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Antioxidant Properties of Methanolic Extract and Fatty Acid Composition of *Centaurea urvillei* DC. subsp. *hayekiana* Wagenitz

Gokhan Zengin[1], Abdurrahman Aktumsek[1*], Gokalp Ozmen Guler[2], Yavuz Selim Cakmak[1] and Evren Yildiztugay[1]

[1]*Department of Biology, Science Faculty, Selcuk University, Konya, Türkiye*

[2]*Department of Biological Education, Ahmet Kelesoglu Education Faculty, Selcuk University, Konya, Türkiye*

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Abstract: *Centaurea* is one of most important genera of Turkey flora. Some members of the genus have been used in Anatolian folk medicine. This study was undertaken in order to examine the in vitro antioxidant activities and fatty acid composition of *Centaurea urvillei* subsp. *hayekiana.* Antioxidative properties of the methanolic extract was assessed by different in vitro experiments including total phenolic and flavonoid content, phosphomolybdenum assay (total antioxidant capacity), free radical scavenging assay, β-caroten/linoleic acid bleaching test system, ferric and cupric ion reducing power. According to the results of antioxidant capacity assays, *C. urvillei* subsp. *hayekiana* showed moderately antioxidant activity in test methods. Fatty acid analysis was performed by GC and thirty-two fatty acids were identified. The oil of *C. urvillei* subsp. *hayekiana* was characterized by higher amounts of C 18:2 ω6 (linoleic acid) and C 18:1 ω9 (oleic acid). The results suggested that *C. urvillei* subsp. *hayekiana* may be utilized as a source of both natural antioxidant and linoleic acid in healthy medicine and food industry.

Keywords: Antioxidant activity; fatty acid; *Centaurea urvillei* subsp. *hayekiana.*

1. Introduction

Free radicals play a vital role in various pathological conditions such as tissue injury, inflammation process and neurodegenerative diseases. Antioxidants have an important role to protect the human body against damage by the free radicals [1]. Research on relationship between antioxidants and prevention of some diseases, such as cardiovascular disease and cancer has been

[*]Corresponding author: E-mail address: aktumsek@selcuk.edu.tr , Tel.: +90 332 223 18 66

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increasing sharply in recent years [2]. However, synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been restricted due to their carcinogenic effect [3,4]. Therefore, investigations of antioxidants are focused on naturally occurring substances, especially plant phytochemicals. Fruits, vegetables and many medicinal plants have antioxidant components, especially phenolic compounds and their consumption has contributed to prevention of destructive processes caused by oxidative stress [5,6].

Turkey flora contained almost 11000 species of which 34.5 % are endemic. The flora is estimated to contain over 3000 aromatic plants [7]. Nevertheless, there is limited knowledge about pharmalogical and biological activities of the aromatic plants. *Centaurea* is one of most important genera of the family Asteraceae. The genus *Centaurea* consist of 400 and 700 species [8-10] and many of them growing in Turkey. [11,12]. Many *Centaurea* species such as *C. depressa*, *C. solsititialis* [13], *C. pulchella* [14] and *C. drabifolia* [15] have significant applications in Anatolian folk medicine. Some *Centaurea* species such as *C. patula, C. pulchella* [16], *C. huber-morathi* [17] and *C. mucronifera* [18] were examined in terms of biological properties. However, there are only a few literatures related to fatty acid profile of *Centaurea* species [16, 19, 20]. *C. urvillei* subsp. *hayekiana* grow naturally in Turkey. However, the antioxidant properties and fatty acid composition of this species previously has been not reported.

The main objectives of this study were to determine total phenolic, flavonoid content, antioxidant properties and fatty acid composition of *Centaurea urvillei* subsp. *hayekiana* which is endemic to Turkey flora.

2. Materials and Methods

2.1. *Plant material*

Centaurea urvillei DC subsp. *hayekiana* Wagenitz was collected in June 2009 from Konya, Turkey. The plant was authenticated by Evren YILDIZTUGAY from Section of Botany, Department of Biology of Selcuk University. The voucher specimens have been deposited in KNYA herbarium at department of Biology, Selcuk University.

2.2. Preparation of methanolic extract

The aerial plant materials were dried at the room temperature and powdered to a fine grain using a laboratory mill. For antioxidant capacity methods, 15 g powdered aerial plant was extracted with methanol at the room temperature with stirring until extraction solvent become colorless. Extract was filtered with Whatman filter paper and methanol was evaporated at 40 °C in rotary evaporator.

2.3. Chemicals

Potassium ferricyanide, ferric chloride, Folin-Ciocalteu's reagent, trichloroacetic acid, methanol, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and methanol were purchased from Merck (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, linoleic acid and Tween 40 were purchased from Sigma Chemical Co. (Sigma–Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents were analytical grade.

2.4. Determination of antioxidant capacity

2.4.1. Assay for total phenolics

The amount of total phenolics content in extract was determined according to Folin-Ciocalteu method [21]. 0.2 µL of sample solution (1mg/mL) were introduced into test tube containing 1 mL of Folin-Ciocalteu's reagent and 2 mL of Na_2CO_3 (7.5%). The final volume was brought up to 7 mL with deionized water. After 2 h incubation at room temperature, the absorbance was measured at 765 nm with spectrophotometer (Shimadzu, UV-1800). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligram per gram of extract (mg GAE/g extract).

2.4.2. Total flavonoid analysis

Total flavonoid content of the extract was determined according to repored method in literature [22]. 0.5 mL of sample solutions (1 mg/mL) was mixed with 2 mL of distilled water and subsequently with 0.15 mL 5% of $NaNO_2$ solution. After 6 min incubation, 0.15 mL of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, followed by adding 2 mL of 4% NaOH solution to the mixture. The mixture was made up to 5 mL with methanol and mixed well. The absorbance was measured at 510 nm after incubation for 15 min. The total flavonoid content was expressed in milligrams of rutin equivalents (RE) per gram of extract.

2.4.3. Determination of total antioxidant capacity by phosphomolybdenum assay

The total antioxidant capacity of extract was evaluated by phosphomolybdenum method according to Prieto et al. (1999) [23]. 0.3 mL of extract solution (1mg/mL) was mixed with 3 mL reagent solution (6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95 °C for 90 min. Then the absorbance of the solution was measured at 695 nm against blank. The antioxidant capacity of extract was evaluated as equivalents ascorbic acid (mg AE/g extract) and trolox (mg TE/g extract).

2.4.4. Scavenging Activity on DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical

The free radical scavenging activity was evaluated according to the method described by the literature [24] with some modifications. 0.5 mL of test samples was mixed with 3 mL 6.10^{-5} M of a methanol solution of DPPH. The reaction mixture was incubated in the dark at room temperature. The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm after 30 min. The inhibition activity was calculated in following way:

$$I(\%)= 100 \times (A_0-A_1)/A_0$$

Where A_0 is the absorbance of the control, A_1 is the absorbance of the extract/standard. %50 of free radical inhibition (IC_{50}) of extract was calculated. The lower the IC_{50} value indicates high antioxidant capacity. BHA and BHT were used as positive controls.

2.4.5. β-carotene-Linoleic Acid Bleaching Assay

The antioxidant activity was performed by slight modifications of the procedure described by Sokmen et al. (2004) [25]. Briefly, 0.5 mg β-carotene was dissolved in 1 mL of chloroform, 25 µL linoleic acid and 200 mg Tween 40 was added. Chloroform was removed by rotary vacuum evaporator and 100 mL distilled water saturated with oxygen were added with vigorous shaking. 2.5 mL of this reaction mixture dispensed into test tubes and 350 µL portion (1 mg/mL) of the extracts were added.

The absorbance was measured at 490 nm immediately. The reaction mixture was incubated at 50 ºC for 2 h and the absorbance was measured again. The same procedure was repeated with synthetic antioxidant (BHT and BHA) and a blank. Inhibition ratio of linoleic acid oxidation was calculated for test sample and synthetic antioxidants.

2.4.6. Reducing Power Activity (Iron (III) to iron (II) reduction)

The ferric reducing power method was applied with slight modifications of the method in the literature [26]. 22.5 mL of extract solution was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%)". This was incubated at 50 C° for 20 min. After the incubation, 2.5 mL of 10% trichloroacetic acid was added. 2.5 mL of the reaction mixture was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1%). The solution absorbance was measured at 700 nm. Increasing absorbance of the reaction mixture indicates increasing reducing power. The same procedure was applied with BHA and BHT. The EC_{50} value (the effective concentration at which the absorbance was 0.5) was calculated for extract, BHA and BHT.

2.4.7. Cupric Ion Reducing Antioxidant Capacity (CUPRAC assay)

The cupric ion reducing capacity was measured according to the method of Apak et al. (2006) [27]. 1 mL $CuCl_2$ (10 mM), 1 mL neocuproine (7.5 mM), and 1mL NH_4Ac buffer (1M, pH 7.0) solutions were added into a test tube. Then, 0.5 mL different concentrations of extract were mixed and total volume was brought up to 4.1 mL with deionized water. The mixture absorbance was recorded against a blank at 450 nm after 30 min incubation at room temperature. The results of the assay were evaluated by using EC_{50} values.

2.5 Oil Extraction

Ten grams of ground sample were extracted for oil, using petroleum ether for 6 h in a Soxhlet system. The solvent was evaporated by rotary evaporator. The obtained oil was esterified to determine fatty acid composition.

2.6. Fatty acid methyl esters (FAMEs) preparation

The fatty acids in the total lipid were esterified into methyl esters by saponification with 0.5 N methanolic NaOH and transesterified with 14% BF_3 (v/v) in methanol [28].

2.7. Gas chromatographic analysis

FAMEs were analyzed on a HP (Hewlett Packard) Agilent 6890N model gas chromatograph (GC), equipped with a flame ionization detector (FID) and fitted to a HP-88 capillary column (100 m length, 0.25 mm i.d. and 0.2 µm thickness). Injector and detector temperatures were set at 240 and 250 ºC, respectively. The oven was held at 160 ºC for 2 min. Thereafter the temperature was increased up to 185 ºC at rate of 4 ºC/min then increased at up to 200 ºC at rate of 1 ºC/min and held at 200 ºC for 46.75 min. Total run time was 70 min. Helium was used as carrier gas (1 mL/min).

Identification of fatty acids was carried out by comparing sample FAME peak relative retention times with those obtained for Alltech and Accu standards. Results were expressed as FID response area in relative percentages. Each reported result is given in the average value of three GC analyses. The results are offered as means±S.D.

3. Results and Discussion

3.1. Antioxidant capacity

Antioxidant capacity of the methanolic extract was examined using seven different assays because evaluation of antioxidant properties of plants cannot be carried out accurately by single universal method.

Total phenolic and flavonoid content were determined as 17.12 mgGAE/g and 36.67 mgRE/g, respectively (Table 1).The total phenolic content of the extract was evaluated by Folin-Ciocalteu assay. The assay is a fast and simple method to rapidly determine the content of phenolics in samples. Plant phenolics present in plants parts have received considerable attention because of their potential antioxidant activity [29,30]. Zengin et al (2010) [16] reported the total phenolic content of *Centaurea patula*, *C. pulchella* and *C. tchihatcheffii* were 25.61, 55.00 and 22.27 mgGAE/g extract in the assay. Total phenolic content of *C. urvillei* subsp. *hayekiana* was observed to be lower than that of the *Centaurea* species. Polyphenol concentration of eight *Centaurea* species was found as ranging from 43.44 to 120.90 mgGAE/L [31].

Total antioxidant capacity was reported as ascorbic acid and trolox equivalents. The total antioxidant capacity of the extract was 39.70 mg AE/g and 143.53 mgTE/g. There is a little information about total antioxidant activity of *Centaurea* species by phosphomolybdate method [16]. The method is utilized for the spectrofotometric quantitation of total antioxidant capacity and employs cost-effective reagents [23]. It based on the reduction of Mo(VI) to Mo(V) in presence of antioxidant compound and subsequent formation of a green phosphate/Mo(V) complex at acidic pH and at higher temperature.

Free radical scavenging activity and inhibition effect on the linoleic acid oxidation of methanol extract are given in Table 2. Free radical scavenging properties of methanolic extract was measured DPPH assay. IC_{50} value was calculated from a calibration curve for extract. The lower IC_{50} value reflects to higher antioxidant activity of plant extracts. IC_{50} value of the extract was determined as 137.06 µg/mL. It seems that the scavenging ability of the sample was less effective than that of BHA (1.57 µg/mL) and BHT (3.56 µg/mL). The values of *Centaurea* species which plants growing in Scotland was found as ranging from 0.018 mg/mL and 0.095 mg/mL, respectively [32]. From the results it can be concluded that *C. urvillei* subsp. *hayekiana* has lower free radical scavenging activity than growing Scotland. As can be seen from the table 2, the inhibition capacity of linoleic acid oxidation of methanolic extract was found as 44.00% which is lower than the inhibition capacity of the positive control BHA and BHT. Some literatures reported that the inhibition capacity of *Centaurea* species such as *C. mucronifera* (35.2%) [18] and *C. ensiformis* (85.15% of ethyl acetate, 72.51% of chloroform extract) [33]

The antioxidant activity of certain plant extracts have been correlated with their reducing powers (Duh 1998) (34). The reducing power of the sample and synthetic antioxidants were assayed and the results are shown in Table 2. In this assay, we investigated the Fe^{3+}–Fe^{2+} transformation in the presence of the extract and EC_{50} (the concentration at which the absorbance is 0.50) was calculated. BHA has the lowest EC_{50} value, that is, the highest reducing power. The extract has lower reducing power than BHT and BHA. Moreover, the CUPRAC assay was performed to determine cupric ion reducing power of the extract. Ascorbic acid was used as a control in the assay and ascorbic acid (EC_{50}: 0.01 mg/mL) has stronger activity than the methanolic extract (EC_{50}: 0.64 mg/mL) (Table 2). CUPRAC method was reported by Apak et al. (2006) [27] as a simple and inexpensive antioxidant capacity assay for plant material. Moreover, result of the assay is correlated with total phenolic content and ferric ion reducing power [35,36] Literature is scarce about reducing power in *Centaura* species. However, some plant species such as *Smilax excelsa* [37], *Chionanthus virginicus* [38] and *Rheum ribes* [35] were examined in these assays.

Table 1. Total antioxidant capacity, total phenolic and flavonoid content of *C.urvillei* subsp.*hayekiana*

Sample	(mg AE /g)[a]	(mg TE/g)[b]	(mg GAE/g) [c]	(mg RE/g)[d]
C. urvillei subsp.*hayekiana*	39.70	143.53	17.12	36.67
Gallic acid	147.20	458.87	-	-

[a] Total antioxidant capacity expressed as ascorbic acid equivalent (mg AE/g extract).
[b] Total antioxidant capacity expressed as trolox equivalent (mg TE/g extract).
[c] Total phenolic content expressed as gallic acid equivalent (mg GAE/g extract).
[d] Total flavonoid content expressed as rutin equivalent (mg RE/g extract).

Table 2. Free radical scavenging capacity, inhibition of the linoleic acid, ferric ion and cupric ion reducing power of *C.urvillei* subsp.*hayekiana* and standard antioxidants

Sample	IC_{50} (µg/mL)[a]	EC_{50} (mg/mL)[b]	Inhibition (%)[c]	EC_{50} (mg/mL)[d]
C.urvillei subsp.*hayekiana*	137.06	1.39	44.00	0.64
BHA	1.57	0.01	94.04	-
BHT	3.56	0.02	96.39	-
Ascorbic acid	-	-	-	0.01

[a] Results of DPPH assay.
[b] Results of ferric ion reducing power.
[c] Results of *β*-caroten/linoleic acid bleaching assay.
[d] Results of CUPRAC assay.

3.2. Fatty acid composition

Thirty-two fatty acids were identified in oil of *C. urvillei* subsp. *hayekiana*. Individual percentages of each fatty acid are given in Table 3. Linoleic acid (C 18:2 ω6) was determined to be major fatty acid (35.92 %) in the oil. Oleic acid (C 18:1 ω9) was detected as the second most abundant fatty acid and percent of oleic acid was 24.10 % in this sample. Oleic and linoleic acid intake have been encouraged by nutritionist and the medical profession because of their ability to lower blood cholesterol levels [39]. Therefore, the oil is expected to be a good source of oleic and linoleic acid. In addition, the content of palmitic acid was found to be 16.24%. Saturated fatty acids (SFAs) represented 26.39% of total fatty acids, with the palmitic acid and stearic acid (3.93%) as the main acids. Total monounsaturated (MUFAs) (25.40 %) and polyunsaturated fatty acids (PUFAs) (48.22%) were higher than saturated fatty acids in the oil. In accordance with our results, Tekeli et al. (2010) [19] reported that linoleic acid was the most abundant fatty acid in the fatty acid composition of six *Centaurea* species. The authors also found palmitic acid content varied from 17.83 % to 25.31 % in six *Centaurea* species. Yildirim *et al.* (2009) [20] were detected fourteen fatty acids and palmitic and oleic acid were found to be the predominant fatty acids in the lipids from 16 *Centaurea* taxa. Palmitic and oleic acid content in the fatty acid composition of *C. urvillei* subsp. *hayekiana* were lower than 16 *Centaurea* taxa. Level of linoleic acid was found in the composition of fatty acid in *C. urvillei* subsp.

hayekiana was similar to the composition reported for some Asteraceae species such as *Telekia speciosa* [40], *Arctium minus* [41] , six *Limonium* species [42] and *Carthamus tinctorius* [43].

Table 3. Fatty acid composition of *C. urvillei* subsp. *hayekiana* (%)

Fatty acids	*C. urvillei* subsp. *hayekiana*
C 8:0	0.13±0.01**
C 10:0	0.04±0.01
C 11:0	0.25±0.01
C 12:0	0.92±0.01
C 13:0	0.05±0.01
C 14:0	1.11±0.01
C 15:0	1.72±0.02
C 16:0	16.24±0.05
C 17:0	1.26±0.04
C 18:0	3.93±0.03
C 19:0	0.09±0.01
C 20:0	0.41±0.04
C 21:0	0.23±0.03
C 22:0	0.04±0.01
ΣSFA*	**26.39±0.13**
C 14:1 ω5	0.05±0.01
C 15:1 ω5	0.06±0.01
C 16:1 ω7	0.11±0.01
C 17:1 ω8	0.12±0.01
C 18:1 ω9	24.10±0.10
C 18:1 ω7	0.78±0.02
C 20:1 ω9	0.18±0.01
C 22:1 ω9	0.01±0.01
ΣMUFA*	**25.40±0.12**
C 18:2 ω6	35.92±0.12
C 18:3 ω6	1.50±0.01
C 18:3 ω3	8.73±0.57
C 20:2 ω6	0.02±0.01
C 20:4 ω6	1.25±0.14
C 20:5 ω3	0.23±0.01
C 22:2 ω6	0.01±0.01
C 22:6 ω3	0.56±0.04
ΣPUFA*	**48.22±0.25**

* SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids
** Values reported are means ±S.D.

4. Conclusion

The present study has demonstrated that extract of *C. urvillei* subsp. *hayekiana* is rich total phenolic, flavonoid content, and the species have moderately antioxidant activity in all of the tested methods. The extract could possess therapeutic effects such as prevent radicals attacks and inhibition lipid oxidation in different areas. Thus, *C. urvillei* subsp. *hayekiana* may be suggested as a new potential source of natural antioxidants and linoleic acid in food and pharmalogical fields. The findings support the view that plants are promising sources of potential antioxidants.

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