Tyrosinase inhibitory properties of phenylpropanoid glycosides and flavonoids from *Teucrium polium* L. var. *gnaphalodes*

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**Abstract**

Objective(s): In food industry, the inhibition of tyrosinase is very important, because this enzyme catalyzes the oxidation of phenolic compounds found in fruits and vegetables into quinones, which contribute to undesirable color and taste of fruits and vegetables. *Teucrium polium* L. var. *gnaphalodes* (Lamiaceae), a wild-growing flowering plant that has many applications in food preparations and traditional medicine. In Persian language, this medicinal herb is called Kalpouregh.

Materials and Methods: 1D- and 2D-NMR experiments were used to determine the chemical structures of the isolated compounds. Antioxidant and tyrosinase inhibitory activities of the isolated compounds were evaluated using DPPH, FRAP and mushroom tyrosinase inhibition assays.

Results: In this research, we isolated two phenylpropanoid glycosides including verbascoside and poliumoside and two flavonoids including jaranol and isorhoifolin using chromatographic techniques. We found promising antioxidant and anti-tyrosinase compounds from *Teucrium polium* L. var. *gnaphalodes*.

Conclusion: To date, different compounds have been isolated and characterized from *T. polium* including terpenoids and flavonoids. But no phytochemical study has been reported from *T. polium* var. *gnaphalodes*. Poliumoside and jaranol showed promising antioxidant and tyrosinase inhibitory activities, respectively.

**Introduction**

Tyrosinase is a kind of oxidase enzyme that involves in reactions of melanin synthesis. In food industry, the inhibition of tyrosinase is very important, because this enzyme catalyzes the oxidation of phenolic compounds found in fruits and vegetables into quinones, which contribute to undesirable color and taste of fruits and vegetables. In addition, quinones reduce the bioavailability of essential amino acids. Arbutin, kojic acid, and hydroquinones have been reported to have tyrosinase inhibitory activity. Also, they have been used in cosmetic industry as whitening composition (1, 2).

The genus *Teucrium* consists of more than 340 species widely distributed around the world (Southwest of Asia, North of Africa, and South and Northeast of Iran). Twelve species occur in Iran including three endemic species. The main compounds reported from the genus *Teucrium* include terpenoids and flavonoids (3, 4). *Teucrium* is called Kalpouregh in Persian language. It has been long used in Iranian traditional medicine to treat stomach disorders, malabsorption, grippe, cold, and was reputable for having hypoglycemic, anti-hyperlipidemia (3), diuretic, analgesic, antipyretic, anti-spasmodic, anti-inflammatory and anti-hypertensive properties. All species of this genus have been showed considerable hypoglycemic and anti-hyperlipidemia properties (5).

A literature review shows that there are a large number of phytochemical studies on the genus *Teucrium* including *T. polium*. Different parts of plants from the genus *Teucrium* have been reported to have monoterpens (6), sesquiterpenes (7), polyphenols and flavonoids such as apigenin and rutin (8, 9) and some fatty acids and steroids such as β-sitosterol and stigmasterol. But there is no

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A phytochemical study on *T. polium* var. *gnaphalodes*. Considering pharmacological benefits of *T. polium* var. *gnaphalodes* and its traditional applications, that are similar to those of *T. polium*, we aimed to characterize its components and primarily evaluate their antioxidant and anti-tyrosinase properties.

In this study, we reported the isolation and structure elucidation of the main constituents of *T. polium* var. *gnaphalodes*, and antioxidant (FRAP and DPPH test) and anti-tyrosinase (mushroom tyrosinase inhibition assay) activities of the main compounds.

**Materials and Methods**

**General experimental procedures**

Preparative HPLC-DAD was performed on a KNAUER liquid chromatograph system consisting of a quaternary pump (Smartline Pump 1000). Detection was carried out using UV/Vis diode array detector (Smartline DAD 2800), and data were processed using EZ Chrom Elite software. The fractions were subjected to reverse-phase HPLC using a gradient method of 20-100 % methanol in water as the eluent including 0.05 % trifluoroacetic acid.

The $^1$H-NMR spectra of the isolated compounds were recorded at 30 °C on a Varian 600 $^1$H-NMR spectrometer. Samples were dissolved in DMSO-$d_6$. The $^1$H-NMR chemical shifts were referenced to the solvent peaks at $\sigma_H 2.50$ ppm.

**Plant material**

The aerial parts of *T. polium* var. *gnaphalodes* were collected in October from north of Iran, Firoozkouh, Alborz Mountains, 2200 meters height. The plant material was identified by Mohammad Reza Joharchi. A voucher specimen (No. 11377) has been deposited at the herbarium of School of Pharmacy, Mashhad University of Medical Sciences.

**Extraction and isolation**

Aerial parts have been dried in room temperature and then have been finely powdered by a miller. The powdered aerial parts (250 g) were extracted in 500 ml of methanol at room temperature for three times each for 24 hr by maceration method. After concentration of extracts with a rotary evaporator and completion of drying of them with a freeze dryer, the obtained extract (37 g) has been conserved in the refrigerator.

10 g of the dried extract was loaded on silica gel column chromatography ($5 \times 50$ cm, normal phase). The column has then been eluted by hexane and then gradual adding of ethyl acetate and methanol to increase mobile phase polarity. The obtained fractions (200 mL each) were compared by TLC and those giving similar spots were combined. Three fractions (A-C) were finally obtained. Then fractions A, B and C were subjected to more purification via HPLC apparatus (C-18 reversed phase with methanol: water solvent system) to obtain pure compound of 1 (21 mg), 2 (9.3 mg), 3 (101.4 mg) and 4 (10.4 mg). Purification of fractions was carried out using an ACE 5 C18 (5 µm, 250 × 21.2 mm) at a flow rate 9 ml/min and linear gradient conditions of 20 % - 100 % MeOH (0.05 % TFA) within 20 min, followed by an isocratic condition of MeOH (0.05 % TFA) for 5 min.

**Antioxidant activity**

**DPPH free radical scavenging assay**

DPPH is a stable radical that is used in a popular method for screening free radical-scavenging ability of compounds or antioxidant activity of plant extracts (10).

Free radical scavenging activity was evaluated by measuring the scavenging activity of the compounds in the solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Briefly, a 0.3 mM solution of DPPH in ethanol was prepared. An aliquot (50 µl) of samples (at four different concentrations (µg/ml) were added to 150 µl of the DPPH solution in each well of a 96-well plate. For blank, only 50 µl of solvent was added to the DPPH solution. The decrease in absorbance was measured at 515 nm after 30 min of incubation at 37 °C using the BioTek micro plate reader (Synergy H4, USA). All tests were performed in triplicate (11) and the data presented as mean of the three values. When a solution of DPPH is mixed with that of a substance, this gives rise to the reduced form diphenylpicrylhydrazyl with the loss of this violet color.

The IC$_{50}$ values were calculated as the concentration of extracts causing a 50% inhibition of DPPH radical. A lower IC$_{50}$ value corresponds to a higher antioxidant activity of sample.

**Ferric reducing antioxidant potential (FRAP) assay**

The FRAP assay was performed according to a previous work (11). FRAP reagent was prepared by adding 10 mL of acetate buffer 300 mM, pH 3.6 (3.1 g sodium acetate trihydrate), to 1.0 mL of ferric chloride hexahydrate 20 mM (dissolved in distilled water) and 1.0 mL of 2,4,6-tri-(2-pyridyl)-s-triazine (TPTZ) 10 mM (dissolved in HCl 40 mM). In a well of a 96-well plate, an aliquot (10 µl) of sample (at five different concentrations (µg/ml) was added to 190 µl of the FRAP solution. After 30 min of incubation at 37 °C, absorbance of the reaction mixture was measured at 593 nm using BioTek micro plate reader (Synergy H4, USA). All tests were carried out in triplicate.

**Mushroom tyrosinase inhibition assay**

Melanin, which is secreted by melanocyte cells, is the major pigment for color of human skin. It may
be overproduced with chronic sun exposure or other hyperpigmentation diseases. Tyrosinase, a copper-containing monooxygenase, is a key enzyme that catalyzes melanin synthesis in melanocytes. Inhibiting this enzyme may be the least invasive procedure for maintaining skin whiteness (10).

Mushroom tyrosinase inhibition assay was performed in the 96-well micro plates. The L-DOPA oxidation activity of tyrosinase was measured by spectrophotometry as described previously with some modifications. Briefly, 160 µl of 5 µM L-DOPA (in 100 µM sodium phosphate buffer pH 6.8) and 20 µl of the same buffer with and without the test sample were placed in the wells of a 96 micro plate, and then 20 µl of mushroom tyrosinase (200 units/ml) were mixed into each well at 37 °C over 30 min. The amount of dopachrome produced in the reaction mixture was measured at 475 using the micro plate reader. Kojic acid was used as positive control of tyrosinase inhibitor.

The 50 % inhibition of tyrosinase activity (IC$_{50}$) was calculated by the use of Prism Graph pad software.

**Results**

Normal-phase column chromatography of the methanol extract of aerial parts, followed by semi-preparative HPLC, afforded four pure known compounds including isorhoifolin (1), jaranol (2), poliumoside (3) and verbascoside (4). We evaluated antioxidant and tyrosinase inhibitory activity of the purified compounds. Jaranol showed the highest tyrosinase inhibitory activity. On the other hand, poliumoside was the best antioxidant among the tested compounds.

Compound 1 was obtained as pale yellow amorphous powder. It was clear from the $^1$H and $^{13}$C NMR data (Table 1) that compound 1 consisted of a flavone nucleus and two sugar moieties.

The $^1$H-NMR spectrum revealed the presence of glucose and rhamnose. The glycoside structure and its connectivity were confirmed by a HMBC experiment, which showed long-range correlations between the signals of H-1" and C-7 and H-1"" and C-6". The β-configuration of the anomeric glucose unit was assigned on the basis of
Table 1. $^1$H-NMR and $^{13}$C-NMR data of compounds 1 and 2 (600 MHz, DMSO-$d_6$)

| Position | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
|----------|------------|------------|------------|------------|
| 2        | -          | 164.8      | -          | 164.5      |
| 3        | 6.83 (s)   | 103.5      | 132.3      |
| 4        | -          | 182.4      | 182.4      |
| 5        | -          | 161.6      | 153.0      |
| 6        | 6.43       | 100.0      | 92.0       |
| 7        | -          | 163.3      | 159.0      |
| 8        | 6.75       | 95.2       | 103.1      |
| 9        | -          | 157.3      | 152.5      |
| 10       | -          | 105.8      | 105.5      |
| 1'       | 5.04 (d, J=7.8) | 121.4      | 121.5      |
| 2',6'    | 7.93 (d, J=8.8) | 129.0      | 128.9      |
| 3',5'    | 6.94 (d, J=8.8) | 116.5      | 116.4      |
| 4'       | -          | 161.7      | 161.7      |
| Glc: 1'' | 5.04 (d, J=7.8) | 100.3      | 60.4       |
| 2''      | 4.54 (s)   | 100.9      |
| 3''      | 3.64 (bs)  | 70.7       |
| 4''      | 3.41 (m)   | 71.2       |
| 5''      | 3.13 (m)   | 72.5       |
| 6''      | 3.44 (m)   | 68.7       |
| Rha: 1'' | 1.06 (d, J=6) | 18.2       |
| 3'-OMe   | -          | -          | 3.72       | 60.4       |
| 7'-OMe   | -          | -          | 3.91       | 56.8       |
| 5'-OH    | -          | -          | 12.90      | -          |
| 4'-OH    | -          | -          | 10.36      | -          |
methylene groups were located at C-3 and 159.0 (C-8) in the HMBC spectrum to the methoxy groups at δ 3.72 and 3.91. Typical NMR spectra of compound 3 showed a bi-amorphous powder. The aromatic part of the spectrum also showed four aromatic protons appearing as two doublets at δ 7.93 with (J = 8.8 Hz) and δ 6.94 with (J = 8.8 Hz) due to ortho-coupled protons, that were assigned to H-2', 6' and H-3', 5', respectively (Figure 1). Also a singlet at δ 6.83 ppm corresponding to H-3, two protons broad singlet at δ 6.75 and 6.43 ppm, assigned to H-8 and H-6, respectively. From these data and by comparing them with the published data (12), compound 1 was identified as isorhoifolin.

Compound 2 was also obtained as yellow amorphous powder. The 1H and 13C NMR data of 2 showed a bi-methoxylated flavone structure for compound 2 (Table 1). The aromatic part of the 1H NMR spectra of compound 2 suggested the presence of a flavone structure. Typical 1H NMR signals of two methoxyl groups at δ 3.72 and 3.91 correlated in the HMBC spectrum to the 13C NMR signals of δ 132.3 (C-3) and 159.0 (C-7), respectively, suggesting that methoxyl groups were located at C-3 and C-7. The spectrum also showed four aromatic protons appearing as two doublets at δ 7.94 with (J = 8 Hz) and δ 6.91 with (J = 8 Hz) due to ortho-coupled protons, that were assigned to H-2', 6' and H-3', 5', respectively (Figure 1). The meta position protons of ring A were also appeared as two singlets in 6.82 and 6.90 ppm. The other 1H and 13C NMR data of compound 2 were in agreement with those of jaranolin (Syn. = kumatakenin) reported in the literature (13, 14).

Compound 3 was also obtained as brownish amorphous powder. 1H and 13C-NMR data indicated the presence of three sugar moieties, due to the presence of signals at δC 102.8 with δH 4.35 (J = 7.9 Hz) for β-glucose moiety and at δC 101.6 with δH 5.01 (d, J = 1) and δC 100.9 with δH 4.47 (d, J = 1) for rhamnose moieties. The 1H NMR spectral data also revealed the presence of trans-olefinic protons attached to tri-substituted benzene ring along with the carbonyl carbon (trans-caffeoyl moiety). The

| Position | δH | δC | δH |
|----------|-----|-----|-----|
| Rha: 1'  | 5.01 | 101.6 | -   |
| 2'       | 3.68 | 70.8 | -   |
| 3'       | 3.28 | 70.7 | -   |
| 4'       | 3.10 | 72.3 | -   |
| 5'       | 3.34 | 69.1 | -   |
| 6       | 0.94 | 18.5 | -   |
| Caf: 1   | -   | 160.0 | -   |
| 2       | 6.17 | 113.8 | 6.17 |
| 3       | 7.44 | 145.9 | 7.43 |
| 4       | -   | 125.9 | -   |
| 5       | 7.01 | 115.1 | 7.09 |
| 6       | -   | 145.9 | -   |
| 7       | -   | 148.9 | -   |
| 8       | 6.73 | 116.3 | 6.74 |
| 9       | 6.95 | 121.8 | 6.95 |
| 6-OH    | 9.11 | - | -   |
| 7-OH    | 9.54 | - | -   |
| DPE: 1   | 3.61 | 70.8 | 3.79 |
| 2       | 3.81 | 35.5 | 2.68 |
| 3       | 2.64 | 329.6 | -   |
| 4       | 6.60 | 116.7 | 6.61 |
| 5       | -   | 145.3 | -   |
| 6       | -   | 143.8 | -   |
| 7       | 6.47 | 115.9 | 6.47 |
| 8       | 6.61 | 119.9 | 6.61 |
| 5-OH    | 8.64 | - | -   |
| 6-OH    | 8.58 | - | -   |

Table 2. 1H-NMR and 13C-NMR data of compounds 3 and 4 (600 MHz- DMSO-d6)
presence of trans-caffeoyl moiety was indicated from the signals at δ_II 7.44 (H-3, J = 15.7 Hz) and δ_II 6.17 (H-2, J = 15.7 Hz) for trans double bond and δ_II 7.01 (H-5), 6.73 (H-8) and 6.95 (H-9) for the tri-substituted benzene ring, which was supported by the 13C NMR spectrum. From the above mentioned data and by comparing these data with the published data (15-17), compound 3 was identified as poliumoside.

Compound 4 was obtained as yellow amorphous powder. The 1H-NMR and 13C-NMR spectral data of 4 were very similar to those of 3 (Table 2) except for the loss of one of rhamnose moieties. Therefore, compound 4 was assumed to have a disaccharide structure. By comparing these data with the published data (18), this compound was also identified as verbascoside. The NMR data of the compounds 1-4 has been summarized in tables 1 and 2. The structure assignments of four compounds were also supported by other 2D NMR experiments including HSQC and HH-COSY.

### Discussion

As shown in Table 3, poliumoside (3) possessed the highest antioxidant capacity in DPPH test with an IC₅₀ of 4.23 µg/ml. In FRAP assay, poliumoside showed the highest activity. It should be pointed out, however, all four compounds, but not jaranol, showed antioxidant activity comparable to the positive control α-tocopherol. Jaranol exhibited the lowest activity among the tested compounds due to the blockage of its two active hydroxyl groups with the loss of one of rhamnose moieties. Therefore, compound 3 was identified as verbascoside. The NMR data of the compounds 1-4 has been summarized in tables 1 and 2. The structure assignments of four compounds were also supported by other 2D NMR experiments including HSQC and HH-COSY.

### Table 3. Antioxidant activities of compounds 1-4 using FRAP and DPPH radical-scavenging activity

| Compound     | FRAP (mmol/g) | IC₅₀ (DPPH radical scavenging activity, µg/ml) |
|--------------|--------------|---------------------------------------------|
| Poliumoside  | 14.32        | 0.042                                       |
| Isorhoifolin | 7.67         | 1.37                                        |
| Verbascoside | 7.49         | 0.53                                        |
| Jaranol      | 1.50         | 12.00                                       |
| α-tocopherol | 1.99         | 0.41                                        |

Poliumoside and verbascoside are structurally characterized with a hydroxyl-phenylethyl moiety attached to a β-glucopyranose through glycosidic linkage. The core structures are often inundated with substituents such as aromatic acids like cinnamic and caffeic acids, and various sugars such as rhamnose, xylose and arabinose attached to the glucose residue through ester or glycosidic linkages. Several pharmacological studies have shown that these compounds possess a broad spectrum of biological activities including antibacterial, antitumor, antiviral, anti-inflammatory, antioxidant and tyrosinase inhibitory actions (20). Studies have also demonstrated that both the number and position of the phenolic hydroxyls play an important role in the antioxidative activity of phenylpropanoid glycosides (21). The length of methylene-chain connected to the benzene ring and the type of phenylpropanoid acids and sugar connected to the aglycone moiety, are major factors affecting the overall activity (20, 22, 23). Overproduced free radicals can often lead to oxidative stress that may result in oxidative injury and diseases such as cardiovascular diseases, retinal ischemia and neurodegenerative diseases (20). Together with searching new antioxidants, there has been increasing interest in replacing synthetic antioxidants with natural ones for safety concern (24).

Verbascoside [first isolated from Verbascum sinuatum (Scrophulariaceae) in 1963 (25)] showed considerable antibacterial activities against of S. aureus strains with the minimum inhibitory concentration (MIC) values ranging from 64 µg/l to 256 µg/l (26). Poliumoside, has also significantly attenuated glutamate-induced neurotoxicity at concentrations ranging from 0.1 to 10 µM. These compounds, having a caffeoyl moiety, showed stronger neuroprotective activity than those of unsubstituted phenylethanoid glycosides (27).

### Table 4. Mushroom anti-tyrosinase activity of compounds 1-4

| Compound     | Verbascoside | Jaranol      | Poliumoside | Isorhoifolin | Kojic acid |
|--------------|--------------|--------------|-------------|--------------|-----------|
| IC₅₀ (mM)    | 0.3240       | 0.04195      | 0.5026      | 0.6701       | 0.0205    |

Tyrosinase inhibitors from Teucrium polium

Baghrati et al

Iran J Basic Med Sci, Vol. 19, No. 8, Aug 2016

809
The role of melanin is to protect the skin against UV light damage by absorbing UV sunlight and removing reactive oxygen species. The key enzyme that is responsible for melanin production is tyrosinase. Hyperpigmentation of the skin occurs due to overactivity of tyrosinase enzyme (28). Many tyrosinase inhibitors find applications in cosmetic products for whitening and depigmentation after sunburn and in the treatment of dermatological disorders related to melanin hyperpigmentation. Due to the harmful effects of commercial skin whitening, natural compounds have been noticed. Among them, phenolic compounds seem to be potent agents (29). Flavonoids are a large class of natural phenolics with various biological activities that occur in many fruits and vegetables (30-34). The studies showed that the presence of the 3, 3’ and 4’-hydroxyl group in flavonoids was essential for high anti-tyrosinase activity, whereas, the presence of a methoxyl group at the C-4’ and C-7 position tended to reduce the anti-tyrosinase activity. Since flavones showed higher antityrosinase activities than flavanones, it was concluded that the presence of the C2-C3 double bond is also essential for tyrosinase inhibitory ability (35). The mechanism of tyrosinase inhibition of flavonoids might also be due to chelating with copper in the active center of tyrosinase enzyme (30). The inhibitory activity of phenylethanoid glycosides could be attributed to the presence of ortho-hydroxyls on the phenolic rings, which give them a property to chelate with metals (29).

Conclusion

*Teucrium polium* var. *gnaphalodes* is a traditional medicinal plant that is used for various ailments. No phytochemical study has been conducted on this variety of *T. polium*. In this study, we have isolated and characterized four compounds including two phenylpropanoid glycosides (verbascoside and poliumoside) and two flavonoids (jaranol and isorhoifolin) from this plant for the first time.

We evaluated antioxidant and tyrosinase inhibitory activity of the purified compounds. Jaranol showed the highest tyrosinase inhibitory activity. On the other hand, poliumoside was the best antioxidant among the tested compounds. Taken together, our findings revealed that *T. polium* var. *gnaphalodes* contained potent antioxidant and tyrosinase inhibitors. Tyrosinase inhibitors of this plant, particularly jaranol, have a potential of application like other tyrosinase inhibitors in cosmetic and food industries.

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Conflict of Interest

The authors declared no conflict of interest.

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