

The Cytotoxicity Evaluation of Seven Species of *Artemisia* on Human Tumor Cell Lines

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Summary

It has been reported that several *Artemisia* species possess cytotoxic activity against different human cell lines.

In this study, the toxicity of the *A. annua*, *A. campestris*, *A. chamaemelifolia*, *A. fragrans*, *A. incana*, *A. persica*, and *A. vulgaris* against human Caucasian hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2) cell lines have been investigated.

Three species of these plants were collected from Golestan and Northern Khorasan provinces, northeast of Iran and the others from Eastern Azerbaijan province, northwest of the country. Different concentrations (25, 50, 100, 200, 400, 800, 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, HepG-2 and Hep-2 were determined by quantitative MTT assay.

The extracts showed significant concentration-dependent toxicity. They showed more toxicity on HepG-2 compared with Hep-2. As HepG-2 cells contain high amounts of metabolizing enzymes, it seems that the active ingredients of the extracts are converted to more toxic metabolites as a result of hepatic metabolism.

Keywords: *Artemisia* spp., Astraceae, Cytotoxicity, Hep-2, HepG-2, MTT assay.

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Introduction

The genus *Artemisia* L. is one of the largest and most widely distributed of the nearly 100 genera in the tribe Anthemideae of the Asterceae (Compositae). This genus 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 capitata 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 (1-5).

The genus in Iran has about 34 species which two of them are endemic to the country (5-7). Plants of the genus contain monoterpenes, sesquiterpenes, sesquiterpene lactones, flavonoides, coumarins, sterols, polyacetylenes Etc. (3,8).

Different species of *Artemisia* showed a vast range of biological effects that including antimalarial (8-10), cytotoxic (11), antibacterial, antifungal (8-10, 12-15) and antioxidant (9-10, 12, 14-19) activities.

Materials and Methods

Plant material

Seven species of *Artemisia* were collected from different parts of Iran (Table 1). Dr. V. Mozaffarian, Research Institute of Forest and Rangelands, Ministry of Jihad-E-Agriculture Iran, was identified these 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

<i>Artemisia</i> species	Location	Collection time
<i>A. annua</i> L.	Islamabad near Maraveh tapeh, Shahrabad road, Northern Khorasan province (height 940 m)	Sep. 15, 2007
<i>A. campestris</i> L.	Kalibar, Eastern Azerbaijan province (height 1650 m)	Aug. 8, 2007
<i>A. chamaemelifolia</i> Vill.	Kalibar, Eastern Azerbaijan province (height 1364 m)	Aug. 8, 2007
<i>A. fragrans</i> Willd.	Kalibar, Eastern Azerbaijan province (height 1450 m)	Aug. 8, 2007
<i>A. incana</i> Druce	Kalibar, Eastern Azerbaijan province (height 1350 m)	Aug. 8, 2007
<i>A. persica</i> Boiss.	Astan Quds Farm, Mashhad, Khorasan Razavi province (height 915 m)	Sep. 19, 2007
<i>A. vulgaris</i> L.	Golestan Forest, 10 km far from Tangrah, Golestan province (height 695 m)	Sep. 12, 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 subcultured in the ratio 1:3 twice per week, completed medium was sterilized by 0.22 µm microbiological filters (Millipore, Ireland) after preparation and kept at 4 °C before using. Passages 1–15 were used for experiments. Cells were seeded at a density of 5000 cells/well in 96-well plates (Greiner, UK). Incubations with various concentrations of the extracts were started 24 h after seeding and continued for 24 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 24 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 (20). 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) (21). Briefly, 200 µL of HepG-2 cells (2.5×10^4 cells per ml of media) were seeded in ten columns of 96-well microplates and incubated for 24 h (37°C, 5% CO₂ air humidified). Then, for 8 columns, 20 µL of prepared concentrations (25, 50, 100, 200, 400, 800, 1600, 3200 µg/mL) of each extract was added to each column and incubated for another 24 h in the same condition. The first 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. 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 (Microplate reader MR 600, Dynatech, USA) at a wavelength of 570 nm. Determination of percent of growth inhibition was carried out using the following equilibrium:

$$\text{Growth inhibition (\%)} = [(C - T)/C] \times 100$$

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 mean \pm 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 for multiple comparisons. A value of $p < 0.05$ was considered significant. InStat for Windows (version 3) software was used for the statistical analysis. Curves were plotted by PRISM (GraphPad, Version 5.00).

Results

Results of the MTT cytotoxicity assay for different concentrations of ethanol extracts of *A. annua*, *A. campestris*, *A. chamaemelifolia*, *A. fragrans*, *A. incana*, *A. persica* and *A. vulgaris* against HepG-2 and Hep-2 cell lines are presented in Figures 1 through 7, 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±SD) values for the above mentioned *Artemisia* species against HepG-2 cells were 235.2±2.1, 118.8±1.8, 44.1±1.2, 963.2±3.1, 102.6±1.9, 511.1±3.4 and 41.0±1.0 and against Hep-2 cells were 959.9±2.1, 345.5±2.0, 318.6±1.1, 174.0±1.6, 255.9±2.3, 696.6±2.7 and 111.9±2.6, respectively. Evaluation of IC₅₀ indicated that the extract of *A. vulgaris* was most toxic against HepG-2 and Hep-2 cell lines.

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 $\mu\text{g/mL}$. Collectively, this toxicity is stronger against HepG-2 compared with Hep-2 cell lines, except for *A. fragrance*. The evaluation of IC₅₀ values implies the highest toxicity for *A. vulgaris* extract against both cell lines. *A. fragrance* and *A. persica* are the least toxic extracts against HepG-2 cells and *A. annua* and *A. persica* the least toxic extracts against Hep-2 cells.

The toxicity of *Artemisia* species on cancer cell lines has been shown *in vitro* (22, 23, 24) and *in vivo* (25, 26). 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 (23) or cell cycle arrest (27, 22). Artesunate, a semi-synthetic derivative from artemisinin, induces apoptosis and necrosis in cancer cells (28). Artemisinin itself produced rapid apoptosis rather than necrosis against human lymphoid leukemia (Molt-4) cells (29). 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.

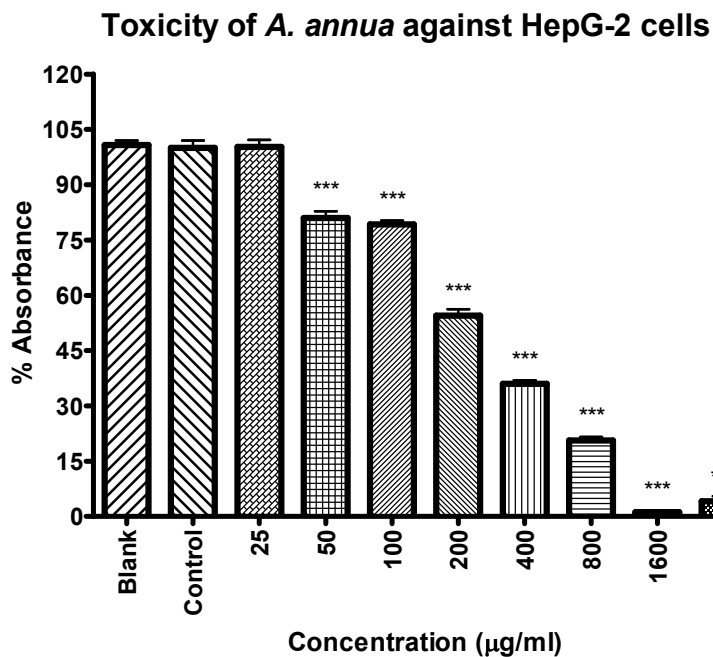
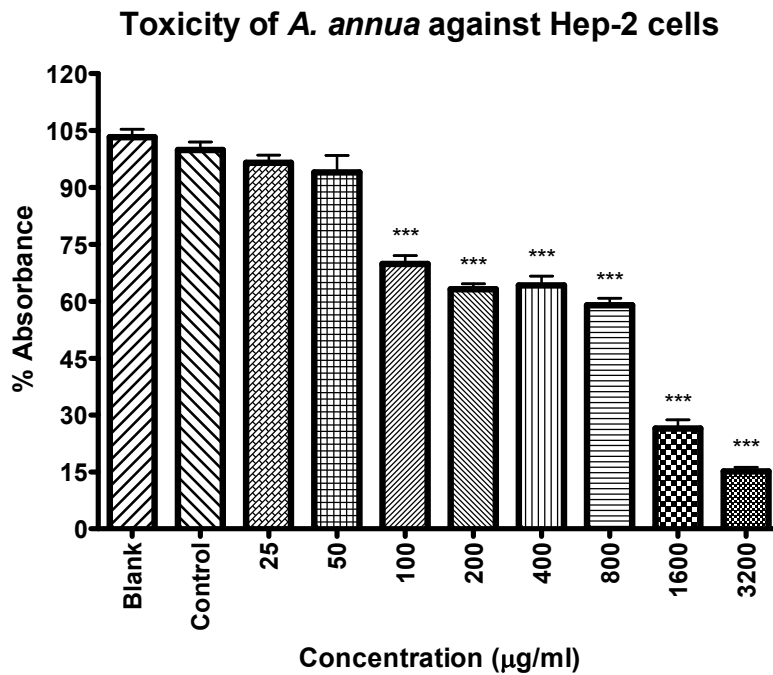


Figure 1- Results of the MTT assay of different concentrations of *Artemisia annua* on Hep2 and HepG2 cells.

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 \pm SEM (n=8 in each concentration).

*** p < 0.001 compared with control column

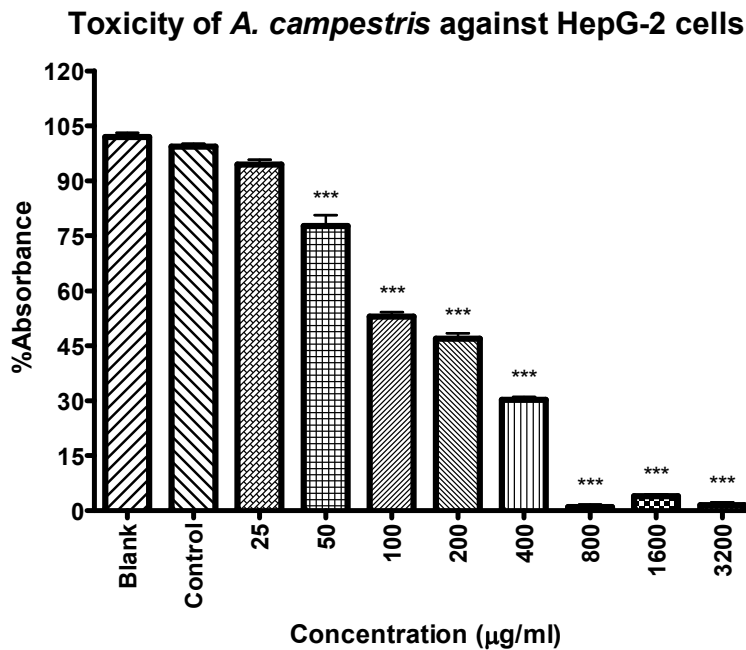
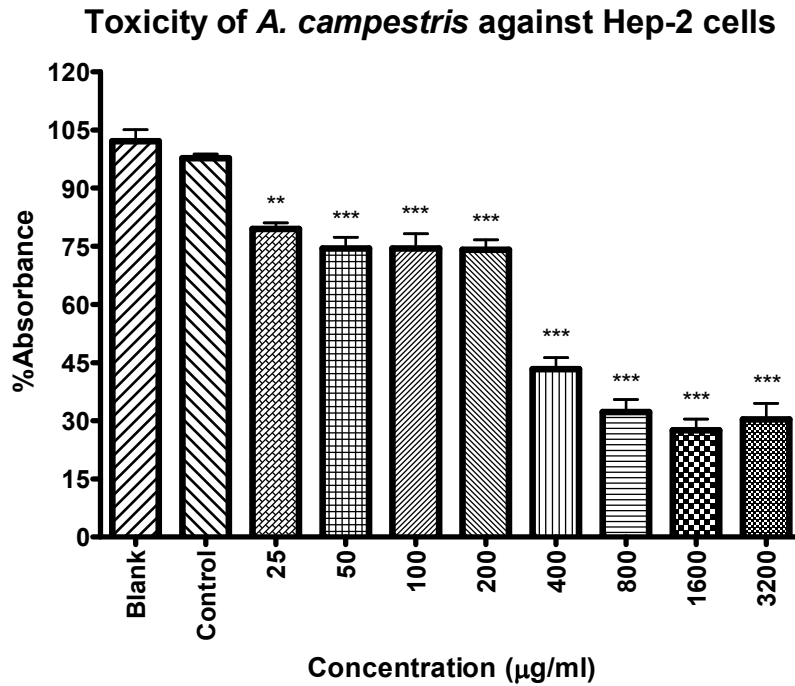


Figure 2- Results of the MTT assay of different concentrations of *Artemisia campestris* on Hep2 and HepG2 cells.

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 \pm SEM (n=8 in each concentration).

* p < 0.05, ** p < 0.01 and *** p < 0.001 compared with control column

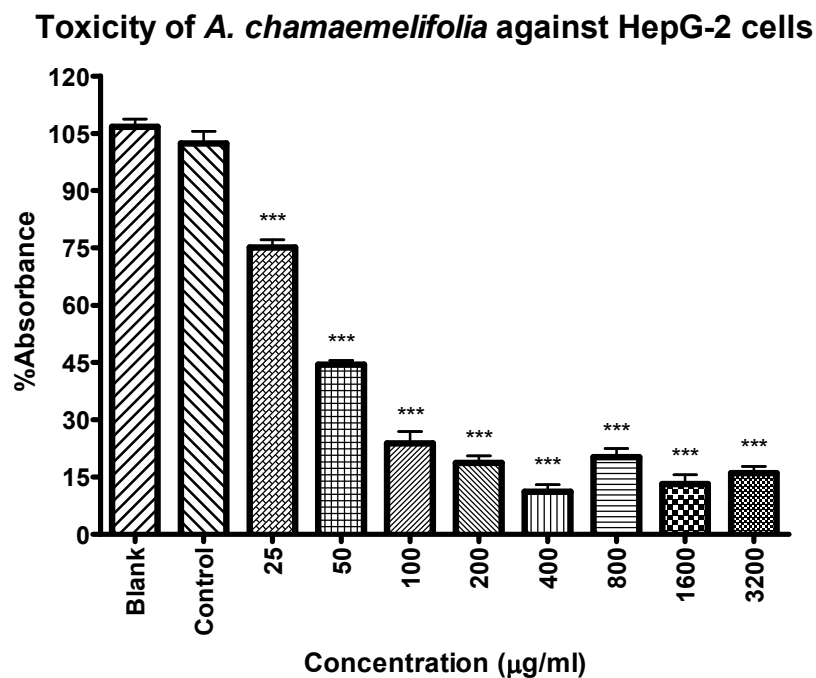
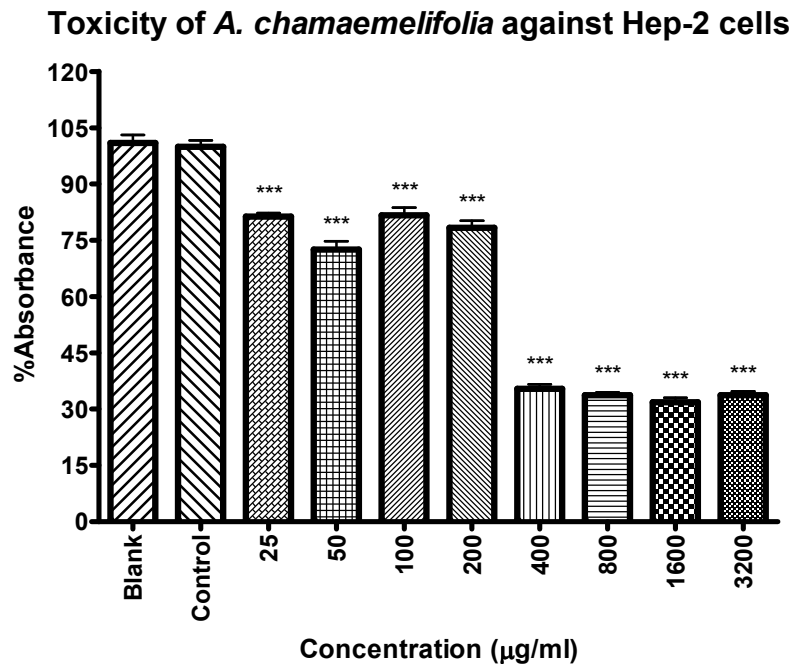


Figure 3- Results of the MTT assay of different concentrations of *Artemisia chamaemelifolia* on Hep2 and HepG2 cells.

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 \pm SEM (n=8 in each concentration).

* p< 0.05, ** p< 0.01 and *** p< 0.001 compared with control column

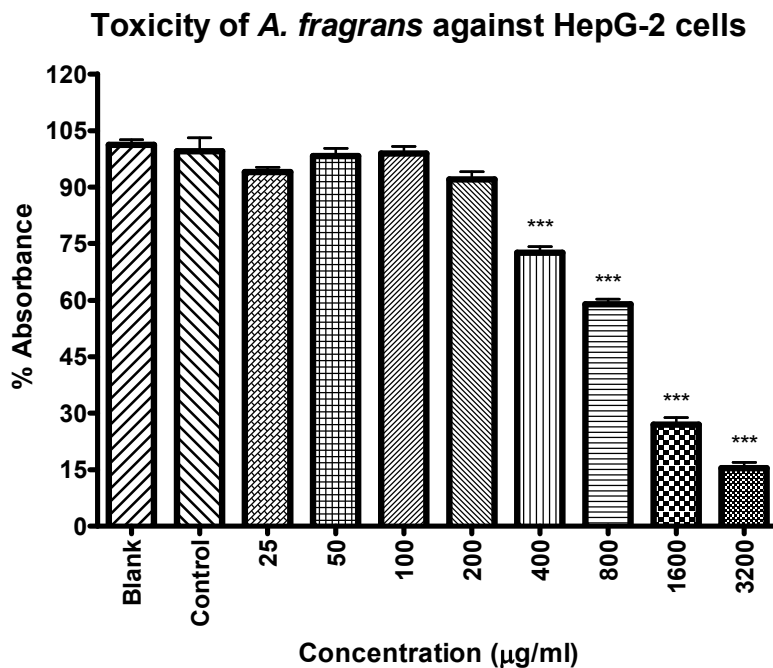
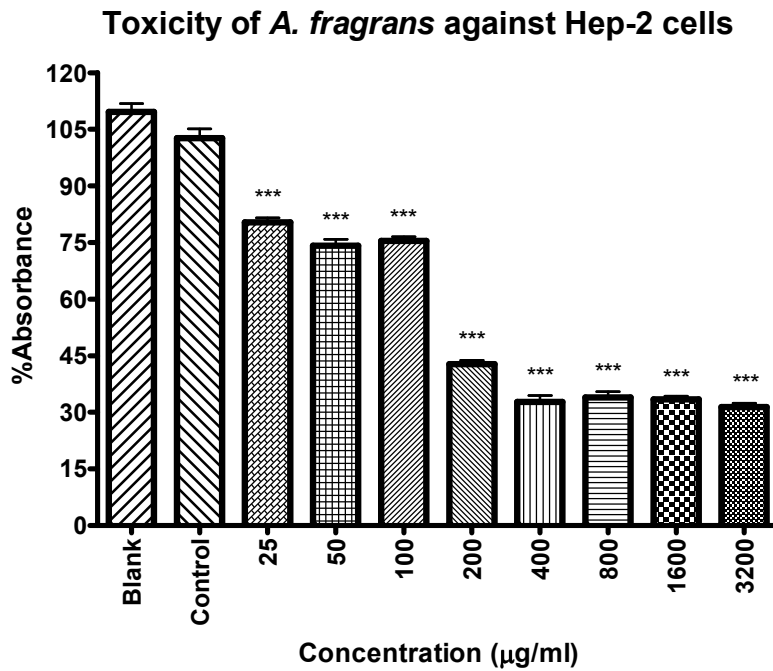


Figure 4- Results of the MTT assay of different concentrations of *Artemisia fragrans* on Hep2 and HepG2 cells.

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).

*** p < 0.001 compared with control column

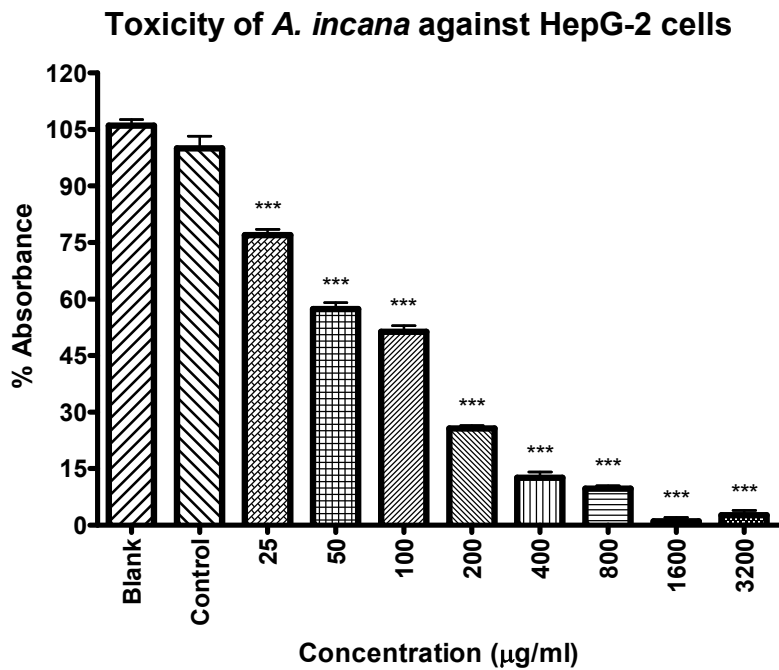
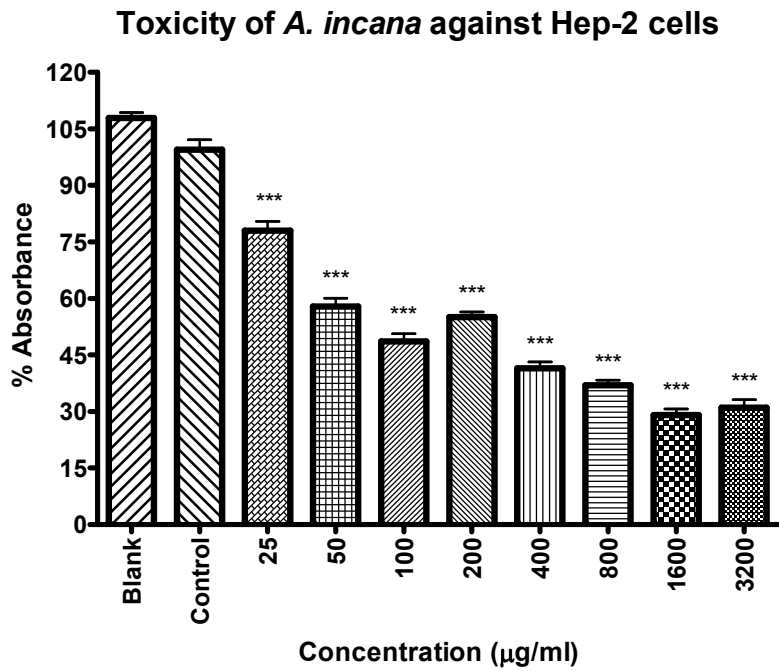


Figure 5- Results of the MTT assay of different concentrations of *Artemisia incana* on Hep2 and HepG2 cells.

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).

*** p< 0.001 compared with control column

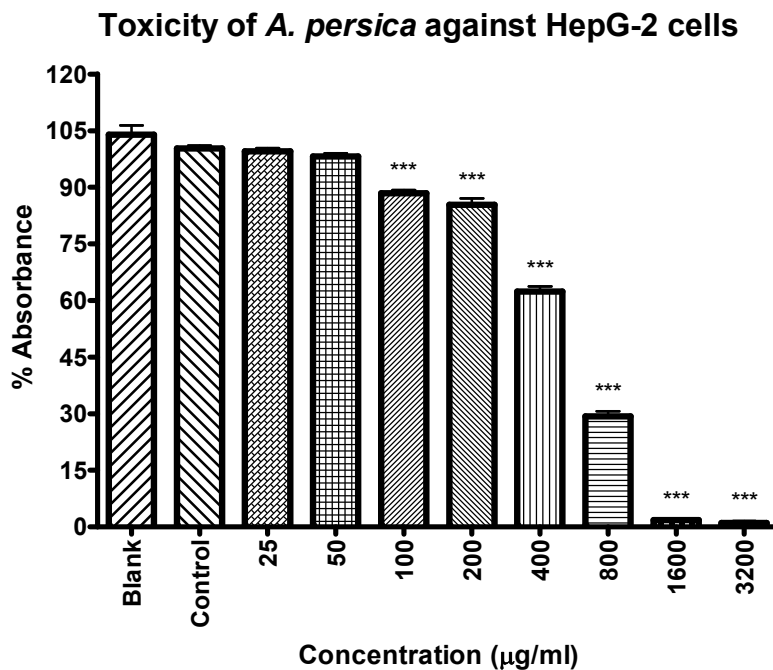
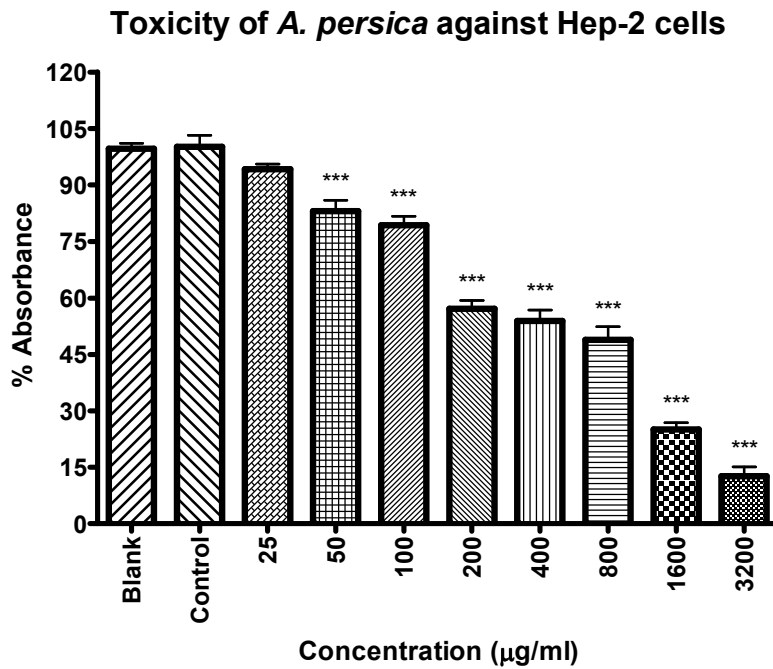


Figure 6- Results of the MTT assay of different concentrations of *Artemisia persica* on Hep2 and HepG2 cells.

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).

*** p < 0.001 compared with control column

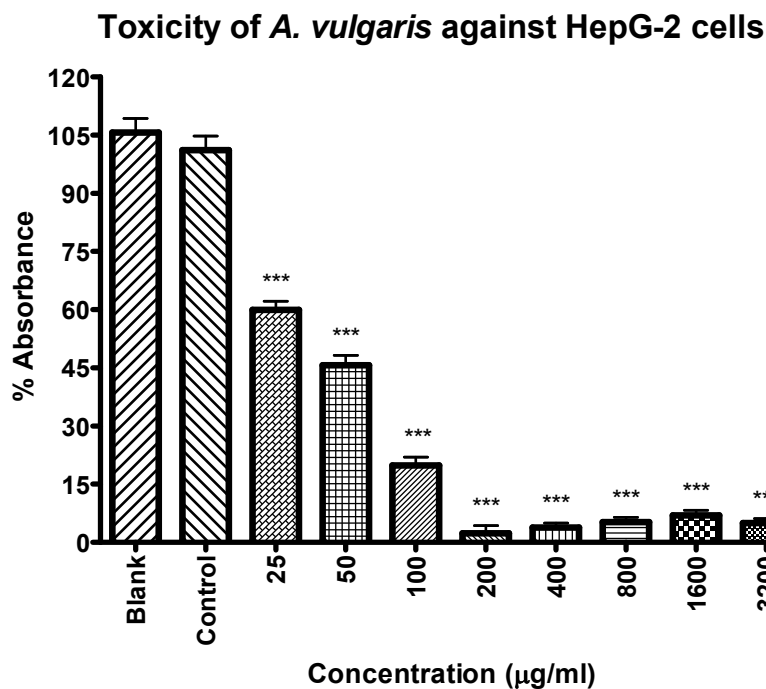
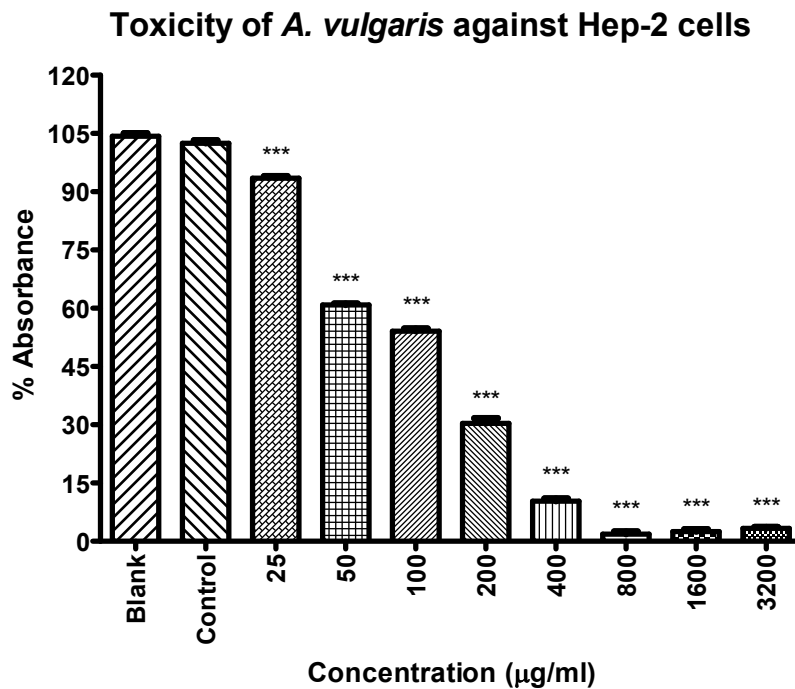


Figure 7- Results of the MTT assay of different concentrations of *Artemisia vulgaris* on Hep2 and HepG2 cells.

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 \pm SEM (n=8 in each concentration).

*** p< 0.001 compared with control column

In our experiments, the toxicity of the extracts was stronger against HepG-2 cells than Hep-2 cells. HepG-2 cells are of liver origin and as reviewed by Knasmuller, *et al.* (30), they express a wide range of phase I enzymes such as cytochrome P450 (CYP) 1A1, 1A2, 2B, 2C, 3A and 2E1, arylhydrocarbon hydrolase, nitroreductase, *N*-demethylase, catalase, peroxidase, NAD(P)H:cytochrome *c* reductase, cytochrome P450 reductase, and NAD(P)H, Quinone oxidoreductase and phase II enzymes such as epoxide hydrolase, sulfotransferase, glutathione S-transferase (GST), uridine glucuronosyl transferase, and *N*-acetyl transferase. Some of these enzymes present in higher concentrations in growing than in confluent cells (31). Our study have been carried out in the growing phase of HepG-2 and Hep-2 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. Because of high metabolic capacity of HepG-2 cells for activation and deactivation of xenobiotics, it is likely that, at least, part of the higher toxicity of the plants extracts against HepG-2 compared with Hep-2 cells be due to the activation reactions of artemisinin or other ingredients of the extracts. This effect has been shown in our previous experiments with rifampin, a drug that has known metabolism by liver cells in the same cell lines (32). More studies should be done to elucidate the uncovered aspects of anticancer toxicity of these plants.

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