

RESEARCH ARTICLE

Anti-cancer Properties of a Sesquiterpene Lactone-bearing Fraction from *Artemisia khorassanica*

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Abstract

Background: *Artemisia* species are important medicinal plants throughout the world. The present *in vitro* study, using a sesquiterpene lactone-bearing fraction prepared from *Artemisia khorassanica* (SLAK), sought to investigate anti-cancer properties of this plant and elucidate potential underlying mechanisms for the effects. **Materials and Methods:** Anti-cancer potential was evaluated by toxicity against human melanoma and fibroblast cell lines. To explore the involved pathways, pattern of any cell death was determined using annexin-V/PI staining and also the expression of Bax and cytochrome c was investigated by Western blotting. **Results:** The results showed that SLAK selectively caused a concentration-related inhibition of proliferation of melanoma cells that was associated with remarkable increase in early events and over-expression of both Bax and cytochrome c. **Conclusions:** The current experiment indicates that *Artemisia* may have anti-cancer activity. We anticipate that the ingredients may be employed as therapeutic candidates for melanoma.

Keywords: *Artemisia khorassanica* - melanoma cell line - apoptosis - bax - cytochrome c

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Introduction

Natural compounds derived from medicinal plants have been traditionally used to treat various diseases or to promote general health; nowadays, they are increasingly being exploited for pharmacologic purposes. Accordingly, numerous investigations have been done to identify plant-derived natural products that can function as anti-cancer mediators to control aberrant cell proliferation (Craig, 1999; Tapsell et al., 2006; Menon et al., 2014).

Artemisia L. (*Astraceae*, *Compositae*) a large heterogeneous genus of evergreen and deciduous shrubs, perennials, and annuals, distributed mainly in the temperate zones of Europe, Asia, and North America. *Artemisia* consisting of \approx 550 species; 43 are found in Iran with two used in traditional medicine (Podlech, 1986; Emami and Aghazari, 2011; Jiang et al., 2013; Liu and Cui, 2013). Different classes of chemicals, e.g., coumarins, terpenes, sesquiterpenes, flavonoids, aromatics, dipeptides, phenolics, coumarins, esterols, germacranolides, guaianolides, secoguaianolides, and polysaccharides have been isolated from *Artemisia* species (Valless et al., 2011). In recent years, increasing evidence has shown that sesquiterpene lactones are one of the constituents that impart a wide-range of pharmacological effects, including anti-cancer and immunomodulatory action (Aveccina, 1998; Kim et al., 2002; Lu et al., 2009; Choi et al., 2011; Yang et al.,

2012).

Among some studies that evaluated produces spectrum of anti-cancer effects of *Artemisia*, few have assessed the impact on various cancer cell types (Firestone 2009; Sun et al., 2010; Choi 2011; Prestap 2012; Tao et al., 2012; Zhang 2012). In a previous study, we reported the anti-inflammatory effects of a sesquiterpene lactone-bearing fraction isolated from *Artemisia khorassanica* (SLAK); those studies showed that SLAK impacted on lipopolysaccharide (LPS)-induced release of nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor (TNF)- α and interleukin (IL)-1 β release, as well as the expression of both iNOS and COX-2 enzymes, by the J774A.1 macrophage cell line (Emami et al., 2010). To expand our understanding if the products of *Artemisia* might also act as anti-cancer agent, the present study sought to clarify the effects of SLAK *in vitro* in various melanoma cell lines and investigating the mechanism of action.

Materials and Methods

Plant materials

Aerial parts of *Artemisia khorassanica* were collected from Chovailly-Bajgirani Northeast Iran (23 Dec 2004). Dr. V. Mozaffarian (Research Institute of Forest and Rangelands, Ministry of Jihad Keshavarzi, Iran)

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confirmed the plant identity and a voucher specimen was deposited in the herbarium at the National Botanical Garden of Iran (TARI, Teheran, Iran).

Isolation of sesquiterpene lactone-bearing fraction

The terpenoid fraction was prepared from the isolated samples as previously described (Iranshahi et al., 2007; Emami et al., 2010). The Herz-Högenauer technique was used to remove chlorophyll and phenolics (by lead (II) acetate precipitation) and to prepare a crude sesquiterpene sample for further chromatographic/spectral investigation. In brief, dried ground plant material (20g) was soaked in dichloromethane (DCM; ~100ml) overnight and the slurry product then filtered and evaporated in *vacuo*. The gummy residue was dissolved in 96% ethanol (~50ml) and heated to improve solubility. Aqueous 5% lead acetate solution was added to precipitate fatty acids, phenolics, and chlorophyll present. The precipitate was removed by filtration through a pad of silica gel (230-400 mesh, Merck, Darmstadt, Germany). The filtrate was concentrated in a waterbath (40-50°C) until a viscous mass developed. An IR spectrum was recorded (as KBr disks) and in CH₂Cl₂ on a Unicam dp 110 spectrometer (Shimadzu Scientific, Tokyo, Japan). ¹H-NMR (500 MHz) spectra of the sample was assessed in deuterated chloroform (CDCl₃, Sigma-Aldrich, Berlin, Germany) using a DRX 500 spectrometer (Bruker, Bremen, Germany). SLAK was dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO was <0.1% in the cell culture medium which has no effect on the experiment carried out in the present study.

Culture and treatment of melanoma cells

Human melanoma cell lines (including MM200, Mel-RM, Me4405) were kind gifts of Dr. S. H. Mousavi (Department of Pharmacology, Mashhad University of Medical Sciences, Mashhad, Iran). A375 cells (NCBI# C136) and human skin fibroblast-like HFFF2 cells (NCBI# C163) were purchased from the National Cell Bank of Iran (NCBI, Tehran). Origin and characteristics of these lines have been reported previously (Zhang et al., 1999; Wua et al., 2005; Mhaidat et al., 2007).

For these experiments, cells were maintained in a 5% CO₂ humidified atmosphere at 37°C as monolayer cultures in DMEM medium (Gibco BRL, Paisley, UK) supplemented with 10% FCS, 100 IU penicillin/ml and 100 µg streptomycin/ml. For use in the experiments, cells were removed from line-specific flasks using 0.25% trypsin-0.05% EDTA solution (this protocol routinely yielded >95% viable cells; determined with trypan blue).

Proliferation inhibition assay

Cell growth and proliferation was determined by an MTT assay (Mosmann 1983; Zamanai Taghizadeh Rabe et al., 2011). Melanoma and fibroblast cells were seeded into 96-well microplates (10⁴ cells/well), treated with increasing concentrations of SLAK (5-300 µg/ml), and then incubated 24hr at 37°C. Cisplatin (Sigma), a potent inducer of growth arrest and apoptosis, was used at 1-300 µg/ml as a reference anti-cancer/cytotoxic agent (Haochuan et al., 2006; Mol et al., 2008; Rakic et al., 2009; Demirci et al., 2014;). At the end of the incubation, the medium in each

well was removed and replaced with fresh medium, and each culture then processed as above to assess levels of formazan present. Cell proliferation inhibition rate was calculated as inhibition (%) = 100 × [1 - (OD_{control} - OD_{treated}) / OD_{control}]. The concentration of SLAK (and cisplatin) that resulted in 50% cell growth inhibition (IC₅₀ value) was calculated using Prism 5 software (Graph Pad, La Jolla, CA) for each cell line (Table 1). All experiments were repeated for at least three times with triplicate samples for each dose tested.

Detection of apoptosis using Annexin-V/propidium iodide (PI) dual staining

Death patterns among the cells were quantified using a fluorescein isothiocyanate-labeled Annexin V (Annexin V-FITC) and propidium iodide (PI) staining method (Emami et al., 2009). Me4405 cells (5 × 10⁵/well) were treated with the designated concentrations of SLAK (5-150 µg/ml) for 24hr, then harvested using trypsin-EDTA solution, washed with PBS, re-suspended in binding buffer, and incubated at room temperature for 5min in the dark with Annexin-V FITC and PI solution (Abcam, Cambridge, MA). The cells were then analyzed (within 1hr) in a FACSCalibur flow cytometer (Becton Dickinson, Carlsbad, CA). All data were analyzed using BD FSC Express 3.0 software. In each case, a minimum of 10,000 events was captured. Results were reported as percentages of Annexin V⁺ (early apoptosis), PI⁺ (necrosis), Annexin V⁺PI⁺ (double positive; late apoptosis), or Annexin V⁻PI⁻ (double negative; non-staining) cells in each population analyzed.

Western blot analysis of treated cancer cells

Cells were lysed in freshly prepared lysis buffer containin 20mM HEPES, pH 7.9, 400mM NaCl, 0.1% Nonidet (N) P-40, 10% glycerol, 1mM sodium vanadate, 1mM sodium fluoride, 1mM dithiothreitol (DTT), 1mM phenylmethylsulphonyl fluoride (PMSF) along with protease inhibitor cocktail (BioVision Inc, CA) for 45min on ice. The protein concentrations of cytosolic extracts were determined using Bradford assay. Equal amounts of protein (30-50 µg/ml) of each cell lysate was dissolved in Laemmli's sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed by transferring proteins from a slab to a sheet of polyvinylidene difluoride membrane at 240mA for 40min at room temperature. The membrane was then blocked overnight at 4°C with 5% skim milk in phosphate-buffered saline (PBS, pH 7.4). After washing with PBS buffer, the membrane was incubated with primary antibodies (BD Pharmingen Co, CA) including anti-human cytochrome c polyclonal antibody or anti-human Bax polyclonal antibody for 2hr at room temperature. After gently rinsing away unbound primary antibody, each membrane was incubated with secondary goat anti-rabbit-HRP-conjugated antibody (KOMA biotech, Seoul, Korea) for 1hr at RT. β-actin protein was used as the housekeeping control. Subsequently, blots were extensively washed with PBS, developed using ECL-detection reagents (Amersham, Cardiff, UK).

Statistical analysis

The significance of differences was evaluated by one-way analysis of variance (ANOVA) and a Bonferroni's *post-hoc* test using SPSS 11.0 software (Chicago, IL). All results were expressed as mean \pm SD. A *p*-value <0.05 was considered statistically significant.

Results

Analysis of *Artemisia* fraction

In IR spectra, a sample showing a strong absorption between ν 1730 and 1780 indicates the presence of a carbonyl moiety. Samples with absorptions more than ν 1760 are considered to bear to a γ -lactone moiety. The *A. khorassanica* extract here displayed strong absorption peak in ν 1765, indicating a high content of sesquiterpene lactones in this terpenoid extract (Figure 1A). In general, diagnostic features in $^1\text{H-NMR}$ spectra of sesquiterpenoid α -methylene γ -lactones are (unsaturated sesquiterpene lactones) two doublets ($J_{\text{B,C}}=1\text{-4Hz}$) which appear above

Table 1. The IC₅₀ values (mean \pm S.D) Determined from the Plot of Percent Cytotoxicity on Melanoma Cell Lines Versus the Concentrations of SLAK or Cisplatin

Cell lines	Mean of IC ₅₀ values ($\mu\text{g/ml}$)	
	SLAK	Cisplatin
MM200	103.15 \pm 1.2	200.3 \pm 3.1
Mel-RM	88.37 \pm 1.9	138.7 \pm 3.8
Me4405	66.51 \pm 1.8	180.3 \pm 1.5
A375	130.03 \pm 1.5	128.6 \pm 3.6
HFFF2	242.92 \pm 0.7	195.8 \pm 2.4

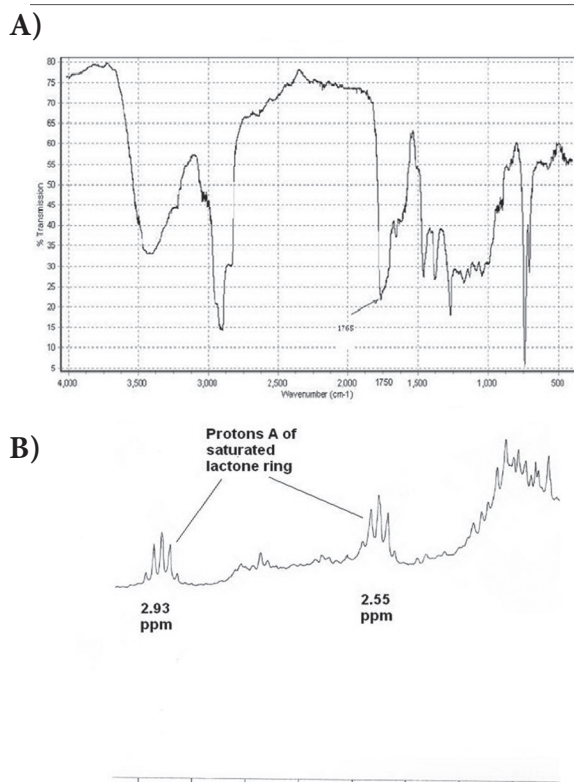


Figure 1. The Terpenoid Fraction of *A. Khorassanica*. (A) IR spectrum showing absorption of carbonyl function at 1765 cm^{-1} ; (B) Expanded $^1\text{H-NMR}$ spectrum showing quintet signals of A Protons of saturated γ -lactone ring

and below 6 ppm. On the other hand, a diagnostic feature in the $^1\text{H-NMR}$ spectra of sesquiterpenoid α -methylene γ -lactones (saturated sesquiterpene lactones) is a quintet between 2.0 and 3.0 ppm (Proton B). As all diagnostic features in the $^1\text{H-NMR}$ spectra were not usually obscured with other signals in the terpenoid extract, the noted signals could be used to determine of the type of γ -lactone present. Overall, the results gleaned from the $^1\text{H-NMR}$ spectra of the sample were also almost in agreement with those from the results of the IR analyses. As shown in Table 1, the main types of sesquiterpene lactone in the *A. khorassanica* terpenoid extract were saturated sesquiterpene lactones. Specifically, the $^1\text{H-NMR}$ spectra of this sample showed there was a quintet signal (Proton A) between 2.0 and 3.0 ppm (Figure 1B); exocyclic methylene protons peaks at 5.5 and 6.2 ppm, indicating α -methylene γ -lactone, were not observed.

Effect of SLAK on melanoma and fibroblast cells

SLAK was evaluated for its *in vitro* growth inhibitory property on four human melanoma cell lines, i.e., MM200, Mel-RM, Me4405, and A375, and also the human skin fibroblast-like cell line HFFF2. For each cell line, there was a linear relationship between growth inhibition rate and SLAK concentration after 24hr of treatment. The inhibitory effect of SLAK on melanoma cell lines (Figure 2A) was significant at doses $>5\mu\text{g/ml}$, reaching $>50\%$ suppression at SLAK levels $>150\mu\text{g/ml}$. In contrast, SLAK up to $75\mu\text{g/ml}$ induced no significant suppression of normal fibroblast cell proliferation (Figure 4B). Table 1 reports the IC₅₀ for SLAK in the melanoma and fibroblast-

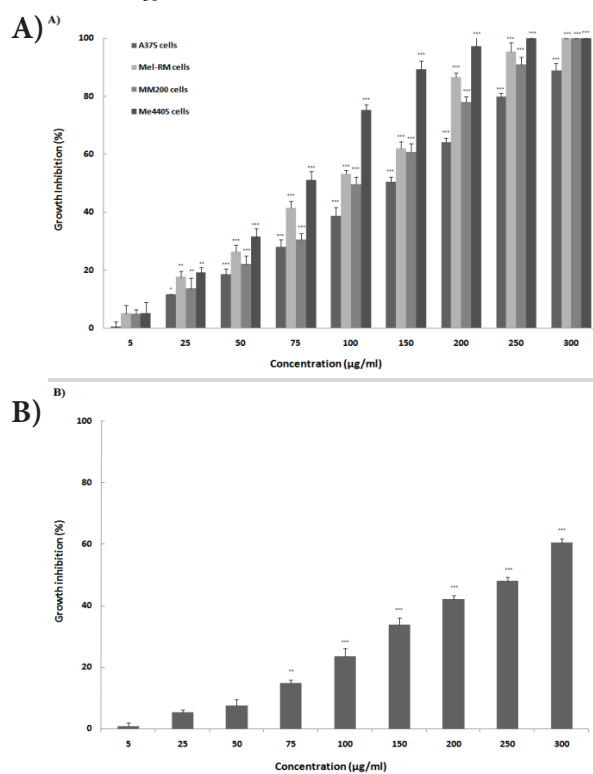


Figure 2. Growth Inhibitory Activity of SLAK (5-300 $\mu\text{g/ml}$) Against. (A) human melanoma cell lines; (B) human skin fibroblast-like cells after 24hr of treatment. Results are shown as mean \pm SEM (n=3). Values significantly different vs control at **p*<0.05; *p*<0.01; ****p*<0.001**

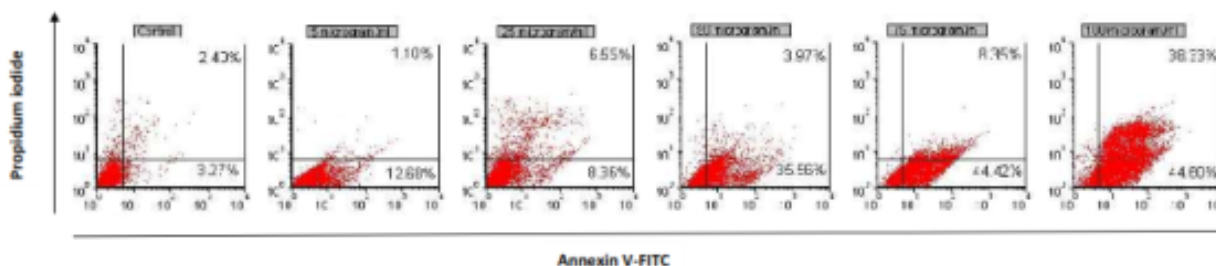


Figure 3. Apoptosis Induced by SLAK in Me4405 Melanoma Cells. Cells were treated with SLAK (25-100µg/ml) for 24hr, then collected/stained with Annexin V-FITC and PI solution. In the flow cytohistograms of apoptosis shown, X-axis is Annexin V-FITC+ cells and Y-axis is PI-labeled populations

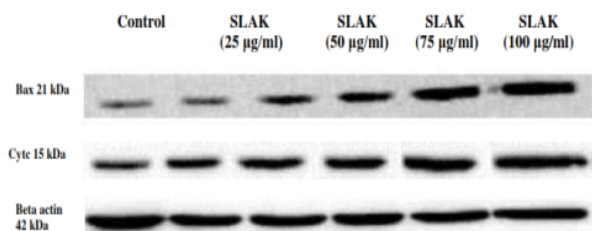


Figure 4. Western Blot Analysis of Extracts Obtained from Me4405 Cells Treated with SLAK (25-100µg/ml) for 24hr. A representative blot from among three independent experiments is shown. Extracts were prepared and analyzed by Western blotting with antibody to Bax and cytochrome c. β-actin was monitored as loading control

cells. As can be seen, there were clearly strong increases in levels of Bax and cytochrome c protein bands at 24hr post-SLAK treatment.

Discussion

While there are often problems associated with the use of synthetic anti-cancer agents, overall, compounds derived from plants are relatively safe and do not cause serious side effects (Kma, 2013). Thus, plant products are superlative candidates for preventive/therapeutic modalities. Many researchers are interested in evaluating natural products that traditionally have been used for their potential biological activities (van der Kooy et al., 2013). The *Artemisia* genus has been used in folk medicine for many years; numerous reports have indicated a vast range of biological effects associated with different *Artemisia* species (Abad Martinez et al., 2012; Choi et al., 2013). Among the various chemical agents found in these plants, sesquiterpene lactones have been proven to impart anti-cancer effects (Tan et al., 1998; Firestone et al., 2009; Firestone 2009; Lu 2009; Zhai 2010; Zhang 2012; Feng 2013).

To evaluate the anti-cancer potential of SLAK, proliferative responses of melanoma cell lines were assessed. SLAK dramatically inhibited the proliferation of four human melanoma cell lines (MM200, Me1-RM, Me4405 and A375), with Me4405 cells the most affected. SLAK also inhibited growth of a human skin fibroblast-like (HFFF2) cell line. To ascertain whether the SLAK-induced inhibition of melanoma cell survival was associated with induction of apoptosis, cell death patterns were evaluated. The results indicated the percentage of Annexin V+ cells (specifically, among Me4405 cells) was significantly increased by SLAK, indicating an increase in the incidence of apoptosis. Other studies have reported the cytotoxic effect of other sesquiterpene lactones in several cell lines occurred via apoptosis induction (Zhaia et al., 2010; Priestap et al., 2012).

Induction of apoptosis can be regulated by several pathways, among them the release of cytochrome c from mitochondria into the cytosol; this is thought to be regulated to a great extent by Bax (Antonsson et al., 1997; Rosse et al., 1998; Sun et al., 2004). As changes in both Bax and cytochrome c expression are associated with induction of cell death (including apoptosis), the effect of SLAK on each was investigated. The data suggested there was a predominant role for Bax and cytochrome

like cell lines. SLAK appeared to exhibit selective toxicity on the melanoma cells. Me4405 cell proliferation was most suppressed in comparison with the other cell lines, with the lowest IC₅₀ value (66.5 [± 1.8]µg/ml). SLAK showed the lowest inhibitory effect on the HFFF2 cells, with an IC₅₀ value of 242.9 [± 0.7]µg/ml. Comparing the IC₅₀ values of cisplatin vs SLAK showed that unlike SLAK, cisplatin exhibited considerable inhibitory effects on the human skin fibroblast-like cells (Table 1).

Induction of apoptosis in melanoma lines by SLAK

To determine if any decrease in cell viability induced by SLAK might be attributable to apoptosis, Annexin-V and PI staining was conducted. SLAK dramatically enhanced the early apoptotic population of melanoma cells (Annexin V+ cells) in a dose-related manner (Figure 3). Specifically, the early apoptotic cell population (right lower quadrant of fluorocytogram) in cells treated with 5, 25, 50, 75, or 100µg SLAK/ml (12.7 [±2], 8.4 [±3], 35.6 [±3], 44.4 [±5], and 44.6 [±4]%, respectively) was significantly increased compared to among untreated cells (3.3 [±0.5]%). Though levels of early apoptotic cells increased significantly due to the treatment, only 100µg SLAK/ml also significantly affected the levels of late apoptosis among the Me4405 cells.

Mechanism of SLAK-induced apoptosis: expression of Bax and cytochrome c

To better understand molecular events involved in any SLAK effect on apoptosis, effects of SLAK on Bax and cytochrome c expression-proteins pivotal for apoptosis - were analyzed. Figure 4 presents a representative Western blot from SLAK-treated (25-100µg/ml) and untreated

c in SLAK-induced apoptosis in the melanoma cells. This outcome would be in line with other reports that suggested an anti-tumor activity of sesquiterpene lactone from several *Artemisia* species was mediated through up-regulation of Bax and cytochrome c proteins (Zhang et al., 2012; Feng et al., 2013). To date, the precise mechanism(s) underlying the pro-apoptotic effect of *Artemisia* in melanoma/other transformed cell lines remains unclear.

In conclusion, a sesquiterpene lactone fraction derived from the aerial portions of *Artemisia khorassanica* (SLAK) imparted considerable anti-cancer potential against human melanoma cell lines. For now, as these studies were all *in vitro*, it remains to be seen if the fraction might also impart anti-tumor activity *in situ*.

We anticipate that constituents of the *A. khorassanica* might be useful in the development of therapeutic approaches toward prevention/treatment of various types of cancer. This study also provides a basis for the potential use of SLAK as a component in any overall anti-cancer therapy. However, further investigations are required to elucidate the precise component responsible for the observed biological activities and effects *in vivo* need to also be evaluated so that the potential broader utility of the SLAK can be more precisely determined.

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