



## Cytotoxicity of *Juniperus excels* and *Salvia mirzayanii* Extracts against HepG2 Cells

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### Abstract

In the recent decades, a lot of efforts have been directed for the discovery and production of anticancer agents. Plant kingdom is one of the main sources which has attracted the attention of investigators for finding new medicines including anticancer agents. In the present study, the cytotoxic effects of the extracts of five plants, native of Iran including *Salvia mirzayanii*, *S. macrosiphon*, *S. multicaulis*, *Juniperus excels*, and *Peganum harmala*, against HepG2 cell lines were investigated. Cytotoxicity was assessed by MTT test, and comparison of the IC<sub>50</sub>s revealed that the extracts of aerial parts of the *J. excelsa* had the strongest cytotoxic effects followed by *S. mirzayanii*, and the extract of the root of *P. harmala* with IC<sub>50</sub>s of 0.54, 1.52, and 2.50 mg/ml, respectively. The extracts of *S. macrosiphon* and *S. multicaulis* had no significant cytotoxic effects against HepG2 cells. The cytotoxic effects of *J. excelsa* seemed to be due to ATP depletion as ATP levels of HepG2 cells incubated for 24 h with 0.5, and 0.7 mg/ml of the extracts of *J. excelsa* was decreased to 47% and 27% of control, respectively. The ATP depletion was time-dependent and ATP was depleted before cytotoxicity ensued. Oxidative stress was not important in cytotoxicity of *J. excelsa* as lipid peroxidation and GSH depletion was not significantly different from control cells. However, cytotoxicity of *S. mirzayanii* was not accompanied by ATP depletion or oxidative stress. The exact mechanism(s) of cytotoxicity of these extracts needs further investigation.

**Keywords:** Cytotoxicity; HepG2 cells; *Juniperus excels*; *Peganum harmala*; *Salvia mirzayanii*; *Salvia macrosiphon*; *Salvia multicaulis*.

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### 1. Introduction

The World Health Organization (WHO)

has estimated that approximately 80% of the World's population depends on traditional medicines for meeting their primary health care needs [1]. Cancer is one of the uncontrolled diseases which is the second cause of deaths worldwide after cardiovascular diseases. The need for new medicines for

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treatment of cancer is necessary. Many cytotoxic anticancer drugs are from plant origin and the plant kingdom is still one of the promising sources for new anticancer drugs [2-6].

Therefore, in the present study, several plants from *Lamiaceae*, *Cupressaceae*, and *Zypophllaceae*, native from Iran with reported antimicrobial or other uses in traditional medicine were selected for study of potential cytotoxic effects against HepG2 cell lines.

The *Lamiaceae* family comprises of about 200 genera and 3000 species. One of the largest genus of the family, *Salvia* L, is represented by over 900 species, and is widely distributed in various regions of the world [7]. *Salvia* species have been used since ancient times for more than sixty different ailments ranging from aches to epilepsy, and mainly to treat colds, bronchitis, tuberculosis, haemorrhage, and menstrual disorders [7, 8]. In this research, three species of *Salvia* were selected to study, including: *S. mirzayanii*, *S. macrosiphon*, and *S. multicaulis*.

Seventy *Juniperus* species (*Cupressaceae*) grow throughout the world. *Juniperus excelsa* is used locally as a traditional remedy for tuberculosis and jaundice in Saudi Arabia. In a study, extracts isolated from *J. excelsa* was found to exhibit a significant activity against several cell lines and Mycobacterium tuberculosis [9]. Sandracopimaric acid isolated from *J. excelsa* has also been found to exhibit antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus durans* [10].

*Peganum harmala* in Iran is grown in regions with less salty soils. Harmine, harmalin, harmolol and vazissin are isolated from *P. harmala*. Harmalin is the most important alkaloid that has bactericide effects. The extract of *P. harmala* seeds has been used in traditional medicine as antimicrobial, antiprotozoal and anticancer, and many studies on the cytotoxic effects of components of seeds have been performed [11, 12]. However,

the effect of *P. harmala* roots is not studied.

Therefore, in the present study, the cytotoxic effects of the extracts of these five plants were evaluated against HepG2 cell lines, as potential anticancer agents. The results suggested that the extracts of *Juniperus excelsa* and *Salvia mirzayanii* have good cytotoxic effects against HepG2 cells.

## 2. Materials and methods

### 2.1. Cells and reagents

Human liver cancer cell line HepG2 was obtained from Pasteur Institute, Tehran, Iran. RPMI-1640 and FBS were from GibCo. Trypan blue, methylthiazolyldiphenyl-tetrazolium bromide (MTT), DTNB, and Na<sub>2</sub>HPO<sub>4</sub> were from Merck, Germany, and thiobarbituric acid was from Sigma Chemical Company, Germany. All other chemicals were of highest quality available in the market.

### 2.2. Plant materials

In this research the aerial parts of three species of *Lamiaceae* family including: *S. mirzayanii*, collected in March 2011 from Bandarabbas, south of Iran, *S. multicaulis* and *S. macrosiphon* collected in July 2011 from countries north of Shiraz, and one species from *Cupressaceae* including *Juniperus excelsa*, collected in August 2010 from Ganoo mountains, Bandarabbas, and

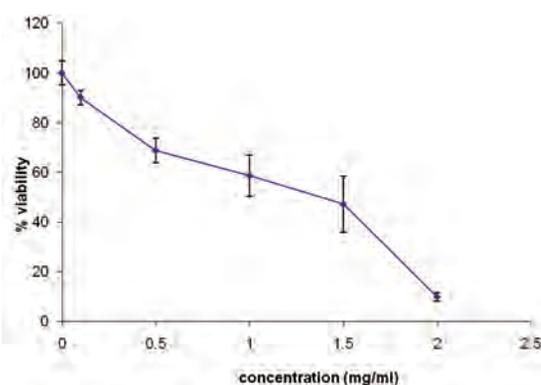


Figure 1. Inhibitory activity of *S. mirzayanii* on HepG2 cell line in different concentration.; (Values are means±S.D).

the roots of one species from *Zypohilaceae* including *Peganum harmala*, collected in July 2010 from Kamfirooz, north of Shiraz, were used. The specified parts of the plants after were dried in the room temperature in the shade after collection. The voucher of each plant is kept in the herbarium of Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

2.2.1. Plant extraction

The dried aerial parts of *S. mirzayanii*, *S. macrosiphon*, *S. multicaulis* and *J. excelsa* were ground into powder, separately, and 25 g from each was weighed and extracted with 90% ethanol in a perculator for 24 h. The dried roots of *P. harmala* was extracted with 90% ethanol for 15 h. The extracts were concentrated by rotary evaporator under reduced pressure and low temperature. The yield of each extract was as follows: *S. mirzayanii*, 26.7%; *S. macrosiphon*, 25.2%; *S. multicaulis*, 17.2%; *J. excelsa*, 24.2% and *P. harmala*, 15.0%.

2.3. Cell culture

Human hepatoma cell lines (HepG2) were cultured and maintained in RPMI-1640, pH 7.3, containing 0.37% NaHCO<sub>3</sub> supplemented with 10% FCS (fetal bovine serum albumin), 1% penicillin and streptomycin (100 IU/ml penicillin and 100 IU/ml streptomycin) in a humidified 5% CO<sub>2</sub>-95% air mixture at 37 °C. The cells were cultured in 10 of the media culture in 50 ml flasks and cultured for 18 h. After 18 h, the media was aspirated and replaced with new RPMI-1640 medium.

2.4. Cytotoxicity assay

2.4.1. Trypan blue assay

Cells were seeded in 96-well microplates (30000 cells/well/90 µl) and routinely cultured in a humidified incubator for 24 h. After 24 h, herbal extracts solutions were added (10 µl/well) in concentrations ranging from 0.5

**Table 1.** Comparison of the IC<sub>50</sub> of three extracts on HepG2 cell line. (Values are means±SD)

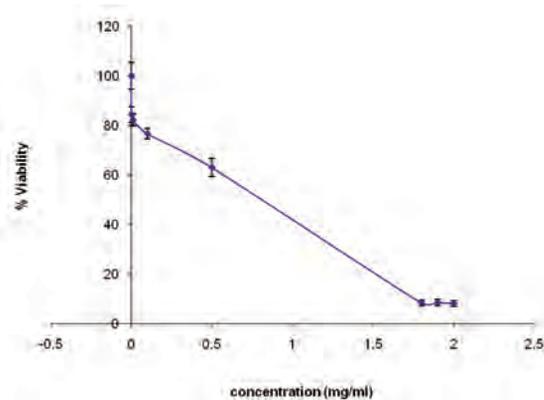
extract	IC <sub>50</sub> (mg/ml)
<i>S.mirzayanii</i>	1.52±0.181
<i>J.excelsa</i>	0.540±0.43
<i>P.harmala</i>	2.5±0.485

mg/ml to 3 mg/ml. Cells were then re-incubated for 48 h. In this test, a control group (RPMI-1640 without herbal extract) and a positive control group (RPMI-1640 with herbal extract and Cisplatin) were also included. After 48 h of incubation, the wells were trypsinized and cells were completely removed from the well. Ten µl sample of the cell suspension was taken and 10 µl trypan blue was added to it and was homogenized by pipeting, then 10 µl from this homogenized solution was taken, and the cells were counted in a hemocytometer lam and a light microscope.

2.4.2. MTT assay

The MTT assay is a test of metabolic competence based upon assessment of mitochondrial performance. It is a colorimetric assay relying on the conversion of yellow tetrazolium bromide (MTT) to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells [13, 14].

Cells were seeded in 96-well microplates (30000 cells/well/90 µl) and routinely cultured in a humidified incubator for 24 h. After 24



**Figure 2.** Inhibitory activity of *Juniperus excelsa* on HepG2 cell line in different concentration; (Values are means±S.D).

**Table 2.** Effect of *J.excelsa* on GSH content of HepG2 cells. (Values are means±S.D)

Addition	Exposure time	
	9 h	24 h
Control	19.1±5.4	15.43±2.5
0.5 mg/ml	17.36±4.62	12.05±1.57
0.7 mg/ml	10.66±1.57	8.44±1.18

h, herbal extracts solutions were added (10 µl/well) in concentrations ranging from 0.5 mg/ml to 3 mg/ml. Cells were then re-incubated for 48 h. After 48 h of incubation, 10 µl MTT solution was added to every well, cells were re-incubated for an additional 4 h. The cell culture media and MTT solution were removed and the cells remained in the bottom of the wells. Then, 100 µl of DMSO was added to each well to dissolve the formazan crystals formed. The absorbance of the converted dye was measured at a wavelength of 570 nm. Five wells were used for each concentration of herbal extracts, and three independent experiments were performed for each extracts.

### 2.5. Measurement of ATP

ATP levels were measured by HPLC method as described originally by [15], using a Shimadzu HPLC system coupled with a dual absorbance UV detector (Model 2487). The mobile phase used was 0.1 M ammonium dihydrogen phosphate (pH 6.0), using the Symmetry Shield C-18 column and a flow rate of 1 ml/min. The peaks of ATP was eluted at retention time of 5.50 min.

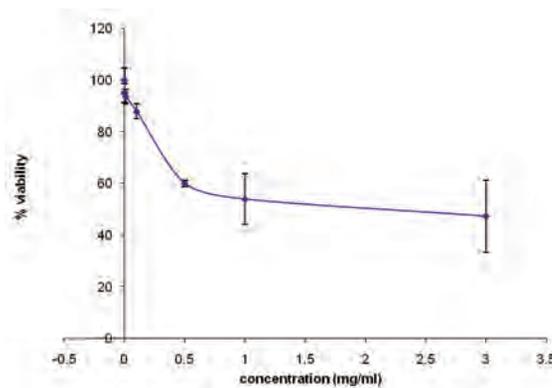
Usually 1 ml of the culture containing  $5 \times 10^6$  of cells was treated with 1 ml of 0.35 perchloric acid plus 1 M Na-EDTA on the ice. Then the mixture was centrifuged at 9000 g for 4 min at 5 °C. The supernatant was then neutralized with 2 M KOH and centrifuged after vortexing. The supernatant was used for measuring ATP. The samples were kept at -70 °C until injection to HPLC.

### 2.6. Measurement of thiobarbituric acid-reactive agents

As a biomarker for lipid peroxidation, concentration of thiobarbituric acid-reactive agents was measured [14]. HepG2 cells ( $5 \times 10^6$  cells/plate) were preincubated in plates for 24 h at 5% CO<sub>2</sub>-95% air at 37 °C. The control cultures were prepared by adding RPMI-1640 without any addition. After incubation with or without extract, the culture medium was removed. After rinsing with 0.5 ml free PBS twice, cells were collected by trypsinization. After determining the viability of the detached cells, 250 µl of 70% (w/v) trichloroacetic acid containing, 1 ml of 0.8% (w/v) thiobarbituric acid with 750 ml deionized water was added to cells and was shaken with vortex. The suspensions were transferred into glass tubes and boiled for 30 min. After cooling to room temperature and centrifugation for 10 min at 5000 rpm, the absorbance of the supernatant was determined at 532 nm.

### 2.7. Measurement of reduced and oxidized glutathione

HepG2 cells ( $5 \times 10^6$  cells/plate) were pre-incubated in plates for 24 h at 5% CO<sub>2</sub>-95% air at 37 °C. Cells were rinsed with PBS and were collected by trypsinization, after determining the viability of detached cells, 200 µl of 20% trichloroacetic acid with 1800 µl of PBS were added to the cell suspension. After shaking with vortex and centrifusing,

**Figure 3.** Inhibitory activity of *Peganum harmala* on HepG2 cell line in different concentration; (Values are means±S.D).

supernatant was divided to two even parts (each 1 ml).

For measurement of reduced glutathione (GSH), two ml of Na<sub>2</sub>HPO<sub>4</sub> (0.3 M) and 0.5 ml of DTNB (0.01 M) were added to 1 ml of the supernatant and was shaken with vortex. The absorbance was then measured at 412 nm [16].

For measurement of oxidized glutathione (GSSG), 1 ml of 5% sodium borohydrid was added to 1 ml of the supernatant, and was incubated for 1 hr in 45 °C, then 0.5 ml of Na<sub>2</sub>HPO<sub>4</sub> (0.3 M) was added to each tube. After neutralization with HCl (2.7 N), 0.5 ml of DTNB (0.01 M) was added and was shaken with vortex. The absorbance was then measured at 412 nm.

### 2.8. Statistical analysis

All values refer to mean±SD of at least three separate experiments. Statistically significant differences between control and experimental groups were obtained using Student's "t" test where two groups were compared and one way ANOVA where more than two groups were compared and controlled by HSD and DUNNET post tests. The Graph Pad Instat 3 and SPSS 11.5 soft wares were used for running the tests. The minimal level of significance chosen was

**Table 3.** Effect of *J.excelsa* on GSSG contact of HepG2 cells (Values are means±S.D)

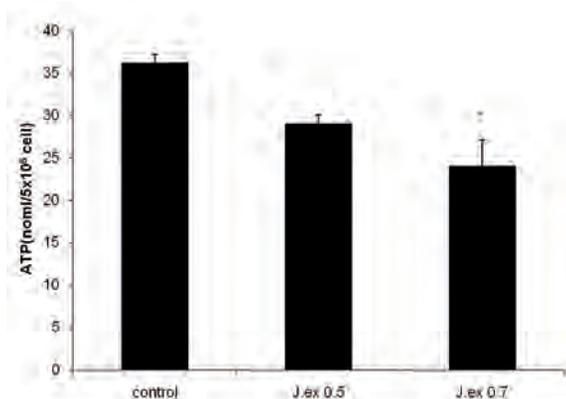
Addition	Exposure time	
	9 h	24 h
Control	9.95±3.33	3.38±1.64
0.5 mg/ml	9.39±1.14	6.06 ± 0.987
0.7 mg/ml	5.98±0.56	5.32 ± 0.745

$p < 0.05$ . For determination of IC<sub>50</sub>, Wilcoxon-Litchefield method was used [17].

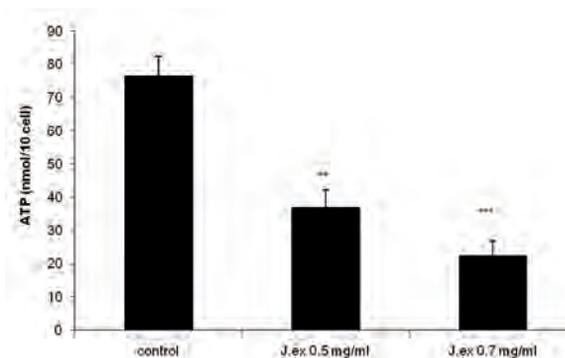
### 3. Results

Incubation of HepG2 cells with different concentrations (0.01 to 2 mg/ml) of the hydro-alcoholic extract of the aerial parts of *Salvia mirzayanii* for 48 h resulted in cytotoxicity with an IC<sub>50</sub> of about 1.52±0.18 mg/ml (Figure 1; Table 1). Incubation of HepG2 cells with 1.5, or 2 mg/ml of this extract for up to 9 h did not affect the ATP content of the cells (data not shown). Incubation with the same concentrations for 9 or 24 h also did not significantly affect GSH and GSSG (Table 2) content of the cells. Lipid peroxidation as measured by production of TBARS was also not significantly affected by *S. mirzayanii* extract (Table 3).

Incubation of HepG2 cells with different concentrations (0.01 to 2 mg/ml) of the hydro-alcoholic extract of aerial parts of *Juniperus excels* for 48 h also resulted in cytotoxicity with an IC<sub>50</sub> of about 0.54±0.04 mg/ml



**Figure 4.** ATP alterations in 9 hours exposure with *J.excelsa*; (Values are means±S.D); \*Significant different from control group ( $P < 0.05$ )



**Figure 5.** ATP alterations in 24 hours exposure with *J.excelsa*; (Values are means±S.D); \*\*Significant different from control group ( $P < 0.01$ ); \*\*\*Significantly different from control group ( $P < 0.001$ ).

(Figure 2; Table 1). Incubation of HepG2 cells with 0.5, or 0.7 mg/ml of the extract of *J. excelsa* for 9 h decreased the ATP content of the cells by 19.4% and 36.1%, respectively (Figure 4). Incubation of HepG2 cells with 0.5, or 0.7 mg/ml of the extract for 24 h, at which time significant cytotoxicity had not occurred, decreased the ATP content of the cells to 26.7% and 46.7% of the original values, respectively (Figure 5). Incubation of the cells with 0.7 mg/ml of the extract for 9 and 24 h also decreased the GSH and increased GSSG content of the cells, significantly (Table 2). Lipid peroxidation as measured by production of TBARS was not significantly induced by *S. mirzayanii* extract (Table 3).

The extract of the roots of *Peganum harmala* was also toxic towards HepG2 cells with an IC<sub>50</sub> of about 2.50±0.48 mg/ml (Figure 3; Table 1), but the extracts of the aerial parts of *Salvia macrosiphon* and *Salvia multicaulis* did not significantly cause cytotoxicity in HepG2 cells with concentrations up to 3 mg/ml (data not shown). Therefore, their effect on biochemical factors of the cells were not assessed.

#### 4. Discussion

Therapeutic utilization of plants is a part of universal human history, and products derived from plants have been used frequently for the treatment or prevention of diseases. In recent years, several herbal products with anticancer effects in various test system have been identified [2-6].

HepG2 cells are human hepatoma cells which have many characteristics of hepatocytes. In the present study, we used HepG2 to examine the cytotoxic effects of five herbal extracts and elucidate the mechanisms responsible for their cytotoxic effects.

*Juniperus excelsa* was the most cytotoxic and caused both ATP depletion and oxidative stress. ATP depletion was both time- and

**Table 4.** Effect of *J. excelsa* on MDA content of HepG2 cells (Values are means±S.D)

Addition	Exposure time	
	9 h	24 h
Control	0.146±0.067	0.142±0.022
0.5 mg/ml	0.054±0.083	0.044±0.021
0.7 mg/ml	0.144±0.024	0.05±0.011

concentration-dependent. ATP depletion by this extract could be due to inhibition of mitochondrial respiration, as ATP content of the cells was highly decreased before cytotoxicity occurred. Oxidative stress was evident as GSH content of the cells was decreased, however, lipid peroxidation was not significant, therefore, oxidative stress may not be an important mechanism cytotoxicity of *J. excelsa* extract in this model.

*Salvia mirzayanii* was also toxic with a reasonable concentration and its toxicity towards HepG2 cells was concentration- and time-dependent, but it did not affect ATP content of the cells neither caused a significant oxidative stress. Therefore, its cytotoxic mechanism of action may be due to other insults including alkylation of vital proteins or damage to DNA. Previous reports also suggested DNA damaging effect of *Salvia officinalis* [18]. Previously, it was shown that *S. mirzayanii* extract has antioxidant and radical scavenging *in vitro* [19, 20]. However, many compounds have been shown to have antioxidant and pro-oxidant effects depending on their concentration.[21, 22]. Clarification of its mechanism of action needs further research which presently is under investigation.

The extracts of the aerial parts of *Salvia macrosiphon*, *Salvia multicaulis*, and the roots of *Peganum harmala* were not highly cytotoxic towards HepG2 cells, therefore, we did not consider for study of biochemical changes.

Taken together the results of the present investigation suggest that *J. excelsa* and *S. mirzayanii* contain compounds which may be useful for preventing cancerous cell growth

and proliferation. The effective compounds and the exact mechanism of cytotoxicity needs to be investigated, and their effect on other cell lines also should be tested.

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