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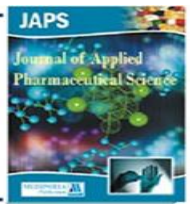

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Determination of antioxidant and antimutagenic activities of *Phlomis armeniaca* and *Mentha pulegium*

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ABSTRACT

This study was designed to determine antioxidant and antimutagenic activities of methanol and *n*-hexane extracts of *Phlomis armeniaca* and *Mentha pulegium* and their phenolic compounds. The extracts were screened for their possible antioxidant and antimutagenic activities by DPPH and ABTS free radical scavenging, reducing power, metal chelating and DNA nicking assays. The methanol extracts of the plants exhibited significant antioxidant activities determined by different assays. *P. armeniaca* showed higher activities in antioxidant assays. Also, the highest phenolic content was observed in *P. armeniaca*. In DNA nicking assay (antimutagenic), all extracts of these plants (20-40 µg/mL) exhibited DNA protecting activities. This study shows that methanol extracts of the plants have higher antioxidant activities than their hexane extract. Moreover, the plants can be used as natural antioxidants and antimutagenic sources.

Keywords: *Phlomis armeniaca, Mentha pulegium*, Antioxidant/antimutagenic activity, phenolic compounds, flavonoid, flavonol.

INTRODUCTION

ROS (Reactive oxygen species) are produced in living systems and the most common sources of ROS arise from leakage of electrons to O_2 from the electron transport chains of mitochondria (Muller, 2001). ROS can damage to biomolecules, resulting in several diseases. Living systems have specific pathways to overcome the adverse affects of various damages. Although sometimes these repair mechanisms fail to keep pace with such deleterious effects (Halliwell, 1995). Moreover, antioxidant agents such as phenolic compounds can decrease harmful effects of ROS (Rice-Evans *et al*., 1997) Phenolic compounds are constituents of both edible and non edible parts of plants (Amarowicz *et al.*, 2010). These compounds were observed in different levels and types in various parts of plants (Konczak *et al.*, 2010). Phenolics have attracted increasing attention for their antioxidant behaviour and beneficial health-promoting effects. It is assumed that many antioxidative phenolic compounds in plants are usually presented in a covalently-bound form (Xu *et al*., 2007).

They can act as antioxidants by donating hydrogen to highly reactive radicals, thereby preventing further radical formation (Lapornik *et al.*, 2005). The Lamiaceae include about 200 genera and 3000 species. Several studies have reported on the antioxidant activity of the Lamiaceae growing in various region of the World (Barros *et al.*, 2010; Kamatou *et al.*, 2010). But there are a few reports about especially South East of Turkey. As far as our survey, there are not so many detailed reports on antioxidant and antimutagenic activities and phenolic compounds of methanol and hexane extracts of *Phlomis armeniaca* and *Mentha pulegium* wildly growing in south east of Turkey. The aim of this study was examined the antioxidant and antimutagenic activities of the methanol and n-hexane extracts of the plants using several tests: DPPH, ABTS, reducing power, metal chelating, *β*-carotene linoleic acid inhibition and DNA nicking assays and was to detect amounts of phenolic by current methods.

MATERIALS AND METHODS

Collection of plant materials

P. armeniaca and *M. pulegium* were collected at flowering stage from Gaziantep-Turkey. Herbarium information of the plant species, which are individually numbered, is listed below:
I- *P. armeniaca*: Kizilhisar Village, meadow-steppe place, Gaziantep-Turkey; 29th July 2008
II- *M. pulegium*: Sof Mountain, Gaziantep-Turkey; 21th May 2008
The voucher specimens have been deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sivas-Turkey (CUFH-Voucher NO:1 AA 4898 and 2-AA 4899, respectively).

Preparation of methanol and hexane extracts

The collected plants were washed with distilled water and dried in air-dark. Then, the plants were powdered by a domestic blender. The air-dried and powdered leaves of the plants (20 g) were extracted successively with 500 ml of the hexane and methanol by using Soxhlet extractor (Gherart, Germany) for 48 h at 40 ˚C. The extracts were then concentrated in vacuo at 40 º C using a Rotary evaporator. Then the extracts were kept in the dark at +4 º C until tested.

ANTIMUTAGENIC ACTIVITY

DNA Nicking Assay

The ability of methanol and hexane extracts to protect super coiled pBR 322 DNA from devastating effects of hydroxyl radicals generated by Fenton's reagent was assessed by the DNA nicking assay described by Lee *et al*. (2002). The reaction mixture contained 1 µl of plasmid DNA. 10 µl Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid and 80 mM $FeCl_3$) followed by the addition of extracts and the final volume of the mixture was brought up to 20 µl using distilled water.

The mixture was then incubated for 30 min at 37 ºC and the DNA was analyzed on 1% agarose gel (prepared by dissolving 0.5 g of agarose in 50 ml of 1X TBE Buffer) followed by ethidium bromide staining. Quercetine was used as a positive control.

ANTIOXIDANT ACTIVITIES

Determination of DPPH free anion radical scavenging activity

DPPH activity of the plant extracts was determined according to the method of Gaulejac *et al*., (1998) with minor changes (Zhao *et al*., 2008). Every plant extract (0.1 ml) was added to 2.9 ml of 0.06 mM methanolic solution of DPPH. The absorbance at 517 nm was measured after the solution had been allowed to stand in the dark for 60 min. The Trolox calibration curve was plotted as a function of the percentage of DPPH radical scavenging activity. The final results were expressed as millimole of TE per gram of dry weight (mmol TE/g dw). Tests were carried out in triplicate.

Determination of ABTS radical cation scavenging activity

The radical scavenging activity of the methanol and hexane extracts against ABTS radical cation was measured using the method of Re *et al*., (1999) with some modifications (Zhao *et al.*, 2008). ABTS was dissolved in water to a 7 mmol/l concentration. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mmol/l potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature (24 ºC) for 12-16 h before use. The ABTS radical cation solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 ºC. Each plant extract (0.1 ml) was mixed with 2.9 ml of diluted ABTS radical cation solution. After reaction at 30ºC for 20 min. the absorbance at 734 nm was measured. The Trolox calibration curve was plotted as a function of the percentage of ABTS radical cation scavenging activity. The final results were expressed as millimole of TE per gram of dry weight (mmol TE/g dw).

Determination of reducing power activity

The determination was carried out as described by Oktay *et al*., (2003). Briefly, 1 ml of the plant extracts was mixed with phosphate buffer (2.5 ml, 0.2 mol/l, pH 6.6) and $K_3Fe(CN)_6$ (2.5 ml, 1%). The mixture was incubated at 50 ºC for 20 min. A portion (2.5 ml) of TCA (10%) was added to the mixture, which was then centrifuged at 10000g for 10 min. The upper layer of solution (2.5 ml) was mixed with deionized water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%). and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. The measurement was compared to a standard curve of prepared ascorbic acid (AA) solution, and the final results were expressed as millimole of AA equivalents (AAE) per gram of dry weight (mmol AAE/g dw).

Determination of metal chelating activity

The chelating activity of the plant extracts for ferrous ions was measured following the ferrozine method with minor modifications (Dinis *et al.* 1994). The reaction mixture contained 0.5 ml of plant extracts and 0.05 ml of $FeCl_2$ (2 mmol/l). After 5 minute, the reaction was initiated by the addition of 5 mmol/l ferrozine (0.1 ml) and the total volume was adjusted to 3 ml with 80% acetone solution.

Then, the mixture was shaken vigorously and incubated at room temperature for 10 min. Absorbance of the solution was measured at 562 nm. The EDTA calibration curve was plotted as a function of the percentage of metal chelating activity. The final results were expressed as millimole of EDTAE per gram of dry weight (mmol EDTAE/g dw).

DETERMINATION OF PHYTOCHEMICALS

Determination of total phenolic content

Folin–Ciocalteu procedure given by Yu *et al.*, (2002) was used to estimate the total phenolic contents in the methanol and hexane extracts of the plants. Following this method, 0.1 ml of fractions was diluted to 1 ml with distilled water. To this solution 0.5 ml of Folin–Ciocalteu reagent (2N, 1:1) and 1.5 ml of 20% sodium carbonate solution was added. The mixture was incubated for 2 h at room temperature. The volume was raised to 10 ml with distilled water and the absorbance of blue colored mixture was measured at 765 nm (Cintra 202 UV- Vis Spectrophotometer, Victoria-Australia). The amount of total phenol was calculated as mg GAE/g from calibration curve of gallic acid standard solution.

Determination of total flavonoids

The flavonoids content was determined by aluminium trichloride method using quercetine as a reference compound (Kumaran and Karunakaran, 2006). This method based on the formation of a complex flavonoid-aluminum having the absorptivity maximum at 415 nm. About 100 µl of plant extracts in methanol (10 mg/ml) was mixed with 100 µl of 20% aluminium trichloride in methanol and a drop of acetic acid (100 %), and then diluted with methanol to 5 ml. The absorption at 415 nm was read after 40 min. Blank samples were prepared from 100 µl of plant extracts and a drop of acetic acid and then diluted to 5 ml with methanol. The absorption of standard quercetine solution (0.5 mg/ml) in methanol was measured under the same conditions. All determinations were carried out in triplicates. The amount of flavonoids in plant extracts in QE was calculated by the following formula:

$$X = (A \,.m_0) / (A_0 \,.\, m)$$

where X is the flavonoid content, mg/mg plant extract in QE. A is the absorption of plant extract solution. A_0 is the absorption of standard quercetine solution, m is the weight of plant extract, mg and m_0 is the weight of quercetine in the solution, mg.

Determination of total flavonols

The amount of flavonols was determined by using quercetine as a reference compound. This method also based on the formation of complex with maximum absorption at 440 nm. About 1 ml of each methanolic plant extract (10 mg/ml) was mixed with 1 ml aluminium trichloride (20 mg/ml) and 3 ml sodium acetate (50 mg/ml). The absorbance at 440 nm was read after 2.5 h. The absorption of standard quercetine solution (0.5 mg/ml) in methanol was measured under the same conditions (Kumaran and Karunakaran 2006). All determinations were carried out in triplicates. The amount of flavonols in the plant extracts was calculated by the same formula of flavonoids:

$$X = (A \,.m_0) / (A_0 \,.\, m)$$

where X is the flavonol content, mg/mg plant extract in QE. A is the absorption of plant extract solution. A_0 is the absorption of standard quercetine solution, m is the weight of plant extract, mg and m_0 is the weight of quercetine in the solution, mg.

RESULT AND DISCUSSION

Figure 1 and Figure 2 shown protecting activity against hydroxyl radical of these plant extracts. The concentration dependent (20–40 µg/mL) antimutagenic effect of the extracts was studied on plasmid DNA pBR322 damage. As can be seen Figure 1, the methanol extracts (20-40 µg/mL) of *P. armeniaca* and *M. pulegium* showed significant reduction in the formation of nicked DNA and increased native form of DNA. The methanol extracts have high amounts of phenolic compounds due to polarity characteristic. And so, these extracts showed significant activities. The phenolic compounds are potential protecting agents against the lethal effects of oxidative stress and offer protection of DNA by chelating redox-active transition metal ions (Prakash *et al.*, 2007). The *n*-hexane extracts (20-40 µg/mL) showed effectively DNA protecting activity (Figure 2). Kaur *et al.*, (2008) reported that chloroform, ethyl acetate and *n*-butanol extracts of *Chukrasia tabularis* showed higher activities on DNA protection when compared to methanol and water extracts. In our study, similar activities on DNA were obtained. It can be said that both methanol and *n*-hexane extracts of tested plants may protect against DNA damage caused by reactive oxygen species.

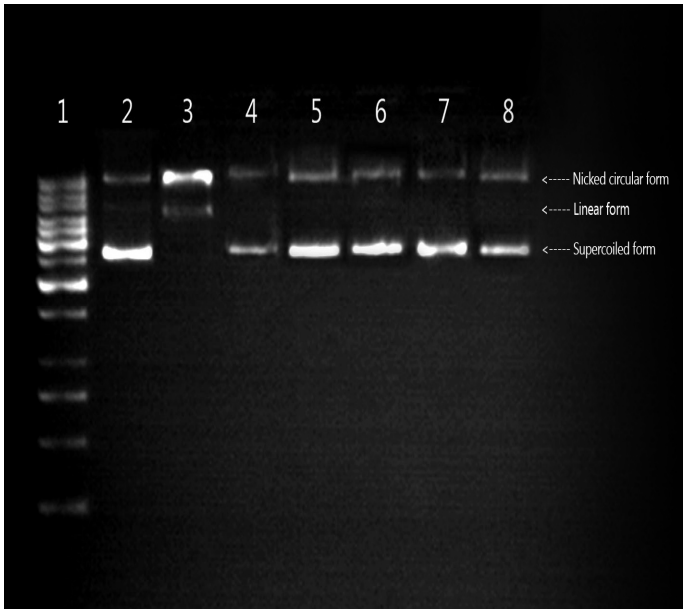

Fig. 1: Protective effect of the methanol extracts (20 and 40 µg/ml, respectively) of plants in DNA nicking caused by hydroxyl radical. Line 1: Marker. Line 2: Control (Distilled water + DNA). Line 3: Negative control (DNA + Fenton's reagent). Line 4: positive control (DNA + Quercetine + Fenton's reagent) line 5 and 6: *P. armeniaca* +DNA+ Fr, Line 7 and 8: *M. pulegium*+DNA+ Fr.

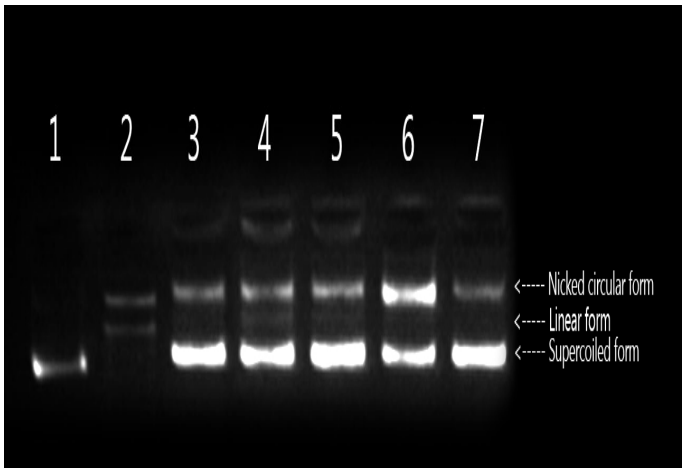

Fig.2: Protective effect of the hexane extracts (20 and 40 µg/ml, respectively) of plants in DNA nicking caused by hydroxyl radical. Line 1: Control (Distilled water + DNA). Line 2: Negative Control (DNA + Fenton's reagent). Line 3: positive control (DNA + Quercetine + Fenton's reagent) line 4 and 5: *P. armeniaca* +DNA+ Fr, Line 6 and 7: *M. pulegium* +DNA+ Fr.

Antioxidant activities

Determination of DPPH free anion radical scavenging activity

The methanol and hexane extracts were subjected to screening for their possible antioxidant activity by DPPH scavenging assay. The DPPH scavenging activities of the methanol extracts from *P. armeniaca* and *M. pulegium* were 45.68 ± 0.12 and 22.45 ± 0.47 mmol TE/g dw, respectively. The hexane extracts showed poorer activity in DPPH assay when compared to methanol extracts. The DPPH activity in hexane extracts was determined as 0.76±0.02 and 0.98±0.01 mmol TE/g dw, respectively.

Determination of ABTS free radical cation scavenging activity

The methanol extracts exhibited a strong radical scavenging activity when reacted with the $ABTS^{+}$ radicals. In contrast, the hexane extracts showed poorer activities when compared to methanol extracts. While the ABTS scavenging activities of the methanol extracts from *P. armeniaca* and *M. pulegium* were determined as 39.76 ± 1.32 and 11.23 ± 0.54 mmol TE/g dw, respectively, activities of hexane extracts were 1.45±0.14 to 2.13±0.10 mmol TE/g dw. It can be suggested that methanol extracts of the plants might have significant effect for free radical scavenging.

Determination of reducing power activity

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al.* 1995). The methanol extracts exhibited significant reducing power activity. The reducing power activity for methanol extracts of *P. armeniaca* and *M. pulegium* was 55.21±0.45 and 35.77±0.73 mmol AAE /g dw, whereas activity of the hexane extracts was 0.81±0.03 and 0.47±0.17 mmol AAE/g dw, respectively. According to our results, it can be said that methanol extracts of plants have significant activities on the reducing power activity.

Determination of metal chelating activity

Chelating agents may inhibit radical generations by stabilizing transition metals consequently reducing free radical damage. In addition, some phenolic compounds exhibit antioxidant activity through the chelating of metal ions (Zhao *et al.,* 2008). The chelating activity of extracts was evaluated against Fe^{2+} for their antioxidative potentials. The methanol extracts exhibited stronger metal chelating activity than the hexane extracts. While chelating activity of the methanol extracts of *P. armeniaca* and *M. pulegium* was 3.35±0.23 and 18.00±031 mmol EDTAE/g dw, activity of the hexane extracts was 1.45±0.01 to 1.57±0.01 mmol EDTAE/g dw, respectively.

Determination of bioactive compounds in plant extracts

Determination of total phenolics (TPC)

The content of total phenolics in the extracts was determined and was expressed as milligrams of gallic acid equivalent per gram of dry weight. Amounts of TPC of methanol extracts were determined in the tested plants. The phenolic amount in methanol extract of *P. armeniaca* was found as 320.37 ± 6.97 mg GAE/g, whereas that of *M. pulegium* was 206.58± 4.54 mg GAE/g. Phenolic content of the hexane extracts was 55.90±1.01 and 50.31±1.13 mg GAE/g. Phenolic compounds are commonly found in both edible and inedible plants and they have been reported to have multiple biological effects including antioxidant activity. The antioxidant activity of phenolic compounds is mainly due to their redox properties which can play an important role in adsorbing and neutralising free radicals quenching singlet and triplet oxygen or decomposing peroxides (Osawa 1994).

Determination of total flavonoid and flavonol contents

Studies on the free radical-scavenging properties of flavonoids have permitted characterization of the major phenolic components of naturally occurring phytochemicals as antioxidants (Rice-evans *et al.,* 1996). While total flavonoid amounts of the methanol extracts was 46.54±1.05 to 77.12± 2.93 mg QE/g dw, that of the hexane extracts was 34.34 ± 1.20 and 40.01± 1.58 mg QE/g dw, respectively. The aromatic rings of the flavonoid molecule allow the donation and acceptance of electrons from free radical species (Kanner *et al.* 1994). Flavonols are a class of flavonoids that have the 3-hydroxyflavone backbone. Their diversity stems from the different positions the phenolic -OH groups. Flavonols are present in a wide variety of fruits and vegetables (Cermak *et al.* 2006). The flavonol contents of the methanol extracts were 22.78 ± 1.80 and 30.28 ± 1.02 mg QE/g dw, that of the hexane extracts were 14.02 ± 1.09 and 20.29 ± 0.65 mg QE/g dw.

CONCLUSION

This study is a detailed report on the antioxidant and antimutagenic activities of methanol and hexane extracts obtained from *P. armeniaca* and *M. pulegium* species of Lamiaceae collected from Turkey.

The extracts were found having various levels of antioxidant activities in all test systems. In antimutagenic activity assay, all of extracts exhibited strong protection effect against hydroxyl radical. Moreover, results showed that the methanol extracts had high antioxidant activities and total phenolic contents. According to the results, the amounts of phenolic contents in extracts are significant for their antioxidant activities. In addition varieties and amounts of phenolic compounds such as flavonoid and flavonol in crude extracts may be important for antioxidant and antimutagenic activities. The antioxidant potentials of these plants could provide a chemical basis for some areas of food industries, health benefits, medicine and pharmacology.

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