

Cytotoxicologic Studies of the Extracts of Iranian *Juniperus Sabina* and *Platycladus Orientalis* on Cancer Cells

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ABSTRACT

Background: Isolation and identification of some potent anti-tumor compounds from medicinal plants, has motivated researchers to screen different parts of plant species for anti-tumor effects. It has been reported that several conifers possess cytotoxic activities on some human tumor cell lines.

Methods: In this study male and female branchlets or fruits of two different species of Iranian conifers were collected from the northern parts of Iran and identified. Hydroalcoholic extracts of them were prepared by percolation. The cytotoxic effects of the extracts on three human tumor cell lines (Hela, KB, and MDA-MB-468) were determined. Different concentrations of extracts were added to cultured cells and incubated for 72 h. Cell survival was evaluated using MTT-based cytotoxicity assay. Cytotoxicity was considered when more than 50% decrease was seen in cell survival.

Results: Although the extracts from *Platycladus orientalis* significantly decreased Hela and MDA-MB-468 cell survival, their effects were not considerable. Extracts from fruit and branchlets of male and female *Juniperus sabina* showed cytotoxic activities against Hela and MDA-MB-468 cells.

Conclusion: It is concluded that extracts of *J. sabin* have cytotoxic effects on cancer cells.

Keywords: *Juniperus sabina*, *Platycladus orientalis*, Cytotoxicity, MTT assay, Cancer cells.

Isolation and identification of some potent anti-tumor compounds such as colchicine, Vinca alkaloids, and recently paclitaxel (taxol®), as one of the most useful anticancer natural products, has encouraged scientists to screen different parts of plant species against cancer cell lines¹⁻³. It has been previously reported that, with the exception of the aril, all parts of the yew plant contain taxine alkaloids and are poisonous. Whether fresh or dried, toxic levels of taxines remain in the plant throughout the year⁴, while maximal concentration occurs during the winter season⁵. However, previous works revealed that different parts of some species of Iranian conifers possess some cytotoxic effects against a number of human tumor cell lines⁶. The potent compounds of some species was podophyllotoxin, while in other species a previously

undescribed but related lignan, silicicolin, now called "desoxy-podophyllotoxin" was the active component. Further investigation on the leaves of several genera of conifers (*Taxus*, *P.*, *Libocedrus*, *Podocarpus*, *Chamacyparis*, and *Callitris*) confirmed the presence of cytotoxic compounds or tumor necrosing substances¹⁻². Cytotoxic effects of *J. sabina* and *P. orientalis* extracts against Hela cells⁷⁻⁹, ethanolic extracts of *J. phenicea*, *J. bermudiana*, *J. communis*, and *Libocedrus decurrens* against KB cell lines have been previously reported¹⁰⁻¹³.

Biological methods (due to their reliability, simplicity, and sensitivity) have been extensively used for evaluation of cytotoxicity of natural products, in the last three decades. As coniferous

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species are among the most genetically diverse of organisms¹⁴, we sought to determine the cytotoxic effects of some Iranian conifers and compare them with other species in the world, using a biological in vitro method.

Materials and methods

1. Plant material

Terminal branchlet of male and female trees and fruits of *J. sabina* and *P. orientalis* were collected from the northern parts of the Iran (Ghozlogh in Golestan province and Sangdehe in Mazandaran province) in September 2000. The plant specimen was identified by the Department of Botany, University of Tehran, and stored at -20 °C before using. A voucher specimen of the plant is deposited in the herbarium of Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

2. Extraction and isolation

50 g of each plant part was chopped and soaked in 75 ml of ethanol (80% V/V) for 24 h and then percolated (5 h, 30 drops/min)¹⁵. The extracts were concentrated by rotary evaporator and dried in an oven at 40°C to give 0.5-0.8 g of solid residue. 0.02 g of these solid residues was dissolved in 100 ml of water containing 0.1% of ethanol, filtered and sterilized using 0.22 µm microbiological filters. Dilution was continued so that the final concentrations of extracts were 10 and 20 µg/ml.

3. Cell lines

Hela (Human cervix carcinoma), KB (Human Caucasian/ epidermal carcinoma), and MDA-MB-468 (Human breast adenocarcinoma) cell lines were purchased from Pasteur Institute of Iran in Tehran. They were grown in RPMI-1640 [each 500 ml of RPMI-1640 was supplemented with 10% of fetal calf serum, 5 ml of penicillin/streptomycin (50 IU ml⁻¹ and 500 µg ml⁻¹ respectively), 5 ml of sodium pyruvate (1 mM), NaHCO₃ (1 g), and 5 ml of L-glutamine (2 mM)]. This completed media was sterilized by 0.22 µm microbiological filters and kept at 4 °C before using.

4. MTT-based cytotoxicity assay

The cytotoxic effect of obtained extracts against previously mentioned human tumor cell lines was determined by a rapid colorimetric assay, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl

tetrazolium bromide (MTT) and compared with untreated controls (Mosmann, 1983). This assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable tumor cells, into an insoluble colored formazan product, which can be measured spectrophotometrically after dissolution in dimethyl sulfoxide (DMSO)¹⁶. Briefly, 200 µl of cells (5 × 10⁴ cells per ml of media) were seeded in 96 microplates and incubated for 24 h (37 °C, 5% CO₂ air humidified). Then 20 µl of prepared concentrations of each extract was added and microplates containing cells and extracts were incubated for another 72 h in the same condition. Doxorubicin was used as a positive control. Absorbance taken from cell grown in the absence of test compound was considered as a negative control. To evaluate cell survival, 20 µl of MTT solution (5 mg/ml in phosphate buffer solution, PBS) was added to each well and incubated for 3 h. Then gently 150 µl of old medium containing MTT was replaced by DMSO and pipetted to dissolve any formed formazan crystals and the plate was read immediately on a microtiter plate reader (StatFax- 2100) at 540 nm. Each extract concentration was assayed in 8 wells and repeated 6 times. Standard curves (absorbance against number of cells) for each cell line were plotted. Intraday and interday variation were determined. Based on standard percent curves, cell survival was calculated. Cell survival in negative control was assumed 100%.

5. Statistical analysis

SIGMASTAT™ (Jandel Software, San Raphael, CA) was used to perform statistical tests. Analysis of variance followed by Duncan test was used to see the differences among groups. Significance was assumed at 5% level.

Results

To evaluate the relationship between number of cells and absorbance, standard curves for Hela, KB, and MDA-MB-468 cell lines were prepared. Findings showed a good relationship between number of cells and absorbance for three tested cell lines ($r^2 = 0.9879, 0.9967, \text{ and } 0.9618$, respectively) (figure 1). Intraday and interday variation for all standard curves were acceptable (coefficient of variation < %20). Doxorubicin as a positive control showed significant inhibitory effects (cell

survival less than 50%) against all tested cell lines. Obtained extracts from the fruits and branchlets of male and female *J. sabina* (10 and 20 µg/ml) showed inhibitory effects against Hela cells. Hydroalcoholic extracts from the fruits and branchlets of male and female trees of *J. sabina* showed inhibitory effects against MDA-MB-468 cells (table 1).

Discussion

New scientific strategies for the evaluation of natural products with biological activities require the implementation of large-scale screening programs. Our laboratory has adopted a microculture assay based on metabolic reduction of MTT to evaluate the cytotoxic effect of plant extracts on different cells. Tetrazolium salt is metabolically reduced by viable cells to yield a blue formosan product measurable by spectrophotometer¹⁷⁻¹⁸. This technique is considered quick and inexpensive for the evaluation of antitumor activity of a large number of natural product extracts^{16, 19}.

Various active compounds (or their semi-synthetic derivatives) derived from medicinal plants have been assessed for their efficacy and tolerability in the treatment of breast cancer. Active compounds from some of these plant species, including paclitaxel, docetaxel (*Taxus baccata*), etoposide (*Podophyllum peltatum*), camptothe-

cine (*Camptotheca acuminata*), vinblastine and vinorelbine (*Vinca rosea*) have well recognized antitumor activity in breast cancer, and have been evaluated in clinical trials²⁰.

In our study, extract from the fruits and branchlets of male and female tree of *Juniperus sabina* reduced percent viability of Hela cells to less than 50. Interestingly, these extracts were more potent against MDA-MB-468 cells (cell survival less than 20%). Although extracts from branchlets and fruits of *Platycladus orientalis* significantly reduced the viability of Hela and MDA-MB-468 cells, it could not reduce cell viability to less than 50%.

The comparison of the viability percent of the cells treated with extracts of these species allows us to conclude that extracts of different parts of *Juniperus sabina* are good candidates for further studies of activity-monitored fractionation to identify their active components.

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Table 1. Cytotoxic effects of hydroalcoholic extracts of different parts of Iranian conifers against 3 cancer cell lines following 72 h continuous exposure to each extract. Values are mean ± SD (n = 6).

Cell lines	Plants						Controls	
	Concentration (µg/ml)	<i>Juniperus sabina</i>			<i>Platycladus orientalis</i>		Positive Doxorubicin	Negative
		Branchlets (female)	Branchlets (male)	Fruits	Branchlets	Fruits		
Hela	10	59.5 ± 7.0 ^{a*}	49.0 ± 2.5 [*]	49.8 ± 4.3 [*]	80.6 ± 5.4 [*]	81.2 ± 9.4 [*]	24.7 ± 1.1 [*]	100 ± 5.4
	20	43.9 ± 8.0 ^{b*}	45.3 ± 2.7 [*]	46.8 ± 4.0 [*]	80.3 ± 6.9 [*]	75.1 ± 3.2 [*]		
KB	10	58.0 ± 5.0 [*]	65.3 ± 8.6 [*]	67.6 ± 11.0 [*]	121.4 ± 12 [*]	107.7 ± 2.0	14.1 ± 1.5 [*]	100 ± 4.6
	20	52.6 ± 3.0 [*]	51.3 ± 2.6 [*]	57.5 ± 9.0 [*]	93.2 ± 2.1	98.9 ± 5.1		
MDA-MB-468	10	20.7 ± 0.7 [*]	17.9 ± 3.0 [*]	19.6 ± 2.0 [*]	79.3 ± 9.4 [*]	64.1 ± 7.4 [*]	3.8 ± 0.7 [*]	100 ± 3.1
	20	14.5 ± 1.1 [*]	13.9 ± 2.5 [*]	15.6 ± 1.0 [*]	62.5 ± 1.6 [*]	50.1 ± 4.8 [*]		

* = p < 0.05

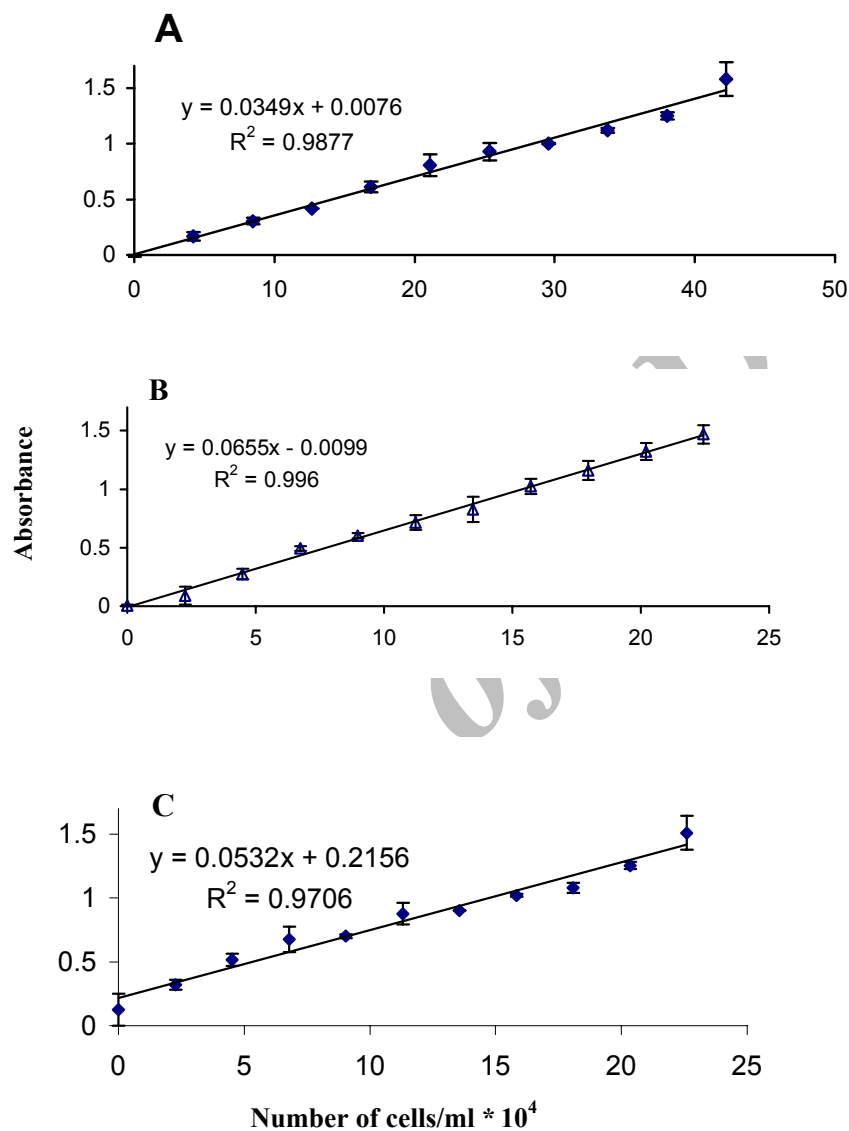


Figure 1. Relationship between cell numbers and absorbance, using MTT assay. Absorbance was determined at 540 nm. Numbers are shown as arbitrary units. A = HeLa, B = KB, C = MDA-MB-468, n = 6.

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