

Cytotoxic Activity and Apoptosis Induction by Gaillardin

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Cytotoxic activity of gaillardin, a sesquiterpene lactone isolated from *Inula oculus-christi* L. (Asteraceae), was assessed in the human breast adenocarcinoma cell line MCF-7, human hepatocellular carcinoma cell line HepG-2, human non-small cell lung carcinoma cell line A-549, and human colon adenocarcinoma cell line HT-29, resulting in IC₅₀ values of 6.37, 6.20, 4.76, and 1.81 µg/mL, respectively, in the microculture tetrazolium-formazan MTT assay. *In vitro* apoptosis-inducing properties of gaillardin were also evaluated in MCF-7 cells with the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. The results suggest gaillardin as a candidate for further studies in cancer therapy

Key words: Gaillardin, TUNEL, MTT Assay

Introduction

Naturally occurring compounds have been reported to prevent tumorigenesis and also to suppress the growth of established tumours (Fulda, 2010). Plants are the most important source of natural products with a broad range of bioactivities. Compounds of herbal origin have proven useful as antitumour agents due to their cytotoxic and apoptosis-inducing activities. Sesquiterpene lactones of the Asteraceae family play an important role in these efforts. Many genera of this family possess cytotoxic and apoptotic activities, among which is the genus *Inula* with various cytotoxic compounds. Pseudoguaianolides and guaianolides from *Inula hookeri* C. B. Clarke (Cheng *et al.*, 2012), britannin from *Inula aucheriana* DC. (Moghadam *et al.*, 2012), bigelovin from *Inula helianthus-aquatica* C. Y. Deng (Zeng *et al.*, 2009), and sesquiterpene lactones from *Inula britannica* L. (Bai *et al.*, 2006) are just few examples of cytotoxic sesquiterpene lactones which have been obtained from different species of *Inula*.

Cytotoxicity of the sesquiterpene lactone gaillardin to mouse fibrosarcoma (WEHI-164) and bovine kidney (MDBK) cells has been previously reported with IC₅₀ values of 15.28 and 11 µg/mL,

respectively (Mosaddegh *et al.*, 2010). The present study reports on the isolation of the sesquiterpene lactone gaillardin from the chloroform extract of *Inula oculus-christi* aerial parts and its cytotoxic activity on human breast adenocarcinoma MCF-7, human hepatocellular carcinoma HepG-2, human non-small cell lung carcinoma A-549, and human colon adenocarcinoma HT-29 cells, as well as the determination of its apoptotic potential in the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. The assay is based on the detection of morphological characteristics which happen during apoptosis, and MCF-7 cells were chosen for the evaluation since changes in these cells can be easily detected using microscopic methods.

Material and Methods

Chemicals and reagents

The *in situ* cell death detection kit, with horseradish peroxidase (POD) (Roche, Mannheim, Germany), was used for assessing apoptosis; Dulbecco's modified Eagles medium (DMEM) and fetal bovine serum (FBS) (Gibco, Auckland, New

Zealand), RPMI 1640 medium, penicillin-streptomycin, MTT [3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide] (Sigma, St. Louis, MO, USA), dimethylsulfoxide (DMSO) (Merck, Hohenbrunn, Germany), Triton X-100 (Sigma), and DAB (diaminobenzidine) (Roche, Indianapolis, IN, USA) were used in cytotoxicity and apoptosis studies.

The solvents and chemicals for isolation of gaillardin were procured from Merck.

Plant material

Inula oculus-christi aerial parts were collected from Golestan province, Iran (June 2009) and were authenticated by Mr. Hamid Moazzeni and Mrs. Atefeh Pirani (botanists), Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran. A voucher specimen is deposited at the TMRC herbarium for future reference.

Extraction

Inula oculus-christi air-dried powder (250 g) was macerated with *n*-hexane (2500 mL) at room temperature for 3 d. Each day the solvent was replaced with fresh solvent. After the third day the residue of the plant material was extracted with chloroform (2500 mL), and the same process continued for 3 d. The concentrated chloroform extract was used for isolation of gaillardin.

Isolation of gaillardin

The isolation of gaillardin followed the procedure of Mosaddegh *et al.* (2010) with some modifications. The chloroform extract (4 g) was chromatographed by vacuum liquid chromatography (VLC): silica gel (40–63 μ m); 1400 mL of mobile phase in each step: *n*-hexane, *n*-hexane/ethyl acetate (3:1, v/v), *n*-hexane/ethyl acetate (2:1), *n*-hexane/ethyl acetate (1:1), ethyl acetate, ethyl acetate/methanol (3:1), ethyl acetate/methanol (2:1), ethyl acetate/methanol (3:1), and methanol. The middle fractions were concentrated (290 mg) and further subjected to solid phase extraction (SPE) on a silica gel column (40–63 μ m; 2.5 x 7.5 cm) eluted with 120 mL of mobile phase in each step: dichloromethane, dichloromethane/ethyl acetate (10:1–1:1), ethyl acetate, ethyl acetate/methanol (3:1), ethyl acetate/methanol (1:1), and methanol.

Gaillardin was collected from the fraction eluted with the mixture of dichloromethane/ethyl acetate, the solvent was evaporated and the resulting crystals were washed with *n*-hexane and ethyl acetate (6:1 and 5:1) to afford 58 mg of gaillardin.

The IR and ¹H NMR spectra of gaillardin were in accordance with previously published data (Mosaddegh *et al.*, 2010; Gonzalez Romero *et al.*, 2001).

Preparation of gaillardin for the MTT assay

Gaillardin was dissolved in DMSO (10 mg/mL) to make a stock solution. Serial dilutions were prepared accordingly from the stock solution to reach the final concentrations (100 μ g/mL, 50 μ g/mL, 25 μ g/mL, 12.5 μ g/mL, 6.25 μ g/mL, and 3.125 μ g/mL, respectively) with DMSO not exceeding 1%.

Cell lines

MCF-7 (human breast adenocarcinoma), HepG-2 (human hepatocellular carcinoma), HT-29 (human colon adenocarcinoma), and A-549 (human non-small cell lung carcinoma) cells were obtained from the Pasteur Institute, Tehran, Iran. MCF-7 cells were maintained in DMEM with 5% FBS, and HT-29 cells were maintained in DMEM with 20% FBS, while the other two cell lines were cultured in RPMI 1640 medium with 10% FBS for optimal growth. All cell lines were treated with 1% penicillin-streptomycin, in a humidified incubator at 37 °C in an atmosphere of 5% CO₂.

MTT assay

The viability of the cells exposed to different concentrations of gaillardin was determined using the MTT colorimetric assay (Alley *et al.*, 1988; Mosaddegh *et al.*, 2010). The cells were seeded in 96-well plates at (per well) 5000 cells for HT-29, 8000 cells for MCF-7, 15000 cells for HepG-2, and 8000 cells for A-549 cells. After 24 h of incubation at 37 °C, the medium was replaced with fresh medium containing different concentrations of gaillardin. After 72 h of exposure of cells at 37 °C to gaillardin, the medium was replaced with fresh medium containing MTT with a final concentration of 0.5 mg/mL. Thereafter the cells were incubated for another 4 h in a humidified atmosphere at 37 °C, the medium containing MTT was then removed, and the remaining formazan crystals were dissolved in DMSO. The absorbance was

recorded at 570 nm with an ELISA reader (TECAN, Salzburg, Austria). Tamoxifen was used as positive control.

To measure the relative viability of the cells (%) related to the negative control (wells without gaillardin) the following formula was applied: relative viability (%) = $[A]_{\text{sample}}/[A]_{\text{control}} \cdot 100\%$, where $[A]_{\text{sample}}$ is the absorbance of a sample treated with gaillardin at a given concentration, and $[A]_{\text{control}}$ is the absorbance of negative control wells (cells in culture medium with 1% DMSO). To calculate IC_{50} values, viability (%) vs. concentrations was plotted by the Microsoft Excel program.

Assessments of apoptosis induction

To detect apoptosis induction in MCF-7 cells, the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) assay (Roche, Mannheim, Germany) was carried out. MCF-7 cells cultured in 96-well plates were treated with gaillardin at 6.25 $\mu\text{g}/\text{mL}$ and incubated for 20 h. The assay was conducted according to the manufacturer's instructions. Briefly, treated MCF-7 cells, which had been blocked by 3% H_2O_2 , were fixed with 4% paraformaldehyde and washed with phosphate-buffered saline (PBS). Cells were then permeabilized using 0.1% Triton X-100. For labeling the fragmented DNA, fluorescein-dUTP, and TdT were added at 37 °C for 1 h. After treating the cells with anti-fluorescein antibody conjugated with horse-radish POD at 37 °C for half an hour, the POD substrate DAB was added (10 min at room temperature). The stained cells were then analysed under a light microscope. Cells receiving only culture medium and 1% DMSO were used as negative control.

Results and Discussion

Various sesquiterpene lactones have been shown to induce apoptosis, such as dehydrocostuslactone from *Saussurea lappa* and *Aucklandia lappa* (Hsu *et al.*, 2009), isodeoxyelephantopin from *Elephantopus scaber*, which inhibits NF- κ B activation and NF- κ B-regulated gene expression (Ichikawa *et al.*, 2006), and vernolide-A from *Vernonia cinerea*, which modulates p53 and caspase-3 gene expression (Pratheeshkumar and Kuttan, 2011). Pratheeshkumar and Kuttan (2011) suggested that apoptosis was induced by activation of p53-induced, caspase-3-mediated proapoptotic signaling and suppression of NF- κ B-induced, bcl-2-mediated survival signaling. Induction of apoptosis by the sesquiterpene lactone salograviolide A from *Centaurea ainetensis* was confirmed by the TUNEL assay (El-Najjar *et al.*, 2008), and the apoptotic potential of the guaianolide thapsigargin from *Thapsia* species has been proven as well (Drew *et al.*, 2008).

In the present study we have evaluated the ability of gaillardin, a sesquiterpene lactone of the guaianolide type, to cause DNA degradation by strand breaks that were discovered by the TUNEL assay. The nuclei of MCF-7 cells exposed to 6.25 $\mu\text{g}/\text{mL}$ gaillardin were stained dark signifying DNA fragmentation and nuclear condensation (Fig. 1).

Reduction of the salt MTT by cellular enzymes to form formazan is an indicator of cell viability. Results of the MTT assay revealed cytotoxic activity of gaillardin with IC_{50} values of 6.37, 6.20, 4.76, and 1.81 $\mu\text{g}/\text{mL}$ against MCF-7, HepG-2, A-549, and HT-29 cells, respectively (Fig. 2). The IC_{50} values of sesquiterpene lactones reported in previous studies vary but those that are below

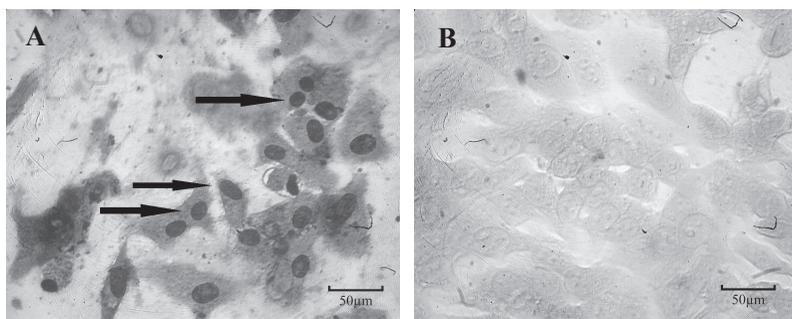


Fig. 1. Results of the TUNEL assay. (A) The arrows point to the condensed nuclei of MCF-7 cells treated with 6.25 $\mu\text{g}/\text{mL}$ of gaillardin; (B) negative control.

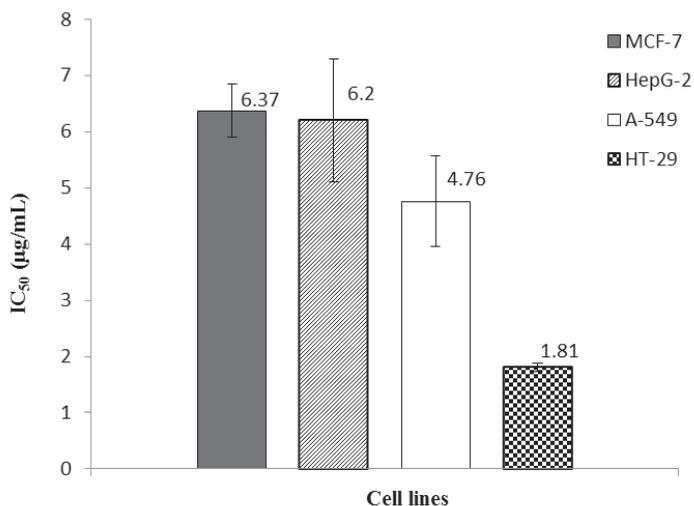


Fig. 2. IC₅₀ values of gaillardin's toxic effect on the cell lines MCF-7, HepG-2, A-549, and HT-29, obtained by the MTT assay. Values represent the mean \pm SD of three separate experiments.

10 μ g/mL are considered as potent agents. This is the case for gaillardin, especially in the HT-29 cell line to which it was highly cytotoxic with an IC₅₀ value of 1.81 μ g/mL.

The cytotoxic activity of gaillardin against MCF-7, MDBK, and WEHI-164 cells has been

reported previously (Mosaddegh *et al.*, 2010). We have now evaluated the toxicity of gaillardin to HepG-2, A-549, and HT-29 along with MCF-7 cells. Gaillardin reduced the cell viability in all four cell lines in a dose-dependent manner (Fig. 3).

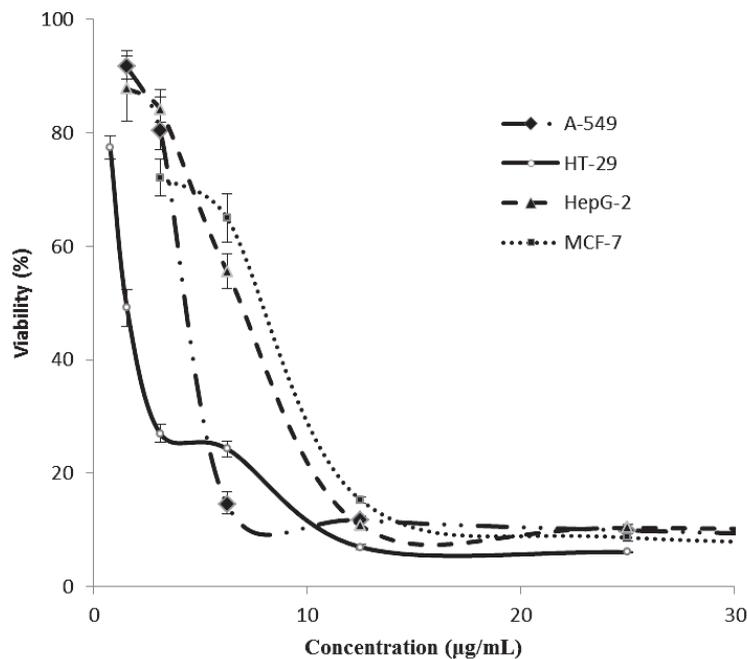


Fig. 3. Dose-response curves of gaillardin's toxic effect on MCF-7, HepG-2, A-549, and HT-29 cells. The cells were treated with gaillardin for 72 h, and the cell viability was determined using the MTT colorimetric assay.

In conclusion, the results of the MTT and TUNEL assays indicate the apoptotic potential of gaillardin and make it likely that its cytotoxic activity results from the induction of cell death by apoptosis; however, further studies are required to understand how gaillardin causes these changes.

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- Alley M. C., Scudiero D. A., Monkes A., Hursey M. L., Czerwinski M. J., Fine D. L., Abbott B. J., Mayo J. G., Shoemaker R. H., and Boyd M. R. (1988), Feasibility of drug screening with panel of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* **48**, 589–601.
- Bai N., Lai C. S., He K., Zhou Z., Zhang L., Quan Z., Zhu N., Zheng Q. Y., Pan M. H., and Ho C. T. (2006), Sesquiterpene lactones from *Inula britannica* and their cytotoxic and apoptotic effects on human cancer cell lines. *J. Nat. Prod.* **69**, 531–535.
- Cheng X. R., Li W. W., Ren J., Zeng Q., Zhang S. D., Shen Y. H., Yan S. K., Ye J., Jin H. Z., and Zhang W. D. (2012), Sesquiterpene lactones from *Inula hookeri*. *Planta Med.* **78**, 465–471.
- Drew D. P., Krichau N., Reichwald K., and Simonsen H. T. (2008), Guaianolides in Apiaceae: perspectives on pharmacology and biosynthesis. *Phytochem. Rev.* **8**, 581–599.
- El-Najjar N., Dakdouki S., Darwiche N., El-Sabban M., Saliba N. A., and Gali-Muhtasib H. (2008), Anti-colon cancer effects of salograviolide A isolated from *Centaurea ainetensis*. *Oncol. Rep.* **19**, 897–904.
- Fulda S. (2010), Modulation of apoptosis by natural products for cancer therapy. *Planta Med.* **76**, 1075–1079.
- Gonzalez Romero M. A., Villaescusa Castillo L., Diaz Lanza A. M., Bartolome Esteban C., and Fernandez Matellano L. (2001), Phytochemistry and pharmacological studies of *Inula montana* L. *Recent Res. Dev. Phytochem.* **5**, 255–268.
- Hsu Y., Wu L., and Kuo P. (2009), Dehydrocostuslactone, a medicinal plant-derived sesquiterpene lactone, induces apoptosis coupled to endoplasmic reticulum stress in liver cancer cells. *J. Pharmacol. Exp. Ther.* **329**, 808–819.
- Ichikawa H., Nair M. S., Takada Y., Sheeja D., Kumar M., Oommen O. V., and Aggarwal B. B. (2006), Iso-deoxyelephantopin, a novel sesquiterpene lactone, potentiates apoptosis, inhibits invasion, and abolishes osteoclastogenesis through suppression of nuclear factor- κ B (NF- κ B) activation and NF- κ B-regulated gene expression. *Clin. Cancer Res.* **12**, 5910–5918.
- Moghadam M. H., Hajimehdipoor H., Saeidnia S., Atoofi A., Shahrestani R., Read R. W., and Mosaddegh M. (2012), Anti-proliferative activity and apoptotic potential of britannin, a sesquiterpene lactone from *Inula aucheriana*. *Nat. Prod. Commun.* **7**, 979–980.
- Mosaddegh M., Moghadam M. H., Ghafari S., Naghibi F., Ostad S. N., and Read R. W. (2010), Sesquiterpene lactones from *Inula oculus-christi*. *Nat. Prod. Commun.* **5**, 511–514.
- Pratheeshkumar P. and Kuttan G. (2011), Vernolide-A, a sesquiterpene lactone from *Vernonia cinerea*, induces apoptosis in B16F-10 melanoma cells by modulating p53 and caspase-3 gene expressions and regulating NF- κ B-mediated bcl-2 activation. *Drug Chem. Toxicol.* **34**, 261–270.
- Zeng G. Z., Tan N. H., Ji C. J., Fan J. T., Huang H. Q., Han H. J., and Zhou G. B. (2009), Apoptosis induction of bigelovin from *Inula helianthus-aquatica* on human leukemia U937 cells. *Phytother. Res.* **23**, 885–891.