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***Original Article***

# Comparison of the cytotoxic effects of different fractions of *Artemisia ciniformis* and *Artemisia biennis* on B16/F10, PC3 and MCF7 Cells

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## *Abstract*

**Background and purpose:** *Artemisia* is one of the well-known herbal medicinal plants for antimicrobial, insecticidal, antioxidant, and antimalarial activities. The antiproliferative effects of dichloromethane extracts of *Artemisia biennis* (*A. biennis*) and *A. ciniformis* and the petroleum ether extract of *A. ciniformis* have been demonstrated previously on human cancerous cell lines. In the current study, further fractionation was carried out on the aforementioned extracts and their cytotoxic effects were evaluated on three human cancer cell lines; B16/F10, PC3, and MCF7. F1 to F16, F1' to F11', and F1" to F10" were resulted from the fractionation of dichloromethane extracts of *A. biennis, A. ciniformis,* and petroleum ether extract of *A. ciniformis,* respectively.
**Experimental approach:** The cytotoxic effects of 16 (F1-F16), 11 (F1'-F11') and 10 (F1"-F10") fractions, on B16/F10, PC3, and MCF7 cell lines were assessed using resazurin to measure viability and propidium iodide staining (sub G1) and flow cytometry to detect apoptosis.
**Findings / Results:** The results showed that, some fractions at 100 µg/mL decreased cell viability. F2" in B16/F10 cells, F2, F4-F6, F10', F11', and F2" in PC3 cells, and F10', F11', and F2" in MCF7 significantly decreased cell viability in a concentration-dependent manner (12.5-50 µg/mL). Among different fractions, F2" demonstrated the most potent cytotoxic effects on cancer cell lines ($P < 0.001$). All of the mentioned fractions (except F11' on PC3 cells) increased the number of apoptotic cells and showed the cytotoxic effects on cancer cells compared with the control group.
**Conclusion and implications:** *A. biennis* and *A. ciniformis* are suggested as the potential sources of cytotoxic phytochemicals. The probable presence of terpenoids, steroids, and alkaloids in the selected fractions is proposed based on the preliminary phytochemical study.

***Keywords:*** Apoptosis; *Artemisia biennis; Artemisia ciniformis*; Cytotoxic; Viability.

## INTRODUCTION

Cancer is known as one of the major leading causes of mortality worldwide especially in less economically developed countries (1). One of the most common types of cancer throughout the world is melanoma, multifactorial disease with increased prevalence (2,3). The incidence of melanoma is 10 to 60 cases per 100,000 populations; in developed countries (4). Melanoma patients have an increased risk of other common cancers such as prostate or breast cancer (5).

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*Artemisia* belongs to the family Asteraceae (Compositae) is one of the well-known herbal medicinal plants (6). As a heterogenous genus, with over 500 diverse species, *Artemisia* is distributed mainly in Europe, Asia, and North America (7). Due to antimicrobial, insecticidal, antioxidant, and antimalarial activities of active compounds of different *Artemisia* species, they are used widely for medical purposes (8). *Artemisia ciniformis* (*A. ciniformis*) Krasch. & Popov ex Poljakov and *A. biennis* Willd. are two species grow in Iran (9). Phytochemical investigations on volatile oil of *A. biennis* led to the identification of camphor and (E)-beta-farnesene as the major components from the plant species growing in Iranian and Canadian habitats, respectively (10,11). Monoterpenoids such as camphor, myrcene, linalool, and sesquiterpenoids like davanone have been reported as the main constituents in the volatile oil of *A. ciniformis* (12,13,14). The leishmanicidal (15), antimalarial (16), free radical-scavenging, and cytoprotective (17,18,19) effects of both species have been previously reported. The *in vitro* cardioprotective (20), antimicrobial and tyrosinase inhibitory effects (21) have been reported from the extracts and essential oil of *A. ciniformis*, as well.

Dichloromethane (DCM) extract of *A. biennis* has shown cytotoxic activities (22,23) and similar effects have been observed for DCM and petroleum ether (PE) extracts of *A. ciniformis* (22,24,25). In continuation of our previous studies (23,25), further fractionation of these extracts was carried out and cytotoxic effects of the fractions were evaluated on B16/F10, PC3, and MCF7 cell lines as three most known types of cancer. B16/F10, PC3, and MCF7 cells are widely used as accepted models for the investigation of melanoma, prostate, and breast cancer, respectively (2).

## MATERIALS AND METHODS

### *Plant material*

The aerial parts of *A. ciniformis* Krasch.& Popov ex Poljakov, and *A. biennis* Willd. were collected from Tandooreh National park, and Zoshk (Razavi Khorasan province, I.R. Iran), respectively. Samples were identified by Dr. Valiollah Mozaffarian (Research Institute of Forest and Rangelands, Tehran, I.R. Iran). The voucher specimens (Nos. 12569 and 12570, respectively) have been deposited at the herbarium, Department of Pharmacognosy, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, I.R. Iran.

### *Extraction and fractionation*

Powdered aerial parts of *A. biennis* (50 g) and *A. ciniformis* (40 g) were successively extracted using PE (40:60) and DCM by maceration method. The concentrated PE extract of *A. ciniformis* (2.15 g) and DCM extracts of both species (3.61 g and 4.60 g, respectively) were fractionated by normal phase vacuum liquid chromatography on silica gel using a step gradient of hexane:ethyl acetate (10: 0 to 0:10) or heptane:ethyl acetate (10: 0 to 0:10). The procedure afforded 16 (F1-F16), 11 (F1'-F11'), and 10 (F1"-F10") fractions from DCM extract of *A. biennis*, DCM extract of *A. ciniformis*, and PE extract of *A. ciniformis*, respectively.

### *Thin-layer chromatographic analysis of selected fractions*

Thin-layer chromatography (TLC) separations of F5, F10', and F2" were performed using commercially available plates (silica gel 60 $F_{254}$-precoated TLC plates; Merck, Germany) and suitable solvent systems at room temperature. For the detection of the main compounds, different spray reagents (Dragendorff, potassium hydroxide, vanillin-sulphuric acid, and Liebermann-Burchard reagents) were used. The detection of the characteristic components was also performed *via* observing the developed chromatograms under long-wave and short-wave UV light (366 and 254 nm, respectively) (26).

### *Cell cultures and treatment*

The mouse skin cancer B16/F10 (C540), the human breast carcinoma MCF7 (C135), and the human prostate carcinoma PC3 (C427) cell lines were obtained from Pasture Institute (Tehran, I.R. 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 (90%) containing 5% $CO_2$ at 37 °C (23).

***Cell proliferation (resazurin) assay***

Resazurin (AlamarBlue®) is an indicator dye which is used to detect the rate of the viability of various human and animal cell lines, bacteria, and fungi (18). B16/F10, MCF7, and PC3 cells were seeded in 96-microwell plate at $4 \times 10^3$ cells per well. After 24 h incubation, cells were treated with 100 µg/mL of 16 (F1-F16), 11 (F1'-F11'), and 10 (F1"-F10") fractions from DCM extracts of *A. biennis* and *A. ciniformis*, and PE extract of *A. ciniformis*. For each cell line, there was a negative control sample, which remained untreated and received an equal volume of the culture medium.Then, based on the results of the previous stage, B16/F10, MCF7, and PC3 cells were subjected to treat with 12.5, 25, and 50 µg/mL of the selected fractions from DCM extracts of *A. biennis* and *A. ciniformis*, and PE extract of *A. ciniformis*. After 24 h incubation, 20 µL AlamarBlue® (0.01% w/v in phosphate-buffered saline, PBS) was added to each well. After 4 h in culture, the absorbance at 570 and 600 nm was measured using the enzyme-linked immunosorbent assay (ELISA) microplate reader (BioTek, Winooski, USA) (19).

***Propidium iodide staining***

Propidium iodide (PI) is a DNA-binding dye that is used to detect the rate of apoptotic cells based on DNA fragmentation that so-called sub-G1 peak by flow cytometry (23). B16/F10, MCF7 and PC3 cells ($2\times10^4$) were seeded in each well of a 12-well plate and after 24 h, incubated with the selected concentration (25 µg/mL) of the potent cytotoxic fractions from DCM extracts of *A. biennis* and *A. ciniformis*, and PE extract of *A. ciniformis* (based on viability). After 24 h incubation, cells were washed with PBS, harvested, and incubated at 4 °C in the dark place with 400 µL of hypotonic buffer (50 µg/mL PI in 0.1% sodium citrate and 0.1% Triton™ X-100) for 30 min before flow cytometric analysis (BD Biosciences, CA, USA) (23).

***Statistical analysis***

Data are presented as means ± SEM and differences were considered significant when $P < 0.05$. One way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test was used for data analysis.

## RESULTS

***TLC analysis of selected fractions***

The developed chromatograms of all selected fractions (F5, F10', and F2") showed fluorescence quenching zones under short-wave UV light while none of them showed fluorescence in UV-366 nm before and after spraying ethanolic potassium hydroxide reagent. The sprayed TLC plates with vanillin-sulphuric acid and Liebermann-Burchard reagents after heating for 10 min at 100 °C were inspected in UV-366 nm or visible and the presence of compounds with visible colors and characteristic fluorescences was noticed. Only in the TLC plate of F5, a dark zone under UV-254 nm appeared as a light orange-brown area, immediately on spraying Dragendorff reagent.

***Cytotoxicity of various fractions***

The cytotoxic potential of 100 µg/mL of 16 (F1-F16), 11 (F1'-F11'), and 10 (F1"-F10") fractions from DCM fractions of *A. biennis* and *A. ciniformis*, and PE fractions of *A. ciniformis* were evaluated on B16/F10, MCF7, and PC3 cells lines using resazurin. As shown in Fig. 1, some fractions (100 µg/mL) decreased cell viability ($IC_{50}$ values of those fractions shown in Table 1). F2" ($P < 0.001$) in B16/F10 cells, F2 ($P < 0.001$), F3 ($P < 0.01$) and F4-F6 ($P < 0.001$), F8'-F9' ($P < 0.01$), F10'-F11' ($P < 0.001$), and F2" ($P < 0.001$) in PC3 cells and F2 ($P < 0.01$), F5-F6 ($P < 0.01$), and F15 ($P < 0.01$), F9' ($P < 0.01$), F10'-F11' ($P < 0.001$) and F2" ($P < 0.001$) in MCF7 significantly decreased cell viability in a concentration-dependent manner (12.5-50 µg/mL) (Fig. 1d-1f). Among different fractions from DCM extract of *A. biennis*, DCM extract of *A. ciniformis*, and PE extract of *A. ciniformis* F2" demonstrated the most potent cytotoxic effects on cancer cell lines.

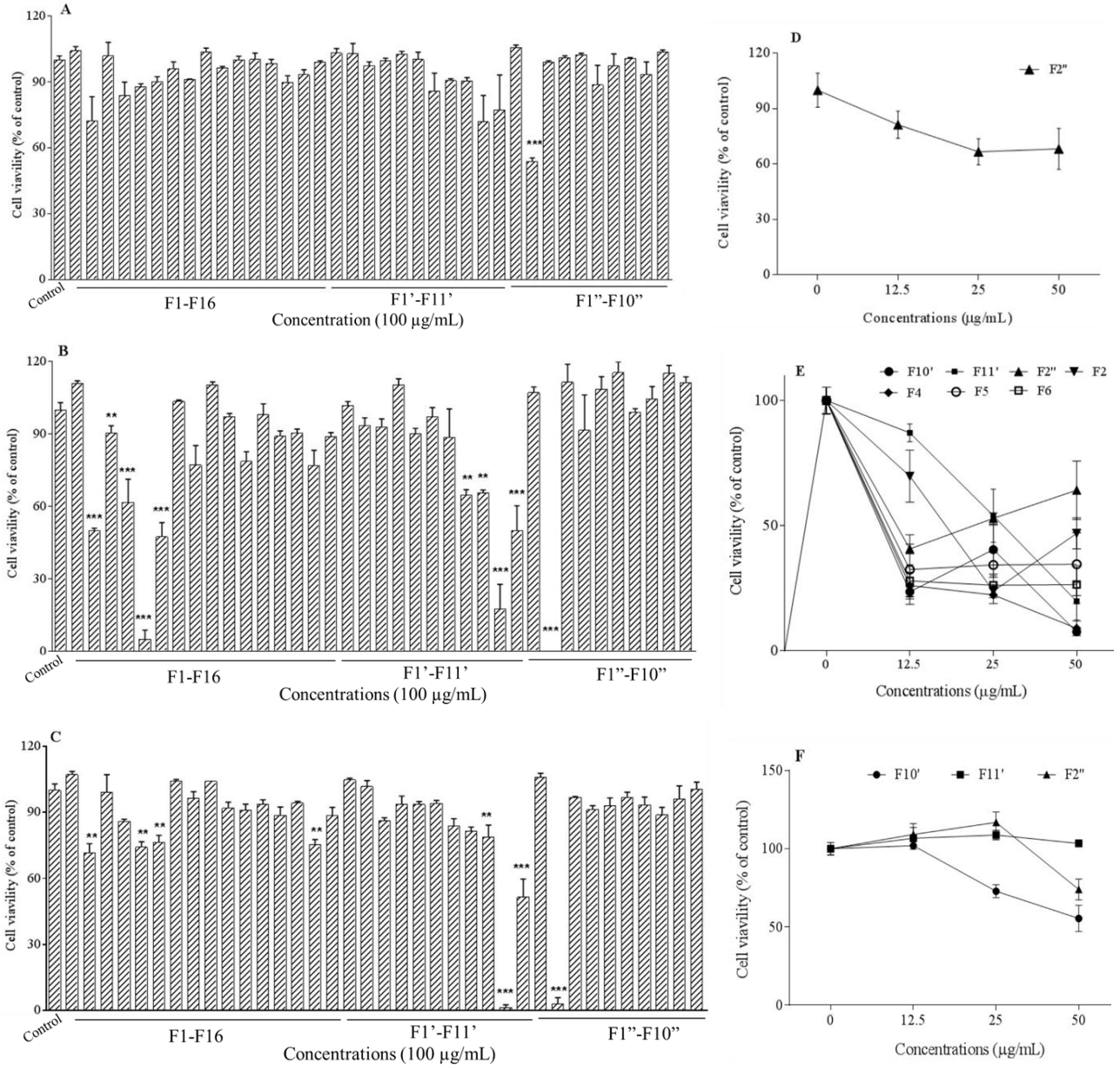


**Fig. 1.** The cytotoxic effects of 100 µg/mL of 16 (F1-F16), 11 (F1'-F11'), and 10 (F1"-F10") fractions from DCM extract of *A. biennis* and of *A. ciniformis*, and PE extract of *A. ciniformis* on (A) B16/F10, (B) PC3, and (C) MCF7 cells. The cytotoxic effects of various concentrations (0-50 µM) of F2" fraction of PE extract of *A. ciniformis* on the viability of (D) B16/F10 cells, (E) F2, F4-F6 fractions of DCM extract of *A. biennis*, F10', F11' fractions of DCM extract of *A. ciniformis* and F2" fraction of PE extract of *A. ciniformis* on the viability of PC3 cells, and (F) F10', F11' fractions of DCM extract of *A. ciniformis* and F2" fraction of PE extract of *A. ciniformis* on the viability of MCF7 cells. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 indicate significant differences compared to the control group. Data represent mean ± SEM, n = 3. DCM, Dichloromethane; PE, petroleum ether.

**Table 1.** The cytotoxic effects of selected fractions from dichloromethane extract of *A. biennis* (F2-F6), and *A. ciniformis* (F10' and F11'), and petroleum ether extract of *A. ciniformis* (F2") on B16/F10, PC3, and MCF7 cells. Data are presented as mean ± SEM.

| Cell lines | $IC_{50}$ (µg/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **F2** | **F4** | **F5** | **F6** | **F10'** | **F11'** | **F2"** |
| B16/F10 | > 70 | > 70 | > 70 | > 70 | > 70 | >7 0 | 53.69 ±2.9 |
| PC3 | 50.07 ± 1.5 | 61.64 ± 13.6 | 7.6 ± 6.6 | 47.35 ± 10.3 | 26.40±12.46 | 56.71±27.6 | 1.54±0.7 |
| MCF7 | >70 | >70 | >70 | >70 | 4.15 ± 1.09 | 51.39 ±11.7 | 8.50 ±2.0 |

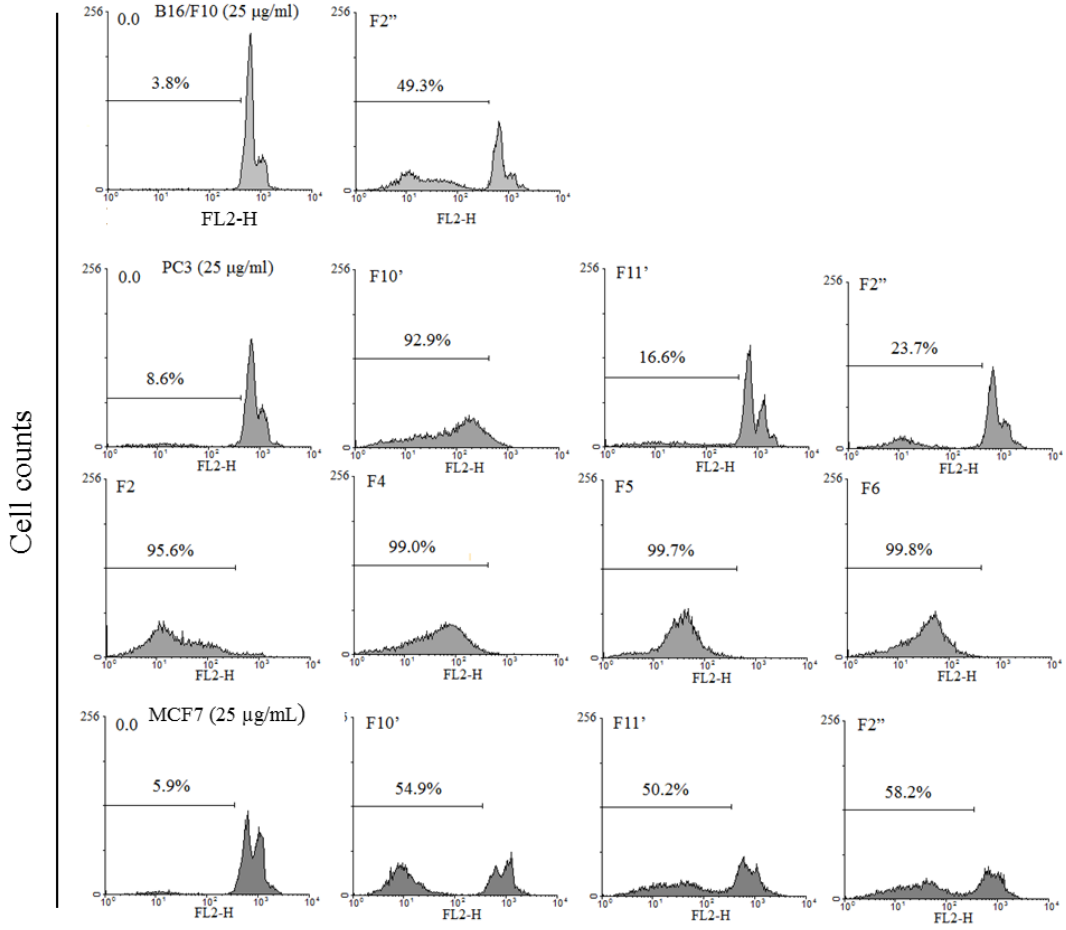


**Fig. 2.** Flow cytometry histograms of apoptosis assays by propidium iodide method of (A) B16/F10, (B) PC3, and (C) MCF7. All components induced cell death. (except for F11' on PC3)

***Apoptosis induction by various fractions***

PI staining was used to detect the antiproliferative effects of selected fraction-based on the results of the previous stage on B16/F10, MCF7, and PC3 cells. All of the mentioned fractions at 25 µg/mL (except for F11' on PC3) increased the number of apoptotic cells and showed the cytotoxic effects on cancer cells compared with those of untreated cells (Fig. 2).

## DISCUSSION

Natural product derivatives comprised approximately more than half of currently-used anticancer agents (27). Cytotoxic activity of different species of Iranian flora like *A. aucheri* (28), *A. persica,* and *A. turcomanica* (29) have been studied. Thirty-seven fractions derived from DCM extracts of *A. biennis* and *A. ciniformis* and PE extract of *A. ciniformis,* were subjected to cytotoxic assay, in this study. Results showed that in B16/F10 cells, a fraction of PE extract of *A. ciniformis* (F2", $P < 0.001$), and in PC3 cells, three fractions of DCM extract of *A. biennis* (F2, $P < 0.001$), F4 ($P < 0.05$)- F6 ($P < 0.001$)), two fractions of DCM extract of *A. ciniformis* (F10'($P < 0.01$), F11'($P < 0.05$)) and a fraction of PE extract of *A. ciniformis* (F2" ($P < 0.01$)) at 12.5-50 µg/mL significantly decreased cell viability in a concentration-dependent manner. Two fractions of DCM extract of *A. ciniformis* (F10', F11') and a fraction of PE extract of *A. ciniformis* (F2") at concentrations of 12.5-50 µg/ml decreased cell viability of MCF7 cells in a concentration-dependent manner, significantly. In flow cytometry, all of the mentioned fractions at 25 µg/mL (except for F11'on PC3) showed the apoptotic effects on cancer cells. Among different fractions from DCM extracts of *A. biennis* and *A. ciniformis,* and PE extract of *A. ciniformis*, F2" demonstrated the most potent cytotoxic and apoptotic effects on cancer cells.

This fraction can be selected as the only active sample on all of the studied cell lines.

Despite many reports about the cytotoxic and apoptotic properties of various extracts of different species of the genus *Artemisia*, few studies have demonstrated the apoptogenic activities of *A. ciniformis* and *A. biennis* (19,23). DCM and ethyl acetate extracts of *A. ciniformis* have been able to exert cytotoxic effects against AGS, MCF-7, and HeLa cell lines (22,24). In another study, the highest toxicity against HL-60 and K562 cell lines was exhibited by DCM and PE extracts of *A. ciniformis*, respectively (25). Cytotoxicity and apoptotic effects of DCM extract of *A. biennis* against K562 and HL-60 cancer cell lines have been previously reported, as well (23). Various types of secondary metabolites have been able to exert cytotoxity toward the cancerous cell lines. The flavonoid jaceosidin from *A. princeps* induced cell death *via* activation Akt apoptosis pathway (30). Koyuncu documented that, phenolic content of the methanol extract of *A. absinthium* L. induced ROS-dependent apoptosis in human colon (DLD-1) and endometrium (ECC-1) cancer cells (31). The isolated sesquiterpenoid lactones from the genus *Artemisia*, like artemisinin and dihydroartemisinin, have been able to induce apoptosis in different cancerous cell lines (32,33). In contrast to the terpenoid extract of *A. ciniformis*, no sesquiterpene lactone has been detected in that of *A. biennis* (34). TLC analysis of the current study showed the probable presence of terpenoids and steroids as the main components of F10' and F2" (from *A. ciniformis*) while in F5 (from *A. biennis*), it seemed the existing of terpenoids and steroids was accompanied by the presence of fewer amounts of alkaloids. There were no evidences about the presence of significant amounts of phenolics in all selected fractions. On the other hand, the cytotoxic potential of davanone-type sesquiterpenoids (35) and the presence of them in the essential oil of *A. ciniformis* (12) may help to justify the results of the current study. Further phytochemical study on the active fractions including F5, F10', and F2" should be performed to recognize the pure component(s) responsible for the cytotoxic activity of the fractions.

## CONCLUSION

The probable presence of terpenoids, steroids, and alkaloids might be related to the reported cytotoxic effects on cancerous cell lines after exposure to selected fractions of PE and DCM extracts of *A. ciniformis* and *A. biennis.* Both species are suggested as the potential sources of cytotoxic phytochemicals.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest for this study.

## AUTHORS' CONTRIBUTION

E. Ramazani performed the experiments, computations, analysis of the data, and wrote the manuscript. Z. Tayarani-Najaran, Y. Shokoohinia, and M. Mojarrab conceived, designed, and supervised the project, wrote the manuscript, provided financial support, and final approval of the manuscript.

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