



## Cytotoxic Effects of Iranian Mistletoe Extract on a Panel of Cancer Cells

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### Abstract

Extracts derived from *Viscum album* have been shown to kill cancer cells *in vitro*. Some studies have noted that different species of this plant collected from around the world displayed cytotoxic effects in different extents. In the present study, we evaluated the effects of Iranian mistletoe extracts on five cancer cell lines. Plants growing on hornbeam tree (*Carpinus betulus*) were collected, air-dried and hydroalcoholic (MeOH-H<sub>2</sub>O with 2% acetic acid) and methanolic extracts were obtained using percolation. Also the plant juice was obtained by pressing. Cytotoxicity of the extracts on a panel of cancer cells (Hela, KB, MDA-MB-468, K562 and MCF-7) were studied using colorimetric MTT assay. Results showed that plant juice was the most cytotoxic fraction on all cancer cells tested (IC<sub>50</sub>=0.0316 mg). The IC<sub>50</sub> of hydroalcoholic and methanolic extracts were 0.1 and 0.316 mg, respectively. These results suggest that alkaloids and huge compounds like viscotoxin and lectins extracted by press or hydroalcoholic solvents were probably responsible for their cytotoxicity. Results also indicated that Hela cells were more resistant while KB cells were more sensitive to the cytotoxic effects of the extracts. It can be concluded that cytotoxicity of Iranian mistletoe extract on the cell lines tested closely depends on the host tree and extraction methods.

**Keywords:** Cancer cells; Cytotoxicity; Mistletoe; MTT assay; *Viscum album*.

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### 1. Introduction

Mistletoe, a semiparasitic plant, growing on Ironwood or hornbeam tree (*Carpinus betulus*), holds interest as a potential anticancer agent because extracts derived from it have been shown to kill cancer cells *in vitro* [1, 2].

It also stimulates immune system both *in vitro* and *in vivo* [2, 3]. Two components of mistletoe, namely viscotoxins and lectins, may be responsible for its anticancer effects or its stimulating activity of the immune system [4-7], although many researches have focused on lectins. The three mistletoe lectins (ML I, II, III) have been identified as the main active principle of its extract. The *in vitro* growth of several types of human cancer cells was stimulated by treatment with low doses of

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the purified lectin ML-I [8].

Mistletoe grows on several types of trees (oak, apple, pine etc.). The chemical composition of its extracts depends on the species of the host tree, the time of harvesting, the method of extract preparation, and the commercial producer [9, 10].

Mistletoe is used mainly in Europe, where commercially available extracts are marketed under a variety of brand names, including Iscador, Isorel etc. All of these products are prepared from *Viscum album*, Loranthaceae (*V. album* L. or European mistletoe).

Most of the researches have been performed on European mistletoe and a few on Korean mistletoe, but there is no report on Iranian mistletoe. The principles of mistletoe therapy were developed by the scientific investigation of Rudolf Steiner, who found that mistletoe constituents stimulate inflammatory processes in the body to combat tumor development [11]. Preclinical studies carried out by Maier and Fiebig showed that European mistletoe extracts inhibited tumor growth in a panel of 16 human tumor cell lines *in vitro* [12]. In another *in vivo* study, Burger et al. concluded that treatment with aqueous mistletoe extract, as an immuno-stimulating agent, did not enhance tumor growth in any of the syngeneic murine tumor models used [13]. Also Gorter and co-workers showed that cytokine was released after incubation of two different *V. album* L. extracts with keratinocytes and cell viability was decreased in a dose dependent manner [14].

In the present study, direct effects of hydroalcoholic or alcoholic extracts and juice of Iranian mistletoe were investigated on the growth of a panel of 5 human tumor cell lines *in vitro*.

## 2. Materials and methods

### 2.1. Plant materials

Leaves and berries of *Viscum album* L. were collected from the northern parts of Iran (Tosiyan village, Siahroud forest in Gilan

province) at an altitude of 350 m in July 2002, and was identified by the botanists at Department of Biology, Isfahan University, Isfahan, Iran. Plant samples were stored at -20 °C. A voucher specimen of the plant (No. 1252) is deposited in the herbarium of Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

Air-dried parts of *V. album* were ground and 50 g of it was extracted with methanol (designated as methanolic extract in the following text) or a mixture of methanol/H<sub>2</sub>O (30:70) containing 2% acetic acid (designated as aqueous extract in the following text) by means of percolation. In another method 50 g of fresh leaves and berries of plant were chopped and pressed by means of special metallic syringe to obtain fresh plant juice (designated as juice in the following text) before using on cell lines.

### 2.2. Phytochemical studies

Preliminary phytochemical screening including the presence of alkaloids, flavonoids, tannins, anthraquinones, saponins and glycosides were performed on extracts and juices of *V. album* as described in our publication [15].

### 2.3. Cell lines and culture

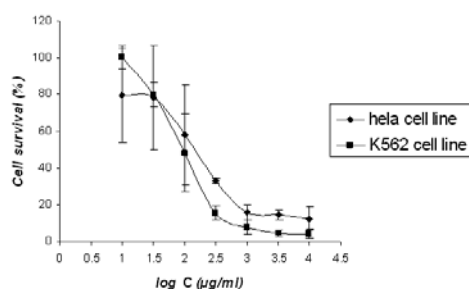
Hela (Human cervix carcinoma), KB (Human Caucasian/ epidermal carcinoma) and MDA-MB-468 (Human breast adenocarcinoma) MCF-7, K562 cell lines were obtained from Pasture Institute of Iran in Tehran. They were grown in RPMI-1640. Each 500 ml of RPMI-1640 was supplemented with 10% of fetal calf serum, 5 ml of penicillin/streptomycin (50 IU/ml<sup>1</sup> and 500 µg/ml<sup>1</sup>, respectively), 5 ml of 1 mM sodium pyruvate, 1 g of NaHCO<sub>3</sub>, and 5 ml of 2 mM L-glutamine. Completed media were sterilized by 0.22 µm microbiological filters after preparation and kept at 4 °C before using.

#### 2.4. Cell viability

The viability of cells was studied by assessment of plasma membrane integrity as determined by trypan blue (0.2 % w/v) exclusion assay. Cells were in their exponential growth phase after 24 h and during next 3 days of incubation. All extracts were added to the cell suspensions after 24 h.

#### 2.5. MTT-based cytotoxicity assay

The cytotoxic effect of the extracts on the 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 [16]. This assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable cells, into an insoluble coloured formazan product, which can be measured spectrophotometrically after dissolution in dimethyl sulfoxide (DMSO) [17]. Briefly, 200  $\mu$ l of cells ( $5 \times 10^4$  cells/ml of media) were seeded in 96 microplates and incubated for 24 h (37 °C, 5% CO<sub>2</sub> air humidified). Then 20  $\mu$ l of prepared concentrations of extract was added and 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 the cell survival, 20  $\mu$ l of MTT solution (5 mg/ml in phosphate buffer solution) was added to each well and incubated for 3 h. Then, gently



**Figure 1.** Dose response curve for HeLa and K562 cell lines following 72 h continuous exposure to aqueous extract of Iranian mistletoe (n=3).

150  $\mu$ l of the 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 3-6 times. Standard curves (absorbance against number of cells) for each cell line were plotted. Intra-day and inter-day variations were determined. Growth inhibition/stimulation was expressed as treated cells/control  $\times$  100 (cell survival %). Cytotoxic activity was defined as inhibition of cell growth to less than 50% (IC<sub>50</sub>). Survival in negative control (medium-treated cells) was assumed 100%. Coefficient of variation [SD/mean  $\times$  100] was below 20% in nearly all experiments.

#### 2.6. Statistical analysis

SPSS software was used to perform statistical tests. One-way ANOVA followed by Post Hoc was used to distinguish the differences among groups. Significance was assumed at 5% level ( $p < 0.05$ ).

### 3. Results

#### 3.1. Phytochemical studies

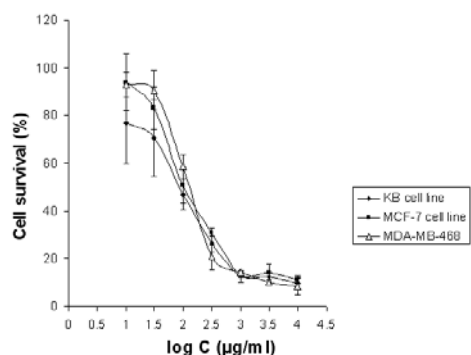
Preliminary phytochemical studies on the plant extracts displayed the presence of alkaloids, tannins, flavonoids and amino acids. Saponins, anthraquinones and glycosides were not determined in the extracts.

#### 3.2. Cytotoxicity of the mistletoe extracts on cancer cell lines

The hydro-alcoholic extract was cytotoxic toward all cell lines tested in a dose-dependent manner (Figures 1 & 2). IC<sub>50</sub> were about  $< 2$  and  $< 2.5$   $\mu$ g/ml for HeLa and K<sub>562</sub> cell lines, respectively (Figure 1). IC<sub>50</sub> for KB, MDA-MB-468 and MCF-7 cell lines were  $< 2$ ,  $< 2.5$  and 2  $\mu$ g/ml, respectively (Figure 2).

#### 3.3. Effect of extraction method on cytotoxicity

Results obtained from these studies showed that the order of cytotoxicity of 3 extracts



**Figure 2.** Dose response curve for KB, MDA-MB-468 and MCF-7 cell lines following 72 h continuous exposure to aqueous extract of Iranian mistletoe (n=3).

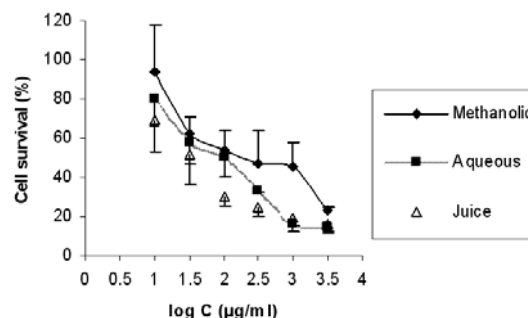
against HeLa cell line were juice > hydroalcoholic extract > methanolic extract. As shown in Figure 3, the  $IC_{50}$ 's were 0.0316, 0.1 and 0.316 mg/ml, respectively.

#### 4. Discussion

Extracts of *V. album* (mistletoe) are widely used as complementary cancer therapies in Europe. Although four species of mistletoe grow in Europe, only the white berried ones are used for cancer treatment. They are harvested from several deciduous hardwood trees (oak, apple and elm) and from coniferous softwood trees (pine and fir). The apple and pine-grown ones are common; the elm- and oak-grown ones, are less common [11]. In this study, we used white berried Iranian mistletoe grown on *Carpinus Betulus* (betulaceae), an oak like tree.

There are several reports indicating that biologically active *V. album* compounds, and thus cytotoxicity or apoptosis-inducing properties, are dependent on the manufacturing process, host trees, different geographical regions and time of harvest [18,19]. For instant European mistletoe has significant antitumor effect, while American *V. album* has no effect [20]. The Korean mistletoe (*V. album* var. *coloratum*) showed better cytotoxic effect than the European mistletoe, and direct injection into tumors showed antitumor properties [21].

Mistletoe contains, among other



**Figure 3.** Dose response curves for HeLa cell line following 72 h continuous exposure to 3 different extracts of Iranian mistletoe (n=6).

constituents, two groups of toxins: Viscotoxins and lectins. Mistletoe lectin (ML) is a ribosome inactivating protein consisting of two chains (A and B) joined by a disulfide bond. The toxicity of ML is mediated by the B-chain, which has lectin activity, interacting with sugar residues of glycoproteins and glycolipids on the surface of target cells [22].

Percolation was used as a safe extraction method to extract most of these active proteins intact. Viscotoxin is a huge compound which is effected by high temperature and can not be extracted by methanol alone, therefore, a mixture of methanol/water and 2% acetic acid was applied to complete the extraction. Acidic extraction solvent could help the extraction of nitrogen containing compounds like alkaloids, viscotoxin and lectins [23].

Five different cell lines were used in these studies for several reasons; HeLa as a resistant cell line and KB as a chemosensitive cell have been used commonly in anti-cancer screening of plant by NCI [24]; MCF-7 and MDA-MB-468 cell lines were used to evaluate the cytotoxic effects of the extracts on breast cancer as a common disease in most societies; K562 cell line was used to show the effect of the extracts on leukemia.

Our results indicated that the hydroalcoholic extract and the juice possess cytotoxic effects against all tested cell lines. These results also indicated that the hydroalcoholic extract of the Iranian *V. album*

is significantly more cytotoxic than its methanolic extract ( $p < 0.005$ ), but there was no significant correlation between the hydroalcoholic extract and the juice. Stein and Berg in their studies provide evidence that immunological reactivity of patients with adverse effects towards Helixoer (an aqueous mistletoe preparation) are seldom [21]. Lyu and co-worker [24] showed that the methanolic extract of the Korean mistletoe had cytotoxic effect indicating that beside lectins, there are some small compounds in this plant which could be extracted by methanol and may be responsible for its cytotoxic properties.

Although cytotoxicity of *V. album* extracts is attributed to its viscotoxin and lectins, but Kuttan suggested that in addition to a direct cytotoxic effect of *V. album* extract, the activation of macrophages may contribute to the overall antitumor activity of the drug [25]. Jurin and others in their studies showed that the combined action of Isorel, *V. album* L. preparation, influencing tumor viability on one hand and the host's immune reactivity on the other hand, seems to be favorable for its antitumor action in vivo [26]. In the case of molecular mechanisms of mistletoe extract, Timoshenko and Gabius in their studies revealed that *V. album* lectins induce superoxide anion release from human neutrophils [27] or generate  $H_2O_2$  [28]. On the other hand, Thionines such as viscotoxin from mistletoe could induce human lymphocytes to generate reactive oxygen intermediates [29]. *V. album* lectins are also cytokine inducers and immunoadjuvant in tumor therapy [14, 30]. Generation of superoxide anion or  $H_2O_2$  could cause cell toxicity. Agglutinins, another *V. album* extract components, inhibited mammalian protein synthesis at nanomolar concentrations [31], increased the secretion of an active form of interleukin-12 and potentiated the cytokine-induced NK activation [32].

In conclusion, the data indicate that juice or hydroalcoholic extracts of the Iranian mistletoe are cytotoxic against a panel of

cancer cells in a dose dependent manner. Iranian mistletoe juice is more cytotoxic than European mistletoe because the host tree are different and also compounds extracted by hydroalcoholic extracts and pressing process are not probably the same.

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