Original Article

Evaluation of Cytotoxic Effect of Different Extracts of *Seidlitzia rosmarinus* on HeLa and HepG2 Cell Lines

Behzad Zolfaghari, Abbas Jafarian[1], Moein Rezaei[1]

From the Department of Pharmacognosy, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, [1]Department of Pharmacology and Toxicology, School of Pharmacy and Pharmaceutical Sciences and Isfahan Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: *Seidlitzia rosmarinus* which is commonly called "Oshnan" or "Eshnan" in Persian belongs to *Chenopodiaceae* family. Conventionally, it is believed that this plant is toxic. This study was aimed to evaluate the cytotoxic effect of *S. rosmarinus* against HeLa and $HepG_2$ cell lines. **Materials and Methods:** *S. rosmarinus* was collected from the desert near Yazd, Iran. Hexane, chloroform, chloroform/methanol (9:1), and butanol extracts of aerial parts of *S. rosmarinus* were prepared. Doxorubicin and dimethyl sulfoxide 10% were used as positive and negative control, respectively. The cytotoxic activity was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **Results:** All extracts significantly and concentration dependently reduced viability of HeLa and HepG2 cells. Hexane, chloroform, and butanol extracts at doses of 200, 500, 750, and 1000 μg/ml significantly reduced HeLa cell viability ($P < 0.05$). Chloroform/methanol extract at doses of 100–500 μg/ml significantly reduced HeLa cell viability ($P < 0.05$). Hexane, chloroform, and butanol extracts at doses of 500, 750, and 1000 μg/ml significantly reduced HepG2 cell viability ($P < 0.05$). Chloroform/methanol extract at doses of 200, 300, 400, and 500 μg/ml significantly reduced HepG cell viability ($P < 0.05$). The most cytotoxic extract was chloroform/methanol extract in both cell lines. Furthermore, in the both cell lines, the second potent extract was chloroform extract. **Conclusions:** It can be concluded from the findings of this study that *S. rosmarinus* is a good candidate for further study to find new cytotoxic agents. Phytochemical investigation on chloroform/methanol extract and their structures is recommended.

Keywords: *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, cytotoxicity, Seidlitzia rosmarinus*

Introduction

Cancer is a term used for a large group of diseases that can happen almost anywhere in the human body. Cancer starts because of abnormal cell growth without stopping. They may spread into surrounding tissues and other organs.[1] In 2012, new cancer cases and cancer deaths increased to 14.1 and 8.2 million, respectively.[2] It is expected that in 2020, 20 million new cancer cases will be diagnosed and 12 million cancer-related deaths occur.[2]

It is shown that plants have been used for the treatment of cancer since a long time ago. Since the cytotoxic effects of plant compounds, such as biflavonoids, bicoumarins, and biquinones, have been proven, the plants have been considered as an acceptable source for the preparation of drugs.[3,4] In recent years, cytotoxic drugs prepared from *Catharanthus roseus*, *Podophyllum peltatum*, and *Taxus brevifolia* are used in the cancer treatment regimens.[5,6] For this reason, scientists have a special interest in medicinal plants for the discovery of new anticancer drugs.

Seidlitzia rosmarinus which is commonly called "Oshnan" or "Eshnan" in Persian belongs to *Chenopodiaceae* family. It is a perennial shrub with white branches, height about 1.5 m, articulated stem, fleshy leaves and cylindrical, a spiral embryo, horizontal grain, winged perianth surrounding the fruits, five stamens flowers, that grow in the salt plains, and generally dispersed in the Persian Gulf countries and Central and Eastern deserts of Iran.[7,8] Conventionally, it is believed that *S. rosmarinus* is a toxic plant. And, it is used as diuretic and laxative.[9-11]

This study was aimed to evaluate the cytotoxic effect of *S. rosmarinus* against HeLa and HePG2 cell lines.

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

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Materials and Methods

Materials

Compounds used were methanol, n-butanol, chloroform, n-hexane, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), NaCl, KCl, NaOH, HCl, H_2SO_4, $NaHCO_3$, Na_2HPO_4 (Merck, Germany), penicillin/streptomycin (Sigma, USA), RPMI-1640, fetal calf serum (FCS), sodium pyruvate, trypsin, L-glutamine (Gibco, Scotland), dimethyl sulfoxide (DMSO) (Fluka, Italy), and doxorubicin (Farmitalia, Italy).

Plant materials

Aerial parts of *S. rosmarinus* were collected from Aghda desert near Yazd, one of the central provinces of Iran, on November 2011. They were identified by Mahboobeh Khatam Saaz as *S. rosmarinus*. A voucher specimen (No. 3532) was deposited at the Department of Pharmacognosy, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences.

Preparation of extracts

100 g of aerial parts of *S. rosmarinus* was finely powdered, and extraction was done by a four-step method. Extraction was started with nonpolar solvent and went forward to completely polar solvent. Extracts were prepared by maceration method using n-hexane, chloroform, chloroform/methanol (9:1), and methanol solvents. Each step lasted 1 day while stirring. To prepare butanol extract, methanol was removed from the filtered methanol extract by evaporating under reduced pressure in a rotoevaporator until two-thirds of the initial volume was removed. Using a separatory funnel, the remaining aqueous extract was partitioned with n-butanol, 1:1 (v: v) overnight at room temperature. The n-butanol extract was collected. After filtration, the resulting extracts were freeze-dried. Then, they were stored at 4°C until use.[12]

Cell lines

HeLa and HepG2 Cell lines were purchased from Pasture Institute, Tehran, Iran. RPMI-1640 medium (supplied with 10% FCS) as used for cell maintenance.

Determination of saponin content

The assay was performed based on erythrocyte lysis in 96-well microtiter plates (Nunc, Wiesbaden, Germany). Briefly, 120 µl of erythrocyte suspension (10^7 cells/ml) and 120 µl of different concentrations (100, 500, and 1000 µg/ml) of *S. rosmarinus* extracts were added to each well. Erythrocytes were incubated in assay buffer alone, and 100% lysis served as negative and positive controls, respectively. They were sonicated for 30 s on ice at the same settings as for *S. rosmarinus* extracts. Each set of samples was pipetted in triplicate. Microtiter plates were placed on the ice-cold surface of a metal block. Finally, microtiter plates were taped with sealing foil and incubated at 15°C in the dark for 3 h. Following incubation, microtiter plates were centrifuged for 5 min at 2000 g at room temperature, and 200 µl of each supernatant was transferred to a 96-well microtiter plate, and its absorption was read at 540 nm.[13]

Preparation of stock solution of extracts

To prepare stock solutions, the extracts of *S. rosmarinus* were dissolved in DMSO, and then the proper amount of distilled water was added. The maximum amount of DMSO was 10% at stock and 1% at the final concentration in the wells.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

MTT assay was used to measure the rate of cell proliferation. This assay is based on the reduction of MTT to a water-insoluble crystal called formazan. The amount of formed formazan is directly related to the number of viable cells. Subsequently, formazan crystals were dissolved in DMSO and measured spectrophotometrically at 540 nm using micro-enzyme-linked immunosorbent assay (ELISA) reader.[14,15] 96-well microplates were seeded with 180 µl of cell suspension (5×10^4 cells/ml) except the first row which loaded with 200 µl of RPMI-1640 medium, considered as blank. Then, they incubated for 24 h (37°C, 5% CO_2 air humidified). After that, 20 µl of each concentration of *S. rosmarinus* extracts was added and incubated for 48 h. After that, 20 µl of MTT solution (with concentration of 5 mg/ml) was added to each well and incubated for 3 h. The medium and MTT discarded and 150 µl DMSO was added to each well to solve formazan crystals. Then, the absorbance was measured by micro-ELISA reader. Doxorubicin 20 µg/ml and DMSO 1% were used for positive and negative controls, respectively. The test was done in tetraplicate and repeated in three different days. Standard curves (absorbance against number of cells) for each cell line were plotted. Intraday and interday variations were determined. Based on the standard curves, the percentage of cell survival was calculated. The percentage of cell survival in DMSO (0.5% as a negative control) was assumed 100.

Statistical analysis

SigmaStat™ (Jandel Software, San Raphael, CA) was used to perform statistical tests. Analysis of variance followed by Student–Newman–Keuls test was used to observe the differences among groups. Significance was assumed at the 5% level.

Results

Using maceration method for extraction, the extract yields of dried masses after evaporation and solvent removal of hexane, chloroform, chloroform/methanol, and butanol extracts of *S. rosmarinus* were 0.35%, 1.2%, 1.48%, and 0.8%, respectively.

A standard curve was set using 100–500 mg/ml of yucca saponin [Figure 1]. Comparison of the absorbance of erythrocyte in the presence of different concentrations of the extracts of *S. rosmarinus* is shown in Table 1.

Standard curves and cell growth curves for HeLa and HepG2 cells are shown in Figures 2 and 3. A good relationship between absorbance and the number of cells was observed for HeLa and HepG2 cell lines (r^2 = 0.981 and 0.982, respectively). Intraday and interday variations for all standard curves were acceptable (%CV <15). Doxorubicin (20 µg/ml), a known cytotoxic antibiotic, as a positive control significantly inhibited the proliferation of both cell lines to <25%. Extracts were considered cytotoxic when cell viability decreased to <50%.

Cytotoxic effects of *Seidlitzia rosmarinus* extracts against HeLa and HepG2 cells

All of the extracts of *S. rosmarinus* significantly and concentration-dependently decreased the viability of HeLa [Figure 2]. Chloroform/methanol extract showed better toxicity. Chloroform and chloroform/methanol extracts of *S. rosmarinus* significantly and concentration-dependently decreased the viability of HepG2 cells at concentration above 200 µg/ml [Figure 3]. Hexane and butanol extracts significantly decreased viability of HepG2 cells at concentrations above 500 µg/ml. HeLa cells were more susceptible to hexane, chloroform, and butanol extracts of *S. rosmarinus* compared to HepG2 cells.

Table 1: Absorbance of erythrocyte in the presence of different concentrations of the extracts of *Seidlitzia rosmarinus*

Type of extract	Concentration (µg/ml)	Absorbance
Butanol	100	1.64±0.04
	500	1.57±0.03*
	1000	1.54±0.04*
Chloroform	100	1.63±0.05
	500	1.60±0.04*
	1000	1.59±0.03*
Hexane	100	1.65±0.03
	500	1.52±0.03*
	1000	1.50±0.04*
Chloroform/methanol	100	1.62±0.04
	500	1.57±0.04*
	1000	1.55±0.05*
Control		1.71±0.03

P<0.05

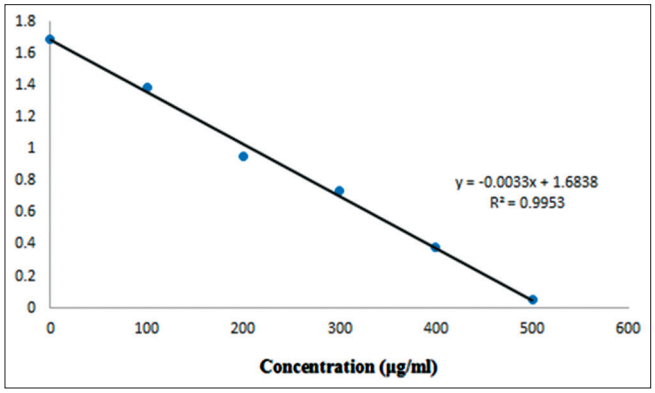

Figure 1: Relationship between absorbance of erythrocyte in the presence of different concentrations of yucca saponin. Absorbance was determined at 540 nm. Data are presented as mean ± standard deviation, *n* = 9

Discussion

An important class of compounds for the treatment of cancer is cytotoxic drugs. Although the search for anticancer agents goes back to more than 3000 years ago, in the last two decades, more attention has been paid to plants, as potential sources of new cytotoxic compounds. More than 3000 plant species have been used in the treatment of cancer.[1,16] It has been shown that some of *Chenopodiaceae* family's plants have antitumor activities. Wang *et al.* showed that *Salicornia bigelovii* Torr. (*Chenopodiaceae* family) has two new nortriterpenoid saponins with cytotoxic activities.[17] *Climacoptera obtusifolia* is another plant from *Chenopodiaceae* family with cytotoxic effect.[17,18] Several studies have indicated that saponins possess cytotoxic and antitumor activity.[1-25]

Conclusion

It has been shown that two compounds isolated from *Suaeda glauca* (*Chenopodiaceae*) had protective effects

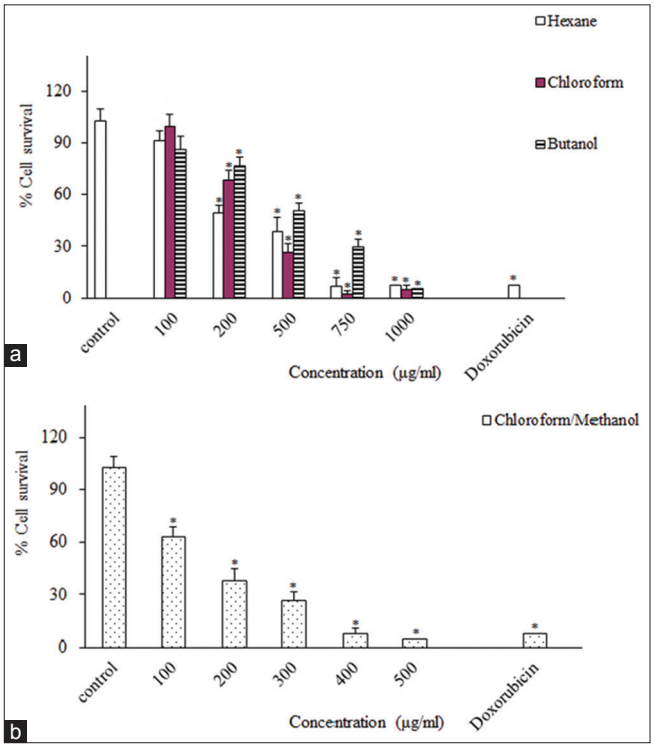

Figure 2: The effects of different extracts of *Seidlitzia rosmarinus* on HeLa cells. Viability of the cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. The percentage cell survival in the control group was assumed 100. (a) hexane, chloroform and butanol extracts. (b) Chloroform/methanol extracts. **P* < 0.05, *n* = 3

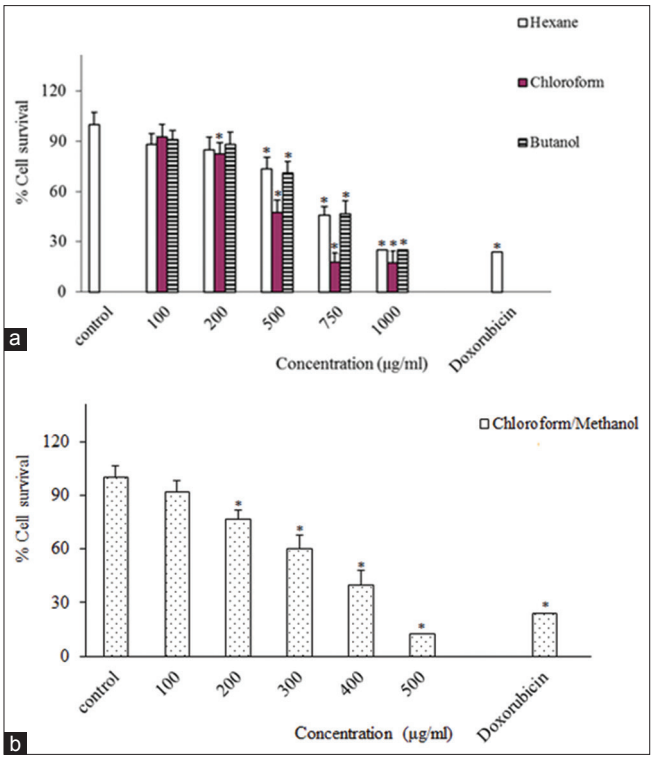

Figure 3: The effects of different extracts of *Seidlitzia rosmarinus* on HepG2 cells. Viability of the cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. The percentage cell survival in the control group was assumed 100. (a) Hexane, chloroform, and butanol extracts. (b) chloroform/methanol extracts. *$P < 0.05$, $n = 3$

on tacrine-induced cytotoxicity in human liver-derived HepG2 cells with the EC50 values of 72.7 ± 6.2 and 117.2 ± 10.5 µm, respectively.[26] Calzada *et al*. isolated five flavonoids (pinocembrin, pinostrobin, chrysin, narcissin, and rutin) from *Teloxys graveolens* (*Chenopodiaceae*), in which two of them (pinocembrin and chrysin) exhibited cytotoxic activity against *E. histolytlica* with inhibitory concentration 50% (IC50) of 82 µg/ml. Furthermore, pinostrobin and narcissin exhibited IC50 of 185 and 17.2 µg/ml, respectively.[27] Considering the toxicity of the plants belong to the *Chenopodiaceae* family and traditional use of this plant,[8-10] we decided to determine cytotoxic activity of the extracts of aerial parts of *S. rosmarinus* against HeLa and HepG2 cell lines using MTT assay.

Doxorubicin, a known cytotoxic agent,[28] as a positive control significantly decreased viability of HeLa and HepG2 cells, indicating the accuracy of the method employed in this experiment ($P < 0.05$).

Our findings indicated that extracts of aerial parts of *S. rosmarinus* significantly and concentration-dependently decreased viability of HeLa and HepG2 cells. Chloroform/methanol extract showed more potency than the other extracts to inhibit cell proliferation, with IC50 of 150 and 350 µg/ml for HeLa and HepG2 cells, respectively. Several studies have been done on sapogenins, and they found to be cytotoxic.[29-31] However, as there was no considerable difference in saponin contents of different extracts of *S. rosmarinus* used in this study, other components of this plant should be responsible for its cytotoxicity of it. Therefore, more phytochemical investigation on chloroform/methanol extract is needed to find the structure of cytotoxic components of *S. rosmarinus*.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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