RESEARCH ARTICLE

Cytotoxic and Apoptotic Effects of Extracts of *Artemisia ciniformis* Krasch. & Popov ex Poljakov on K562 and HL-60 Cell Lines

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Abstract

***Artemisia*, as one of the largest genera in the tribe Anthemideae of the Asteraceae comprises an important part of Iranian flora. While cytotoxic and apoptotic properties have already been reported for some species of the genus there is not any report on cytotoxic effects of *A. ciniformis*. Petroleum ether (40-60), dichloromethane, ethyl acetate, ethanol and ethanol-water (50:50) extracts of the aerial parts of *A. cinformis* were subjected to cytotoxic and apoptotic evaluations on two cancer human cell lines (K562 and HL-60) and on J774 normal cells. Among multiple extracts evaluated for cytotoxicity, dichloromethane (CH_2Cl_2) and petroleum ether (PE) extracts were shown to possess the highest anti-proliferative effects on HL-60 and K562 cells with IC_{50} values of 31.3 and 25.5 μg/ml respectively. Apoptosis induction verified by sub-G1 peaks was seen in flow cytometry histograms. Increase in the amount of Bax protein, formation of DNA fragments, and cleavage of PARP to 24 and 89kDa sub units all confirmed induction of apoptosis by *A. cinformis* extracts. Taken together according to the result of the present study some extracts of *A. cinformis* could be considered as sources for natural cytotoxic compounds and further mechanistic and phytochemical studies are recommended to fully understand the underlying mechanisms of cnacer cell death as well as identification of responsible phytochemicals.**

Keywords: *Artemisia ciniformis* - asteraceae leukemic cell lines - petroleum ether extract - dichloromethane extract

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Introduction

Increase in prevalence of cancer cases has been associated with the expansion of industrial, chemical and radioactive pollutants, also the gap between human being and nature (Colditz and Wei, 2012; Fasinu and Orisakwe, 2013). Despite the progress in the field of medicinal chemistry and development in the synthesis of several new therapeutic compounds, the role of plants and phytochemicals can not be ignored in pharmaceutical sciences (Cerella et al., 2014). Due to the presence of compounds with cytotoxic and anti-tumor activity in some plants and significant efficacy of plant derived compounds in inhibition and treatment of cancer, as well as the amazing diversity of natural compounds in plants, researches to find new phytochemicals and to evaluate their biological effects is essential and a research priority (Tayarani-Najaran et al., 2011; Wegiera et al., 2012; Sehitoglu et al., 2014).

Artemisia is a genus belonging to the family Asteraceae Compositae with commonly fragrant species. Some species such as *A. dracunculus* L. are kitchen plants and used for culinary purposes. *A. absinthium* L. and *A. vulgaris* L. have long been implicated as medicinal herb, while some others are cultivated as ornamental species (Bora and Sharma, 2011). Various species of *Artemisia* have been extensively studied for their biological activities. Among the about 400 species of the genus, 34 species are grown in Iran, with two being endemic to this country (Ghahreman and Attar, 1999; Emami and Aghazari 2011). Some species of the genus *Artemisia* have been utilized widely for food and drug purposes in both traditional and modern markets. They have value as food additives for culinary purposes, antimicrobial or antiviral (Aniya et al., 2000; Kordali et al., 2005a; 2005b), antihepatotoxic, anti-malarial and anti-inflammatory agents (Gilani et al., 2005; Bora and Sharma 2011; Abad et al., 2012). *Artemisia* is a rich source of plant phytochemicals, including flavonoides, coumarins, sterols, polyacethylenes, mono- and sesquiterpenes, and sesquitrpene lactones (Tan et al., 1998; Bora and Sharma, 2011). *Artemisia ciniformis* Krasch. & Popov ex Poljakov. is one of *Artemisia* species growing wildly in Iran (Ghahreman and Attar, 1999). Volatiles from the aerial parts of *A. ciniformis* were identified as myrcene

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(19.3%), linalool (13.5%), camphor (13.1%) and davanone (12.6%) (Rustaiyan et al., 2007). Another study on the composition of the essential oil of *A. ciniformis* has revealed the presence of davanone (29.6%), myrcene (14.4%), camphor (10.6%), *p*-cymene (9.6%), and linalool (8.6%) (Firouzni et al., 2008). Dichloromethane fraction of *A. ciniformis* showed the highest cytotoxicity on the gastric cancer cell line in comparison with six other *Artemisia* species (Emami et al., 2010). Further study on *A. ciniformis* extracts showed the outstanding cytotoxicity of dichloromethane fraction against a wide range of cancer cell lines (Taghizadeh Rabe et al., 2011). Significant *in vitro* leishmanicidal activity for the ethanol extract of *A. ciniformis* has been reported (Emami et al., 2012). Nontoxic concentration of ethyl acetate, ethanol and ethanol-water (1:1 v/v) extracts of *A. ciniformis* have shown protective effect on H_2O_2-induced cytotoxicity in H9c2 cells (Mojarrab et al., 2013). According to the cytotoxic effects reported for *A. ciniformis*, it is of value to study the mechanisms responsible for this cell toxicity. To the best of our knowledge, there has not been any similar study done on the different fractions of *A. ciniformis* and its mechanism on cell death. As apoptosis is the desired pathway of cell death in cancer treatment we have designed this study to elucidate if apoptosis is involved in growth inhibitory activity of *A. ciniformis* extracts.

Materials and Methods

Reagents and chemicals

AlamarBlue® (resazurin) from Sigma (Saint Louis, MO, USA); RPMI-1640 and FBS from Gibco; β-actin and PARP antibodies, anti-rabbit IgG and HRP linked antibody from CellSignaling technology (Boston, USA); ECL Western blotting detection reagent from Bio-RaD (USA); the fluorescent probe propidium iodide (PI), protease inhibitor cocktail, phosphatase inhibitor cocktail, sodium citrate, Triton X-100, phenylmethylsulfonyl fluoride and QuantiPro BCA Assay Kit from Sigma (Steinheim, Germany); All the solvents used for extraction were purchased from Caledon and Scharlau.

Plant material

Aerial parts of *A. ciniformis* were collected from Tandoureh National Park (Razavi Khorasan province, Iran) in September 2010. Samples were identified by Dr. V. Mozaffarian (Research Institute of Forest and Rangelands, Tehran, Iran). The voucher specimen (No. 12569) has been deposited in the herbarium, Department of Pharmacognosy, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

Preparation of extracts and fractions

The dried powdered aerial parts (80 g) of *A. ciniformis* were extracted with petroleum ether (40-60), dichloromethane, ethyl acetate, ethanol and ethanol-water (1:1 v/v) respectively (sequential maceration with 3× ca. 0.8 L of each solvent). The extracts were filtrated with filter paper and dried using rotary evaporator at a reduced pressure at a temperature below 45 0C to yield 4.14, 9.08, 0.37, 2.54 and 16.87 g of each extract.

Cell cultures and treatment agent

HL-60 and K562 human leukemic cancer cell lines were purchased from Pasture Institute (Tehran, Iran) and preserved in RPMI-1640 medium with 10% v/v fetal bovine serum and 100 u/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5 % CO_2 and 95% of air.

In vitro Cell Viability

The alamarBlue® is composed of resazurin, a reagent which can be reduced in live cells indicating the rate of viability in culture cells. Once resazurin is converted to reduced form (resorufin) the ratio of red color product is proportional to live cells (O'Brien et al., 2000). About 5×10^4 K562 and 10^5 HL-60 cells were seeded in each well of 96-microwell plate and subjected to treatment with various concentrations of each extract of *A. ciniformis*. J774 cell line was selected to use as non-malignant cell line. After 48h incubation, alamarBlue® was added to each well according to the manufacturer's instructions. After 4h incubation, the cell viability was determined by reading the absorbance at 570 nm and 600 nm using an ELISA microplate reader (Awareness, Palm City, FL, USA). The cytotoxicity of *A. ciniformis* extracts was expressed as IC_{50}, which was calculated using Graph Pad Software (Graph Pad prism 5 software) and presented as mean±SEM of three separate experiments with three replicates for each concentration of *A. ciniformis* extracts.

PI staining

The method was performed according to the previously reported assay (Tayarani-Najaran et al., 2013; Nicoletti et al., 1991). Briefly, 10^6 K562 and HL-60 cells were seeded in each well of a 24-well plate and treated with petroleum ether (12.5, 25 and 50 μg/ml) and CH_2Cl_2 (25, 50 and 100 μg/ml) extracts of *A. ciniformis* for 48h. 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 plus 0.1% Triton X-100) before flow cytometric analysis using a flow cytometer (Becton Dickinson). 104 events were acquired with flow cytometer.

Western blotting analysis

About 10^7 HL-60 cells were treated with different concentrations of petroleum ether and CH_2Cl_2 extracts (12.5, 25 and 50 µg/mL of the first extract, 25, 50 and 100 µg/mL of the second one) for 48h. The cells rinsed and harvested using cool PBS for three times, the cell pellet was resuspended in a lysis buffer containing 50 mM Tris-HCl (PH=7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.2% SDS, 1% Protease inhibitor cocktail, 1% phosphatase inhibitor cocktail and 1 mM phenylmethylsulfonylfluoride and left on ice for 30 min. After centrifugation at 10000 rpm for 20 min at 4ºC, the cell lysate was gathered and protein concentration was determined according to the Bio-Rad Protein Assay kit. Balanced amount of proteins was subjected to 12% SDS-page (W/V). The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and subjected to immunoblotting using Bax, β-actin and PARP antibody

as primary antibodies while anti-rabbit IgG and HRP-linked antibody were used as secondary antibodies. Bcl-2. Bax protein band and PARP cleavage in HL-60 cells were detected by enhanced chemiluminescence using the 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

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

Results

Cytotoxicity of various extracts

Different extracts of *A. ciniformis* were examined for cytotoxic potential on K562, HL-60 and normal cell lines (J774). These cells incubated in 37°C and 5% CO_2 with various concentrations of different extracts of *A. ciniformis* (25- 100 μg/ml) for 48h.

Results demonstrated that some extracts decreased cell viability in a concentration-dependant manner. Among different extracts, CH_2Cl_2 extract demonstrated the highest cytotoxicity on HL-60 cells while petroleum ether extract exhibited the most potent cytotoxic activity on K562 cells, but minimal effect on normal cells (Figure 1). IC_{50} values (μg/mL) for different extracts of *A. ciniformis* in HL-60 and K562 cells are presented in Table 1.

Table 1. IC_{50} values (μg/mL) for Different Extracts of A. Ciniformis in HL-60 and K562 Cell Lines.

	Extracts				
Cell line	PE	CH_2Cl_2	EtOAc	EtOH	EtOH/H_2O (50:50)
K562	25.53	>200	>200	>200	>200
HL-60	105.20	31.33	165.20	>200	>200

Apoptosis induction by CH_2Cl_2 and petroleum ether extracts

Apoptosis in K562 and HL-60 cell lines was detected with flow cytometry by the aid of PI staining test. Cells incubated with various concentrations of CH_2Cl_2 (25, 50 and 100 μg/ml) and petroleum ether (12.5, 25 and 50 μg/ml) extracts of *A. ciniformis* for 48h. Comparison of Sub-G1 peak of treated cells to that of untreated control cells in flow cytometry histograms confirmed the induction of apoptosis in treated cells (Figure 2).

Western blotting

The cleavage of 116 kDa PARP-1 to 89 and 24 kDa fragments was used as an indicator of apoptosis. 48h treatment of HL-60 cells with different concentrations (25, 50 and 100 μg/ml) of CH_2Cl_2 extract resulted in cleavage of aforementioned protein. The same result was recorded after treatment of K562 cells with different concentrations (12.5, 25 and 50 μg/ml) of petroleum ether extract (Figure 3). Bax proteins play a determining role to control the release of cytochrome c and to initiate the apoptosis via the mitochondrial pathway (Renault and Manon, 2011). Different concentrations of dichloromethane (25, 50 and 100 μg/ml) and petroleum ether (12.5, 25 and 50 μg/ml) extracts enhanced the expression of Bax protein in HL-60 and K562 cells cells in a concentration-dependent manner respectively (Figure 3).

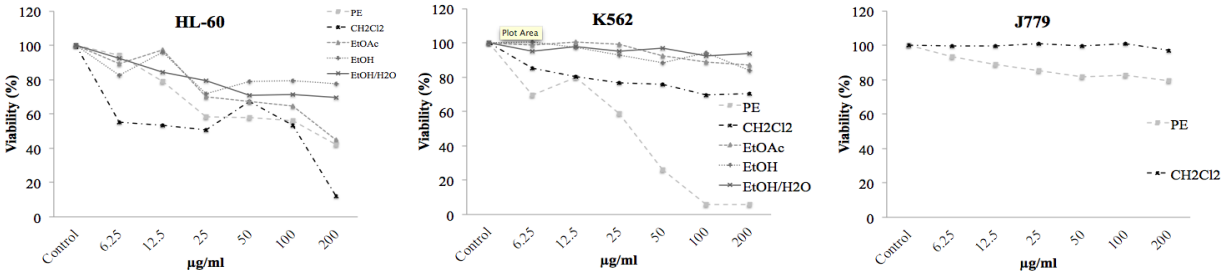

Figure 1. The Dose-Dependent Effects of Different Extracts of *A. ciniformis* on the growth of K562 and HL-60 cells as well as normal J774 cells. PE, CH_2Cl_2 and EtOAc extracts exhibited cytotoxic activity against apoptosis-proficient HL-60 and apoptosis-resistant K562 cells with much less cytotoxic effects on normal J774 cells. Values were mean±SEM of at least three independent experiments, each in triplicates

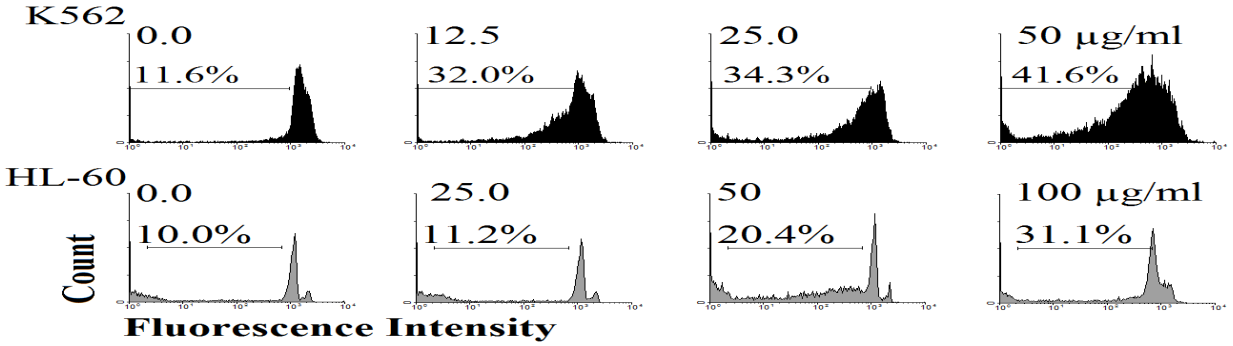

Figure 2. PI Staining and Flow Cytometric Analysis of CH_2Cl_2 Extract (25, 50, 100 μg/ml) Induced Apoptosis in HL-60 cells and Petroleum Ether Extract (12.5, 25, 50 μg/ml) Induced Apoptosis in K562

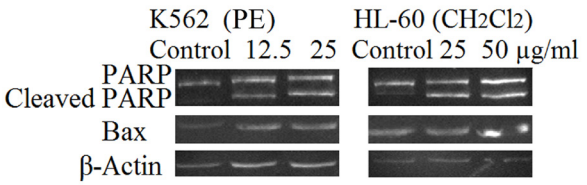

Figure 3. Proteolytic Cleavage of Poly (ADP-ribose) Polymerase (PARP) and Increasing Expression of Bax Protein in HL-60 after 48h Exposure to CH_2Cl_2 Extract (25, 50 µg/ml) and K562 Cells after 48h Exposure to Petroleum ether Extract (12.5 and 25 µg/ml). β-Actin was used as a loading control. All Western blots were representative of three independent experiments

Discussion

This is the first mechanistic study on the cytotoxicity and apoptosis induction by *A. ciniformis*. However, other studies have been done on the cytovtoxicity of the different species of the plant *Artemisia*. Concentration dependent cytotoxicity of the essential oil of *A. indica* against four cancer cell lines A-549, THP-1, Caco-2 and HEP-2 has been reported (Rashid et al., 2013). Essential oils of *A. absinthium* which contained higher amounts of trans-sabinyl acetate and cis/trans-thujones exhibited more toxicity on brine shrimp (Judzentiene et al., 2012). Moreover, the essential oils of *A. capillaris* and *A. herba-alba* are regarded as good candidates for further study, because they showed significant anti-proliferative activity against the human oral cancer and acute lymphoblastic leukaemia (CEM) cell lines respectively (Cha et al., 2009; Tilaoui et al., 2011). Dichloromethane extracts of *A. annua* exhibited higher cytotoxicity in comparison with methanol extracts against HeLa cancer cells (Efferth et al., 2011). The CH_2Cl_2 fraction from *A. sacrorum* was more cytotoxic than eight other fractions towards HepG2, HT-29 and MCF-7 cells (Piao et al., 2012). Ethanol extracts of *A. argyi, A. annua* and *A. afra* exhibited activity against P388 murine leukemia, molt-4 human leukemia, U937, and HeLa cancer cell lines, respectively (Lee and Vairappan, 2011; Singh et al., 2011; Spies et al., 2013). Similar studies on potential use of *Artemisia* genus and its active constituents in cancer therapy reported the synergistic cytotoxicity of artesunate from the traditional Chinese herb *Artemisia annua* in combination with triptolide or allicin in pancreatic cancer or osteosarcoma cell lines respectively (Liu and Cui, 2013; Jiang et al., 2013).

In our study, we have shown that petroleum ether and CH_2Cl_2 extracts from *A. ciniformis* were cytotoxic and apoptotic against HL-60 and K562 cells. The highest cytotoxic effect of these two extracts among the other solvent extracts used in this study shows the ability of petroleum ether and CH_2Cl_2 solvents to extract potent phytochemicals, which may be due to the semi/non polarity nature of the solvents.

In the present study, some extracts of *A. ciniformis* showed apoptosis inducing activity that has confirmed the need for further analytical and mechanistic studies of the plant. As we expected the cytotoxicity of the extracts on HL-60 cells were more than that was observed in K562 cells. This is associated with the differences between these two cell lines (Munker et al., 1997). K562 cells lack Fas receptors and are apoptosis resistant while HL-60 cells are apoptosis proficient.

Once apoptosis cascade is started, signals from either extrinsic or intrinsic pathway promote enzymatic activation of substrates. Caspase are cysteine enzymes, which play the most important role in apoptosis promotion. Each of the extrinsic or intrinsic pathways recruits their own mediators. While caspase 9 is the mediator of the mitochondrial pathway, death receptors join to caspase 8 to activate the enzyme activation. Caspase 8 can affect mitochondria via truncation of Bid, which interrupts the membrane potential. The mitochondrial membrane potential is also affected by changes in the balance of presented in the bilayer membrane space of pro-apoptotic and anti-apoptotic proteins mitochondria. Both the extrinsic and connect via activation of 3, which create changes in critical nuclear enzyme activity and DNA degradation that lead to programmed mode of cell death (Parsons et al., 2010). In our study *A. ciniformis* could increase the amount of Bax protein and the cleaved form of PARP.

Our results are in agree with previous reports about the potency of CH_2Cl_2 extracts of different species, which may point to the similar chemicals present in the various species of the genus *Artemisia* that are responsible for cytotoxic effects.

Taken together, we can introduce *A. ciniformis* as a potent plant with cytotoxic effects against human leukemia cell lines. However, further analytical and mechanistic studies on the plant and its various species are essential to fully identify the chemicals responsible for this cytotoxic effect and to understand the pathway affected by the plant.

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