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SCREENING OF SOME BIOLOGICAL ACTIVITIES OF *ALYSSUM FULVESCENS* VAR. *FULVESCENS* KNOWN AS EGE MADWORT

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In this research, the phenolic composition, antioxidant, antibacterial and cytotoxic activities of the methanolic extracts obtained from *Alyssum fulvescens* var. *fulvescens* aerial parts known as Ege kuduzotu in western Turkey, were firstly investigated. The antioxidant activity of the extract was determined by DPPH, metal chelating, phosphomolybdenum, β -carotene/linoleic acid and ferric reducing power assays. Moreover, total phenolic and flavonoid contents in the extract were investigated. The brine shrimp (*Artemia salina* L.) lethality test was used to investigate for the possible cytotoxic activity of the extract. Microdilution broth method was used to study antibacterial potency of extract against Gram-positive and Gram-negative bacteria. The extract exhibited good biological activities. Total phenolic and flavonoid contents in the extract were significantly correlated with antioxidant potentials. HPLC analysis showed that chlorogenic acid was the major phenolic in extract tested. The results indicated that the extract of *A. fulvescens* var. *fulvescens* may be considered as a potential source of biological agents and *in vivo* investigations are needed to test the biological effects of *A. fulvescens* var. *fulvescens*.

Keywords: *Alyssum fulvescens* var. *fulvescens* – antioxidant activity – cytotoxicity – phenolic composition – HPLC

INTRODUCTION

The family Brassicaceae consists of about 400 genera and 4000 species, and includes several genera such as *Crambe*, *Sinapis*, *Thlaspi*, *Alyssum* and *Brassica*. Many Brassicaceae species are known for their use in folk medicine as antimicrobial agent for relief of biliary colic and wound sores [11]. The genus *Alyssum* L. is one of the biggest genera, which include many species in Turkey. It is represented by 90 species and 54 of them are endemic [5].

The name *Alyssum* is of Greek derivation: *a* meaning not, and *lyssa* alluding madness, rage or hydrophobia. Accordingly, the names Madwort and *Alyssum* both refer to the plant's reputation as an officinal herb in Europe and North America. An infusion concocted from the leaves and flowers was reputed to have been administered as a specific antidote against madness or the bite of a rabid dog. Some sources have

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suggested the use of *Alyssum* tea as a sedative for allaying anger [8]. Shimer [27], has indicated that *Alyssum* was used by the Greeks as a remedy for hiccoughs. Although there are no reports of the medicinal uses of *Alyssum fulvescens* Sibth. & Sm. var. *fulvescens*, the aerial parts of *A. constelletum* Boiss. are used in tea form as a demulcent in Turkey [18]. Brassicaceae plants have evolved the ability to synthesis a wide range of sulfur-containing secondary metabolites, including glucosinolates and indole-type phytoalexins [3]. Previous studies have shown that *Alyssum* genus contains glucosinolates, hydrocarbons, fatty acids and phenolics [10, 23, 30].

As it is known, the secondary metabolites such as polyphenols, flavonoids, terpenes, saponins and alkaloids are primarily responsible constituents that contributes the biological potential of various plants [16]. Therefore, this study was centered upon the analysis of the phenolic content, antibacterial, antioxidant and cytotoxic activity of the methanolic extract from *A. fulvescens* var. *fulvescens*. Several studies have been published in which antioxidant, antibacterial and cytotoxic activities of different plant species are studied but as far as our literature survey could as certain, there are no reports on the biological activities and phenolic composition of *A. fulvescens* var. *fulvescens*. Thus, this study is the first report on this plant.

MATERIALS AND METHODS

Plant material and extraction procedure

A. fulvescens var. *fulvescens* was collected in April 2013 from İzmir-Turkey (Yamanlar Mountain, 800 m) and identified in our laboratory (Voucher No: C. OZAY1003). Ten g of air-dried aerial parts of the plant were extracted with 100 mL methanol in a shaker water bath at 50 °C for 6 h. The extraction was repeated twice at the same condition [24]. The extract was filtered with Whatman No. 1 filter paper and methanol was evaporated at 40 °C by using a rotary evaporator. Residual part of the extract was dissolved with water and the water in the extract was lyophilized. Anhydrous extract was stored at –20 °C until analysis.

Antioxidant activity

Phosphomolybdenum method

The total antioxidant capacity of extract was evaluated by phosphomolybdenum method according to Prieto et al. [25], 0.3 mL of extract solution (1 mg/mL) was mixed with 3 mL of reagent solution. 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 the extract was expressed as equivalents of ascorbic acid (mgAEs/g).

DPPH free-radical scavenging activity

The effect of methanol extract on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was estimated according to Wu et al. [31]. Four ml of the DPPH's 0.004% methanolic solution was mixed with 1 mL (0.2–1.0 mg) of the extracts, and their absorbances were measured at 517 nm after incubation for 30 min at room temperature in dark. BHT and BHA were used as standarts.

Reducing power activity (iron (III) to iron (II) reduction)

The ferric reducing power method was applied with slight modifications of the method of Oyaizu [22]. Various concentrations of extract (2.5 mL) were mixed with 2.5 mL, 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After, 2.5 mL of 10% trichloroacetic acid was added. The 2.5 mL of the reaction mixture was mixed with 2.5 mL distilled water and 0.5 mL of 0.1% ferric chloride. The solution absorbance was measured at 700 nm. EC₅₀ of the plant extract was then determined. BHT and BHA were used as standarts.

β-Carotene-linoleic acid assay

In this assay, antioxidant capacity of extract was determined by slight modifications of the procedure described by Sokmen et al. [29]. Antioxidant activity (AA) was calculated in following way:

$$AA: [1 - (A_0 - A_t / A_0^{\circ} - A_t^{\circ})] \times 100$$

where A₀ is the initial absorbance of the sample, A_t is the initial absorbance of the control, A₀[°] is the sample's absorbance after 120 min, and A_t[°] is the control's absorbance after 120 min.

Metal chelating activity on ferrous ions

The metal chelating activity on ferrous ions was determined by the method described by Aktumsek et al. [1] and expressed as equivalents of EDTA (mg EDTAEs/g).

Total phenolic content (Folin–Ciocalteu assay)

Total phenolic content of the extract was analyzed via Folin–Ciocalteu method which gallic acid was used as a standart [28]. One mL of extract solution (1 mg) was added to 46 mL of distilled water and 1 mL of Folin–Ciocalteu reagent and was mixed properly. After 3 min, the mixture was added to 3 mL of sodium carbonate (2%) and shaken intermittently for 2 h. The absorbance was read at 760 nm and the total phenolic content was expressed as equivalents of gallic acid (mgGAEs/g).

Total flavonoid content

Total flavonoid content of the extract was determined by the aluminium colorimetric method [2] and expressed as equivalents of quercetin (mg QEs/g).

Quantification of phenolic compounds by RP-HPLC

HPLC analysis was carried out on Shimadzu LC-20AT Prominence (Shimadzu, Japan). Separations were carried out at 30 °C on C-18 reversed-phase column (250 mm × 4.6 mm length, 5 µm particle size). The mobile phases were A: 3.0% formic acid in distilled water and B: methanol. Methanol was used to dissolve samples, and then 20 µL of this solution was injected into the column. Phenolic composition of the extract was determined according to the method of Caponio et al. [4] with slight modification. Gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid and cinnamic acid were used as standard. The differentiation and quantitative analysis were made by comparing the standards. The quantity of each phenolic compound was expressed as mg per gram of the extract.

Antibacterial activity

The extract was tested against different microorganisms, including *P. aeruginosa*, *E. coli*, *S. enteritidis*, *M. luteus*, *S. aureus* and *B. subtilis* by means of microdilution broth method [20]. Bacterial strains were cultured on Mueller Hinton agar for 24 h, at 37 °C. Test strains were suspended in Mueller Hinton broth to give a final density of 5×10^5 cfu/ml and these were confirmed by viable counts. Dilutions ranging from 4.50 mg/mL to 100 mg/mL of the extract were prepared in a 96-well microtiter plate. Plates were incubated at 37 °C for 24 h. Bacterial density was then measured at 610 nm to determine minimum inhibitory concentration (MIC). Antimicrobial activities of gentamicin and oxacillin were determined as positive controls in parallel experiments.

Brine shrimp (Artemia salina L.) lethality test

Brine shrimp lethality test (BSLT) was applied to analyze the possible cytotoxic activity of the extracts. *A. salina* eggs were incubated in seawater at 28 °C. After incubation for 24 h, larvae were collected and kept for an additional 24 h under the same conditions to reach the mature larvae stage. Ten larvae were drawn with a pasteur pipette and placed in every test tube containing 4.5 mL of brine solution. In each experiment, 0.5 mL of the plant extract was mixed with 4.5 mL of brine solution and kept at 25 °C for 24 h under the light and then dead nauplii were counted [14]. Experiments were held together with control and five different concentrations (10–1000 µg/mL) of the extract in a set of three tubes per dose. Analysis of the data was carried out by EPA Probit Analysis Program to find the LC₅₀ values.

RESULTS

Five methods, including β-carotene/linoleic acid bleaching method, phosphomolybdenum method, DPPH radical scavenging activity, ferric reducing power and metal chelating activity were used to measure antioxidant activity in this study. The results of the antioxidant activities of *A. fulvescens* var. *fulvescens* are summarized in Table 1. Total flavonoid and phenolic contents of the extract were also evaluated as quercetin and gallic acid equivalents, respectively (Table 2).

Free radical scavenging activity of methanolic extract of *A. fulvescens* var. *fulvescens* was measured using DPPH assay. IC₅₀ value of extract was 0.123 mg/mL. IC₅₀ values of BHA and BHT were 0.030 and 0.045 mg/mL, respectively (Table 1). IC₅₀ value is inversely related to antioxidant ability of the extract. It seems that the scavenging ability of the extract was less effective than that of BHA and BHT. Reducing power activity was evaluated by using EC₅₀. The EC₅₀ value of extract was found to be 0.133 mg/mL (Table 1). Lower EC₅₀ value indicates higher antioxidant properties. Metal ion chelating activity of extract toward ferrous ions was also investigated. The chelating activity of extract was evaluated using EDTA as a standard (Table 1). The extract showed good chelating activity with the value of 15.64 mgEDTAEs/g. Total

Table 1
Antioxidant activities of methanolic extract from *A. fulvescens* var. *fulvescens* (mean±SD)

Sample	β-Carotene-linoleic acid assay (%)	DPPH assay (IC ₅₀ mg/mL)	Ferric reducing power activity (EC ₅₀ mg/mL)	Metal chelating activity (mgEDTAEs/g) ^a
<i>A. fulvescens</i>	75.08±0.04 ^b	0.123±0.003	0.133±0.002	15.64±0.16
BHA	90.02±0.10	0.030±0.007	0.051±0.004	nt
BHT	91.05±0.33	0.045±0.006	0.052±0.002	nt

^aEDTAEs – EDTA equivalents; nt – no tested; ^bValues expressed are means±S.D.

Table 2
Total antioxidant capacity, total phenolic and flavonoid contents
of methanolic extract from *A. fulvescens* var. *fulvescens*

Sample	TPC ^a	TFC ^b	TAC ^c
<i>A. fulvescens</i>	35.03±0.04 ^d	18.45±0.03	68.21±0.07

^aTotal phenolic content (TPC) expressed as gallic acid equivalents (mgGAEs g⁻¹).

^bTotal flavonoid content (TFC) expressed as quercetin equivalents (mgQEs g⁻¹).

^cTotal antioxidant capacity (TAC) expressed as ascorbic acid equivalents (mgAEs g⁻¹).

^dValues expressed are means±S.D.

antioxidant activity of extract was investigated by phosphomolybdenum assay. Phosphomolybdenum method is based on the reduction of Mo (IV) to Mo (V) by the antioxidants and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. In this assay, total antioxidant activity of extract was determined as 68.21 mgAEs/g (Table 2). Inhibition capacity of oxidation of the linoleic acid of extract was found as 75.08% (Table 1). Antioxidant activities of BHA and BHT were investigated in this assay. Inhibition activities of the linoleic acid of BHA and BHT were detected as 90.02% and 91.05%, respectively.

Total phenolic and flavonoid content were determined as 35.03 mgGAEs/g and 18.45 mgQEs/g, respectively (Table 2). As a part of the study, a rapid high performance liquid chromatographic method using a reversed-phase column and a diode array detector method was established. Using HPLC analysis, the quantitative results of identified phenolic compounds of *A. fulvescens* var. *fulvescens* are listed in Table 3.

Table 3
Phenolic components in the methanolic extract
from *A. fulvescens* var. *fulvescens* (mg/g extract) (mean±SD)

No	Phenolic components	<i>A. fulvescens</i>
1	Gallic acid	0.09±0.02
2	Protocatechuic acid	0.04±0.03
3	4-Hydroxybenzoic acid	0.15±0.04
4	Chlorogenic acid	0.84±0.03
5	Vanillic acid	0.27±0.01
6	Caffeic acid	0.52±0.02
7	<i>p</i> -Coumaric acid	0.02±0.01
8	Ferulic acid	0.13±0.02
9	Cinnamic acid	0.11±0.01

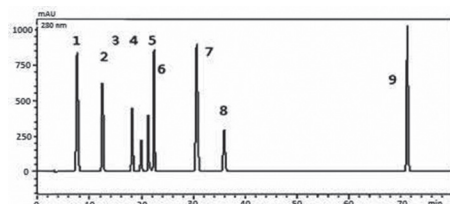


Fig. 1. HPLC chromatogram of standards: 1. Gallic acid; 2. Protocatechuic acid; 3. 4-Hydroxybenzoic acid; 4. Chlorogenic acid; 5. Vanillic acid; 6. Caffeic acid; 7. *p*-Coumaric acid; 8. Ferulic acid; 9. Cinnamic acid

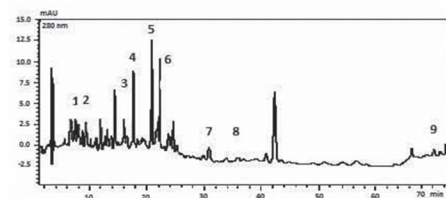


Fig. 2. HPLC chromatogram of phenolic components in the methanolic extract from *A. fulvescens* var. *fulvescens*, where: 1. Gallic acid; 2. Protocatechuic acid; 3. 4-Hydroxybenzoic acid; 4. Chlorogenic acid; 5. Vanillic acid; 6. Caffeic acid; 7. *p*-Coumaric acid; 8. Ferulic acid; 9. Cinnamic acid

Table 4

The LOD and LOQ values of phenolic components analyzed with HPLC

No	Phenolic components	Retention time (RT), min	LOD (mg/L)	LOQ (mg/L)
1	Gallic acid	7.8	0.014	0.083
2	Protocatechuic acid	12.2	0.035	0.109
3	4-Hydroxybenzoic acid	18.1	0.018	0.072
4	Chlorogenic acid	19.9	0.015	0.065
5	Vanillic acid	22.1	0.112	0.164
6	Caffeic acid	23.0	0.013	0.060
7	<i>p</i> -Coumaric acid	30.3	0.010	0.055
8	Ferulic acid	35.7	0.012	0.058
9	Cinnamic acid	71.1	0.011	0.056

Major phenolic compound was determined as chlorogenic acid (0.84 mg/g extract). HPLC chromatogram of phenolic standards and phenolic compounds in the extract were shown in Figures 1 and 2, respectively. Values for limit of detection (LOD) and limit of quantification (LOQ) were calculated for identification and quantification of phenolic compounds in the extract (Table 4).

The antibacterial activities of *A. fulvescens* var. *fulvescens* were evaluated by microdilution broth method. Increased concentrations of extracts caused decrease in survival of bacterial cells. The obtained results are shown in Table 5. Compared to Gram-negative bacteria tested, Gram-positive bacteria were less sensitive to the extract because the MIC values of the extract were observed as 95.4 mg/mL for *B. subtilis* and 83.2 mg/mL for *S. aureus*.

The BSLT was used for the preliminary screening for cytotoxicity of extract, as this assay has shown a good correlation with cytotoxicity assays with human cell lines and mouse bioassays [15]. The percentage of lethality was calculated from the

Table 5

MIC values (mg/mL) of methanolic extract from *A. fulvescens* var. *fulvescens* against standard bacteria

Microorganisms	<i>A. fulvescens</i>	Gentamicin	Oxacillin
<i>Escherichia coli</i> (ATCC 25922)	25	3.12	–
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	3.12	6.25	–
<i>Salmonella enteritidis</i> (PT4)	–	1.56	–
<i>Staphylococcus aureus</i> (ATCC 25923)	83.2	–	6.25
<i>Bacillus subtilis</i> (NRRL B-209)	95.4	–	3.12
<i>Micrococcus luteus</i> (NRRLB-1013)	50	–	6.25

mean death shrimp larvae of extracts treated tubes and control. The degree of lethality was found to be directly proportional to the concentration of the extract. The results of the BSLT are presented in Fig. 3. The extract of *A. fulvescens* var. *fulvescens* showed significant cytotoxicity with LC_{50} value of 37.71 $\mu\text{g/mL}$ compared to *Lepidium sativum* L., which is from the same family with LC_{50} value of 262.46 $\mu\text{g/mL}$ [32].

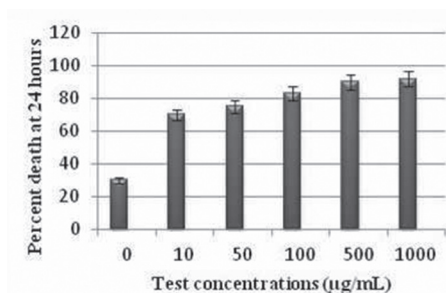


Fig. 3. The effects of methanolic extract from *A. fulvescens* var. *fulvescens* on the death percentage of brine shrimps

DISCUSSION

There are several ways to determine the antioxidant activities due to the complex nature of plant chemicals [7]. Measuring total phenolics and flavonoids is also important in association with their antioxidant activity, because phenolic compounds such as flavonoids and phenolic acids are known as powerful chain breaking antioxidants and may contribute directly to antioxidative activity [26]. Fe^{3+} reduction is often used to measure electron donation activity which is an important mechanism of antioxidants [6]. Therefore, in order to assess the electron-donating power of the extract, its ability to reduce Fe^{3+} was investigated. Ferrous ions are known as the most effective

pro-oxidants in lipid peroxidation due to their high reactivity. In relation to this, the ability of substances or plant extracts to chelate iron can be important mechanism for antioxidant property [17].

The results of total phenolic and flavonoid content showed a similar tendency to the antioxidant abilities of the extract. In this context, the high content of total phenolics in the extract might be explain the strong antioxidant ability of *A. fulvescens* var. *fulvescens*. These results are in accordance with other reports in the literature, which showed strong relationship between antioxidant activities and total phenolic content [9, 24]. Similar results were reported for some *Alyssum* species such as *A. homolocarpum* (F&M.) Boiss by Souri et al. [30]. In a previous study, where 35 selected plants from Spain were compared in terms of their antioxidant activity, total phenolic content and skin care properties, *A. serpyllifolium* Desf. subsp. *lusitanicum* Dudley & Silva leaf extract has exhibited high antioxidant activity and phenolic contents [19]. The highest phenolic compound found in in methanolic extract of *A. fulvescens* var. *fulvescens* was chlorogenic acid. Chlorogenic acid is well known as metal chelator and lipid peroxidation inhibitor in relation to its biological activities [21]. These data supported that the biological activities of *A. fulvescens* var. *fulvescens* could be attributed to its phenolics compounds. A previous study has revealed that *A. maritimum* (L.) Lam. leaf extracts act as a natural quorum sensing inhibitor with slight inhibition [13].

Brine shrimp lethality bioassay is a simple, high throughout cytotoxicity test of bioactive chemicals, toxins and herbal extracts. It is based on the killing ability of test compounds on a simple zoological organism-brine shrimp (*A. salina* L.) [12]. It is concluded that BSLT can be suggested as a valid method to evaluate the cytotoxic activity of plant extracts.

In conclusion, such detailed studies on phenolic composition and biological activities of methanolic extract from *A. fulvescens* var. *fulvescens* aerial parts has been conducted for the first time. Based on biological activity assays, antioxidant, antibacterial, and cytotoxic activity of the extract was revealed. The extract can be beneficial due to its biological activity, and further studies are needed to be performed to identify biologically active compounds such as glucosinolates as well as phenolic compounds.

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