Evaluation of In Vitro Cytotoxic Effects of Juniperus foetidissima and Juniperus sabina Extracts Against a Panel of Cancer Cells

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Abstract

Isolation of some potent anti-tumor compounds from medicinal plants has motivated researchers to screen different parts of plant for their anti-tumor effects. It has been reported that several species of conifers posses’ cytotoxic activities on some tumor cell lines. Here branchlets and berries of Juniperus foetidissima and J. sabina were collected, dried and ethanol extracts of them obtained using percolation. Extracts were dried in reduced pressure and cytotoxic effects of different concentrations (5, 10, 20 μg/ml) were evaluated by MTT assay against three tumor cell lines (Hela, KB, MDA-MB-468), using ELISA at 540 nm. The extracts of the branchlets of male and female of J.foetidissima and berries extract of J. sabina showed inhibitory activities against KB cells. Extracts of male branchlets of J. foetidissima and berries extract of J. sabina were cytotoxic (cell survival less than 50%) against Hela cell line. Regarding to MDA-MB-468, only the extract of male branchlets of J. foetidissima was cytotoxic. Extracts of J. sabina were not cytotoxic at tested concentrations. According to the results obtained by MTT assay, KB cells seem to be much more sensitive than the other cell lines.

Keywords: J. foetidissima; J. sabina; MTT assay; Hela, KB, MDA-MB-468.

Introduction

Conifers are a small group of the flora of Iran (8 species from 8000 species). All aromatic Iranian conifers belong to Cupressaceae family. In Iran this family consists of one species of Platyclusus, one species of Cupressus and five species of Juniperus.

Juniperus species are the second most diverse genus of conifers. The genus Juniperus consist of approximately 67 species and 28 varieties. This genus is divided into three sections: Caryocedrus Edlicher(with only one species); Juniperus (syn: Oxycedrus Spach with 12 species) and Sabina (Miller) Spach (with 55 species) (1).

Two examined species of Iranian Juniperus, J. sabina and J. foetidissima, belong to later section. J. sabina L. (Cupressaceae) is normally low shrub with procumbent or obliquely ascending branches, or rarely a small tree to about 4m, monoecious or dioecious. J. sabina, named “Maymarz” in Persian, generally distributed in central and southern Europe, Anatolia, the

Caucasus, the southern mountains of Asian Russia, Siberia, Mongolia and southwest of Asia (2, 3). *J. sabina* is a medicinal plant which is used in folk medicine as an abortive (4). It’s lignanes have antineoplastic and antiviral activity (5). *J. sabina*’s essential oil has shown antibacterial (6) and antifungal (7) activity.

*J. foetidissima* Willd. (Cupressaceae) is a tree with 5-15 m high, crown slender conical, branched to the ground. This species, named “Arduj” in persian, found in mountains of Greece, Albania, Yugoslavia, Asia Minor to Transcaucasus (2,3). *J. foetidissima* is also a medicinal plant with antifungal activity (8).

Following our main project to screen Iranian medicinal plant for their cytotoxic effects, these species were evaluated. Several investigators have shown that the leaves of several genera of conifers (*Taxus, Platycladus, Libocedrus, Podocarpus, Chaemacyparis and Callitris*) possess cytotoxic compounds or tumor necrosing substances (9). Also, cytotoxic effects of *Juniperus sabina* and *Platycladus orientalis* extracts against Hela cells (7), ethanol extracts of *J. phenicea, J. bermudiana, J. communis* and *Libocedrus decrrens* against KB cell lines (10) have been previously reported. Our previous studies revealed that different parts of Iranian conifers possess cytotoxic effects on some tumor cell lines (11-13). It is believed that some lignans such as podophyllotoxin and desoxypodophyllotoxin are responsible for this effect. In this study we sought to evaluate the cytotoxic effects of different parts of *J. excelsa* and *J. polycarpos* on Hela, KB and MDA-MB-468 cell lines.

**Experimental**

**Plant material**

The branchlets of male and female of and berries of *J. sabina* were collected from Aliabad Katol (2000 m, Golestan province, north of Iran); *J. foetidissima* was collected from Vinaq in Arasbaran (1950 m, East Azarbayejan province, northwest of Iran) in September 2001. The plants were identified by the Department of Forestry, University of Tehran, Iran. The plant materials were stored at -20 °C before use. Voucher specimens of the plants (No. 1414-1415) were deposited in the herbarium of School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

**Extraction and isolation**

Fifty g of each plant part was crushed and soaked in 75 ml of ethanol (80% V/V) for 24 h and then percolated (5 h, 30 drops/min) (14). The extracts were concentrated by a rotary evaporator and dried in an oven at 40 °C to give 0.5-0.8 g of solid residue. Twenty mg of solid residues were dissolved in one ml of ethanol and diluted to 100 ml with distilled water and filtered through 0.22 μ microbiological filters. Dilution was continued so that the final concentrations of extracts were 5, 10 and 20 μg/ml (12).

**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, Tehran, Iran.

**Maintenance of human cell lines**

Cells were grown in RPMI-1640 [supplemented with 10% of fetal calf serum, penicillin/ streptomycin (50 IU ml⁻¹ and 500 μg ml⁻¹ respectively), sodium pyruvate (1 mM), NaHCO₃ and l-glutamine (2 mM)]. Completed media was sterilized using 0.22 μ microbiological filters and kept at 4 °C prior to use. Cells were maintained and grown in RPMI 1640 up to 15 subcultures. A sample of each cell lines was frozen and kept under liquid nitrogen for future studies.

**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 (15). 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 dissolving in
Cytotoxicity of Juniperus foetidissima and J. sabina

Cells (5 × 10⁴ cells per ml of media) were seeded in 96-well 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. The first column of each microplate was assumed as negative control (containing no extracts or doxorubicin). To evaluate cell survival, 20 μl of MTT solution (5 mg/ml in phosphate buffer solution) 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. Absorbance was then determined at 540 nm by ELISA plate reader. 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 variations were determined. Based on standard curves percent cell survival was calculated. Percent of cell survival in ethanol treated cells (1% as negative control) was assumed 100.

**Statistical analysis**

SIGMASTAT™ (Jandel Software, San Raphael, CA) was used to perform statistical tests. Anova was used to see the differences among groups. Significance was assumed at the 5% level.

**Results**

A good relationship between absorbance and the number of cells was observed for Hela, KB and MDA-MB-468 cell lines, (r² = 0.9789, 0.9852 and 0.9919, respectively). Intraday and interday variations for all standard curves were acceptable (%CV < 15). Doxorubicin (20 μg/ml), a known cytotoxic antibiotic, as a positive control significantly inhibited the proliferation of all tested cell lines to less than 25%. Extracts were considered cytotoxic when cell viability decreased to less than 50%.

**Cytotoxic effect of extracts against Hela cells**

As shown in Figure 1, hydroalcoholic extract of male branchlets of *J. foetidissima* (20 μg/ml), was cytotoxic against Hela cells; whereas other plant samples were not cytotoxic at tested concentrations.

**Cytotoxic effect of extracts against KB cells**

Hydroalcoholic extracts of the terminal branchlets of male and female and berries of *J. foetidissima* in all tested concentration showed an excellent inhibitory effects against KB cells (IC₅₀ < 5 μg/ml; Figure 2). However, cytotoxicity was seen at highest concentration (20 μg/ml) of berries extract of *J. sabina* (Figure 2).

**Cytotoxic effect of extracts against MDA-MB-468 cells**

In the case of MDA-MB-468 cells only the...
highest concentration (20 μg/ml) of obtained extract from branchlets of *J. foetidissima* was cytotoxic (Figure 3); whereas with extracts of *J. sabina* IC$_{50}$ was not obtained.

### Discussion

Cytotoxic compounds are one of the most important classes of drugs used for cancer treatment. There have been several researches to get new cytotoxic agents. In this regard compounds such as colchicine, *Vinca* alkaloids and paclitaxel isolated from medicinal plants showed considerable promises. Studies on different genera of conifers showed the presence of cytotoxic compounds or tumor necrosing substances (9). Furthermore, cytotoxicity of some Iranian conifers has been shown in our previous studies (11-13). To evaluate the cytotoxicity of the other genera of Iranian conifers, this study was conducted.

In the present study, MTT assay was used for evaluation of cytotoxic activity of conifers. As seen in Figure 2, hydroalcoholic extracts of all tested parts of *J. foetidissima* showed cytotoxic effects comparable to that of doxorubicin (positive control) against KB cells (IC$_{50}$ < 5 μg/ml). Lignans, neolignans and flavonoid glycosides in *juniperus* species are responsible for their antibacterial and cytotoxic properties.
(16, 17). Substances that induce apoptosis toward human promyelocytic leukemia HL-60 cells have been extracted from leaves of Juniperus taxifolia (18). The results of a study conducted by Topcu and co-workers (19) showed that diterpenes and sesquiterpenes extracted from the berries of J. excelsa had cytotoxic activity against a panel of cell lines [human colon cancer cell line (LNCaP), KB-V (+VLB) and KB-V (-VLB)] and Mycobacterium tuberculosis. Emami and colleagues (20) showed that extracts obtained from leaves and fruits of Iranian J. foetidissima possesses good antioxidant activity. Also, it has been revealed that antioxidants have beneficial effects in cancer (21). So it can be concluded that J. foetidissima can be effective in cancer by different mechanisms.

Emami and Aslì (22) found that flavonoids are the major components of J. foetidissima extract while their presence in J. Sabina was not that much. J. Sabina had no cytotoxic effect against all cell lines used in this study. Comparing the cytotoxicity of J. foetidissima and J. sabina may lead to conclusion that this effect is related to its flavonoid contents.

Our preliminary findings showed that KB cells were the most sensitive cells which are consistence with the findings that susceptibility of cells to cytotoxic compounds is variable (23). To evaluate the apoptosis mechanism of these extracts more experiments such as TUNEL and Annexin V assays are underway.

Acknowledgment

This study was supported by a grant from the Research Council of Isfahan University of Medical Sciences, Isfahan, Iran (No. 78303). The authors are thankful to the late Dr. K. Javanshir, University of Tehran for identification of the plant material.

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