Iranian Journal of Pharmaceutical Research (2009), 8 (4): 281-286
Received: November 2008
Accepted: January 2009

Original Article

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

Hojjat Sadeghi-aliabadi[a,c*], Ahmad Emami[b], Morteza Saidi[a], Babak Sadeghi[a] and Abbas Jafarian[c]

[a]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.* [b]*Department of Pharmacognosy, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.* [c]*Isfahan Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Isfahan, Iran.*

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 *Platycladus*, 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

* Corresponding author:
E-mail: sadeghi@pharm.mui.ac.ir

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 Pasture 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), $NaHCO_3$ 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

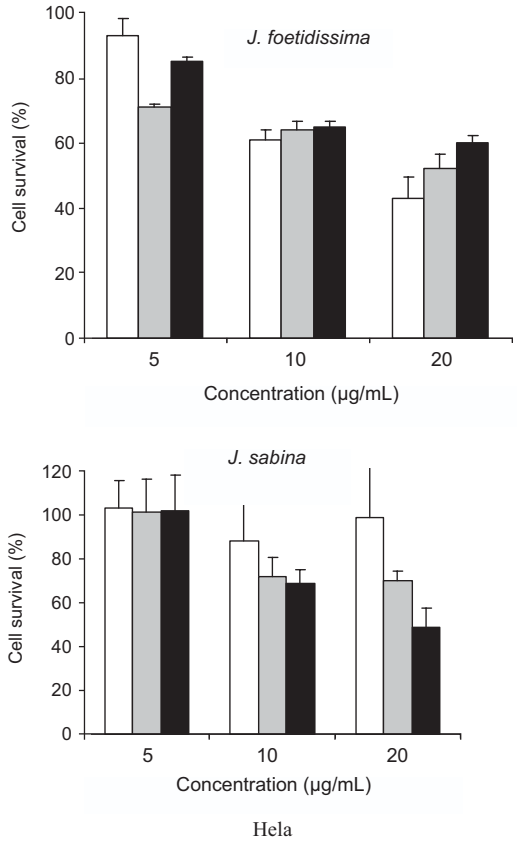


Figure 1. Cytotoxic effects of different concentrations (5, 10, 20 µg/ml) of *J. foetidissima* and *J. sabina* extracts against Hela cells. Cell viability was assessed using MTT assay. Percent cell survival in control group was assumed 100. Data are shown as Mean ± SD, n = 6, male branchlets □, female branchlets ■, berries ■.

dimethyl sulfoxide (DMSO). Briefly, 200 µl of cells (5 × 10^4 cells per ml of media) were seeded in 96-well microplates and incubated for 24 h (37 °C, 5% CO_2 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. Analyze-of-variance followed by Dunkan test 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, (r^2 = 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

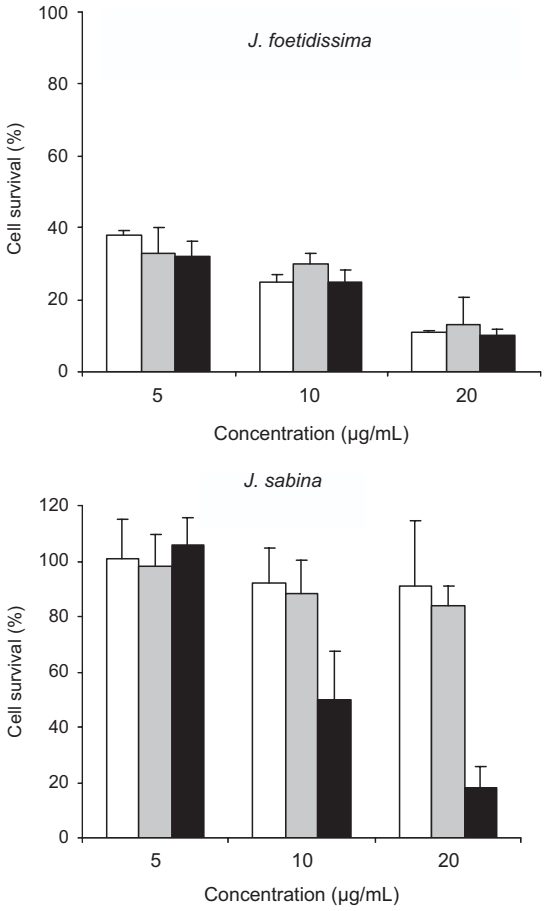
As shown in Figure 1, hydroalcoholic extract of male branchlets of *J.foetidissima* (20 µg/ml), was cytotoxic against Hela cells; whereas other plant samples were not cytotoxic at tested concentrations.

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 (IC_{50} < 5 µg/ml; Figure 2). However, cytotoxicity was seen at highest concentration (20 µg/ml) of berries extract of *J. sabina* (Figure 2).

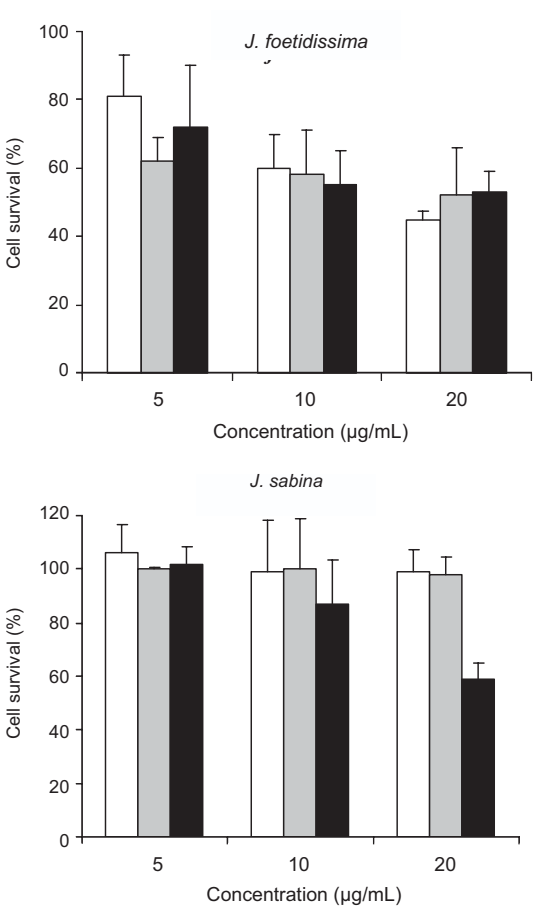
Cytotoxic effect of extracts against MDA-MB-468 cells

In the case of MDA-MB-468 cells only the



KB

Figure 2. Cytotoxic effects of different concentrations (5, 10, 20 µg/ml) of *J. foetidissima* and *J. sabina* extracts against **KB** cells. Cell viability was assessed using MTT assay. Percent cell survival in control group was assumed 100. Data are shown as Mean ± SD, n = 6, male branchlets □, female branchlets ■, berries ■.



MDA-MB-468

Figure 3. Cytotoxic effects of different concentrations (5, 10, 20 µg/ml) of *J. foetidissima* and *J. sabina* extracts against **MDA-MB-468** cells. Cell viability was assessed using MTT assay. Percent cell survival in control group was assumed 100. Data are shown as Mean ± SD, n = 6, male branchlets □, female branchlets ■, berries ■.

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 *Mycobactrium tuberculosis*. Emami and colleagues (20) showed that extracts obtained from leaves and fruits of Iranian *J. foetidissima* posses 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 Asili (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.

References

(1) Adams RP. *Juniperus* of the world. Trafford Publishing Co. Vancouver (2004) P. 1

(2) Assadi M. *Cupressaceae* In: Assadi M, Khatamsaz M, Maassoumi AA and Mozaffarian V. (eds.) *Flora of Iran*. Vol. 21, Research Institute of Forests and Rangelands, Tehran (1998) 19-28

(3) Sabeti H. *Forests, Trees and Shrubs of Iran.* Ministry of Information and Tourism Press, Tehran (1975) 420-5

(4) Alm T. Juniperus sabina in Norwegian folk tradition. *Sevenbom Juniperus sabina i folketradisjonen i Norge* (2003) 61: 186-9

(5) San Feliciano A, Gordaliza M, Miguel Del Corral JM, Castro MA, Garcia-Gravalos MD and Ruiz-Lazaro P. Antineoplastic and antiviral activities of some cyclolignans. *Planta Medica* (1993) 59: 246-9

(6) Akimov Yu A, Kharchenko GI, Krylova AP and Belova NN. Antimicrobe action of terpenes from savin (*Juniperus sabina* L.). *Appl. Biochem. Microbiol.* (1977) 13: 139-41

(7) San Feliciano A, Miguel Del Corral JM, Gordaliza M and Castro A. Lignans from *Juniperus sabina*. *Phytochemistry* (1990) 29: 1135-8

(8) Balaban M, Atik C and Ucar G. Fungal growth inhibition by wood extracts from *Juniperus foetidissima* and *J. oxycedrus*. *Holz als Roh - und Werkstoff* (2003) 61: 231-2

(9) Van Uden W, Homan B, Woerdenbag HJ, Pras N, Malingré TM, Wichers HJ and Harkes M. Isolation, purification, and cytotoxicity of 5-methoxypodophyllotoxin, a lignan from a root culture of *Linum flavum*. *J. Nat. Prod.* (1992) 55: 102-10

(10) Cairnes DA, Ekundayo O and Kingston DG. Plant anticancer agents. X. Lignans from *Juniperus phoenicea*. *J. Nat. Prod.* (1980) 43: 495-7

(11) Sadeghi-aliabadi H, Emami SA, Saeidi M and Jafarian A. Cytotoxic effects of the extracts of Iranian Taxus baccata and Cupressus horizentalis on cancer cells. *Iranian J. Pharm. Res.* (2003) 2: 107-10

(12) Jafarian-Dehkordi A, Emami SA, Saeidi M and Sadeghi H. Cytotoxicologic studies of the extracts of Iranian *Juniperus sabina* and *Platycladus orientalis* on cancer cells. *J. Res. Med. Sci.* (2004) 5: 7-11

(13) Emami SA, Sadeghi-aliabadi H, Saeidi M and Jafarian A. Cytotoxic evaluation of Iranian conifers on cancer cells. *Pharmaceutical Biology* (2005) 43: 299-304

(14) List H and Schmidt P. *Technologie Pflanzlicher Arzneizuberitungen*. Wissenschafliche Verlagsgesellschaft mbH Stuttgart, (1984) 140

(15) Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* (1983) 65: 55-63

(16) San Feliciano A, Gordaliza M, Miguel del Corral JM, Castro MA, Garcia-Gravalos MD and Ruiz-Lazaro P. Antineoplastic and antiviral activities of some cyclolignans. *Planta Medica* (1993) 59:246-9

(17) Gao R, Gao C, Tian X, Yu X, Di X, Xiao H and Zhang X. Insecticidal activity of deoxypodophyllotoxin, isolated from *Juniperus sabina* L, and related lignans against larvae of Pieris rapae L. *Pest Manag. Sci.* (2004) 60:1131-6

(18) Muto N, Tomokuni T, Haramoto M, Tatemoto H, Nakanishi T, Inatomi Y, Murata H and Inada A. Isolation of apoptosis- and differentiation-inducing substances toward human promyelocytic leukemia HL-60 cells from leaves of *Juniperus taxifolia*. *Biosci.*

Biotechnol. Biochem. (2008) 72:477-84

(19) Topcu G, Erenler R, Cakmak O, Johansson CB, Celik C, Chai HB and Pezzuto JM. Diterpenes from the berries of *Juniperus excelsa*. *Phytochemistry* (1999) 50:1195-9

(20) Emami SA, Asili J, Mohagheghi Z and Hassanzadeh MK. Antioxidant activity of leaves and fruits of Iranian conifers. *eCAM* (2007) 4: 313-9

(21) Jeong JM, Choi CH, Kang SK, Lee IH, Lee JY and Jung H. Antioxidant and chemosensitizing effects of flavonoids with hydroxy and/or methoxy groups and structure-activity relationship. *J. Pharm. Pharm. Sci.* (2007) 10:537-46

(22) Emami SA and Asili J. phytochemical evaluation and antimicrobial activity of essential oils of Iranian conifers. Pharm D thesis, School of Pharmacy, Mashhad University of Medical Sciences (1997) 33-95

(23) Suzuki F, Hashimoto K, Ishihara M, Westman G, Samuelsson K, Kawase M, Motohashi N and Sakagami H. Tumor-specificity and type of cell death induced by phenoxazines. *Anticancer Res.* (2007) 27: 4233-8

This article is available online at http://www.ijpr-online.com