Antioxidant Activity and Total Phenolic Content of *Gagea fibrosa* and *Romulea ramiflora*

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ABSTRACT: *Free radicals involved in a number of diseases due to the oxidative damage to DNA, lipids, and proteins and which can result in failure of cellular functions. Dried plant bulbs and leaves methanolic and ethanolic extracts were prepared. Total antioxidant activities of the methanolic extracts of Romulea ramiflora and Gagea fibrosa were determined using β-caroten-linoleic acid assay. Romulea-Bulb-Ethanol (RBE) extract showed the highest (89.64 ± 1.25%) but, Romulea-Leaves-Ethanol (RLE) extract showed the lowest (33.23 ± 1.13%) antioxidant activity. The lowest antioxidant activity of the Gagea leaves extract (GLE - Gagea-Leaves-Ethanol, 46.18 ± 0.09%) was higher ($p < 0.05$) then Romulea Leaves Extract (RLE - 33.23 ± 1.13%). This may be related with the structure of the plant's leaves cells. The DPPH radical scavenging assay was used to determine the antiradical activities. Gagea-Leaves-Methanol extract (GLM - 61.16 ± 1.46%) has higher DPPH radical scavenging activity than Romulea-Bulb-Methanol extract (RBM - 51.27 ± 0.94%). Generally leaf extracts have the highest free radical scavenging activity. The total phenolic content of extracts was determined using to the Folin-Ciocalteu method. RBE has the highest phenolic content (74,12 ± 4,6 mg/100 g plant extract). R. ramiflora and G. fibrosa have the antioxidant and free radical scavenging activities. These properties are due to the number of the hydroxyl groups of the phenolic compounds, but not amount of them.*

KEY WORDS: *Romulea ramiflora, Gagea fibrosa, antioxidant activity, DPPH, β-caroten-linoleic acid assay, Folin-Ciocalteu method.*

INTRODUCTION

Oxygen free radicals, classified under the more general term of Reactive Oxygen Species (ROS) which includes non-radical species such as hydrogen peroxide, are highly reactive transient chemical species formed in all tissues during normal aerobic cellular metabolism, with the potential to initiate damage to the various intracellular components (nucleic acids, lipids, proteins) on which normal cell functioning depends. Free radicals provoked by various environmental chemicals as well as endogenous metabolism are involved in a number of

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diseases like tumors, inflammation, shock, atherosclerosis, diabetes, infertility, gastric mucosal injury, brain disfunction, cancer and ischemia due to the oxidative damage to DNA, lipids, and proteins and which can result in failure of cellular functions. Free radicals and ROS are controlled in biological systems by some enzymes possessing antioxidant activities such as superoxide dismutase and peroxidase [1-3].

The most active dietary antioxidants belong to the family of phenolic and polyphenolic compounds. Phenolic antioxidants are reported to quench oxygen-derived free radicals as well as the substrate-derived free radicals by donating a hydrogen atom or an electron to the free radical and the antioxidant activity of phenolics in several systems has indicated that they were as active as Buthylated hydroxyaniscle (BHA) or Buthylated hudroxytoluane (BHT) Antioxidant effects of sage (*Salvia officinalis*) have been attributed to the main phenolic components-rosmarinic acid, a caffeic acid derivative, and carnosic acid. Polyphenol antioxidants have protective effects against different diseases, including cardiovascular, inflammatory and neurological diseases, as well as cancers [4,5].

Plant materials contain numerous types of antioxidants with varied activities. The methanol extract of *Plantago bellardii* could be used as a source of natural antioxidants [6]. A number of studies demonstrated antioxidant properties of ginseng extracts and some of its selected ingredients, convincing evidence of the free radical scavenging activity of ginseng is still obscure [7]. Flavonoids are also widely encountered in the plants tested as the most active radical scavengers [8]. The multiple pharmacological properties of flavonoids, such as anti-inflammatory, antibiotic and cardiovascular activities [9] are, to a large extent, linked to their polyphenolic and hence radical scavenging nature [10].

Romulea is a genus of about 80 species of flowering plants in the Iris family distributed in Europe, the Mediterranean, and South Africa. The genus name refers to the legendary Rome founder Romulus, and alludes to the abundance of one of the species in the Roman countryside [11]. *Gagea* is a Eurasian genus of petaloid monocots, with a few species in North Africa, comprising between 70 and approximately 275 species depending on the author. Lloydia thought to be the closest relative of *Gagea* consists of 12–20 species that have a mostly eastern Asian distribution [12].

There is no research has been reported about the antioxidant activities of *Gagea* and *Romulea*. The objective of this study was to investigate the total phenolics, and the antioxidant activities of *Gagea fibrosa* (Desf.) Schultes&Schultes fil. (Fam: Liliaceae) and *Romulea ramiflora* Ten subsp. *ramiflora* (Fam: Iridaceae) bulbs and leaves, methanolic and ethanolic extracts.

EXPERIMENTAL SECTION

Materials

Gagea fibrosa (Desf.) Schultes&Schultes fil. (Fam: Liliaceae) and *Romulea ramiflora* Ten subsp. *ramiflora* (Fam: Iridaceae) species were collected in the spring from Kötekli locality, near Muğla province, in Turkey. The fresh underground and above ground parts of the plants samples were cleaned and dried in the shadow for extraction.

Chemicals

Na_2CO_3, methanol, ethanol, β-carotene, chloroform, ascorbic acid, Tween-20, linoleic acid were obtained from E. Merck (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT) was obtained from Sigma Chemical Co. (St. Lois, MO). Other chemicals used were of analytical grade.

Extraction and plant extract preparations

Dried plant parts (bulbs and leaves) were pulverized. Each ground sample was transferred into a beaker. Ethanol and methanol were added in the ratio of 1:10 and they were put in waterbath at 55ºC for 6 h [13]. The extraction mixture was separated from the residue by filtration through Whatman No: 1 filter paper. The plant residue was re-extracted twice with ethanol and methanol. After the filtration two extracts were combined. The residual solvent of methanol and ethanol extracts of samples were removed under reduced pressure at 48-49°C using a rotary evaporator (BUCHI, rotavapor R-210/R-215, Germany). The water extract was lyophilized using a freeze dryer (Thermosavant Modulyo D, USA). Extracts were produced in duplicates and used to assay the antioxidant activity.

Plant extracts

Gagea (G), Bulb-Methanol (GBM), Bulb-Ethanol (GBE), Leaf-Methanol (GLM), Leaf-Ethanol (GLE). *Romulea* (R),

Bulb-Methanol (RBM), Bulb-Ethanol (RBE), Leaf-Methanol (RLM), Leaf-Ethanol (RLE).

Determination of total phenolic content

The total phenolic content (μg of PEs[a] / mg of extract) of extracts was determined using to the Folin-Ciocalteu method [14, 15]. Briefly, 0.75 mL of Folin–Ciocalteu reagent (1:9; Folin-Ciocalteu reagent: distilled water) and 100 mL of sample (5 mg/mL) were put into a test tube. The mixture was allowed to stand at room temperature for 5 min. 0.75 mL of 6% (w/v) Na_2CO_3 was added to the mixture and then mixed gently. The mixture was homogenized and allowed to stand at room temperature for 90 min. Total polyphenol content was determined using a spectrophotometer at 725 nm. The standard calibration (0.01–0.05 mg/mL) curve was plotted using gallic acid. The total phenolic content was expressed as Gallic Acid Equivalents (GAE) in milligrams per 100 g plant extract.

Antioxidant activity

β –Carotene-linoleic acid assay

β-carotene bleaching assay was carried out according to the method developed by *Wettasinghe & Shahidi* [16]. One milliliter of β-carotene solution (0.2 mg/mL chloroform) was pipetted into a round-bottom flask (50 mL) containing 0.02 ml of linoleic acid and 0.2 mL of 100% Tween 20. The mixture was then evaporated at 40°C for 10 min using a rotary evaporator (BUCHI, Germany) to remove chloroform. After evaporation, the mixture was immediately diluted with 100 mL of distilled water. The distilled water was added slowly to the mixture with vigorous agitation to form an emulsion. Five mL aliquots of the emulsion were transferred into different test tubes containing 0.2 ml of samples in 80% methanol at 1 mg/mL. The mixture was then gently mixed and placed in a water bath at 50°C for 2 h. Absorbance of the sample was measured every 30 min for 2 h at 470 nm using a spectrophotometer (UV-VIS Spectrophotometer, Chebios). Blank solution was prepared, containing the same concentration of sample without β-carotene. All determinations were performed in triplicate. The total antioxidant activity was calculated based on the following equation:

$$AA = \left[1 - \frac{A_0 - A_t}{A_0^0 - A_t^0}\right] \times 100 \qquad (1)$$

where AA is antioxidant activity, A_0 and $A°_0$ are the absorbance values measured at the initial incubation time for samples and control, respectively. While A_t and $A°_t$ are the absorbance values measured in the samples or standards and control at t = 120 min.

DPPH assay

Effect of extract on 2,2-diphenyl-1-picrylhdrazyl (DPPH) free radical was measured, based on *Lee et al.* [17]. Positive control was prepared by mixing 4 mL of ascorbic acid (0.05 mg/mL) and 1 mL of DPPH (0.4 mg/ml), whereas negative control was prepared by mixing distilled water with 1 mL of DPPH. 4 mL of the extract (a final concentration of 20 mg/mL) were added to 1 mL DPPH. The mixture was gently homogenized and left to stand at room temperature for 30 min. Absorbance was read using a spectrophotometer (UV-VIS Spectrophotometer, Chebios) at 520 nm. The ability of extract to scavenge DPPH free radical was calculated using the following equation:

$$\text{Scavenging activity } (\%) = \left[\frac{A_{(-ve)} - A_S}{A_{(-ve)} - A_{(+ve)}}\right] \times 100 \qquad (2)$$

where, A_s is the absorbance of the sample, A(-ve) and A(+ve) are the absorbance values of negative and positive controls, respectively. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted of inhibition percentage against extract concentration. Tests were carried out in triplicate.

RESULTS AND DISCUSSION

Determination of total antioxidant activity

Total antioxidant activities of many plants were investigated by researchers. Many sources of antioxidants of plant origin have been studied in recent years [6,9,13]. Plant materials contain numerous types of antioxidants with varied activities [18,19]. Effects of microwave and conventional cooking methods were studied on total phenolics and antioxidant activity of pepper, squash, green beans, peas, leek, broccoli and spinach. Total antioxidant activity ranged from 12.2% to 78.2%. After cooking, total antioxidant activity increased or remained unchanged depending on the type of vegetable but not type of cooking [20]. *Xu et al.* [21] were investigated the antioxidant activities of brown pigment, extract of n-hexane and extract of supercritical carbon dioxide extraction of black sesame seeds in their study.

Tepe et al. [22], were investigated the antioxidant activities of the methanolic extracts of 5 *Allium* species which spread in Turkey. In this study total antioxidant activity of the plants were found 60–70%.

Total antioxidant activities of the methanolic extracts of *Romulea ramiflora* and *Gagea fibrosa* were determined using β-caroten-linoleic acid model system. The bleaching mechanism of β-carotene is a free radical mediated phenomenon resulting from the formation of hydroperoxides from linoleic acid oxidation. In the absence of antioxidant, β-carotene will undergo rapid discoloration. The addition of the antioxidant containing extracts can protect the extent of β-carotene orange color by neutralizing the peroxide products which were formed from linoleic acid. Hence the high absorbance values indicated that highest antioxidant activity. The measurement was carried out at 30 min intervals for 120 min (t=120). In this time the decrease of the absorbance values is indicated that the reduction of the antioxidant activity.

Romulea ramiflora Ten subsp. r*amiflora* bulb ethanolic extracts (RBE) and *Gagea fibrosa* (Desf.) Schultes & Schultes fil. leaf methanolic (GLM) extracts showed the highest antioxidant activity. The mean antioxidant activity of RBE and GLM were 89.64 ± 1.25% and 72.54 ± 0.4%, respectively. Both plants leaf methanolic extracts showed slightly low, but leaf ethanolic extracts showed lowest antioxidant activity. Mean antioxidant activity of these plant extracts were, GLM (72.54 ± 0.4%) > RLM (68.43 ± 0.6%) > GLE (46.18 ± 0.09%) > RLE (33.23 ± 1.13%) respectively. Results of ANOVA analysis indicated that antioxidant activity of RBE (89.64 ± 1.25%) and RBM (63.56 ± 0.56 %) is significantly higher ($p < 0.05$) than GBE (54.17 ± 1.06 %) and GBM (60.89 ± 1.25 %) respectively (Fig. 1).

The extracts which obtained from same plant using same solvent have nearly the same antioxidant activities. These results indicated that the underground and over ground parts of the plants have the same amount of phenolic compounds during the flowering time.

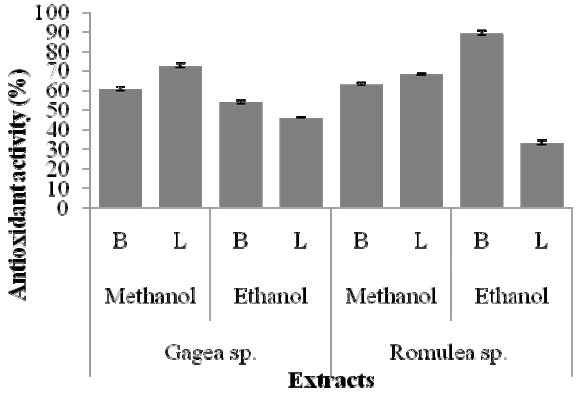

Fig. 1: Antioxidant activity of methanol and ethanol extracts determined with β-carotene-linoleic acid model system Leaf (L), Bulb (B).

Determination of free radical-scavenging activity

The DPPH radical scavenging assay modified according to *Connan et al.* [23] and *Turkmen et al.* [24] was used to determine the antiradical activities in extracts and BHA and BHT were used as positive controls.

The free radical scavenging activity of the extracts is related with hydrogen atom or electron donation abilities and the conformations of the antioxidant compounds of the extracts. DPPH, a stable free radical with a characteristic absorption at 517 nm, was used as free radical to study the radical scavenging effects of some natural products [25].

Free radical scavenging capacities of the extracts and were tested by DPPH.

Free radical scavenging effects results were defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50% in 30 minutes (IC_{50}). Lowest IC_{50} values indicated that the highest antioxidant capacity. For this reason, firstly residual DPPH value was determined and then the ratio of the DPPH value which used was calculated in the system. In this investigation, efficiencies of the GLE and GBE were found 37.32% and 40.66% respectively. These results indicated that, GBE has the highest free radical scavenging activity. Also the DPPH radical scavenging activity of BHT was assayed for comparison. The scavenging activity of RLM was not effective than BHT (90.73%). But it should be noted that GLM (61.16 ± 1.46%) has higher DPPH radical scavenging activity than RBM (51.27 ± 0.94%). Generally leaf extracts have the highest free radical scavenging activity (Fig 2). Our results suggested that phenolic content increased the free radical scavenging activities.

Determination of total phenolic content

In the last decade a number of publications have been published in which antioxidant capacity of plant material, so as antioxidant characteristics of phenol compounds are

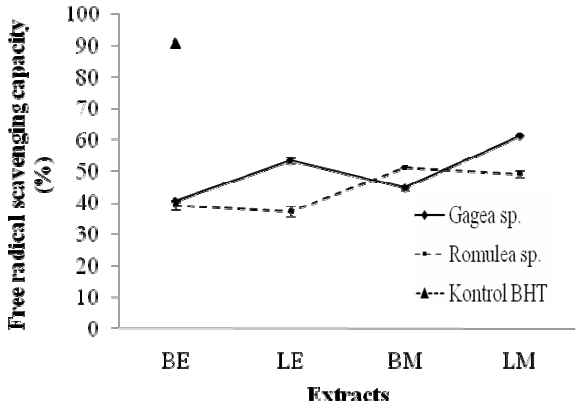

Fig. 2: The free radical scavenging capacity of the extracts with methanol, ethanol, acetone and benzine through DPPH method. Butylated HydroxyToluene (BHT), Leaf-Ethanol (LE), Bulb-Ethanol (BE), Leaf-Metanol (LM), Bulb-Methanol (BM).

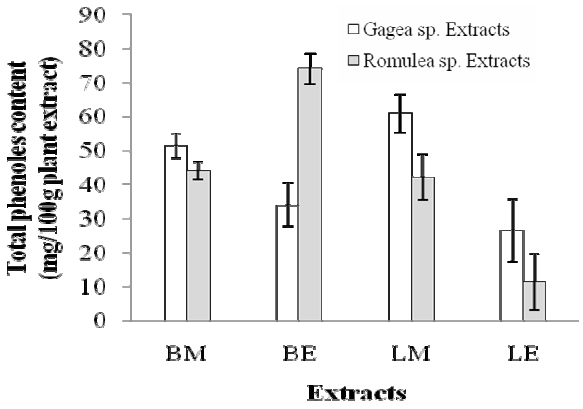

Fig. 3: Total phenolic content (µg of PEs / mg of extract) of extracts from different parts of G. fibrosa and R. ramiflora using various solvents. Leaf-Ethanol (LE), Bulb-Ethanol (BE), Leaf-Methanol (LM), Bulb-Methanol (BM).

tested, through different methods [26]. The responses of the methods are about the number of the hydroxylic groups in phenolic compounds. Because of this, it is difficult to compare final results, even though there are the same plant species. The total phenolic content of the plant extracts is shown in Fig. 3. Among all plant extracts, RBE had the highest phenolic content (74.12 ± 4.6 mg/100 g plant extract), followed by GLM (60.86 ± 5.6 mg/100 g), GBM (51.42 ± 3.7 mg/100 g), RBM (44.13 ± 2.4 mg/100 g), RLE (11.32 ± 8.4 mg/100 g), GBE (34.13 ± 6.3 mg/100 g), GLE (26.57 ± 9.3 mg/100 g) and RLM (42.19 ± 6.7 mg/100 g). The results indicated that RBE (74.12 ± 4.6 mg/100 g) has the highest and the RLE (11.32 ± 8.4 mg/100 g) has the lowest phenolic contents. Other extracts phenolic contents were GLM > GBM > RBM > RLM > GBE > GLE respectively and they have nearly the same amounts of phenolic contents.

CONCLUSIONS

Extracts of *Romulea ramiflora* Ten subsp. r*amiflora* and *Gagea fibrosa* (Desf.) Schultes & Schultes fil bulb and leaves methanolic and ethanolic extracts exhibited varying degrees of total phenolic content, and antioxidant activity. The extracts, should be beneficial as an antioxidant protection system and they are required to isolate and identify the secondary metabolites responsible for their antioxidant activity.

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