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Antioxidant potential of *Helichrysum plicatum* DC. (Asteraceae)

Tatjana Kadifkova Panovska[1] and Svetlana Kulevanova[2]

[1]*Institute of Applied Biochemistry, Faculty of Pharmacy, Skopje, Macedonia*
[2]*Institute of Pharmacognosy, Faculty of Pharmacy, Skopje, Macedonia*

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Abstract

The present study describes the ability of different extracts of *H. plicatum* obtained from flowers, stems and leaves, to act as natural antioxidants in different *in vitro* experimental models in which free radical reactions are involved: inhibition of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, inhibition of hydroxyl radicals and protection of β-carotene-linoleic acid model system.

Investigate extracts showed radical scavenging activity with IC_{50} from 6 to 11 mg/ml. The extracts are capable to reacting with $OH^{\bullet}$ radical with inhibition of its production ranged between 33-58%. The high preventive activity against the bleaching of beta-carotene (15-49% of initial value after 120 minutes) was also observed. The antioxidative activity of the extracts in the experimental systems was compared with that of reference substances: luteolin, quercetin, BHA, BHT and sylimarin (the main agent of the well-known milk thistle – *Silybum marianum* L.).

Results of this study suggest that *Helichrysum plicatum* represent a natural source with antioxidant potential.

Key words: *Helichrysum plicatum*, flavonoids, DPPH, hydroxyl radicals, lipid peroxidation.

Introduction

Helichrysum species (Asteraceae) have been well known plants in folk medicine for hundreds of years. One of them, *Helichrysum plicatum* DC. is widely distributed throughout the whole territory of Balkan Peninsula. Water extract (infuse) of this plant have been used for treatment of gastric and hepatic disorders, usually in combination with other plants with similar effects (1). Phytochemical screening (HPLC) of the *Helichrysum plicatum* from Macedonia proved the presence of apigenin and naringenin as free aglycones and glycosides of apigenin, naringenin, quercetin and kaempferol in the flowers as well as quercetin and luteolin glycosides and free luteolin in stems and leaves (2). Its antioxidant and scavenging properties have not been studied previously. Among phytochemicals, flavonoids deserve a special mention due to their free radical scavenging activities and *in vivo* biological activities that are being investigated by many researchers (3,4,5,6,7). The goal of research on antioxidative characteristics of plant extracts is to discover a potential replacement for synthetic antioxidants (BHT, BHA), which cause unwanted processes after prolonged used.

The evaluation of plant extracts antioxidant capacity is not easy task, as many methods can be used to determine this activity, and substrates, conditions, analytical methods, and concentrations can affect the estimated activity (8). This paper reports a study in which antioxidative activity of methanolic, ethyl acetate, and after hydrolysis extracts are tested. This effect was followed by three complementary *in vitro* methods: evaluation the free radical scavenging capacity (DPPH method) (9), inhibition of hydroxyl radicals production (10) and protection of β-carotene-linoleic acid system (11).

Materials and Methods

Plant material

The aerial parts of *Helichrysum plicatum* DC. were collected on the Golac Mountain, Eastern Macedonia, in the flowering period of the plant, during the summer of

*Prof. Dr. Tatjana Kadifkova Panovska, Institute of Applied Biochemistry, Department of Toxicology, Faculty of Pharmacy, Vodnjanska 17, 1000 Skopje, Macedonia.
E-mail: taka@ff.ukim.edu.mk
Phone: ++389 02 3126 032 Fax: ++389 02 3123 054

2001. Voucher specimens were deposited at the Herbarium of the Institute of Pharmacognosy, Faculty of Pharmacy, Skopje, Macedonia.

Reagents

The reagents used were of highest purity (>99.95 purity) and were purchased from Sigma Chemical Co (Germany). Authentic samples of luteolin, quercetin, sylimarin, BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) were the products of Extrasynthese (France).

Preparation of plant extracts

Dried powdered plant material (flowers, stems and leaves separately) was cut into small pieces and extracted in the ratio 1:10 (w/v) by three procedures: a. with methanol (M-extract); b. with ethanol-water (7:3), then evaporated until water remains and extracted with ethyl acetate (EA-extract); c. hydrolysis with HCl, aglycones extracted with ethyl acetate (H-extract). All extracts were dried over anhydrous sodium sulfate, filtered and concentrated under vacuum up to concentration of 1 g per 1 mL of extract. M-extract was further diluted with methanol; EA and H extracts with ethyl acetate, in order to obtained 0.01 g mL^{-1} solutions used in the experiments.

Free radical scavenging activity in 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) assay

The antioxidant activity using the DPPH assay was assessed by the method of Brand-Williams et al. (9). A test sample solution (200 μL) was added to 4 mL of 100 mmol L^{-1} ethanolic DPPH. After vortexing, the mixture was incubated for 10 minutes at room temperature and the absorbance at 517 nm was measured. The difference in absorbance between a test sample and a control (ethanol) was considered as activity. The activity was shown as IC_{50} value (50% of inhibitory concentration in mg mL^{-1}). Luteolin, quercetin, BHA, BHT and sylimarin (100 μg mL^{-1} in ethanol) were used as reference substances. All values are shown as the mean of three measurements.

Evaluation of the hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was carried out by measuring the competition between deoxyribose and the extracts for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. Attack of the hydroxyl radical on deoxyribose led to TBARS (thiobarbituric acid-reactive substances) formation (10). The extracts were added to the reaction mixture containing 2.8 mmol L^{-1} deoxyribose, 100 μmol L^{-1} $FeCl_3$, 104 μmol L^{-1} EDTA, 100 μmol L^{-1} ascorbic acid, 1 mmol L^{-1} H_2O_2 and 230 mmol L^{-1} phosphate buffer (pH 7.4), making a final volume of 1.0 mL. One milliliter of thiobarbituric acid TBA (1%) and 1.0 mL trichloroacetic acid (TCA 2.8%) were added to test the tube and incubated at 100ºC for 20 min. After cooling, absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Reactions were carried out in triplicate. In the series of control experiments, reference substances: luteolin, quercetin, BHA, BHT and sylimarin (100 μg mL^{-1} in phosphate buffer-pH 7.4) were used instead of the extract solution. The reaction mixture was incubated at 37ºC for 1 h.

Evaluation of antioxidant activity

The antioxidant activity of the extracts was evaluated using a β-carotene/linoleate model system (11). A solution of β-carotene was prepared by dissolving 2.0 mg of β-carotene in 10 mL of chloroform. One milliliter of this solution was then pipette into a round-bottom flask. After chloroform was rotary evaporated at 40 ºC under vacuum, 20 mg of purified linoleic acid, 200 mg of Tween 40 emulsifier and 50 mL of distilled water were added to the flask with vigorous shaking. Aliquots (5 mL) of this emulsion were transferred into a series of tubes containing 2 mg of each extract or 2 mg of BHA (butylated hydroxyanisole) for comparison. An aliquot (5 mL) of emulsion without any further additions was used as control. As soon as the emulsion was added to each tube, the zero time absorbance was read at 470 nm. Subsequent absorbance readings were recorded at 10-min intervals by keeping the sample in a water bath at 50 ºC until the visual color of β-carotene in the control sample had disappeared (about 120 min).

Results and discussion

Interest in the search for new natural antioxidants has grown dramatically over the past years because reactive oxygen species production and oxidative stress have been shown to be linked to ageing related illnesses (12). Also the restrictions to use of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), due to their toxicity (13) has been an important incentive for such research. Numerous plants have been examined for antioxidant activity. According to this, antioxidant properties of *Origanum* spp. (aerial parts) have been studied relatively well over the past years (14,15,16). Also, *Urtica* sp., has been shown to have antioxidant activity (17). On the other hand, *Helichrysum* species have not been investigated systematically for their potential health-benefiting properties.

Free radical scavenging activity

This method is based on the reduction of DPPH, a stable free radical. Because of the odd electron of DPPH, it gives a strong absorption maximum at 517 nm by visible

spectroscopy (purple color). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, that is, a free radical scavenging antioxidant, the absorption strength is decreased, and the resulting discoloration is stehiometric with respect to the number of electrons captures (18). This reaction has been widely used to test the ability of compounds to act as free-radical scavengers of hydrogen donors and to evaluate the antioxidant activity of food and plant extracts (19-21).

Free radical scavenging activity (FRSA) of *Helichrysum* extracts was determined by comparing with activities of substances such as luteolin, quercetin, BHA, BHT and sylimarin (reference substances), which possess some antioxidant potential. In Table 1. FRSA values obtained for plant extracts together with reference substances in DPPH assay are shown. Various extracts showed different activity in this assay. Methanol extract derived from stems and leaves showed the highest inhibitory activity with IC_{50} of 6 mg mL^{-1}. The lowest DPPH scavenging activity was shown by ethyl acetate extract derived from flowers (IC_{50} 11 mg mL^{-1}). The other *Helichrysum* extracts demonstrated similar DPPH scavenging activity (8-9 mg mL^{-1}). When compared to the reference substances, the *Helichrysum* extracts were found to be less efficient in radical scavenging. Luteolin, quercetin, BHA, and sylimarin interacted intensively with DPPH (IC_{50}: 0.09, 0.06, 0.152 and 1.96 mg mL^{-1}, respectively) while BHT possessed moderate scavenging properties (3.52 mg mL^{-1}). The scavenging effects can probably be attributed to the flavonoids present in the examined extracts, but could also be the result of the activity of other secondary metabolites, such as volatile oils, carotenoids, and vitamins, that in the case may contributed to the antioxidant capacity (16,17).

Table 1. DPPH radical scavenging activity of the *Helichrysum plicatum* extracts against luteolin, quercetin, BHA, BHT and sylimarin as standards.

	IC_{50}[a] (mg ml^{-1})
Flower extracts	
Methanol	9 ± 0.7[b]
Etanol:water	11 ± 2.2
After hydrolysis	8 ± 1.9
Stem and leaf extracts	
Methanol	6 ± 3.0
Etanol:water	8 ± 0.5
After hydrolysis	8 ± 2.4
Reference substances	
Luteolin	0.09 ± 0.01
Quercetin	0.06 ± 0.02
Sylimarin	1.96 ± 0.03
BHA	0.152 ± 0.002
BHT	3.52 ± 0.02

[a] IC_{50} denotes the extract concentration at which the absorbance shows 50 % of control
[b] All values are shown as the mean ± SD of more than 3 measurements

Hydroxyl radical scavenging activity

The Fenton reaction describes the oxidation of H_2O_2 by Fe^{2+} to $OH^{\bullet}$ and Fe^{3+}. In the model employed in this experiment, the production of $OH^{\bullet}$ induced oxidation of the deoxyribose, which in turn reacted with TBA to produce a TBA reactive chromofore that was detectable at 535 nm, thus enabling assessment of antioxidant activity of plant extracts (10).

Fig. 1 presents the results of the effects of examined *Helichrysum* extracts, reference substances (luteolin, quercetin, BHA, BHT and sylimarin) as well as control solution on $OH^{\bullet}$ radical production. The results show that all extracts of *Helichrysum plicatum* inhibited the production of $OH^{\bullet}$ radicals. The strongest inhibitory activity was exhibited by the H-extract derived from stems and leaves (58%). When compared to the reference substances, luteolin, quercetin, BHA, BHT and sylimarin (48%, 42%, 52%, 47% and 43%, respectively), the extracts had slightly lower activity, except of H-extract derived from stems and leaves with greater activity than BHT.

The findings demonstrate hydroxyl radical scavenging potential of *Helichrysum* extracts against Fenton reaction induced $OH^{\bullet}$ generation, showing similar performance with luteolin, quercetin, BHA, BHT and sylimarin. This activity is mainly due to the redox properties of flavonoids, which

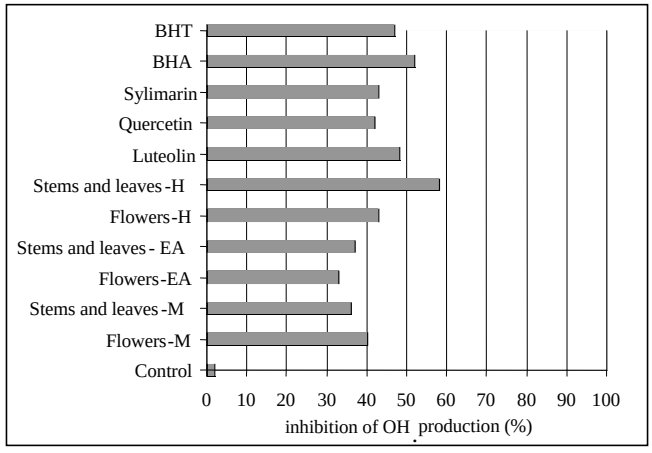

Fig. 1. Effects of *Helichrysum plicatum* extracts against luteolin, quercetin, BHA, BHT, sylimarin as standards and control on the *in vitro* $OH^{\bullet}$ production.

allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may also have a metal chelating potential (22). The slight quantitative differences in the amounts of flavonoids (2) might also explain the minor differences between the activities of the extracts.

Antioxidant activity

For the screening of antioxidant potential of *Helichrysum* extracts, β-carotene/linoleate model system has been applied (11). This test involves a reaction between a potential antioxidant, β-carotene and linoleic acid. β-Carotene undergoes rapid discoloration in the absence of an antioxidant. The presence of antioxidant compounds can hinter the extent of β-carotene destruction by neutralizing the linoleate free radical and any other free radicals formed within the system.

The antioxidant activity of each extract of *Helichrysum plicatum*, BHA and control is presented in Fig. 2. According to the preventive activity against bleaching of β-carotene, M-extract (46%) derived from flowers and H-extract (44%) from stems and leaves are the most promising

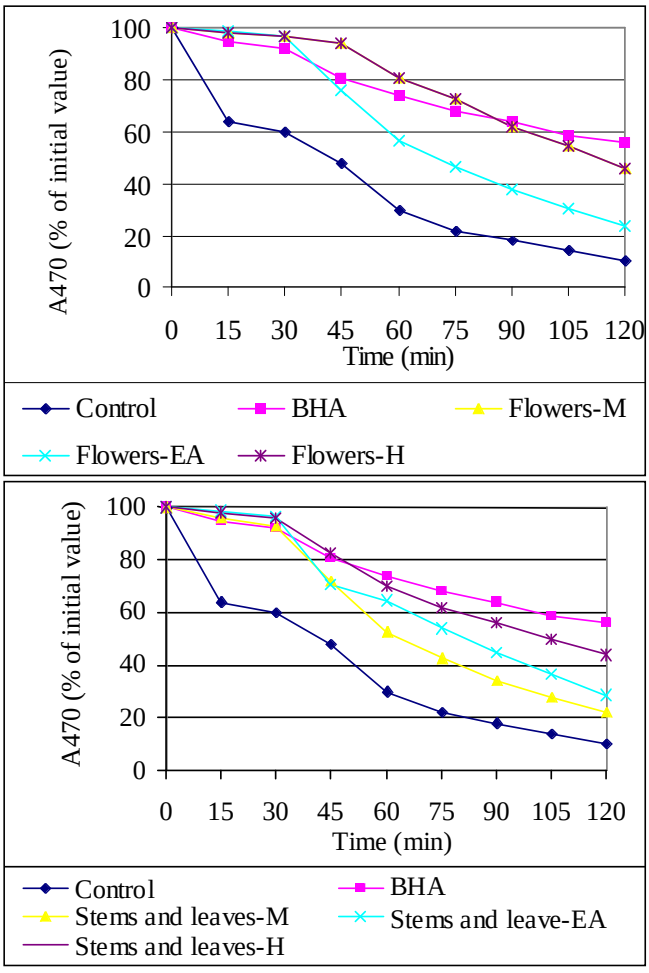

Fig. 2. Effect of *Helichrysum plicatum* extracts and control on oxidation of the β-carotene-linoleic acid model system.

Helichrysum extracts. M-extract (22%) and EA-extract (29%) derived from stems and leaves showed short and weak inhibition of bleaching, close to that of flowers EA-extract (24%). H-extract (15%) from flowers was found to be less efficient in this model system. The inhibitory effect on β-carotene bleaching of the all *Helichrysum* extracts was lower than that of BHA.

The antioxidant activity of *Helichrysum plicatum* extracts has been attributed to various mechanisms, among which is neutralizing β-carotene destruction. Also, we can suggest that there is always no linear correlation between antioxidant activity and the content of flavonoids (2). This indicates that the concentration of flavonoids is not the only factor related to the antioxidant activity. Possible synergism of flavonoids with other components present in the extracts may be responsible for this observation.

Conclusion

Results of this study suggested that *Helichrysum plicatum* DC. could be regarded as a good source for natural antioxidant. Its extracts exhibit potent free radical scavenging, hydroxyl radical scavenging and antioxidant activity *in vitro*. The information from this study can explain the traditional use and the further development of these extracts into new pharmaceuticals.

The data reported here can be consider as the first information on the antioxidant properties of Macedonian *Helichrysum plicatum*. Further studies are needed to evaluate the *in vivo* potential of these extracts in animal models.

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Резиме

Антиоксидативен потенцијал на *Helichrysum plicatum* DC. (Asteraceae)

Татјана Кадифкова Пановска[1] и Светлана Кулеванова[2]

[1]*Институт за применета биохемија, Фармацевтски факултет, Скопје Македонија*
[2]*Институт за фармакогнозија, Фрамацевтски факултет, Скопје, Македонија*

Клучни зборови: *Helichrysum plicatum*, флавоноиди, DPPH, хидроксил радикал, липидна пероксидација.

Опишана е способноста на различни екстракти од цветови, стебла и листови на *H. plicatum* да делуваат како природни антиоксиданси во различни *in vitro* експериментални модели во кои се вклучени реакции со слободни радикали: инхибиција на DPPH (1,1-diphenyl-2-picrylhydrazyl) радикалот, инхибиција на хидроксил радикали и заштита на β-каротен-линоленска киселина модел системот. Испитуваните екстракти покажуваат активност за фаќање на слободни радикали со IC_{50} од 6 до 11 mg/ml. Екстрактите се способни да реагираат со OH• радикалот со инхибиција на продукција меѓу 33-58%. Изразена превентивна активост покажуваат и кон промените на β-каротенот (15-40% од почетната вредност, после 120 минути). Антиоксидативната активност на екстрактите во експерименталните системи е споредувана со референтните супстанции: лутеолин, кверцетин, BHA, BHT и силимарин (главниот активен принцип на растението млечен трн - *Silybum marianum* L.*)*. Резултатите од испитувањето укажуваат дека *Helichrysum plicatum* претставува природен извор со антиоксидативен потенцијал.