CYTOTOXIC AND APOPTOGENIC PROPERTIES OF DRACOCEPHALUM KOTSCHYI AERIAL PART DIFFERENT FRACTIONS ON CALU-6 AND MEHR-80 LUNG CANCER CELL LINES

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Abstract

Dracocephalum kotschyi is one of the Iranian medicinal plants. The aim of this study was to assess in vitro, the cytotoxic, antiproliferative and apoptotic effects of this plant against lung cancer cell lines (Calu-6 and Mehr-80). Cell viability was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following 24 and 48 h exposure to (12.5 – 200 µg/mL) of plant methanolic extract, dichloromethane (CH2Cl2), ethyl acetate (EtOAc), hexane, water fractions and the essential oil (EO). Further fractionation of primary total methanolic extract revealed luteolin (F10) as the major component. The chemical constituents of EO were identified using gas chromatography linked mass spectrometry (GC MS) analysis. The percentage of apoptotic cells was determined using propidium iodide (PI) staining of DNA fragments by flow cytometry (sub G1 peak). Morphological changes in cells were identified. The most effective fractions were CH2Cl2 fraction, EO and luteolin (F10) (IC50: 79.1 ± 5.36, 62.2 ± 6.94 and 56.32 ± 6.58 µg/mL in Calu-6 cells and 124.2 ± 5.78, 88.13 ± 4.29 and 78.32 ± 6.59 µg/mL in Mehr-80 cell line after 48 h respectively). The DNA fragmentation assay and morphological microscopy also confirmed these data. Trans-citral (geraniol) (15.65%), eucalyptol (9.32%), limonene (7.95%), beta-linalool (4.64%), neryl-acetate (3.67%) and myrcene (3.36%) were identified as the major constituents of the EO. Dracocephalum kotschyi different fractions and compounds have shown significant cytotoxic activities against Calu-6 and Mehr-80 cells.

Rezumat

Dracocephalum kotschyi este o plantă medicinală originară din Iran. Scopul prezentului studiu a fost de a determina in vitro, efectele citotoxice, antiproliferative și apoptotice ale acestei plante pe linile celulară Calu-6 și Mehr-80. Viabilitatea celulară a fost cuantificată prin testul cu 3-(4,5-dimethylthiazol-2-yl)-2,5-difeniltetrazolium (MTT) (12.5 - 200 pg/mL) după expunerea timp de 24 și 48 de ore la extracte metanolice, extracte cu dichlormetan (CH2Cl2), extracte cu acetat de etil (EtOAc), extracte cu hexan și la uleiul esențial (EO). Prin fracționarea ulterioră a extractului metanolic primar total s-a evidențiat luteolina (F10) drept componentă majoră. Componenții chimici ai uleiului esențial au fost identificați folosind gaz cromatografia cu spectrometrie de masă (GC-MS). Prin citometrie în flux s-a determinat procentul de celule apoptotice prin colorarea fragmentelor de ADN cu iodo de propidium (PI), observându-se și modificările morfologice ale celulelor. Efectele cele mai relevante au fost observate la extractul cu dichlormetan CH2Cl2, uleiul esențial și luteolina (F10). Aceste date au fost confirmate și prin testul de fragmentare a ADN-ului și microscopia morfologică. În uleiul esențial au fost identificate următoarele componente majore: trans-citral (geraniol) (15,65%), eucaliptol (9,32%), limonene (7,95%), beta-linalool (4,64%), neryl-acetat (3,67%) și mircenc (3,36%).

Keywords: Dracocephalum kotschyi, Calu-6, Mehr-80, MTT

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Introduction

Cancer is one of the most important health problems worldwide [47, 48]. According to World Cancer Report 2014, cancer was the most prevalent cause of death and it was counted 8.2 million deaths in 2012 [50]. Lung cancer is known as one of the most fatal types of cancer (1.59 million deaths per year). The National Lung Association reported increasing (about 3.5%) in the number of deaths due to lung cancer since 1999 to 2012 and it continues to be one of the most common causes of cancer death [8]. Cigarette smoking has been understood as a major cause of lung cancer [16]. Based on the therapeutic purposes, lung cancer was classified into two classes: non-small-cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC) [1]. The subtypes of NSCLC are adenocarcinoma, squamous-cell carcinoma and large-cell carcinoma [20]. Large-cell carcinoma (LCC) is a heterogeneous group of undifferentiated malignant neoplasms which originates from epithelial cells of the lung. There are four types of large-cell carcinoma: large-cell neuroendocrine carcinoma (LCNEC), large-cell carcinoma with neuroendocrine differentiation (LCCND), large-cell carcinoma with neuroendocrine morphology (LCCNM), and classic large-cell carcinoma (CLCC) [52]. Large cell neuroendocrine carcinoma of the lung (LCNEC) is a highly malignant neoplasm that arises from transformed epithelial cells originating in tissues within the pulmonary tree [53]. The major problem in the treatment of SCLC and NSCLC lung cancers is the resistance to chemotherapy and research studies should be focused on discovering new and more effective therapeutic agents for their treatment. Many natural extracts [4, 40, 34, 54] and natural active compounds have shown anticancer activities such as β-himachalene [39], apigenin [46], luteolin [3, 15, 29, 42, 45], xanthomicrol [2, 22], d-limonene [38], geranial [59] etc. so this is the main reason why the current direction in cancer therapy researches should be focused on the discovery of new natural products or molecules with cytotoxic properties on different cancer cell lines and to use nanotechnology to design targeted drugs for tumour cell for an effective anticancer treatment [33, 37]. Dracocephalum kotschyi Boiss, from Labiatae family, is an endemic plant in Iran [14, 17]. There are some reports on the antinociceptive [18, 43], antihyperlipidemic [11], antitumour [22, 32], antioxidant [25, 26, 41], anti-hypoxic, and immunomodulatory activities of genus Dracocephalum [58]. D. kotschyi, in Iranian folk medicine, was used as antispasmodic and analgesic remedy [22]. D. kotschyi has shown immune inhibitory [13], anticancer, antiinflammatory, antifungal or antibacterial effects [9, 10, 57]. Some chemotherapeutic drugs are botanical metabolites or semi-synthetic derivatives [27]. Spinal-Z as an anticancer agent is extracted from this plant [30, 48]. In 2014, Talari et al. showed that D. kotschyi extract (250 µg/mL) induced reactive oxygen species (ROS) formation, mitochondrial membrane permeabilization (MMP), and mitochondrial swelling and cytochrome c release only in tumoural but not non tumoural hepatocyte [51]. Studies showed that some methoxylated flavonoids from D. kotschyi as xanthomicrol [22] and calycopterin [12, 17] have great anticancer properties. The leaf extract of D. kotschyi showed a higher cytotoxic effect than Spinal-Z, against A172, A2780-s, HL60, KB, K562, MCF-7, Saos-2, Hela, A2780-cp, A549, A375 and HFFF-P16 cell lines [22]. In 2008, Faham et al., reported that calycopterin inhibited proliferation of lymphocyte in a dose-dependent manner (IC50 = 1.7 µg/mL) [13]. Moghaddam et al. isolated eight flavonoid aglycones from the aerial parts of D. kotschyi; luteolin, naringenin, apigenin, isokaempferide, cirsimaritin, penduletin, xanthomicrol and calycopterin. The methoxylated hydroxyflavones (cirmimaritin, penduletin, xanthomicrol, calycopterin) showed selective activities against tumour cells [32]. The aim of this study was to examine the cytotoxic effects of fractionated D. kotschyi extracts on Calu-6 and Mehr-80 cancer cell lines and to find other compounds with significant anticancer properties in this plant. In this study we choose two cell lines of NSCLC: Calu-6 and of LCNEC: Mehr-80. There are few reports evaluating the cytotoxic effects of some agents against Calu-6 as a NSCLC and Mehr-80 as a LCNEC. Mehr-80, a neuroendocrine variant of LCC, was established from the peritoneal fluid of a 39-years-old woman from Iran and the doubling time for this cell line was reported to be 29 hours [48]. Varamini et al., in 2008, demonstrated high cytotoxic activity of Ruta graveolens extract against Mehr-80 (IC50 = 46.2 µg/mL) [56].

Materials and Methods

Reagents

Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution, dimethyl sulphoxide (DMSO), Triton X 100 in analytical grade were purchased from St. Louis, MO, USA (Sigma), also antibiotic solution (penicillin and streptomycin), RPMI-1640 medium, and fetal bovine serum (FBS) were obtained from Grand Island, NY, USA (Gibco).

Plant material

The aerial parts of the plant were collected from Bojnurd, Iran, and the surrounding areas in North Khorasan, Iran, and the authenticity of the specimen was confirmed by researchers of the Research Center of Natural Product Health, North Khorasan University of Medical Sciences, Iran. The herbarium code of the plant was 36-I-2.
Preparation of the methanolic extract and fractions
According to the previously used method [31] in order to obtain the most effective fraction of the extract, the aerial parts of plant were shade-dried, ground to powder and stored. The shade-dried (100 g) powder was suspended in absolute methanol (5 × 250 mL). After 5 days, the solution was filtered and the solvent was evaporated to dryness in vacuum at 45°C. The residue was dissolved in H2O to get a 95% aqueous solution and then n-hexane, dichloromethane, and finally ethyl acetate were used as extraction solvent. All the fractions, under a vacuum, were evaporated to dryness, and then stored at 4°C until further analysis. Figure 1 shows a partitioning scheme of the methanolic extract. Each of these fractions was tested separately.

**Figure 1.**
Partitioning scheme of the methanolic extract, hexane, CH2Cl2 and EtOAc

Fractionation of methanolic extract by medium pressure liquid chromatography (MPLC) and characterization of pure fraction (F10) by high performance liquid chromatography (HPLC).
For evaluating the major bioactive compound(s) of the plant, the methanolic extract (total primary extract) was fractionated by silica gel column chromatography using a mixture of petroleum ether/ethyl acetate with increasing polarity [6, 7]. The obtained fractions were pooled according to TLC profiles and evaporated. Then the extracts were repeatedly subjected to reversed-phase semi-preparative HPLC. HPLC separations were performed on a Shimadzu, Japan semi prep. The system consisted of 2 mL injector loop and C18, 4.6 × 250 mm 5 μm column (Shimadzu), (pH = 3) and Diode Array (Shimadzu) detector operating at 254 nm. According to HPLC and TRLC results the major compound extracted was luteolin (F10 fractionated from the methanolic extract) and this compound was tested as well.

Extraction of the essential oil
The essential oil was extracted according to the method previously described [31]. Briefly, the aerial parts of the plant (350 g) were grounded and then hydro distilled (by a Clevenger apparatus) for 4 h. After separating the upper oily layer of the extract, it was dried with anhydrous sodium sulphate.

Cell lines assays
A human pulmonary adenocarcinoma cell line (Calu-6), human lung cancer cell line (Mehr-80) and mouse fibroblast cell line (L929; as a non-malignant cell line) were purchased from the Pasteur Institute of Iran. Cells were maintained at 37°C in a humidified atmosphere (95%) containing (5%) CO2. All cell lines were cultured in (RPMI-1640) with 10% v/v FBS, 100 unit/mL penicillin and 100 mg/mL streptomycin [43]. Cell lines were grown in tissue culture (100 mm) dishes and harvested with a solution of trypsin-EDTA while in the logarithmic phase.

Cell viability assay
The MTT reduction assay was used to evaluate the cytotoxicity of the isolated compounds [44]. Briefly, the cells were placed in 96 well culture plates with various concentrations (12.5 - 150 μg/mL) of hexane, dichloromethane, ethyl acetate and methanolic extracts, the tenth fraction that extracted by preparative MPLC that we called F10, as well as the essential oil of the plant. The cultured plates were incubated at 37°C and 5% CO2 for 24 and 48 h. 20 μL of MTT solution in PBS (phosphate-buffered saline) (5 mg/mL) was added to each well and the cells were incubated under the same conditions for 3 h at 37°C. The medium was removed, thereafter, in order to dissolve the formazan; DMSO (100 mL) was added to each well. The absorbance was measured at 490 nm (630 nm as a reference) using an ELISA reader (Start Fax 2100; Awareness Technology Inc., Fisher Bioblock Scientific, Tournai, Belgium). Three independent experiments (8 replicates for each one) were carried out. To calculate the concentration of the methanolic extract and fractions which resulted in a 50% reduction of cell viability, IC50 value (the half maximal inhibitory concentration), the following formula was used:

\[ \text{% inhibition} = \frac{(\text{control abs} \times \text{sample abs})}{(\text{control abs})} \times 100. \]

DNA fragmentation assay
All cell lines were incubated in a 6 well plate (1 × 10^5 cell/well) and were treated with 150 μg/mL (the optimal concentration) of methanolic extract, fractions and EO for 48 h (time point) at 37°C and 5% CO2. Floating and adherent cells were harvested and incubated for 4 h at 4°C in the dark. Finally, 750 mL propidium iodide (PI) saline solution (50 μg/mL. PI in 0.1% sodium citrate plus 0.1% Triton X 100, PBS) was added to the samples and the samples were analysed by flow cytometry using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The sub-G1 peak was analysed by FACScan using Cell Quest software (BD Biosciences) [44].
Gas chromatography-mass spectrometry (GC-MS) analysis

The essential oil was analysed using GC-MS, by a Shimadzu QP2010SE chromatograph mass spectrometer (qp 2010 ultra; serial no. 01139; Shimadzu, Kyoto, Japan) operating at 70 eV ionization energy, equipped with a HP 5 capillary column (serial no. 1107908; Restek Corp., Bellefonte, PA, USA; phenyl methyl siloxane, 30 m x 0.25 mm; with 0.25 µm film thickness) with helium as the carrier gas, flow rate 1.5 mL/min and a split ratio of 1:20. The acquisition mass range was 35 - 200 and the scan time was 0.5 sec/scan. For the determination of retention indices, retention times of n-alkanes were used as standards that were injected after the sample under the same conditions. The compounds were identified by comparison of the retention indices (RRI, HP 5) as well as by comparison of their mass spectra with the Wiley and Mass Finder 3 libraries or with the published mass spectra [24].

Statistical analysis

Data were expressed as mean ± SD for at least three independent determinations in triplicate for each experimental point. One-way analysis of variance (ANOVA) and Tukey-Kramer post-hoc test were used for data analysis. A p-value less than 0.05 was considered to be statistically significant.

Results and Discussion

Effects of different fractions and essential oil of *Dracocephalum kotschyi* on cell viability

Cytotoxicity of *D. kotschyi* was examined after 24 and 48 h, at concentrations of 12.5 - 150 µg/mL. The viabilities of Calu-6 and Mehr-80 cells were significantly inhibited by EO, CH2Cl2 and F10 fractionated from methanolic extract (luteolin) in a time-dependent manner as shown in Table I. The most cytotoxic activity was observed after 48 h of treatment; this indicates that there was a delay in reaching the maximum effect in both cell lines. After 48 h exposure in L929 cells (as control normal like cells), there were shown no significant cytotoxic activities from all fractions and the methanolic extract (IC50 > 200 µg/mL), except the EO (IC50 = 97.54 ± 10.23, < 100). In Calu-6 cell line, CH2Cl2 was the most effective fraction (IC50 = 79.1 ± 5.36, 48 h); this fraction was more effective than Hexane fraction (IC50 after 48 h, 136 ± 15.7) and primary methanolic extract (IC50 after 48 h, 148 ± 12.9).

EO and F10 fractionated from methanolic extract were the most effective ones (IC50 after 48 h were 62.2 ± 6.94 and 56.32 ± 6.58, respectively). In Mehr-80 cells, IC50 values after 48 h were 124.2 ± 5.78, 158.1 ± 16.3, 88.13 ± 4.29 and 78.32 ± 6.59, for CH2Cl2 fraction, hexane fraction, EO and F10 fractionated from methanolic extract, respectively. Therefore EO was more effective than CH2Cl2 fraction in both cell lines.

### Table I

<table>
<thead>
<tr>
<th>Treatment agent</th>
<th>IC50 in L929 cells</th>
<th>IC50 in Calu-6 cells</th>
<th>IC50 in Mehr-80 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH2Cl2 fraction</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Hexane fraction</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Essential oil</td>
<td>115.7 ± 8.35</td>
<td>97.54 ± 10.23</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>F10</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Methanolic extract</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
</tr>
</tbody>
</table>

The tenth fraction extracted by preparative MPLC (luteolin). The IC50 values were quantified by MTT assay. The data are presented as the means ± standard deviation (n = 5). ** represent differences in compare with L929 cells (p < 0.01). ## and ### represents differences between Calu-6 and Mehr-80 cells (p < 0.01 and p < 0.001 respectively).

As shown in Table I, in Mehr-80 cells compared to the L929 cells methanolic extract, EO, two fractions (CH2Cl2 and hexane) F10 fractionated from the methanolic extract exhibited more significant cytotoxic effects (p < 0.05, p < 0.001, p < 0.001 and p < 0.001 respectively). Similar effects could be seen also for Calu-6 cells (p < 0.01, p < 0.001, p < 0.001 and p < 0.001 respectively). There was a significant difference in the IC50 values of the EO in the Calu-6 cells compared to the Mehr-80 cells (62.2 ± 6.94 and 88.13 ± 4.29, respectively; p < 0.001). These results showed that the plant extract, fractions and essential oil exerted more potent inhibitory effects on Calu-6 cells compared to Mehr-80 cells.

Effects of the extract, different fractions and EO of *D. kotschyi* on cell apoptosis

The proportion of apoptotic cells was measured by flow cytometry after PI staining, aiming to detect the sub-G1 peak resulting from DNA fragmentation. The sub G1 peak was observed at 48 h following treatment of the cells with the methanolic extract, different fractions and EO (Figure 2). Our results indicated that Hexane fraction, the methanolic extract and the ethyl acetate fraction as well as F10 at the same concentration (150 µg/mL) were the
most effective inducers of apoptosis among the plant extracts examined in Calu-6 cell line (Table II). Morphological changes induced by different extracts and fractions were captured by using an inverted microscope (Olympus CKX41) and shown in Figure 3.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mehr-80</th>
<th>Calu-6</th>
<th>L929</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image" alt="Control" /></td>
<td><img src="image" alt="Control" /></td>
<td><img src="image" alt="Control" /></td>
</tr>
<tr>
<td>Methanol Extract</td>
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<td><img src="image" alt="Methanol" /></td>
<td><img src="image" alt="Methanol" /></td>
</tr>
<tr>
<td>CHCl₂ fraction</td>
<td><img src="image" alt="CHCl₂ fraction" /></td>
<td><img src="image" alt="CHCl₂ fraction" /></td>
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<tr>
<td>Hexane fraction</td>
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<td><img src="image" alt="Hexane fraction" /></td>
<td><img src="image" alt="Hexane fraction" /></td>
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<tr>
<td>Ethyl acetate fraction</td>
<td><img src="image" alt="Ethyl acetate fraction" /></td>
<td><img src="image" alt="Ethyl acetate fraction" /></td>
<td><img src="image" alt="Ethyl acetate fraction" /></td>
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<tr>
<td>Essential oil</td>
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<td><img src="image" alt="Essential oil" /></td>
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</tr>
</tbody>
</table>
Figure 2.
Flow cytometry histograms of apoptosis assays by PI method in apoptotic cells. The sub G1 peak as an indicative of apoptotic cells was observed at 48 h in cells treated with the extract, fractions and the EO of *Dracocephalum kotschyi*.

Table II
Effects of the extract, fractions and essential oil of *Dracocephalum kotschyi* on the sub-G1 cell population (apoptosis, %) in the Calu-6 and Mehr-80 cell lines following 48 h of exposure.

<table>
<thead>
<tr>
<th>Treatment agent</th>
<th>Mehr-80 cells</th>
<th>Calu-6 cells</th>
<th>L929 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.26 ± 0.13</td>
<td>1.26 ± 0.23</td>
<td>6.3 ± 0.25</td>
</tr>
<tr>
<td>CH2Cl2 fraction</td>
<td>97.63 ± 0.35</td>
<td>82.53 ± 0.19</td>
<td>31.2 ± 1.95</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>96.16 ± 0.4</td>
<td>83.13 ± 2.23</td>
<td>75.5 ± 1.8</td>
</tr>
<tr>
<td>Hexane fraction</td>
<td>95.1 ± 0.18</td>
<td>61.93 ± 1.38</td>
<td>63.5 ± 1.9</td>
</tr>
<tr>
<td>Essential oil</td>
<td>98.26 ± 0.24</td>
<td>96.3 ± 0.26</td>
<td>43.1 ± 0.92</td>
</tr>
<tr>
<td>F10</td>
<td>72.7 ± 0.33</td>
<td>82.74 ± 1.2</td>
<td>66.8 ± 2.34</td>
</tr>
<tr>
<td>Methanolic extract</td>
<td>95.83 ± 0.07</td>
<td>68.03 ± 0.3</td>
<td>75.4 ± 2.47</td>
</tr>
</tbody>
</table>
Morphological changes induced by the extract, the fractions, F10 and the essential oil of *Dracocephalum kotschyi* in the L929, Calu-6 and Mehr-80 cell lines following 48 h of exposure.

**Chemical composition of EO**

From the hydro-distillation of the dried powder of the plant it was obtained a pale yellow-coloured oil with a pleasant aroma with a yield of 0.45% (v/w). In EO there where identified 36 components (81.77% of the EO) (Table III). Trans-citral (15.65%), eucalyptol (9.32%), geranyl-acetate (9.12%), limonene (7.95%), beta-linalool (4.64%), neryl-acetate (3.67%) and myrcene (3.36%) were the major constituents of the EO. Among the identified components, 20 components (56.32%) were oxygenated monoterpenes, 4 components (1.44%) were sesquiterpene hydrocarbons, 10 components (21.41%) were monoterpene hydrocarbons, 1 component (1.52%) was a ketone and 1 component (1.08%) was an ester.

**Table III**

<table>
<thead>
<tr>
<th>NO.</th>
<th>Name of compound</th>
<th>Classes of essential oil compound</th>
<th>Ret. time</th>
<th>Area %</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>alpha-Thujene</td>
<td>monoterpene hydrocarbon</td>
<td>5.651</td>
<td>0.63</td>
<td>800.59</td>
</tr>
<tr>
<td>2</td>
<td>alpha-pinene</td>
<td>monoterpene hydrocarbon</td>
<td>5.786</td>
<td>2.07</td>
<td>800.67</td>
</tr>
<tr>
<td>3</td>
<td>sabinene</td>
<td>monoterpene hydrocarbon</td>
<td>6.453</td>
<td>2.24</td>
<td>900.04</td>
</tr>
<tr>
<td>4</td>
<td>beta-pinene</td>
<td>monoterpene hydrocarbon</td>
<td>6.529</td>
<td>1.44</td>
<td>900.076</td>
</tr>
</tbody>
</table>
Our data demonstrated that partially non-polar fractions from *D. kotschyi* extract exert potent cytotoxic effects on Calu-6 and Mehr-80 cells. The most effective fraction was the CH$_2$Cl$_2$ one followed by EtOAc fraction and then the methanolic extract. Furthermore plant EO showed suitable cytotoxic effects. The water (aqueous) fraction did not exhibit any significant anticancer activity in any cell lines (IC$_{50}$ > 200 μg/mL). Luteolin, the major compound of the total extract, exhibited significant anticancer activity. All the effective fractions had more potent inhibitory effects on Calu-6 cells compared to Mehr-80 cells. The critical time point for cytotoxic activity was 48 h following exposure; this indicated that there was a delay in reaching the maximum effect in both cell lines. DNA fragmentation analysis and morphological changes in cells supported the data extracted from cytotoxicity identification in MTT assay. In many studies, essential oil components had shown dose dependent anti-proliferative effects on cancer cells, which make them potentially interesting for experimental cancer treatments. Eucalyptol showed cytotoxic effects on drug-resistant H1299 cells (lung carcinoma) through protection against H$_2$O$_2$ action and reduction of intracellular oxygen radicals [35].

D-limonene [38], geranial (trans-citral) [28, 59] and (citral-1) have been shown to exert cytotoxic effects against cancer cell lines. Citral is a random mixture of two stereoisomers; geranial (trans-cital) and neral (cis-citral). It is confirmed that geranial is significantly more potent than neral and citral and autophagy is the major mechanism of its tumour growth inhibitory effect in p53-null 4T1 cells [59]. Geranyl acetate (3, 7-dimethyl- 2, 6-octadiene-1-ol acetate) as a natural constituent from more than 60 different essential oils has been reported to possess *in vitro* and *in vivo* antitumor activities [36, 55]. Moreover F10 fractionated from methanolic extract, luteolin, showed significant cytotoxic effects on both cell lines, Calu-6 and Mehr-80, respectively (IC$_{50}$ = 56.32 ± 6.58 and 78.32 ± 6.59 μg/mL).
Studies have shown that luteolin exerted cytotoxic activities on different lung carcinoma cells (human A549, human A431 squamous cells, human CH27 and GLC4 lung cancer cells) with IC_{50} ranging from 1 to 15 μg/mL [45]. In previous studies, the compounds identified in D. kotschyi essential oil from different areas were alpha-pinene, limonene [19], geranial (19.63%) [5], Citral, myrcene, caryophyllene, terpinyl acetate [21], limonene, verbene, α-terpineol, perillyl alcohol, caryophyllene [18], α-pinene, caryophyllene oxide, terpenin-4-ol, germacrene D [23], limonene-10-al, 1-dimethoxy decane, methyl geranate, geranial and neral [5, 19]. D. kotschyi different fractions and compounds have shown significant cytotoxic activity against Calu-6 and Mehr-80 cells with acceptable IC_{50} values (< 100 μg/mL) but different from those reported by previous studies [45]. This could be due to environmental factors that affect plant growth and metabolism during different stages of growing period such as geographical variations.

Conclusions

EO, CHCl3 and hexane fractions showed the highest cytotoxic effect on both Calu-6 and Mehr-80 cells. EtOAc fraction was more effective than the methanolic extract in Mehr-80 cells (p < 0.05), while in Calu-6 cells, they showed non-significant cytotoxic effects. Trans-citral (geranial) was the major constituent of plant EO as a documented anti-cancer agent. Luteolin was the major compound that was extracted from Dracocephalum kotschyi with considerable cytotoxic activities in lung cancer cell lines, Calu-6 and Mehr-80.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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