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. ciniformis* 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 (CH₂Cl₂) and petroleum ether (PE) extracts were shown to possess the highest anti-proliferative effects on HL-60 and K562 cells with IC₅₀ 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 subunits all confirmed induction of apoptosis by *A. ciniformis* extracts. Taken together according to the result of the present study some extracts of *A. ciniformis* could be considered as sources for natural cytotoxic compounds and further mechanistic and phytochemical studies are recommended to fully understand the underlying mechanisms of cancer cell death as well as identification of responsible phytochemicals.

Keywords: *Artemisia ciniformis* - asteraceae leukemic cell lines - petroleum ether extract - dichloromethane extract
(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%) (Firoozni 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,O₂-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, phenylmethylsulfonylfluoride 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 3x 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°C 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 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ 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 5x10⁵ K562 and 10⁵ 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 IC₅₀, 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, 10⁶ 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 CH₃Cl (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 10⁷ HL-60 cells were treated with different concentrations of petroleum ether and CH₃Cl 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 4°C, 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 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% CO₂ 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, CH₂Cl₂ 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). IC₅₀ values (µg/mL) for different extracts of *A. ciniformis* in HL-60 and K562 cells are presented in Table 1.

Apoptosis induction by CH₂Cl₂ 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 CH₂Cl₂ (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).

**Table 1. IC₅₀ values (µg/mL) for Different Extracts of A. Ciniformis in HL-60 and K562 Cell Lines.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PE</th>
<th>CH₂Cl₂</th>
<th>EtOAc</th>
<th>EtOH</th>
<th>EtOH/H₂O (50:50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>25.53</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>HL-60</td>
<td>105.20</td>
<td>53.13</td>
<td>165.20</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

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 CH₂Cl₂ 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).
Cl2 extracts of different species, which induces apoptosis in KB cells via which contained higher amounts of CH2Cl2, 7058 HL-60 cells were more than that was observed in K562 plant. As we expected the cytotoxicity of the extracts on polarity nature of the solvents. phytochemicals, which may be due to the semi/non petroleum ether and CH methanol extracts against HeLa cancer cells (Efferth et al., 2009; Tilaoui et al., 2011). Dichloromethane extracts of leukemia (CEM) cell lines respectively (Cha et al., 2009). Essential oils of A. annua exhibited higher cytotoxicity in comparison with methanol extracts against HeLa cancer cells (Efferth et al., 2011). The CH2Cl 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. atra 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 artemesate from the traditional Chinese herb Artemisia annua in combination with triptolide or allicin in pancreatic cancer or osteosarcoma cell lines (Piao et al., 2012). The CH2Cl2 extracts of A. ciniformis were cytotoxic and that are responsible for 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 CH2Cl2 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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