Anticancer effects of various Iranian native medicinal plants on human tumor cell lines*

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In this study the antineoplastic activity of methanolic extracts of six medicinal plants that are native to Iran, including *Galium mite, Ferulago angulata, Stachys obtusicrena, Cirsium bracteosum and Echinophora cinerea* was investigated. Different tumor cell lines were exposed to the extracts and cytotoxic analysis using MTT colorimetric assay was performed. Quantification of percentage of cells undergoing apoptotic changes by flow cytometry, and DNA fragmentation analysis on sensitive cell lines was then carried out. Results obtained indicated that almost all of the extracts more or less had the capacity to decrease the proliferation of tumor cells. Among the plants, the highest activity against K562 leukemia cell line was found for *E. cinerea and C. bracteosum* with IC50 less than 20 μg/ml followed by *G. mite* with IC50 of 39.8 μg/ml. *F. angulata* and *E. cinerea*, mostly inhibited Jurkat cells proliferation (IC50 less than 8 μg/ml). Fifty percent inhibition of Fen bladder cell carcinoma due to exposure to *F. angulata* and *E. cinerea* was found at concentrations of nearly 180 μg/ml. A549, a lung carcinoma cell, was mostly affected by *S. obtusicrena* (IC50 more than 200 μg/ml). In flow cytometry analysis, *C. bracteosum, E. cinerea, F. angulata* and *S. obtusicrena* extracts demonstrated no remarkable effects on the cell cycle profile of K562 and Jurkat cells. Moreover, in DNA fragmentation analysis of treated cells, no ladder formation was detected. In contrary, *G. mite* caused more than 40% apoptosis in the K562 and Jurkat cells. In DNA fragmentation analysis *G. mite* extract produced ladder formation in these cells. In conclusion, these results indicated that the extracts used in this study have anti tumor activity particularly against the leukemia cell lines and that apoptosis is the possible cause of cell death observed due to the extract of *G. mite*.

Key words: medicinal plants, cytotoxic activity, apoptosis

Some of the most useful antineoplastic drugs have been extracted from plants [1–4]. Vinblastin, vincristine, etoposide and taxol are plant-derived compounds that have been approved for use as to anticancer drugs [5]. During our course of study on different medicinal herbs we found some inhibitory effect of several plants on tumor cell proliferation. These plants that are native to Iran include *Galium mite, Ferulago angulata, Stachys obtusicrena, Cirsium bracteosum* and *Echinophora cinerea.*

Galium includes about 400 species and is the main herbaceous genus of the Rubiaceae and consists of mostly temperate species [6]. Among different species, *G. mite* is one that is native to Iran. Aerial parts of *G. mite* are traditionally used as sedative, tonic and stomachic.

The genus *Ferulago* (Apiaceae) is represented by 40 species in the world. Eight species exist in Iran of which three are endemic [7]. The plants of the *Ferulago* have been employed against ulcers, snake bite, as well as headache and diseases of the spleen [8]. *F. angulata* is a perennial, herbaceous plant that grows in different areas of Iran including southern parts and is used for infectious diseases [9]. *Stachys* (Lamiaceae) is distributed in mild regions of the Mediterranean and south-west Asia. About three hundred Stachys species are known [7, 10]. It is represented by thirty-four species in the flora of Iran which eighteen of them are endemic [11]. *S. obtusicrena* (Boiss) is one of the endemic species which has been used in Iranian folk medicine for treatment of inflammatory diseases and tumors [12]. *C. bracteosum*

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(Compositae) is endemic specie that grows in different parts of Iran and has been employed against ulcers, insect bite, as well as diarrhea in folk medicine [13]. *Echinophora* (Umbelliferae, subfamily Apioideae) is represented in the flora of Iran by four species including two endemic ones [9, 13]. One of the endemic species, *E. cinerea* (Boiss.) is found in some central and western provinces of the country [9]. Fresh and dried aerial parts of *E. cinerea* and some other species are added to cheese and yoghurt for flavoring.

The aim of the present study is to investigate the antitumor activity of these extracts on different tumor cell lines.

Material and methods

Preparation of the extracts. Aerial parts of the plants were collected from Booshehr and Yasuj areas in the south of Iran. Plants were identified by Dr. Azizollah Jafari from the Department of Botany, Central Research of Natural Resource and Animal Husbandry, Yasuj, Iran, and a voucher specimen was deposited there. The aerial parts were air dried in shade, powdered and defatted with petroleum ether for 4 hours. A methanol extract was obtained by maceration of the plant in 3x1500 ml methanol at room temperature for 48 hours. The methanol extract was filtered and concentrated under reduced pressure. The yield (w/w) of extracts was 8.3% for *G. mite*, 27.2% *F. angulata,* 15.1% *S. obtusicrena,* 12.3% *C. bracteosum* and 12.2% for *E. cinerea*. Dried extracts were later dissolved in DMSO followed by RPMI medium to obtain 20 mg/ml and mixed at 37 °C for 20 minutes. This solution was centrifuged to remove insoluble ingredients, and then the supernatant was passed through 0.22 µm filters for sterilization. The solution was diluted with the medium and prepared at different concentrations.

Cell lines. Tumor cell lines including, A549 (lung carcinoma), Fen (bladder carcinoma), K562 (myelogenous leukemia) and Jurkat (T cell leukemia) obtained from Iranian cell bank were used in this study. All the cell lines were kept in RPMI 1640 medium (Sigma, St, Louis, USA) supplemented with 10% fetal calf serum (Gibco, Germany) in culture flasks at 37 °C in 5% humidified CO_2 incubator. The cells were fed until confluence ($2x10^6$) and were expanded by trypsinization (for adherent cells) and subcultured at lower numbers in new culture flasks. Viability of cells was determined by trypan blue dye test.

MTT Colorimetric assay. A colorimetric assay using 3-4,5-dimethylthiazoyl-2, 5-diphenyltetrazolium bromide (MTT) was performed. Briefly, cells were added onto the flat-bottomed micro culture plates in the presence or absence of various concentrations of the extracts (in triplicate) and incubated at 37 °C in a 5% humidified CO_2 incubator for 48 hours. Then, 10 µl of MTT (5 mg/ml, Sigma) was added to each well and incubation was continued for a further 4 hours at 37 °C. 100 µl/well of solubilization solution containing isopropanol and 10% SDS in 0.01 M HCl was added into each well. After complete solubilization of the dye, plates were read at 570 nm on an ELISA reader. The reference wavelength was 690 nm. The mean optical density (OD) ±SD for each group of replicates was calculated. Percent inhibition of cells exposed to various treatments was obtained as follows. % Inhibition = 100 – [(Test OD / Non-treated OD) x 100]. Non-treated cultures in all experiments contained the medium but not the extract.

Analysis of cell cycle changes. Cell lines ($2x10^6$) were seeded into a 24 well plate and treated with the appropriate concentration of the extracts for 48 hours. Cells were then centrifuged and fixed in 70% ethanol. After washing, cells were resuspended in 1 ml of PBS containing 10 mg/ml RNase and 1mg/ml propidium iodide (PI, Sigma) and incubated for 1 hour at 37 °C in the dark. After that cells were analyzed on a FACScalibur flow cytometer (Becton-Dickinson, USA).

DNA fragmentation analysis. The isolation of fragmented DNA from cells cultivated in 24 well plates was carried out according to the procedure of Hermann et al with some modifications [14]. In brief, cells ($2x10^6$) were treated with the plant extract for 48 hours and then collected by centrifugation (2000 g, 10min). The pellet was resuspended in 0.5 ml DNA lysis buffer (2% SDS, 10 mM EDTA, 10 mM Tris-Hcl, pH 8.5). The lysate was immediately incubated with 0.1 mg/ml proteinase k (Sigma) and then incubated for 3 hours at 37 °C. After addition of isopropanol the DNA was precipitated with 70% ethanol. Then the suspension was centrifuged and DNA treated with 100 µl of 10 mM Tris-HCl pH 7.5 and 0.5 mg/ml RNase A (Boehringer Mannheim, Germany) at 37 °C for 24 hours. The sample was then loaded into a 2% agarose gel and electrophoresed. The DNA band pattern was visualized under UV light using ethidium bromide staining.

Results

Effect of the extracts on proliferation of tumor cells. In order to study the possible anti neoplastic activity of the extracts, *in vitro* cytotoxic analysis using MTT colorimetric assay on different tumor cells was performed. Results obtained are presented in Figure 1.

F. angulata. A relatively dose dependent inhibitory effect of this plant on the proliferation of all tested cell lines was detected. The Strongest growth inhibitory effect was observed for Jurkat cell line (IC50; 7.9 µg/ml) followed by K562 (IC50; 97.7 µg/ml).

G. mite. This extract exhibited growth inhibitory effect particularly on K562 and Jurkat cell lines under experimental conditions used in 48 hour treatment. IC50 obtained for K562 cell line was 39.8 µg/ml and for Jurkat was more than 200 µg/ml. Treatment of other cell lines with the extract decreased the proliferation but the percent inhibition was less than 30% at 200 µg/ml.

S. obtusicrena. A dose-dependent inhibitory effect of this extract on tumor cell lines was detected. According to 50% inhibition of cell proliferation, the order of sensitivity of the

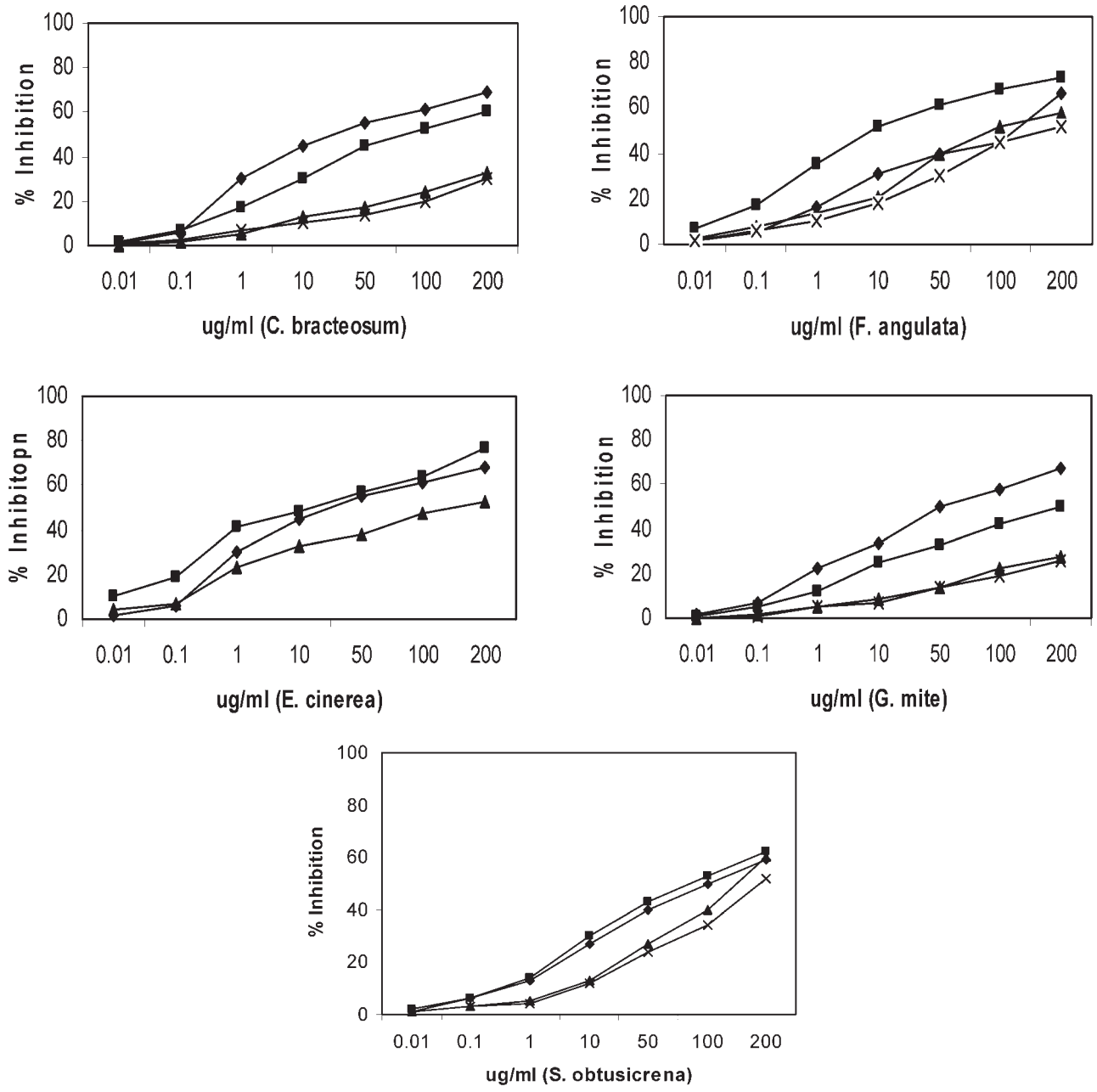

Figure 1. Effect of different concentrations of the extracts on tumor cells growth. Values represent the mean of three experiments. SD was less than 2.1 in all experiments. K562 (♦), Jurkat (■), Fen (▲) and A549 (×).

cell lines to this extract was Jurkat > K562 > Fen > A549. The extract at concentration of 102 µg/ml caused 50% inhibition of Jurkat cells growth.

C. bracteosum. This plant caused 50% inhibition of K562 cells growth at a concentration of 19.9 µg/ml. At 93.3 µg/ml of the extract 50% of Jurkat cells were also affected. Treatment of Fen and A549 cells slightly decreased the proliferation.

E. cinerea. This plant produced a strong proliferation inhibition in Jurkat cells on the concentration and time of exposure (IC50; 6.9 µg/ml). The cytotoxicity was not restricted to this cell line, since other cell lines particularly K562 were also sensitive to this extract.

Effect of the extracts on cell cycle changes of tumor cell lines. To investigate if the inhibitory effect of the extracts on the cell lines observed in this study was due to induction of apoptosis, cell cycle analysis on sensitive cell lines was performed. Quantification of percentage of cells undergoing apoptotic changes for all the extracts is shown in Figure 2.

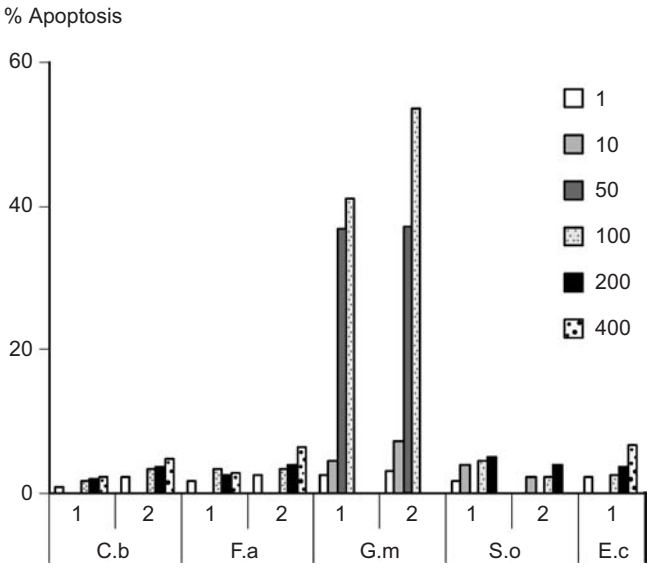

Figure 2. Percent of apoptotic cells accumulated in sub-G1 phase in cell cycle analysis by flow cytometry after treatment of Jurkat (1) and K562 (2) cells with 1–400 µg/ml of different extracts for 48 hours. C.b – *Cirsium bracteosum*, F.a – *Ferulago angulata*, G.m – *Galium mite*, S.o – *Stachys obtusicrena*, E.c – *Echinophora cinerea*

F. angulata. Extract of 1 to 200 µg/ml of this herb was chosen for Jurkat and K562 cell cycle analysis using PI staining. At 200 µg/ml, the percentage of death of K562 and Jurkat cells was fewer than 4%. Addition of more extract to Jurkat cell culture (400 µg/ml) slightly elevated the number of apoptotic cells to 6.3±0.5%.

G. mite. Incubation of K562 cells with this extract resulted in an accumulation of cells in the G1 region. The number of cells in S phase decreased as well, indicating the ability of the extract to induce apoptosis in these cells. Similar result was obtained for the effect of the extract on Jurkat cells. The changes in cell cycle profile induced by this extract are shown in Figure 3. Quantification of cell apoptosis by flow cytometry at 100 µg/ml of the extract showed that 53.4±0.6% and 40.7±1.8% of K562 and Jurkat cells presented an apoptotic peak, respectively. These changes are suggestive of apoptosis.

S. obtusicrena. Amounts of apoptotic cells even at highest concentration of this extract (200 µg/ml) were not more than 5% for both k562 and Jurkat cell lines.

C. bracteosum. In this case, the maximal apoptotic effect observed on the cell lines was 4.8% for K562. Amount of apoptotic Jurkat cells at highest concentration of the extract (200 µg/ml) was 2.31±0.1.

E. cinerea. The maximal apoptotic effect of this extract was 6.5% on Jurkat cells at 200 µg/ml.

Effect of the extract on the appearance of DNA ladder in treated cells. To confirm the effect of extracts on the induction of apoptosis in the cell lines, the extracts were examined for the internucleosomal DNA fragmentation as a characteristic feature of apoptosis. Among different extracts, *F. angulata, S. obtusicrena, C. bracteosum* and *E. cinerea* at different concentrations (up to 800 µg/ml) showed no fragmented DNA in the

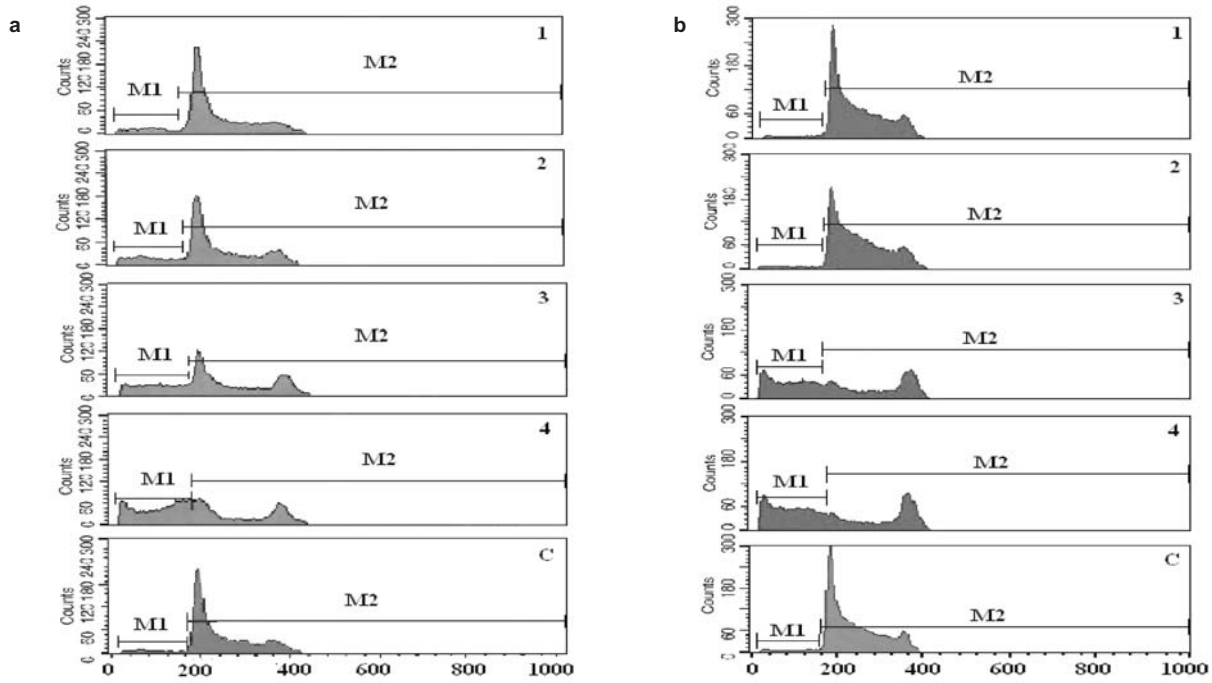

Figure 3. Flow cytometry analysis of K562 (a) and Jurkat (b) cell lines treated with the 1 = 1, 2 = 10, 3 = 50, 4 = 100 µg/ml of *G. mite*. C – non-treated cells.

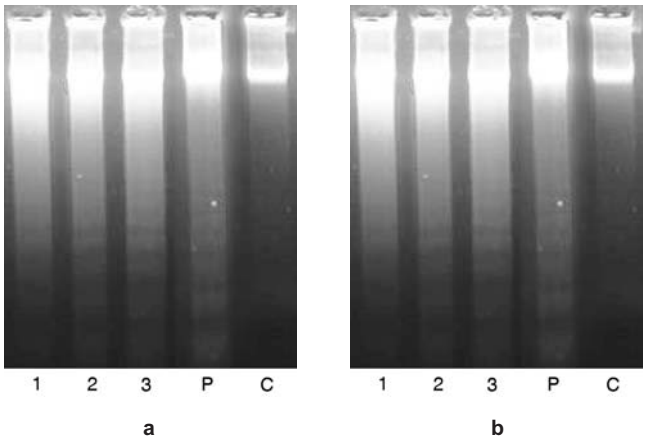

Figure 4. Electrophoretic separation of fragmented DNA from K562 (a) and Jurkat (b) cell lines after treatment with 1 = 50, 2 = 100 and 3 = 200 µg/ml of G. mite. P – Doxorubicin (1 µg/ml) as positive control, C – non-treated cells.

agarose gel electrophoresis. In the case of *G. mite*, the ladder formation was detected after exposing K562 and Jurkat cells with 50 to 200 µg/ml of the extract and as shown in Figure 4, increased DNA fragmentation was dose dependent.

Discussion

Several studies have demonstrated that plant extracts possess various biological activities including anti tumor activity [15–18]. In the present study methanolic extracts of five herbal plants were examined for the antiproliferative activity against different tumor cell lines. We found that all of the extracts more or less have the capacity to decrease the proliferation of tumor cells and that the non-adherent cells were more sensitive than adherent cells to these extracts. Among the plants, the highest activity against K562 which is a myelogenous leukemia cell line was for *E. cinerea, C. bracteosum* with IC50 less than 20 µg/ml followed by *G. mite* with IC50 of 39.8 µg/ml. Jurkat, a T cell leukemia cell line, was more sensitive to *E. cinerea* and *F. angulata* with IC50 less than 8 µg/ml, and *C. bracteosum* with IC50 of 93.3 µg/ml. Average inhibition of Jurkat cells treated with *G. mite* was at values slightly more than 200 µg/ml. The highest cytotoxic activity against Fen bladder carcinoma cells was observed for *F. angulata* and *E. cinerea* with IC50 slightly less than 200 µg/ml. A549 lung carcinoma cell line was almost resistant to *G. mite* and *C. bracteosum*. A549 showed less than 36% inhibition at the highest concentration of these extracts.

The results presented, clearly indicate that the extracts used in this study have anti tumor activity particularly against the cells that are originated from leukemia. Leukemia is one of the most common malignancies causing death worldwide that, though, chemotherapy is the standard method of treatment for the patients, but it has not been fully effective and therefore development of new agents is still important to reduce the rate of mortality.

It has been shown that the mechanism of action of anti neoplastic agents can be due to two distinct processes of necrosis or apoptosis in the cells. Cell death by necrosis is a more passive form of cell death that is characterized by organelle and cell swelling, loss of membrane integrity, rupture of the plasma membrane and cell lysis [19]. Necrosis is often associated with extensive tissue damage and an intense inflammatory response [20]. Apoptosis, on the other hand, is an active process that involves the activation of various cell signaling cascades which results in characteristic morphological and biochemical changes such as chromatin condensation, DNA fragmentation, membrane blebbing, and cell shrinkage [21]. The cell is eventually broken down into smaller membrane-bound vesicles termed apoptotic bodies that become engulfed by surrounding cells without initiating an inflammatory response [22]. As results of our study obtained by flow cytometry revealed, although *C. bracteosum, E. cinerea, F. angulata* and *S. obtusicrena* extracts demonstrated strong anti tumor activity against K562 and Jurkat cells in the growth inhibition assay, they showed no remarkable effects on the cell cycle profile of the treated cells. Moreover, in DNA fragmentation analysis of treated cells, the nucleosome production had no difference with the non-treated cells. These results in conjunction with the observation that in our experiments we found a decrease in the viability of cells treated with these extracts (data not shown) indicates that apoptosis is not the cause effect of cell death due to these extracts and therefore necrosis might be the underlying mechanism. In contrast, leukemic cells used in this study showed the characteristic features of apoptosis due to exposure to *G. mite* in both flow cytometry and DNA fragmentation analysis. *G. mite* caused nearly 53% apoptosis in the K562 and 40% in Jurkat cells indicating the ability of the extract to induce apoptosis in both these leukemic cell lines. Neither the cytotoxicity of these medicinal plants nor the induction of apoptosis has been previously reported. Apoptosis of tumor cells represents a protective mechanism against neoplastic transformation and development of tumors and manipulation of apoptosis has been introduced as a novel and promising strategy for cancer chemoprevention [23–24].

In conclusion, we observed the anti tumor activity of several medicinal plants against different tumor cell lines. Among the plants, *G. mite* exerts its activity through induction of apoptosis the process that may favour this herb for further studies.

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