Research Article Open Access

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The biological activities of *Arabis alpina* L. subsp. *brevifolia* (DC.) Cullen against food pathogens

https://doi.org/10.1515/chem-2018-0104
received February 15, 2018; accepted June 23, 2018.

Abstract: In this study, *in vitro* biological activities of both methanol and ethanol extracts of *Arabis alpina* subsp. *brevifolia* were investigated. Also, the phenolic components of this plant was examined in this study. The extracts were tested against the eight strains of food pathogens for their antimicrobial activities by utilizing minimum inhibitory concentration (MIC) and disc diffusion assay. The non-enzymatic antioxidant activities were determined according to scavenging of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The phenolic compounds were analyzed by high performance liquid chromatography (HPLC). The main component was ellagic acid for the methanol extract of stem-leaf, rutin for the ethanol extract of stem-leaf, and 2,5-dihydroxybenzoic acid for the methanol and ethanol extracts of fruit-flower. The ethanolic extracts of leaves revealed antibacterial activities against *Salmonella* Typhimurium (7 mm) while the ethanolic extracts of flowers demonstrated no activity against the test pathogens. The methanolic extracts of leaf-flower showed antibacterial activities against *S.* Typhimurium (7 mm). No activity was observed against *C. albicans*. The MIC value for four test bacteria was 13000 µg/mL. The ethanol extracts of *A. alpina* subsp. *brevifolia* exhibited the highest DPPH inhibition (76%). This study showed that *A. alpina* subsp. *brevifolia* possesses antioxidant and antimicrobial activities.

Keywords: *Arabis*; Antimicrobial activity; Antioxidant activity; Food-borne pathogens; HPLC.

1 Introduction

The *Brassicaceae* family is one of the broad-spreaded and richly diverse families within the plant kingdom and contains many species (*Arabidopsis*, *Brassica*, *Boechera*, *Thellungiella*, *Camelina*, *Raphanus* and *Arabis*) that have economic, scientific and agricultural importance [1-3]. This family comprises 49 tribes, 325 genera and 3740 species which mainly spread in the temperate region of the world, and Turkey is extremely rich in terms of the diversity of this family, as well [4-6]. In the family, the genus of *Arabis* L. containing about 60 species is represented by 17 species in the *Flora of Turkey and the East Aegean Islands* and by 22 species (24 taxa) in Turkey [7-9]. *Arabis alpina* L. in the mountain habitats of Anatolia, which is distributed on the alpine habitats, so named alpine rock-cress, emerges as model species in the ecological and evolutionary researches which have been conducted in recent years [10,11]. As a perennial herb, *A. alpina* L. subsp. *brevifolia* (DC.) Cullen is one of the two subspecies (*brevifolia* and *caucasica*) and generally grows on rocks and screes in Turkey [12].

Brassicaceae family has received a great deal of attention in recent years because of its antioxidative and antimicrobial properties [13-16]. It is one of the most studied family due to its rich phytochemical contents [17]. They have great importance in terms of preventing oxidative damage [18] and diseases like cancer, Alzheimer's and Parkinson's [19]. Karakoca et al. [20] report high chlorogenic acid in the methanolic root extract of *Isatis floribunda*, which is used to reduce the relative risk of cardiovascular disease, diabetes mellitus, and Alzheimer's disease [21-23]. Ethanol extracts of the leaf and seed of *Eruca sativa* have shown anti-secretory, cytoprotective, and anti-ulcer effects against gastric lesions [24]. Recent studies have indicated that the essential oils of the root-stem-leaf-fruit of *Eruca vesicaria* subsp. *longirostris* have shown significant antimicrobial activity against *S. aureus*, *B. subtilis*, *B. amyloliquefaciens*, *E. coli*, *P. aeruginosa*, *S. enterica*, and *C. albicans* [25]. Similarly, Rani et al. [26] report that the crude water extracts of the seed of *Eruca sativa* posses highest antibacterial activity against *Enterobacter agglomerans* and *Hafnia alvei*.

In scope of my knowledge, the present study is the first attempt to investigate *A. alpina* L. subsp. *brevifolia* (DC.) Cullen. The objectives of this study are to determine the

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phenolic compound composition of this plant, to examine antimicrobial activities of the various extracts of above-ground organs (leaf, flower) of the plant against various food pathogens, and to determine antioxidant capacity of the extracts.

2 Methods

2.1 Plant material

The samples of *A. alpina* subsp. *brevifolia* were collected from Burdur/Turkey (37°41′44.82″N, 30°20′43.50″E; 1157m asl.) in its blooming season in May 2015. The identification of the specimens was made according to *Flora of Turkey and the East Aegean Islands* [12,27], and the samples were deposited at the Botanical Research Laboratory of the Biology Department, Mehmet Akif Ersoy University (voucher number: Balpinar, 1551).

2.2 Microorganisms

In order to specify *in vitro* antimicrobial activities, the following food pathogens were utilized in this study: *Bacillus subtilis* RSKK245, *Candida albicans* RSKK02029, *Escherichia coli* ATCC11229, *Enterococcus faecalis* ATCC8093, *Listeria monocytogenes* ATCC7644, *Salmonella* Typhimurium RSKK19, *Staphylococcus aureus* RSKK2392 and *Yersinia enterocolitica* NCTC11174. These microorganisms were supplied from ATCC (American Type Culture Collection, USA), RSKK (Refik Saydam National Type Culture Collection, Turkey) and NCTC (North Central Texas College, USA).

2.3 Cultivation of microorganisms

Among these food-borne pathogens, the yeast *C. albicans* was cultivated in Sabouraud Dextrose agar plates (SDA, Merck) at 30°C for 24 h, and the other bacteria were in Mueller-Hinton agar plates (MHA, Merck) at 37°C for 24 h.

2.4 Extraction process

The above-ground organs of plant were washed 2-3 times in running water and once in sterile water. The plant materials were divided into their pieces (stem, leaf and flower). Next, they were air-dried and milled by using a blender. The homogenized fine powder was stored at 4°C in a room which is away from natural sunlight until extraction process. These materials (40 g) were extracted separately in a soxhlet apparatus (Isotex) with 250 ml methanol and ethanol solvents. Time of extraction process was 4-8 h. The purpose of preparing both methanol and ethanol extracts of the plant is that the presence of polar groups (e.g. phenolics, alcoholoids etc.) which shows antimicrobial activity and are highly soluble in these two solvents. The obtained extracts were evaporated by using an evaporator and transferred into the sterilized falcon tubes which contain their own solvents (i.e. the material extracted in methanol was placed in falcon tubes containing methanol; the material extracted in ethanol was transferred in the tubes containing ethanol). The extracts prepared in the concentration of 200 mg/ml were kept under refrigerated conditions until the analysis.

2.5 HPLC analysis of phenolic compounds in the extracts

The HPLC analysis (described by Caponio et al. [28]) that was slightly modified was used to determine phenolic components. A modular Shimadzu Prominence Auto Sampler (SIL 20 ACHT) HPLC system (Shimadzu, Kyoto, Japan) comprised of a LC-20AT pump, a CTO-10ASVp column oven, a SPD-M20A DAD detector, a 20ACBM interface was utilized in the analysis. The flow rate of run was maintained at 0.8 mL/min, and the detection wavelength was 240 nm for ellagic acid, 260 nm for epicatechin, 280 nm for gallic acid, 3,4-dihydroxybenzoic, 4-hydroxybenzoic, caffeic, cinnamic acids and naringin, 320 nm for 2,5-dihydroxybenzoic, chlorogenic, vanillic, p-coumaric, ferulic acids, and 360 nm for rutin and quercetin. A Zorbax Eclipse XDB-C18 (4.6×250 mm) 5 µm column was used and its temperature was 40°C. The separation was executed by using a gradient program with a two-solvent system (solution A was 3% formic acid, solution B was methanol). Injection volume was 100 µL. Data were acquired by Shimadzu LC Solution software.

2.6 *In vitro* antimicrobial assay

In order to determine antimicrobial activities, disc diffusion assay was used. The organic solvents of this study were ethanol and methanol. The turbidity of the active cultures was equalled to 0.5 McFarland (1.5 x 10^8 cfu/mL), and then they were inoculated to the plates (0.1 ml) under aseptic conditions. The plant extracts (45

µL from 200 mg/mL concentration) were ingrained into blank discs (6mm) and they were placed on plate surface. After the incubation, the diameter of inhibition zones was measured in millimeters. The solvents of extraction (ethanol and methanol) were defined as negative control group while ampicillin (10 µg), tetracycline (30 µg) and nystatin (100 µg) were used as positive control group [29]. All measurements were performed in triplicate parallel cultures, and the values obtained were given in average.

2.7 Determination of minimal inhibitory concentration

Broth dilution method was employed for determination of MIC values of the extracts. The active culture concentrations were standardized to 0.5 McFarland and all experiments were conducted in 2 mL Mueller-Hinton Broth. Serial dilutions, each of their concentrations was 13000; 6500; 3250; 1625; 812.5 µg/mL, were prepared, and the same amounts of active cultures (100 µL) were inoculated into each of them [30,31]. After incubation at 37°C for 24 h and the lowest concentration was defined as MIC value.

2.8 Non-enzymatic antioxidant assay

The determination of antioxidant activity was accomplished by using DPPH radical scavenging assay. The absorbance of the extracts was measured at a wavelength of 515 nm in an UV/Vis spectrophotometer (Optizen POP, Korea) [32]. The methanol DPPH solution was used as control. Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid; Sigma) was used as reference standard and the results were given through the equivalent of mM Trolox/g DW (TE).

Ethical approval: The conducted research is not related to either human or animal use.

3 Results

The analysis of phenolic acid compounds in *A. alpina* subsp. *brevifolia* was performed utilizing the HPLC technique. A total of 15 phenolic standards were used and 14 phenolic components were determined in this study (Table 1). The results of the analysis showed that the major components were ellagic acid, which was followed by rutin, caffeic acid and epicatechin, for the methanolic stem-leaf extract, rutin for the ethanolic stem-leaf extract, 2,5-dihydroxybenzoic acid for the methanolic fruit-flower extract, and 2,5-dihydroxybenzoic acid for the ethanolic fruit-flower extract. The chromatogram for the standards and the HPLC chromatograms of the various extracts are shown in Figure 1 and Figure 2, respectively.

Table 1: Phenolic compositions of the various extracts from *A. alpina* subsp. *brevifolia*.

	The various extracts of *A. alpina* subsp. *brevifolia* (µg/mg)			
Phenolic compounds	A	B	C	D
Gallic acid	0.96	2.91	3.14	1.96
3,4-dihydroxybenzoic acid	0.65	2.69	3.4	2.25
4-hydroxybenzoic acid	2.71	5.98	19.19	20.62
2,5-dihydroxybenzoic acid	141.76	353.33	1458..09	14672.68
Chlorogenic acid	7.73	24.23	32.7	83.22
Vanillic acid	49.79	110.08	124.71	109.14
Epicatechin	212.86	73.01	663.83	136.25
Caffeic acid	278.37	315.72	847.90	543.91
p-coumaric acid	(nd)	(nd)	(nd)	(nd)
Ferulic acid	8.07	12.04	1.23	0.01
Rutin	300.43	1238.77	582.93	(nd)
Ellagic acid	319.01	493.19	726.41	157.09
Naringin	119.60	167.27	32.80	267.31
Cinnamic acid	25.09	17.98	6.72	13.30
Quercetin	131.374	155.39	289.904	322.974

A: methanol stem-leaf extract, B: ethanol stem-leaf extract, C: methanol fruit-flower extract, D: ethanol fruit-flower extract, (nd): not determined

Using disc diffusion assay, which it was utilized as a method for determining antimicrobial activity in this study, it was determined whether the various extracts of some parts of *A. alpina* subsp. *brevifolia* would show activity or not against the food-borne pathogens by measuring their inhibition zone. The results indicated that no activity was observed in the ethanol extract of the flowers while the ethanol extract of the leaves revealed antibacterial activities against *S.* Typhimurium RSKK19 (7 mm). The methanol extracts of the leaves and the flowers showed antibacterial activities against *S.* Typhimurium RSKK19 (7 mm). Moreover, neither ethanol nor methanol extracts of the plant parts exhibited antifungal activity against *C. albicans* RSKK02029. The results are illustrated in Table 2.

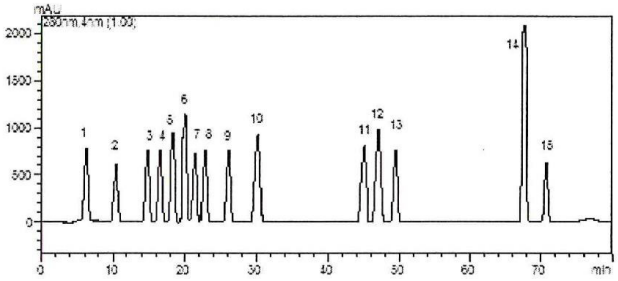

Figure 1: Standard chromatogram; 1 = gallic acid, 2 = 3,4-dihydroxybenzoic acid, 3 = 4-hydroxybenzoic acid, 4 = 2,5-dihydroxybenzoic acid, 5 = chlorogenic acid, 6 = vanillic acid, 7 = epicatechin, 8 = caffeic acid, 9 = p-coumaric acid, 10 = ferulic acid, 11 = rutin, 12 = ellagic acid, 13 = naringin, 14 = cinnamic acid, 15 = quercetin.

The other test applied in this study was MIC which was conducted in order to determine the antibacterial activity [30,31]. According to the results, the MIC value of *A. alpina* subsp. *brevifolia* extracts was determined to be 13000 µg/mL (Table 3).

In order to characterize the non-enzymatic antioxidant activities of the plant extracts, DPPH assay was used. The highest DPPH scavenging capacity was 76.3% in the ethanol extracts of the flowers-fruits-seeds of *A. alpina* subsp. *brevifolia*. Its trolox equivalent was 2.134 mM/g DW (Table 4).

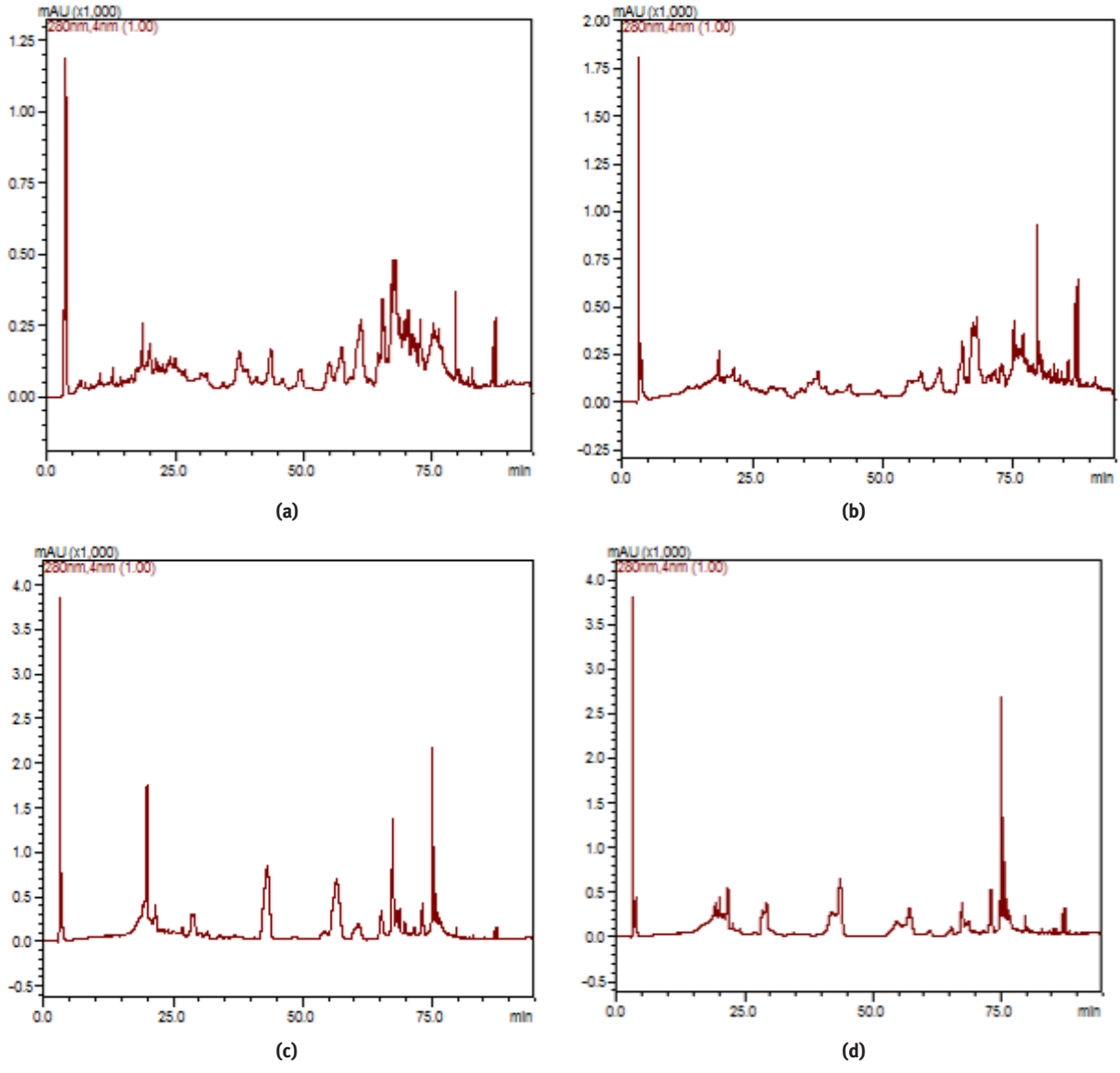

Figure 2: HPLC chromatograms of *A. alpina* subsp. *brevifolia*. a: methanol stem-leaf extract, b: ethanol stem-leaf extract, c: methanol fruit-flower extract, d: ethanol fruit-flower extract.

Table 2: Antimicrobial activities of *A. alpina* subsp. *brevifolia* against food-borne pathogens (200 mg/mL).

	Inhibition zone (mm)						
	Extracts of Plant Parts				Antibiotics		
Microorganisms	Leaf Ethanol	Flower	Leaf Methanol	Flower	TE	NS	A
Bacillus subtilis RSKK245	(-)	(-)	(-)	(-)	nt	nt	10±0.001
Staphylococcus aureus RSKK2392	(-)	(-)	(-)	(-)	nt	nt	10±0.001
Salmonella Typhimurium RSKK19	7±0.01	(-)	7±0.01	7±0.01	14±0.001	nt	nt
Enterococcus faecalis ATCC8093	(-)	(-)	(-)	(-)	nt	nt	-
Escherichia coli ATCC11229	(-)	(-)	(-)	(-)	14±0.001	nt	nt
Listeria monocytogenes ATCC7644	(-)	(-)	(-)	(-)	nt	nt	12±0.001
Yersinia enterocolitica NCTC11174	(-)	(-)	7±0.01	(-)	20±0.001	nt	nt
Candida albicans RSKK02029	(-)	(-)	(-)	(-)	nt	7±0.001	nt

(-): no inhibition, nt: not tested, TE: tetracycline (30 µg), NS: nystatin (100 µg), A: ampicillin (10 µg)

Table 3: Minimum inhibitory concentrations of *A. alpina* subsp. *brevifolia* (µg/mL).

Microorganisms	LE	FE	LM	FM
Salmonella Typhimurium RSKK19	13000	(nt)	13000	13000
Yersinia enterocolitica NCTC11174	(nt)	(nt)	13000	(nt)

LE: leaf ethanol extract, FE: flower ethanol extract, LM: leaf methanol extract, FM: flower methanol extract, (nt): not tested

Table 4: DPPH radical scavenging activities of *A. alpina* subsp. *brevifolia* extracts.

Radical scavenging activity	Stem-Leaf		Flower-Fruit-Seed	
	Ethanol	Methanol	Ethanol	Methanol
DPPH (%)	0	70.0	76.3	74.7
Trolox equivalent (mM/g DW)	0	1.96	2.1	2.06

DW: dry weight

4 Discussion

The present analysis showed the presence of tannins (ellagic acid, epicatechin), flavonoids (rutin) and phenolic acids (caffeic acid, 2,5-dihydroxybenzoic acid) in various extracts of the different parts of *A. alpina* subsp. *brevifolia* (Table 1). Recent pharmacological studies on tannins have revealed that they have antibacterial [33,34], anticarcinogenic [35], and antioxidant [36,37] properties. Flavonoids have been previously associated with antioxidant properties [38]. Among all phenolic compounds identified in *A. alpina* subsp. *brevifolia*, 2,5-dihydroxybenzoic acid showed the highest level, and it has remarkable antioxidant characteristics against oxidative stress [39].

Because of its economic value, *Brassicaceae* has been in the centre of attraction among the studies of antimicrobial activity. For instance, Razavi et al. [40] and Esmaeili et al. [19] reported there were 27.7 and 20 mm zones for *B. subtilis* in the methanol extracts of *Crambe orientalis* (*Brassicaceae*) and *Malcolmia africana* (*Brassicaceae*), respectively. Moreover, Razavi et al. [40] reported the presence of 23 mm activity zone for *E. coli* while Esmaeili et al. [19] found no activity against the same bacteria. In the studies by Karakoca et al. [20] regarding the flower ethanol extracts of *Isatis floribunda* (*Brassicaceae*), it was detected 12.9 mm activity zone for *S. aureus* and 13.5 mm for *E. coli*. In the present study, 7 mm zone was obtained against the gram negative bacteria *S.* Typhimurium while there was no activity against *E. coli*. However, these bacteria are more complex regarding the structure of cell membrane in comparison to the gram positive bacteria [25]. Generally it was difficult to determine striking antimicrobial activity against Gram negative bacteria. Another study supports my results. For example, Esmaeili et al. [19] found no inhibition zone against the second Gram negative bacteria at issue.

The results of this study showed that the MIC value was 13000 µg/mL both in the ethanolic extracts of the leaves and the methanolic extracts of flowers against *S.*

Typhimurium RSKK19 and in the methanolic extracts of the leaves against *Y. enterocolitica* NCTC11174. In the studies of *Crambe orientalis* (*Brassicaceae*), this value was measured as 500 µg/mL for *E. coli* by Razavi *et al.* [40]. MIC result of this study is higher than Razavi's result. The possible reason of their finding may come from a phytochemical called isothiocyanates which is responsible for some biological activities [40]. Grosso et al. [41] found the MIC value of 125 mg/mL in the methanol extracts of *Capsella bursa-pastoris* for *S.* Typhimurium and *E. coli*. The MIC result of this study is better than the result of Grosso et al. [41].

In this study, the highest inhibitory percentage of DPPH was 76.3% and the trolox equivalent was 2.1 mM/g DW. The highest DPPH scavenging activity was determined by Karakoca et al. [20] in the flower extracts of *Isatis floribunda* (89.6%). Omri et al. [25] recorded the radical activity as 56.3% in the leaf extracts and 83.6% in the fruit extracts of *Eruca vesicaria* subsp. *longirostris*. These results are similar to result of the present study. The studies conducted on the members of *Brassicaceae* family report that there is a high correlation between antioxidant activity and polyphenolic contents [25].

In the present study, the phenolic components of *A. alpina* subsp. *brevifolia* is reported for the first time. It indicates that this species is an interesting source of polyphenols and phenolic acids. This study is also the first attempt in utilizing antimicrobial tests on the various extracts of the different body parts (leaf, flower) of *A. alpina* subsp. *brevifolia*. Besides, it differs from the others in terms of the variety of bacteria used in the tests. The findings obtained in this research reveal that the extracts of the leaves of *A. alpina* subsp. *brevifolia* generally indicate higher antimicrobial activity than the extracts of the flowers, and that the extracts of the flower-fruit-seed of this plant have a high antioxidant capacity (Table 2 and 4). The data of this study indicate that *A. alpina* subsp. *brevifolia* may be used as a potential source of natural antioxidant and antibacterial agents. Nevertheless, I am of the opinion that *Brassicaceae* family, which is rich in isothiocynate derivatives, should be further investigated in *in vivo* and *in vitro* studies.

Acknowledgments: This study was supported by Mehmet Akif Ersoy University Scientific Research Projects Coordination Unit (No. 0338-NAP-16). No sponsors have got involved in any stage of the study; which are collection, analysis and interpretation of data, and manuscripting of the study and making decision on publishing the results. The author thanks Assoc. Prof. Dr. Gulten OKMEN for her contributions in the laboratory studies.

The HPLC analysis of phenolic compounds in the extracts was conducted in Scientific and Technological Research and Application Center at Mehmet Akif Ersoy University (Burdur-Turkey); (Protocol no: E.5045).

Conflict of interest: Authors state no conflict of interest.

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