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Short communication

Cytotoxic Activity of *Juniperus excelsa* M. Bieb. Leaves Essential Oil in Breast Cancer Cell Lines

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Abstract

Background and objectives: *Juniperus excelsa* is a flowering plant that has been applied as traditional medicine for treatment of various disorders such as dysmenorrhea, bronchitis and colds, jaundice and tuberculosis. The aims of the present study were analyzing *J. excelsa* essential oil and investigation of its cytotoxic activity on three breast cancer cell lines. **Methods:** *Juniperus excelsa* leaves were collected from Dena mountains, located in the south-west of Iran. The composition of the essential oil of was analyzed by gas chromatography-mass spectrometry (GC/MS). Cytotoxic activity was evaluated using MTT assay. **Results:** Forty-one components, related to 99.83% of the total oil, were identified. Monoterpene hydrocarbons represented the major components of the volatile oil while α-pinene (73.27%) was the major component. The essential oil showed significant cytotoxic activity against breast cancer cell lines MCF-7 (IC_{50}=0.084 µg/mL), MDA-MB-231 (IC_{50}=0.090 µg/mL) and T-47D (IC_{50}=0.124 µg/mL). **Conclusion:** The analysis of *J. excelsa* oil revealed α-pinene and cedrol as the main compounds of the volatile oil that could justifiy its remarkable cytotoxic effect against the tested cell lines.

Keyword: Cytotoxic; *Juniperus excelsa*; MTT assay

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Introduction

Juniperus excelsa M. Bieb (Greek juniper) is a flowering plant of the Cupressaceae family [1], which is scattered around Mediterranean region, South East Europe, Caucasus, Iran, Iraq and the Arabian Peninsula. One of the most common habitats of the plant is the alpine regions including Alborz and Zagros mountains [2,3]. Different species of *Juniperus* have been applied as medicinal plants for a long time in folk and traditional medicine of Lebanon. The plant has been used as a remedy for treatment or relief the symptoms of dysmenorrhea, cough, bronchitis and colds, jaundice, tuberculosis and as an emmenagogue in Lebanon [4] and these effects have been established by recent studies [5,6]. Essential oil of *J. excelsa* is exploited in traditional medicine of Pakistan for aromatherapy. It is notable that the essential oil is used in a wide variety of products such as scent masks, soaps, candles, lotions, cosmetics and fragrances [7]. Previous studies showed that the essential oil possessed strong antioxidant, antimicrobial, antifungal, disinfectant, and insect-repellent properties [8-13]. Recently, some studies have pointed out that the volatile oil has antimicrobial activity against the Gram-positive

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Staphylococcus aureus and the dermatophyte *Trichophyton rubrum* [5,6]. α-Pinene as the main component of the essential oil [14] which has exhibited antioxidant, anticholinesterase and cytotoxic activities [15-17].

The extract of *J. excelsa* has revealed cytotoxic activity in previous studies [18]. Recent reports have indicated that the essential oil of *J. excelsa* berries and leaves possessed cytotoxic effect against various human cancer cell lines such as CEM/ADR5000, LU1, COL2, KB and LNCaP cells [19,20]; but, there is no report on cytotoxic activity of the essential oil against breast cancer cell lines. In the present study, chemical composition of the essential oil of *J. excelsa* was analyzed by GC/MS. Moreover, cytotoxic activity of the essential oil was investigated on three breast cancer cell lines **(**MCF-7, MDA-MB-231 and T47D).

Material and Methods

Plant collection

The leaves of *Juniperus excelsa* M. Bieb. were collected from Dena mountains located in Kohgiluyeh and Boyer Ahmad province, Iran, in July 2015. The plant was identified by Dr. Yousef Ajani. A voucher specimen was deposited at the Herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (6859 TEH).

Isolation of the essential oil

The essential oil of dried *J. excelsa* leaves (200 g) was obtained by hydro-distillation, using a Clevenger type apparatus for 4 h. The essential oil was dried with anhydrous sodium sulfate, then stored at 4 °C until succeeding tests [21,22].

Gas chromatography mass spectroscopy

Qualitative and quantitative analysis of the essential oil was performed on an Agilent GC/MS system and used Helium as the carrier gas with 1 mL/min flow rate, 1:25 split ratio, a 30 m length capillary column (DB-5) equipped with flame ionization detector (FID). The column temperature was set at 280 °C but was initially held at 50 °C for 5 min, then increased to 280 °C at a rate of 3 °C/min held for 10 min, and eventually increased to 280 °C before injection. Injection and detection temperatures were 280 °C and 300 °C, respectively.

GC/MS was carried out using an Agilent GC with a quadruple detector, on capillary column DB-5 (GC); Helium as carrier gas; flow rate 1 mL/min; the column temperature was kept at 50 °C for 5 min, then raised to 280 °C at a rate of 3 °C/min and kept constant at 280 °C for 10 min. Mass spectra were recorded at 70 eV ionization energy. Retention indices were determined relative to the retention time of n-alkanes series in the same conditions. The compounds were identified based on their mass spectra, Kovats retention indices (KI) were compared with data in Wiley library and literatures and standards of the main components [23,24].

Cytotoxic activity by MTT assay

The cytotoxicity of the essential oil was studied by MTT (3-[4, 5-dimethyl-thiazole-2-yl] - 2, 5 diphenyl-2H-tetrazolium bromide) assay on three human breast cancer cell lines, MCF-7, MDA-MB-231 and T47D. The cell lines were provided from Pasture Institute of Iran, Tehran, Iran. The cells were cultured in RPMI 1640 medium (Biosera, England) containing sodium bicarbonate and N-Hydroxyethylpiperazone-n-2 Ethanesulfonic Acid (HEPES, Biosera, England) supplemented with 10% fetal bovine serum (FBS; Biosera, England) and 1% antibiotics including streptomycin (100 µg/mL) and penicillin (100 U/mL).

The cells were incubated at 37 °C in air atmosphere with 5% carbon dioxide and passaged by trypasinization. After that, the cells were counted by trypan blue exclusion method and finally the cytotoxic activity of the samples were measured by the colorimetric method of MTT (Sigma-Aldrich, USA) assay [25].

Different concentrations of the essential oil were prepared in 10% DMSO/ethanol solution. The final concentrations in each well were 0.005, 0.05, 0.5 and 2.5 µg/mL. The solutions were completely dissolved in the medium, so that there was no particle or sediment. The cells suspensions (200 µL) were seeded into the wells and the 96-well plates incubated in air atmosphere with 5% CO_2 at 37 °C overnight. Five µL of various concentrations of the volatile oil added to the well and incubated again for 24 h. Then MTT reagent (0.5 mg/mL) and the media were added per well and incubated for 4 h. Final percentage of DMSO in the culture medium was less than 1%.

The medium was discharged and 200 µL of pure DMSO was added per well. Finally, the absorbance was measured at 545 nm by

microplate reader. Etoposide (Ebewe Pharma, Austria was used as the positive control while the mixture of the cells, medium and DMSO was applied as the negative control.

Statistical analysis

All results were recorded as mean ± standard deviation (SD) of triplicate tests and statistical analysis was conducted using Microsoft Excel 2016.

Results and Discussion

The hydro-distilled volatile oil of *J. excelsa* leaves yielded 4.3 % v/w. Forty-one compounds were detected in the essential oil of *J. excelsa* leaves. Major components of the essential oil were α-pinene (73.27%) and α-cedrol (5.53%) (table 1).

Monoterpene hydrocarbons were the major compounds of the essential oil (78.52%).

Table 1. Chemical composition of *Juniperus excelsa* M. Bieb leaves essential oil

NO	Compound	Percentage	KI_C	KI_R
1	Tricyclene	0.25	922	926
2	α-Pinene	73.27	936	939
3	Camphene	0.47	948	953
4	β-Pinene	0.77	974	980
5	β-Myrcene	1.26	991	991
6	δ −3-carene	0.41	1010	1011
7	O-cymene	0.66	1018	1022
8	Limonene	1.19	1023	1031
9	α-Terpinolene	0.24	1087	1088
10	α-Pinene oxid	0.13	1093	1095
11	L-Linalool	0.77	1097	1098
12	Limonene oxid	0.41	1141	1139
13	Hexyl butanoate	0.05	1192	1191
14	Hexyl isovalerate	0.38	1241	1241
15	2-tert-butylphenol	0.10	1249	NA
16	Isobornyl acetate	0.36	1287	1285
17	*trans*-Sabinyl acetate	0.13	1291	NA
18	δ –Elemene	0.13	1337	1339
19	Hexyl hexanoate	0.77	1381	1383
20	β-Bourbonene	0.10	1384	NA
21	β-Elemene	0.30	1390	1391
22	Sibirene	0.10	1401	1400
23	β-Cedrene	1.87	1418	1418
24	β-Caryophyllene	1.18	1418	1418
25	γ-Elemene	0.74	1433	1433
26	(E)-β-Farnesene	0.36	1456	1458
27	Germacrene-D	1.22	1480	1480
28	α-Amorphene	0.22	1481	1483
29	α-Muurolene	0.25	1498	1500
30	Cuparene	0.32	1503	1502
31	γ-Cadinene	1.02	1513	1513
32	δ −Cadinene	1.04	1524	1524
33	Elemol	0.11	1548	1549
34	Germacrene B	1.86	1556	1556
35	Ledol	0.12	1565	NA
36	Germacrene d-4-ol	0.70	1574	1574
37	Caryophyllene oxide	0.24	1580	1581
38	Viridiflorol	0.34	1590	NA
39	α-Cedrol	5.53	1596	1596
40	β-Cadinol	0.21	1640	1640
41	α-Cadinol	0.25	1649	1653
	Total	**99.83**		
	Monoterpene hydrocarbons	78.52		
	Oxygenated monoterpene	1.31		
	Sesquiterpene hydrocarbons	10.71		
	Oxygenated sesquiterpene	7.63		
	Aliphatic ester	1.56		
	Other	0.1		

KI_C: Calculated Kovats Index; KI_R: reported Kovats Index ; NA: Not Available

Other ingredients were sesquiterpene compounds (10.71%), oxygenated sesquiterpenes (7.63%), aliphatic esters (1.56%) and oxygenated monoterpenes (1.31%).α-Pinene (73.27%), α-cedrol (5.53%), β-cedrene (1.87%) and germacrene B (1.86%) were identified as the main compounds of the volatile oil. As observed in table 2, our findings demonstrated that monoterpene compounds were the main components of *J. excelsa* leaves essential oil similar to other studies. Previous researches on this species revealed that α-pinene was the major component of leave and berry of *J. excelsa* essential oil, whereas, major compounds of *J. excelsa* fruit essential oil collected from Oman were limonene and terpinolene [20]. Moreover, as shown in table 2, collection time was an important factor in the amount of ingredients [26]. The effect of various concentrations of the essential oil on proliferation of MCF-7, MDA-MB-231 and T47D cells was evaluated. As can be seen in table 3, the best activity was obtained in MCF-7with IC_{50} =0.084±0.011 µg/mL by the volatile oil however the essential oil of *J. excelsa* leaves demonstrated potent cytotoxic activity on all examined cell lines.

Table 2. Comparison of major compounds of *Juniperus excelsa* M. Bieb. essential oil from different regions

Place of Collection	Plant part	Time of collection	Major component*	Monoterpene compounds (%)	Oxygenated monoterpene (%)	Sesquiterpene compounds (%)	Oxygenated sesquiterpe (%)	Aliphatic ester	Ref
Dena mountains (Iran)	Leaves	July	α-Pinene	78.52	1.31	10.71	7.63	1.56	This study
R. Macedonia (Lake Dojran)	Leaves	Late autumn	α-Pinene, Cedrol,	40.96	0.34	16.11	25.28	-	[14]
R. Macedonia (Lake Dojran)	Berries	late autumn	α-Pinene,	80.05	0.06	3.54	0.04	-	[14]
R. Macedonia (Lake Ohrid)	Leaves	late autumn	Sabinene, *trans*-Sabinyl acetate	42.80	26.28	15.03	3.71	-	[14]
R. Macedonia (Lake Ohrid)	Berries	late autumn	Sabinene	80.33	10.51	3.45	0.12	-	[14]
Lebanon	Berries	October	α-Pinene	97.3	0.8	-	-	-	[4]
Oman	Fruit	November	Limonene, Terpinolene	70.747	3.649	4.97	0.444	0.896	[28]
Isparta, Southwestern Turkey	Leaves	June	α-Pinene, Cedrol	44.9	5.2	8.1	-	2.1	[18]
Isparta, Southwestern Turkey	Berries	June	α-Pinene, Cedrol	44.7	28	3.1	0.1	1.7	[18]
Alborz Mountains (Iran)	Foliage	April	α-Pinene	75.5	15.6	6.7	0.5	0.7	[25]
Alborz Mountains (Iran)	Foliage	August	α-Pinene, Trans-verbenol, Germacrene B,	19.5	40	19.5	2.8	3.6	[25]
Alborz Mountains (Iran)	Foliage	November	α-Pinene,	63.86	20.4	11.7	0.3	0.4	[25]
Alborz Mountains (Iran)	Berries	April	α-Pinene, Trans-pinocarveol, Trans – verbenol,	15.2	67.6	8.2	6.3	3.4	[25]
Alborz Mountains (Iran)	Berries	August	α-Pinene	84.2	0.7	10.9	1	0.8	[25]
Alborz Mountains (Iran)	Berries	November	α-Pinene, Germacrene B	69.7	8.9	17.3	1.5	1.4	[25]

*: more than 10%

Table 3. Cytotoxic activity of the *Juniperus excelsa* volatile oil

Sample	MCF-7 IC_{50} µg/mL	T-47D IC_{50} µg/mL	MDA-MB-231 IC_{50} µg/mL
J. excelsa essential oil	0.084 ± 0.011	0.124 ± 0.074	0.090 ± 0.061
Etoposide*	16.082 ± 0.095	18.286 ± 0.064	19.639 ± 0.149

*The positive control

According to the reports available reports, *J. excelsa* essential oil indicated moderate cytotoxic activity against different cancer cell lines such as lung cancer (LU1), colon cancer (COL2), HeLa cancer (KB), prostate cancer (LNCaP) and leukemia (CEM/ADR5000) cell lines [19,20]. The comparison of our finding with results of previous studies demonstrated that the essential oil had more potent cytotoxic activity against MCF-7, MDA-MB-231 and T47D cell lines. Moreover, there are other researches that reported cytotoxic activity of extract of *Juniperus* species. Sadeghi Aliabadi et al., reported that hydro alcoholic extract of *J. excelsa* had significant cytotoxic effect against KB, Hela and MDA-MB-468 cells [27]. In addition, total extract and fractions of *J. excelsa* demonstrated significant cytotoxic activity against MCF-7 cells [18]. Our data showed that essential oil of *J. excelsa* leaf had potent cytotoxic effects against MCF-7, T47D and MDA-MB-231 cells comparable with etoposide as the positive control. Previously, cytotoxic activity of α-pinene, as the major component of the volatile oil, had been shown. It is suggested that the considerable cytotoxic property of the volatile oil could be associated with the presence of α-pinene [16,17,28]. However, additional researches are needed to determine how the essential oil acts with potent cytotoxic activity against the tested cell lines. Hence, *J. excelsa* essential oil could be applied as a natural source for pharmaceutical aspects.

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Author contributions

Study concept and design: Mahdieh Eftekhari and Mohammad Reza Shams Ardekani; analysis and interpretation of data: Mahnaz Khanavi and Aysheh Enayati; statistical analysis: Tahmineh Akbarzadeh and Elahe Karimpour Razkenari; drafting of the manuscript: Mahdieh Eftekhari and Mahnaz Khanavi

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

GC/MS: Gas chromatography mass spectroscopy; MTT: 3-[4, 5-dimethyl-thiazole-2-yl] - 2, 5 diphenyl-2H-tetrazolium bromide**;** DMSO: Dimethyl sulfoxide