SUPPLEMENTARY MATERIAL

Investigation of *In Vitro* Cytotoxic, Mutagenic and Anti-Mutagenic Effects of Shirazolide Extracted from *Jurinea leptoloba*

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Shirazolide is an elemanolide isolated from *Jurinea leptoloba* DC. The aim of this study was to determine the cytotoxic, mutagenic and anti-mutagenic properties of shirazolide from *J. leptoloba* DC *in vitro*. Cytotoxicity was measured using a modified MTT (3-(4,5-di methyl thiazol-2-yl)-2,5-di phenyltetrazolium bromide) assay on normal human lymphocytes and tumor HeLa cells, showing that the cytotoxicity of shirazolide is much higher for HeLa cells than for normal lymphocytes. Mutagenic and anti-mutagenic activities of shirazolide were evaluated using the *Salmonella typhimurium* tester strains TA98 and TA100 showing anti-mutagenic properties against the former strain under metabolic activation.

Keywords: Shirazolide; Cytotoxicity; Anti-mutagenic; *Salmonella typhimurium*

Experimental

Plant material

Aerial parts of *J. leptoloba* were collected in August 2011 south of Shiraz, Iran. The voucher number was 324R, deposited at the herbarium of the Department of Botany, Shahid Beheshti University, Tehran, Iran.

Extraction and isolation

Air dried aerial parts of *J. leptoloba* (550 g) were extracted twice with 6 L each of diethylether/methanol/petroleum ether (1:1:1) by immersion in the solvent at room temperature for 72 h. The extract obtained was evaporated to dryness (86 mg), defatted with methanol (46 mg) and separated by column chromatography over silica gel. The dimensions of the chromatographic column were 1200 × 30 mm. Silica gel 70-230 mesh ASTM (merck 774) was used for column chromatography. Silica gel was thoroughly mixed with hexane and was packed into a column. After the column was packed with Silica gel, a slurry of the extract/silica gel and hexane was prepared, and the slurry was very carefully added to the column. The column was eluted with 360 mL each of hexane / diethyl ether and petroleum ether (1:3) / methanol, respectively to give 24 fractions. The polarity of the mobile phase was increased by changing the ratio of non-polar to polar solvents. The fractions obtained with diethyl ether and petroleum ether (1:3) were evaporated to dryness (28 mg). The residue was rechromatographed (Column dimensions: 800 × 20 mm) on silica gel (230-400 mesh ASTM from merck) with 180 mL each of hexane / diethyl ether and petroleum ether (1:3) / methanol, to give 10 fractions. IR spectrum were taken from all fractions. The most obvious characteristics of shirazolide are three signals that are related to carbonyl groups in the IR spectrum [1775 nm (γ -lactone); 1725 nm (C=O ester); 1710 (C=O)]. Fraction 5 showed signals related to shirazolide. Fraction 5 was rechromatographed (Column dimensions: 800 × 20 mm) on silica gel (230-400 mesh ASTM from merck) with 180 mL of the same solvents to give 6 fractions. ¹H-NMR spectrum were taken from all fractions. Fractions 3 and 4 afforded shirazolide. The structure of shirazolide was elucidated by FT-IR (Shimadzu, Japan) and 500 MHz ¹H-NMR (Brucker, Germany) (Figure S1).

Cytotoxicity assay

The human cervical carcinoma HeLa cell line, NCBI code No. 115 (ATCC number CCL-2), and the human lymphocyte cell line NCBI, code No. 124 (ECACC number 91112124) were obtained from Pasteur Institute (Tehran, Iran). The HeLa cells were grown in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10 % fetal bovine serum (FBS), 1 % (w/v) glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, were grown in RPMI 1640 supplemented with 10 % FBS, 1 % (w/v) glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were cultured in a humidified atmosphere (90-95% relative humidity) at 37°C in 5 % CO₂ in air. Cytotoxicity was measured using a modified cell viability assay for cancer cells and normal human lymphocytes with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Allahghadri et al., 2010; Lau et al. 2004). Briefly, the cells (5×10^4) were seeded in a 96-well plate, each well containing 100 µL of RPMI medium supplemented with 10 % FBS, 1 % (w/v) glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. After 24 h of adhesion, a serial of doubling dilution of the sample was added in triplicate to wells over the range of 1.0-0.005 µL/mL. The final concentration of ethanol in the culture medium was maintained at 0.5 % (volume/volume) to avoid toxicity of the solvent (Sylvestre et al., 2005). After 2 days, 10 µL of a solution (of 5 mg/mL MTT) in PBS (Phosphate buffered saline) was added, and the plates were incubated for an additional 4 h. The medium was discarded, and the formazan blue formed was dissolved with 100 µL dimethyl sulphoxide (DMSO) and the optical density measured at 490 nm using a microplate reader. The cell viability curves were calculated taking into account control cells incubated in the presence of 0.5 % ethanol alone. Cytotoxicity was expressed as the concentration of drug inhibiting cell growth by 50 % (IC₅₀).

Preparation of metabolic activation system (S9 Mixture)

The S9 metabolic activator was prepared just before use by combining 500 µL phosphate buffer (0.2 mol/L), 130 µL deionized water, 100 µL aqueous KCl (0.33 mol/L) solution, 80 µL aqueous MgCl₂ (0.1 mol/L) solution, 100 µL S9 fraction (Moltox, Boone, NC), 50 µL glucose-6-phosphate (0.1 mol/L) and 40 µL NADP (0.1 mol/L).

Toxicity determination

The concentration of sample was selected based on a preliminary toxicity test by *S. typhimurium* strains TA100 and TA98 with and without S9. For the toxicity test, 12 mL of nutrient agar and 0.50 mL of metabolic activation (S9) mix or buffer (Phosphate buffer 0.2M, pH 7.4), 0.01 mL of the test chemical dilution and 0.1 mL of an overnight culture of the *Salmonella* strain were then added in tubes. The contents of the test tubes were then mixed and poured onto the surface of Glucose minimal agar plates. (The Glucose minimal agar, consisting of 1.5 % agar, 0.02 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 % Citric acid, 1 % K_2HPO_4 , 0.35 % $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$ and 2 % Glucose). The plates were inverted and placed in a 37°C incubator for 48 h. The colonies were then counted, and the results were expressed as the number of revertant colonies per plate. Comparisons of bacterial counts on test compound plates with bacterial counts on control plates were used to determine growth inhibition (Mortelmans and Zeiger, 2000). Experiments were performed in triplicate.

Mutagenicity and anti-mutagenicity test

Mutagenic activity was evaluated by the *Salmonella*/microsome assay, using the *Salmonella typhimurium* tester strains TA98 and TA100, with (+S9) and without (-S9) metabolism, using the pre-incubation method (Maron and Ames, 1983). It is important that the same number of bacteria is used in the preliminary toxicity assay as well as in the definitive mutagenicity assay (Mortelmans and Zeiger, 2000).

Salmonella cultures were inoculated 15-18 h prior to performing the experiment. Top agar melt supplemented with 0.05 mM histidine and biotin was maintained at 43°C to 48°C. To the 13×100 mm sterile glass tubes maintained at 43°C, add in the following order with mixing after each addition. Each test was performed using a single batch of reagents, media and agar (Mortelmans and Zeiger, 2000). The top agar, consisting of 0.6 % agar and 0.6 % NaCl, is one of the most critical medium components in the Ames test because it contains the trace amount of histidine (0.05 mM) for limited growth. It also contains biotin at a concentration of 0.05 mM which is in excess of what is needed for the growth of the *Salmonella* strains. Using pre-incubation, we studied the effect of metabolic activation. In condition without metabolic activation, 0.01 mL of each concentration of test ingredient, negative control or positive control was added to 0.5 mL of 0.1 M phosphate buffer (pH 7.4) and 0.1 mL of each *Salmonella* strain (approximately $1/6 \times 10^6$ cells/mL), and then incubated at 37°C for 20 min. After shaking incubation, 2 mL of top agar

was added to the incubation mixture according to the strains and then poured on to a plate containing minimal agar. The plates were incubated at 37°C for 48 hours, and the revertant colonies were counted manually. In the presence of metabolic activation, 0.5 mL of freshly prepared S9 mix instead of 0.1 M phosphate buffer (pH 7.4) were added to the incubation mixture. Other procedures were performed in the same way (Mortelmans and Zeiger, 2000). All experiments were performed in triplicate. After incubation at 37°C for 48 hours, colonies were counted and the results expressed as the number of revertant colonies per plate. The standard mutagens used as positive controls in experiments without the S9 mix were 2-nitrofluorene for TA98, sodium azide for TA100. In experiments with S9 activation, 2-aminoanthracene was used with TA98 and TA100. DMSO served as negative (solvent) control (Mortelmans and Zeiger, 2000). The percentage of mutations was calculated using the following formula:

$$(T/M)*100$$

T: The number of revertant colonies in the presence of shirazolide

M: The number of revertant colonies in the presence of mutagen

The number of colonies that had been grown up was deducted from the numerator and denominator.

Shirazolide: IR $\nu_{\max}^{CHCl_3}$ cm^{-1} : 1775 (γ -Lactone); 1725 (C=O ester); 1710 (C=O). $^1\text{H-NMR}$ Spectral result: δ = 5.81, dd (H_1); δ = 5.34, d (H_2); δ = 5.31, d ($\text{H}_{2'}$); δ = 5.36, s (H_3); δ = 5.28, s ($\text{H}_{3'}$); δ = 3.02, dd (H_5); δ = 3.71, dd (H_6); δ = 2.91, dddd (H_7); δ = 5.27, ddd (H_8); δ = 3.03, dd (H_9); δ = 2.46, dd ($\text{H}_{9'}$); δ = 6.18, d (H_{13}); δ = 5.61, d ($\text{H}_{13'}$); δ = 4.98, dt (H_{15}); δ = 4.87, dt ($\text{H}_{15'}$); δ = 6.17, qq ($\text{H}_{3'}$); δ = 2.03, dq (H_4); δ = 1.90, dq (H_5).

Figure S1. FT-IR and $^1\text{H-NMR}$ Spectral data of Shirazolide

Table S1. Cytotoxicity assay of shirazolide on HeLa and lymphocyte cells.

Shirazolide Dilutions (µg/mL)	% Viable HeLa cells	% HeLa cells Death
control	100	0
7	45.69 ± 5.08	54.30
14	34.21 ± 8.61	65.78
28	18.49 ± 7.19	81.50
IC ₅₀ (µg/mL)	2.80	

Shirazolide Dilutions (µg/mL)	% Viable Lymphocyte cells	% Lymphocyte cells Death
control	100	0
1400	71.58 ± 3.08	28.41
2800	66.58 ± 2.61	33.41
5600	60.06 ± 8.17	39.93
IC ₅₀ (µg/mL)	9202.22	

Table S2. Percent of mutagenicity (M) and anti-mutagenicity (A) of shirazolide to *S. typhimurium* (TA98, TA100) with and without S9.

	Dilution (mg/plate)	Percent of mutagenicity (M)			
		TA 100 M-S9	TA 100 M+S9	TA 98 M-S9	TA 98 M+S9
Shirazolide	1.2	68.37 ± 0.83	30.00 ± 2.04	58.69 ± 1.77	8.57 ± 1.17
		Percent of anti-mutagenicity (A)			
	1.2	TA 100 A-S9	TA 100 A+S9	TA 98 A-S9	TA 98 A+S9
		92.26 ± 1.95	54.17 ± 4.17	27.27 ± 1.13	89.66 ± 0.36