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Cytotoxic effect of *Cousinia verbascifolia* Bunge against OVCAR-3 and HT-29 cancer cells

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ABSTRACT

Introduction: Little information is available about phytochemical and biological properties of *Cousinia* genus. In a primary study, seven *Cousinia* species including *C. verbascifolia* showed cytotoxic activity ranged between 18.4 ± 0.59 to 87.9 ± 0.58 µg/mL. To the best of our knowledge, no other biological studies have been conducted on this plant. Therefore, in this study the cytotoxic effect of *Cousinia verbascifolia* Bunge against OVCAR-3 and HT-29 cancer cells was evaluated.
Methods: Filtration and *in vacuo* concentration of methanol extract resulted in a green gum which was subjected on reverse column chromatography. Semi polar fraction (41.3 g) eluted with water: methanol (20:80), was then subjected on a silica gel column chromatography using hexane/acetone and resulted in 11 fractions. Finally, cytotoxic activities against ovarian and colon cancer cells were determined at a wavelength of 570 nm by Matrix metalloproteinase protein (MTT) standard method.
Results: None of the fractions showed highly cytotoxic activity. Based on NCI, fractions Fr. 1, Fr. 2, Fr. 4, Fr. 5, Fr. 6, Fr. 8 and Fr. 10 showed moderately cytotoxicity with IC_{50} values ranged between 119 to 190 µg/mL against OVCAR-3 cells. Fractions Fr. 1, Fr. 2, Fr. 6, Fr. 7 and Fr. 8 showed moderately cytotoxic activity ranged between 118 to 194 µg/mL against HT-29 cells. Fr. 10 and Fr. 11 showed no cytotoxic activity.
Conclusion: Due to the inhibitory properties of extract and its fractions on cancer cells, identification of responsible compounds possessing cytotoxic effects for generating possible new approach in medicinal chemistry are recommended.

Implication for health policy/practice/research/medical education:
In the present study, we evaluated the cytotoxicity properties of *Cousinia verbascifolia* fractions on cancer cells of ovarian and colon cancer cells. Moderated toxicity of fractions indicated that this plant has the value of more phytochemical consideration to found anti-cancer lead compounds and generating new achievements in medicinal chemistry.
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Introduction

Cancer, a term applied to a group of different diseases can be generally described as an uncontrolled multiplication of cells at any part of the body. These abnormal cells can create expansive masses, spread to vital organs and eventually cause patient death (1). Cytotoxic compounds are the majority of drugs currently used for cancer chemotherapy. The first step of developing new antitumor compounds is testing a wide range of compounds both natural and synthetic with the aim of discovering molecules of useful properties. Plant kingdom provides a rich and wonderful source of biologically active compounds. There are about 250 000-500 000 plant species on earth, out of which only about 20% have been submitted to biological and phytochemical screening. Besides, plant species investigated for anticancer potentials are also a very small fraction (2,3). So, a huge number of plant species still remain to be studied and described in terms of biological and phytochemical profiles.
Cousinia, one of the largest genera of Asteraceae, with 600-

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700 species, is one of the most diverse genera in central and south west Asia. About 250 species of the genus are distributed throughout Iran, comprising About 200 endemic species (4-7). *Cousinia verbascifolia* Bunge, is a monocarpic biennial plant, up to 45 cm high, stems simple or branched from the base, leaves lyrate with spiny-dentate margin, flowers 50-150; corolla pink, rose or purple, 20-35 mm long. The flowering period of the plant is May to July. This plant is endemic to Kopetdagh (NE Iran and S Turkmenistan), open areas, scrublands and stony slopes (6). Little information is available about its phytochemical and biological properties. Phytochemical studies on some *Cousinia* species provide some information about their chemical constituents including: acetylenes, triterpenes, steroids, sesquiterpene lactones, and flavonoids (8-17). In a previous study, the cytotoxic activity against Fibrosarcoma WEHI 164 cancer cells, as well as antibacterial and Matrix metalloproteinase protein (MMP) inhibitory effects of a total ethanol extract of seven *Cousinia* species including *C. verbascifolia* were tested (18). In this study total extract of these plants showed cytotoxic activity with IC_{50} values ranged between 18.4 ± 0.59 to 87.9 ± 0.58 µg/mL.

To the best of our knowledge, no other biological studies have been conducted on this plant. Therefore, in this study the cytotoxic effect of *Cousinia verbascifolia* Bunge against OVCAR-3 and HT-29 cancer cells was evaluated.

Materials and methods

Plant material

Flowering aerial parts of *C. verbascifolia* from Asteracea family were collected from Salehabad, Torbate-Jam, Khorassan-Razavi province, Iran in May 2013 and authenticated by Dr. Iraj Mehregan. A voucher specimen (No. 2838) was deposited at the Herbarium unit of the Pharmacognosy Department, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

Extraction and fractionation

Aerial parts of plant material (3.5 kg) were air-dried in the shade and reduced to fine particles using an electric mill. The powdered plant was extracted exhaustively via percolation using methanol as solvent at a flow rate of 4 ml/min for 10 days. The extract was concentrated under reduced pressure by a rotary evaporator at a temperature not exceeding 50°C. The concentrated extract (450 g) was mixed with appropriate amount of Celite, left to dry, sieved to obtain fine homo sized particles. It was subjected to Medium Pressure Liquid Chromatography (MPLC) on C-18 column and water: methanol (0→100) as mobile phase. Aqueous fraction eluted with water: methanol (70:30) containing polar contents and fraction eluted by methanol (100%) containing fats and chlorophyll were excluded from the study. Semi-polar fraction (41.3 g) eluted with water: methanol (20:80), was subjected on a silica gel column chromatography (400 g silica gel, 7×30 cm) using hexane/acetone (97:3) as mobile phase with increasing acetone to 100%.

MTT viability assay

Human ovarian carcinoma cancer cell line OVCAR-3, and colon carcinoma cancer cell line HT-29 were obtained from Pasteur Institute of Iran. The cell lines were grown in RPMI-1640 media supplemented with 10% fetal calf serum, 100 U/ml + 100 µg/ml penicillin and streptomycin at 37°C in 5% CO_2 condition. Cell viability was determined by colorimetric assay, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and compared with untreated controls. Cells were seeded at 5×10^3 cells/well in 96-well plates. After incubation, compounds at concentrations of 5, 50, and 500 µg/ml were added and incubated again for 48 h. Then, MTT (5 mg/ml in PBS) was added to each well and the cells were incubated for 4 hours. The supernatants were aspirated and 200 µl of dimethyl sulfoxide was added to each well. The plates were shaken for 10 minutes and using colorimetric method, the absorbance was read by the microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm (19).

$$\text{Cell viability} = \frac{\text{Average absorbances of triplicate treated cells}}{\text{Average absorbances of control cells}} \times 100$$

The criteria used to categorize the cytotoxicity of *C. verbascifolia* fractions against OVCAR-3 and HT-29, based on U.S. National Cancer Institute (NCI) and Geran protocol was as follows: $IC_{50} \leq 20$ µg/ml = highly cytotoxic, IC_{50} ranged between 21 and 200 µg/ml = moderately cytotoxic, IC_{50} ranged between 201 and 500 µg/ml = weakly cytotoxic and $IC_{50} > 501$ µg/ml = no cytotoxicity (20-23).

Statistical analysis

All samples were presented as mean ± SD for three measurements. Unpaired Student's t test was used to calculate $P<0.05$ for each compound against the control (+ve). The dose-response curves were plotted to enable the calculation of IC_{50} for each sample.

Results

Semi-polar fraction obtained from MPLC was subjected to silica-gel column chromatography (CC) and eluted with n-hexane: acetone with increasing polarity. Isolated fractions were organized by TLC profile into 11 combined fractions (Fr. 1-Fr. 11). The fractions were concentrated by a rotary evaporator at 40°C and stored in refrigerator at -20°C before running biological assays (Table 1).

To investigate the cytotoxic effects of different factions of the *C. verbascifolia*, on the cell viability, OVCAR-3 and HT-29 cell lines were incubated with the concentrations of 5, 50, and 500 µg/mL of fractions 1-11. None of the fractions showed significant cytotoxic effect ($P<0.05$) at concentrations of 5, and 50 µg/ml against OVCAR-3 cell line. Fr. 1-Fr. 10 showed significant cytotoxic effect at concentration of 500 µg/ml against OVCAR-3 cell lines ($P<0.05$). Fractions 1-11 inhibited growth of HT-29 cells in a concentration-dependent manner. Fr. 1, Fr. 2 and Fr. 4-Fr. 11 showed significant cytotoxic effect at concentration of 50 and 500 µg/ml against HT-29 cell

Table 1. Solvent system employed for column chromatography

Combined Fraction	n-hexane: acetone
Fr. 1	93:7
Fr. 2	88:12
Fr. 3	80:20
Fr. 4	80:20
Fr. 5	70:30
Fr. 6	50:50
Fr. 7	50:50
Fr. 8	50:50
Fr. 9	0:100
Fr. 10	0:100
Fr. 11	Methanol (100%)

lines (P<0.05). Cytotoxicity activities of Fr. 1-11 against cancer cell lines: OVCAR-3 and HT-29 cells are displayed in Figure 1.

A fitted dose-response curve was plotted to enable the calculation of the concentrations that could kill 50% of the cells (IC_{50}). The IC_{50} was calculated for all of the fractions with cytotoxic activities (Table 2). The cytotoxicity data based on IC_{50} values are displayed in Table 2.

A fitted dose-response curve were plotted to enable the calculation of the concentrations that kill 50% of the cells (IC_{50}). The criteria used were as follows: IC_{50}<20 µg/ml (high cytotoxic activity), IC_{50}: 20-100 µg/ml (moderate cytotoxic activity), IC_{50}: 201-500 µg/ml (weak cytotoxic activity), IC_{50}>500 µg/ml (no cytotoxic activity).

Discussion

Fr. 1-Fr. 10 inhibited growth of OVCAR-3 and HT-29 cells in a concentration-dependent manner. None of the fractions showed high cytotoxic activity. Fractions Fr. 1, Fr. 2, Fr. 4, Fr. 5, Fr. 6, Fr. 8 and Fr. 10 showed moderate cytotoxic activity against OVCAR-3 cells according to a criteria based on NCI and Geran protocols (22,23).

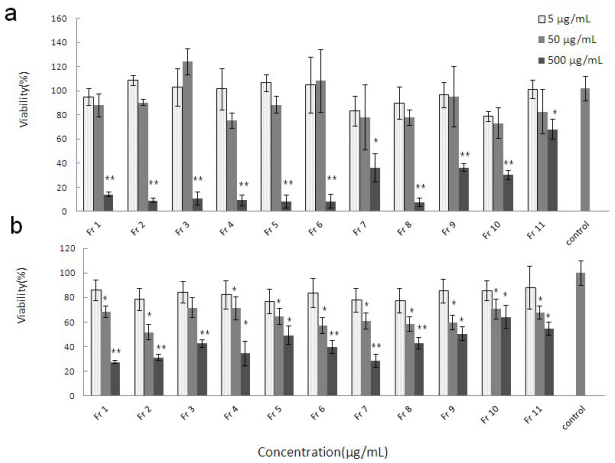

Figure 1. Cytotoxic effect of different fractions: F1-F11 of C. *verbascifolia* against ovarian OVCAR-3 (a) and colon HT-29 (b) cancer cell lines. Cells were treated with different concentrations of fractions 1-11 for 48 hours, and Cytotoxicity was assessed by MTT assay. Results (mean ± SD) were calculated as percent of corresponding control values. *P<0.05, **P<0.001 are significant. Statistical analysis was performed by unpaired Student's t test.

Fractions Fr. 1, Fr. 2, Fr. 6, Fr. 7 and Fr. 8 showed moderate cytotoxic activity against HT-29 cells. The highest cytotoxic activities were observed for Fr. 4, and Fr. 8, with IC_{50} values of 120-121 µg/mL against OVCAR-3 and for Fr. 2 with IC_{50} values of 73.4 µg/mL against HT-29 colon cancer cells. The lowest cytotoxic effects were observed for Fr.10 against HT-29 and Fr. 11 against both cell lines with IC_{50} values more than 500 µg/mL.

Despite the large body of work on taxonomy, systematics, and phylogeny studies of *Cousinia* species, their biological and phytochemical studies are very limited. Based on previous studies, some species of *Cousinia* including *C. verbascifolia* as reported by Shahverdi *et al.*, have shown cytotoxic, MMP inhibitory and antbacterial activities (18). In this study ethanol extract of *C. verbascifolia* exhibited high cytotoxic effect against fibrosarcoma cell line (WEHI

Table 2. IC_{50} Values of *C. verbascifolia* fractions against ovarian (OVCAR-3) and colon (HT-29) cancer cell lines

	OVCAR-3		HT-29	
Fraction	IC_{50} ± SD (µg/mL)	Cytotoxic activity	IC_{50} ± SD (µg/mL)	Cytotoxic activity
Fr. 1	162.73±11.9	Moderate	125.4±8.3	Moderate
Fr. 2	138.68±0.84	Moderate	73.4±8.4	Moderate
Fr. 3	221.3±0.35	Weak	370.2±24.8	Weak
Fr. 4	119.92±12.15	Moderate	214.8.5±20.8	Weak
Fr. 5	149.26±10.61	Moderate	481.6±45.3	Weak
Fr. 6	190.09±63.64	Moderate	162.5±18.2	Moderate
Fr. 7	232.08±15.87	Weak	118±11.2	Moderate
Fr. 8	121.46±18.38	Moderate	194.6±15.2	Moderate
Fr. 9	289.52±28.78	Weak	392.3±38.9	Weak
Fr. 10	171±16.06	Moderate	>500	No
Fr. 11	>500	No	>500	No

A fitted dose-response curve were plotted to enable the calculation of the concentrations that kill 50% of the cells (IC_{50}). The criteria used were as follows: IC_{50}<20 µg/ml (high cytotoxic activity), IC_{50}: 20-100 µg/ml (moderate cytotoxic activity), IC_{50}: 201-500 µg/ml (weak cytotoxic activity), IC_{50}>500 µg/ml (no cytotoxic activity).

164) with IC_{50} value of 18.4 ± 0.59. In contrast, the ethanol extract of *C. shulabadensis*, exhibited weak cytotoxicity with IC50 value of 304.5 ± 0.61 µg/mL.

Comparatively, among the plants from the group "*Arctium-Cousinia* complex"; *Arctium*, the closely related genus to *Cousinia* (6), has showed considarable cytotoxic activity against cancer cell lines. Recently, arctigenin, a lignane-9,9'-olide structure isolated from *Arctium lappa*, showed tumor specific cytotoxicity against lung, liver and stomach cancer cells (23).

Phytochemical assessments in *Cousinia species* revealed the presence of sesquiterpene lactones (SLs) mainly guaianolides and oxygenated bisabolene derivatives, and flavonoids (11-13,18). SLs possess a wide range of biological activities including cycotoxic, and antitumor effects (9,10). So, the cytotoxic properties observed in *C. verbascifolia* less polar fractions like Fr. 2 and Fr. 4 could be contributed to the presence of SLs. Cytotoxic activities of semi polar fractions like Fr. 8 might be due to the presence of flavonoids, lignanes or other phenolic compounds. Fr. 10 and Fr. 11 as polar fractions showed no cytotoxicities.

Conclusion

Due to the inhibitory properties of extract and its fractions on cancer cells, identification of responsible compounds possessing cytotoxic effects for generating possible new approach in medicinal chemistry are recommended.

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Authors' contributions

SES participated in most of the study. MG carried out the design and contributed to data analysis and writing and finalizing the manuscript. MH participated in most of the experiments and in manuscript writing. LM carried out the MTT assay.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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