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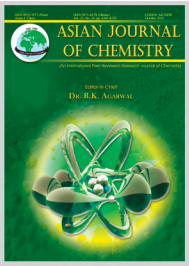

Determination of Antioxidant Capacities of *Phlomis pungens* Willd. var. *hispida* Hub.-Mor.

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The genus *Phlomis* L. belongs to the Lamiaceae family and encompasses 100 species native to Turkey, North Africa, Europe and Asia. It is a popular herbal tea enjoyed for its taste and aroma. *Phlomis* species are used to treat various conditions such as diabetes, gastric ulcer, hemorrhoids, inflammation and wounds. In this study, ethanol and water extracts were prepared from powdered *P. pungens* flowers and leaves. Antioxidant activities were measured by 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical scavenging, 1,1-diphenyl-2-picryl-hydrazyl free radical ($DPPH^{\bullet}$) scavenging, superoxide anion ($O_2^{\bullet-}$) radical scavenging, reducing activity and ferrous metal chelating activity assays. In conclusion, *P. pungens* flowers and leaves had effective $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging, superoxide anion radical scavenging, reducing power and metal chelating activities when compared with BHA, BHT and α-tocopherol as reference antioxidants.

Key Words: Phlomis pungens, Antioxidant capacity, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, Superoxide radical.

INTRODUCTION

Plants are potential sources of natural antioxidants because they contain phenolic compounds such as phenolic acids, flavonoids, tannins and phenolic diterpenes[1]. Numerous reports of antioxidant extracts from medicinal plants have appeared, strongly inspired by an increasing consumer interest in natural healthy diets[2,3]. The genus *Phlomis*, perennial herbs of the family Lamiaceae, consists of more than 100 species distributed in Africa, Asia and Europe[4]. The genus *Phlomis* L. is represented by 34 species in Turkish flora[5]. Some of the *Phlomis* species are used as tonic and stimulant in Anatolia[6]. A number of *Phlomis* species have medicinal characteristics. Furthermore, there is evidence indicating various antimicrobial[7], antiinflammatory, immunosuppressive[8] and free radical scavenging properties[9,10] for *Phlomis* species. Previous phytochemical investigations of the genus *Phlomis* have shown that they contain iridoids, flavonoids, phenylpropanoids, phenylethanoids, lignans, neolignans, diterpenoids, alkaloids and essential oils[11,12]. In regard to phenolic acids, a wide variety of caffeic acid derivatives, including acteoside and forsythoside B from some *Phlomis* species have been identified[10,13-15].

The free-radical theory of aging postulates that damage caused by the reactions of free radicals, called reactive oxygen species, is responsible for the aging process and that this damage may be mitigated by the action of the antioxidants[16]. The hypothesis is that the use of antioxidants that scavenge reactive oxygen species provides biological resistance to free radicals, retards the process of aging and decreases the risk of age-associated degenerative diseases, such as cancer, cardiovascular diseases, immune system decline and brain dysfunction[17]. The aim of this study is to determine antioxidant capacities of *Phlomis pungens* water and ethanol extracts of leaves and flowers.

EXPERIMENTAL

Extraction procedures: *Phlomis pungens* leaves and flowers were obtained from Sivrice, Elazig in Turkey. For ethanol extraction, 25 g powder of *P. pungens* leaves and flowers ground into a fine powder in a mill and were mixed five times with 100 mL of ethanol. Extraction continued until the extraction solvents became colourless (total solvent volume 500 mL). The obtained extracts were filtered over Whatman no.1 paper and the filtrate was collected, then ethanol was removed by a rotary evaporator at 50 ºC. For preparation of water extraction, 25 g powder of *P. pungens* leaves and flowers ground into a fine powder in a mill and was mixed with 500 mL boiling water by magnetic stirrer during 10 min. Then the extract was filtered over Whatman no.1 paper and then water was removed by a rotary evaporator[18] at 50 ºC.

2,2-Azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), ethanol, $K_2S_2O_8$, NaH_2PO_4, 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH), nitroblue tetrazolium (NBT), NADH, phenazine methosulphate (PMS), $FeCl_2$, ferrozine, $K_3[Fe(CN)_6]$, trichloroacetic acid (TCA), $FeCl_3$.

$ABTS^{\bullet+}$ radical scavenging capacity: ABTS also forms a relatively stable free radical, which decolourizes in its non-radical form[19]. The spectrophotometric analysis of $ABTS^{\bullet+}$ radical scavenging capacity was determined according to the method of Re *et al*.[20]. $ABTS^{\bullet+}$ was produced by reacting 2 mM ABTS in H_2O with 2.45 mM $K_2S_2O_8$, stored for 12 h at room temperature in the dark. The $ABTS^{\bullet+}$ solution was diluted to give an absorbance of 0.750 ± 0.025 at 734 nm in 0.1 M sodium phosphate buffer (pH 7.4). Then, 1 mL of $ABTS^{\bullet+}$ solution was added to 3 mL of *P. pungens* extracts in ethanol at 100 µg/mL concentrations. The absorbance was recorded for 0.5 h, after the mixing and percentage of radical scavenging were calculated for each concentration relative to a blank containing no scavenger. The extent of decolourization is calculated as percentage reduction of absorbance. The scavenging capability of test compounds was calculated by the following equation:

$$ABTS^{\bullet+} \text{ scavenging effect } (\%) = [(A_0 - A_1)/A_0] \times 100$$

where, A_0 is the absorbance of control and A_1 is the absorbance in the presence of the sample of *P. pungens* extracts or standards.

$DPPH^{\bullet}$ radical scavenging capacity: The free radical scavenging capacity of *P. pungens* extracts was measured by 2,2-diphenyl-1-picryl-hydrazil ($DPPH^{\bullet}$)[21]. Briefly, 0.1 mM solution of $DPPH^{\bullet}$ in ethanol was prepared and 1 mL of this solution was added to 3 mL of *P. pungens* extracts solution in water at 100 µg/mL concentration. Absorbance at 517 nm was determined after 0.5 h against a blank solution containing the ethanol. Lower absorbance of the reaction mixture indicates the higher free radical scavenging activity. When a hydrogen atom or electron was transferred to the odd electron in $DPPH^{\bullet}$, the absorbance at 517 nm is decreased proportionally to the increase of non-radical forms of DPPH[22]. The capability to scavenge the $DPPH^{\bullet}$ radical was calculated by the following equation:

$$DPPH^{\bullet} \text{ scavenging effect } (\%) = [(A_0 - A_1)/A_0] \times 100$$

where, A_0 is the absorbance of control reaction and A_1 is the absorbance in the presence of the sample of *P. pungens* extracts.

Superoxide anion scavenging capacity: Measurement of superoxide anion scavenging capacity of *P. pungens* extracts was based on the method described by Liu *et al*.[23] with slight modification. One milliliter of nitroblue tetrazolium (NBT) solution (156 mmol/L NBT in 100 mmol/L phosphate buffer, pH 7.4), 1 mL NADH solution (468 mmol/L in 100 mmol/L phosphate buffer (pH 7.4) and 100 µL of sample solution of *P. pungens* extracts in water were mixed. The reaction was started by adding 100 µL of phenazine methosulphate (PMS) solution (60 mmol/L PMS in 100 mmol/L phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 ºC for 5 min and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture shows increase in superoxide anion scavenging capacity. The percentage inhibition of superoxide anion generation was calculated by the following formula:

$$\text{Superoxide anion scavenging effect } (\%) = [(A_0 - A_1)/A_0] \times 100$$

where, A_0 is the absorbance of the control and A_1 is the absorbance of *P. pungens* extracts or standards[24].

Metal chelating activity: The chelating of ferrous ions by the *P. pungens* extracts and standards was estimated by the method of Dinis *et al*.[25]. Briefly, extracts 100 µg/mL was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was started by addition of 5 mM ferrozine (0.2 mL) and the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated by the following formula:

$$\text{Metal chelating } (\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of control and A_1 is the absorbance in the presence of the sample of *P. pungens* extracts or standards. The control does not contain $FeCl_2$ and ferrozine, complex formation molecules.

Total reduction activity: The samples prepared for ferric thiocyanate method was used for the present and other antioxidant assays. The reducing activities of *P. pungens* extracts were determined by the method of Oyaizu[26]. The capacity of *P. pungens* extracts to reduce the ferric-ferricyanide complex to the ferrous-ferricyanide complex of Prussian blue was determined by recording the absorbance at 700 nm after incubation. Simply, different concentrations of *P. pungens* extracts (50-250 µg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1 %). The mixture was incubated at 50 ºC for 20 min. Aliquots (2.5 mL) of trichloroacetic acid (10 %) were added to the mixture, which was then centrifuged for 10 min at 1000×g. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1 %) and the absorbance was measured at 700 nm in a spectrophotometer. In the control, the sample was replaced with an equal volume of ethanol. Increased absorbance of the reaction mixture indicates grater reduction capability[27].

RESULTS AND DISCUSSION

In this study, the antioxidant activity of *P. pungens* extracts was compared to BHA, BHT, gallic acid, quercetin, pyrocatechol, α-tocopherol and trolox. The antioxidant activity of the extracts, α-tocopherol, trolox, BHA and BHT was also evaluated in a series of the following *in vitro* tests: ABTS radical, DPPH free radical and superoxide anion radicals scavenging, total reducing activity and metal chelating activity.

$ABTS^{\bullet+}$ radical scavenging capacity: Radical scavenging activities are very important due to the deleterious role of free radicals in foods and biological systems. Excessive formation of free radicals accelerates the oxidation of lipids in foods and decreases food quality and consumer acceptance[28]. The improved technique for the generation of $ABTS^{\bullet+}$ described here involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through reaction between ABTS and potassium persulfate. As shown in Table-1, *P. pungens* extracts had $ABTS^{\bullet+}$ radical scavenging activity. The scavenging effect of *P. pungens* extracts and standards on the $ABTS^{\bullet+}$ decreased in the order: BHA > BHT > α-tocopherol > *P. pungens* flower water extract > *P. pungens* flower ethanol

TABLE-1
ABTS, SUPEROXIDE, DPPH SCAVENGING AND METAL CHELATING ACTIVITY OF *P. pungens* EXTRACTS AND SOME STANDARD ANTIOXIDANTS (%)

Samples	% ABTS scavenging activity (100 µg/mL)	% Superoxide anion scavenging activity (100 µg/mL)	% Metal chelating activity (100 µg/mL)	% DPPH• scavenging activity (100 µg/mL)
Control	0	0	0	0
Phlomis pungens - leaf ethanol extract	89.8	66.4	9.82	72.98
Phlomis pungens - leaf water extract	90.5	97.7	20.98	77.19
Phlomis pungens - flower ethanol extract	90.5	55.5	8.7	65.79
Phlomis pungens - flower water extract	90.6	98.3	23.43	73.68
BHA	99.9	99	50.22	-
BHT	97.3	88	27.01	81.44
α-Tocopherol	96.9	81	30.8	-
Trolox	-	-	-	92.27
Gallic Acid	-	-	-	90.75
Quercetin	-	-	-	77.18
Pyrocatechol	-	-	-	80.51

extract > *P. pungens* leaf water extract > *P. pungens* leaf ethanol extract, which were 99.9, 97.3, 96.9, 90.6, 90.5, 90.5 and 89.8 %, respectively, at the 100 µg/mL concentration.

DPPH• radical scavenging capacity: The model of scavenging the stable DPPH• radical is a widely used method to evaluate antioxidant activities in a relatively short time compare with other methods. The effect of antioxidants on DPPH• radical scavenging was presumed to be due to their hydrogen donating ability. DPPH• is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[29]. The reduction capability of DPPH• radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The decrease in absorbance of DPPH• radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progresses, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discolouration from purple to yellow. Hence, DPPH• is usually used as a substrate to evaluate antioxidative activity of antioxidants. The scavenging effect of *P. pungens* extracts and standards on the DPPH• decreased in the order: trolox > gallic acid > BHT > pyrocatechol > *P. pungens* leaf water extract > quercetin > *P. pungens* flower water extract > *P. pungens* leaf ethanol extract > *P. pungens* flower ethanol extract, which were 92.27, 90.75, 81.44, 80.51, 77.19, 77.18, 73.68, 72.98 and 65.79 %, respectively, at the 100 µg/mL concentration (Table-1).

Superoxide anion scavenging capacity: Superoxide anion radical included in free radical species is a factor that can induce aging and destruct the cell membrane and it can be generated by oxidative stress. They are produced *in vivo* by electron leakage from the mitochondrial electron transport chain, by activated phagocytes[30] and in the conversion of xanthenes to uric acid[31]. Superoxide anions are precursor to active free radicals that have the potential to react with biological macromolecules, thereby inducing tissue damage[32]. It has also been implicated in several pathophysiological processes due to its transformation into more reactive species, such as hydroxyl radical that initiate lipid peroxidation. Superoxide has also been observed to directly initiate lipid peroxidation[33]. It has also been reported that antioxidant properties of some flavonoids are effective, mainly *via* scavenging of superoxide anion radical[34]. Superoxide anion plays an important role in formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA[35].

In this method, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. Antioxidants are able to inhibit the blue NBT formation[36,37]. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Table-1 shows that the percentage inhibition of superoxide radical generation by 100 µg/mL concentration of *P. pungens* extracts and standards were found to be similar. As can be seen in Table-1, the scavenging effect of *P. pungens* extracts and standards on the percentage inhibition of superoxide anion radical generation by 100 µg/mL concentration in the order: BHA > *P. pungens* flower water extract > *P. pungens* leaf water extract > BHT > α-tocopherol > *P. pungens* leaf ethanol extract > *P. pungens* flower ethanol extract which were 99, 98.3, 97.7, 88, 81, 66.4 and 55.5 % superoxide anion radical scavenging activity, respectively.

Metal chelating activity: Transition metals have a major role in the generation of free oxygen radicals in living organisms. Iron exists in two distinct oxidation states; ferrous (Fe^{2+}) or ferric ions (Fe^{3+}). The ferric ion (Fe^{3+}) is the relatively biologically inactive form of iron. However, it can be reduced to the active Fe^{2+}, depending on the conditions, particularly pH[38] and oxidized back through Fenton type reactions, with production of hydroxyl radicals; or Haber-Weiss reactions with superoxide anions[39,40]. The production of these radicals can lead to lipid peroxidation, protein modification and DNA damage. Chelating agents may inactivate metal ions and potentially inhibit the metal-dependent processes[41].

Phenolic compounds are natural chelating agents in fresh foods, in addition to ascorbic acid, phosphorylated compounds and proteins[40,42]. Also, the production of highly reactive oxygen species, such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals is also catalyzed by free iron through Haber and Weiss[43]. Free iron is known to have low solubility

and a chelated iron complex, such as EDTA-Fe and has greater solubility in solution, which can be contributed solely from the ligand. Furthermore, chelated iron, such as EDTA-Fe, is also known to be active, since it can participate in iron-catalyzed reactions[40].

Ferrous ion chelating activities of *P. pungens* extracts, α-tocopherol, BHA and BHT are shown in Table-1. The chelating effect of ferrous ions by *P. pungens* extracts and standards was determined according to the method of Dinis *et al*.[25]. Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals *via* the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$). Fe^{3+} ion also produces radicals from peroxides, although the rate is tenfold less than that of Fe^{2+} ion[44]. Fe^{2+} ion is the most powerful pro-oxidant among various species of metal ions[32]. Chelation of the ferrous ions by *P. pungens* extracts was estimated by the ferrozine assay. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is inhibited and the red colour of the complex fades. By measuring the colour reduction, therefore, it is possible to estimate the chelating activity of the co-existing chelator[45]. In this assay, the natural compound interfered with the formation of the ferrozine-Fe^{2+} complex, suggesting that it has chelating activity and captures ferrous ions before ferrozine.

In fact, as shown in Table-1, *P. pungens* extracts disrupted the Fe^{2+}-ferrozine complex at 100 µg/mL concentration. *P. pungens* extracts exhibited, respectively, 43.08, 28.34, 25.13 and 25.44 % chelating of ferrous ion at the above concentration. On the other hand, at the 100 µg/mL concentration, the percentages of metal chelating capacity of BHA, BHT and α-tocopherol were found to be 72.99, 37.94 and 39.28 %, respectively. The metal scavenging effects of those samples decreased in the order of BHA > *P. pungens* flower water extract > α-tocopherol > BHT > *P. pungens* leaf ethanol extract > *P. pungens* leaf water extract > *P. pungens* flower ethanol extract.

Total reduction activity: Fig. 1 depicts the reducing activity of the *P. pungens* extracts and standards (BHA, BHT, α-tocopherol) using the potassium ferricyanide reduction method. For the measurements of the reductive activity, the Fe^{3+}-Fe^{2+} transformation was investigated in the presence of *P. pungens* extracts using the method of Oyaizu[26]. The reducing activity of *P. pungens* extracts, α-tocopherol, BHA and BHT increased with increasing concentration of samples. As can be seen in the Fig. 1, *P. pungens* extracts showed more effective reducing activity than control at different concentrations. Reducing power of *P. pungens* extracts and standard compounds are as follows: BHA > BHT > *P. pungens* leaf water extract > α-tocopherol > *P. pungens* leaf ethanol extract > *P. pungens* flower ethanol extract > *P. pungens* flower water extract.

Natural antioxidants have biofunctionalities such as the reduction of chronic diseases like DNA damage, mutagenesis, carcinogenesis, *etc*. and inhibitions of pathogenic bacteria growth, which are often associated with the termination of free radical propagation in biological systems[46,47]. Thus, for medicinal bioactive components, antioxidant capacity is widely used as a parameter. A number of assays have been introduced to measure the total antioxidant activity of pure compounds[48]. Many studies have shown that natural antioxidants in plants are closely related to their biofunctionalities such as the reduction of chronic diseases and inhibition of pathogenic bacteria growth, which are often associated with the termination of free radical propagation in biological systems[49]. Thus, antioxidant capacity is widely used as a parameter to characterize food or medicinal plants and their bioactive components.

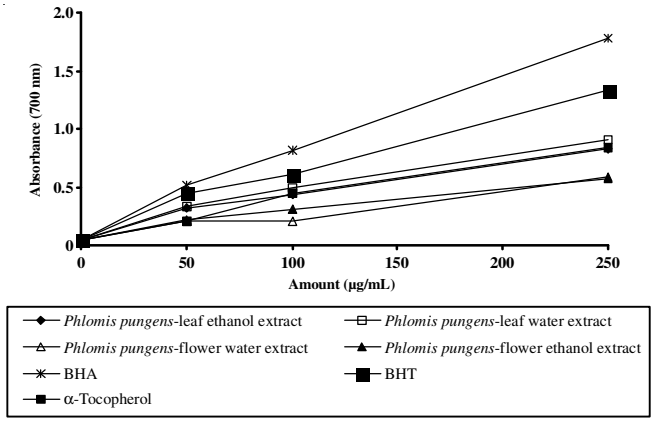

Fig. 1. Total reduction activity of *P. pungens* extracts and some standard antioxidants

Conclusion

This study demonstrated the potential antioxidant properties of the *P. pungens* extracts, from Sivrice, Elazig in Turkey. According to data of the present study, *P. pungens* extracts were found to be effective antioxidants in different *in vitro* assays including reducing power, $DPPH^{\bullet}$ scavenging, $ABTS^{\bullet+}$ scavenging and superoxide anion radical scavenging and metal chelating activities when compared to standard antioxidant compounds, such as synthetic antioxidants (BHA, BHT), α-tocopherol, a natural antioxidant and trolox, which is a water-soluble analogue of α-tocopherol.

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