

Original Research Paper

Phytochemical screening and evaluation of antioxidant activities of *Dracocephalum kotschy* and determination of its luteolin content

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Abstract

Objective: *Dracocephalum kotschy* (Lamiaceae family) has been used in traditional medicine for stomach and liver disorders, headache and congestion. In the present study, we have investigated phytochemical properties and antioxidant activities of dichloromethane, ethyl acetate and methanol extracts of *D.kotschy*.

Material and Methods: Antioxidant activities of extracts were evaluated using the integration of HPLC-DPPH and ferric reducing antioxidant power (FRAP) methods. In addition, the luteolin content was determined using HPLC method.

Results: The highest antioxidant activity was observed for the methanol extract (among the three tested extracts) showing 50% DPPH scavenging activity at 4.85 µg/ml as compared to butylated hydroxy toluene (BHT) and ascorbic acid (3.00 µg/ml, 0.97 µg/ml). Also, luteolin was detected in methanol extract; it was identified by comparing its retention time and DAD spectra with standard and it was one of antioxidant components of this plant. In addition, the antioxidant activity of methanol extract was higher than BHT, in FRAP assay. Total phenolic content was in the range of 11.62-22.29 mg Gallic acid /gram of dry extract and flavonoid content was in the range of 3.97-5.042 mg Quercetin/ gram of extract for dichloromethane, ethyl acetate and methanol extracts. The quantity of luteolin in *D.kotschy* was found to be 1061.005 µg/g of dried plant.

Conclusion: The results of this investigation indicated that luteolin plays major role in the antioxidant activity of the plant.

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Introduction

The plant of *Dracocephalum kotschyi* (Lamiaceae family) is one of the three endemic species growing in North Khorasan, Iran (Rechinger, 1986). The genus of *Dracocephalum* has been used in traditional medicine for stomach and liver disorders, headache and congestion (Mirheydar, 1995). *D.kotschyi* has some medicinal properties such as anti-spasmodic, analgesic, anti-hyperlipidemic and immuno modulatory activities (Sajjadi et al., 1998; Amirghofran et al., 2000). One hundred compounds such as terpenoids, flavonoids and alkaloids were isolated from this genus and identified (Zeng et al., 2005). Phenolic compounds such as caffeic acid, chlorogenic acid, phenylpropanoids and flavonoids in *Dracocephalum* genus are probably responsible for the antioxidant activity (Sultan et al., 2008). *Dracocephalum* is a source of flavonoids and terpenoids such as luteolin, apigenin, oleanolic acid, ursolic acid, geranial, neral, limonene-10-al and rosmarinic acid (Zeng et al., 2005; Gohari et al., 2003; Saeidnia et al., 2004; Fattahi et al., 2013; Saeidnia et al., 2005). Rosmarinic acid and phenolic compounds contribute to antioxidant activity of *Dracocephalum moldavica* (Weremczuk-Jezyna et al., 2013). *D. moldavica* contains polar compounds such as luteolin, rosmarinic acid and apigenin; and demonstrates high antioxidant activity in all the antioxidant assays but its antioxidant activity was not as potent as the positive controls (Dastmalchi et al., 2007). Antioxidant activity of *D. Kotschyi* was mostly due to the flavonoids such as luteolin, apigenin, cirsimaritin, xanthomicrol and rosmarinic acid (Fattahi et al., 2013). From the oils of *D. kotschyi*, oxygenated monoterpenes were reported and the major components were geranial, limonene and 1, 1-dimethoxy decane (Gohari et al., 2007). Limonene and α -terpineol are responsible for anti-

nociceptive properties of the essential oil of *D. Kotschyi* and methoxylated flavones such as apigenin, luteolin, isokaempferid, cirsimaritin, penduletin and xanthomicrol are responsible to anticancer effects (Golshani et al., 2004; Jahanian et al., 2005; Moghaddam et al., 2012). Luteolin has multiple biological properties such as anti-inflammatory, antioxidant and anticancer activities (Ashokkumar and Sudhandiran, 2008; Park et al., 2012). Methoxylated flavonoids in *D.kotschyi* have anticancer effects (Jahanian et al., 2005). The effect of flavonoids on inhibition of tumor cells has been reported for *D.kotschyi* and flavonoids of this plant were most effective chemicals (Moghaddam et al., 2012). Luteolin can induce apoptosis in tumor cells such as epidermoid carcinoma, pancreatic tumor, leukemia and lung cancer (Jahaniani et al., 2005).

The aim of this study was phytochemical screening and evaluation of antioxidant activity of *D. Kotschyi* and determination of its luteolin content.

Materials and Methods

Chemicals and reagents

The following chemicals were purchased: methanol (Chromasolv, Sigma-Aldrich), dichloromethane (Chromasolv, Sigma-Aldrich), n-hexane (Chromasolv, Sigma-Aldrich), ethyl acetate (Chromasolv, Sigma-Aldrich), folin-Ciocalteu reagent (Sigma-Aldrich), Na₂CO₃ (anhydrous powder, Sigma-Aldrich), Gallic acid (Fluka), aluminum chloride (anhydrous powder, Sigma-Aldrich), quercetin (Sigma-Aldrich), luteolin (Sigma-Aldrich), 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich), ascorbic acid (European pharmacopoeia (EP) reference standard, Fluka), BHT (Sigma-Aldrich), distilled water (Water for chromatography LiChrosolv).

High performance liquid chromatography Conditions

High performance liquid chromatography (HPLC) was run using a LC-6AD pump (Shimadzu, Kyoto, Japan) connected to a SPD-M20A diode array detector (Shimadzu) and the column was analytical Shim-pack ODS-A, 4.6 × 250 mm, 5 µm (Shimadzu, Japan).

Plant material

The plant was collected in June 2014 from the mountains of North Khorasan Province of Iran. The plant was identified by Natural Products & Medicinal Plants Research Center, North Khorasan University of Medical Sciences, Bojnurd, Iran and a voucher specimen (No. 36-1-2) was deposited in the Herbarium of Natural Products & Medicinal Plants Research Center.

Preparation of plant extracts

The aerial parts of the plant were dried under shade at room temperature and then cut into small pieces. About 100 g of the sample was macerated in methanol, dichloromethane and ethyl acetate at room temperature for 48 hr separately. Solvents removal under vacuum at 40 °C gave the crude extracts (Prachayasittikul et al., 2008).

Phytochemical analysis

In order to determine the presence of flavonoids, methanol extract (1 g) was dissolved in 10 ml distilled water and then magnesium powder (100 mg) was added to 2–3 ml of the solution and then 0.5 ml of HCl was added to the mixture. The presence of flavonoids is characterized by the formation of a pale pink to red color within 2 min (Markham, 1982).

Test for phenol

Here, 2ml of extract solution was added to 2 ml of methanol and few drops of ferric chloride solution and it was observed for coloration (Kardong et al., 2013).

Test for saponin

To determine the presence of saponins, methanol extract (1 g) was diluted with 10 ml distilled water in a test tube. After shaking for 2 min, the presence of saponin was confirmed by the formation of froth which was stable for at least 30 min (Hostettmann and Martso, 1995).

Test for tannin

The presence of tannin was evaluated by dissolving methanol extract (1 g) in 10 ml distilled water. Then, the solution was divided into 2 portions. NaCl (10%) and aqueous gelatin solutions (1%) were added to them. Tannin was detected by formation of sediment. The tannin content of the extract is proportional to the amount of sediment. As a confirmation test, one drop of extract was placed on a paper and sprayed with 5% ferric chloride solution. Formation of blue or dark green spots is indicative of the presence of tannin (Evans, 2009).

Evaluation of antioxidant activity

HPLC analysis for DPPH radical scavenging

The antioxidant activities of extracts were described by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity of extracts (Chandrasekar et al., 2006). Fresh DPPH stock solution at a concentration of 2.5 mM was prepared. Then, 100 µl of plant extract at several concentrations (125-500 µg/ml) was added to 100 µl of DPPH solution (final concentration 250µM/200µl). The mixture was vortexed for few seconds and left in the dark for 20 min at room temperature. After that, 20 µl of the sample was injected to HPLC. The blank was prepared by adding 100µlof methanol to 100µlof DPPH stock solution. Analyses were carried out using a Reversed-phase (RP) column (250mm×4.6 mm, 5 µm). Isocratic elution was carried out with methanol/water (80:20, v/v) at a flow rate of 1 ml/min. The DPPH peaks were monitored at 517 nm and 325 nm.

The difference in the reduction of DPPH peak area (PA) between the blank and the sample was used for determining the percent radical scavenging activity of the sample by using the following formula (1):

$$\text{Radical scavenging (\%)} = \left(\frac{\text{PA blank} - \text{PA sample}}{\text{PA blank}} \right) \times 100 \quad (1)$$

Ascorbic acid and butylatedhydroxy toluene (BHT) were used as positive controls. Absorbance inhibition (AI) and 50% effective concentration (EC₅₀) values were calculated using Graph Pad Prism software, version 5.01 (Graph Pad Software Inc., San Diego CA, USA) (Chen et al., 2013).

Ferric reducing antioxidant power (FRAP) assay

The FRAP reagent contained from 2,4,6-tripyridyl-s-triazine (TPTZ) solution (10 mM) in 40 mM HCl, 20 mM FeCl₃.6H₂O and 0.3 M acetate buffer with pH 3.6. Here, 3 ml of FRAP reagent was mixed with 100 µl of each sample and then, the tubes were incubated at 37 °C for 10 min in a water bath. The absorbance of samples was measured at 593 nm. Aqueous solutions of FeSO₄.7H₂O (0-1 mM) were used to plot the calibration curve. FRAP values were expressed as mmol Fe (II) per gram dried extract (Xu et al., 2010).

Total phenolic determination

The total phenolic content of *D. Kotschy* was determined by using Folin-Ciocalteu reagent. Briefly, 100 µl of extract (1000 mg/L) was mixed with 500 µl of diluted Folin-Ciocalteu reagent (1/10). Sodium carbonate (Na₂CO₃) (20%, 1.5 ml) was added to each tube after 1 min of reaction, then tubes were vortexed and incubated for 120 min at room temperature. The absorbance was read at 760 nm. Gallic acid (50 to 500 mg/l) was used as a reference standard for plotting calibration curve. The analyses were done in triplicates. The content of total phenolic compounds expressed as mg gallic acid

equivalent (GAE)/g of dry extract (Hayouni et al., 2007).

Total flavonoid determination

For determination of total flavonoid content, 0.5 ml of the extract was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. Then, tubes were incubated at room temperature for 30 min and the absorbance of the reaction mixtures was measured at 415 nm. All experiments were repeated three times and values as mean standard deviation in terms of flavonoid content (quercetin equivalent: QE per dry weight of extract). The calibration curve was plotted using 12.5 to 100 µg/ml of quercetin in methanol (Chang et al., 2002).

Quantitative analysis of luteolin from aerial parts of *Dracocephalum kotschy*

The amount of luteolin in *D. Kotschy* was evaluated by HPLC method. Luteolin standards were dissolved in methanol to yield concentrations of 0.5- 0.001mg/ml. Methanol extract of *D.kotschy* was prepared at concentrations of 0.05 and 0.2 mg/ml. Mobile phase was a mixture of methanol:H₂O:acetic acid (50:45:5, V/V) at a flow rate of 1 ml/min and the peaks were monitored at 290 nm (Jung et al., 2009). The solutions were filtered through a 0.45 µm membrane filter. Evaluation of each point was repeated three times and each calibration curve was fitted by linear regression. Linearity was determined by the calibration curves obtained from the HPLC analyses of the standard solutions of luteolin. The range of the appropriate amount of sample was then determined. The slope and the other features of the calibration curve were calculated by linear regression method.

Results

The extraction yield depends on solvents, time and temperature of extraction. In the present study, the extraction yield for the different solvents (Table 1) stratified in the following order:

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methanol> dichloromethane> ethyl acetate. The presence of compounds such as saponins, flavonoids and tannins in the methanol extract of *D.kotschyi* was investigated and the presence of flavonoids and absence of saponins and tannins were recorded. The regression equation for determination of total phenolic content was $y= 3.724x+0.061$ ($R^2 =0.985$). In the present study, the total phenolic contents of extracts were in the range of 11.62-22.29 mg GAE/g extract (Table1), while total flavonoid contents were determined based on the equation $y= 0.004x+0.006$ ($R^2= 0.996$) obtained from calibration curves as quercetin equivalent (QE, mg/g dry material). Methanol extract contained a

higher content of total phenolic and flavonoid contents as compared to ethyl acetate and dichloromethane extracts (Table 1). In the present study, antioxidant activity was evaluated by using two different methods namely, DPPH and FRAP. The results of both methods were shown in Table 1. The radical scavenging effects of the extracts were concentration-dependent. The results were reported as EC_{50} , which is defined as the amount of antioxidant required to inhibit 50% of DPPH free radicals and in this method, it is well-known that the lower EC_{50} has the higher anti-radical activity (Magalhães et al., 2008). EC_{50} values of extracts were shown in Table 1.

Table 1. Extraction yield, total phenolic and flavonoid contents, antioxidant properties of *Dracocephalum kotschyi* extracts and positive controls.

Extracts	Extraction yield (%)	Total phenolic (Gallic acid equivalents mg/g of dry extract)	Total flavonoid (Quercetin equivalents mg/g of dry extract)	EC_{50} via HPLC-DPPH ($\mu\text{g/mL}$)	FRAP value (mmol Fe^{2+} /g dry plant)
Dichloromethane	2.42%	11.62±0.02	3.97±0.007	252.48±3.07	trace
Ethyl acetate	0.7%	17.3±0.06	4.78±0.105	9.64±1.09	2.515±0.012
Methanol	11.11%	22.29±0.04	5.042±0.04	4.85±1.36	32.357±0.032
BHT				3.0±0.09	14.3±0.018
Ascorbic acid				0.97±1.62	81.6±0.002
Luteolin				3.6± 0.8	

Discussion

The antioxidant activity is generally attributed to phenolic compounds in plant extracts (Babbar et al., 2011). The redox properties of phenolic compounds enable them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Rice-Evans, 1996). Solvent polarity plays an important role in extraction of phenolic compounds. In this regard, methanol was a better solvent in extraction of phenolic compounds (Hernandez et al., 2009). Antioxidant activity is due to chemical structures of compounds, which allow them to act as reducing agents (Nour et al., 2014). DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a stable free radical. In the presence of antioxidant compounds, DPPH can accept an electron or a

hydrogen atom from them, to be converted to a more stable DPPH molecule. In the present study, ascorbic acid and BHT had the highest radical scavenging activities. Among the three tested extracts, methanol extract with the highest amount of phenolic and flavonoid contents exhibited the strongest antioxidant activity ($EC_{50}=4.85 \mu\text{g/ml}$) compared to ethyl acetate and dichloromethane extracts. The most active compounds of *Dracocephalum* species are flavonoids, such as luteolin, quercetin and apigenin. These flavonoids appear to play important roles in the biological activities (Fattahi et al., 2013). In addition, these flavonoids have anti-bacterial properties (Toit et al., 2009). The difference in the antioxidant activity of extracts may be attributed to the difference in the total phenolic and flavonoid contents

(Singh et al., 2007). Recently, pre-column reaction of samples with DPPH radical followed by HPLC-DAD analysis was developed for identification of antioxidant compounds in biological samples. The conjugated system in the molecular structure would be destroyed due to the reaction of compounds with DPPH, so that the peak areas of compounds with potential antioxidant effect in the HPLC chromatograms would be significantly reduced or disappeared (Figure 1). Figure 2 shows the chromatograms of methanol extract with and without DPPH treatment monitored at 325 nm (Koleva et al., 2002).

The peak areas obviously decreased after spiking the DPPH solution. It was found that the peak area of luteolin was reduced to more than half and this compound had scavenging effect on DPPH radical. The decrease in peak areas was 80.68% for luteolin. In the current investigation, the DPPH scavenging activity of luteolin was detected (Table 1). In other study on *Dracocephalum multicaule*, the results indicated that this plant possesses a marked antioxidant and radical scavenging activity with an EC₅₀ value of 156.5 µg/ml (Mandegary et al., 2014).

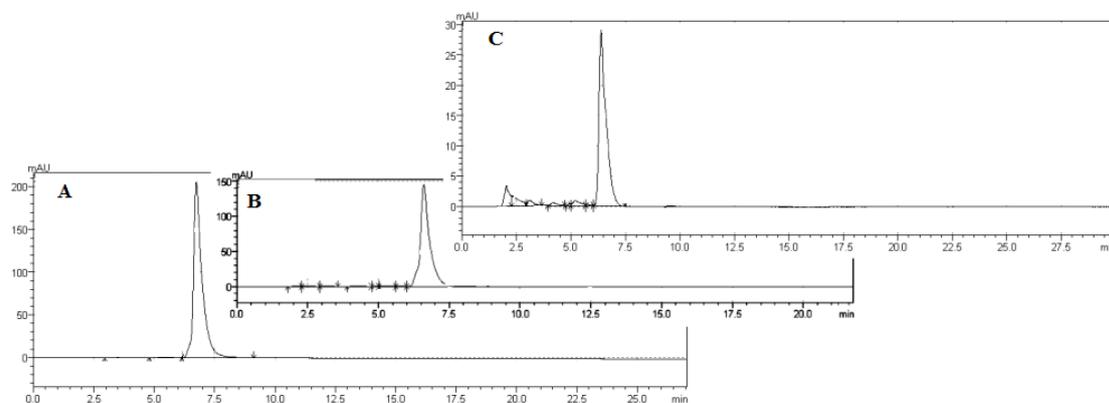


Figure 1. HPLC chromatograms of DPPH monitored at 517 nm: (A) Blank; (B) After incubation with methanol extract (0.125 mg/ml); (C) After incubation with methanol extract (0.25mg/ml). The mobile phase was a mixture of methanol and H₂O (80:20 V/V).

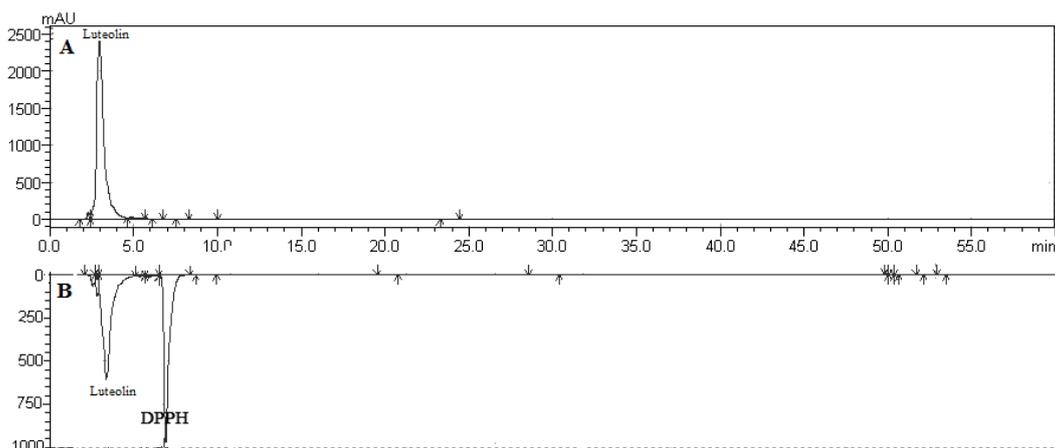


Figure 2. HPLC-DAD chromatogram of *Dracocephalum kotschy* was monitored at 325 nm. (A) Before reaction with DPPH free radicals, (B) After reaction with DPPH free radicals. The mobile phase was a mixture of methanol-H₂O (80:20 V/V).

In FRAP assay, the antioxidant activity was evaluated by oxidation-reduction potential. In this method, antioxidants

react with the ferric tri-pyridyl-triazine complex (Fe (III)-TPTZ) and produce the intense blue color of ferrous tri-pyridyl-

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triazine complex (Fe (II)-TPTZ) (Gülçin, 2012). The antioxidant activities were expressed as the concentrations of antioxidant having a ferric reducing ability (Gülçin, 2012). In this study, methanol extract with the highest FRAP value (and the lowest amount of EC₅₀) had the highest antioxidant activity but it was weaker than ascorbic acid.

Recently, luteolin was isolated from the methanol extract of *Dracocephalum kotschyi* (Gohari et al., 2003; Fattahi et al., 2013). Therefore, a method based on reversed phase HPLC separation combined with UV spectrophotometric detection was

developed in this report for quantitative analysis of luteolin in this plant. The mobile phase was a mixture of methanol:H₂O: acetic acid (50:45:5 V/V), as well as the other chromatographic conditions, showed high performance in the separation of the luteolin. Retention time of luteolin in this condition was 6.6 min (Figure 3) and the representative linear equation was $y=44825x+14683$ ($n=6$; $R^2=0.999$). The relative content of luteolin in methanol extract of *Dracocephalum kotschyi* was 9550 µg/g of extract and 1061.005 µg per dried plant, respectively.

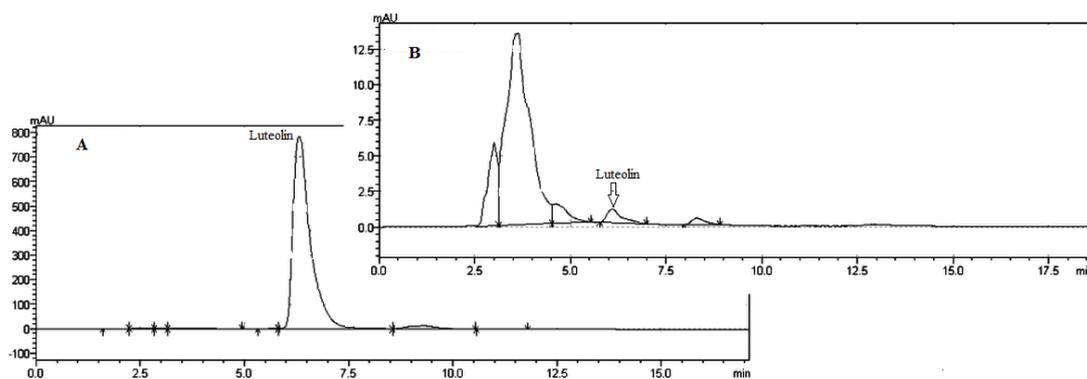


Figure 3. HPLC-DAD chromatograms of (A) luteolin (standard compound); (B) *Dracocephalum kotschyi* were monitored at 290 nm. The mobile phase was a mixture of methanol-H₂O-acetic acid (50:45:5 V/V).

The result of the present study showed that the extract which had the highest amount of flavonoid and phenolic compounds (methanol extract), exhibited the greatest antioxidant activity and luteolin was responsible for antioxidant activity for this plant. The findings of this study support this view that some medicinal plants are promising sources of potential antioxidant and flavonoids and could be used as preventive agents for some diseases.

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Conflict of interest

There is no conflict of interests.

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