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'In vivo' and 'in vitro' activity of *Larrea divaricata* Cav. on EL-4 cells

Roberto Davicino[1], Rosario Alonso[1] and Claudia Anesini[1,2]

Abstract

Larrea divaricata is a plant widely used in folk medicine in Argentina. It has been demonstrated that an aqueous extract of *L. divaricata* possesses a biphasic effect on cell proliferation, at low concentrations exerts a stimulatory action and at high concentrations exerts anti-proliferative effects upon the T lymphoma BW 5147; therefore, we propose in this paper to test the effect of the extract 'in vitro' and 'in vivo' in another T-cell lymphoma named EL-4. It was analyzed 'in vitro' cell proliferation by tritiated thymidine uptake and the effect of the extract on tumors induced in mice analyzing tumor progression and survival.The results showed that the aqueous extract induced the proliferation of tumor cells at all the concentrations studied. The results 'in vivo' showed that the aqueous extract stimulated significantly the size of tumors and that untreated mice lived longer than those treated. It is important to be very careful when plant extracts are selected for the treatment of several diseases. Consequently, before using a plant extract, specific scientific studies must be undertaken on different models to certificate therapeutic and adverse effects. Moreover, it can be said that *L. divaricata* has a specific anti-tumor mechanism of action depending on the targets.

Keywords

Larrea divaricata, EL-4, T lymphoma, proliferative effect, 'in vivo'

Introduction

Larrea divaricata Cav. (Zygophyllaceae) is a plant widely distributed in north-west, center, and south-east of Argentine.[1] In folk medicine, *L. divaricata* has the following claims and uses: healing sores and wounds, rheumatism, inflammation of respiratory and intestinal tract, gastric disturbance, venereal diseases, as tonic, as corrective, as antiseptic, stimulating expectorant, emetic, arthritis, cancer, tuberculosis, common cold, and rubefascient.[2]

It was demonstrated that at low concentration *L. divaricata* (0.5 μg/ml) showed pro-proliferative effect on a lymphoma cell line (BW5147), which was due to the activation of lipoxygenase metabolism.[3] On the other hand, it has been demonstrated that at high concentrations (10-1000μg/mL) it has an anti-proliferative activity upon the same cells. Recently, Davicino et al. (2009)[4] showed the participation of hydrogen peroxide (H_2O_2) in the anti-proliferative effect of *L. divaricata* on BW 5147 cells. Furthermore, it was shown that the extract exerts the activation of extracellular signal–regulated kinases 1 and 2 (ERK1/2) pathway and the subsequent translocation of nuclear factor κB (NF-κB) to the nucleus leading to the induction of inducible nitric oxide sintase (iNOS) and the consequent increase of nitric oxide (NO) level, which in turn activates P-38 pathway leading to apoptosis. Finally, it was observed that BW5147 lymphocytes need to have low levels of H_2O_2 to allow the proliferation; this requirement can be accomplished by maintaining a low Mn^{2+}-containing superoxide dismutase (Mn^{2+} SOD) and high peroxidase (Px) activities.

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L. divaricata increased the activity of Mn^{2+} SOD which leads to increase in H_2O_2.[5] The majority compound found in the resin of the leaves is nordihydroguaiaretic acid (NDGA).[5] Nevertheless, this compound is not related with the anti-proliferative effect exerted by the extract on BW 5147 cells.[7]

Taking into account that the aqueous extract of *L. divaricata* exerts anti-proliferative action upon BW5147 T lymphoma cells, it is proposed in this paper to test the effect of the extract not only 'in vitro' but also, 'in vivo' on another murine T-cell lymphoma named EL-4. Also, NDGA was quantified and its effect was studied in order to determine its contribution in the effect exerted by the aqueous extract.

Materials and methods

Plant material and extract

Leaves of *Larrea divaricata* Cav. were collected in the province of Cordoba, Argentina, and identified using morphological, anatomical and histochemical analysis. A voucher specimen was deposited in the Museum of Pharmacobotany, School of Pharmacy and Biochemistry, University of Buenos Aires.

An aqueous extract (EA) of the leaves was prepared as follows: air-dried leaves (750 mg) were extracted for 10 min with boiling distilled water (10 mL), heated for a further 45 min at 56°C with mechanical agitation and let to rest during 72 h at 5°C. The extract was filtered, centrifuged at 3.500 rpm for 15 min, sterilized through a 0.22 μm membrane, lyophilized, aliquoted and stored at –20°C until use.[8]

HPLC analysis

The high-performance liquid chromatography (HPLC) analysis was performed in a Varian Pro Star instrument with UV photodiode array detector. A column C18 Gemini (150 mm × 4.6 mm and 5 μm ID) was used. The mobile phase was A: Water: acetic acid (98:2) B: Methanol: acetic acid (98:2); the gradient was from 15% B to 40% B in 30 min; 40% B to 75% B in 10 min; 75% B to 85% B in 5 min and 100% B in 5 min, leave 10 min 100% B and back to initial conditions. Flow 1.2 mL/min, room temperature. Detection: with UV 260 nm. The samples were analyzed with the Varian Prostar 430 Autosampler HPLC Galaxy Software provided by Varian S.A.[9]

Pure standards: NDGA and quercitrine were obtained from Carl Roth (Karlsruhe, Germany).

Cell culture conditions

The tumor cell line EL-4 (Institute fur Virologie und Immunobiologie der Universitat Wurzburg, Germany) is a T cell lymphoma induced by 9, 10-dimethyl-1, 2-benzanthracene in C57BL/6N mice. Cells were cultured at optimal concentrations of 5×10^5 cells/mL in RPMI 1640 medium (Gibco, New York, USA) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and antibiotics (Sigma, San Diego, California, USA).[4]

Proliferation assays

The effects of different concentrations of aqueous extract (10, 100, 1000 μg/mL) and NDGA (1×10^{-7}, 1×10^{-6} and 1×10^{-5} M) on tumor lymphocytes proliferation were evaluated. Cell proliferation was assayed by the uptake of tritiated thymidine ([3H]TdR; Sigma). Cells were cultured in presence or absence of aqueous extract during 24 and 48 h and then pulsed with [3H]TdR (20 Ci/mmol) for the last 6 h as previously described.[10] Results were expressed as cpm.

Induction of tumors in mice

Tumors were induced in 155 females C57BL/6N mice (25−30 g), by subcutaneous inoculation with 5×10^5 cells per mL. Animals were housed and cared for at the Animal Resource Facilities, Faculty of Pharmacy and Biochemistry, National University of Buenos Aires, in accordance with institutional guidelines. Tumor size was measured at 10 days with a caliper and their growth parameters were tested. Five animals were randomly chosen for histological examination. To do this, these animals were necropsied when tumors were palpable and measurable; the tumors were analyzed in accord to their histological appearance.[11]

'In vivo' activity of aqueous extract

Mice were divided in two groups: (1) Seventy-five animals treated by oral administration with aqueous extract of *L. divaricata* (33.4 mg/kg) and (2) Eighty animals received only the vehicle (control). Groups 1 and 2 were administered during 7 days beginning 5 days after the tumor appearance.[11]

Statistic analysis

Data are the average of duplicate samples performed by triplicate. The data were recorded as mean value

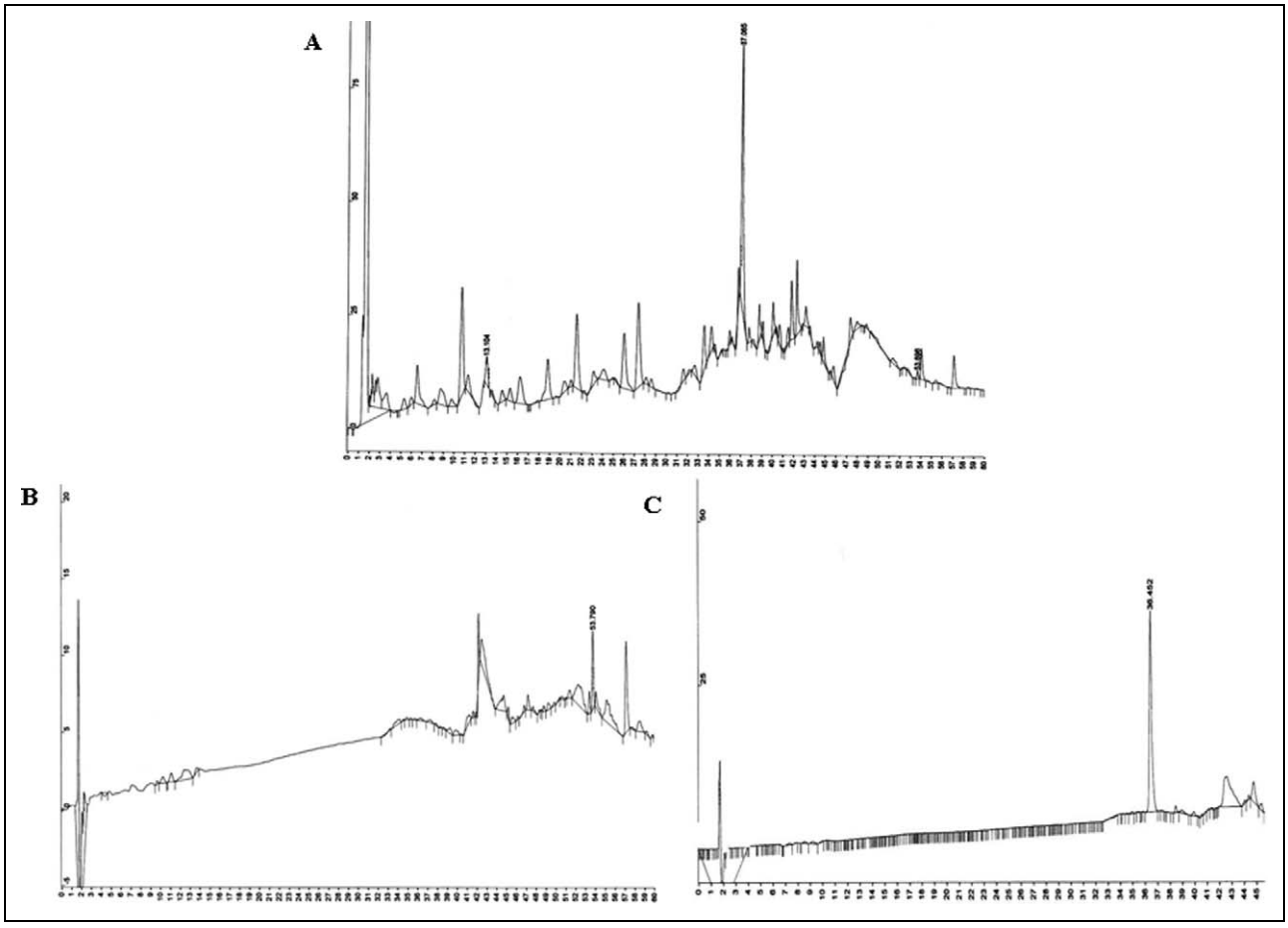

Figure 1. HPLC analysis of aqueous extract (EA) obtained from *L. divaricata* Cav. A). Chromatogram of EA; where the nordihydroguaiaretic acid (NDGA) content represents the 0.3% w/w of the dry extract. There are also a polar derivate from NDGA (0.26 g % w/w) and a monoglicoside of quercetin (0.18 g % w/w) expressed as quercitrin. B, Chromatographic profile of NDGA standard. C, Chromatographic profile of quercitrin. For samples and standards the same running conditions were used.

$\pm$ standard error of mean (SEM). One-way analysis of variance was performed by ANOVA followed by comparisons with Dunnett test. A $p \leq 0.05$ was considered statistically significant.[12]

Results

HPLC analysis

L. divaricata EA was resolved with HPLC and in the chromatographic profile three peaks are shown: A peak corresponding to NDGA (0.30 g % w/w) with retention time at 53.696 min, a second peak corresponding to a polar derivate from NDGA (0.26 g % w/w) with retention time at 13.104 min and a third peak belonging to a monoglicoside of quercetin (0.18 g % w/w) with retention time of 37.065 min (Figure 1A). The third peak was expressed as quercitrin. In Figure 1B is shown the chromatographic profile of NDGA standard and in Figure 1C the chromatographic profile of quercitrine.

Proliferation assays

It can be seen in Figure 2A that the aqueous extract of *L. divaricata* induced significantly ($p \leq 0.05$) the cell proliferation in all assayed concentrations after 24 h of incubation. This effect was also observed after 48 h of incubation principally with 10 and 100 μg/mL, meanwhile, 1000 μg/mL did not modify basal proliferation, but the maximum stimulation was observed at 24 h of cell incubation (Figure 2B). On the other hand NDGA induced cell proliferation only at 24 of cell incubation, in relation to the concentrations. At 48 h NDGA did not modify basal cell proliferation

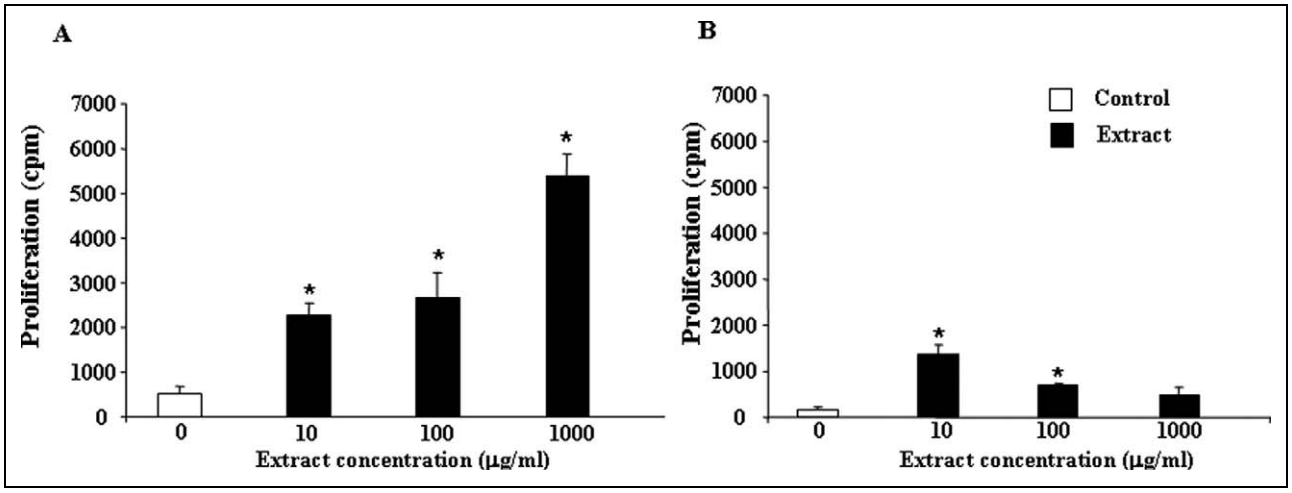

Figure 2. Effect of an aqueous extract of *L. divaricata* upon EL-4 cells proliferation. The cells were incubated with different concentrations of extract from 10, 100 and 1000 µg/mL during 24 h (A) and 48 h (B). Results were expressed as mean ± SEM of three experiments made by triplicate. *$p \leq 0.05$ with respect to control.

Table 1. 'In vitro' effect of NDGA on extract response and on EL-4 proliferation[a]

A

	Proliferation (Cpm)	
Additions	24 h	48 h
Control	506 ± 20	166 ± 8
NDGA 1 × 10−7 M	1100 ± 80[b]	155 ± 10
N.DGA 1 × 10−6 M	1430 ± 100[b]	160 ± 15
NDGA 1 × 10−5 M	2900 ± 130[b]	179 ± 14

B

	Proliferation (Cpm)			
Additions	Extract alone	Extract + NDGA 10^{-5} M	Extract alone	Extract + NDGA 10^{-5} M
Extract 10 µg/mL	2247 ± 240[b]	947 ± 100[c]	1380 ± 150*	191 ± 49[c]
Extract 1000 µg/mL	5391 ± 500[b]	3800 ± 200[d]	500 ± 155*	260 ± 70

Abbreviation: NDGA: nordihydroguaiaretic acid.

[a] Cells were incubated during 24 or 48 h in presence of different concentrations of NDGA (A) or in presence of extract alone or with NDGA 10^{-5} M (B). The concentrations of NDGA analyzed corresponded to the concentrations found in the concentrations of the extract. Results were expressed as mean ± SEM of three experiments made by triplicate.

[b] $p < 0.01$ significant differences between control and NDGA or extract in accordance to ANOVA plus Dunnett's multiple comparison.

[c] $p < 0.01$ significant differences between extract and extract plus NDGA, in accord with ANOVA plus Dunnett's multiple comparison test.

[d] $p < 0.05$ significant differences between extract and extract plus NDGA, in accord with ANOVA plus Dunnett's multiple comparison test.

* $p < 0.01$ significant differences between extract and 48 h control in accord with ANOVA plus Dunnett's multiple comparison test.

(Table 1A). When NDGA and extract were associated, a decrease in the hiper-proliferative effect of the extract was observed (Table 1B). The reversion was observed not only at 24 h but also at 48 h of cell incubation.

'In vivo' activity of aqueous extract.

To perform the 'in vivo' assays 33.4 mg/kg of the aqueous extract was administered to mice; this dose was calculated to reach in tissues a concentration of 1000 µg/mL. In Figure 3, a control and treated tumor evolution, as a representative graphic, can be seen. The aqueous extract significantly increased ($p \leq 0.05$) the size of tumors after 5 days of treatment. After six and a half days of treatment all of the animals died. Table 2 shows the effect of the aqueous extract of *L. divaricata* on some

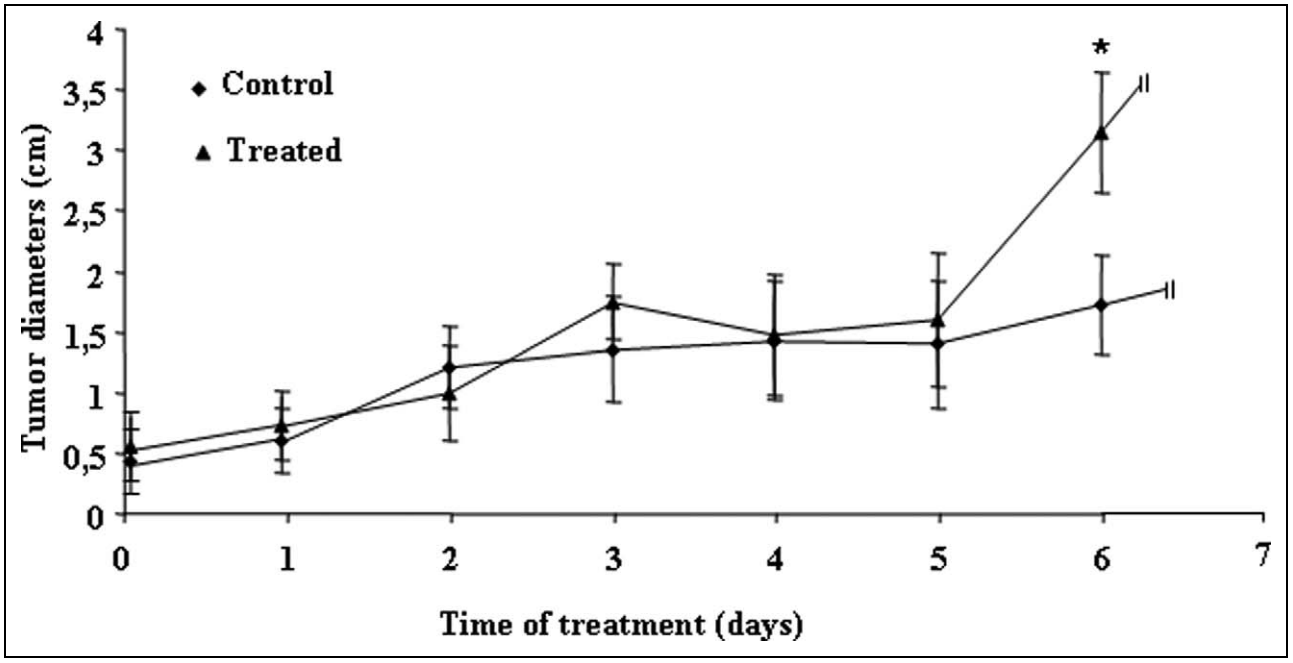

Figure 3. Representative evolution of tumor progression of a control (◆) and extract-treated mice (▲). Tumors were induced in 155 females C57BL/6N mice (25−30 g), by subcutaneous inoculation with 5×10^5 cells per mL. Mice were divided in two groups: (1) Seventy-five animals treated by oral administration with aqueous extract of *L. divaricata* (33.4 mg/kg) and (2) Eighty animals received only the vehicle (control). The tumors were observed and measured during 7 days. The size of tumors was expressed in cm and the progression was registered with the time. Results were expressed as mean ± SEM of three experiments made by triplicate. *$p \leq 0.05$ with respect to control.

Table 2. Effect of the aqueous extract of *L. divaricata* on some growth parameters of tumor and survival time

Tumor parameters[a]	Control	Treated
Total tumors	80	70
Stabilization	30 (37.5%)	10 (14.28%)
Regression	− (0%)	− (0%)
Progression	50 (62.25%)	60 (85.72%)
Sunrival time[b]	6.5 ± 1	5 ± 1[c]

[a] The results are expressed as percentage of tumors that decreased, increased or remained static in control and extract-treated mice.
[b] The results are expressed in days as mean ± SD of 20 control and 2O treated mice.
[c] $p \leq 0.05$ vs control.

growth parameters such as survival time. The results demonstrated that while 37.5% of untreated tumors showed stabilization, only 14.28% of treated achieved this state. Besides, the 85.72% of treated tumors progressed in comparison with untreated ones, in these animals a 60.25% of progression was observed. On the other hand, the survival time of control animals was longer than treated mice ($p \leq 0.05$).

Discussion

In this work, the hiper-proliferative action of an aqueous extract of *L. divaricata* is demonstrated in a lymphoma T line EL-4 'in vitro' and 'in vivo.' *L. divaricata* aqueous extract induced cell proliferation (Figure 2) and decreased the survival time of the treated animals (Figure 3). The aqueous extract increased 'in vitro' cell proliferation in all concentrations analyzed. Previously, it was demonstrated that, the aqueous extract of *L. divaricata* (the same as used in this work) also increases BW 5147 cell proliferation, but only at low concentrations; meanwhile, at high concentrations, a decrease in cell proliferation is observed.

The mechanism involve in the pro-proliferative action of the extract on BW 5147 cells is leukotrienes production (principally LTB4) which in turn induce cell proliferation.[3] The participation of LTs in the modulation of cell proliferation is known. It was reported that the participation of LTB4 in the pathogenesis of head and neck carcinoma's induced proliferation of oral squamous cell cancer.[13] Moreover, LTB4 stimulated the proliferation of the colon cancer cell lines HT-29 and HCT-15, this effect was inhibited by a competitive antagonist of LTB4, SC-41930.[14]

What is more, LTB4 stimulates growth of human pancreatic cancer, related to MEK/ERK pathway activation.[15]

As NDGA, a lipoxigenase inhibitor, could reverse the effect of the extract in EL-4 (Table 1B), the proliferative action of the extract could be related to LTs production.

On the other hand, previously it was determined that the extract induced H_2O_2 and cyclic adenosine monophosphate (cAMP) production in BW 5147 cells, both effectors are related to the antiproliferative action exerted by the extract.[4] On the contrary, in EL-4, the extract did not produce anti-proliferative effect. This could be related to the fact that, in some tissues, H_2O_2 induces the expression of c-fos mRNA, through LTs synthesis, and consequently conduces cell to proliferate, although, in others tissues the increase in H_2O_2 is related to apoptosis.[4,16] So, the induction of H_2O_2 production could be related to LTs production and cell proliferation in EL-4 cells. Moreover, other biochemical and genetic differences between the two type of cells could be implicated in the diverse effects exerted by the extract. While, BW 5147 is a line obtained from a spontaneous tumor, EL-4 is obtained from chemically induced tumors. While, BW5147 tumors are sensitive to corticosteroids, EL-4 tumors are not.[17] Also, both type of cells, differ in the intracellular signals related to the control of cell proliferation. It is important to note that concanavalin A or PMA (PKC activators) induces a decrease in EL-4 cells proliferation conducting cells to an arrest in G1 and G2/M phase, meanwhile, on BW 5147, an increase of cell proliferation is observed. By other way, meanwhile mitogen-activated protein kinases (MAPK) pathway is related to the proliferation of BW 5147 cells, in EL-4 cells, this pathway is related to death.[18-20] Furthermore, as well, the p38 kinase is not essential to inhibit cell proliferation in EL-4 cells, it plays a major role in BW 5147 cells apoptosis.[21] What is more, some authors reported that *B7-1* gene-modified EL-4 lymphoma could induce more effective immunity against wild-type EL-4 'in vitro and in vivo,'[22] suggesting the importance of some molecules in the function of these cells.

The compounds present in the extract were identified and quantified by HPLC. The major compound identified was NDGA, nevertheless, other compounds were identified and quantified, such as a monoglicoside of quercetin expressed as quercitrin and a polar derivate from NDGA. Previously NDGA was found in the aqueous extract from *L. divaricata*.[6] As well, NDGA is not implicated in the antiproliferative effect of the aqueous extract in BW 5147 cells[3] and appears to be one of the compounds involved in the pro-proliferative effect exerted by the extract upon EL-4 cells (Table 1A). NDGA is a well-known liopxigenase inhibitor, which conduces to decrease LTs levels. However, we demonstrated here that NDGA induced EL-4 proliferation and not cell inhibition. An explanation of this phenomenon could be the misbalance between LTs and prostaglandins (Pgs) production. So, it is hypothesized that NDGA induced Pgs production, which in turn also could present proliferative action in these cells. It has been demonstrated that pulmonary and colon carcinomas produced high levels of Pgs and that ciclooxigenase inhibitors can block cell proliferation.[23,24] The increase in cell proliferation induced by NDGA was lower than induced by the extract, this suggests that LTs were more proliferative than Pgs. On the other hand, when the extract and NDGA were associated, NDGA reversed the effect of the extract on cell proliferation, confirming the participation of LTs in the proliferative response of the extract. The fact that NDGA partially reversed the hiper-proliferative effect of the extract suggested that there occurred a misbalance of Pgs production and that these Pgs could exert a proliferative action.

In conclusion, depending on the intracellular signal triggers during cell proliferation, the effect of a same extract could be different. Because of this, it is very important to study the mechanisms elicit by cells to control cell proliferation as well as the mechanisms exerted by the extracts on these pathways. These actions together could be useful in the selection of the medicinal plant to be used in a particular case.

Future studies must be performed to reveal the mechanisms elicit by EL-4 in the balance of proliferation-apoptosis, studying the participation of LTs and Pgs in this balance.

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