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Determination of Phenolics and Flavonoid Contents, Antioxidant Capacity and Major Flavonoids Structure in *Teucrium perscicum* Boiss

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Abstract: Phytochemical and bioactivity studies of *Teucrium persicum* Boiss. have been investigated on the aerial parts of the plant. About 2 flavonoids, 5-hydroxy 3, 7, 4-trimethoxyflavone and 5-hydroxy 7, 3', 4'-trimethoxyflavone were purified and identified from chloroform partition by spectroscopy methods. The crude extracts and isolated compounds were screened for their antioxidant activities using DPPH radical-scavenging, FRAP and Reducing power methods. Methanol extract and isolated flavonoids were found to be the most antioxidant active portions.

Key words: *Teucrium persicum* boiss, antioxidant property, 5-hydroxy 3, 7, 4'-trimethoxyflavone, 5-hydroxy 7, 3', 4'-trimethoxyflavone, total phenolic content, total flavonoid content

INTRODUCTION

Some damages to the human body were occurred by free radicals because of the disruption of membrane fluidity, lipid peroxidation, oxidative protein denaturation, DNA and platelet function alterations which have been linked to some diseases such as inflammation, atherosclerosis, cancer, tissue damage in rheumatoid arthritis and aging. Therefore, antioxidants are vital substances because of the protection ability (Sharififar *et al*., 2009; Abdolghaffari, 2010; Rohman *et al*., 2010).

Recently, there have been big attempts to find safe and potent antioxidants from natural sources especially plants. Lamiacae is included of species with antioxidant activity. Teucrium, edible and medicinal herbs belongs to the Lamiaceae and is represented by 12 species in the flora of Iran (Mozaffarian, 1997). *T. persicum* Boiss is one of them and is found abundantly in Fars province, Iran. It is endemic. There are numerous reports about the biological activities of Teucrium and it has been shown to possess hypolipidemic, anti-inflammatory, anti-nociceptive, anti-hypertensive, anti-bacterial, anti-rheumatoid and hypoglycemic effects (Sharififar *et al*., 2009; Abdolghaffari *et al*., 2010).

Traditionally, Teucrium is used in the treatment of cough, bloat, amnesia, polydipsia, joint pain, wound healing and etc.

Within the antioxidant compounds, flavonoids and phenolics, widely distributed in plants have received considerable attention because of their physiological effect like antioxidant, anti-inflammatory, antitumor activities and low toxicity compared with those of synthetic phenolics antioxidant such as BHA (Butylated Hydroxyanisole), BHT (Butylated Hydroxytoluene) and propyl gallate (Kumar *et al*., 2008; Rohman *et al*., 2010).

MATERIALS AND METHODS

Plant materials: Aerial parts of *T. persicum* Boiss. were collected from Lar mountain in Fars province, Iran in September 2009. A voucher specimen (No. 397) was deposited at the Central Herbarium of Medicinal Plants (ACECR), Iran. The aerial parts of plants were cleaned, dried in the shade, at room temperature, powdered and finally stored in the dark at 25°C.

Extraction and isolation: The aerial parts of plant (1000 g) were extracted using percolation method by methanol at room temperature. Solvents were removed by drying under reduced pressure at 35°C in a rotary evaporator. The residue (125 g) was dispersed in water and extracted successively with $CHCl_3$ and ACOEt, yielding 85 and 8 g fractions, respectively.

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The chloroform partition was fractionated on the silica gel column, using gradient mixtures of hexane-ethyl acetate (starting from 100% hexane). Fraction 23 was chromatographed on a silica gel preparative TLC divided into two portions (A and B) and subsequent research was conducted on the portion B. Fraction 23B (500 mg) was subjected to silica gel column eluting with hexane-ethyl acetate mixtures (stepwise gradient, 6% ethyl acetate). Two yellow compounds 23BYF and 23BYD were obtained (8 and 10 mg, respectively).

Determination of total phenolic and flavonoid content: The Total Phenolic Contents (TPC) were measured by a photometric assay using Folin-Ciocalteu reagent, according to Ghafar *et al.* (2010). Absorbance was measured at 725 nm and gallic acid was used as a standard phenolic compound for the calibration curve (20-200 mg^{-1}, $y = 0.003 \times 0.027$, $R^2 = 0.991$). Total phenolic content was expressed as mg gallic acid equivalents per gram dry weight of plant (mg GA/g DW).

The Total Flavonoid Contents (TFC) were calculated according to Lamison and Carnet. The absorbance of the final mixture, pink was measured against prepared reagent blank at 367 nm (El-Far and Taie, 2009). Quercetin was used for calibration curve ($y = 0.6542 \times - 0.0037$, $R^2 = 0.943$). TFC of the extract and fractions were expressed as mg quercetin equivalents per gram of sample (mg Qr/g DW).

Antioxidant activity

2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay: DPPH antioxidant assay is based on the measurement of the reducing ability of antioxidants toward DPPH radical. The ability can be evaluated by measuring the decrease of its absorbance and the reaction is monitored by a spectrometer at 517 nm. This assay was carried out according to Tofighi *et al.* (2009). Inhibition of DPPH free radical in percent is calculated as:

$$\text{Inhibition}(\%) = \left(\frac{1 - \text{Abs of remaining DPPH}}{\text{Abs of initial DPPH}}\right) \times 100$$

The concentration that causes 50% decrease in the initial DPPH radical concentration is defined as IC_{50}. BHA and α-tocopherol were used as reference substances (Tofighi *et al.*, 2009).

Ferric Reducing Antioxidant Power assay (FRAP): The FRAP assay is based on the ability of sample to reduce Fe^{3+} in Tripyridyltriazine (TPTZ) solution to Fe $^{2+}$and create blue colored complex Fe^{2+}-TPTZ. The FRAP assay was used to estimate the antioxidant potential of samples, according to Benzie and Strain (1996). The absorbance was taken at 593 nm (Benzie and Strain, 1996; Huang *et al.*, 2005; Hong *et al.*, 2008).

Reducing power assay: The reducing power was measured according to Hinneburg *et al.* (2006). This method is based on the abilities of sample to reduce ferri cyanide to ferro cyanide.

The absorbance of the reaction output, blue colored complex $(Fe^{3+})_4$ $[Fe^{2+}(CN^{-})_6]_3$ was measured at 700 nm (Hinneburg *et al.*, 2006; Rohman *et al.*, 2010). BHA was used to produce the standard calibration curve (5-60 μg mL^{-1}, $y = 0.006x + 0.058$, $R^2 = 0.997$). The reducing powers of samples were expressed as μg BHA equivalent per g DW.

Statistical analysis: All tests were repeated three times and data was expressed as mean±SD. Statistical analysis, plots and fittings were carried out by using Excel 2007.

RESULTS AND DISCUSSION

Two flavonoids were isolated from chloroform fraction. Compound 23BYF, 2-(3,4-dimethoxyphenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one was isolated as a yellow amorphous powder (Fig. 1). The information from the ^{13}C-NMR spectrum displays 18 signals due to: one carbonyl, three methoxy, six methine as well as eight quaternary carbon atoms indicate this to be an aromatic compound. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.60 (s, 1H, H3), 6.39 (bs, 1H, H6), 6.51 (bs, 1H, H8), 7.36 (bs, 1H, H2'), 7.00 (d, 1H, J = 8.3, H5'), 7.55 (d, 1H, J = 8.3, H6'), 3.98 (s, 3H), 3.90 (s, 3H), 4.00 (s, 3H). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ164, 104.7, 182.4, 105.57, 162.2, 98.07, 165.5, 92.68, 157.7, 123.8, 108.8, 152.4, 149.2, 111.17, 120.11, 56.11, 55.82, 56.11 (ppm).

Compound 23BYD, 2-(4-methoxyphenyl)-5-hydroxy-3,7-dimethoxy-4H-chromen-4-one had the molecular formula $C_{18}H_{16}O_6$ (Fig. 1). ^{13}C-NMR shows one carbonyl, three methoxy as well as eight quaternary carbon atoms. ^{1}H-NMR ($CDCl_3$, 500 MHz): 6.56 (bs, 1H, H6), 6.6 (bs, 1H, H8), 7.87 (d, 2H, J = 8.2, H3', H5'), 7.02 (d, 2H, J = 8.2, H2', H6'), 3.99 (s, 3H), 3.91 (s, 3H), 3.94 (s, 3H). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 162.6, 106.1, 182.6, 111.57, 158.7, 104.1, 164.01, 90.5, 153.23, 123.58, 114.5, 128, 153.08, 55.81, 56.11, 60.08 (ppm).

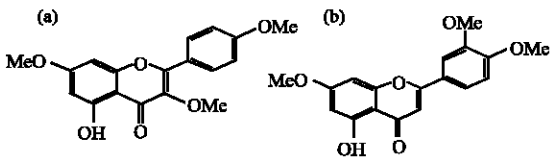

Fig. 1: Flavonoids isolated from *T. persicum*; a) 5-hydroxy 3, 7, 4'-trimethoxyflavone; b) 5-hydroxy 7, 3', 4'-trime thoxyflavone

Table 1: UV-visible absorption spectroscopy of flavonoids in methanol with shift reagents

Compounds	MeOH	$AlCl_3$	$AlCl_3$/HCl	NaOAc	NaOAc/H_3BO_3
23BYF[a]	340	388	388	340	340
	238	365	357	238	238
	-	290 (sh)	290 (sh)	-	-
	-	270	270	-	-
23BYD[b]	332	360	358	332	332
	277	297	295	277	277

[a]5-hydroxy 7, 3', 4'-trimethoxyflavone; [b]5-hydroxy 3, 7, 4'-trimethoxyflavone

Table 2: Antioxidant activity of isolated flavonoids and extract/partitions of Aerial part of *T. persicum*

Parameters	DPPH IC50, mg/cc	FRAP µmol Fe2+/g DW	RPA µg BHA/g DW
Methanol	0.270	868.0±15.0	141.2±18.0
Chloroform	1.450	159.6±11.0	30.5±8.00
Ethyl acetate	0.850	659.0±18.0	70.8±10.0
Water	0.570	248.0±9.00	85.1±16.0
23BYF[a]	0.085	-----	----
23BYD[b]	0.109	-----	----

Data presented is mean±SD from three different experiments. [a]5-hydroxy 7, 3', 4'-trimethoxyflavone; [b]5-hydroxy 3, 7, 4'-trimethoxyflavone

UV spectrum of methanolic solution of flavonoids supported the flavone structure (Table 1). Bathochromic shift with $AlCl_3$ and its stability in the presence of HCl relates to 5-hydroxyl (Mabry *et al*., 1970). The stability of the spectrum in the presence of NaOAC relates to lacking 7-hydroxyl and its stability in the presence of NaOAC/H_3BO_3 shows lack orto-dihydroxy.

Based on UV, ^{1}H-NMR and ^{13}C-NMR data, compound 23BYD was identified as 5-hydroxy 3,7,4'-trimethoxyflavone and compound 23BYF was recognized as 5-hydroxy 7, 3', 4'-trimethoxyflavone (Markham, 1982; Dong *et al*., 1999; Citoglu *et al*., 2005; Aiyama *et al*., 2010). This is the first report on the presence of these flavonoids in the aerial parts of *T. persicum*.

Antioxidant tests: DPPH has been usually used in the determination of antioxidant activity of compounds and plant extracts (Sharififar *et al*., 2009). The IC_{50} values for DPPH assay of the samples have been shown in Table 2. The methanol extracts of *T. persicum* and its partitions could reduce the stable free purple-colored radical DPPH into the yellow-colored DPPH-H. The most effectiveness was obtained with methanol extract (IC_{50} = 0.27 mg mL^{-1}), though it was less effective than BHA (IC_{50} = 0.016 mg mL^{-1}) and α-tocopherol (IC_{50} = 0.015 mg mL^{-1}). It followed by water, ethyl acetate and chloroform partitions (0.57, 0.85 and 1.45, respectively).

The isolated flavonoids, 5-hydroxy 3, 7, 4'-trimethoxyflavone and 5-hydroxy 7, 3', 4'-trimethoxyflavone, from chloroform partition also have shown to be active antioxidants (IC_{50} of 0.085 and 0.109 mg mL^{-1}, respectively). The results are completely compatible with the extract of *T. polium*, *T. chamaedrys* and *T. montanum* that possessed inhibitory activity with IC_{50} of 10, 11 and 10 mg mL^{-1}, respectively (Kadifkova-Panovska *et al*., 2005).

Table 3: Total Phenolic (TPC) and Flavonoid (TFC) content of extract/ partitions of aerial part of *T. persicum*

Parameters	TPC mg GA/g DW	TFC mg Qr/g DW
Methanol	72.1±3.20	2.19±0.20
Chloroform	10.5±0.90	1.45±0.30
Ethyl acetate	15.05±1.2	0.21±0.10
Water	23.4±2.00	0.18±0.11

Data presented is mean±SD from three different experiments

Reducing capacity: The reducing capacities of extracts of *T. persicum* were determined according to the FRAP and reducing power assay. In the FRAP assay, an aqueous solution of ferrous sulphate (50-500 µmol mL^{-1}, y = 0.002x-0.025, R^2 = 0.993) was obtained as a standard curve and the antioxidant potential of the samples were determined using the line equation. The results were expressed as µmol Fe^{2+} equivalents per g DW and are shown in Table 2. These results were compatible with DPPH radical scavenging and indicated that the reducing power of methanolic extract (868±15 µmol Fe^{2+}/g DW) of *T. persicum* is more than the FRAP value for other portions (Chloroform: 159.6±11 µmol Fe^{2+}/g DW, Ethyl acetate: 659±18 µmol Fe^{2+}/g DW, Water: 248±9 µmol Fe^{2+}/g DW).

Results of reducing power assay are shown in Table 2. Reducing power values point to a considerably higher reducing power of methanolic extract (141.2±18 µg BHA/g DW) compared to the water, ethyl acetate and chloroform portion (85.1±16, 70.8±10, 30.5±8 µg BHA/g DW, respectively).

Total phenolic and flavonoids content: Phenolic and flavonoid components are important secondary metabolites in plants. It is noted that Folin-Ciocalteu assay is not specific to polyphenols (Ghafar *et al*., 2010; Rohman *et al*., 2010).

As shown in Table 3, the methanolic extract revealed the highest content of total phenolics (72.1±3.2 mg GAE/g DW), approximately three fold more than the water portion and four fold higher than ethyl acetate and seven-fold greater than chloroform partitions (23.4±2.00, 15.05±1.2 and 10.5±0.9 mg GAE/g DW, respectively). The data on phenolic content of crude methanolic extract were approximately three fold more than the average values found for *T. arduini* flower (30.49±1.00 mg GAE/g DW) and leaf (23.39±3.60 mg GAE/g DW) (Samec *et al*., 2010).

Table 3 shows that among tested samples, methanol extract has the highest total flavonoids content (2.19±0.2 mg Qr/g DW) followed by chloroform, ethyl acetate and water partition (1.45±0.3, 0.21±0.1, 0.18±0.11 mg Qr/g DW, respectively). Kadifkova Panovska showed TFC varied in different *Teucrium* species and ranged from

0.15- 0.20% (Kadifkova-Panovska *et al*., 2005) that it is in agreement with the results. The results of antioxidant activities are compatible with TPC and TFC.

CONCLUSION

As far as we know, there is no information regarding the utilization of *T. persicum* as a source of natural antioxidant. Therefore, the aim of this study was to estimate *T. persicum* as a source of natural antioxidants using different extracting solvents to determine their antioxidant capacities. Because of the important roles of the total phenolics and flavonoids as antioxidants, the amounts of total phenolics and flavonoids in the extracts/fractions were also determined. In the present research, we wish to report the isolation and structural elucidation of two flavonoids from the aerial parts of *T. persicum*, together with their antioxidant activities.

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