The Anticancer Activity of Five Species of *Artemisia* on Hep2 and HepG2 Cell Lines

Seyed Ahmad Emami, Nasser Vahdati-Mashhadian*, Remisa Vosough and Mohammad Bagher Oghazian

Drug Research Centre, School of Pharmacy, Mashhad University of Medical Sciences (MUMS), Mashhad, I R Iran

Summary

It has been reported that several *Artemisia* species (Astraceae) possess cytotoxic activity against different human cell lines. In this study, the toxicity of the *A. kulbadica, A. sieberi, A. turanica, A. santolina* and *A. diffusa* against human Caucasian hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2) cell lines have been investigated.

These plants were collected from Khorasan province, northeast of Iran. Different concentrations (200, 400, 600, 800, 1000, 1200, 1600 and 3200 µg/mL) of ethanol extract of each sample were prepared. The cytotoxic effects of these concentrations against two human tumor cell lines Hep2 and HepG2 were determined by quantitative MTT assay.

Results showed concentration- and time-dependent toxicity. In all extracts, toxic effects were significantly higher on HepG2 cells compared with Hep2 cells. HepG2 cells are rich in phase I and phase II metabolic enzymes and it is probable that metabolic activation of some active ingredients of the extracts were converted to more toxic metabolites and caused more toxicity in these cells.

Keywords: Hep2, HepG2, *Artemisia* spp., Hepatotoxicity, MTT assay.

*** Author of correspondence:**

Nasser Vahdati-Mashhadian
Drug research center
School of Pharmacy
Mashhad University of Medical Sciences
Mashhad, 91775-1365, Iran
E-mail: vahdatin@mums.ac.ir
Fax No. +98-511-8823251

Introduction

The genus *Artemisia* is one of the largest and most widely distributed of the nearly 100 genera in the tribe Anthemideae of the Asterceae (Compositae). This is a large and heterogeneous genus, numbering over 400 species distributed mainly in the temperate zone of Europe, Asia and North America. These species are Perennial, biennial and annual herbs or small shrubs, frequently aromatic. Leaves are alternate, capitula small, usually pendent, racemose, paniculate or capitate inflorescences, rarely solitary. Involucral bracts stand in few rows, receptacle flat to hemispherical, without scales, sometimes hirsute. Florets are all tubular, Achenes obvoid, subterete or compressed, smooth, finely striate or 2-ribbed; pappus absent or sometimes a small scarious ring (1-3).

The genus in Iran has about 34 species which two of them are endemic to the country (3-6). These plants contain monoterpenes, sesquiterpenes, sesquiterpene lactones, flovonoides, coumarins, sterols, polyacetylenes etc. (1).

Artemisia species have been shown to have wide range of pharmacological and toxicological effects, including antimalarial (7, 8), cytotoxic (9), antifungal (10-12) and antioxidant (12-14) activities.

In this study, the anticancer activity of five species of the genus against two human cancer cell lines (Hep2 and HepG2) was investigated.

Materials and Methods

Plant material

Five species of *Artemisia* were collected from different parts of Iran (Table 1). Dr. V. Mozaffarian, Research Institute of Forest and Rangelands, Ministry of Jahad-E-Agriculture Iran, confirmed the identity of the plants. Voucher specimens of the species have been deposited in the Herbarium of National Botanical Garden of Iran (TARI).

Table 1- Characteristics of collected *Artemisia* species

***Artemisia* species**	**Location**	**Collection time**
A. diffusa Krasch. ex Poljak.	Mazdavand, Khorasan Razavi province (height 900 m)	Aug. 19, 2007
A. kulbadica Boiss. & Buhse	Islamabad, Shahrabad road, Northern Khorasan province (height 907 m)	Aug. 5, 2007
A. santolina Schrenk	Daghe Akbar, Southern Khorasan province (height 1460 m)	Sep. 20, 2007
A. sieberi Besser	Samie Abad, Khorasan Razavi province (height 909 m)	Sep. 15, 2007
A. turanica Krash	Samie Abad, Khorasan Razavi province (height 808 m)	Sep. 15, 2007

Cell cultures and treatments

HepG-2 and Hep-2 cells were purchased from Pasteur Institute Collection of Cell Cultures, Tehran, Iran and were cultured in DMEM (Sigma) supplemented with 10% FBS (Gibco, USA), L-glutamine (2 mM, Jaber ibn Hayan, Iran), penicillin (100 IU/mL, Jaber ibn Hayan, Iran) and streptomycin (100 µg/mL, Jaber ibn Hayan, Iran) under standard conditions and was sterilized by 0.22 µm microbiological filters (Millipore, Irland) after preparation and kept at 4°C before using. The cells were subcultured in the ratio 1:3 twice per week. Passages 1–15 were used for experiments. Cells were seeded at a density of 5000 cells/well in 96-well plates (Nunc, Denmark). Incubations with various concentrations of the extracts were started 24 h after seeding and continued for 24 and 48 hours.

Extraction Procedure

The shade dried aerial parts of each species (100 g) were chopped in small pieces and then crushed into powder by a blinder. Each sample was macerated in ethanol 70% (v/v) for 48 hours and then extracted by a percolator. The extracted solutions were concentrated at 50°C under reduced pressure to dryness.

Cytotoxicity assay

The cytotoxic effect of obtained extracts against previously mentioned human tumor cell lines was determined by a rapid colorimetric assay, using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, Germany] 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 dimethyl sulfoxide (DMSO, Significancema, Germany) (16). Briefly, 200 µL of HepG-2 cells (2.5×10^4 cells per ml mL of media) were seeded in ten columns of 96-well microplates and incubated for 24 h (37°C, 5% CO_2 air humidified). Then, for 8 columns, 20 µL of prepared concentrations (200, 400, 600, 800, 1000, 1200, 1600 and 3200 µg/mL) of each extract was added to each column and incubated for another 24 and 48 h in the same condition. Two columns of each microplate were specified to blank (containing only DMEM) and control (containing 5% ethanol 96%), respectively. To evaluate cell survival, 20 µL of MTT solution (5 mg/mL in phosphate buffer solution) was added to each well and incubated for 3-4 h. Then, almost all old medium containing MTT was gently replaced by 200 µL of DMSO and 20 µL of glycine buffer (0.1 M, Biogen, Iran) and then pipetted to dissolve any formed formazan crystals. The first column contained blank (20 µL glycine, 200 µL DMSO) and last column contained culture without cells. Finally, the microplates were incubated at room temperature for 30 min. The same procedure was carried out for Hep-2 cells as well.

The absorbance of each well was measured by an ELISA reader (STAT FAX 303, USA) at a wavelength of 570 nm. Determination of percent of growth inhibition was carried out using the following equilibrium:

$$\text{Cell survival (\%)} = T/C \times 100$$

Where C is the mean absorbance of control group and T is the mean absorbance of test group.

Statistical analysis

The data were expressed as standard error of mean (SEM) of 8 independent experiments. Wherever appropriate, the data were subjected to statistical analysis by one-way analysis of variance (one-way ANOVA) followed by Tukey-Kramer test and Paired-Sample T Test for multiple comparisons. A value of $p<0.05$ was considered significant. SPSS 11.5 software was used for the statistical analysis. Curves were plotted by Graphpad Prism 4 Demo.

Results

24-hour exposure

Results of the MTT cytotoxicity assay for different concentrations of ethanol extracts of *A. kulbadica, A. diffusa, A. sieberi, A. santolina and A. turanica* against HepG-2 and Hep-2 cell lines are presented in Figures 1 through 5, respectively. Results showed a concentration-dependent toxicity for all the extracts and the overall toxicity on HepG-2 cells is more than that on Hep-2 cells. The calculated IC50 values for the above mentioned *Artemisia* species against Hep2 cells were 951.11, 1054.75, 231.59, 1093.82 and 975.54 and against HepG2 cells were <200, 520.70, <200, 514.82 and 259.49, respectively. Evaluation of IC50 indicated that the extract of *A. kulbadica* was most toxic against HepG2 and *A. sieberi* was the most toxic against Hep2 cells.

48-hour exposure

Results have shown a similar pattern to 24-hour exposure experiments with higher toxicity, possibly due to longer exposure (results not shown).

The order of survival cells are as follows:

HepG2 (After 24 h):

A. diffusa > *A. santolina* > *A. turanica* > *A. sieberi* > *A. kulbadica*

Hep2 (After 24 h):

A. santolina > *A. kulbadica* = *A. turanica* = *A. diffusa* > *A. sieberi*

According to graphs, Minimum toxic dose of extracts of *Artemisia* as follows:

A. kulbadica=400 µg/ml *A. sieberi*=800 µg/ml

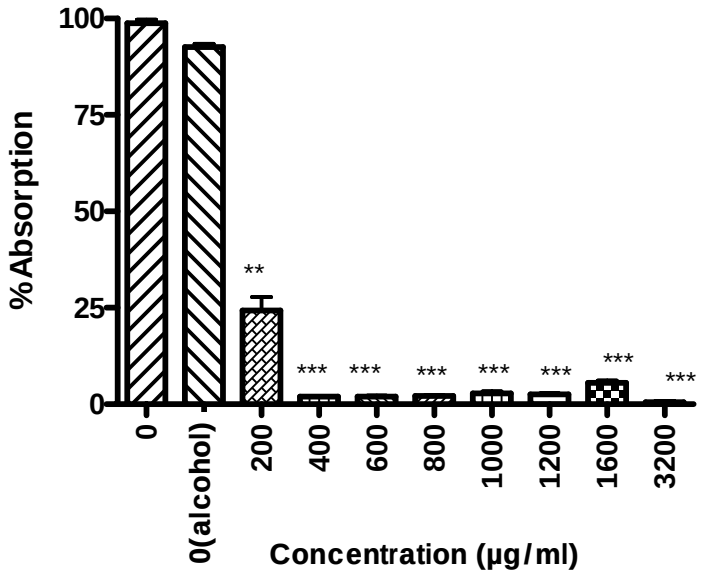
A. turanica=1000 µg/ml *A. santolina*=1600 µg/ ml

A. diffusa=1600µg/ml

Discussion

In our investigation, the *in vitro* toxicity of the ethanol extracts of some Iranian *Artemisia* species were shown against two cancer cell lines: Hep-2 and HepG-2. This is a concentration-dependent effect in the range of 200 through 3200 µg/mL. Collectively, this toxicity is stronger against HepG-2 compared with Hep-2 cell lines.

The toxicity of *Artemisia* species on cancer cells has been shown *in vitro* (17-19) and *in vivo* (7, 20). Also, we previously have shown a similar effect for some other Iranian *Artemisia* species (21). Artemisinin, the active ingredient of Artemisia annua, showed cell toxicity against human lymphoid leukemia (Molt-4) cells (22). Cesquiterpenlactones, terpenoids and flavonoids are other antitumor substances extracted from *Artemisia* species (23, 24). Artesunate, a semisynthetic derivative of artemisinin, showed both in vitro and in vivo anticancer effects (25). It also may induce antiangiogenic effects that may contribute to its anticancer effects. The predominant effect of the extracts or the active ingredients of *Artemisia* species is apoptosis. They induce apoptosis in various cell lines via activation of caspases, depolarization of the mitochondrial membrane potential and down-regulation of Bcl-2 expression (18) or cell cycle arrest (26, 27). Hu, et al. observed morphological changes typical of apoptosis, including condensed chromatin and a reduction in volume with exposure of another human hepatoma cell line to components of *Artemisia capillaries thunberg* (26). Kim et al. (2007) suggested the use of *Artemisia fukudo* as a preventive measure against cancer (27). Toxicity of *Artemisia* species on HepG2 observed through inhibition of transeaminase (23).



Toxicity of *A. Kulbadica* against

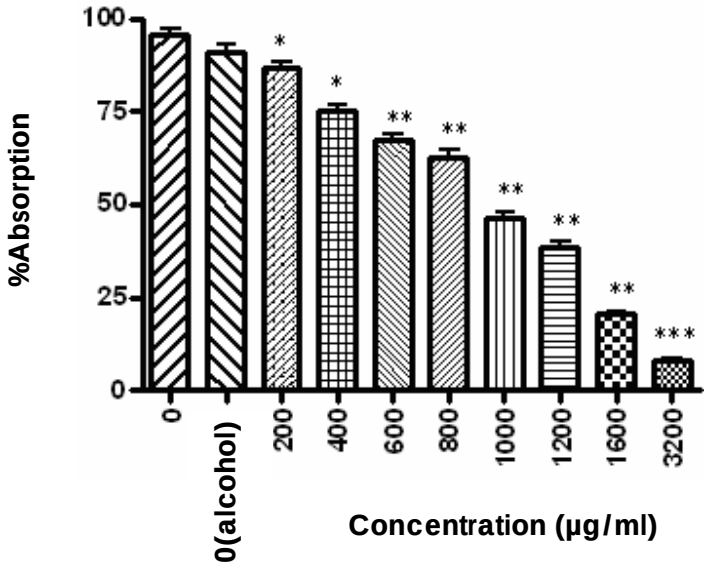
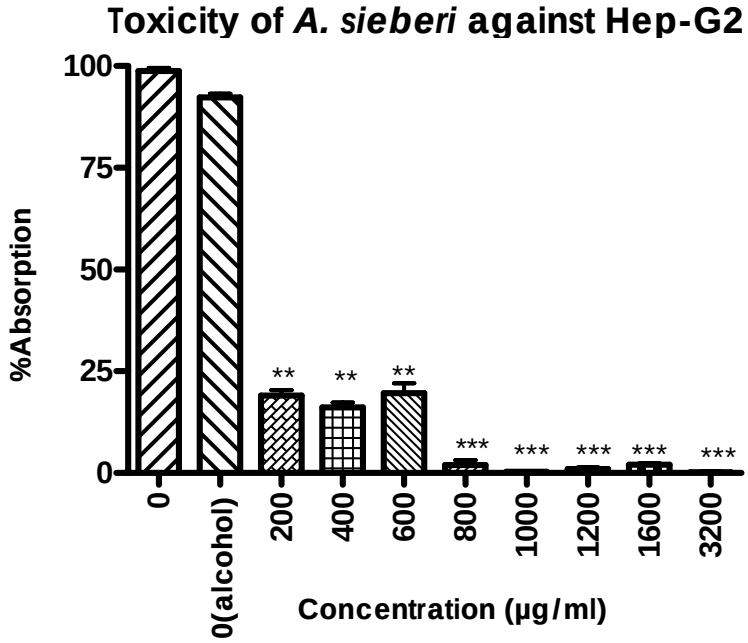


Figure 1- Results of the MTT assay of different concentrations of *Artemisia kulbadica* on HepG2 and Hep2 cells.

The cell lines were exposed to the plant extract for 24 hours and the results of MTT viability assay at 570 nm were shown as mean ± SEM (n=8 in each concentration).

(*: 0.01 < *p* value < 0.05 و **: 0.001 < *p* value < 0.01 و ***: *p* value < 0.001)



Toxicity of *A. sieberi* against Hep2

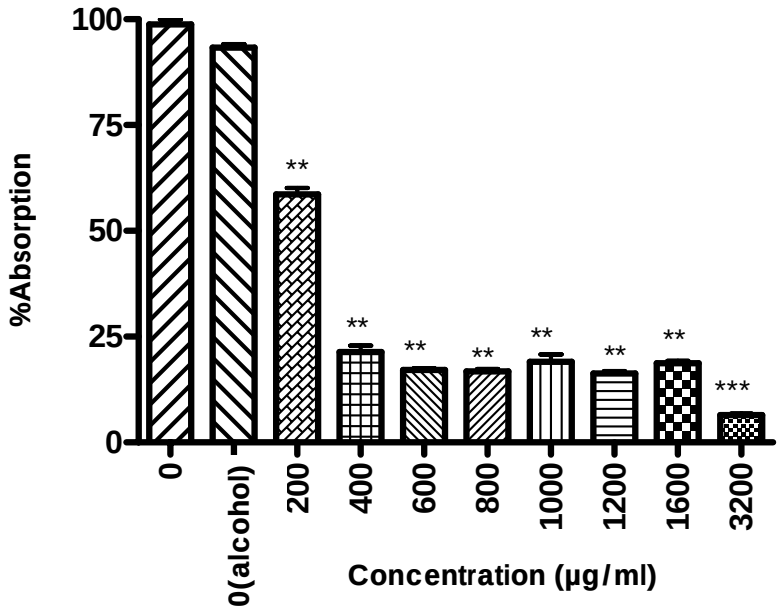


Figure 2- Results of the MTT assay of different concentrations of *Artemisia sieberi* on HepG2 and Hep2 cells.

The cell lines were exposed to the plant extract for 24 hours and the results of MTT viability assay at 570 nm were shown as mean ± SEM (n=8 in each concentration).

(**: 0.001 < *p* value < 0.01 و ***: *p* value < 0.001)

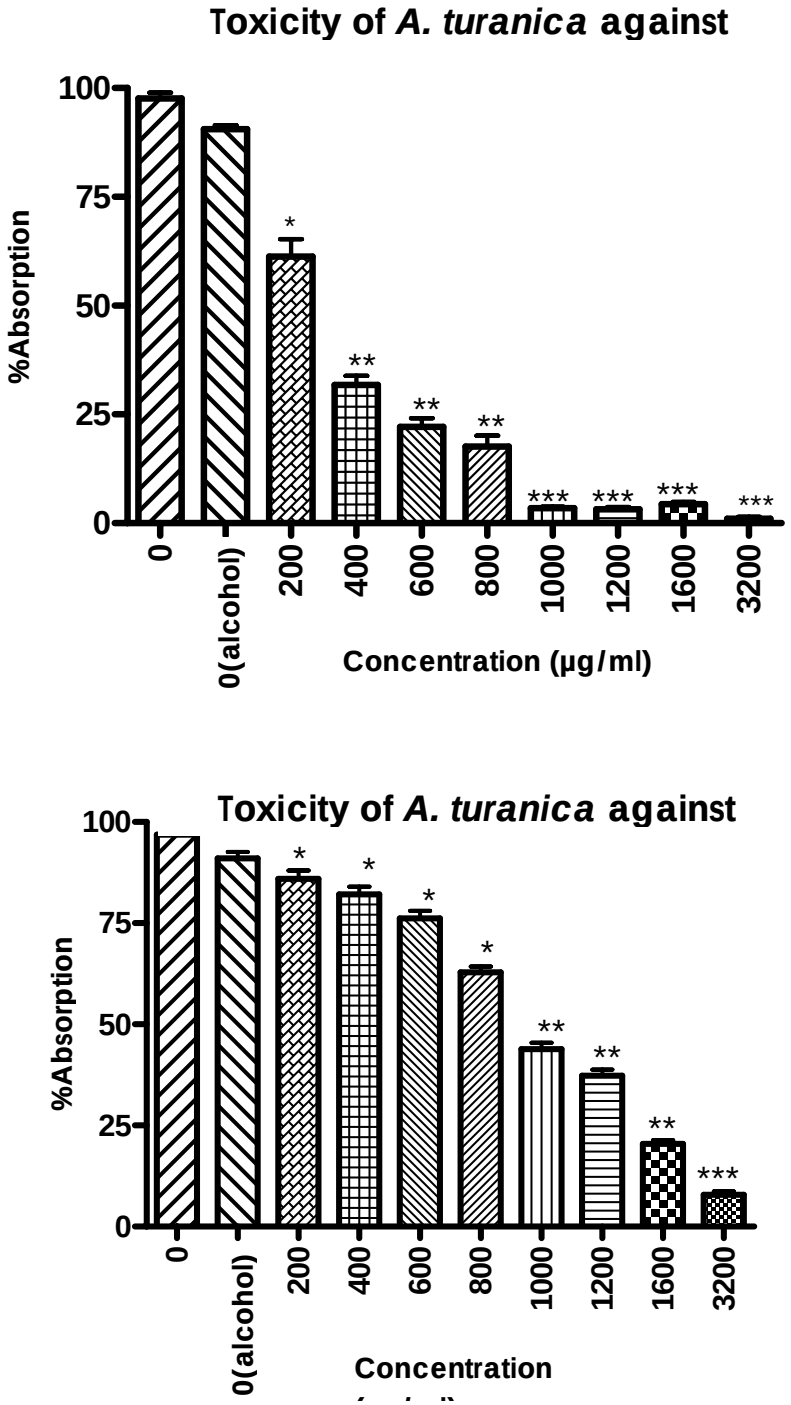


Figure 3- Results of the MTT assay of different concentrations of *Artemisia turanica* on HepG2 and Hep2 cells.

The cell lines were exposed to the plant extract for 24 hours and the results of MTT viability assay at 570 nm were shown as mean ± SEM (n=8 in each concentration).

(*: 0.01 < *p* value < 0.05، **: 0.001 < *p* value < 0.01، ***: *p* value < 0.001)

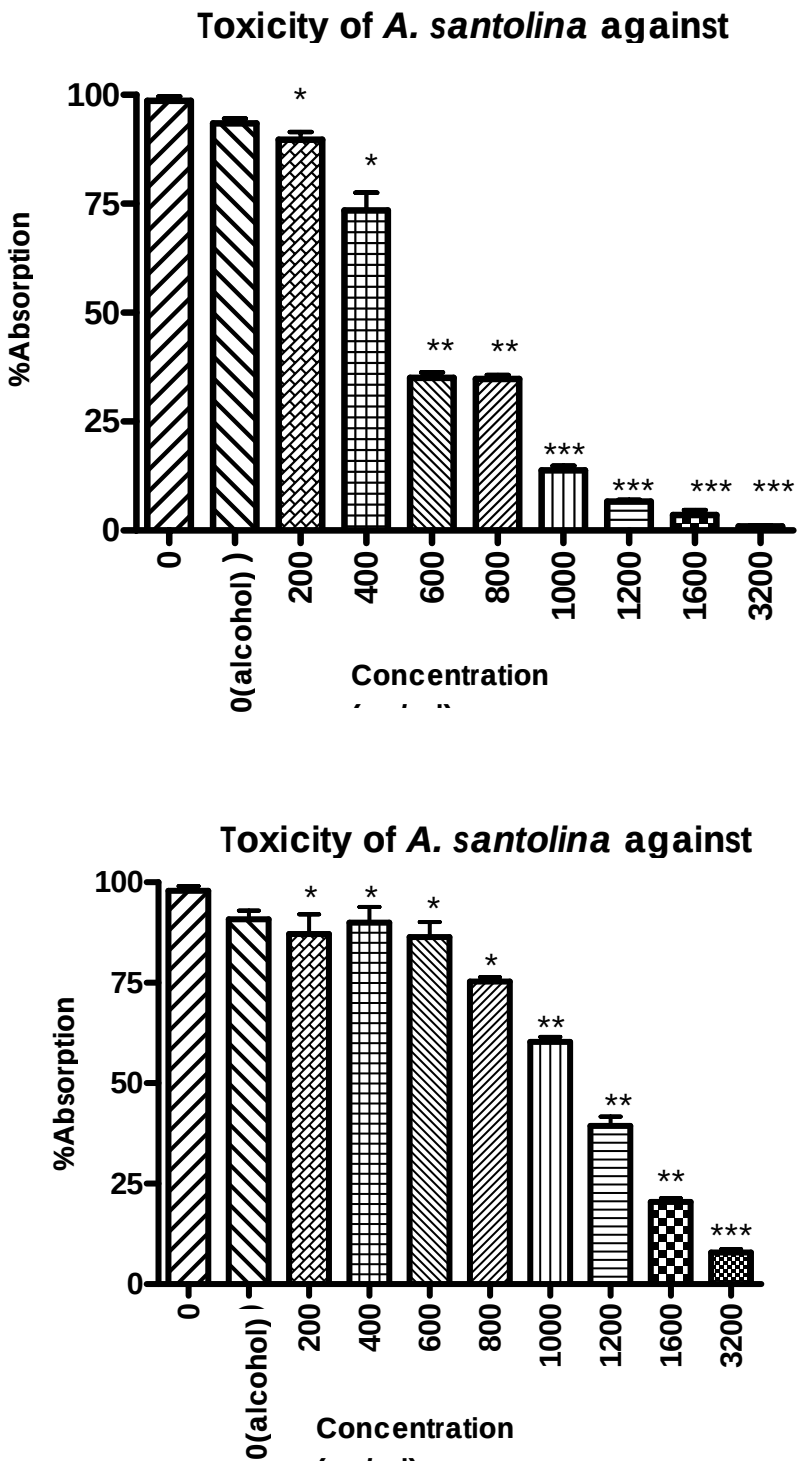


Figure 4- Results of the MTT assay of different concentrations of *Artemisia santolina* on HepG2 and Hep2 cells.

The cell lines were exposed to the plant extract for 24 hours and the results of MTT viability assay at 570 nm were shown as mean ± SEM (n=8 in each concentration).

(*: 0.01 < *p* value < 0.05، **: 0.001 < *p* value < 0.01، ***: *p* value < 0.001)

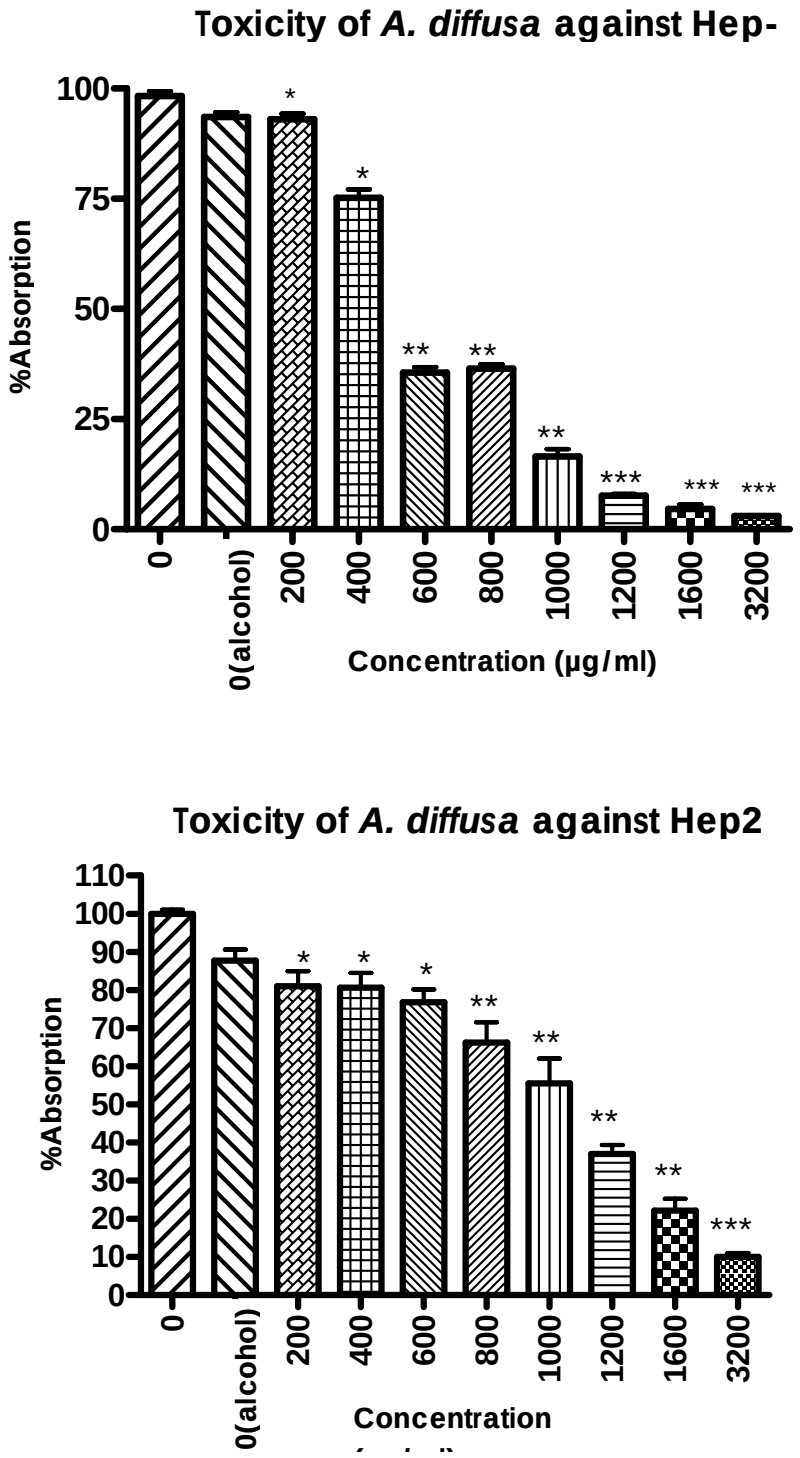


Figure 5- Results of the MTT assay of different concentrations of *Artemisia diffusa* on HepG2 and Hep2 cells.

The cell lines were exposed to the plant extract for 24 hours and the results of MTT viability assay at 570 nm were shown as mean ± SEM (n=8 in each concentration).

(*: 0.01 < *p* value < 0.05 و **: 0.001 < *p* value < 0.01 و ***: *p* value < 0.001)

Artemisinin itself produced rapid apoptosis rather than necrosis against human lymphoid leukemia (Molt-4) cells (22). Based on these previous studies, the mechanism of toxicity of *Artemisia* species is predominantly apoptosis, although, necrosis is also another possibility, especially in higher concentrations.

In our experiments, the extracts showed more potent activity against HepG2 cells than Hep2 cells. HepG2 cells are of liver origin and express a wide range of phase I and phase II enzymes such as cytochrome P450 enzymes, hydrolase, nitroreductase, catalase, peroxidase, NAD(P)H:cytochrome *c* reductase, cytochrome P450 reductase, epoxide hydrolase, sulfotransferase, glutathione S-transferase (GST), and *N*-acetyl transferase (28). Some of these enzymes present in higher concentrations in growing than in confluent cells (29). Our experiments were carried out in the growing phase of HepG2 and Hep2 cells, thus a high metabolic activity could be estimated. Eupatilin, a pharmacologically active flavone derived from *Artemisia* species, is extensively metabolized by cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) enzymes in human liver microsomes. Activation reactions with rifampin have been shown in HepG2 cells, a drug that undergo metabolic activation in the liver and causes hepatotoxicity (30). In our previous study, similar results were observed in these two cell lines (31). Because of high metabolic capacity of HepG2 cells for activation and deactivation of xenobiotics, part of the higher toxicity of the plants extracts against HepG-2 compared with Hep2 cells may be due to the activation reactions of ingredients of the extracts. Complementary studies will elucidate the uncovered aspects of anticancer toxicity of these plants.

Acknowledgement

The authors would like to thank the authorities in research council of Mashhad University of Medical Sciences and School of Pharmacy (MUMS) for their supports.

References

1- Mucciaralli M, Maffel M. Introduction of the genus In: Wright CW. ed. *Artemisia*, Taylor and Francis, London, 2002: 1-50.
2- Polyakov P. *Artemisia* In: Shishkin BK, ed. Flora of the USSR (English translation), vol. 29, Bisnen Singh Scientific Books, Koeringstein, Germany, 1995: 488-489.
3- Podlech D. Compositae VI Anthemideae In: Rechinger KH, ed. Flora Iranica, No. 158, Graz, Akademische Druck-u, Verlagsansalt, 1986: 159-223.
4- Ghahreman A, Attar F. Biodiversity of plant species in Iran, vol. 1, Tehran University Publication, Tehran, 1999: 41-42.
5- Emami SA, Aghazari F. Les Phanerogames endemiques de la flore d' Iran, publication de l' Universite' d' Iran des Sciences Medicales, Téhéran, 2008: 349.
6- Mozaffarian V. Compositae: Anthemideae & Echinapeae. In: Assadi M, ed. Flora of Iran No. 59, Research Institute of Forests and Rangelands 2008: 199-261 (In Persian)
7- Efferth T. Willmar Schwabe Award 2006: Antiplasmodial and Antitumor Activity of Artemisinin - From Bench to Bedside, Planta Med 2007; 73: 299-309.
8- Allen PC, Danforth HD, Augustine PC. Dietary modulation of avian coccidiosis. Int J Parasitol 1998; 28: 1131-1140.

9- Zheng GQ. Cytotoxic terpenoides and flavonoides from *Artemisia annua*. Planta Med 1994; 60: 54-57.
10- Farzaneh M, Ahmadzadeh M, Hadian J, Tehrani AS. Chemical composition and antifungal activity of the essential oils of three species of *Artemisia* on some soil-borne phytopathogens. Commun Agric Appl Biol Sci 2006; 71: 1327-1333.
11- Kordali S, Kotan R, Mavi A, Cakir A, Ala A, Yildirim A, Agric J. Determination of the chemical composition and antioxidant activity of the essential oil of Artemisia dracunculus and of the antifungal and antibacterial activities of Turkish *Artemisia absinthium, A. dracunculus, Artemisia santonicum,* and *Artemisia spicigera* essential oils. Food Chem 2005; 53: 9452-9458.
12- Kordali S, Cakir A, Mavi A, Kilic H, Yildirim A. Screening of chemical composition and antifungal and antioxidant activities of the essential oils from three Turkish *Artemisia* species. J Agric Food Chem 2005; 53: 1408-1416.
13- Szeto YT, Benzie IFF. Is the yin-yang nature of Chinese herbal medicine equivalent to antioxidation-oxidation? J Ethnopharmacol 2006; 108: 361-366.
14- Bhatt LR, Lim JA, Chai KY, Kang JI, Oh HK, Baek SH. Antioxidant and antimicrobial activities of essential oil from *Artemisia vulgaris.* Nat Prod Sci 2006; 12: 226-231.
15- Mosmann T (1983): Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55-63.
16- Carmichale J, De Graff WG, Gazdar AF, Mina JD, Mitchell JB. Evaluation of a tetrazolium based semiautomated colorimetric assay: Assessment of chemosensitivity testing. Cancer Res 1987; 47: 936-942.
17- Willoughby Sr JA, Sundar SN, Cheung M, Tin AS, Modiano J, Firestone GL. Artemisinin blocks prostate cancer growth and cell cycle progression by disrupting Sp1 interactions with the cyclin-dependent kinase-4 promoter and inhibiting CDK4 gene expression. J Biol Chem 2009; 284:2203-13.
18- Sarath VJ, So CS, Young DW, Gollapudi S. *Artemisia princeps* var orientalis induces apoptosis in human breast cancer MCF-7 cells. Anticancer Res 2007; 27:3891-3898.
19- Mercer AE, Maggs JL, Sun XM, Cohen GM, Chadwick J, O'Neill PM. Park BK. Evidence for the involvement of carbon-centered radicals in the induction of apoptotic cell death by artemisinin compounds. J Biol Chem 2007; 282:9372-9382.
20- Lai H, Singh NP. Oral artemisinin prevents and delays the development of 7,12-dimethylbenz[a]anthracene (DMBA)-induced breast cancer in the rat. Cancer lett 2006; 231:43-8.
21- Tan RX, Zheng WF, Tang HQ. Biologically active substances from genus *Artemisia*. Planta Med 1998; 64: 295-302.
22- Singh NP, Lai HC. Artemisinin induces apoptosis in human cancer cells. Anticancer Res 2007; 24: 2277-2280.
23- Suffiness M, Pezzuto JM. Methods in plant biochemistry, Academic Press, Sandiego, 1991: 71-131.
24- Wang Q, Wu Lu, Li AY, Zhao Y, Wang NP. Experimental studies of antitumor effect of artesunate on liver cancer. China Journal of Chinese Materia Medica 2001; 26: 707-708, 720.
25- Li PC, Lam E, Roos WP, Zdzienicka MZ, Kaina B, Efferth T. Artesunate derived from traditional Chinese medicine induces DNA damage and repair. Cancer Res 2008; 68:4347-4351.

26- Hu YQ, Tan RX, Chu MY, Zhou J. Apoptosis in human hepatoma cell line SMMC-7721 induced by water-soluble macromolecular components of *Artemisia capillaries* Thunberg. China Journal of Chinese material medica 2001; 26: 707-708, 720.
27- Kim KN, Lee JA, Yoon WJ, Kim JY, Song GP, Park SY. The cytotoxicity of *Artemisia fukudo* extracts against HL-60 cells. Journal of the Korean Society of Food Science and Nutrition 2007; 36: 819-824.
28- Knasmüller S, Parzefall W, Sanyal R, Ecker S, Schwab C, Uhl M, Mersch-Sundermann V, Williamson G, Hietsch G, Langer T, Darroudi F, Natarajan AT. Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. Mutat Res 1998; 402: 185–202.
29- Alexandre E, David P, Viollon C, Wolf P, Jaeck D, Azimzadeh A, Nicod L, Boudjema K, Richert L. Expression of cytochromes P-450 2E1, 3A4 and 1A1/1A2 in growing and confluent human HepG2 hepatoma cells-effect of ethanol. Toxicol In Vitro 1999; 13: 427-435.
30- Vahdati-Mashhadian N, Jaafari MR, Nosrati A. Differential toxicity of rifampin on HepG2 and Hep2 cells using MTT test and electron microscope. Pharmacologyonline 2007; 3: 405-413.
31- Vahdati-Mashhadian, N., Emami, S. A., Oghazian, M. B. and Vosooq, R. (2009). The cytotoxicity evaluation of seven species of *Artemisia* on human tumor cell lines. Pharmacologyonline 1: 229-242.