Original Article

Cytotoxicity of different extracts of arial parts of *Ziziphus spina-christi* on Hela and MDA-MB-468 tumor cells

Abbas Jafarian, Behzad Zolfaghari[1], Kobra Shirani

Departments of Pharmacology and Toxicology, [1]Pharmacognosy, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: It has been shown that plants from the family *Rhamnaceae* possess anticancer activity. In this study, we sought to determine if *Ziziphus spina-christi*, a species from this family, has cytotoxic effect on cancer cell lines.

Materials and Methods: Using maceration method, different extracts of leaves of *Z. spina-christi* were prepared. Hexane, chloroform, chloroform-methanol (9:1), methanol-water (7:1) methanol, butanol and water were used for extraction, after preliminary phytochemical analyses were done. The cytotoxic activity of the extracts against Hela and MDA-MB-468 tumor cells was evaluated by MTT assay. Briefly, cells were seeded in microplates and different concentrations of extracts were added. After incubation of cells for 72 h, their viability was evaluated by addition of tetrazolium salt solution. After 3 h medium was aspirated, dimethyl sulfoxide was added and absorbance was determined at 540 nm with an ELISA plate reader. Extracts were considered cytotoxic when more than 50% reduction on cell survival was observed.

Results: Hexane, chloroform, chloroform-methanol, butanol, methanol-water and aqueous extracts of *Z. spina-christi* significantly and concentration-dependently reduced viability of Hela and MAD-MB-468 cells. In the both cell lines, chloroform-methanol extract of *Z. spina-christi* was more potent than the other extracts.

Results: From the finding of this study it can be concluded that *Z. spina-christi* is a good candidate for further study for new cytotoxic agents.

Key Words: Cytotoxicity, Hela, MDA-MB-468, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, *Ziziphus spina-christi*

Address for correspondence:
Dr. Abbas Jafarian, Department of Pharmacology and Toxicology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran. E-mail: jafarian@pharm.mui.ac.ir

Received: 22-08-2012, **Accepted:** 23-09-2012

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.125727

INTRODUCTION

Cancer, one of the leading causes of death worldwide, accounts for about 13% of all deaths in 2008.[1] During the last decades, we are encountering with increase in the incidence of cancer.[2] Despite the progress in our knowledge about cancer, recent strategies for cancer treatment were not very effective. Therefore, scientists are still trying to find new compounds for the treatment of this disease. It has been shown that plants provide

How to cite this article: Jafarian A, Zolfaghari B, Shirani K. Cytotoxicity of different extracts of arial parts of *Ziziphus spina-christi* on Hela and MDA-MB-468 tumor cells. Adv Biomed Res 2014;3:38.

important resources for a large number of compounds with anti-tumor activity.[3-5] In recent years, a significant percentage of anti-cancer drugs are in clinical trials that are dependent on natural resources.[6]

During the last few decades, chemo preventive and chemotherapeutic compounds including colchicine, vincristine, vinblastine, podophyllotoxin and taxol were isolated from various plants and used against various types of cancers.[7-10]

Cytotoxic activity of plants from family *Rhamnaceae* has been shown in several studies.[5,11] The family of *Rhamnaceae* consists of about 50-60 genera and approximately 870-900 species. They are flowering plants, mostly trees, shrubs and some vines with worldwide distribution, but are more common in the subtropical and tropical regions. Ziziphus, one of the genus of the family of *Rhamnaceae*, consist of more than 40 species of spiny shrubs and small trees.

It has been shown that *Ziziphus jujube* mill, a member of the genus Ziziphus, possess different effects including antimicrobial, antioxidant, immunostimulant, antidiabetic, antihyperglycemic and anticancer effects.[12,13]

One of the active component of *Ziziphus* species e.g., *Z. mauritiana, Z. rugosa* and *Z. oenoplia* is betulinic acid. Studies have shown that betulinic acid selectively inhibited the growth of human melanoma cell line *in vitro* and it is in clinical or preclinical development for cancer therapy.[5]

Z. spina–christi L., commonly known by the Persian name, "Konar" or "Sedr", is an armed shrub or tree, which widely growth in the southern of Iran. The plant fruit is edible sweet drupe and its leaves have been used as stomachic, emollient, antiulcer, disinfectant and antifungal in the Iranian traditional medicine. *Z. spina–christi* is well known among people as a good hair and body detergent.[14-17] The phytochemical and pharmacological studies of the *Z. spina–christi* have been the subject of some research in the recent years. These studies confirmed that essential oil such as geranyl acetate, methyl hexadecanoate and methyl octadecanoate, peptide and cyclopeptide alkaloids like spinanine-A, tanines, sterols like β-sitosterol, flavonoids such as rutin and quercetin derivatives, triterpenoid sapogenins and saponins such as betulinic acid are the main constituents of the leaves of *Z. spina–christi*.[15,18,19] Biological and pharmacological tests have shown antibacterial, antiviral and antidiabetic effects of the extracts or fractions of the leaves of this plant.[14,16,17]

Limited studies have shown that crude extract of *Z. spina-Christi* had cytotoxic effect on tumor cell lines.[11] In this study, we used solvents with different polarity for extraction of ingredients of *Z. spina-christi* and evaluated their cytotoxic effects on Hela and MDA-MB-468 cells.

MATERIALS AND METHODS

Materials

Compounds used were: Methanol, butanol, chloroform, hexane, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), NaCl, KCl, NaOH, HCl, H_2SO_4, $NaHCO_3$, Na_2HPO_4 (Merck, Germany), penicillin/streptomycin (Sigma, USA), RPMI1640, fetal calf serum (FCS), sodium pyruvate, trypsin, L-glutamine (Gibco, Scotland), dimethyl sulfoxide (DMSO) (Fluka, Italy), doxorubicin (Farmitalia, Italy).

Plant material

The leaves of *Z. spina-christi* were bought from local market of Isfahan, Iran and identified in the School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

Extraction and isolation

One hundred grams of dried powder was extracted by 300 ml of hexane using maceration method.[4] The residue was subjected to more extraction using 300 ml of chloroform, chloroform-methanol (9:1), methanol-water (7-1) and methanol, respectively. Butanolic and aqueous extracts were then obtained from the methanolic extract. The extracts were concentrated by a rotary evaporator and freeze dryer. The extracts were dried in vacuum oven at 40°C for 5 days. Then they were stored at 4°C until used. Ten mg of the solid residues were dissolved in 100 µl of DMSO and diluted to 2 ml with phosphate buffer saline (PBS) and filtered through 0.22 µ microbiological filters. Dilution was continued so that the final concentrations of extracts were 10, 100, 200, 500, 1000 and 5000 µg/ml.

Preliminary phytochemical analyses

The presence of alkaloids, glycosides, flavonoids, tannins, saponins and anthraquinones in the leaves of *Z. spina-christi* was tested using standard qualitative methods described earlier.[20,21]

Cell lines

Hela (human cervix carcinoma) and MDA-MB-468 (Human breast carcinoma), cell lines were purchased from the cell bank of Pasture Institute, Tehran, Iran.

Maintenance of human cell lines

Cells were grown in RPMI-1640 [supplemented with 10% of FCS, penicillin/streptomycin (50 IU ml^{-1} and 500 µg ml^{-1}, respectively), sodium pyruvate (1 mM),

$NaHCO_3$ and L-glutamine (2 mM)]. Using 0.22 μ microbiological filters, completed media was steril ized 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.

Cytotoxicity evaluation of extracts

Cytotoxic effect of the extracts with different polarity against Hela and MDA-MB-468 human tumor cell lines was determined by a rapid colorimetric assay, using MTT.[22] In this assay soluble MTT is metabolized by mitochondrial enzyme activity of viable tumor cells, into an insoluble colored formazan product. Subsequently formazan were dissolved in DMSO and measured spectrophotometrically at 540 nm. Briefly, 200 μl of cell suspension (5* 10^4 cells per ml of media) was seeded in 96-well microplates and incubated for 24 h (37°C, 5% CO_2 air humidified). Then 20 μl of each concentration of different extracts was added and microplates containing cells and extracts were incubated for another 48 h in the same condition. Doxorubicin was used as a positive control. For the negative control wells contained only the cells in the media with no extracts or doxorubicin. To evaluate cell survival, 20 μl of MTT solution (5 mg/ml in PBS) 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 enzyme-linked immunosorbent assay (ELISA) plate reader. Each extract concentration was assayed in 4 wells and repeated 3-times. Standard curves (absorbance against number of cells) for each cell line were plotted. Percent of cell survival in DMSO (0.5% as negative control) was assumed 100.

Statistical analysis

To perform statistical tests, SIGMASTAT™ (Jandel Software, San Raphael, CA) was used. Analyze-of-variance followed by Student-Newman-Keuls test was used to see the differences among groups. Significance was assumed at the 5% level.

RESULTS

Preliminary phytochemical studies

Phytochemical analysis of the extracts of leaves of *Z. spina-christi* is shown in the Table 1. The results show the presence of alkaloid, tannin, flavonoid and saponin. However, cardiac glycosides and anthraquinone were not detected.

Cytotoxic effect of the extracts of *Z. spina-christi*

The standard curves for Hela and MDA-MB-468 cell lines showed a good linear relationship between absorbance and the number of cells (r^2 = 0.979 and 0.980 respectively) [Figures 1 and 2]. Doxorubicin (20 μg/ml), a known cytotoxic antibiotic, as a positive control significantly inhibited the growth of Hela and MDA-MB-468 cell lines to less than 25%. Extracts were considered cytotoxic when cell viability decreased to less than 50%.

The cytotoxic effect of different extracts of *Z. spina-christi* on Hela and MDA-MB-468 cells were evaluated using MTT-based cytotoxicity method. Hexane, chloroform, chloroform-methanol, butanol, methanol-water and aqueous extracts of *Z. spina-christi* significantly and concentration-dependently reduced viability of Hela and MAD-MB-468 cells ($P < 0.05$, Figures 3 and 4). In the both cell lines, chloroform-methanol extracts of *Z. spina-christi* were more potent than the other extracts.

DISCUSSION

Cancer is one of the most important causes of death in many countries. During the last decades, scientists have paid more attention in discovery of new anticancer drugs. Different classes of anticancer drugs are used for cancer treatment among which cytotoxic drugs are very important. Due to the presence of various complex chemical substances such as alkaloids, flavonoids, terpenoids, saponin and phenolic compounds in plants, they are important sources for cytotoxic agents. It has been shown that more than 3,000 species of plants contain anticancer compounds.[7] It has been shown that some species of *Ziziphus* possess anticancer properties.[2]

In this study, we studied the cytotoxic effects of different extracts of *Z. spina-christi* on Hela and MDA-MB-468 tumor cell lines, using MTT assay. Hela and MDA-MB-468 are two well known tumor cell lines, which have been widely used for evaluation of cytotoxicity.[22-25] Standard curves for Hela and MDA-MB-468 cells were plotted that showed good relationship between absorbance and number of cell. Doxorubicin, a known cytotoxic drug, significantly

Table 1: Phytochemical analysis of the leaves of *Z. spina christi*

	Type of test	Control	*Z. spinaa christi*
Alkaloids	Wagner	*Datura metel*	+
	Mayer	*Datura metel*	+
Cardiac glycosides	Baljet	*Digitalis nervosa*	-
	Cad	*Digitalis nervosa*	-
Tannins	Ferric chloride	*Thea scinesis*	++
Flavonoids	Wilson-Tabuk	*Crataegus curvisepela*	+
Saponins	Foam test	*Ziziphus spina* Christi	+++
Anthraquinones	Borntrager's	*Cassia ovata*	-

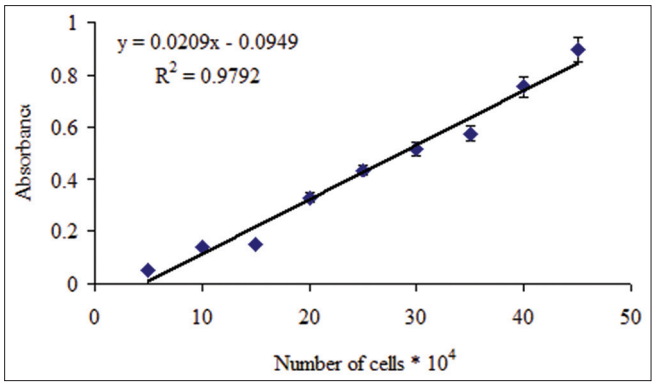

Figure 1: Relationship between absorbance and number of Hela cells Number of viable cells was assessed using MTT assay. Absorbance was determined at 540 nm. Data are presented as mean ± SD, $n = 5$

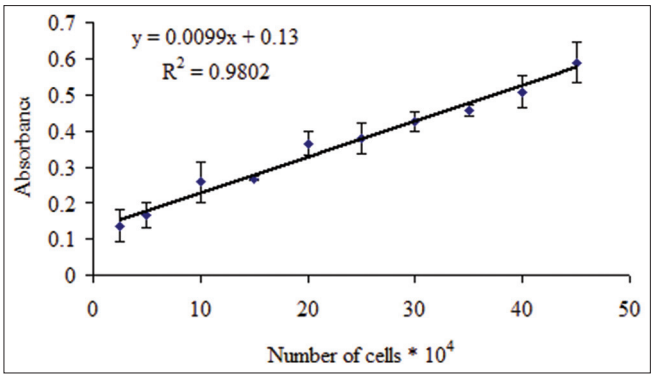

Figure 2: Relationship between absorbance and number of MDA-MB-468 cells Number of viable cells was assessed using MTT assay. Absorbance was determined at 540 nm. Data are presented as mean ± SD, $n = 5$

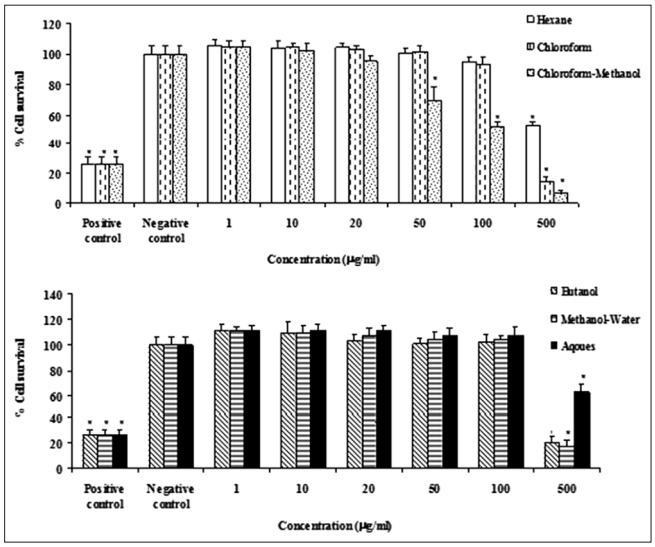

Figure 3: The effects of different extracts of *Ziziphus spina-christi* on Hela cells viability of the cells was determined by MTT assay. Percent cell survival in the control group was assumed 100. (a) hexane, chloroform and chloroform-methanol extracts. (b) Butanol, methanol-water and aqueous extracts. * = $P < 0.05$, $n = 3$

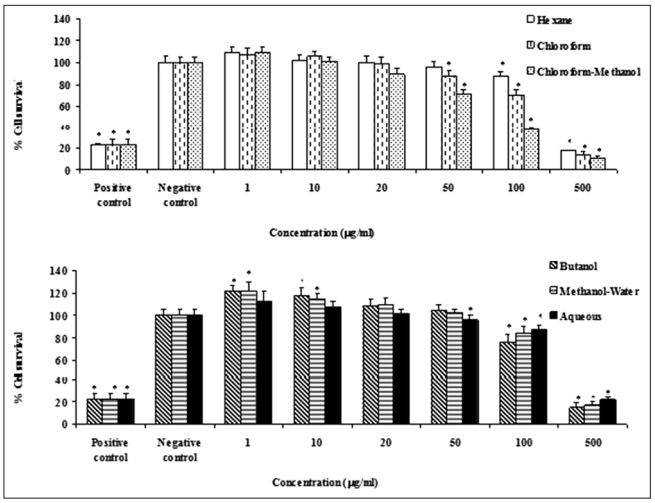

Figure 4: The effects of different extracts of *Ziziphus spina-christi* on MDA-MB-468 cells viability of the cells was determined by MTT assay. Percent cell survival in the control group was assumed 100. (a) hexane, chloroform and chloroform-methanol extracts. (b) Butanol, methanol-water and aqueous extracts. * = $P < 0.05$, $n = 3$

reduced the viability of the both cell lines indicating the validity of the method employed. In order to fractionize compounds with different polarities in this plant, chloroform, chloroform-methanol (9:1), butanol, methanol, methanol-water (7-1) and water were used for extraction. All tested extracts showed significant cytotoxicity against Hela and MDA-MB-468 cell.

However, they were more effective against MDA-MB-468 cells. Several studies have shown that susceptibility of different tumor cell lines to cytotoxic agents is different.[22,26] The order of potency of the extracts against Hela cells was as follow: Chloroform-methanol > chloroform > methanol-water = butanol > hexane >> aqueous. For MDA-MB-468 cells the pattern was: Chloroform-methanol > chloroform = butanol > hexane > aqueous > methanol-water. The cytotoxic effects of chloroform-methanol extract of *Z. spina-christi* may be associated to the presence of triterpenoid saponin glycoside, cyclopeptide alkaloids such as spinanine A, flavonoides such as rutnine and quercetin.[18,27] Also, the better extraction of alkaloids such as jubanin A, amphibian H and spinanine A may explain the better effect of chloroform and chloroform-methanol extracts of *Z. spina-christi* on both tested cell lines.[28] It hase been shown that some alkaloids possess good cytotoxic activity against different cell lines[29,30] The cytotoxic effect of butanolic extract could be explained by the presence of saponin such as betulic acid in this extract.[31]

From the findings of this study it can be concluded that *Z. spina-christi* contain compounds with cytotoxic activity which makes it a promissing candidate for

further study to get new cytotoxic agents.

ACKNOWLEDGMENT

This study was supported by a grant from the Research Council of Isfahan University of Medical Sciences, Isfahan, Iran.

REFERENCES

1. Available from: http://www.who.int/mediacentre/factsheets/fs297/en [Last accessed on 2013].
2. Meghna RA, Narshimha R, Minoo HP. Anti-tumor activity of four ayurvedic herbs in Dalton lymphoma ascites bearing mice and their short-term *in vitro* cytotoxicity on DLA-cell-line. Afr J Tradit Complement Altern Med 2008;5:409-18.
3. Hoffman EJ. Cancer and the search for selective biochemical inhibitors. Washington DC: CRC Press; 1999. p. 2-18, 57-96.
4. Evans WC. Trease and Evans Pharmacognosy. 15th ed. London: Sounders; 2002. p. 394-406.
5. Kaur R, Kapoor K, Kaur H. Plants as a source of anticancer agents. J Nat Prod Plant Resour 2011;1:119-24.
6. Cragg GM, Newman DJ. Antineoplastic agents from natural sources: Achievements and future directions. Expert Opin Investig Drugs 2000;9:2783-97.
7. Graham JG, Quinn ML, Fabricant DS, Farnsworth NR. Plants used against cancer-an extension of the work of Jonathan Hartwell. J Ethnopharmacol 2000;73:347-77.
8. Srivastava V, Negi AS, Kumar JK, Gupta MM, Khanuja PS. Plant-based anticancer molecules: A chemical and biological profile of some important leads. Bioorg Med Chem 2005;13:5892-908.
9. Mahboobi S, Sellmer A, Beckers T. Development of tubulin inhibitors as antimitotic agents for cancer therapy. Studies Nat Prod Chem 2006;33:719-50.
10. Höfle G, Bedorf N, Steinmetz H, Schomburg D, Gerth K, Reichenbach H. Epothilone A and B – novel 16-membered macrolides with cytotoxic activity: Isolation, crystal structure and conformation in solution. Angew Chem Int Ed Engl 1996;35:1567-9.
11. Mervat MA, Gendy EL. *In vitro*, evaluation of medicinal activity of egyptian honey from different floral sources as anticancer and antimycotic infective agents. J Microbial Biochem Technol 2010;2:118-23.
12. Fernanda B, Daniela J. Homoharringtonine and related compounds. Toxicon 2005;45:459-66.
13. Cragg GM, Kingston DG. Newman D. Anticancer agents from natural products. Boca Raton, Florida: Brunner-Routledge Psychology Press, Taylor and Francis Group; 2005. p. 47-70.
14. Abdel-Wahhab MA, Omara EA, Abdel-Gali MM, Hassan NS, Nada SA, Saeed A, *et al*. A Zizyphus Spina-Christi extract protects against aflatoxin B_1-Intitiated hepatic carcinogenicity. Afr J Tradit Complement Altern Med 2007;4:248-56.
15. Shahat AA, Pieters L, Apers S, Nazeif NM, Abdel-Azim NS, Berghe DV, *et al*. Chemical and biological investigations on *Zizyphus spina-christi* L. Phytother Res 2001;9:593-7.
16. Amal H, Eman IA. Effect of *Zizyphus* leaves extract on mice suffering from ehrlich ascites carcinoma. Nature Science 2010;8:234-44.
17. Saied SA, Gebaur J, Hammer K, Buerkert A. *Ziziphus spina-christi* (L.) Willd: A multipurpose fruit tree. Genet Rosour Crop Evol 2008;55:929-37.
18. Anthony C. Dweck FLS FRSC FRSH – Technical Editor. A review of *Zizyphus spina-christi*. Personal Care 2005.
19. Sudhersan C, Hussain J. *In vitro* clonal propagation of a multipurpose tree, *Ziziphus spina-christi* (L.) Desf. Turk J Bot 2003;27:167-71.
20. Trease GE, Evans WC. A textbook of pharmacognosy. London: Bailliere Tindall and Company Publishers; 1983. p. 343-83.
21. Sofowora A. Medicinal plants and traditional medicine in Africa. Ibadan: Spectrum Books Ltd; 1993. p. 150.
22. Jafarian A, Emami SA, Saeidi M, Sadeghi H. Cytotoxicologic studies of the extract of iranian juniperus sabina and platycladus orientalis on cance cells. J Res Med Sci 2004;9:7-11.
23. Sadeghi-Aliabadi H, Emami A, Saidi M, Sadeghi B, Jafarian A. Evaluation of *in vitro* cytotoxic effects of *Juniperus foetidissima* and *Juniperus sabina* extracts against a panel of cancer cells. Iran J Pharm Res 2009;8:281-6.
24. Wang X, Yu B, Wu Y, Lee RJ, Lee LJ. Efficient down-regulation of CDK4 by novel lipid nanoparticle-mediated siRNA delivery. Anticancer Res 2011;31:1619-26.
25. Papanastasiou AD, Sirinian C, Kalofonos HP. Identification of novel human receptor activator of nuclear factor-kB isoforms generated through alternative splicing: Implications in breast cancer cell survival and migration. Breast Cancer Res 2012;14:R112.
26. Monajemi R, Oryan S, Haeri-Roohani A, Ghannadi A, Jafarian A. Cytotoxic effects of essential oils of some Iranian Citrus peels. Iran J Pharm Res 2005;3:183-7.
27. Pawlowska AM, Camangi F, Bader A, Braca A. Flovonides of *Zizyphus jujuba* L. and *Zizyphus spina-christi* (L.) Willd (Rhamnaceae) fruits. Food Chem 2009;2:585-862.
28. AbDEl-Galil FM, Jissry MA. Cyclopeptidealkaloids from *Z.spina-christi*. Phytochemistry 2001;3:1348-9.
29. Guo LL, He HP, Di YT, Li SF, Cheng YY, Yang W, *et al*. Indole alkaloids from ervatamia chinensis. Phytochemistry 2012;74:140-5.
30. Chanakul W, Tuchinda P, Anantachoke N, Pohmakotr M, Piyachaturawat P, Jariyawat S, *et al*. Cytotoxic alkaloids from stems, leaves and twigs of *Dasymaschalon blumei*. Fitoterapia 2011;82:964-8.
31. Benammar C, Hichami A, Yessoufou A, Simonin A, Belarbi M, Allali H, *et al*. Zizyphus lotus L. (Desf.) modulates antioxidant activity and human T-cell proliferation. BMC Complement Altern Med 2010;10:54.

Source of Support: Research Council of Isfahan University of Medical Sciences, Isfahan, Iran. **Conflict of Interest:** None declared.