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Effects of root, shoot, leaf and seed extracts of seven *Artemisia* species on HIV-1 replication and CD4 expression

Hassan Mohabatkar[1*], Mandana Behbahani[1], Mohammad Reza Rahimi Nejad[2]

[1]*Department of Biotechnology, Faculty of Advanced Sciences and Technologies, University of Isfahan, Isfahan 81746-73441, Iran*

[2]*Faculty of Science, University of Isfahan, Isfahan 81746-73441, Iran*

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ABSTRACT

Objective: To investigate the effects of flower, leaf, shoot and root extracts of seven *Artemisia* species on peripheral blood mononuclear cells (PBMCs) toxicity and HIV-1 replication.
Methods: The studied *Artemisia* species were *Artemisia absinthium, Artemisia khorasanica, Artemisia deserti, Artemisia fragrans, Artemisia aucheri, Artemisia sieberi* and *Artemisia vulgaris*. The activity of these plant extracts on HIV-1 replication and CD4 expression was performed by HIV-1 p24 antigen kit and flow cytometry respectively.
Results: The results demonstrated that flower extracts of all species increased PBMCs number more than shoot, leaf and root extracts. However, the frequency of CD4 expression in PBMC was not increased in the presence of all flower extracts. The flower extracts of all species had inhibitory effect on HIV-1 replication.
Conclusions: In conclusion, the results demonstrated that flower extracts of *Artemisia* species are good candidates for further studies as anticancer agents.

1. Introduction

Artemisia species (Asteraceae family) are small herbs that grow in dry and semi-dry regions[1]. Five hundred species of this genus are widely distributed in Asia, Europe and North America. It has been reported that most species of this genus are found in Iran[2,3]. Researchers throughout the world have approved the great medicinal values of the genus *Artemisia*. Pharmacological investigations of *Artemisia* species have led to the detection of novel biologically active compounds such as monoterpenes, sesquiterpenes, lactones, flavonoids and coumarins from *Artemisia annua*[1,4,5]. This plant has been used in Iranian traditional medicine as anti-infectious and anti-bacterial agent[6]. The extract of some *Artemisia* species such as *Artemisia absinthium* (*A. absinthium*), *Artemisia vulgaris* (*A. vulgaris*), *Artemisia incana, Artemisia fragrans* (*A. fragrans*) and *Artemisia spicigera* have been reported to have potent cytotoxic effects on breast cancer cells[7-9]. Some studies reported that essential oils obtained from *Artemisia* species have strong inhibitory activities against the pathogenic bacteria[10,11]. However, there is no scientific study available about anti-HIV-1 activity of Iranian *Artemisia* species. Therefore, we became eager to assess *Artemisia* species with respect to their anti-HIV-1 activity. Several studies have been reported on anti-HIV-1 and antibacterial activity of natural products. However, current anti-HIV-1 drugs have a lot of disadvantages including resistance and toxicity[12]. So the development of drugs that inhibit diverse steps of viral replication and improve immunologic parameters is essential. Screening anti-HIV compounds from natural products may be one of the effective ways to discover new drugs. The present study is focused to evaluate the effect of different part extracts of Iranian *Artemisia* species on HIV-1 replication and

*Corresponding author: Hassan Mohabatkar, Department of Biotechnology, Faculty of Advanced Sciences and Technologies, University of Isfahan, Isfahan 81746-73441, Iran.
Tel: +98 3137934391
Fax: +98 3137932342
E-mail: h.mohabatkar@ast.ui.ac.ir
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peripheral blood mononuclear cell (PBMC) proliferation.

2. Materials and methods

2.1. Plant material

Seven species of *Artemisia* [*A. absinthium, Artemisia khorasanica* (*A. khorasanica*), *Artemisia deserti* (*A. deserti*), *A. fragrans, Artemisia aucheri* (*A. aucheri*), *Artemisia sieberi* (*A. sieberi*) and *A. vulgaris*] were collected from Isfahan and Azerbaijan agriculture centers, Iran in October 2012. These species were recognized after a series of taxonomic revisions. The voucher specimens were deposited at the Herbarium of National Botanical Garden of Iran.

2.2. Preparation of extract

All species were separated into flower, leaf, shoot and root parts. The plant material was carefully dried and powdered. The dried plant samples (50 g) were separately placed in a stopped conical flask and macerated with 250 mL methanol (98% v/v. Merck, Germany) at room temperature (25–28 ˚C) for 72 h with occasional stirring. The solvent was filtered and evaporated in a vacuum rotary evaporator at 45 ˚C. The residue was dried by a freeze dryer (Zirbus, Germany).

2.3. Cells and viruses

Blood samples were collected from three healthy donors into ethylene diamine tetraacetic acid and sodium heparin tubes. PBMCs were isolated by Lymphodex density centrifugation. The cells were cultured in RPMI-1640 media supplemented with 15% fetal bovine serum, penicillin solution (100 µg/mL), streptomycin (100 µg/mL) and L-glutamine (2 mmol/L). All of the reagents were purchased from Gibco (Germany). The PBMCs were incubated in 37 ˚C and 5% CO_2 condition. A single-cycle HIV-1 stock was obtained from Pasture Institute, Tehran. The viruses were stored in medium containing dimethyl sulfoxide (DMSO) in liquid nitrogen (-70 ˚C).

2.4. Human PBMC cytotoxicity assay

The cellular toxicity of PBMCs were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at concentrations of 0, 10, 50, 100, 250, 500, 1 000 and 2 000 µg/mL[13]. The extracts were initially dissolved in DMSO at subtoxic concentrations (maximum of 0.019%). The cells were grown in a 96-well plate at a density of 5×10^5 cells per well. After 2 h, cells were treated with different concentrations of samples and incubated for 48 h. Later, 25 µL of the MTT solution (5 mg/mL) was added to each well, and plate was re-incubated for 2 h. Finally, 50 µL of PrOH/HCl/TX (0.04 mol/L HCl in 2-propanol plus 10% Triton X-100) was added to solubilize the formed formazan crystals. The plate was again re-incubated for 12 h and amount of formazan crystal was determined by measuring the absorbance at A540 nm using a microplate spectrophotometer (Awareness Technology Inc., stat fax 2100).

2.5. Analysis of CD4 by flow cytometry

The FACScan flow cytometer was used to determine the percentage of CD4 lymphocyte value and the intensity of CD4 expression in PBMCs in the presence of eight *Artemisia* flower extracts. The cells were cultured in 24-well plates for 72 h at 37 ˚C. Then cells were washed and stained with phycoerythrin conjugated monoclonal antibody specific to human CD4 (Cyto Matin Gene, Iran) for 20 min at 4 ˚C.

2.6. Anti-HIV-1 activity

Anti-HIV-1 activity of the extracts was carried out as described previously by Behbahani[14]. Briefly, PBMCs were plated in 24-well plates at a concentration of 6×10^5 cells per well. Then, PBMCs were infected with 0.5 multiplicity of infection of HIV-1 and flower extracts of seven species of *Artemisia* at three concentrations (1 000, 500 and 250 µg/mL). Then cells were incubated at 37 ˚C for 12 h. Then infected cells were washed and overlaid with medium and mentioned extracts. Zidovudine (AZT) (2.5, 1 and 0.5 µg/mL) was used as positive control. After 3 days of incubation, the overlay medium was used to detect and quantify HIV-1 p24 core protein via HIV-1 p24 antigen kit. At the end, supernatant was transferred to the coated 96-well plate for the p24 assay. The protocol was followed as described by the manufacturer, with absorbance measured at 450 nm.

2.7. Statistical analysis

Each experiment was performed in triplicate and repeated three times. The experiments were performed using completely randomized design and results were analyzed by One way ANOVA using SPSS software (version 22). The half-maximal cytotoxic concentration (CC_{50}) and half maximum effective concentration (EC_{50}) values were estimated using regression line analysis in Microsoft Excel 2007.

3. Results

3.1. Human PBMC cytotoxicity assay

Different parts of these seven species of *Artemisia* were tested

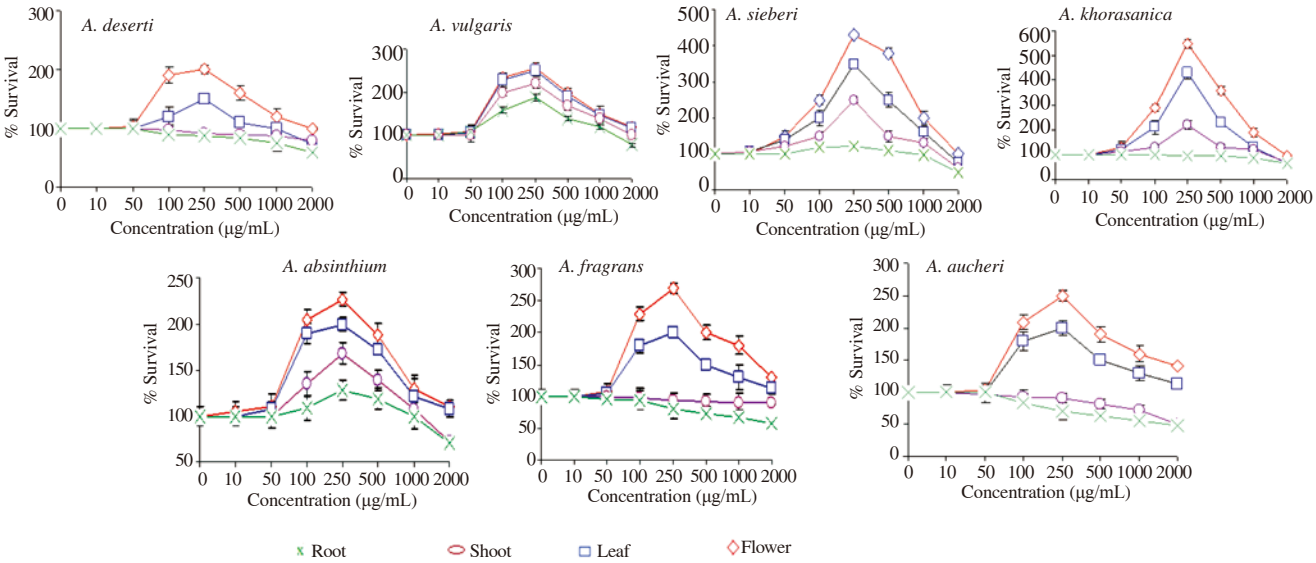

Figure 1. Effect of different extracts of seven *Artemisia* species on PBMC proliferation.
Each bar represents the mean ± SD of three independent experiments.

for cytotoxicity against PBMCs (Figure 1). The results showed that the extracts of different parts of *Artemisia* species increased PBMC number in a dose dependent manner up to 250 μg/mL. The flower extracts of all species increased PBMCs number more than shoot, leaf and root extracts. The increasing effect was respectively followed by leaf, shoot and root extracts of all species. Among these seven species, flower extracts of *A. sieberi* and *A. khorasanica* strongly increased PBMC's number up to 4.3 and 5.5 folds respectively. Flower and leaf extracts of these species didn't have any cytotoxicity up to 2 000 μg/ml. The root and shoot extracts showed cytotoxicity at 2 000 μg/mL and CC_{50} of all extracts was attained at > 2 000 μg/mL.

3.2. Effect of flower extract on expression of CD4

The effects of flower extract of these seven species of *Artemisia* on frequency and the average mean fluorescent intensity (MFI) of $CD4^+$ T cells in PBMCs were summarized in Table 1. The results showed that the flower extracts of these species had no effect on the frequency and the MFI values of $CD4^+$ T cells in PBMCs.

Table 1
Effect of flower extracts of *Artemisia* species on the MFI and frequency of $CD4^+$ T cells in PBMC.

Sample	$CD4^+$ cells	
	Frequency (%)	MFI
Control (treated with DMSO)	41.07	29.31
A. absinthium	40.40	30.05
A. khorasanica	39.49	30.56
A. deserti	41.25	31.42
A. fragrans	40.24	31.51
A. aucheri	40.84	31.82
A. sieberi	39.08	31.72
A. vulgaris	42.67	33.72

3.3. Antiviral activity of flower extracts of Artemisia species

The anti-HIV-1 activity of flower extracts of *Artemisia* species was investigated at concentrations of 250, 500 and 1 000 μg/mL. Results (Figure 2) showed that among these species, flower extracts of *A. vulgaris* and *A. absinthium* inhibited HIV-1 replication in a dose dependent manner. The EC_{50} values of *A. vulgaris* and *A. absinthium* extracts were 350 and > 1 000 μg/mL respectively. The other extracts did not show any antiviral activity. As a control, AZT, a potent HIV-1 inhibitor, showed higher anti-HIV-1 activity.

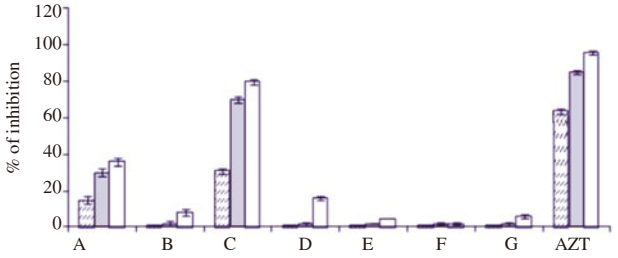

Figure 2. Effects of flower extracts of *Artemisia* species at different concentrations of 250 (hatched column), 500 (gray column) and 1 000 μg/mL (white column) on HIV-1 replication in PBMCs.
A: *A. absinthium*; B: *A. fragrans*; C: *A. vulgaris*; D: *A. deserti*; E: *A. aucheri*; F: *A. sieberi*; G: *A. khorasanica*. AZT (during infection) at concentrations of 0.5 (hatched column), 1 (gray column) and 2.5 μg/mL (white column) have been used as positive control. The 50% inhibitory concentration (EC_{50}) of each extract was calculated using regression line. Each bar represents the mean ± SD of three independent experiments.

4. Discussion

The results of this study demonstrated that flower extract of *A. absinthium* and *A. vulgaris* have potent anti-HIV activities. The

anti-HIV activity of these plants has been reported here for the first time.

Rabe and van Staden demonstrated that the extracts of *A. absinthium* and *A. vulgaris* can prevent growth of bacteria[15]. These species have also been proved to inhibit the growth of breast cancerous cells in a concentration dependent way[16]. According to literature, some compounds such as tannins, flavonoids and terpenoids were identified in these two species[16,17]. Some flavonoids and terpenoids compounds have been previously reported as potent anti-HIV-1 agents due to their significant anti-HIV-1 activity and low toxicity. These compounds could inhibit reverse transcription process, virus entry and integrase and protease activities[18-20]. Our results also confirmed that flower extracts of all species could increase PBMC proliferation. But all species do not have any effects on frequency and MFI values of CD4 expression in PBMC. So these species may increase the values of other lymphocyte cells. Some results demonstrated that stimulation of T cells *in vivo* could be related with a decrease in viral replication. Additional evidence suggested that potent T cell stimulation may improve production of chemokines and other antiviral cytokines[21]. Nevertheless, further studies are needed to prove the mechanism of these extracts. In conclusion, all the data demonstrated that the extracts of *A. absinthium* and *A. vulgaris* might be good candidates for *in vivo* evaluation of anti-HIV-1 therapies.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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