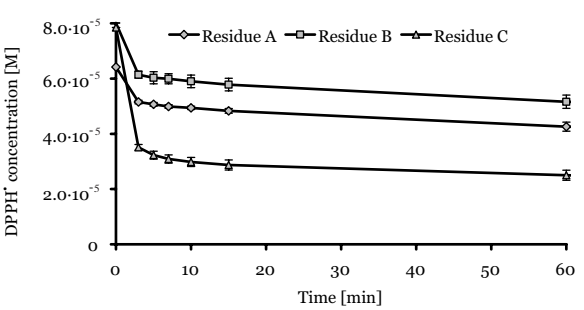

Fig. 1. Antiradical activity of residues A, B and C expressed as the decrease of DPPH· (2,2-diphenyl-1-picrylhydrazyl radical) concentration (see caption of Table II).

Table II. Antiradical activity of residues A, B and C expressed as the decrease of DPPH• (2,2-diphenyl-1-picrylhydrazyl radical) concentration calculated according to equation:
$c_{DPPH\cdot} = \frac{A_{515\,nm} + 2.58 \cdot 10^{-3}}{12509}$; $c_{DPPH\cdot}$ – concentration of DPPH• radical [M]; $A_{515\,nm}$ – absorbance of sample.
The exact DPPH• concentration in the reaction medium was calculated from a calibration curve with the equation: $A_{515\,nm} = 12509 \times c_{DPPH\cdot} - 2.58 \times 10^{-3}$, as determined by linear regression (Brand-Williams *et al.*, 1995).

Time of reaction [min]	Residue A	Standard deviation	Residue B	Standard deviation	Residue C	Standard deviation
0	$6.42 \cdot 10^{-5}$	0	$8.01 \cdot 10^{-5}$	0	$7.85 \cdot 10^{-5}$	0
3	$5.15 \cdot 10^{-5}$	$6.7 \cdot 10^{-7}$	$6.14 \cdot 10^{-5}$	$1.3 \cdot 10^{-6}$	$3.51 \cdot 10^{-5}$	$1.0 \cdot 10^{-6}$
5	$5.07 \cdot 10^{-5}$	$6.8 \cdot 10^{-7}$	$6.03 \cdot 10^{-5}$	$2.2 \cdot 10^{-6}$	$3.23 \cdot 10^{-5}$	$1.4 \cdot 10^{-6}$
7	$4.99 \cdot 10^{-5}$	$6.8 \cdot 10^{-7}$	$5.99 \cdot 10^{-5}$	$1.8 \cdot 10^{-6}$	$3.09 \cdot 10^{-5}$	$1.5 \cdot 10^{-6}$
10	$4.94 \cdot 10^{-5}$	$6.4 \cdot 10^{-7}$	$5.90 \cdot 10^{-5}$	$2.3 \cdot 10^{-6}$	$2.98 \cdot 10^{-5}$	$1.6 \cdot 10^{-6}$
15	$4.83 \cdot 10^{-5}$	$8.3 \cdot 10^{-7}$	$5.78 \cdot 10^{-5}$	$2.3 \cdot 10^{-6}$	$2.87 \cdot 10^{-5}$	$1.8 \cdot 10^{-6}$
60	$4.26 \cdot 10^{-5}$	$1.6 \cdot 10^{-6}$	$5.16 \cdot 10^{-5}$	$2.4 \cdot 10^{-6}$	$2.50 \cdot 10^{-5}$	$1.8 \cdot 10^{-6}$
180	$3.52 \cdot 10^{-5}$	$1.4 \cdot 10^{-6}$	$4.51 \cdot 10^{-5}$	$2.6 \cdot 10^{-6}$	$1.82 \cdot 10^{-5}$	$1.6 \cdot 10^{-6}$
1440	$2.11 \cdot 10^{-5}$	$2.5 \cdot 10^{-6}$	$2.61 \cdot 10^{-5}$	$2.5 \cdot 10^{-6}$	$0.33 \cdot 10^{-5}$	$4.4 \cdot 10^{-7}$

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