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Fractions of a chloroform extract of ajenjo leaves (*Artemisia mendozana* DC. var. *mendozana*) inhibit the proliferation, viability and clonogenicity of B16-F0 melanoma cells

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Abstract: The ajenjo, *Artemisia mendozana* DC. var. *mendozana* (Asteraceae), grows in the Andean foothills of Mendoza and San Juan, Argentina, and is used as a medicinal plant for its antispasmodic and antifungal properties. The aim of this work was to obtain fractions of a chloroform extract of ajenjo leaves and to evaluate the *in vitro* effects on proliferation, viability and clonogenicity of B16-F0 melanoma cells. Using a silica gel chromatography column, 120 fractions were collected and grouped according to the chromatographic profile in 9 main fractions (F1–F9). Their major compounds identified were: terpenes (F1), terpenes and sesquiterpene lactones (F2–F3), sesquiterpenes (F4–F6) and phenols and sesquiterpenes (F7-9). B16-F0 cells were incubated for 72 h with DMSO (vehicle) or 0.1 mg/ml F1–F9. At 72 h of culture, F1 decreased both the growing index (GI) and cell viability. F2 and F3 both decreased GI and only F3 decreased clonogenic activity. F4 and F5 both decreased GI. Only F5 decreased cell viability and F4 decreased clonogenicity. Consequently, fractions F6–F8 did not affect any of the cell parameters assayed, while F9 decreased cell viability and inhibited clonogenicity.

Introduction

Argentina has a rich floral biodiversity; the native medicinal flora consists of 1529 taxa. One hundred and fifteen of these taxa are endemic and the Asteraceae is the richest family among medicinal taxa and medicinal endemic species (Barboza *et al.*, 2009).

Artemisia is a genus of small herbs and shrubs found in temperate regions. It belongs to the diverse family Asteraceae, one of the most numerous plant groupings, which comprises about 1000 genera and over 20000 species. Within this family, *Artemisia* belongs to the tribe Anthemideae, which comprises over 100 genera and more than 1200 species, mainly found in Asia, Europe and America (Bora and Sharma, 2011).

Artemisia species are most commonly shrubs, rarely perennial herbs and more rarely annual or biennial herbs. Many *Artemisia* species have economic value in several medical fields, as an antihelminthic (*A. santonicum* and related taxa; Valles and McArthur, 2001), *A. annua* and *A. mexicana* as antimalarial (Dhingra *et al.*, 2000; Malagon *et al.*, 1997). Artemisinin (obtained from *A. annua*) selectively kills human breast cancer cells (Singh and Lai, 2001).

Artemisia mendozana DC *mendozana* (Fig. 1), popularly known as ajenjo, is a medicinal plant of the Andean foothills of Mendoza and San Juan provinces, Argentina (Márquez, 1999). Leaves, flowers and shoots are employed in folk medicine as infusions to treat liver and stomach aches (Bustos *et al.*, 1996). The antibacterial properties of the ethanolic extract of *A. mendozana* inhibited *in vitro* the growth of several Gram-positive and Gram-negative bacteria (Feresin *et al.*, 2000); also the essential oil of *A. mendozana* has antiviral (Duschatzky *et al.*, 2005) and trypanocidal activities (Cimador *et al.*, 2019).

The aim of this work was to test the *in vitro* effects of fractions of an ajenjo chloroform extract on the proliferation, viability and clonogenicity of mouse melanoma B16-FO cells.

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Materials and Methods

Plant material

Aerial parts from wild plants of ajenjo. *A. mendozana DC* var. *mendozana*, were collected in the hills near the Potrerillos town (Luján de Cuyo, Mendoza: 32°57'S, 69°11'W, ~1400 masl) on April 2016. A voucher specimen was deposited at the Herbarium Ruiz Leal (MERL) under n° 38214.

Chloroform extract

Ajenjo leaves (300 g) were washed with chloroform for 20 s and the chloroform suspension was dried to obtain a chloroform crude extract pellet. In order to partially eliminate phenolic compounds from the extract, the pellet was suspended with chloroform and alkalized with 1% sodium hydroxide (pH = 8). A colored upper phase of phenolic salts was removed and the suspension was acidified with hydrochloric acid (1%), washed with distilled water and dried in a rotary evaporator. The pellet containing a high concentration of medium polarity compounds (medium polarity compounds pellet) was loaded to a silica gel chromatography column.

Silica gel chromatography

Afterwards, 1.5 g of medium polarity compounds pellet were suspended in 5 ml of mobile phase (5:2 v/v hexane : ethyl acetate) and seeded in a column containing 33 g of Silica gel (230–400 mesh) equilibrated with petroleum ether. The separation was performed isocratically with the mobile phase. Column fractions of 5 ml each were collected and analyzed by thin layer chromatography (TLC). Eluted fractions were grouped according to the similarity of their chromatographic profiles in main fractions. Retention factor (Rf), distance traveled by the spot/distance traveled by mobile phase, were established for each spot for the purpose of comparing the fractions obtained.

Thin layer chromatography (TLC)

Aluminum sheets, 10 × 10 cm Silica gel 60 F254 with fluorescent indicator UV_{254} (Merck, Germany), were loaded with 2 µL of each fractions and cholesterol (employed as Rf reference); a mixture of hexane: ethyl acetate (5:3, v/v) was employed as mobile phase. The chromatograms were visualized under UV 254 nm and a solution of p-anisaldehyde was employed for the chemical identification of spots.

Cells, treatments and assays

B16-F0 murine melanoma cells were a kind gift of Dr. Gabriel Rabinovich (IBYME, CONICET, Buenos Aires). They were cultured in RPMI medium (RPMI1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 50 U/ml of penicillin, 50 µg/ml streptomycin, 50 µg/ml ciprofloxacin) in a humidified incubator with 5% CO_2 at 37° C. Cells were harvested after reaching a confluence of 70–80% and 1 × 10^4 cells were plated in 12 well plates with RPMI medium and left for 15 h for adhesion.

For cell proliferation, viability and clonogenic survival assays, the medium was removed, the cells washed with PBS, incubated with RPMI medium and treated with 10 µl of 10 mg of main fractions/ml of DMSO (0.1 mg/ml final fraction concentration) 72 h. For controls, 10 µl of the vehicle (DMSO) was added.

For cell proliferation assays a direct cell count in each well was made every 24 h, during 72 h since the start of treatments, using a Nikon Eclipse TE 2000U inverted microscope (Nikon, Japan), by means of a 20× objective lens. Five independent experiments were performed. The results were expressed as the growing index (GI, number of cells found in a particular case over the number of cells seeded at time 0 of treatment).

For clonogenic survival assay, cells that had been treated for 72 h, were trypsinized, and were seeded in 12 well multiwell plates (250 cells/well) and were cultured in RPMI medium for 10 days (at this time the colonies were large enough to be clearly appreciated). Then the colonies were fixed with 100% methanol for 30 min, and were stained with a filtered 0.5% crystal violet water solution (Sigma-Aldrich, USA) for 10 min, washed, and counted. Percent clonogenicity was calculated as the number of colonies over number of cells seeded, times 100. Three independent experiments were performed.

For cell viability assays, 5 × 10^3 B16-F0 cells/well were seeded in 96 well multiwell plates and treated 72 h as mentioned above. For MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay, cells were washed with PBS and 0.2 ml RPMI medium containing 0.5 mg/ml MTT (Sigma-Aldrich, USA) was added to each well and incubated at 37°C for 2 h. Then, DMSO was added to dissolve the MTT-derived formazan which was quantified by measuring the absorbance at 562 nm wavelength (Liu *et al.*, 1997).

Statistical analysis

The data shown are the mean ± SEM of 3–5 independent experiments. Statistical analysis was performed using 2-way ANOVA and 2-tails Student's *t* test (Prism 5, GraphPad Software Inc., USA). The differences were considered significant for: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Results

Fractions obtained by column chromatography of chloroform extract

One hundred and twenty fractions, 5 mL each, were collected and the fractions showing a similar chromatographic profile on TLC were mixed and the solvent evaporated to yield 9z main fractions (dubbed as main fractions F1–F9) (Figs. 2A and 2B).

The qualitative composition and relative quantity of main fractions F1–F9 are shown in Tab. 1. A first observation under UV 245 nm (Fig. 2A) and after the chemical development (Fig. 2B) indicated the following: main fraction F1 presented a blue spot with 90% relative intensity (RI) of Rf = 0.96, corresponding to terpenes (Fig. 2B, line 1. Main fractions F2 and F3 showed two spots, one of terpenes (blue colored, Rf = 0.96) and the other of sesquiterpene lactones (brown colored, Rf = 0.64). RIs corresponded to 30% and 40% terpenes and 60% and 70% sesquiterpene lactones for the main fractions F2 and F3 respectively (Fig. 2B, lines 2, 3). Main fractions F4 and F5 showed a purple spot (Rf = 0.44), corresponding to sesquiterpenes. RIs were 70% and 85% of sesquiterpenes for F4 and F5, respectively (Fig. 2B, lines 4, 5). Main fraction F6 also showed a purple spot of sesquiterpenes, and another unidentified spot (gray, 0.30 Rf) (Fig. 2B, line 6). Main

FIGURE 1. Aerial parts of ajenjo, *Artemisia mendozana* DC. var. *mendozana*.

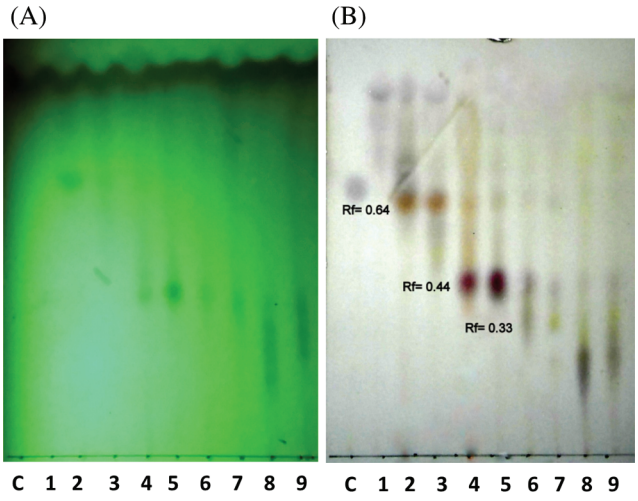

FIGURE 2. TLC of main fractions of the ajenjo chloroform extract. (A, B) Chromatogram of F1–F9, cholesterol employed as reference substance. (A) Plate visualized under at UV-254 nm and (B) plate revealed with p-anisaldehyde. Rf positions (Rf = 0.64, Rf = 0.44 and Rf = 0.33) are indicated.

TABLE 1

Qualitative chromatographic profile of the chloroform fractions of the extract of ajenjo leaves

Compound groups	Main fraction	Column fractions collected	Qualitative chromatographic profile	Cellular effects		
				Cell Proliferation	Cell viability	Clonogenicity
Terpenes	1	15–28	90% Terpenes (Rf = 0.96)	Decreased	Decreased	No changed
Terpenes-sesquiterpene-lactones	2	29–34	30% Terpenes (Rf = 0.96) 70% Sesquiterpene lactones (Rf = 0.68)	Decreased	No changed	No changed
	3	35–44	40% Terpenes (Rf = 0.96) 60% Sesquiterpene lactones (Rf = 0.68)	Decreased	No changed	Decreased
Sesquiterpenes	4	45–53	70% Sesquiterpenes (Rf = 0.44)	Decreased	No changed	Decreased
	5	54–61	85% Sesquiterpenes (Rf = 0.44)	Decreased	Decreased	No changed
	6	62–71	60% Sesquiterpenes (Rf = 0.44) 40% Unidentified compounds (Rf = 0.30)	No changed	No changed	No changed
Sesquiterpenes-phenols	7	72–80	50% Sesquiterpenes (Rf = 0.44) 50% phenols (Rf = 0.33)	No changed	No changed	No changed
	8	81–96	10% Sesquiterpenes (Rf = 0.44) 50% phenols (Rf = 0.33) 40% Unidentified compounds (Rf = 0.24)	No changed	No changed	No changed
	9	97–120	10% Sesquiterpenes (Rf = 0.44) 30% Phenols (Rf = 0.33) 60% Unidentified compounds (Rf = 0.24)	Decreased	Decreased	Decreased

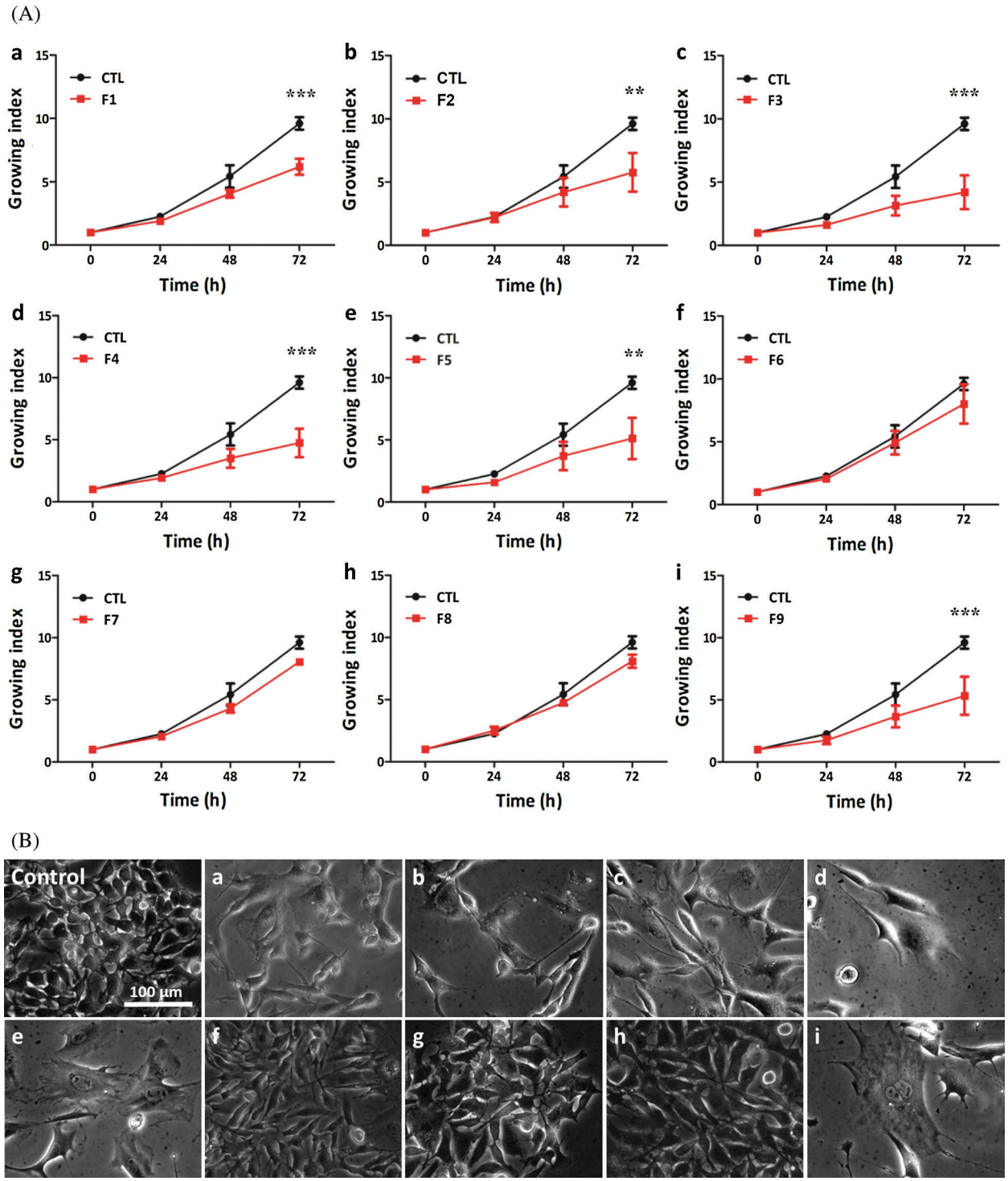

FIGURE 3. Main fractions of the ajenjo chloroform extract inhibit B16-F0 cell proliferation. (A, B) B16FO cells were incubated with 0.10 mg/ml of fractions: F1 (a), F2 (b), F3 (c), F4 (d), F5 (e), F6 (f), F7 (g), F8 (h), F9 (i) and with DMO, for 72 h and counted every 24 h. (A) The number of cells was expressed as a growing index (GI) (number of cells at a given time/number of cell at 0 time of incubation). Data are the mean ± SE of 5 independent experiments $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control group (10 µl DMSO). (B) Representative images of the cells at 72 h of treatment.

fraction F7 also showed a purple spot of sesquiterpenes (50%) and another Rf = 0.33 yellow spot of phenols (Fig. 2B, line 7).

Finally, main fractions F8 and F9 contain sesquiterpenes (10% in both), phenols (50% and 30% respectively) and a Rf = 0.24 green spot of unidentified compounds (40% and 60% respectively) (Fig. 2B, lines 8, 9).

According to the characteristic of the compounds in the main fractions (see Fig. 2 and Tab. 1), they were classified in 4 groups: terpenes group (F1), terpenes-sesquiterpene lactones group (F2–F3), sesquiterpenes group (F4–F6) and sesquiterpenes-phenols group (F7–F9).

With the purpose of analyzing whether main fractions alter cell proliferation, cell viability and clonogenic capacity, B16-F0 cells were separately cultured in presence of each of the nine main fractions. The main results were summarized in Tab. 1.

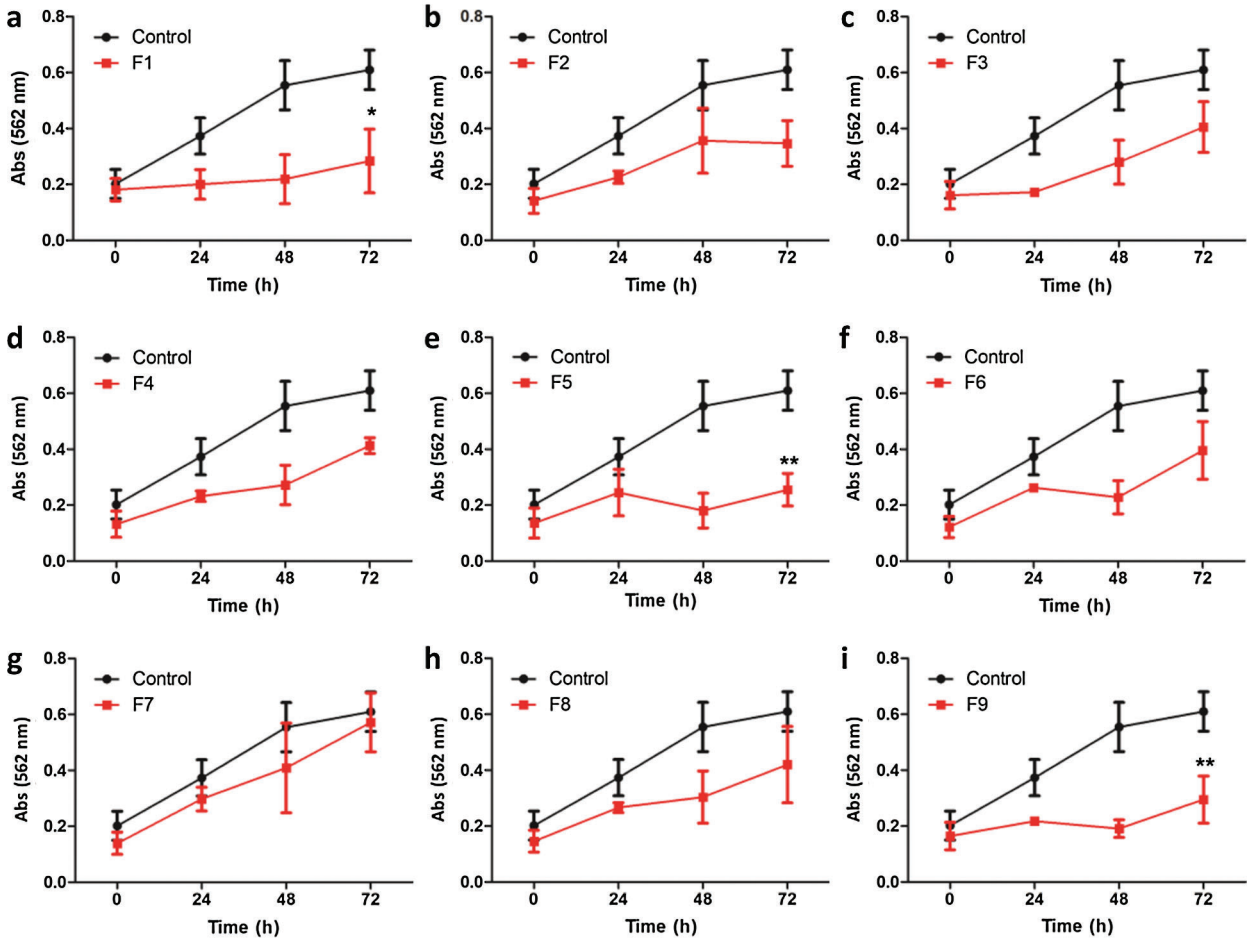

FIGURE 4. Main fractions of the ajenjo chloroform extract inhibit B16-F0 cell viability. B16FO cells were incubated with 0.10 mg/ml of fractions: F1 (a), F2 (b), F3 (c), F4 (d), F5 (e), F6 (f), F7 (g), F8 (h), F9 (i) and with DMSO, for 72 h. Every 24 h MTT-derived formazan was assayed at 562 nm wavelength. Data are expressed as the absorbance (562 nm) mean ± SE of 4 independent experiments $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control group (DMSO).

Effect of terpenes group (F1)

The treatment of B16-F0 cell with F1 decreased both GI (36%) (Fig. 3A,a) and cell viability (53%) (Fig. 4a), indicating that the F1 containing terpenes (90% RI) inhibited cell proliferation and generated cell dead.

Effects of terpene-sesquiterpene lactones group (F2 and F3)

The treatment of B16-F0 cell with F2 and F3 both decreased GI (40% and 56% respectively) (Fig. 3A,b,c) and only F3 decreased clonogenic activity (43.9%) (Figs. 5A and 5B, F3).

These results indicate that the combination of terpenes and sesquiterpene lactones inhibit cell proliferation. It is deemed that the inhibition of cell proliferation and the decrease of clonogenicity require a terpenes relation higher than 30% RI.

Effects of sesquiterpenes group (F4–F6)

The treatment of B16-F0 cell with F4 and F5 both decreased GI (51% and 47% respectively) (Fig. 3A,d,e). Only F5 decreased cell viability 58% (Fig. 4e) and F4 decreased clonogenicity (15.1%) (Figs. 5A and 5B, F4). F6 did not affect any parameter assayed (Figs. 3A,f, 4f, 5A and 5B, F6). These results indicate that sesquiterpenes in a RI of 85% (as those in F5) are needed to inhibit cell proliferation and generate cell dead.

F4, which contained 70% RI of sesquiterpenes (Tab. 1) inhibited cell proliferation and affected cell clonogenicity, while F5, containing 85% RI of sesquiterpenes (Tab. 1) inhibits cell proliferation and also affected cell viability. Apparently, a sesquiterpene higher RI than 70% was needed to affect cell viability.

Regarding F6, which contained 60% RI of sesquiterpenes and 40% RI of unidentified compounds (Rf = 0.3) (Tab. 1) did not inhibit cell proliferation nor cell clonogenicity. Also, F6 did not affect cell viability. It is possible that a high RI of sesquiterpenes are needed to alter these cellular parameters, because F5 has higher RI of sesquiterpenes (60%) than that of F6, and that only F5 inhibited cell proliferation and cell clonogenicity. Another possibility is that the unidentified compounds (0.3 Rf) in F6, may interfere with sesquiterpene activity.

Effects of sesquiterpenes-phenols group (F7–F9)

Only F9 decreased cell viability (43%) (Fig. 4i) and inhibited clonogenicity (9.3%) (Figs. 5A and 5B, F9). Neither a high IR of sesquiterpenes in F7 (50%) nor a high IR of phenols in F8 (50%) had effect in the cell parameters assayed. Then, it is possible that the unidentified compound (Rf = 0.24) found in F9, with 60% RI, inhibited both cell viability and clonogenicity. It is possible that same cells died in apoptosis and others were arrested in senescence. It is interesting to mention that cell treated with F9 had an increased cell size (Fig. 3B,i) and it is known that senescent cells look flat and with an enlarged cytoplasm (Hwang *et al.*, 2009).

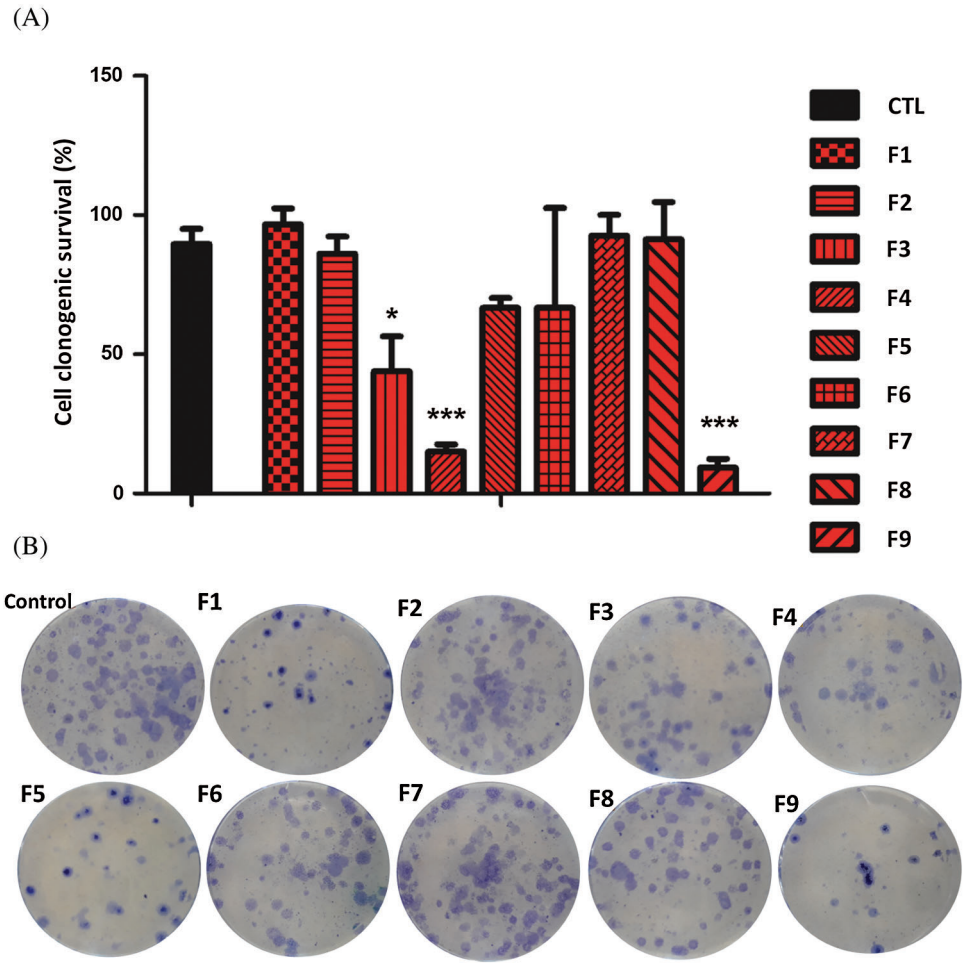

FIGURE 5. Main fractions of ajenjo chloroform extract inhibit clonogenic capacity of B16B0 cells. (A, B) B16-F0 Cells after incubation for 72 h with 0.1 mg/ml of F1-9 fractions and DMSO, were seeded in multiwell plates. After 10 days of culture, the surviving colonies were stained with crystal violet and the number assayed. (A). Data are expressed as the mean % cell clonogenic survival ± SE of 3 independent experiments $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control group (10 µl DMSO) (B). Representative images of B16-F0 cell colonies.

Discussion

Although the studies with *Artemisia* species show predominantly the bioactivity of fractions with a single type of medium polarity compounds, as sesquiterpene lactones, the effects of two types of compounds together were also shown. This is in parallel with the known interaction of phenolic compounds (flavonoids) with artemisinin, which improves the effectiveness of the treatment of parasitic diseases such as malaria and cancer (Ferreira *et al.*, 2010).

Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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