

BIOACTIVITY OF MAJOR COMPONENTS FROM THE SEEDS OF *BUNIUM PERSICUM* (BOISS.) FEDTCH

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ABSTRACT

Zire Kermani (*Bunium persicum*) is an Iranian plant which is commonly used as antispasmodic, carminative, anti obesity and lactogage. The essential oil and different extracts of the seeds of this plant were evaluated for antioxidant activity by three complementary methods: DPPH assay, β -carotene bleaching and ammonium thiocyanate methods. The oil and methanolic extract of *B. persicum* exhibited the highest antioxidant activity with $IC_{50} = 23.4 \pm 1.6$ and $45.7 \pm 3.6 \mu\text{g mL}^{-1}$ in DPPH assay and the most inhibition of β -carotene oxidation and lipid peroxidation. The GC/MS analysis of the essential oil of *B. persicum* indicated the γ -Terpinene (46.1%) and cuminaldehyde (15.5%) as the major components of the oil. Furthermore the active methanol extract of the plant was fractionated by column chromatography to afford several fractions, of which in more purification, kaempferol, caffeic acid and *p*-coumaric acid were found to be the antioxidant components of this extract. These results confirm the *in vitro* antioxidant and radical scavenging activity of the essential oil and methanolic extract of *B. persicum*, which warranty the use of the plant in many traditional uses.

Keywords: *Bunium persicum*; Apiaceae; seeds; antioxidant activity; essential oil, kaempferol, phenolic acids.

INTRODUCTION

Oxidative damages have been known as an important cause of many of diseases. Free radicals attack cell membrane which may trigger different disorders. Antioxidants can react with these free radicals and prevent from lipid peroxidation of cell membrane (Lai, Chou *et al.*, 2001). Plant- derived antioxidants can scavenge the oxygen free radicals and protect cell by counteracting reactive oxygen species (ROS). The health promoting effects of plants have found to be due to some of bioactive substances such as essential oils, flavonoids and phenolic compounds which have antioxidant activity (Liu, 2003).

Bunium persicum (Boiss.) B. Fedtsch. (syn. *Carum persicum* Boiss.), a grassy plant, is endemic to Iran. This plant belongs to the Apiaceae family and is predominantly found in the mountainous regions in Kerman, Iran (Rechinger, 1989). The seeds of this plant have been widely used as an additive in food stuff such as in bread cooking, rice and yoghurt for its carminative, anti dyspepsia and antispasmodic effect (Zargari, 1996). Anti fungal effect of this plant has also been reported (Sekine *et al.*, 2007). Regarding the pleasant flavour and taste of the seeds of *B. Persicum*, our aim was to identify the major antioxidant compounds of the essential oil and methanolic extract of the plant by various complementary methods.

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MATERIALS AND METHODS

Plant material

The seeds of *Bunium persicum* (Boiss.) Fedtch. (Apiaceae) were collected in June and July, 2006, from the wild plants which grow in the Koochpayeh, Kerman, Iran. The taxonomic identification of the plant was confirmed by Dr. Mozaffarian, Department of Botany of the Research Institute of Forests and Rangelands (TARI), Tehran, Iran. A voucher specimen (KF 1141) was deposited in the Herbarium center of the Kerman Faculty of Pharmacy, Kerman, Iran.

Chemicals

Diphenylpicrylhydrazil (DPPH), β -carotene, linoleic acid and butylated hydroxyl toluene (BHT) were prepared from Sigma Chemical Co (Germany). Caffeic acid and *p*-coumaric acid, Rothchrom-Chr, 97% (Roth, Iran). The other used materials were also of analytical grade. The silica gel G₆₀ pre coated plates (35-70 μm , Merck, Iran) was used for analytical thin-layer chromatography.

Phytochemical screening

The seeds of *Bunium persicum* were studied for the presence of flavonoids, saponins, alkaloids and tannins (Trease, 2002).

Extraction and essential oil isolation

Dried seeds (500g) of *B. persicum* were grounded and extracted by percolation method with petroleum ether, chloroform, methanol and water successively. Solvents

were removed in a rotary evaporator and extracts were concentrated to dryness. The essential oil of the plant (100 g) was isolated using water-distillation method with cleavenger apparatus and stored at 2-8°C until testing.

Gas chromatography/mass spectrometry analysis

GC analysis of the oil was done by a Shimadzu QP 5000 (FID) chromatograph HP-5 MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm). Helium was used as carrier gas at a flow rate 1 mL/min (split ratio 1:20) with injection volume of 0.2 μL. Injector and detector temperatures were set at 220 and 290 °C, respectively. Oven temperature was kept at 50 °C for 3 min, gradually raised to 160 °C at 3 °C/min, held for 10 min and finally raised to 240 °C at 3 °C/min. GC/MS analysis was carried out using a Shimadzu QP 5050 operating at 70 eV ionization energy, equipped with an HP-5 capillary column (phenyl methyl siloxane, 30 m × 0.25 mm, 0.25 μm film thickness) with Helium as the carrier gas (split ratio 1:20). Retention indices were determined by using retention times of n-alkanes that had been injected after the oil under the same chromatographic conditions. The components were identified based on the comparison of their relative retention time and mass spectra with those of standards, Wiley 2001 library data of the GC-MS system and literature data (Adams, 2001). Alkanes were used as reference points in the calculation of relative retention indices (RRI).

Antioxidant activity

DPPH assay and DPPH assay on TLC

The method of Burits was used for evaluation of inhibitory activity of *B. persicum* against DPPH radical (Burits *et al.*, 2001). Fifty microliters of each concentration of the methanolic extract was added to 5 ml of methanol solution of DPPH (0.004%), 30 min incubated at room temperature and absorbance was measured at 517 nm. BHT was used as positive control. The percent of inhibition was calculated in following ways following:

$$I\% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$$

A_{blank} is the absorbance of the control reaction which contains all of the components without the tested sample), and A_{sample} is the absorbance of the tested sample. IC_{50} (concentration of the sample which induces 50% inhibition) of each sample was calculated from the graph plotting inhibition percentage against extract concentration. TLC assay of DPPH scavenging was done as the same reference.

B- carotene bleaching test

A mixture of an ethanolic solution of linoleic acid (10 ml of 2 mg/ml) and acetone solution of β-carotene (10 ml, 2mg/ml) with molten agar (10 ml, 1.2% solution in boiling water) was provided, followed by shaking to give an orange color. 25 mL of the molten agar was added to

each petri dish, stored in dark place and allowed the agar to set. Each ethanolic extract (1mg) was poured into the holes punched into the agar and the petri dishes were incubated at 45°C for 4 h. Samples with antioxidant activity exhibited a zone of color retention around the hole after incubation. The zone diameter was measured using vernier calipers (Graven *et al.*, 1992). BHT was used as positive control.

Ammonium thiocyanate method (ATC)

A solution of sample in deionized water (1mg/mL) was added to a mixture of a buffered emulsion of linoleic acid (2.5ml, 0.02M, pH 7.0 in phosphate buffer) with phosphate buffer (2ml, 0.2M, pH 7.0). At various intervals during the incubating at 37°C, aliquots of 0.1ml was tested. For determination of oxidation, 4.7 mL of ethanol 75%, 0.1 mL of a solution of 30% ammonium thiocyanate solution and 0.1mL of ferrous chloride 0.02M in 3.5% HCl were added sequentially. The absorbance was measured at 500nm after 3 minutes by a spectrophotometer (Shimadzu, 1200, Japan) indicates the peroxide value (Masude *et al.*, 1992). The linoleic acid without any extracts was used as control.

Separation of the antioxidant compounds from the methanolic extract

One half of the methanolic extract of the plant was fractionated by column chromatography using silica gel 60 (70-230 mesh) as stationary phase, using different composition of petroleum ether- chloroform (9:1, 8:2, 5:5, v/v); chloroform- EtOAc (9:1, 8:2, 5:5, v/v), EtOAc- MeOH (9:1, 8:2, 5:5, v/v) and MeOH to give three major fractions (BP1-BP3). Fraction BP3 which showed the most antioxidant activity was further purified by TLC method using silica gel with EtOAc / water/ formic acid/ glacial acetic acid (100:26:11:11) and afforded fractions 1-3 and 3-7. The more purification of fractions 1-3 (the most antioxidant active fractions) was carried out by Sephadex LH-20 with methanol to give two major compounds (compound A and B).

The second half of methanolic extract was concentrated to dryness, re-dissolved in 50 mL distilled water and extracted three times with diethyl ether, ethyl acetate and n-butanol sequentially. All of the extracts were evaporated to dryness using N₂. Ethyl acetate and n-butanol extracts were concentrated and dissolved in 80% methanol. n-Butanol extract (500 mg) was hydrolyzed in 30 mL HCl (1 mol L⁻¹) for 1 h in a boiling water bath and then was treated with diethyl ether. Separation of phenolic acids was varied out on TLC plates using dichloromethane/ toluene/ formic acid (5:4:1). Authentic phenolic acids were used as standard. The presence of phenolic acids of the methanolic extract of the plant was confirmed by comparing their R_f values with that one of the standard. These isolated phenolic acids were evaluated for antioxidant activity again using DPPH assay on TLC.

STATISTICAL ANALYSIS

Data was reported as mean \pm standard deviation. The group means were compared using the one-way ANOVA test and the Tukey post hoc test was applied to compare the means. Values were determined to be significant when $p < 0.05$.

RESULTS AND DISCUSSION

Primary phytochemical studies of the plant confirmed the presence of flavonoids and tannins in the plant.

Hydrodistillation of *B. persicum* seeds yielded the pale yellow-colored essential oil (8.3% v/w). Twenty-four compounds were identified in the oil which constitute about 97.20% of this oil. This oil was composed of hydrocarbon and oxygenated monoterpenes (95.9%) (table1). γ -Terpinene was identified as the major component of the *B. persicum* essential oil. The other main components of this oil were found to be cuminaldehyde (15.5%), ρ -cymene (6.7%) and limonene (5.9%). In all of the three antioxidant methods, the plant essential oil has shown the highest antioxidant activity among the tested samples.

Table 1: The result of Chemical composition of *B. persicum* essential oil by GC/MS¹

| Peak No. | Component | KI ² | Composition (%) |
|----------|-------------------------------|-----------------|-----------------|
| 1 | α - Thujene | 926 | 0.4 |
| 2 | α - Pinene | 936 | 2.7 |
| 3 | Sabinene | 968 | 1.0 |
| 4 | β -Pinene | 976 | 2.5 |
| 5 | Myrcene | 990 | 1.8 |
| 6 | ρ -Cymene | 1016 | 6.7 |
| 7 | α -Terpinene | 1019 | 1.3 |
| 8 | σ -Cymene | 1021 | 0.2 |
| 9 | Limonene | 1029 | 5.9 |
| 10 | γ - Terpinene | 1060 | 46.1 |
| 11 | α - Terpeneolene | 1087 | 0.9 |
| 12 | ρ -Mentha-3-ene-7-al | 1138 | 0.9 |
| 13 | Terpinene-4-ol | 1160 | 0.2 |
| 14 | α - Terpeneol | 1168 | 2.2 |
| 15 | ρ -Mentha-1,3 diene-7-al | 1176 | 0.2 |
| 16 | Cuminaldehyde | 1243 | 15.5 |
| 17 | Cuminy alcohol | 1265 | 7.4 |
| 18 | β - Caryophyllene | 1419 | 0.2 |
| 19 | γ -Eleman | 1435 | 0.1 |
| 20 | β - Bisabolene | 1478 | 0.5 |
| 21 | β - Selinene | 1488 | 0.1 |
| 22 | Myristicin | 1491 | 0.1 |
| 23 | Germacrene B | 1558 | 0.1 |
| 24 | Dillapiol | 1631 | 0.2 |
| Total | | | 97.2 |

¹Diluted 1/100 in acetone v/v, Relative percentages of the compounds were obtained electronically from FID area percent data.

²Kovats index on non-polar HP-5 MS column in reference to *n*-alkanes.

In DPPH assay, the oil and all of extracts of *B. persicum* significantly inhibited DPPH radical in comparison to control ($p < 0.05$). These samples have notably reduced the DPPH radical to diphenylpicrylhydrazil with an IC₅₀ of 23.4 ± 1.6 and $36.1 \pm 2.8 \mu\text{g mL}^{-1}$ respectively in comparison to BHT (IC₅₀ = $20.3 \pm 1.9 \mu\text{g mL}^{-1}$) (table 2). The oil and methanolic extract of *B. persicum* also exhibited the highest antioxidant activity with the mean zone of color retention of 26.2 ± 0.8 and $18.7 \pm 1.5 \text{ mm}$ respectively in the β -carotene bleaching test (table 2). This activity is comparable to one of BHT ($30.4 \pm 1.2 \text{ mm}$).

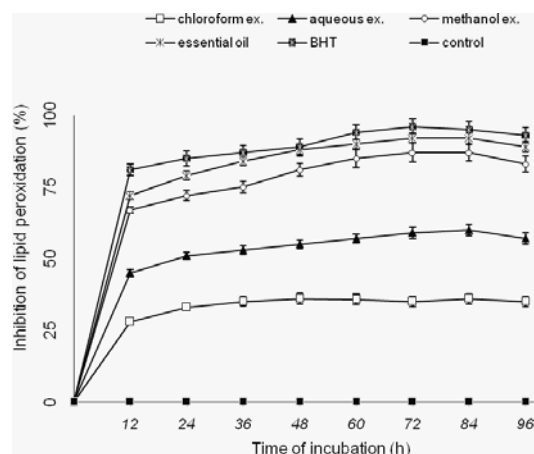


Fig. 1: Antioxidant activity of the essential oil and various extracts from *B. persicum* using ammonium thiocyanate method

Data represent means \pm SD of three independent experiments performed in triplicate. All tested samples in the time of incubation were significantly different from control ($p < 0.001$).

The antioxidant activity of the essential oil and different extracts of *B. persicum*, in ATC test have shown in fig. 1. These results showed that all of the samples have shown the most inhibition of lipid peroxidation in 72 h after incubation. The essential oil and methanolic extract of *B. persicum* strongly inhibited oxidation of linoleic acid (%93.5 and 88.0% inhibition respectively). Antioxidant activity of γ -terpinene as the major component in this oil has been reported in the literature (Ruberto and Baratta, 2000). The essential oils rich of monoterpene hydrocarbons show higher antioxidant activity. This activity is attributed to the presence of methylene groups in monoterpene hydrocarbons which is strongly active (Tepe *et al.*, 2005). The fraction A, from methanolic extract, gave a yellow amorphous powder: mp $279\text{-}280^\circ\text{C}$. The methanol UV spectrum of "A" exhibited two major absorption bands at 265nm (band II) and 367nm (band I) and one shoulder at 322nm. Decomposition of the compound A in NaOMe reagent confirmed the presence of free hydroxyl groups at carbons of C-3 and C-4'. A bathochromoc shift of 25 nm in band I with AlCl_3 and AlCl_3/HCl indicated the free hydroxyl groups at C-5

Table 2: The yield of extraction and antioxidant activity of the essential oil and various extracts of *Bunium persicum* using two methods of free radical scavenging capacities measured in DPPH assay and antioxidant activity in terms of mean zone of β -carotene color retention (mm).

| Sample | Yield of extract or oil (%) | IC ₅₀ for DPPH scavenging activity ^a ($\mu\text{g mL}^{-1}$) | Zone of β -carotene color retention (mm) ^a |
|-------------------------|-----------------------------|--|---|
| Essential oil | 8.3 | 23.4 \pm 1.6 ^b | 26.2 \pm 0.8 ^b |
| Petroleum ether extract | 6.4 | 45.7 \pm 3.6 ^b | 13.1 \pm 1.6 ^b |
| Chloroform extract | 3.1 | 79.6 \pm 6.2 ^b | 4.2 \pm 0.2 |
| Methanol extract | 7.4 | 36.1 \pm 2.8 ^b | 18.7 \pm 1.5 ^b |
| Aqueous extract | 1.3 | 49.8 \pm 4.1 ^b | 6.7 \pm 1.1 |
| BHT | - | 20.3 \pm 1.9 ^b | 30.4 \pm 1.2 ^b |
| Control (methanol) | - | 0 | 0 |

^a Means \pm SD of three independent experiments performed in triplicate.

^b Different from the control ($p < 0.001$)

position. A bathochromic shift of 20nm in band II with NaOAc indicating the free hydroxyl groups at C-7 position. The IR spectrum of compound A in KBr showed absorbance bands at 3451:3350 indicating the presence of hydroxyl group (OH), and absorbance band at 1671 presenting carbonyl group (C=O), and several bands at 2950 (CH), 1611 (C-C), 1671 and 825 cm^{-1} . The ¹H-NMR spectrum of the compound A in dimethylsulfoxide-d₆ (400 MHz, DMSO-d₆) exhibited one proton signal at δ : 6.23 (1H, J= 1.95 Hz) and at 6.45 (1H, d, J= 1.95 Hz) indicating the protons at C-6 and C-8. The signals of protons of 3', 5' presented at 6.95 (2H, d, J= 8.4 Hz) and the signals for protons of 2', 6' were observed at 8.1 (2H, d, J=8.3). The appearance of a signal at 12.47 (1H, brs) was assigned to a hydroxyl proton at C-5. In ¹³C-NMR spectrum in 100.0 MHz, DMSO-d₆, signals at δ : 174.90 indicated the presence of C-4. The carbon resonance of C-7, C-5 and C-4' were observed at δ : 165.8, 158.7 and 159.3. Three carbon resonances were observed at 154.1, 147.2 and 137.1 were attributed to C-9, C-2 and C-3 respectively. The signals of C-2', 6', C-1', C-1', C-3', 5', C-10, C-6 and C-8 were assigned at δ : 128.5, 120.4, 116.5, 102.8, 96.0 and 93.6. The melting point and spectral data (UV, IR, ¹H and ¹³C NMR) of the compound A were correlated to those one of reference value and was identified as kaempferol (Markham and Chari, 1982) which show antioxidant activity. The other compound (B), exhibited no antioxidant activity.

The hydrolyzed butanol fraction from methanolic extract of *B. persicum* seeds was analyzed by TLC. R_f value of the fractions in comparison to the reference standard revealed the presence of caffeic acid and *p*-coumaric acid in hydrolyzed butanol fraction. These two major compounds have shown antioxidant activity in DPPH assay on TLC.

Regarding the presence of kaempferol and phenolics in the seeds of *B. persicum*, the antioxidant activity of the methanolic extract may be due to these compounds. The antioxidant and DPPH radical scavenging activities of flavonoids and phenolics have been reported previously (Sharififar et al., 2009; Sharififar et al., 2007; Sharififar et al., 2007; Thériault et al., 2006; Yassa et al., 2005).

The seeds of *B. persicum* have widespread usage in Iranian diet and no toxicity has been reported for this plant and has health promotional effect for human even with the lesser antioxidant activity in comparison to synthetic antioxidants such as BHT.

CONCLUSION

In conclusion, *B. persicum* seeds, rich in phenolic compounds and γ -terpinene are an efficient free radical scavenger. The essential oil and methanolic extract show similar activity in three *in vitro* antioxidant assays. In the essential oil and methanolic extract the presence of γ -terpinene and phenolic compounds determine the antioxidant activity respectively.

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