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ORIGINAL ARTICLE

# Antiproliferative and Apoptotic Activities of the Medicinal Plant *Ziziphus obtusifolia*

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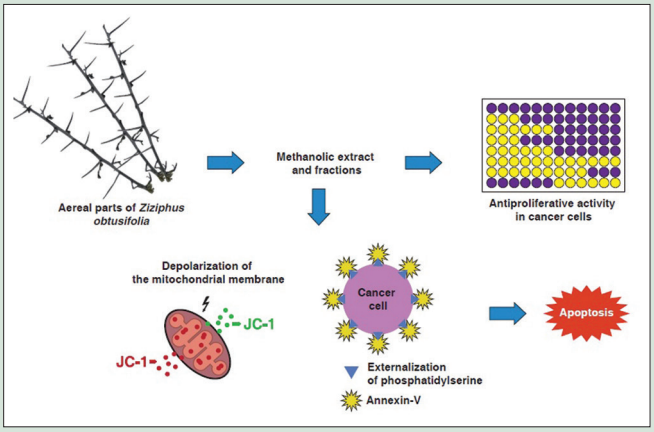
## ABSTRACT

**Background:** *Ziziphus obtusifolia* is a spiny shrub found in Northwest Mexico desert, with traditional medicinal use to treat several diseases including cancer. **Objective:** The aims of the present study were to evaluate the antiproliferative and apoptotic activities of the aerial parts of this plant. **Materials and Methods:** The methanol extract and its fractions were prepared using several solvents. The antiproliferative activity was evaluated by the (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) (MTT) assay on HeLa, A549, RAW 264.7, M12.C3.F6, and L-929 cell lines, and the apoptotic activity using Annexin V and (5,50,6,60-tetra-chloro-1,10,3,30-tetra-ethylbenzimidazol-carbocyanine iodide) staining. The most active fraction was further separated by column chromatography. **Results:** The most active fraction was hexane with an $IC_{50}$ of 90.4 μg/mL against RAW 264.7, 94 μg/mL against M12. C3.F6, 165.5 μg/mL against HeLa and 187.7 μg/mL against A549 cell lines. In apoptotic activity assays the methanol extract and its *n*-hexane fraction were found to induce mitochondrial depolarization in HeLa cells (83 and 87% respectively), and both induced the externalization of the phosphatidylserine increasing the percentage of cells in early apoptosis from 1.4% in untreated control cells, to 1.9% and 3.5% for methanol extract and *n*-hexane fraction-treated cells, respectively, statistically different for the total percentage of apoptotic cells ($P < 0.05$). **Conclusions:** These results show that *Z. obtusifolia* has antiproliferative and apoptotic activities *in vitro* and confirms its use in traditional medicine.

**Key words:** Antiproliferative activity, apoptotic activity, *Ziziphus obtusifolia*

## SUMMARY

The methanol extract and its fractions using several solvents were evaluated in the antiproliferative activity by the MTT assay on HeLa, A549, RAW 264.7, M12.C3.F6, and L-929 cell lines, and the apoptotic activity using Annexin V and (5,50,6,60-tetra-chloro-1,10,3,30-tetra-ethylbenzimidazol-carbocyanine iodide) staining. The most active fraction against cell lines was hexane. In apoptotic activity assays, the methanol extract and its *n*-hexane fraction were found to induce mitochondrial depolarization. This results we showed that *Ziziphus obtusifolia* has antiproliferative and apoptotic activities *in vitro*.




**Abbreviations Used:** DMEM: Dulbecco's modified eagle's medium, DMSO: Dimethyl sulfoxide, MTT: (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium), JC-1: (5,50,6,60-tetra-chloro-1,10,3,30-tetra-ethylbenzimidazol -carbocyanine iodide), FBS: Fetal bovine serum, CAPE: Caffeic acid phenethyl ester, PBS: Phosphate-buffered saline.

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## INTRODUCTION

Cancer represents a main public health problem worldwide. It is estimated that in 2012 alone, the global burden of cancer reached 14.1 million and the number is projected to rise in the next 20 years to 22 million new cases a year. The most commonly diagnosed cancer types in the world in 2012 were lung, breast, and colorectal. The types that caused the greatest number of deaths were lung, liver and stomach.[1] Currently, more of the 100 types of cancer are primarily treated with chemotherapy, but due mainly to the side effects, the search for new treatments continues. An invaluable source of chemical structures with different anticancer effects are the plant-derived natural compounds.[2]

The *Ziziphus* genus in the *Rhamnaceae* family, consist of about 100 species of deciduous or evergreen trees and shrubs throughout the world.[3] Some species have been known to possess ethnomedicinal properties such as *Ziziphus jujuba* (L) Lam., *Ziziphus mauritiana* Lam., *Ziziphus spina-cristi*, *Ziziphus lotus* (L.) Lam., and *Ziziphus spinosa* (Bunge) Hu ex F. H which are used in the traditional Chinese medicine.[4] Some triterpene compounds have been isolated from *Z. jujuba*: β-sitosterol, stigmasterol, and



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campesterol. Stigmasterol is intensively investigated due to its beneficial effects on health.[5]

One of the species considered as a potential source of compounds for cancer treatment is *Z. obtusifolia* a spiny shrub occurring in Texas, Arizona, New Mexico, and Northern Mexico. *Z. obtusifolia* has traditional medicinal use in Mexico against some diseases such as cancer, diabetes, chest diseases, eye diseases, syphilis, gonorrhea, and stomach infections.[6-8] There are no scientific reports about the anticancer activity of *Z. obtusifolia*. In this work, the antiproliferative and apoptotic activities of a methanol extract from stems and thorns of *Z. obtusifolia* and its fractions obtained with solvents of different polarity were evaluated against cancer cell lines.

## MATERIALS AND METHODS

### Plant material

The plant was collected in Hermosillo, Sonora, Mexico (29°5'0.92" N, 111°3'0.22" W), on November 2014. Jesús Sánchez Escalante, Taxonomist of the University of Sonora Herbarium, identified the specimen (voucher specimen no. 20365). The aerial parts of the specimen were dried at room temperature in the shade and then grounded and the powder stored at 4°C.

### Methanol extract and fractions

Methanol extract of *Z. obtusifolia* was prepared with the dried powder from the aerial parts of the plant (in a 1/10 w/v proportion) in methanol for 10 days by maceration with periodic agitation.[9] The extract was concentrated with a rotary evaporator at 40°C under reduced pressure, and it was then fractionated with solvents of increasing polarity: First *n*-hexane, then ethyl acetate, and finally ethanol. Then, stock solutions were prepared at 80 mg/mL in dimethyl sulfoxide (DMSO). Test concentrations were prepared in Dulbecco's modified eagle's medium with 5% of fetal bovine serum.

### Cell lines

The HeLa (human cervical carcinoma), A549 (human alveolar adenocarcinoma), and L-929 (murine subcutaneous connective tissue) cell lines were obtained from the American Type Culture Collection. Dr. Emil A. Unanue (Pathology and Immunology Department, Washington University in St. Louis MO) provide the RAW 264.7 (murine macrophages, Abelson leukemia virus-transformed) and M12. C3.F6 (murine B-cell lymphoma) cell lines.

### Cell viability assay

Antiproliferative assay was evaluated by the (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) (MTT) reduction assay with minor modifications.[10-12] Briefly, a cellular suspension with $1 \times 10^4$ cells per well, were loaded (50 µL) in 96-well plates and incubated 24 h at 37°C in a 5% $CO_2$ atmosphere, then 50 µL of extracts at different concentrations were added at each well, and the plate was incubated for 48 h. Previously, extracts were dissolved in DMSO and subsequently diluted in the culture medium. At 44 h of incubation time, cells were washed with phosphate-buffered saline (PBS), then 100 µL of culture medium and 10 µL of a 5 mg/mL MTT solution were added to each well. Viable cells reduced tetrazolium salt to formazan crystals which were dissolved in acidic isopropyl alcohol, and the absorbance was measured with an ELISA plate reader (Multiskan EX, Thermo Scientific, Waltham, MA, USA), at 570 nm (test wavelength) and 650 nm (reference wavelength). Viable cells were expressed as percentage, where the optical density measured from DMSO-treated cells was considered 100% proliferation. Antiproliferative activity of extract and fractions was reported as $IC_{50}$ values calculated with GraphPad Prism 5 (GraphPad Software, INC., La Jolla, CA, USA). Treatments were compared using one-way ANOVA for a significant difference.

### Apoptosis detection

#### *Annexin V-propidium iodide staining*

This assay was carried out using the previously described method for Annexin V and propidium iodide by Rascón *et al*.[12] HeLa cells ($4.5 \times 10^5$ cells/mL) were stimulated for 24 h with the methanolic extract (0.4 mg/mL) or its hexanic fraction (0.2 mg/mL). We used as apoptosis positive control caffeic acid phenethyl ester (CAPE) at 120 mM. Then, stimuli were removed and after trypsination cells were washed with cold PBS three times by centrifugation 200 ×*g*, 7 min, 4°C, resuspending the cell pellet in an Annexin V-FITC solution in binding buffer with a subsequent 10-min incubation in the dark at room temperature. Then, cells were washed twice with PBS, resuspended and analyzed by flow cytometry within 1 h in a FACS Canto II cytometer (BD Systems, San Jose, CA, USA).

#### *Measurement of the mitochondrial membrane breakdown*

In this assay, the cationic lipophilic fluorochrome (5,50,6,60-tetra-chlo ro-1,10,3,30-tetra- ethylbenzimidazol-carbocyanine iodide) (JC-1) was used, and the mitochondrial membrane breakdown was detected using flow cytometry. A $4.5 \times 10^5$ HeLa cells/mL suspension was stimulated with methanolic extract (0.4 mg/mL) and its *n*-hexane fraction (0.2 mg/mL) for 24 h using. Then, cells were trypsinized and washed twice with PBS, the JC-1 solution (5 mg/ml) was added and cells incubated at 37°C in the dark for 15 min. Finally, cells were washed two times in PBS, resuspended in culture medium, and immediately analyzed by flow cytometry. As a positive control of mitochondrial membrane potential breakdown a CAPE solution at 120 mM was used.[12]

### Purification of the n-hexane fraction

The *n*-hexane fraction (9 g) was separated by silica-gel (200–400 mesh) column gravity chromatography to obtain the active fractions. In this process, a glass column (length of 70 cm and 4.5 cm internal diameter) was used and eluates were obtained with a step-wise gradient of *n*-hexane-ethyl acetate solvents (0, 5, 10, 15, 20, 50 and 100% of ethyl acetate). The flow rate was 2.5 mL/min and 250 mL fractions were collected (named F1 to F156). Using silica gel $GF_{254}$ thin-layer chromatography plates (Merck, Darmstadt, Germany), the fractions composition were monitored and plates were revealed with 10% sulfuric acid and hot plate.

## RESULTS AND DISCUSSION

### Antiproliferative activity

The antiproliferative activity of the methanol extract and its solvent fractions were tested on different cell lines by the MTT assay; the methanol extract was tested in a concentration range of 50–400 µg/mL and the solvent fractions of 6.25–50 µg/mL. The methanol extract was more active on RAW 264.7 cells ($IC_{50}$ value = 187.78 ± 4 µg/mL) than on L-929 ($IC_{50}$ > 400 µg/mL) [Table 1].

Fractions from the methanol extract were obtained using consecutively *n*-hexane, then ethyl acetate and finally ethanol. The antiproliferative activity of fractions were tested and the *n*-hexane fraction was more active on RAW 264.7 and M12.C3.F6 cancer cell lines ($IC_{50}$ values 90.4 ± 0.3 and 94 ± 2 µg/mL, respectively) and for the ethyl acetate fraction ($IC_{50}$ values 72.5 ± 1.8 and 142 ± 1.5 µg/mL, respectively), while the ethanol and residual fractions had $IC_{50}$ values >400 µg/mL. The L-929 cells were not affected with the treatment with *n*-hexane chromatographic fractions at the concentrations tested, whereas the lowest $IC_{50}$ on cancer

**Table 1:** Evaluation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide of antiproliferative activity (mean±standard deviation) of solvent fractions obtained from methanol extract of *Ziziphus obtusifolia*

| | Cell lines ($IC_{50}$) | | | | |
|---|---|---|---|---|---|
| | HeLa | A549 | RAW 264.7 | M12.C3.F6 | L-929 |
| Methanol extract | 396±11.3 | ND | 187.8±4.0 | 223±2.5 | ND |
| *n*-hexane fraction | 165.5±2.7 | 187.7±7 | 90.4±0.3 | 94±2 | 105.1±4.8 |
| Ethyl acetate fraction | 203.9±0.8 | 315.7±8.1 | 72.5±1.8 | 142±1.5 | 254.7±14 |
| Ethanol fraction | ND | ND | 382.5±17 | ND | ND |
| Residual fraction | ND | ND | ND | 318±2 | ND |

$IC_{50}$: 50% inhibitory concentration with values in μg/mL (*n*=3); ND: Activity not determined at highest concentration tested (400 μg/mL)

cell lines were for F64 against RAW 264.7 with <6.25 μg/mL, F113 against HeLa and RAW 264.7 (18.3 and 17.6 μg/mL, respectively) [Table 2].

## Apoptosis detection

Several apoptotic events were evaluated in cells treated with the methanolic extract of *Z. obtusifolia* and its *n*-hexane fraction. When phosphatidylserine is located on the cell surface of cancer cells, an apoptotic event is considered[13] because its usual localization is on the inner leaflet of cytoplasmic membrane; it was detected by staining HeLa cells with Annexin V-FITC and propidium iodine (PI) and analyzed by flow cytometry.

Percentage of early apoptotic events was increased in HeLa cells from 1.4% in control-untreated cells to 1.9 when treated with methanolic extract and 3.5% with *n*-hexane fraction. Treatment with *n*-hexane fraction resulted significantly more effective than methanol extract and CAPE, with percentages of total apoptosis of 26.2, 22.3, and 21.8%, respectively ($P < 0.05$) [Table 3]. Cells treated with this extracts died by apoptosis and early events of this process were detected; as shown in Figure 1 more cells were in late apoptosis in treated cells than in the untreated cells in the upper-right quadrant (Annexin V positive-PI positive).

## Depolarization of mitochondrial membrane potential

Another consideration for the detection of apoptotic activity was when the treatment with the methanol extract or its *n*-hexane fraction collapses the mitochondrial membrane potential, and the fluorescence of JC-1 stain (which normally emits mitochondrial red fluorescence with a little green fluorescence), changes by shifting its fluorescence emission from red to green when cell apoptosis occurs and was detected in FL-1 channel as green fluorescence.[13] The mitochondrial membrane potential depolarization is indicated by a decrease in the red/green fluorescence ratio for HeLa cells treated with these extracts, as compared with the control. As shown in Figure 2, the treatment with *n*-hexane fraction had more effect in the Red/green ratio (87% less than the control), which indicates the depolarization of the mitochondrial membrane.

There is a large number of traditional benefits of *Ziziphus* plants since ancient times. Leaves, fruits, seeds, and barks of these plants have been medicinally used.[14] Traditional Chinese medicine also involves the use of *Ziziphus* extracts for cancer treatment and other therapeutic potential.[4] The principal bioactive compounds identified are Vitamin C, flavonoids, phenolics, triterpenic acids, and polysaccharides.

Another studies made in *Z. jujuba* fruits have another pharmacological effects, such as anticancer, and anti-inflammatory.[15] In the traditional medicine in Sonora, Mexico, the Mayo ethnic group uses *Z. obtusifolia* for cancer treatment. There are no scientific studies that demonstrate which compounds are responsible from its anticancer properties. Some bioactive compounds were found in other species from the same genus. In *Z. mauritiana*, betulinic acid and oleanolic acid were identified,

**Table 2:** Evaluation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide of the antiproliferative activity (mean±standard deviation) of the chromatographic fractions from *n*-hexane fraction obtained by open column with silica gel

| | Cell lines (IC50) | | | | |
|---|---|---|---|---|---|
| | HeLa | A549 | RAW 264.7 | M12.C3.F6 | L-929 |
| F19[a] | 27.4±0.8 | 40±5 | 20.2±0.4 | >50 | ND |
| F44[a] | 40.5±2 | ND | NA | 33±0.3 | ND |
| F61[a] | 39.4±2.8 | ND | 20.9±2.8 | 43.7±1.4 | ND |
| F64[a] | 29.5±2.1 | 36.8±0.6 | <6.2 | 22.9±0.3 | ND |
| F113[a] | 18.3±1.6 | ND | 17.6±1 | 32.9±0.7 | ND |

[a]F19, F44, F61, F64 and F113: Chromatography fractions from the *n*-hexane fraction. $IC_{50}$: 50% inhibitory concentration (μg/ml, mean±SD, *n*=3); ND: Not determined at highest concentration tested (50 μg/ml); SD: Standard deviation

both with cytotoxic activity for some specific cancer cell lines.[14,16] In other studies, the activity of triterpenes against some cancer cell lines are reported, and the mechanism by which compounds such as ursolic acid and belutilinic acid induce cell death have been studied. In another investigation, selective activity of *Z. jujuba* extracts was found for the growth inhibition of some cancer cell lines and triterpenic acids were found present in the most cytotoxic extracts that inhibited cell growth and induced apoptosis in two breast cancer cell lines.[17]

With this knowledge, we studied the antiproliferative activity of these extracts in murine (RAW 264.7 and M12.C3.F6) and human (A549 and HeLa) cancer cell lines using the murine normal cell line L-929 as a control. We found that the *n*-hexane fraction showed antiproliferative activity with $IC_{50}$ values of 90 and 94 μg/ml, against RAW 264.7 and M12.C3.F6 cancer cell lines, respectively [Table 1], and showed $IC_{50}$ values of 165 and 187 μg/ml against HeLa and A549, respectively. We decided to evaluate the silica gel-chromatographic fractions of the *n*-hexane fraction, resulting in $IC_{50}$ values <30 μg/mL [Table 2]. Several reports in the literature exemplify susceptibility of different tumor cell lines to cytotoxic agents found in other *Ziziphus* species. In *Z. mauritiana*, the active fractions illustrate enhanced antiproliferative activity against A549 cells,[4] and *Z. spina-cristi* showed efficient cytotoxic potency toward the HeLa cell line.[18] In other study, in human cancer cell lines: HeLa, Jurkat (T-cell leukemia) and HEp-2 (larynx carcinoma), water extract of dried jujube fruits in concentration of 0–1 mg/mL was used. They found induction of apoptosis in this cancer cell lines, and that HEp-2 was the most sensitive to this treatment with $IC_{50}$ of 0.1 μg/mL.[19]

The evasion of apoptosis is a characteristic of tumor cells and helps with the chemoresistance. During apoptosis cells show shrinkage and membrane blebbing. Furthermore, the phosphatidylserine change to the outer leaflet of membrane cell and this event is used as a marker in apoptosis.[20] In our study, the Annexin V-Propidium iodide and JC-1 staining were used to detect apoptosis. The methanol extract and the *n*-hexane fraction were inducers of the externalization of phosphatidylserine [Figure 2], and the results show that cells treated with

**Table 3:** Percentage of HeLa cells in apoptosis induced by treatments with methanolic extract and its n-hexane fraction of *Ziziphus obtusifolia*

| | Live cells | Dead cells | Early apoptosis | Late apoptosis | Total apoptotic cells |
|---|---|---|---|---|---|
| Solvent control DMSO | 96.3±0.14 | 0.7±0 | 1.4±2.8 | 1.6±0.14 | 3.0 |
| Methanolic extract | 67.4±6.1 | 10.6±1.5 | 1.96±1.9 | 20.3±4.3 | 22.3* |
| *n*-hexane fraction | 61.4±5.7 | 12.1±1.47 | 3.5±1.1 | 22.9±5.5 | 26.4* |
| CAPE | 64.5±3.7 | 13.5±1.7 | 2.5±0.07 | 19.3±1.8 | 21.8* |

*$P$<0.05. Culture cells treated with 0.4 or 0.2 mg/ml of methanolic extract and *n*-hexane fraction in DMSO. Control positive of apoptosis: CAPE (120 mM). CAPE: Caffeic acid phenethyl ester; DMSO: Dimethyl sulfoxide

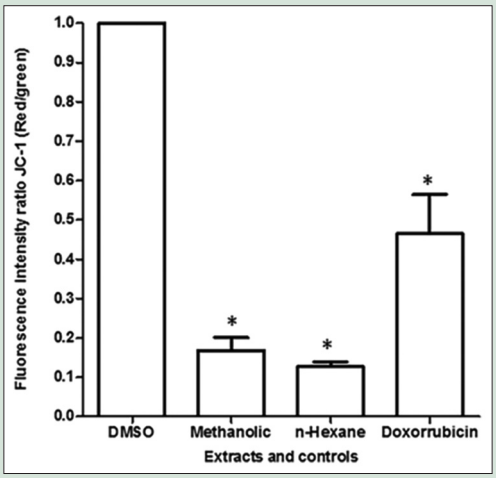


**Figure 1:** Comparison of percentages of apoptosis induced by methanolic extract and its *n*-hexane fraction of three-independent experiments in HeLa cells, using caffeic acid phenethyl ester and DMSO as negative and positive controls, respectively. *Statistical significance on the comparison with the negative control, $P < 0.05$

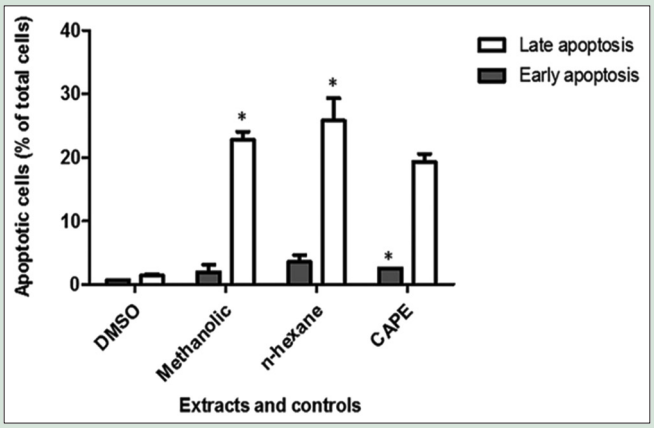


**Figure 2:** Fluorescence intensity ratio (Red/green) for (5,50,6,60-tetra-chloro-1,10,3,30-tetra-ethylbenzimidazol-carbocyanine iodide) due to the depolarization of the mitochondria membrane of three separated experiments using HeLa cells treated with 0.4 and 0.2 mg/mL of methanol extract and its *n*-hexane fraction respectively for 24 h, and Doxorubicin and Dimethyl sulfoxide as positive and negative control, respectively. ANOVA * for significant differences with respect to control, $P < 0.05$ (Tukey's test).

the methanol extract and the *n*-hexane fraction died by apoptosis 7-fold with respect to the control. The intrinsic and the extrinsic pathways are the main signaling pathways of apoptosis.[21] For the intrinsic pathway, a protein (Bid Puma or Noxa) can activate another proteins such as Bax or Bak which can permeabilize the mitochondria membrane, and then, cytochrome c can oligomerize with Apaf-1, and then, the formation of apoptosome occurs, and caspase-9 is activated, then cleave to caspases-3 and-7 and both cleave targets for the cell death. In the extrinsic pathway, the death ligand (Fas ligand, TNF, or TRAIL) can be activated with death receptor (Fas, TNF receptor, or DR4/DR5) leads to the recruitment of FADD and pro-caspase-8. Then, caspase-8 activates itself, and it activates the effector caspases-3 and-7 resulting in cell death or cleaves Bid to induce Bax/Bak activation which in turn activates the intrinsic apoptotic pathway.[22] During the early stage of apoptosis, the loss of the mitochondrial potential occurs,[12] evidenced here when the red/green fluorescence intensity was reduced compared with the controls without treatment [Figure 2]. In this investigation, the early apoptosis and the antiproliferative activity detected are consistent results. We found for the first-time evidence of antiproliferative and apoptotic activities of *Z. obtusifolia* reporting the molecular events of apoptosis induced by the extracts of this plant.

## CONCLUSIONS

The present study highlights the antiproliferative potential of extracts from the aerial parts of *Z. obtusifolia* providing promising baseline information for its traditional medicinal property and possibility of its extract as a potent agent for the development of natural anticancer agents. Chemical characterization of the responsible compounds of this antiproliferative activity needs further investigation.

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## Conflicts of interest

There are no conflicts of interest.

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