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Original article

In vitro anti-proliferative and apoptotic activity of different fractions of *Artemisia armeniaca*

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Anti-proliferative properties have been reported for certain species of the genus *Artemisia*. In this study, we investigated the cytotoxic and apoptotic effects of *n*-hexane, CH_2Cl_2, EtOAc, *n*-BuOH and H_2O fractions obtained from a crude methanol extract of *A. armeniaca* on two myeloid cell lines, apoptosis-proficient HL60 cells and apoptosis-resistant K562 cells; in addition, J774 cells were used as a control. Among the solvent fractions of *A. armeniaca*, the CH_2Cl_2 fraction was found to have the largest anti-proliferative effect on cancer cells. The IC_{50} values obtained using an MTS assay for the CH_2Cl_2 fraction were 75 and 130 µg/ml for HL-60 cells and K562, respectively. The control cells were not significantly affected by this fraction. A flow cytometry histogram of cells treated with the CH_2Cl_2 fraction of *A. armeniaca* revealed a sub-G1 peak. DNA fragmentation, increased protein levels of Bax and cleavage of the poly (ADP-ribose) polymerase (PARP) protein confirmed the induction of apoptosis in cells after a 48-h exposure to the CH_2Cl_2 fraction. Our results corroborate the cytotoxic and apoptotic effects of the CH_2Cl_2 fraction of *A. armeniaca* on K562 and HL-60 cancer cell lines.
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Introduction

In recent years, the prevention and treatment of cancer has been one of the most imperative topics in medical sciences. Screening of bioactive substances for their ability to be used as anti-cancer agents has led to the identification of many natural chemotherapeutic agents. Induction of apoptosis in cancer cells has been developed as an indicator for the ability of naturally derived active components to treat cancer (Shu et al., 2010). Apoptosis in cancer cells can be triggered through several tightly regulated signalling pathways, such as the receptor, mitochondrial and mitogen-activated protein kinase (MAPK) pathways. Caspases are the main promoters of apoptosis and are activated in all pathways. The cleavage of 116 kDa PARP-1 to 89 and 24 kDa fragments induced by caspase 3 activation is an indicator of apoptosis. Caspase 9 activation is a marker of increases in pro-apoptotic proteins, such as Bax, in the outer layer of the mitochondria; furthermore, activation of caspase 8 is triggered following death receptor signals (Mousavi et al., 2008).

Artemisia is a large and diverse genus of plants with approximately 400 species belonging to the Asteraceae family. Some species of the genus have been used as food additives,

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and as anti-malarial, anti-hepatotoxic, (Bora and Sharma, 2011; Gilani et al., 2005), anti-inflammatory (Abad et al., 2012), anti-microbial and anti-viral agents (Aniya et al., 2000; Kordali et al., 2005a; Kordali et al., 2005b). There are 34 reported species of this genus in Iran, and two of these species are endemic to the country (Emami and Aghazari, 2011; Ghahreman and Attar, 1999).

Diverse chemical components, such as flavonoids, coumarins, sterols, polyacetylenes, monoterpenes, sesquiterpenes and sesquiterpenes lactones, have been found in plants from the *Artemisia genus* (Bora and Sharma, 2011; Tan et al., 1998). Previous phytochemical studies on the aerial parts of *A. armeniaca* showed the presence of simple and prenylated coumarins (Mojarrab et al., 2010; Mojarrab et al., 2011; Rybalko et al., 1976; Szabo et al., 1985) and some not certain known flavonoids (Mojarrab et al., 2011). α-Pinene and 1,8-cineole were identified as the major components of the hydro-distilled oils from the aerial parts of *A. armeniaca* (Kazemi et al., 2010); however, non-terpene hydrocarbons were reported as the main constituents of this essential oil in another study (Mojarrab et al., 2013). *A. armeniaca* Lam. is a perennial subshrub of the genus *Artemisia* which is largely distributed in the north western part of Iran (Mozaffarian, 2008).

The extracts of the species from the same genus have shown anti-proliferative effects by inducing apoptosis and/or stopping the cell cycle progression (Choi et al., 2013; Lee et al., 2013; Park et al., 2008; Rabe et al., 2011; Tin et al., 2012).

Collectively, our study was designed to determine if various fractions of *A. armeniaca* can inhibit the progression of leukemic malignancies by examining the sensitivity of apoptosis-proficient HL-60 and apoptosis-resistant K562 cells to cytotoxic insults (Martins et al., 1997). Both cell lines are p53 deficient and K562 cells are null for the Fas/CD95/APO-1 receptor (Martins et al., 1997).

The study also explored the possible mechanism(s) of the apoptosis mediated by *A. armeniaca* fractions. To our knowledge, we are the first to demonstrate that the CH_2Cl_2 fraction of *A. armeniaca* crude extract induces apoptosis in human leukaemia cells through a mitochondrial and caspase-dependent pathway.

Materials and methods

Plant material

The aerial parts of *Artemisia armeniaca* Lam., Asteraceae, were collected from Arasbaran in the Eastern Azarbaijan Province, northwest of Iran. Voucher specimen (No. TBZ-fph 528) was deposited in the Herbarium of School of Pharmacy at Tabriz, University of Medical Sciences in Tabriz, Iran. The dried and ground aerial parts (300 g) were percolated with methanol (MeOH) at room temperature. The whole extract was filtered and the solvent was evaporated under reduced pressure at 45-50 °C to obtain a crude methanol extract (28 g). The dried crude extract was then resolved in 95% methanol and partitioned successively in *n*-hexane, methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), and H2O. The *n*-Hexane, CH_2Cl_2, EtOAc and *n*-BuOH fractions were evaporated under vacuum to yield 17, 13.5, 12.4 and 12 g of each dried fraction, respectively. Freeze drying of the water fraction afforded 9.6 g of dried residue. The dried fractions were stored at 4 °C until subsequent analysis. The partitioning scheme of the *A. armeniaca* methanol extract is presented in Fig. 1. All of the isolated fractions were dissolved in dimethylsulfoxide (DMSO) to a final concentration of 50 mg/ml before use in cytotoxicity and apoptosis assays.

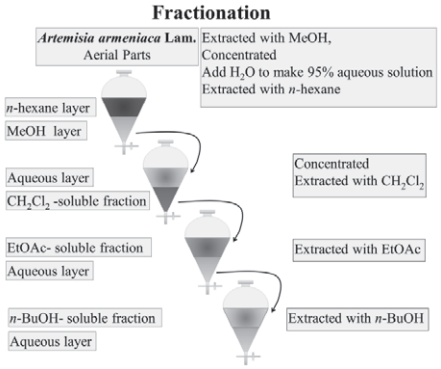

Fig. 1 - Partitioning scheme of *n*-hexane, CH2Cl2, EtOAc, *n*-BuOH and H2O fractions obtained from the crude methanolic extract of *Artemisia armeniaca*.

Reagents and chemicals

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was purchased from Promega (Madison, WI, USA). RPMI-1640 and FCS were purchased from Gibco. Lympholyte®-H was purchased from Cedarlane (Canada). β-Actin and poly (ADP-ribose) polymerase (PARP) antibodies, anti-rabbit IgG and HRP linked antibody were obtained from Cell Signaling Technology (Boston, USA). The ECL Western blotting detection reagent was purchased from Bio-Rad (USA). The fluorescent propidium iodide (PI), protease inhibitor cocktail, phosphatase inhibitor cocktail, sodium citrate, Triton X-100, phenylmethylsulfonyl fluoride and QuantiPro BCA Assay Kit were obtained from Sigma (Steinheim, Germany). All the solvents (analytical grade) were purchased from Dr. Mojallali (Tehran, Iran).

Cell cultures and treatments

The human leukemic cancer cell lines HL-60 and K562 were obtained from the Pasteur Institute (Tehran, Iran) and maintained in RPMI-1640 medium with 10% v/v foetal bovine serum, 100 µ/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Assays

In vitro *cell proliferation*

The MTS assay is based on the reduction of the novel tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-

carboxymehoxy phenyl)-2H-tetrazolium inner salt (MTS) by the mitochondrial dehydrogenase from metabolically active cells. The compound is reduced to the coloured water-soluble formazan which can be detected at a wavelength of 490 nm.

Approximately 5×10^4 K562 and 10^5 HL-60 cells were seeded in each well of a 96-microwell plate and treated with various concentrations of each fraction of *A. armeniaca*. The J774 cell line was used as the non-malignant control. After a 48-h incubation, the CellTiter 96®AQueous one solution containing MTS and the electron coupling reagent phenazinemethosulfate was added to each well according to the manufacturer's instructions. After 3 h in culture, cell viability was determined by measuring the absorbance at 490 nm using an ELISA microplate reader (Awareness, Palm City, FL, USA). The cytotoxicity of the methanol extract and fractions of *A. armeniaca* was calculated using Graph Pad Prism 5 software. The data are presented as the mean ± SEM of three independent experiments completed in triplicate for each sample concentration.

For each concentration and time course study, there was a control sample which remained untreated and received an equal volume of the solvent.

PI Staining

Apoptotic cells were detected using PI staining of small DNA fragments followed by flow cytometry. It has been reported that a sub-G1 peak that is reflective of DNA fragmentation can be observed following the incubation of cells in a hypotonic phosphate-citrate buffer containing a quantitative DNA-binding dye, such as PI. Apoptotic cells that have lost DNA will take up less stain and appear on the left side of the G1 peak in the histogram. Briefly, 10^6 K562 and HL-60 cells were each seeded in wells of a 24-well plate and treated with the CH_2Cl_2 fraction of *A. armeniaca* at different concentrations (50, 75 and 100 µg/ml) for 48 h. Floating and adherent cells were then harvested and incubated at 4 °C overnight in the dark with 750 µl of a hypotonic buffer (50 µg/ml PI in 0.1% sodium citrate with 0.1% Triton X-100). Next, flow cytometry was completed using a FACScan flow cytometer (Becton Dickinson). A total of 104 events were acquired with FACS.

Western blotting analysis

Approximately 10^7 HL-60 and K562 cells were treated with 37.5 and 75 µg/ml of the CH_2Cl_2 fraction of *A. armeniaca* for 48 h. The cell lysate was then prepared (Tayarani-Najaran et al., 2013), and the protein concentration was determined using a Bio-Rad Protein Assay kit. Equal amounts of protein were subjected to 12% (W/V) SDS-PAGE. The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and subjected to immunoblotting using primary antibodies for Bax, β-actin and an anti-rabbit IgG and HRP-linked antibody as secondary antibodies. Protein levels of Bcl-2 and Bax and cleavage of PARP in K562 and HL-60 cells were detected by enhanced chemiluminescence using an ECL Western blotting detection reagent. Images were quantified using Gel-pro Analyser V.6.0 Gel Analysis software (Media Cybernetics, Inc, Bethesda, MD)

Statistical analysis

A one-way analysis of variance (ANOVA) and the Bonferroni *post hoc* test were used for data analysis. All the results are expressed as the mean ± SEM, and *p* values below 0.05 were considered statistically significant.

Results

Cytotoxicity of A. armeniaca *fractions*

The crude methanol extract and multiple fractions of *A. armeniaca* were examined for their cytotoxic potential in K562, HL-60 and normal cell lines (J774). These cells were incubated for 48 h at 37 °C and 5% CO2 with various concentrations of the extract and its different fractions (6.25-200 µg/ml). The results demonstrated that the crude methanol extract and derived fractions decreased cell viability in a concentration-dependent manner (Fig. 2). Among all the fractions tested, the CH_2Cl_2 fraction exhibited the largest cytotoxic effects on cancer cells; in addition, there were limited adverse effects on normal cells. In HL-60 cells, the IC_{50} values for the methanol extract, *n*-hexane and CH_2Cl_2 fractions of *A. armeniaca* were 309.1, 160.9, and 74.95 µg/ml, respectively. In K562 cells, the IC_{50} values for the methanol extract, *n*-hexane and CH_2Cl_2fractions were 285.5, 155.4 and 130.7 µg/ml, respectively.

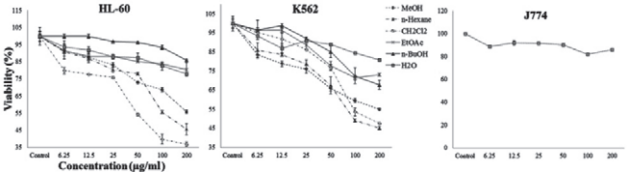

Fig. 2 - The dose-dependent effects of the methanol extract and *n*-hexane, CH_2Cl_2, EtOAc, *n*-BuOH and H_2O fractions on the growth of K562, HL-60 and normal J774 cells. All fractions exhibited cytotoxic activity against apoptosis-proficient HL-60 and apoptosis-resistant K562 cells, with IC_{50} values ranging from 74.95 to > 450 µg/ml. A lesser degree of cytotoxicity was observed for normal J774 cells. Values are the mean ± SEM of at least three independent experiments that were performed in triplicate.

Apoptosis induction using the CH_2Cl_2 fraction

Apoptosis in K562 and HL-60 cell lines was detected with flow cytometry using PI staining. Cells were incubated for 48 h with various concentrations (0, 50, 75 and 100 µg/ml) of the CH_2Cl_2 fraction of *A. armeniaca* methanol extract. Analysis of the sub-G1 peak in flow cytometry histograms revealed the induction of apoptosis in treated cells when compared with untreated control cells (Fig. 3).

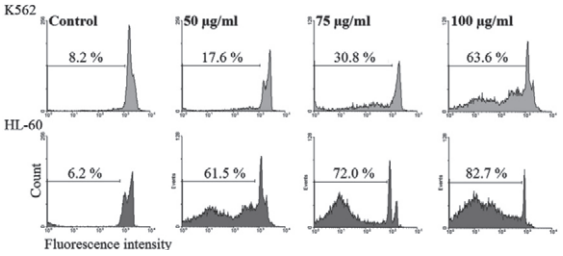

Fig. 3 - The effects of the CH_2Cl_2 fraction (0, 50, 75 and 100 µg/ml) on apoptosis in K562 and HL-60 cells using PI staining and flow cytometry.

Western blotting with HL-60 and K562 cells

In HL-60 and K562 cells, cells, PARP-1 was clearly cleaved to produce 89 kDa and 24 kDa fragments after a 48-h treatment with the CH_2Cl_2 fraction (Fig. 4). The Bax protein is critical for controlling cytochrome c release and apoptosis initiation via the mitochondrial pathway (Suzuki et al., 2000). The CH_2Cl_2 fraction increased Bax levels in HL-60 and K562 cells in a concentration-dependent manner (Fig. 4).

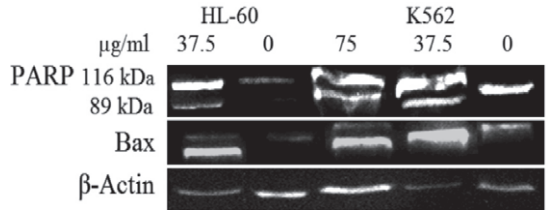

Fig. 4 - HL-60 and K562 cells show increased proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) and Bax protein levels after a 48-h exposure to the CH_2Cl_2 fraction of *Artemisia armeniaca* (37.5 and 75 µg/ml). β-Actin was used as a loading control. All Western blots are representative of 3 independent experiments.

Discussion

Considering the safety and efficacy of phytochemicals, molecular mechanistic research has focused on plants with cancer prevention potential (Shu et al., 2010). Changes in cell proliferation and cell death are basic determining factors in the pathogenesis of cancer. Anti-cancer phytochemicals can act as cell cycle and apoptosis regulators or as anti-oxidative stress and anti-inflammatory agents (Hanahan and Weinberg, 2000).

In the present study, the methanol extract and different fractions of *A. armeniaca* were evaluated for possible cytotoxic/apoptotic activity. Due to the intrinsic differences between HL-60 and K562 cell lines (Martins et al., 1997), the CH_2Cl_2 fraction induced apoptotic cell death at different IC_{50} values.

The CH_2Cl_2 fraction obtained from the methanol extract of *A. armeniaca* was also evaluated for apoptotic potential. According to our findings, the CH_2Cl_2 fraction increased DNA fragmentation in HL-60 and K562 cells as shown by the low fluorescent intensity of PI stained cells in the flow cytometry histogram. These data suggest the fraction induced apoptosis. Apoptotic cell populations (sub-G1 peak) of cells treated with the CH_2Cl_2 fraction (50, 75 and 100 µg/ml) increased in a concentration-dependent manner. Caspases promote the intrinsic (mitochondrial) and extrinsic (death receptor) pathways of apoptosis. poly (ADP-ribose) polymerase (PARP) is a downstream target of caspases, and its cleavage can effect both the extrinsic and intrinsic pathways of apoptosis. The activation of the cytochrome c/Apaf-1/caspase-9 complex is a response to mitochondrial outer-membrane permeabilisation, and the release of pro-apoptotic factors is upstream of mitochondrial-dependent apoptosis (Wang, 2001). The balance between the BCL-2 superfamily of pro-apoptotic and anti-apoptotic members is vital to cell survival. Once pro-apoptotic multi-BH domain proteins Bax and Bak are homo-oligomerised they form an opening in the mitochondria outer membrane (Suzuki et al., 2000). Proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) and an increase in Bax protein levels in K562 cells support the apoptosis-inducing properties of the CH_2Cl_2 fraction of *A. armeniaca*.

Artemisia annua (Singh and Lai, 2004), (Ravizza et al., 2008), *A. absinthium* and the acetone extract of *A. afra* (IC_{50} 2.65 ± 1.05, 6.54 ± 1.06 and 6.98 ± 1.06 µg/ml in MCF-7, HeLa and HT-29 cells, respectively) (Feridberg, 2009), the CH_2Cl_2 extract of *A. diffusa* (IC_{50} 71 and 42 µg/ml in HeLa and HT-29 cells, respectively), the CH_2Cl_2 fractions of *A. biennis*, *A. ciniformis* (IC_{50} 35, 94 and 29 µg/ml in AGS, HT-29 and MCF-7 cells, respectively), *A. santolina* (IC_{50} 91 µg/ml in HT-29 cells) and the EtOAc extract of *A. vulgaris* (IC_{50} 57 µg/ml in MCF-7 cells) (Rabe et al., 2011) were all effective in inhibiting the proliferation of a panel of cell lines. The IC_{50} values obtained in this study for the CH_2Cl_2 fraction of *A. armeniaca* (74.95 and 130.7 µg/ml in HL-60 cells and K562 cells, respectively) are comparable with those reported for other species of the genus *Artemisia*.

Artemisinin, a chemical compound extracted from *A. annua*, exhibits *in vitro* and *in vivo* anti-cancer activity (Efferth et al., 2011). Eupatilin, a naturally occurring flavonoid isolated from *A. princeps*, inhibits the growth of human endometrial cancer cells via G2/M phase cell cycle arrest through the up-regulation of p21, inhibition of mutant p53 and activation of the ATM/Chk2/Cdc25C/Cdc2 checkpoint pathway (Cho et al., 2011). Hispidulin, an active compound of *A. vestita*, targets the VEGF receptor 2-mediated PI3K/Akt/mTOR signalling pathway in endothelial cells, leading to the suppression of pancreatic tumour growth and angiogenesis (He et al., 2011). Essential oil isolated from *A. iwayomogi* has the ability to stimulate the MAPK-mediated signalling pathway triggered by mitochondria- and caspase-dependent mechanisms (Cha et al., 2009). Jaceosidin, isolated from *A. princeps*, modulates the ERK/ATM/Chk1/2 pathway, leading to the inactivation of the Cdc2-cyclin B1 complex, followed by G2/M cell cycle arrest in endometrial cancer cells (Lee et al., 2013).

Using solvents with varying polarity enables the isolation of phytochemicals that correspond to the nature of each solvent. The results observed with the *A. armeniaca* crude extract and the *n*-hexane and CH_2Cl_2 fractions confirmed the presence of potent non/semi-polar phytochemicals in this plant.

The use of crude plant extracts in biological assays provides the synergistic and antagonistic evaluation of the phytochemicals present. To better understand the biological activity related to each chemical constituent(s) and reduce possible side effects, further bio-assays on individual pure compounds are necessary. Moreover, biological evaluation of single components within plants eliminates the recognised inconsistencies found in extract preparations (Bandaranayake, 2006).

Authorship

ML (Pharm. D. Student) contributed in conducting the laboratory work. MM contributed in collecting plant sample, confection of herbarium, analysis of the data and drafted the manuscript. JA contributed to editing of the manuscript and conducting the laboratory work. SAE and ZT designed the study, supervised the laboratory work and contributed to critical reading of the editing manuscript. All the authors have read the final manuscript and approved the submission.

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