ORIGINAL ARTICLE

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 (CH_2Cl_2), 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 CH_2Cl_2 fraction, EO and luteolin (F10) (IC_{50}; 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 (geranial) (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 liniile celulare Calu-6 și Mehr-80. Viabilitatea celulară a fost cuantificată prin testul cu 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazoliu (MTT) (12,5 - 200 pg/mL) după expunerea timp de 24 și 48 de ore la extracte metanolice, extracte cu diclormetan (CH_2CI_2), extracte cu acetat de etil (EtOAc), extracte cu hexan și la uleiul esențial (EO). Prin fracționarea ulterioară a extractului metanolic primar total s-a evidențiat luteolina (F10) drept componentă majoră. Componenții chimici ai uleiului esențial au fost identificați folosind gaz cromatografia cuplată cu spectrometria de masă (GC-MS). Prin citometrie în flux s-a determinat procentul de celule apoptotice prin colorarea fragmentelor de ADN cu iodura de propidium (PI), observându-se și modificările morfologice ale celulelor. Efectele cele mai relevante au fost observate la extractul cu diclorometan CH_2CI_2, uleiul esențial și luteolină (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%), limonen (7,95%), beta-linalool (4,64%), neryl-acetat (3,67%) și mircen (3,36%).

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

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 since1999 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 neuro-endocrine 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 focus 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 *Labiateae* 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 anti-bacterial 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 (IC_{50} = 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 (cirsimaritin, 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 (IC_{50} = 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 36-1-2.

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 45ºC. The residue was dissolved in H_2O 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 4ºC until further analysis. Figure 1 shows a partitioning scheme of the methanolic extract. Each of these fractions was tested separately.

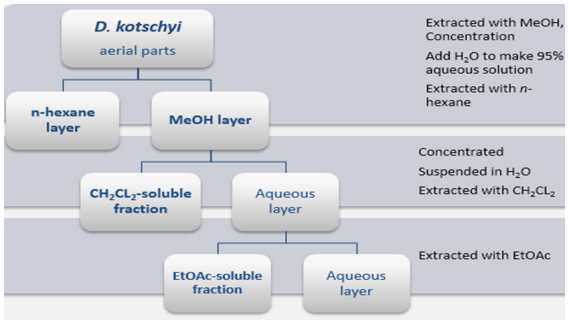


Figure 1.
Partitioning scheme of the methanolic extract, hexane, CH_2Cl_2 and EtOAc

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 TLC 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 37ºC in a humidified atmosphere (95%) containing (5%) CO_2. 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 37ºC and 5% CO_2 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 37ºC. 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, IC_{50} value (the half maximal inhibitory concentration), the following formula was used:

$$\% \text{ inhibition} = ((\text{control abs} \times \text{sample abs})/(\text{control abs})) \times 100.$$

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 37ºC and 5% CO_2. Floating and adherent cells were harvested and incubated for 4 h at 4ºC 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 - 300 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 were 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, $CH2Cl_2$ 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 (IC_{50} > 200 µg/mL), except the EO (IC_{50} = 97.54 ± 10.23, < 100). In Calu-6 cell line, CH_2Cl_2 was the most effective fraction (IC_{50} = 79.1 ± 5.36, 48 h); this fraction was more effective than Hexane fraction (IC_{50} after 48 h, 136 ± 15.7) and primary methanolic extract (IC_{50} after 48 h, 148 ± 12.9). EO and F10 fractionated from methanolic extract were the most effective ones (IC_{50}s after 48 h were 62.2 ± 6.94 and 56.32 ± 6.58, respectively). In Mehr-80 cells, IC_{50} values after 48 h were 124.2 ± 5.78, 158.1 ± 16.3, 88.13 ± 4.29 and 78.32 ± 6.59, for CH_2Cl_2 fraction, hexane fraction, EO and F10 fractionated from methanolic extract, respectively. Therefore EO was more effective than CH_2Cl_2 fraction in both cell lines.

Table I

IC_{50} values of the methanolic extract, fractions and essential oil of *Dracocephalum kotschyi* in the L929, Calu-6 and Mehr-80 cell lines following 24 and 48 h of exposure

	IC_{50} in L929 cells		IC_{50} in Calu-6 cells		IC_{50} in Mehr-80 cells	
Treatment agent	24 h	48 h	24 h	48 h	24 h	48 h
CH_2Cl_2 fraction	> 200	> 200	125 ± 2.12	79.1 ± 5.36###	> 200	124.2 ± 5.78###
Ethyl acetate fraction	> 200	> 200	> 200	168.75 ± 0.53###	> 200	> 200
Hexane fraction	> 200	> 200	> 200	136 ± 15.7##	> 200	158.1 ± 16.3###
Essential oil	115.7 ± 8.35	97.54 ± 10.23**	63.57 ± 3.69	62.2 ± 6.94###	94.25 ± 7.24	88.13 ± 4.29###
F10	> 200	> 200	67.28 ± 7.54	56.32 ± 6.58###	83.65 ± 4.98	78.32 ± 6.59###
Methanolic extract	> 200	> 200	199.5 ± 18.03	148 ± 12.9###	> 200	> 200

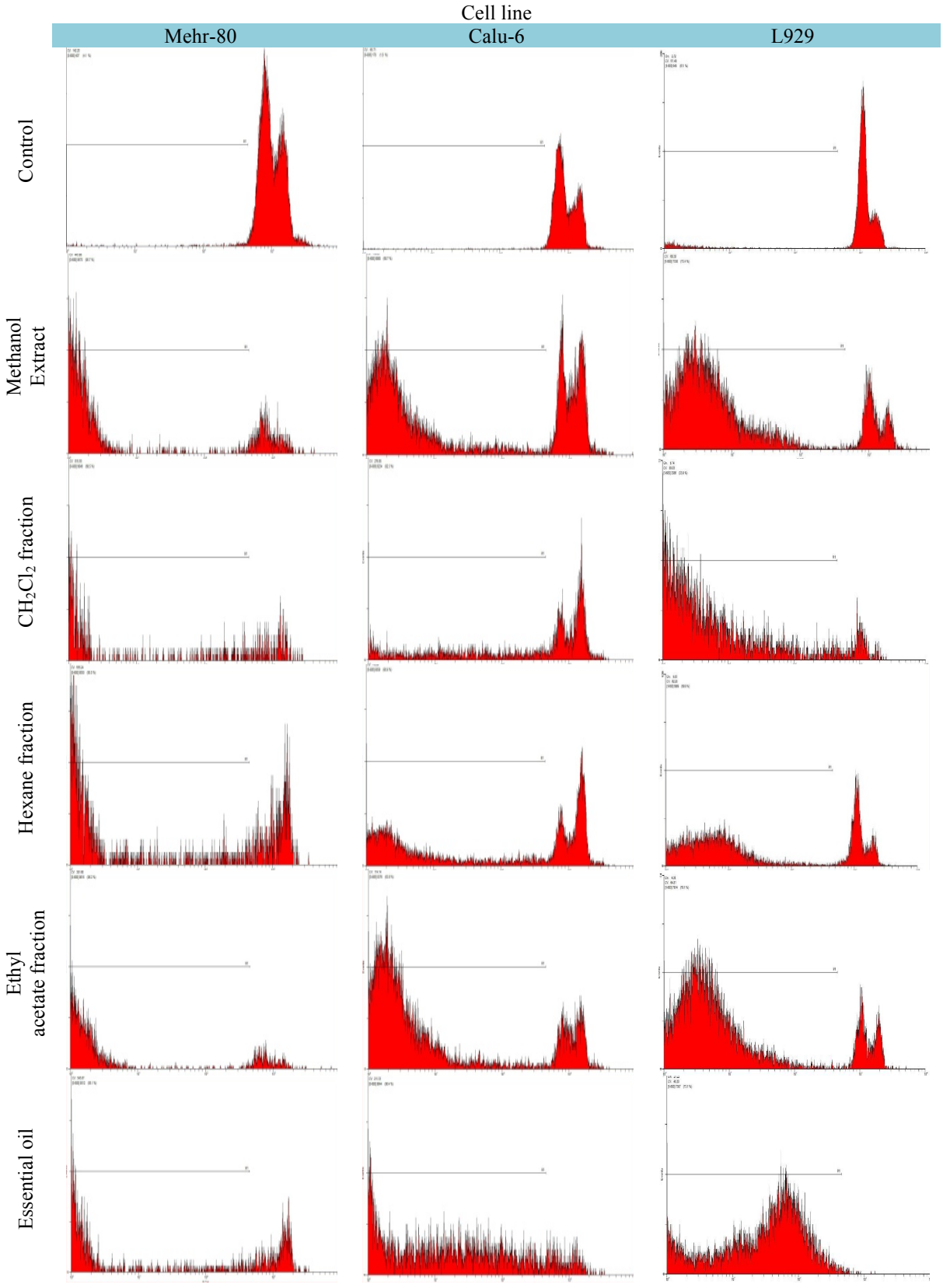
The tenth fraction extracted by preparative MPLC (luteolin). The IC_{50} values were quantified by MTT assay. The data are presented as the means ± standard deviation (n = 5). ** represent differences in compare with L929 cells ($p < 0.01$). ## and ### represents differences between Calu-6 and Mehr-80 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 (CH_2Cl_2 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 IC_{50} 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.



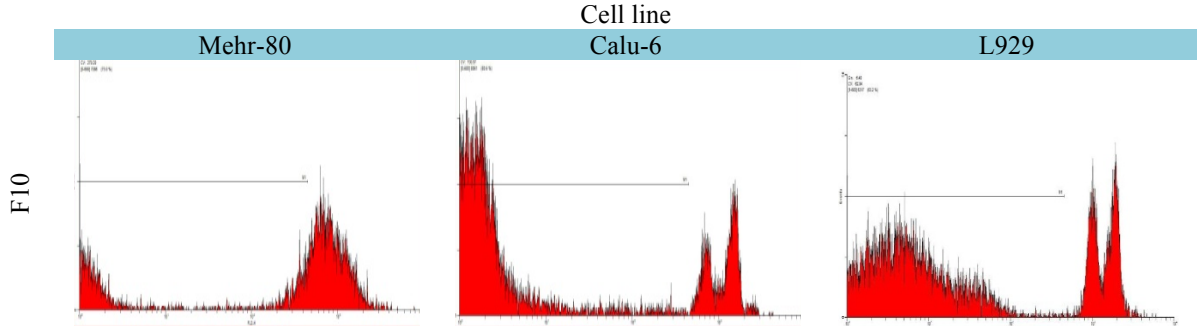


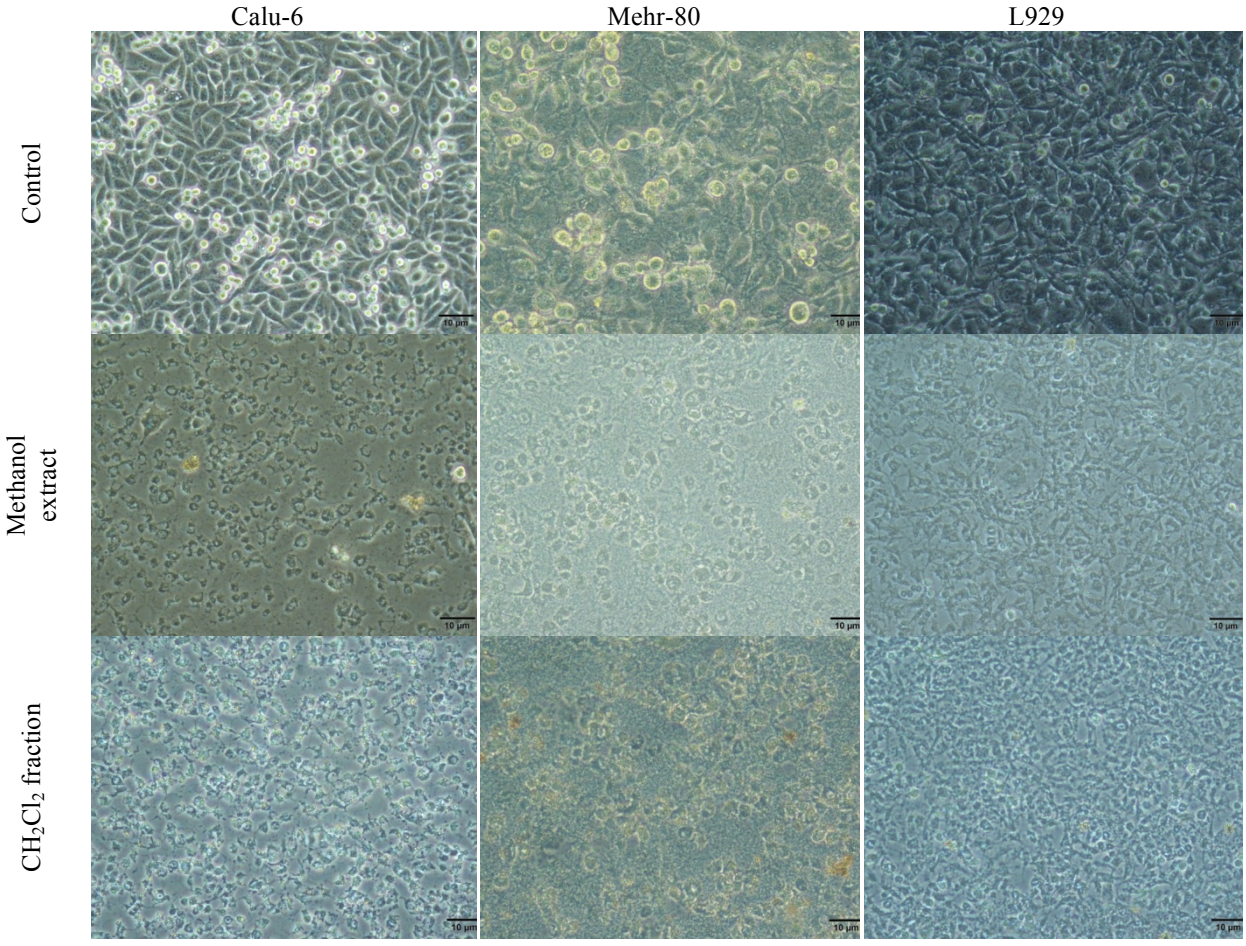
Figure 2.

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.

	Percentage of apoptotic cells %		
Treatment agent	Mehr-80 cells	Calu-6 cells	L929 cells
Control	4.26 ± 0.13	1.26 ± 0.23	6.3 ± 0.25
CH2Cl2 fraction	97.63 ± 0.35	82.53 ± 0.19	31.2 ± 1.95
Ethyl acetate fraction	96.16 ± 0.4	83.13 ± 2.23	75.5 ± 1.8
Hexane fraction	95.1 ± 0.18	61.93 ± 1.38	63.5 ± 1.9
Essential oil	98.26 ± 0.24	96.3 ± 0.26	43.1 ± 0.92
F10	72.7 ± 0.33	82.74 ± 1.2	66.8 ± 2.34
Methanolic extract	95.83 ± 0.07	68.03 ± 0.3	75.4 ± 2.47



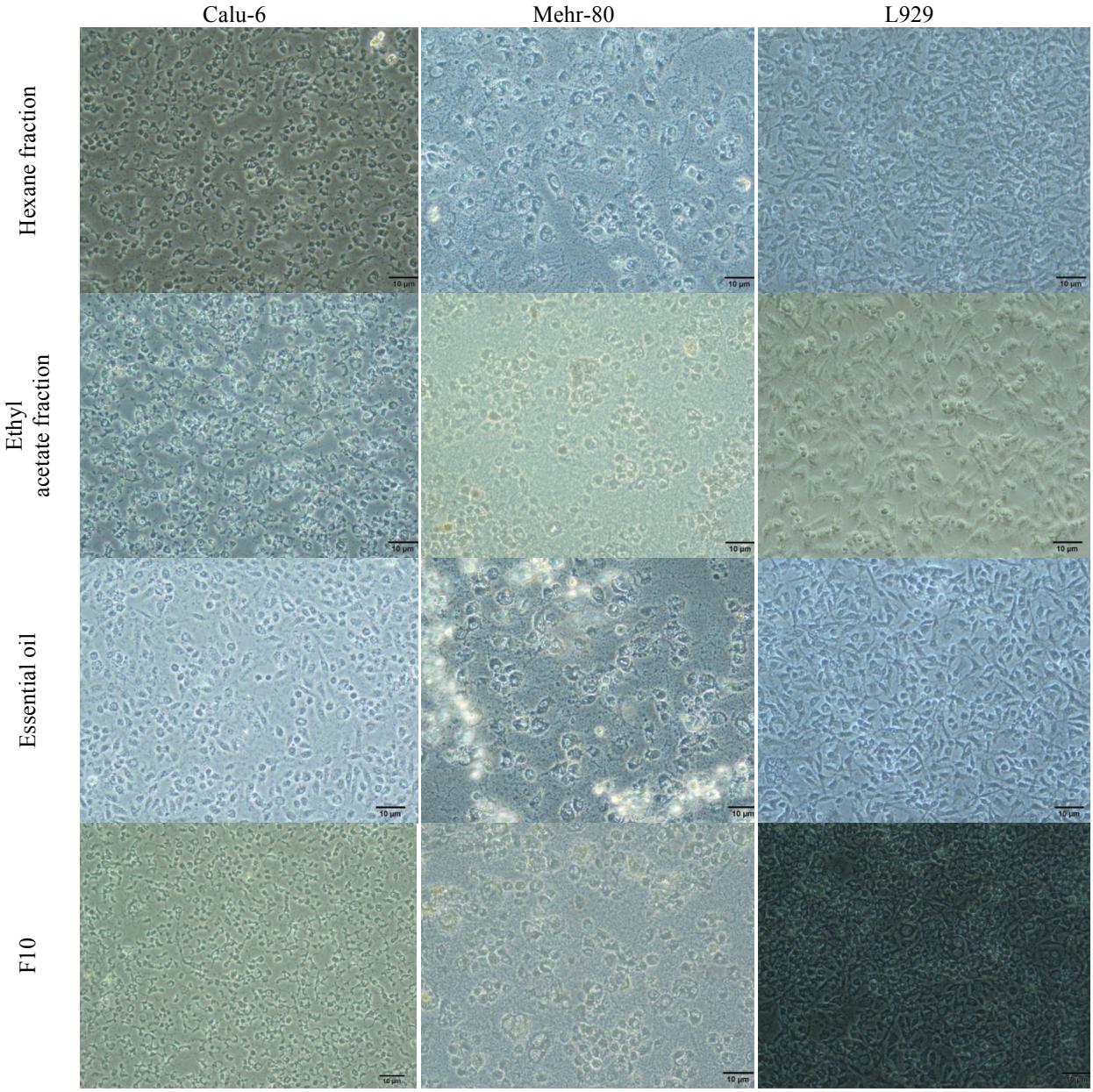


Figure 3.
Morphological changes induced by the extract, the fractions, F10 and the essential oil of *Dracocephalum kotschyi* in the L929, Calu-6 and Mehr-80 cell lines following 48 h of exposure.

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

Chemical composition of the essential oil of *Dracocephalum kotschyi* from North Khorasan, Iran

NO.	Name of compound	Classes of essential oil compound	Ret. time	Area %	RI
1	alpha-Thujene	monoterpene hydrocarbon	5.651	0.63	800.59
2	alpha-pinene	monoterpene hydrocarbon	5.786	2.07	800.67
3	sabinene	monoterpene hydrocarbon	6.453	2.24	900.04
4	beta-pinene	monoterpene hydrocarbon	6.529	1.44	900.076

NO.	Name of compound	Classes of essential oil compound	Ret. time	Area %	RI
5	2-methyl-2-hepten-6-one	Ketone	6.629	1.52	900.13
6	myrcene	monoterpene hydrocarbon	6.704	3.36	900.17
7	alpha-terpinene	monoterpene hydrocarbon	7.179	0.77	900.43
8	p-cymene	monoterpene hydrocarbon	7.317	1.35	900.51
9	limonene	monoterpene hydrocarbon	7.414	7.95	900.56
10	eucalyptol	oxygenated monoterpene	7.474	9.32	900.59
11	trans-beta-ocimene	monoterpene hydrocarbon	7.669	0.32	900.70
12	gamma-terpinene	monoterpene hydrocarbon	7.909	1.28	900.83
13	trans-sabinene hydrate	oxygenated monoterpene	8.083	1.34	900.93
14	linalool oxide	oxygenated monoterpene	8.167	0.72	900.98
15	piperitenone	oxygenated monoterpene	8.6	0.34	1000.23
16	beta-linalool	oxygenated monoterpene	8.682	4.64	1000.27
17	carene-4-epoxy-trans	oxygenated monoterpene	10.199	0.78	1100.16
18	isomenthone	oxygenated monoterpene	10.327	0.71	1100.24
19	terpinene-4-ol	oxygenated monoterpene	10.713	2.61	1100.47
20	alpha-terpineol	oxygenated monoterpene	11.103	1.82	1100.71
21	methyl-benzoate	Ester	11.575	1.08	1200.00
22	nerol	oxygenated monoterpene	12.337	0.43	1200.50
23	carvone	oxygenated monoterpene	13.033	0.21	1200.96
24	geraniol	oxygenated monoterpene	13.232	2.36	1300.09
25	piperitone	oxygenated monoterpene	13.366	0.37	1300.18
26	trans-citral	oxygenated monoterpene	13.943	15.65	1300.59
27	neryl-acetate	oxygenated monoterpene	16.204	3.67	1500.25
28	geranyl-acetate	oxygenated monoterpene	16.668	9.12	1500.62
29	beta-bourbonene	sesquiterpene hydrocarbon	16.796	0.41	1500.72
30	limonen-10-yl-acetate	oxygenated monoterpene	17.247	0.91	1600.08
31	verbenone	oxygenated monoterpene	17.419	0.23	1600.22
32	alpha-humulene	sesquiterpene hydrocarbon	18.175	0.25	1600.85
33	germacrene-D	sesquiterpene hydrocarbon	18.665	0.51	1700.27
34	delta-cadinene	sesquiterpene hydrocarbon	19.408	0.27	1700.92
35	caryophyllene oxide	oxygenated monoterpene	20.768	0.46	1900.17
36	humulene oxide	oxygenated monoterpene	21.377	0.63	1900.76
Total compounds		81.77%			
monoterpene hydrocarbon		21.41%			
oxygenated monoterpene		56.32%			
hydrocarbon sesquiterpene		1.44%			
other		2.6%			

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_2Cl_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_2O_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-citral) 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 showed 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, verbenone, α-terpineol, perillyl alcohol, caryophyllene [18], α-pinene, caryophyllene oxide, terpinen-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, CH_2Cl_2 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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References

1. Abbas A., Aster J., Kumar V., Robbins – Fondamenti di Patologia e di Fisiopatologia. Edra Masson, Milano, 2013; 1-900.
2. Abbaszadeh, H., Ebrahimi, S.A., Akhavan, M.M., Antiangiogenic Activity of Xanthomicrol and Calycopterin, Two Polymethoxylated Hydroxyflavones in Both *In Vitro* and *Ex Vivo* Models. *Phytotherapy Research*, 2014, 28(11): 1661-1670.
3. Androutsopoulos V.P., Tsatsakis A.M., Benzo[a]-pyrene sensitizes MCF7 breast cancer cells to induction of G1 arrest by the natural flavonoid eupatorin-5-methyl ether, *via* activation of cell signaling proteins and CYP1-mediated metabolism. *Toxicol. Lett.*, 2014; 230(2): 304-313.
4. Apostolou A., Stagos D., Galitsiou E., Spyrou A., Haroutounian S., Portesis N., Trizoglou I., Wallace Hayes A., Tsatsakis A.M., Kouretas D., Assessment of polyphenolic content, antioxidant activity, protection against ROS-induced DNA damage and anticancer activity of *Vitis vinifera* stem extracts. *Food Chem. Toxicol.*, 2013; 61: 60-68.
5. Asghari G., Keyhanfard N., Seasonal variation of mono-and sesquiterpenoid components in the essential oil of *Dracocephalum kotschyi* Boiss. *Research Journal of Pharmacognosy*, 2014; 1(4): 41-47.
6. Bălăşoiu L., Calina D., Docea A.O., Patru E., Vlase L., Bubulica M.V., Popescu H., Determination of cardiac glycosides in *Scilla bifolia* (*Liliaceae*) by two different analytical techniques: Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography - Mass Spectrometry (HPLC-MS). *Journal of Medicinal Plant Research*, 2013; 7(42): 3131-3138.
7. Bălăşoiu M.L., Călina D., Vlase L., Bubulică M.V., Chirigiu L., Quantitative Determination of Caffeic Acid, Chlorogenic Acid and Luteolin-7-glucoside from *Scilla bifolia* by HPLC-MS. *Rev. Chim.*, 2014; 65(3): 290-294.
8. Cho Y.K., Kim G., Park S., Sim J.H., Won Y.J., Hwang C.H., Yoo J.Y., Hong H.N., Erythropoietin promotes oligodendrogenesis and myelin repair following lysolecithin-induced injury in spinal cord slice culture. *Biochemical and Biophysical Research Communications*, 2012; 417(2): 753-759.
9. Cordell G.A., Beecher C.W., Pezzuto J.M., Can ethnopharmacology contribute to the development of new anticancer drugs?. *Journal of ethnopharmacology*, 1991; 32(1-3): 117-133.
10. Cox P.A., Balick M.J., The ethnobotanical approach to drug discovery. *Scientific American*, 1994; 270(6): 82-87.
11. Ebrahim Sajjadi S., Movahedian Atar A.M., Yektaian A., Antihyperlipidemic effect of hydro-alcoholic extract, and polyphenolic fraction from *Dracocephalum kotschyi* Boiss. *Pharmaceutica acta Helvetiae*, 1998; 73(3): 167-170.
12. Esmaeili M.A., Farimani M.M., Kiaei M., Anti-cancer effect of calycopterin *via* PI3K/Akt and MAPK signaling pathways, ROS-mediated pathway and mitochondrial dysfunction in hepatoblastoma cancer (HepG2) cells. *Mol. Cell Biochem.*, 2014; 397(1): 17-31.
13. Faham N., Javidnia K., Bahmani M., Amirghofran Z., Calycopterin, an immunoinhibitory compound from the extract of *Dracocephalum kotschyi*. *Phytotherapy research*, 2008; 22: 1154-1158.
14. Fattahi M., Nazeri V., Sefidkon F., Zamani Z., Palazon J., The effect of pre-sowing treatments and light on seed germination of *Dracocephalum kotschyi* Boiss: An endangered medicinal plant in Iran. *Hortic. Environ. Biotechnol.*, 2011; 52: 559-566.
15. Fenga C., Costa C., Caruso E., Raffa L., Alibrando C., Gangemi S., Docea A.O., Tsatsakis A.M., Current evidence on the protective effect of dietary

polyphenols on breast cancer. *Farmacia*, 2016; 64(1): 1-12.
16. Flouris A.D., Poulianiti K.P., Chorti M.S., Jamurtas A.Z., Kouretas D., Owolabi E.O., Tzatzarakis M.N., Tsatsakis A.M., Koutedakis Y., Acute effects of electronic and tobacco cigarette smoking on complete blood count. *Food Chem. Toxicol.*, 2012; 50(10): 3600-3603.
17. Ghahreman A., Flore d'iranica en couleur naturelle, Faculty of Science. University of Tehran, 1987; 432.
18. Golshani S., Karamkhani F., Monsef-Esfehani H.R., Abdollahi M., Antinociceptive effects of the essential oil of *Dracocephalum kotschyi* in the mouse writhing test. *J Pharm Pharm Sci*, 2004; 7(1): 76-79.
19. Hassani M., Farahpour M., Mahdavi M., Hassani L., Study of essantial oil content and composition of *Dracocephalum Kotschyi* in different stages of plant growth in Mazandaran Province. *Advances in Environmental Biology*, 2013: 1584-1588.
20. Horn L., Pao W., Johnson D., Neoplasms of the lung. In Harrison's principles of internal medicine, 18th Edition, McGraw-Hill Companies, Inc, USA.
21. Girish H.V., Vinod A.B., Manjula D.R., Dhananjava B.I., Satish K.D., Senthil D., *In-vitro* cytotoxic effect of *Canthium dicoccum* on different cancer cell lines. *Int. J. of Pharmacy and Pharmaceutical Sci.*, 2015; 7(1): 133-135.
22. Jahaniani F., Ebrahimi S.A., Rahbar-Roshandel N., Mahmoudian M., Xanthomicrol is the main cytotoxic component of *Dracocephalum kotschyii* and a potential anti-cancer agent. *Phytochemistry*, 2005; 66: 1581-1592.
23. Javidnia K., Miri R., Fahham N., Mehregan I., Composition of the essential oil of *Dracocephalum kotschyi* Boiss. from Iran. *Journal of Essential Oil Research*, 2005; 17: 481-482.
24. Juergens U.R., Dethlefsen U., Steinkamp G., Gillissen A., Repges R., Vetter H., Anti-inflammatory activity of 1.8-cineol (eucalyptol) in bronchial asthma: a double-blind *placebo*-controlled trial. *Respir. Med.*, 2003; 97(3): 250-256.
25. Kamali H., Khodaverdi E., Hadizadeh F., Ghaziaskar S.H., Optimization of phenolic and flavonoid content and antioxidants capacity of pressurized liquid extraction from *Dracocephalum kotschyi via* circumscribed central composite. *J. of Supercritical Fluids*, 2016; 107: 307-314.
26. Kamali M., Khosroyar S., Jalilvand M.R., Evaluation of phenolic, flavonoids, anthocyanin contents and antioxidant capacities of different extracts of aerial parts of *Dracocephalum kotschyi*. *Jnkums.*, 2014; 6(3): 627-634.
27. Katzung B.G., Masters S.B., Trever A.J., Basic and clinical pharmacology 12th Edition, McGraw-Hill Companies, Inc, San Francisco, 2012; 949-977.
28. Liu Y., Whelan R.J., Pattnaik B.R., Ludwig K., Subudhi E., Rowland H., Claussen N., Zucker N., Uppal S., Kushner D.M., Terpenoids from *Zingiber officinale* (Ginger) induce apoptosis in endometrial cancer cells through the activation of P53. *PLoS One*, 2012; 7(12): e53178.
29. Lu J., Li G., He K., Jiang W., Xu C., Li Z., Wang H., Wang W., Wang H., Teng X., Teng L., Luteolin exerts a marked antitumor effect in cMet-over-expressing patient-derived tumor xenograft models of gastric cancer. *J Transl. Med.*, 2015; 13(42): 1-11.
30. Mahmoudian M., Rahbar N., Hoormand M., Ebrahimi S., Madadkar Sobhani A., Cytotoxicity of *Peganum harmala* L. seeds extract and its relationship with contents of ß-carboline alkaloids. *Razi Journal of Medical Sciences*, 2002; 8(26): 432-437.
31. Memariani T., Hosseini T., Kamali H., Mohammadi A., Ghorbani M., Shakeri A., Spandidos D.A., Tsatsakis A.M., Shahsavand S., Evaluation of the cytotoxic effects of *Cyperus longus* extract, fractions and its essential oil on the PC3 and MCF7 cancer cell lines. *Oncology Letters*, 2016; 11(2): 1353-1360.
32. Moghaddam G., Ebrahimi S.A., Rahbar-Roshandel N., Foroumadi A., Antiproliferative activity of flavonoids: influence of the sequential methoxylation state of the flavonoid structure. *Phytotherapy research*, 2016; 26(7): 1023-1028.
33. Neagu M., Piperigkou Z., Karamanou K., Engin A.B., Docea A.O., Constantin C., Negrei C., Nikitovic D., Tsatsakis A., Protein bio-corona: critical issue in immune nanotoxicology. *Arch. Toxicol.*, 2017; in press.
34. Olaru O.T., Venables L., Van de Venter M., Nitulescu G.M., Margina D., Spandidos D.A., Tsatsakis A.M., Anticancer potential of selected *Fallopia Adans* species. *Oncol. Lett.*, 2015; 10(3): 1323-1332.
35. Ozkan A., Erdogan A., Membrane and DNA damaging/protective effects of eugenol, eucalyptol, terpinen-4-ol, and camphor at various concentrations on parental and drug-resistant H1299 cells. *Turkish Journal of Biology*, 2013; 37: 405-413.
36. Paik S.Y., Koh K.H., Beak S.M., Paek S.H., Kim J.A., The Essential Oils from *Zanthoxylum schinifoliumpericarp* induce apoptosis of HepG2 human hepatoma cells through increased production of reactive oxygen species. *Biol. Pharm. Bull.*, 2005; 28(5): 802-807.
37. Piperigkou Z., Karamanou K., Engin A.B., Gialeli C., Docea A.O., Vynios D.H., Pavão M.S., Golokhvast K.S., Shtilman M.I., Argiris A., Shishatskaya E., Tsatsakis A.M., Emerging aspects of nano-toxicology in health and disease: From agriculture and food sector to cancer therapeutics. *Food Chem. Toxicol.*, 2016; 91: 42-57.
38. Rabi T., Bishayee A., d-Limonene sensitizes docetaxel-induced cytotoxicity in human prostate cancer cells: Generation of reactive oxygen species and induction of apoptosis. *Journal of Carcinogenesis*, 2009; 8(9): 1-18.
39. Saab A.M., Guerrini A., Sacchetti G., Maietti S., Zeino M., Arend J., Gambari R., Bernardi F., Efferth T., Phytochemical Analysis and Cyto-toxicity Towards Multidrug-Resistant Leukemia Cells of Essential Oils De-rived from Lebanese Medicinal Plants. *Planta Med.*, 2012; 78(18): 1927-1931.
40. Sahpazidou D., Geromichalos G.D., Stagos D., Apostolou A., Haroutounian S.A., Tsatsakis A.M., Tzanakakis G.N., Hayes A.W., Kouretas D., Anti-carcinogenic activity of polyphenolic extracts from grape stems against breast, colon, renal and thyroid cancer cells. *Toxicol. Lett.*, 2014; 230(2): 218-224.

41. Sajadi E., Naderi G., Ziaee R., Antioxidant Effects of Some Medicinal Plants. *Journal of Kermanshah University of Medical Sciences (J Kermanshah Univ Med Sci)*, 2004; 8(2): 1-17.
42. Samy R.P., Gopalakrishnakone P., Ignacimuthu S., Anti-tumor promoting potential of luteolin against 7,12-dimethylbenz(a)anthracene-induced mammary tumors in rats. *Chemico-Biological Interactions*, 2006; 164(1-2): 1-14.
43. Santos F., Rao V., Antiinflammatory and antinociceptive effects of 1, 8-cineole a terpenoid oxide present in many plant essential oils. *Phytotherapy Research*, 2000; 14(4): 240-244.
44. Buteică A.S., Mihăescu D.E., Grumezescu A.M., Vasile B.S., Popescu A., Călina D., Mihăiescu O.M., The citotoxicity of (non)magnetic nano-particles tested on *Escherichia coli* and *Staphylococcus Aureus*. *Dig. Jour. Nanomater. Biostructur.*, 2010; 5(3): 651-655.
45. Sevastre B., Sarpataki O., Olah N.K., Stan R.L., Taulescu M., Marcus I., Cătoi C., Anti-tumor effect of *Euonymus Europaeus* on Ehrlich tumor cells *in vivo*. *Farmacia*, 2014; 62(5): 907-917.
46. Shao H., Jing K., Mahmoud E., Huang H., Fang X., Yu C., Apigenin sensitizes colon cancer cells to antitumor activity of ABT-263. *Molecular Cancer Therapeutics*, 2013; 12(12): 2640-2650.
47. Siegel R.L., Miller K.D., Jemal A., Cancer statistics, 2015. *CA: A Cancer Journal for Clinicians*, 2015; 65(1): 5-29.
48. Siegel R.L., Miller K.D., Jemal A., Cancer statistics, 2016. *CA: A Cancer Journal for Clinicians*, 2016; 66(1): 7-30.
49. Sobhani A.M., Ebrahimi S.A., Mahmoudian M., An *In vitro* evaluation of human DNA topo-isomerase I inhibition by *Peganum harmala* L. seeds extract and its a-Carboline alkaloids. *J Pharm. Pharmaceut. Sci.*, 2002; 5(1): 19-23.
50. Stewart B., Wild C.P., World cancer report 2014. Lyon: International Agency for Research on Cancer, 2015.
51. Talari M., Seydi E., Salimi A., Mohsenifar Z., Kamalinejad M., Pourahmad J., *Dracocephalum*: novel anticancer plant acting on liver cancer cell mitochondria. *BioMed research international*, 2014; 2014(Article ID892170): 1-10.
52. Travis W.D., Colby T.V., Corrin B., Shimosato Y., Brambilla E., In Collaboration with Sobin L.H. and Pathologists from 14 Countries. World Health Organization International Histological Classification of Tumours. Histological Typing of Lung and Pleural Tumours. 3rd EdnSpringer-Verlag, 1999.
53. Travis W.D., Harris C., Pathology and genetics of tumours of the lung, pleura, thymus and heart. Feance: IARC Press, Lyon, 2004.
54. Trifan A., Sava D., Bucur L.A., Mihai C.T., Aprotosoaie A.C., Cioancă O., Hăncianu M., Miron A., Antioxidant and cytotoxic activities of *Phyllophora pseudoceranoides* (Gmelin) new. *et* Tayl. *Farmacia*, 2016; 64(4): 502-506.
55. Ueda S., Masutani H., Nakamura H., Tanaka T., Ueno M., Yodoi J., Redox control of cell death. *Antioxidants and Redox Signaling*, 2002; 4: 405-414.
56. Varamini P., Soltani M., Ghaderi A., Cell cycle analysis and cytotoxic potential of *Ruta graveolens* against human tumor cell lines. *Neoplasma*, 2009; 56(6): 490-493.
57. Wu J., Wu Y., Yang B.B., Anticancer activity of *Hemsleya amabilis* extract. *Life Sciences*, 2002; 71(18): 2161-2170.
58. Zeng Q., Jin H.Z., Qin J.J., Fu J.J., Hu X.J., Liu J.H., Yan L., Chen M., Zhang W.D., Chemical Constituents of Plants from the Genus *Dracocephalum*. *Chemistry & Biodiversity*, 2010; 7: 1911-1929.
59. Zeng S., Kapur A., Patankar M.S., Xiong M.P., Formulation, Characterization, and Antitumor Properties of Trans- and Cis-Citral in the 4T1 Breast Cancer Xenograft Mouse Model. *Pharmaceutical Research*, 2015; 32(8): 2548-2558.