**SUPPLEMENTARY MATERIAL**

**Oxidative stress protective effect of *Dracocephalum multicaule*** **essential oilagainst human cancer cell line**

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Abstract

In this study, we report that the antioxidative and protective effect of essential oil of *Dracocephalum multicaule* on K562 cells. Our results showed that monoterpenoids including oxygenated and hydrocarbons ones with 71.5 and 28.3 %, respectively, were the principal essential oil of *D. multicaule*. Perilla aldehyde (71.5%) and limonene (28.1%) were identified as the main components. Antioxidant studies based on DPPH assay indicated that the *D. multicaule* essential oil possesses a marked antioxidant and radical scavenging activity with an IC50 value of 438.2 µg/ml. Pre-treatment with essential oil and main constituents protected K562 cells 49.5% against H2O2 induced oxidative damage throughout increasing the activities of antioxidant enzymes and glutathione content in K562 cells. Taken together, *D. multicaule* essential oil and its main compounds especially in combinatory condition with ratio of 7:3 with high antioxidant properties may be able to protect cells against oxidative stress induced by H2O2 through antioxidative mechanisms.

*Keywors:* Antioxidant activity; Anticancer activity; *Dracocephalum multicaule*; Essential oil; Perilla aldehyde;

**Experimental**

***Plant material***

The aerial parts of *D. multicaule* were collected from West Azarbaijan province of Iran, in June 3, 2008. A voucher specimen (MPH-1307) has been deposited in Medicinal Plants and Drugs Research Institute Herbarium of Shahid Beheshti University (MPH), Tehran, Iran.

***Essential oil isolation and analysis***

Air-dried plant material (50g) were hydrodistilled for 2.5 h using a Clevenger type apparatus. The essential oil was dried over anhydrous Na2SO4 and then was kept in sealed vials at 4οC until analysis and tests. GC analysis was carried out on a Thermoquest-Finnigan Trace GC instrument equipped with a capillary DB-5 fused silica column (60 m × 0.25 mm i.d., film thickness 0.25 μm). The details of GC and GC-MS analyses have already been published (Sonboli et al*.,* 2008). The constituents of the oil were identified by calculation of their retention indices under temperature-programmed conditions for *n*-alkanes (C6–C24) and the essential oil on DB-5 column. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library or with authentic compounds and confirmed by comparison of their retention indices with those of reported data in the literature (Adams, 2007).

***DPPH and β-Carotene-linoleic acid bleaching assay***

The DPPH radicals scavenging activity was determined according to published reference (Blois, 1958). The β-carotene bleaching assay was carried out to determine the antioxidant activityof samples based on Miller's method (Miller 1971)

***Cell culture and cell viability***

The human leukemia K562 cells were cultured in RPMI-1640 medium supplemented with FBS (10%, v/v), streptomycin (100 µg/ml) and penicillin (100 U/ml). The cells were incubated under 5% CO2 humidified atmosphere at 37 ºC. Cell viability was estimated using the MTT assay.

***Lactate dehydrogenase (LDH) release assay***

The LDH activity was assayed spectrophotometrically according to instructions given by the kit's manufacturer (Pars Azmun, Iran).

***Assay of intracellular ROS levels***

The production of ROS was monitored using 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining regarding the Lebel method (Lebel et al., 1992). Briefly, in the present study, cells were pretreated with various concentrations of essential oil and major compounds and after 24 h; the cells were treated with 10 µM DCFH-DA for 30 min. Then the compound-treated cells were washed twice with PBS to remove the extracellular compounds, and DCFH-DA green fluorescence was detected using flow cytometry (Partec PAS, Germany).

***Measurement of antioxidant defense factors***

The intracellular GSH content was measured using spectrofluorometer (Osseni et al., 2000). For the assay of the GPx and GR activities, treated cells (105) were suspended in PBS and centrifuged at 300*g* for 5 min to pellet cells. Cell pellets were resuspended in 20 mM Tris, 5 mM EDTA, and 0.5 mM mercaptoethanol, sonicated, and centrifuged at 3000*g* for 15 min. the activities of GPx and GR were determined regarding the methods have been previously described (Gunzler et al., 1974; Goldberg and Spooner, 1978).



**Figure S1.** Protective effect of the essential oil and main compounds of *Dracocephalum multicaule* (single or binary at ratio of 7:3 in mixture) on cellular LDH release (■) and intracellular ROS generation (□). Values are mean ± SD (n=3). Significantly different from H2O2-treated cells (*P*< 0.05); C: Control; HP: Hydrogen peroxide; other abbreviations are presented as in Table S2.



**Figure S2.** Protective effect of the essential oil and main compounds of *Dracocephalum multicaule* (L, single or binary at ratio of 7:3 in mixture) on antioxidant defenses: GSH content (■) and activities of GPx (□) and GR ( ) in K562 cells.Values are mean ± SD (n=3). Significantly different from H2O2-treated cells (*P*< 0.05), Abbreviations are presented as in Figure S1.

Table S1. Essential oil composition of *Dracocephalum multicaule.*

|  |  |  |
| --- | --- | --- |
| Compound | RI | % |
| β-Pinene | 974 | 0.1 |
| Myrcene | 981 | 0.1 |
| Limonene | 1026 | 28.1 |
| Perilla aldehyde | 1192 | 71.5  |
|  |  |  |
| Monoterpene hydrocarbons |  | 28.3 |
| Oxygenated monoterpenes |  | 71.5 |
| Total (4 Comps.) |  | 99.8 |

RI, retention indices relative to C6 - C24 *n*-alkanes on the DB-5 column.

Table S2. Antioxidant activities of *Dracocephalum multicaule* essential oiland its main constituents.

|  |  |  |
| --- | --- | --- |
| Sample | DPPH (IC50) (μg/ml) | β-carotene bleaching (%) |
| Essential oil (EO) | 438.2 ±4.5 | 54.2 ± 3.4 |
| Perilla aldehyde (PA)  | >1000 | 39.3 ± 1.8 |
| Limonene (L) | 824.1 ± 3.7 | 43.4 ± 2.1 |
| PA/ L (1:1, v/v) | 943.2 ± 1.2 | 41.7 ± 1.7 |
| PA/ L (3:7, v/v) | 741.5 ± 2.5 | 40.5 ± 3.3 |
| PA/ L (7:3, v/v) | 624.7 ± 3.4 | 47.2 ± 2.1 |
| BHT | 21.34 ± 1.4 | 100 |

Values are given as average ± SD.

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