





Cholinesterase Inhibitory, Anti-oxidant and Anti-tyrosinase Activities of Three Iranian Species of *Dracocephalum*

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Abstract

Background and objectives: *Dracocephalum* species are mentioned in Iranian traditional medicine for enhancement of cognitive performance. In the present study, the acetyl cholinesterase inhibitory and butyryl cholinesterase inhibitory activities as well as the anti-oxidant and anti-tyrosinase effects of three Iranian *Dracocephalum* species (*D. kotschyi*, *D. multicaule*, *D. polychaetum* was analyzed). **Methods:** The extractions were performed stepwise with hexane, chloroform, ethyl acetate (EtOAc), methanol (MeOH) and water. AChE and BChE inhibitory properties were measured by a microplate assay. Total phenolic content of all extracts were also evaluated and anti-oxidant activities of the extracts were assessed using DPPH, FRAP assays. Tyrosinase inhibitory activity was measured using the modified dopachrome method with L-DOPA as the substrate. **Results:** The results showed that the EtOAc extract of *D. multicaule* and MeOH extract of *D. kotschyi* were the most active anti-oxidant and anti-tyrosinase extracts which showed the highest amounts of phenolic compounds. *Dracocephalum multicaule* demonstrated the most considerable activity in AChE inhibition and *D. polychaetum* the highest activity in BChE inhibition. The aqueous extract of *D. multicaule* inhibited both AChE and BChE. **Conclusion:** *Dracocephalum multicaule* can be suggested as a proper natural candidate for improvement of cognitive disorders.

Keywords: antioxidants; anti-tyrosinase; butyrylcholinesterase; cholinesterase inhibitors; *Dracocephalum*

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Introduction

Dracocephalum is a genus belonging to the Lamiaceae family which is found abundantly in central Asia, Iran, Turkey and Europe [1,2]. Recently, the *Dracocephalum* species and their chemical constitution have drawn much attention due to their various biological effects including

their anti-oxidant, anti-inflammatory, anti-hypoxic and immunomodulatory activities [3]. The plants of this genus typically contain terpenoids and flavonoids [4]. Autoxidation of lipids, which can be triggered by light, temperature, oxygen and some other factors,

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decreases the quality of foods that contain fats. In addition, it is clear that the consumption of such foods is associated with aging, heart disease, stroke, and cancer; therefore, antioxidants should be indispensable ingredients in foods. Possible toxicity of synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), has raised interest for investigation of natural substitutes over the last years [5].

In the Iranian traditional medicine (ITM), *Dracocephalum* species are used not only for their memory improvement capacity, but also for their anti-anxiety and analgesic effects [6]. Although *Dracocephalum* species have shown great potential in the treatment of cognitive disorders in ITM, their effects on cholinesterase enzymes have not yet been investigated in detail. Acetylcholinesterase inhibition correlates with an increase in the concentration of acetylcholine in synaptic area which is presently the most accepted and recognized therapeutic marker in the development of cognitive enhancers [7]. Therefore, the study of the anti-cholinesterase effects of these species and comparison of their activities seems to be of great importance.

Tyrosinase is one of the multifunctional enzymes containing copper that is found in fungi, plant materials, and animal tissues. Tyrosinase has been reported to be a major contributor to the neurotoxicity created by dopamine associated with neurodegeneration in Parkinson's disease so the inhibition of tyrosinase could be a treatment for Parkinson's disease [8]. Tyrosinase is also responsible for melanin synthesis [9]. Many tyrosinase inhibitors are used in depigmenting preparation and whitening cosmetics [10]. In addition, the compounds that increase melanogenesis may protect human skin from ultraviolet irradiation damage [11]. For example, ascorbic acid, arbutin and kojic acid have been utilized as cosmetic agents [12-14]. However, a recent study has shown that kojic acid has carcinogenic action [15]. So, a natural alternative for kojic acid as a tyrosinase inhibitor is of high interest. Besides, the formation of melanin is detrimental to the color quality of plant-derived foods. Consequently, tyrosinase inhibitors can be used as food additives.

There are seven species of *Dracocephalum* growing natively in Iran. In this study, we selected three (*Dracocephalum kotschyi* Boiss., *Dracocephalum multicaule* Montbr. & Auch., and *Dracocephalum polychaetum* Bornm.)

from different ecological conditions for a comparative investigation of their biological effects.

Material and Methods

Chemicals

Acetylcholinesterase (AChE) from *Electrophorus electricus*, butyrylcholinesterase (BChE) from Equine Serum, acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCl), Tyrosinase, L-Tyrosine, 5,5'-dithiobis-bis-nitrobenzoic acid (DTNB), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and gallic acid all purchased from Sigma-Aldrich (St. Louis, MO, USA), Folin-Ciocalteu reagent, anhydrous sodium carbonate, ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), sodium sulfate, sulfuric acid, phosphoric acid, potassium dihydrogen phosphate, potassium hydrogen diphosphate, hydrogen peroxide, sodium carbonate, Na-K-tartrate, cupric sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and the solvent in analytical grade were provided by Merck (Germany).

Plant material

The aerial parts of *D. multicaule*, *D. polychaetum* and *D. kotschyi* were collected from Ardebil, Hezar Mountain near Kerman and around Zanjan, respectively during flowering in May to July 2015. The plants were identified by Prof. F. Attar and the samples were kept at the herbarium of School of Science, University of Tehran (Tehran, Iran) with the Voucher specimens of 45869 TUH, 45866 TUH and 45879 TUH, respectively.

Extraction

Dried aerial parts of each *Dracocephalum* species were pulverized and extracted with hexane by maceration at room temperature for 24 h. The residue was extracted by chloroform (CHCl_3) in the same condition and followed by ethyl acetate (EtOAc), methanol (MeOH) and water to obtain five extracts with different polarities.

Total phenolics content

The total phenolics content was determined by employing a previously published method [16] with a slight modification. All extract samples were dissolved in methanol; 0.25 mL of the sample solution (1 mg/mL) was mixed with 1 mL of diluted Folin-Ciocalteu reagent in 50% MeOH-water (1:9) and shaken vigorously. After 3 min, Na_2CO_3 the aqueous solution (0.75 mL, 1%

w/v) was added and the solution was kept for 2 h for incubation at room temperature. Then the sample absorbance was measured at 760 nm. Gallic acid was used as the standard and the 5 concentrations of 75 to 250 µg/mL were used to obtain the trend line. The total phenolics content was expressed as gallic acid equivalent (µg GAE/mg extract).

Free radical scavenging activity

The effect of the samples on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical was estimated according to Sarikurkcu [17]. One ml of the different extracts in methanol at different concentrations (5, 10, 15, 20, 25, 50, 100, 200, 300, 400 µg/mL) was added to 2 mL of DPPH (0.5 mM). The mixture was shaken well and left at room temperature for 30 min. Then, the absorbance was measured at 517 nm with a Shimadzu UV spectrophotometer (Japan). Torolox was used as the standard and each experiment was conducted three times. The IC₅₀ value of each sample was determined. The percentage of DPPH scavenging was calculated using the following formula:

(%) DPPH scavenging or Percentage of inhibition = $(A_0 - A_1) / A_0 \times 100$.

A₀: Absorbance of the blank (MeOH+DPPH), A₁: Absorbance of sample.

FRAP assay

The FRAP (ferric reducing antioxidant power) assay was performed as described by Aktumsek et al. [18] with some modifications. All extract samples were dissolved in methanol. Sample solution, 0.1 mL (1 mg/mL) was added to a premixed FRAP reagent (3 mL) which contained acetate buffer (0.3 M pH: 3.6), 2,4,6 tris (2-pyridyl)-s-triazine (TPTZ) (10 mM) in 40 mM HCl and ferric chloride (20 mM) with a ratio of 10:1:1 (v/v/v). Then the sample absorbance was read at 593 nm after 30 min incubation at 37 °C. FRAP activity was expressed as FeSO₄ (7H₂O) equivalent (FSE mg/g extract).

Anti-tyrosinase activity

Tyrosinase inhibitory activity was measured using the modified dopachrome method with L-DOPA as the substrate [19] with a slight modification. All extract samples were dissolved in DMSO. Phosphate buffer (180 µL 1.15 M pH 6.8) was added to the sample solution (100 µL, 100 µg/mL) mixed with 1 mL of L-DOPA (1.5 mM) in a 96-well microplate and the absorbance

was measured at 492 nm. The reaction then initiated by adding a tyrosinase solution (100 µL, 1000 U/mL) and it was incubated for 20 min at 37 °C and the absorbance was measured again. Kojic acid and the sample solvent (DMSO) were used as positive control and the blank, respectively. The absorbance of the blank was subtracted from the sample and the percentage of tyrosinase inhibitory activity was calculated using the following formula:

$$\% \text{ tyrosinase inhibitory activity} = 100 - [(\Delta SM / \Delta BL) \times 100]$$

ΔSM: difference of sample absorption before and after adding tyrosinase enzyme

ΔBL: difference of blank absorption before and after adding tyrosinase enzyme

Antiacetylcholine esterase and antibutyrylcholine esterase assay

The microplate assay for AChE and BChE inhibitory activity was modified from the assay described by Adhami et al. [20]; 125 µL of 3 mM DTNB, 25 µL of 15 mM ATCI or BCTI, and 50 µL of buffer were added to a 96 well plate followed by 25 µL of sample (1 mg/mL) dissolved in 10% MeOH/buffer. The absorbance was measured by a Bio-Rad microplate reader model 3550 UV (Richmond, CA, USA) at 405 nm five times every 13 s. Then 25 µL of 0.22 U/mL AChE or 0.11 U/mL BChE solution was added and the absorbance was measured again at the same wavelength eight times every 13 s after 10 min incubation. Galantamine and sample solvent (methanol) were used as positive control and the blank, respectively. The percentage of inhibition was calculated using the following formula:

$$\% \text{ AChE \& BChE Inhibitory} = 100 - [(\Delta SM / \Delta BL) \times 100]$$

ΔSM: difference of sample absorption before and after adding enzyme (AChE or BChE)

ΔBL: difference of blank absorption before and after adding enzyme (AChE or BChE)

Results and Discussion

The total phenolics contents of the 3 species of *Dracocephalum* extracts were defined using Folin-Ciocalteu method. The calibration curve of gallic acid was measured using five different concentrations of gallic acid. The equation was obtained as $y = 0.0054x + 0.3105$ ($R^2 = 0.9854$). Total phenolics content of the extract was

expressed as μg gallic acid equivalent (μg GAE/g extract). It was revealed that the total phenolics contents of different fractions of the three species varied greatly based on the solvent used. The three highest total phenolics content belonged to the EtOAc extract of *D. multicaule*, MeOH extract of *D. Kotschyi* and CHCl_3 extract of *D. multicaule* (table 1). Among the three *Dracocephalum* species, the total phenolics content of *D. multicaule* was the highest (table 1). This assay examines reduction of radical solution in the presence of a hydrogen-donating antioxidant. Free-radical-scavenging activities of *Dracocephalum* extracts were analyzed by DPPH assay. Trolox was used as the positive control; $\text{IC}_{50} = 7.45 \mu\text{M}$ and the result have been presented in table 2. The results indicated that the MeOH extract of *Dracocephalum* species and the EtOAc extracts of *D. multicaule* and *D. kotschyi* presented the highest activities, respectively. On the other hand, Hexane extract of *D. kotschyi* and aqueous extract of *D. polychaetum* showed the lowest antioxidant activities.

Reducing capacity is normally an indicator of electron donation, which is one of the most essential antioxidant mechanisms. FRAP assay is a simple, fast and reproducible test that measures the Fe^{3+} to Fe^{2+} transformation capacity. The calibration curve for gallic acid was drawn and the formula was obtained as $y = 0.0039x + 0.5437$. The trend line of FeSO_4 was determined

as standard (R^2 : 0.9921) and the reducing power of *Dracocephalum* species extracts was expressed as mg ferrous sulfate equivalent (FSE)/g extract. The result showed the EtOAc extract of *D. multicaule* has the strongest reducing power, then the MeOH extract of *D. kotschyi* and the EtOAc extract of *D. kotschyi*, respectively (table 3). The reducing capacities of the different extracts were in agreement with their total phenolic contents.

Tyrosinase is a polyphenol oxidase enzyme containing copper that plays an important role in melanogenesis. Tyrosinase converts L-tyrosine to L-DOPA and oxidizes L-DOPA to dopachrome that results in melanin pigments production. Although melanin production is essential for the prevention of UV damage to the eyes, skin and hair, overproduction of melanin can make pigment variations (e.g. melisma, freckles, etc.) as well as neurodegenerative disorders (e.g. Parkinson's disease). The results have been presented in table 4. The current research revealed that all the examined extracts could inhibit the oxidation of catalyzed L-DOPA by tyrosinase enzyme. MeOH extract of *D. kotschyi* and EtOAc extract of *D. multicaule* showed the highest tyrosinase inhibitory activities. According to the result (table 5) EtOAc, water and MeOH extracts of *D. multicaule* represented higher activities of AChEI, respectively.

Table 1. Total phenolics content of *Dracocephalum* species as μg gallic acid /g extract

Plant species	Hexane extract	CHCl_3 Extract	EtOAc extract	MeOH extract	Aqueous extract
<i>Dracocephalum kotschyi</i>	32.0 \pm 0.3	47.5 \pm 0.1	73.0 \pm 0.2	98.5 \pm 0.1	17.3 \pm 0.1
<i>Dracocephalum multicaule</i>	29.5 \pm 0.1	94.0 \pm 0.5	524.5 \pm 0.3	92.9 \pm 0.8	85.5 \pm 0.5
<i>Dracocephalum polychaetum</i>	29.0 \pm 0.4	78.0 \pm 0.2	82.4 \pm 0.5	19.6 \pm 0.2	17.6 \pm 0.2

Table 2. IC_{50} value of free radical scavenging activity of *Dracocephalum* species extracts ($\mu\text{g}/\text{mL}$) in DPPH assay

Plant species	Hexane extract	CHCl_3 Extract	EtOAc extract	MeOH extract	Aqueous extract
<i>Dracocephalum kotschyi</i>	1584.0 \pm 0.0	354.0 \pm 0.3	47.2 \pm 0.4	23.2 \pm 0.2	643.3 \pm 0.8
<i>Dracocephalum multicaule</i>	428.5 \pm 0.7	152.5 \pm 0.2	25.0 \pm 0.9	91.0 \pm 0.2	707.7 \pm 0.5
<i>Dracocephalum polychaetum</i>	712.0 \pm 0.3	166.9 \pm 0.9	50.5 \pm 0.1	118.9 \pm 0.7	5346.0 \pm 0.1

IC_{50} for Vitamin E: 1.91

Table 3. Reducing power of *Dracocephalum* species extracts as mg FSE/g extract in FRAP assay

Plant species	Hexane extract	CHCl_3 extract	EtOAc extract	MeOH extract	Aqueous extract
<i>Dracocephalum kotschyi</i>	17.73 \pm 0.1	12.12 \pm 0.2	50.39 \pm 0.7	80.75 \pm 0.6	6.65 \pm 0.2
<i>Dracocephalum multicaule</i>	9.93 \pm 0.3	14.63 \pm 0.3	229.92 \pm 0.1	46.18 \pm 0.8	21.9 \pm 0.9
<i>Dracocephalum polychaetum</i>	16.43 \pm 0.5	13.96 \pm 0.8	27.05 \pm 0.5	8.24 \pm 0.1	4.3 \pm 0.1

Table 4. Tyrosinase activity of *Dracocephalum* species extracts (%)

Plant species	Hexane extract	CHCl_3 extract	EtOAc extract	MeOH extract	Aqueous extract
<i>Dracocephalum kotschyi</i>	16.2 \pm 0.1	27.9 \pm 0.05	43.3 \pm 0.05	51.0 \pm 0.03	11.7 \pm 0.1
<i>Dracocephalum multicaule</i>	19.8 \pm 0.2	23.3 \pm 0.07	50.0 \pm 0.05	34.0 \pm 0.05	24.5 \pm 0.07
<i>Dracocephalum polychaetum</i>	6.5 \pm 0.1	35.0 \pm 0.08	38.1 \pm 0.1	39.5 \pm 0.04	36.3 \pm 0.2

IC_{50} for Kojic acid: 15 mM

However, EtOAc extract of *D. multicaule* was much more active compared with the other two. For BChE inhibitory effect, the EtOAc and MeOH extracts of *D. polychaetum* were the most active extracts, respectively. It seemed that *D. polychaetum* was a stronger BChE inhibitor than *D. multicaule* and both were stronger than *D. kotschyi*.

Table 5. AChE and BChE inhibitory activity of *Dracocephalum* species extracts (1 mg/mL)

Extracts	Species	AChE inhibition %	BChE inhibition %
Hexane	<i>D. kotschyi</i>	18.0±0.04	40.8±0.1
	<i>D. multicaule</i>	23.0±0.1	41.4±0.2
	<i>D. polychaetum</i>	10.0±0.3	39.7±0.05
Chloroform	<i>D. kotschyi</i>	17.7±0.1	40.4±0.02
	<i>D. multicaule</i>	27.6±0.5	43.5±0.06
	<i>D. polychaetum</i>	48.6±0.04	42.5±0.05
Ethyl acetate	<i>D. kotschyi</i>	63.0±0.5	47.0±0.05
	<i>D. multicaule</i>	97.0±0.2	15.0±0.3
	<i>D. polychaetum</i>	41.1±0.6	62.7±0.1
Methanol	<i>D. kotschyi</i>	42.6±0.07	45.0±0.2
	<i>D. multicaule</i>	51.1±0.05	41.5±0.4
	<i>D. polychaetum</i>	48.1±0.1	61.2±0.25
Water	<i>D. kotschyi</i>	30.7±0.3	11.1±0.08
	<i>D. multicaule</i>	51.9±0.02	46.8±0.09
	<i>D. polychaetum</i>	42.3±0.3	21.7±0.09
Standard	Galantamin	9.2	45.5±0.1

Previously, other researchers have only reported the antioxidant activity of *D. kotschyi* and *D. polychaetum* [21,22]. Our research showed EtOAc extract of *D. multicaule* and then MeOH extract of *D. kotschyi* were the most active antioxidant extracts. The great free-radical activities of the EtOAc and MeOH extracts can be explained by the high level of phenolic compounds. Previous reports have shown phenolic constituents greatly contributed to the antioxidant activity in medicinal plants [23,24].

Tyrosinase has been reported to be a major contributor to the neurotoxicity created by dopamine associated with neurodegeneration in Parkinson's disease so the inhibition of tyrosinase could be a treatment for Parkinson's disease [25]. This is the first investigation on the anti-tyrosinase activity of *Dracocephalum* species. EtOAc extract of *D. multicaule* and then MeOH extract of *D. kotschyi* represented the highest anti-tyrosinase activities which are in agreement with their highest total phenolic contents among all the other examined extracts. These findings are in agreement with published data of Lee and Choi [26,27]. In general, the current research revealed that EtOAc was a proper solvent for phenolic enrichment in this method of extraction.

This finding confirmed those reported by Elzaawely et al. [28] and Khole et al. [29].

Inhibition of AChE is a treatment strategy for Alzheimer's disease (AD), senile dementia, ataxia, and myasthenia gravis. The only article related to cholinesterase inhibitory effect of *Dracocephalum* is from Mandegari et al. [30] which indicated that the MeOH extract of *D. multicaule* could inhibit AChE. In this study, five different extracts of three species of *Dracocephalum* were examined for both AChE and BChE inhibition. All polar extracts (EtOAc, MeOH and water) of *D. multicaule* were the most potent AChE inhibitors while the polar extracts of *D. polychaetum* showed greater BChE inhibitory activity.

The present research was the first investigation on cholinesterase inhibitory activity as well as anti-oxidant and anti-tyrosinase activities of the three species of *Dracocephalum* from Iran. According to the present study, *D. multicaule* and *D. polychaetum* showed inhibitory effects on AChE and BChE, respectively which introduces them as natural candidates for detailed investigation for treatment of cognitive disorders. In addition, *D. multicaule* and *D. kotschyi* can be considered as great sources of natural antioxidants and anti-tyrosinase that can be replaced with synthetic ones such as kojic acid.

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Author contributions

Maryam Khodaie was the main responsible person for the practical work as a PhD student. Yaghub Amanzadeh and Morteza Piralı were responsible for plant collection and supervising the PhD student. Bio-assays were performed under the supervision of Mohammad Ali Faramarzi. Hamid Reza Adhmi was responsible for extractions and fractionations and also project coordinating. All the authors have approved the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

AChE: acetylcholinesterase; BChE: butyrylcholinesterase; ATCI: acetylthiocholine iodide; BTCl: butyrylthiocholine iodide; DTNB: 5,5'-dithiobis-bis-nitrobenzoic acid; DPPH: 1,1-diphenyl-2-picrylhydrazyl; TPTZ: 2,4,6-Tris(2-pyridyl)-s-triazin; ITM: Iranian traditional medicine; CHCl₃: chloroform; EtOAc: ethylacetate; MeOH: methanol; FSE: ferrous sulfate equivalent; GAE: gallic acid equivalent