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Antiproliferative Activity of *Larrea Divaricata Cav.* on Lymphoma Cell Line: Participation of Hydrogen Peroxide in Its Action

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ABSTRACT

***Larrea divaricata* is a plant used in Argentina. The aim was to analyze the participation of H_2O_2 in the antiproliferative effect of *L divaricata* on BW5147 cells. Different studies were performed: proliferation, viability, nitrite production, apoptosis, oxygen reducing activity, NF-κB translocation, H_2O_2 production, H_2O_2 scavenging activity, and effect on exogenous superoxide dismutase. The extract decreased proliferation and induced apoptosis through P-38 and ERK pathways. The extract activated Mn^{2+}–SOD, increasing H_2O_2, which was implicated in the antiproliferative mechanism by the increase of Nitric oxide (NO). New drugs, which can increase H_2O_2, could be a therapeutic strategy for the treatment of cancer.**

INTRODUCTION

Larrea divaricata Cav. (Zygophyllaceae) is a plant that grows in South America and is widely distributed in Argentina. It is used in folk medicine for the treatment of a number of disorders due to its anti-inflammatory and antirheumatic properties (1). The aqueous extract of its leaves possesses well-documented antitumoral and immunomodulatory activities (2, 3), antimicrobial properties (4, 5), and an antioxidant activity demonstrated on peroxidase secretion of rat salivary glands (6). In a previous work, it was demonstrated that an aqueous extract prepared from leaves of *Larrea divaricata Cav.* exerts antiproliferative action on a lymphoma cell line (3).

By other way, there are evidences indicating that hydrogen *peroxide* (H_2O_2) and the superoxide anion can influence the growth and death of animal cells (7). Previously, it was shown that low concentrations of H_2O_2 (10^{-9} M–10^{-12} M) exert a stimulatory action on BW5147 cell proliferation. In contrast, H_2O_2 treatment of cells with concentrations of about 10^{-4} M causes activation of NF-κB, phenomenon related to apoptosis and decrease in cell proliferation, by the induction of Nitric oxide (NO) level. By other way, it has been demonstrated that some tumor cells can produce variable levels of H_2O_2 according to the activity of antioxidant enzymes, such as the Mn^{+2}-dependent superoxide dismutase (SOD). SOD, catalase (CAT), and peroxidase (Px) are the major antioxidant intracellular enzymes in mammalian cells that protect cells from the damage by reactive oxygen species (ROS) (8). It was also observed that BW5147 cells need to have low levels of H_2O_2 to allow the proliferation; this requirement can be accomplished by maintaining a low Mn^{2+}–SOD and high Px activities. Low expression of antioxidant enzymes has been observed in numerous solid tumors and the increase in Mn^{+2}–SOD expression induced the inhibition of breath cancer cells (9, 10). It is also known that, Mn^{+2}–SOD suppress the HER2/neu oncogene, which in turn inhibits breast cancer cell proliferation (11). Moreover, the anticancer effects of Mn^{+2}–SOD can be reverted by the overexpression of catalase and peroxidase supporting that the anticancer effect exerted by Mn^{+2}–SOD is mediated by H_2O_2 (12, 13).

In these cell types, the development of drugs producing an increase in H_2O_2 levels could be a therapeutic strategy, clinically useful for the treatment of cancer (14).

In view of these facts, the aim of this study was to analyze the participation of H_2O_2 in the antiproliferative effect of the aqueous extract of *L. divaricata* on BW5147 cells, thereby studying the pathways related to the control of cell proliferation. The

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effect of the extract on the enzymes that modulate H_2O_2 level (SOD, CAT, and Px) and on NO were also studied.

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 of the leaves was prepared in the following manner: 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 for 72 hr at 5°C. The extract was filtered and centrifuged at 3.500 rpm for 15 min. Supernatants were filter-sterilized through a 0.22 μm filter, lyophilized, aliquoted, and stored at −20°C until used (5).

Cell culture conditions

The tumor cell line BW5147 (Institute fur Virologie und Immunobiologie der Universitat Wurzburg, Germany) is a T-cell lymphoma cell line that expresses the H-2k haplotype, is $CD3^+$, and has a TCR$\alpha\beta$ as determined by flow cytometric analysis. Cells were cultured at optimal concentrations of 3×10^5 cells/ml in RPMI 1640 medium (GIBCO, NY, USA) supplemented with 10% fetal calf serum (FCS), 2 mm glutamine, and antibiotics (15, 16).

Proliferation assays and viability studies

The effects of different concentrations of aqueous extract on tumoral lymphocytes proliferation were evaluated in the absence or presence of 5×10^{-6} M, PD98059 (Sigma, San Diego, USA), an inhibitor of MEK-1/2, 10×10^{-6} M, and/or SB 203580 (Calbiochem, San Diego, USA), a P-38 inhibitor. Cell proliferation was evaluated by the uptake of tritiated thymidine ($[^3H]$TdR). Cells were cultured for 24 hr and then pulsed with $[^3H]$TdR (20 Ci/mmol) for the last 6 hr as described previously [16]. Results were expressed as counts per minute (cpm). Data represent the media ± SEM of three experiments performed in triplicate.

Viability assay

Cell viability of *L. divaricata*-treated cells was determined by the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma). Briefly, tumoral lymphocytes (3×10^5 cells/ml) were incubated alone or in presence of different concentrations of aqueous extract (10 μg/ml, 100 μg/ml, 1,000 μg/ml) in 100 μl of RPMI 1640 containing 10 μL of 5 mg/ml MTT (Sigma, CA, USA) for 1 hr and 24 hr. The purple formazan formed was solubilized by the addition of acidic isopropanol. The absorbance was measured using a microplate reader (Microplate Reader Benchmark Bio-Rad) at 570 nm. Untreated cells were used as control for viability (100%) and results were expressed as percentage of viability relative to control.

Total nitrite determination

The effect of H_2O_2 on total nitrites production by BW5147 cells was determined using the Griess reagents (17). Briefly, cells were incubated with different concentrations of extract (10 μg/ml, 100 μg/ml, and 1,000 μg/ml) alone or in presence of 10^{-3} M of a nonselective nitric oxide synthase (NOS) inhibitor, L-N monomethyl arginine (LNMMA), or/and 5×10^{-6} M of an inhibitor of MEK-1/2 (PD98059) for 1 hr and 24 hr. After incubation, culture supernatants were collected and centrifuged at 800 × g for 10 min, and then incubated with the Griess reagent for 20 min in dark and measured at 540 nm. Total nitrites were calculated by interpolation in a standard curve made with known concentrations of nitrites.

Apoptosis assays

To determine whether the extract induced apoptosis in tumoral lymphocytes, cells were incubated during 24 hr in presence or absence of aqueous extract at different concentrations (10 μg/ml, 100 μg/ml, and 1,000 μg/ml) alone or in the presence of 5×10^{-6} M of PD98059 inhibitor and/or 10×10^{-6} M of SB 203580. Cells were then washed twice with PBS, resuspended in binding buffer (10 mm HEPES, 140 mm NaCl, 2.5 mm $CaCl_2$, pH 7.4) at a concentration of 1×10^6 cells. Aliquots of 1×10^5 cells were incubated with annexin V-FITC (Sigma, San Diego, USA) and propidium iodide (Sigma, San Diego, USA) for 15 min at room temperature in the dark. Samples were shaken gently, diluted with binding buffer, and analyzed by flow cytometry within 1 hr. A control of apoptosis was done, incubating the cells in the same conditions, but without FCS for 48 hr.

Oxygen-reducing activity: SOD

This assay is based on the property of compounds possessing a SOD activity to inhibit the spontaneous oxidation of adrenaline to adenochrome by the reduction of oxygen. Adrenaline rapidly undergoes autooxidation at pH 10.7 producing adrenochrome, which is a pink colored product that can be measured spectrophotometrically at 480 nm. It is possible, thus, to monitor SOD activity by monitoring the production of adenochrome with a spectrophotometer in kinetic mode. Each cell supernatant (50 μL) after 1-hr and 24-hr incubation with aqueous extract (10 μg/ml, 100 μg/ml, and 1,000 μg/ml) was diluted with 910 μL of sodium buffer phosphate (0.05 M, pH 10.7) and 40 μL of adrenaline (final concentration 2 mm), and the absorbance was recorded immediately. Results were expressed as units (U) of SOD activity/ml, where 1 unit (U) of SOD inhibits the autooxidation of adrenaline by a50% (18).

NF-κB assays

The effect of the extract (1,000 μg/ml) on nuclear factor kappa-B (NF-κB) translocation in tumoral cells was determined

by the Cayman Chemical NF-κB (p65) (Cat. 10007889) transcription factor assay in a nonradioactive, sensitive method for detecting specific transcription factor; DNA-binding activity in nuclear extracts was prepared according to instructions given on the kit.

Release of H_2O_2

The determination of H_2O_2 was carried out by incubating tumoral cells (3×10^5 cells/ml) with extract (10 μg/ml, 100 μg/ml, and 1,000 μg/ml) and a solution of 0.56 mM of diaminobencidine tetrahydrochloride (DAB) containing 140 mM of NaCl, 10 mM of potassium phosphate, 5.5 mM of dextrose, and 0.01-mg/ml type-II horseradish peroxidase (HRP) (Sigma, San Diego, USA). After 1-hr incubation, the reaction was stopped by the addition of 10 ml of 4 N NaOH and the absorbance was measured at 650 nm in a microplate reader (Microplate Reader Benchmark. Bio-Rad, CA, USA). Results were expressed as nanomoles of $H_2O_2/10^6$ cells. A standard curve of known molar concentrations of H_2O_2 in buffered phenol red was run in each test. The quantity of H_2O_2 was determined in absence or presence of 6 mM of sodium azide (SOD inhibitor) (14).

Hydrogen peroxide scavenging activity: peroxidase activity

Supernatants were incubated in Krebs-Henseleit buffer (pH 7.4) containing NaCl 125 mM, KCl 4.0 mM, NaH_2PO_4 0.5 mM, $MgCl_2$ 0.1 mM, $CaCl_2$ 1.1 mM, and glucose 5.0 mM, bubbled with 95% O_2 and 5% CO_2 at 37°C. The peroxidase activity was determined by the method described by Herzog and Fahimi (19). Briefly, 25 μL of each sample were incubated with 950 μL of DAB (5×10^{-4} M) and 25 μL of H_2O_2 (a solution of 30% H_2O_2 Parafarm R, diluted 1/86 in distilled water) reaching a final volume of 1,000 μL. A DAB solution without H_2O_2 was used as reaction blank. In all cases the reaction was initiated by the addition of H_2O_2 and the change in absorbance readings was recorded at 30 s intervals for 5 mins using a Shimadzu recording spectrophotometer UV-240 (graphic printer PR-1) set at 465 nm. The Δ absorbance/min was calculated. A calibration curve of peroxidase concentration vs. Δ absorbance/min was plotted using HRP thereby obtaining a linear relationship in the range of 1.95×10^{-3} to 2.5×10^{-5} U/ml. The activity of samples was calculated by interpolation in the standard curve and corrected accordingly.

Effect of extract on activity of exogenous superoxide dismutase (eSOD)

This assay is based on the property of SOD to inhibit the spontaneous oxidation of adrenaline to adenochrome by the reduction of oxygen (18). It is thus possible to monitor eSOD activity by observing the production of adenochrome with a spectrophotometer in kinetic mode (480 nm). eSOD (150 U/ml) (14) was incubated for 15 min and 60 min in presence or absence of aqueous extract (10 μg/ml, 100 μg/ml, and 1,000 μg/ml), respectively. After incubation, 50 μL of sample was diluted with 910 μL of sodium buffer phosphate (0.05 M, pH 10.7) and 40 μL of adrenaline (final concentration 2 mM), and the absorbance was recorded immediately. Results were expressed as units (U) of SOD activity/ml, where 1 U of SOD inhibits the autooxidation of adrenaline by a 50%.

Statistical analysis

Data was analyzed by the Student's t test, one way ANOVA, and Dunnett's test. Differences were considered significant when $p \leq .05$ (20).

RESULTS

First, the effect of the aqueous extract of *L. divaricata* on tumoral cell proliferation was studied. As it can be seen in Figure 1(a), the aqueous extract decreased the cell proliferation in a concentration-response relationship ($p < .01$). Moreover, the effect on cell viability was studied at two different incubation time, i.e., 1 hr and 24 hr. At 1 hr the extract decreased only significantly ($p < .01$) cell viability at higher concentrations (100 μg/ml and 1,000 μg/ml), but at 24 hr the decrease in cell viability was observed with all analyzed concentration ($p < .01$) (Figure 1(b)). To study the participation of ERK and P-38 pathways in the observed responses, the antiproliferative effect of the extract was studied in the presence of SB 203580 (Sigma), a P-38 inhibitor and PD 98059 (Sigma), a MEK-½ inhibitor. It can be seen in Figure 1(c) that the MEK-½ inhibitor decreased basal cell proliferation *per se* ($p < .01$), and P-38 inhibitor did not modify it. The P-38 inhibitor reverted the effect exerted by extract in cell proliferation in all concentrations analyzed ($p < .01$).

Taking into account the relation of P-38 pathway with apoptosis, and that the P-38 inhibitor reverted the effect of the extract on cell proliferation, the induction of apoptosis was studied. The MEK-½ inhibitor decreased cell viability *per se* and produced early apoptosis, also P-38 inhibitor induced early apoptosis ($p < .05$) (see Table 1). The extract induced ($p < .01$) early apoptosis significantly in comparison to basal values at concentration the of 1,000 μg/ml and decreased cell viability ($p < .05$), this effect was reverted by P-38 inhibitor, MEK-½ inhibitor, and P-38 and MEK-½ inhibitors used together ($p < 0.01$). The low concentration analyzed, i.e., 10 μg/ml did not induce apoptosis (Figure 2 and Table 1)

To analyze if the antiproliferative effect of the extract and the induced apoptosis were mediated by the endogenous production of H_2O_2, cells were incubated in the presence of different concentrations of aqueous extract (10 μg/ml, 100 μg/ml, and 1,000 μg/ml). The extract significantly increased ($p < .01$) the H_2O_2 level at 100 μg/ml and 1,000 μg/ml (see Figure 3(a)). To analyze the participation of SOD in the increase of H_2O_2 amount, the same experiment was performed in the presence of sodium azide, a Mn^{+2}–SOD inhibitor. In Figure 3(b) it can be seen that sodium azide reverted ($p < .01$) the effect of aqueous extract of H_2O_2 production; even so at the concentration of 10 μg/ml it was able to increase H_2O_2 production, but not in a significant manner.

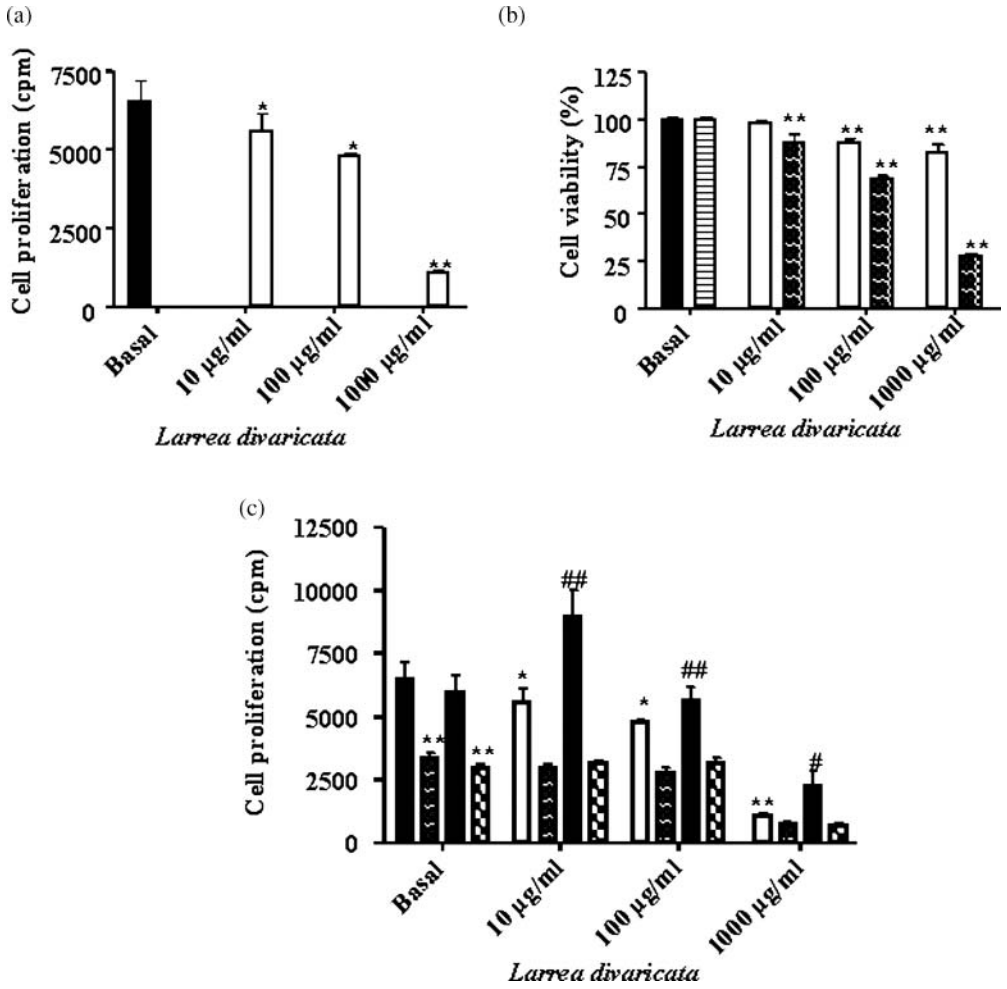

Figure 1. Effect of aqueous extract and MEK-1/2 and P-38 inhibitors on cell proliferation and cell viability after 24 hr incubation. (a) Tumoral cells were treated with or without aqueous extract (10, 100, and 1,000 μg/ml). (b) Tumoral cells were incubated with or without aqueous extract plus sodium azide. (c) Cells were incubated with or without aqueous extract (10, 100, and 1,000 μg/ml) in presence of MEK inhibitor (5×10^{-6} M), P-38 inhibitor (10×10^{-6} M), or both. Results were expressed as mean $\pm$ SEM of three experiments made in triplicate. $^{*}p < .05$, $^{**}p < .01$, show significant differences with respect to normal basal value. $^{\#}p < .05$, $^{\#\#}p < .01$, show significant differences between tumoral basal values and P-38 inhibitor treatment.

Taking into account that H_2O_2 can increase nitric oxide (NO) levels, the participation of NO in the effect of the extract was studied. To do this, NO level was measured in the presence of different concentrations of extract (10 μg/ml, 100 μg/ml, and 1,000 μg/ml) alone or in presence of MEK-1/2 inhibitor, LNMMA, and two inhibitors used together. It can be observed in Figure 4(a) that only 1,000 μg/ml increased NO production significantly ($p < .01$) at 1-hr incubation and this effect was reverted by MEK-1/2 inhibitor, LNMMA, as well as by two inhibitors used together ($p < .05$). At 24-hr incubation extract did not increase NO levels (data not shown). In order to study whether NF-κB was related to the antiproliferative action of the extract and the induction of NO level, the effect of aqueous extract upon NF-κB was evaluated. In Figure 4(b) it can be observed that the extract produced a significant nuclear translocation of NF-κB ($p < .01$).

To relate the production of H_2O_2 with SOD activation (enzyme involved in H_2O_2 formation), the effect of *L. divaricata* extract on cellular SOD and Px activities was studied. The cells were incubated during 1 hr and 24 hr with different concentrations of aqueous extract (10 μg/ml, 100 μg/ml, and 1,000 μg/ml). The results obtained showed that at 1 hr of incubation *L. divaricata* increased the SOD activity in higher concentrations (100 μg/ml and 1,000 μg/ml) ($p < .01$), but the basal SOD activity could not be detected by this method. At 24 hr and 1,000 μg/ml concentration (Figure 5(b)) the SOD activity with respect to basal activity increased significantly ($p < .01$). The Px activity got decreased significantly ($p < .05$) by the extract only after 24 hr of treatment, this effect was exerted by all analyzed concentrations (Figure 5(d)). To study the possible mechanism by which the extract increased cell SOD activity, the effect of the extract on the activity of an exogenous SOD (eSOD)

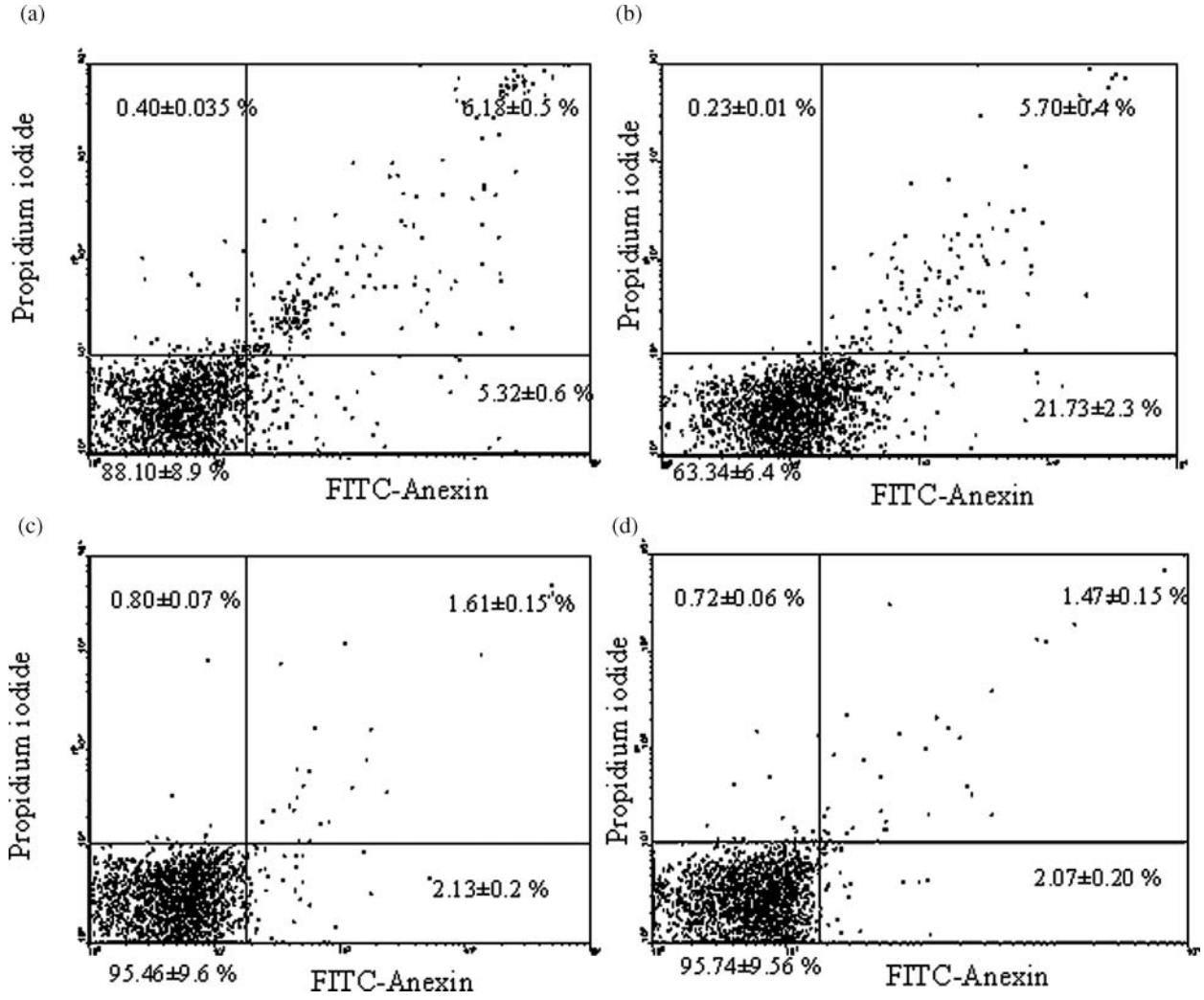

Figure 2. Dot plot analysis of BW5147 cells stained with annexin–V FITC and propidium iodide treated with aqueous extract (1,000 μg/ml) under different conditions. (a) Untreated cells (basal). (b) Treated with aqueous extract (1,000 μg/ml). (c) Treated with aqueous extract (1,000 μg/ml) plus P-38 inhibitor (10×10^{-6} M). (d) Treated with aqueous extract (1,000 μg/ml) plus ERK $^1/_2$ inhibitor (5×10^{-6} M). Upper left panel (necrosis), upper right panel (late apoptosis or necrosis), low left panel (viable cells), and low right panel (early apoptosis).

was studied. The results have been summarized in Figure 5(e) and (f). These results showed that the aqueous extract of *L. divaricata* increased significantly ($p < .01$) the activity of eSOD at 15 min of incubation at all analyzed concentrations (Figure 5(e)), whereas at 60 min of incubation only the concentration of 1,000 μg/ml increased eSOD activity (Figure 5(f)).

DISCUSSION

The aqueous extract of *L. divaricata* presented antiproliferative action on BW5147 cells, this effect has already beenobserved by Anesini *et al*. (2, 3). The extract decreased cell viability in relation to the concentrations and the time of incubation (Figure 1(b)). Different authors have shown the antiproliferative effect of plant extracts; among others Barreiro Arcos *et al.* (21) demonstrated that dichloromethane and ethanol extracts obtained from *Tilia cordata* Mill. flowers induced apoptosis on tumoral cells (BW5147 lymphoma). Both extracts exerted a selective action, showing no cytotoxic effect in normal T-cells (21). Mehrotra *et al.* (22) demonstrated the anti-lymphoproliferative potential of ethanolic extract of *B. diffusa in vitro* (22).

It is known that the responses of eukaryotic cells to external stimuli are partially regulated by the activation of the tree major MAP kinase signaling pathways: kinases 1 and 2 (ERK), Jun N-terminal kinase (JNK), and P38 MAPK. These kinases are known to mediate many of the cellular processes related to cell growth, survival, and death. Principally, the ERK pathway is activated by mitogenic stimuli, such as growth factors, cytokines, and phorbol esters and plays a major role in regulating cell growth and differentiation (23). It is shown that the P-38 MAPK activity may be regulated by oxygen species and

Table 1. Apoptosis Induced by *L divaricata* on BW5147 Tumoral Cells

	Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis (%)
Apoptosis: positive control	10.97 ± 1	1.79 ± 2	61.99 ± 6	25.25 ± 2.5
Basal	88.10 ± 8.9	5.32 ± 0.6	6.18 ± 0.5	0.40 ± 0.035
Inh. P38	76.74 ± 7.5*	12.20 ± 1.3*	9.60 ± 0.9	1.46 ± 0.2
Inh. MEK-1/2	67.99 ± 6.8*	13.85 ± 1.4*	15.69 ± 1.6	2.46 ± 0.25
Inh. P38 + Inh MEK-1/2	67.31 ± 6.5	18.20 ± 1.7	12.40 ± 1.3	2.09 ± 0.2
Larrea divaricata 1,000 μg/ml	63.34 ± 6.4*	21.73 ± 2.3**	5.70 ± 0.4	0.23 ± 0.01
Larrea divaricata 1,000 μg/ml + Inh. P38	95.46 ± 9.6#	2.13 ± 0.2##	1.61 ± 0.15	0.80 ± 0.07
Larrea divaricata 1,000 μg/ml + Inh. MEK	95.74 ± 9.56#	2.07 ± 0.20 ##	1.47 ± 0.15	0.72 ± 0.06
Larrea divaricata 1,000 μg/ml + Inh. P38 + Inh. MEK	91.05 ± 9#	6.54 ± 0.6 ##	1.83 ± 0.2	0.67 ± 0.07
Larrea divaricata 100 μg/ml	75.69 ± 7.4	7.03 ± 0.78	7.29 ± 0.7	0.99 ± 0.09
Larrea divaricata 100 μg/ml + Inh. P38	93.24 ± 9.2	1.88 ± 0.2	3.69 ± 0.34	1.19 ± 0.12
Larrea divaricata 100 μg/ml + Inh. MEK	90.47 ± 9	2.29 ± 0.2	5.86 ± 0.6	1.38 ± 0.1
Larrea divaricata 100 μg/ml + Inh. P38 + Inh. MEK	76.92 ± 7.5	4.28 ± 0.42	16.11 ± 1.5	2.69 ± 0.25
Larrea divaricata 10 μg/ml	90.82 ± 9	5.11 ± 0.6	3.29 ± 0.2	0.79 ± 0.05
Larrea divaricata 10 μg/ml + Inh. P38	94.43 ± 9.5	1.28 ± 0.2	3.51 ± 0.3	0.78 ± 0.06
Larrea divaricata 10 μg/ml + Inh. MEK	90.02 ± 9.2	2.65 ± 0.24	6.12 ± 0.5	1.21 ± 0.2
Larrea divaricata 10 μg/ml + Inh. P38 + Inh. MEK	91.15 ± 8.3	2.15 ± 0.3	5.71 ± 0.6	0.99 ± 0.01

Results represent the percentage of cells in different stages of apoptosis of a three representative experiments realized separately. *$p < .05$, significantly different with respect to basal value; **$p < .01$, significantly different with respect to basal value; #$p < .05$, significantly different with respect to values of viable cells after treatment with L. divaricata, 1,000 μ g / ml. ##$p < .01$, significantly different with respect to values of early apoptosis of cells treated with L. divaricata, 1,000 μg / ml.

NO, which, in turn, inversely regulates the expression of cyclin D1, is implicated in G1 progression in hepatocytes and other proliferating cell types (24). In order to investigate the mechanism of the antiproliferative action of the extract, the pathways related to cell growth such asP38 and ERK were analyzed. P-38 MAPK and ERK are believed to be redox-dependent biomolecules that modulate cell proliferation, survival, and death (25). First the inhibitors of these ways were studied alone. The MEK-1/2 inhibitor decreased the cell proliferation *per se*; this could be related to the fact that these cells used this pathway for proliferation (26) (Figure 1(c). Furthermore, the P-38 inhibitor did not modify basal cell proliferation, these results are in agreement with the results obtained by Miura *et al.* (27). Only P-38 inhibitor reverted significantly the antiproliferative response of the extract (Figure 1(c)). These results suggested that the extract exert the anti-proliferative action through P-38 pathway. Some authors have documented antiproliferative effect by plant extract (28–30).

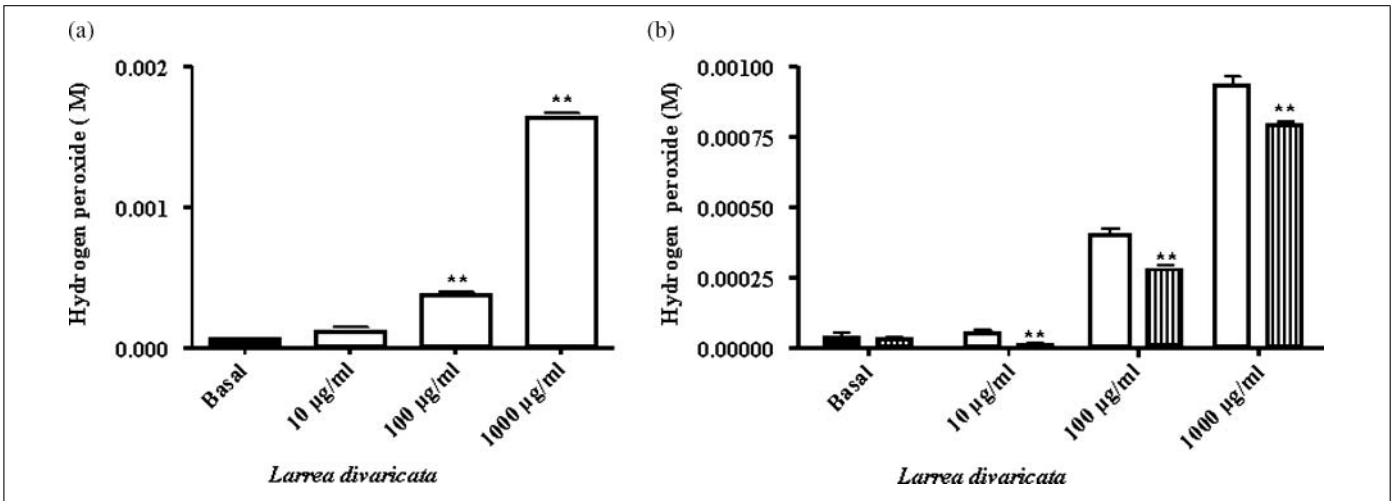

Figure 3. Determination of H_2O_2 levels in tumoral lymphocytes treated with aqueous extract of *L divaricata* (10, 100, and 1,000 μg/ml). In (a) the level of H_2O_2 in basal cells and in treated cell was determined. Results represent the mean ± SEM of three experiments performed in triplicate. **$p < .01$ shows significant differences with respect to basal cells according to ANOVA plus Dunnett's test. In (b), cells were incubated with aqueous extract of *L divaricata* (10, 100, and 1,000 μg/ml) plus sodium azide. Bars represent the mean ± SEM of three experiments performed in triplicate. **$p < .01$ shows significant differences with respect to basal values according to ANOVA plus Dunnett's test.

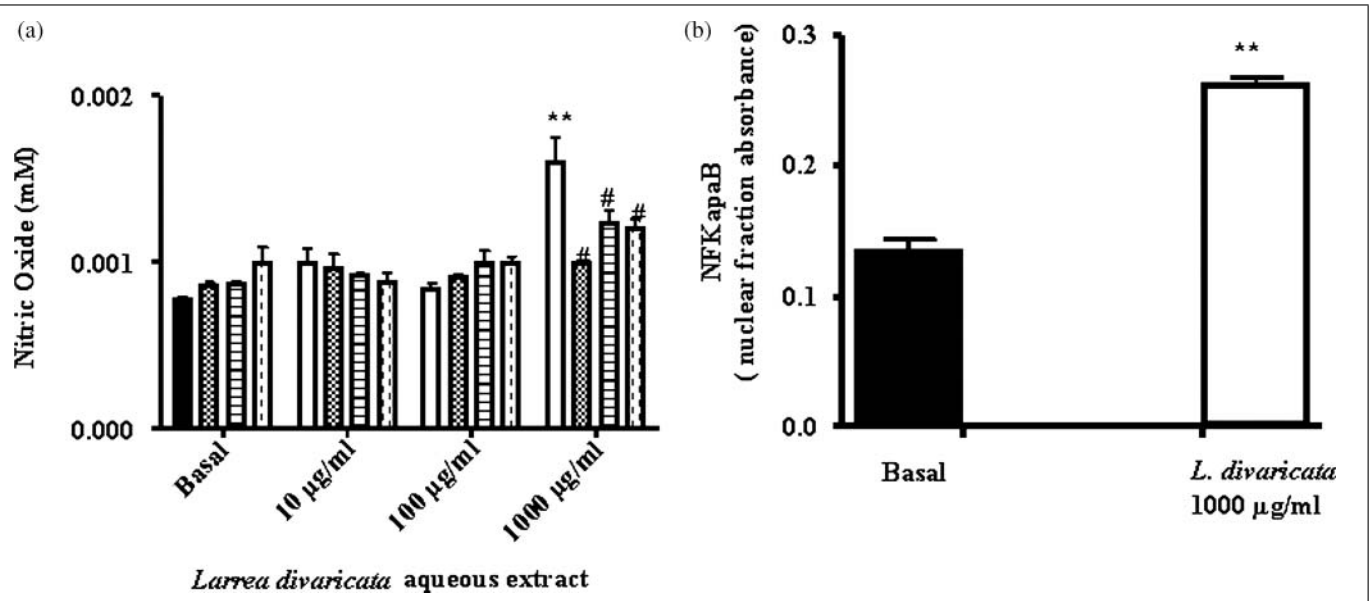

Figure 4. Effect of aqueous extract of *L divaricata* on total nitrites and NF-κB translocation. (a) Cells were treated with different concentrations of *L divaricata* (10, 100, and 1,000 μg/ml) during 1 hr, alone or in presence of LNMMA (1×10^{-3} M), MEK-$^1/_2$ inhibitor (5×10^{-6} M), LNMMA plus Erk $^1/_2$ inhibitor. (b) NF-κB translocation was determined as nuclear absorbance in tumoral cells in absence or presence of *L divaricata* (1,000 μg/ml). $^{**}p < .01$ is significantly different with respect to basal value according to ANOVA plus Dunnett's test. $^{\#}$Significant differences between *L divaricata* alone and in presence of inhibitors according to Student's *t* test.

The antiproliferative and cytotoxic effect of the aqueous extract is, in fact, related to the induced apoptosis, principally at 1,000 μg/ml. The effect on apoptosis was reverted by P-38 and MEK-$^1/_2$ inhibitor (Table 1 and Figure 2), suggesting the participation of these pathways in the effect of extract. The activation of ERK pathway is related to the death via production of NO (31); this is in accordance with our results. At 1,000 μg/ml, the extract increased NO level and this effect was reverted by MEK-$^1/_2$ inhibitor (Figure 4(a)). Moreover, the increase in NO levels could be related with a significant nuclear translocation of NF-κB induced by the extract (Figure 4(b)). NF-κB is a nuclear transcription factor probably related to the induction of NOS and NO production (32). It is known that the MEK/ERK pathway is related to the NO production and that NO in turn induces apoptosis. Besides, NO can directly induce cytochrome c release through mitochondrial membrane potential loss leading to cell death by apoptosis via the activation of JNK/SAP and P-38 MAPK pathways (33, 34). Moreover, the cytotoxic effects of NO on some tumor cells are due to the accumulation of P53 and increase in Bax/Bcl-Xl, both effects leading DNA damage (35). We have demostrated the increase in NO levels after treatment with plant extracts (36, 37).

The extract increased endogenous production of H_2O_2 at 100 and 1,000 μg/ml (Figure 3(a)). To analyze the participation of SOD in the increase of H_2O_2, the same experiment was done in presence of sodium azide. It can be seen that sodium azide reverted the effect of the aqueous extract on H_2O_2 production (Figure 3(b)). It was found that tumor cells displayed low level of H_2O_2 (32). Previous results showed that BW5147 cells can modulate the production of H_2O_2 maintaining low SOD activity and a high Px activity (32). Moreover, low levels of Mn^{2+}–SOD have been observed in invasive breast carcinomas more frequently than in *in situ* carcinomas or non-neoplastic epithelia (38). Either the activation or the over expression of Mn^{2+}–SOD may lead to changes in the superoxide (O_2^-)/H_2O_2 balance, causing changes in the redox state affecting signal transduction pathways and finally modulating cell proliferation (39).

The increase in H_2O_2 level induced by the extract could be related to the effect exerted by it on cell SOD and Px activities. The extract significantly increased SOD activity at 1 and 24 hr of cell incubation (Figure 5(a) and (b)), meanwhile, the Px activity was decreased after 24 hr treatment (Figure 5(d)).

In order to analyze the mechanism of action by which *L. divaricata* increased SOD activity, the effect of the extract was studied on an exogenous SOD. The extract only increased the activity of eSOD at 1,000 μg/ml, suggesting an interaction with the active or alosteric site of the enzyme, which contributed to its activation (Figure 5(e) and (f)). These results are in agreement with Chaurasia *et al.* (40) who showed that *Withania somnifera* root extract increased the activities of antioxidant enzymes, SOD, and CAT in mice (40). Ali (41) demonstrated that *Rhazya stricta* in a dose-dependent manner significantly increased SOD activity and GSH concentration in a kidney cortex of rats (41). Badami *et al.* (42) showed that *Striga orobanchioides* increased levels of SOD in liver of rats (42).

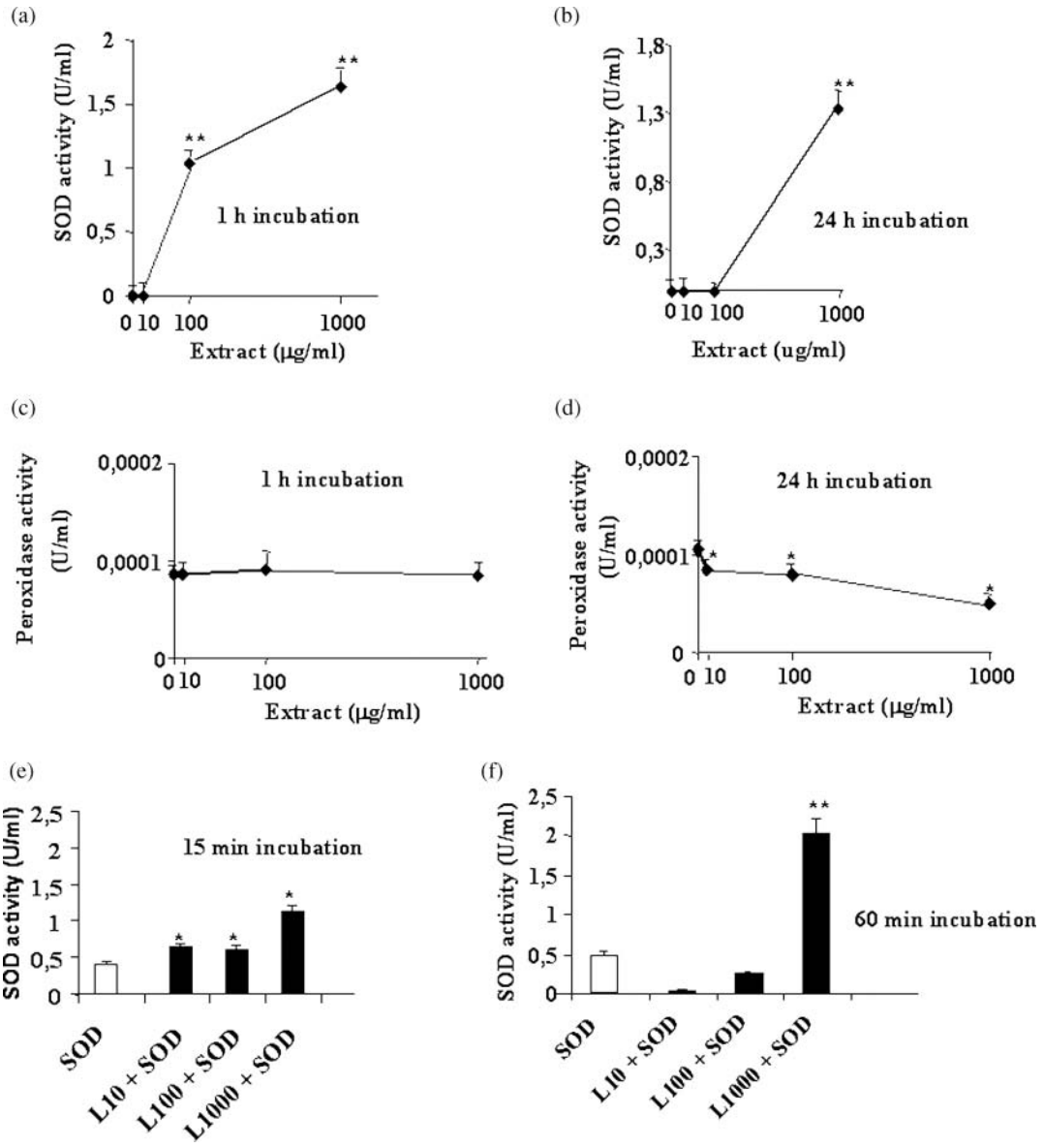

Figure 5. Effect of *L divaricata* on the activity of SOD, Px, and aSOD. Cells were incubated with *L divaricata* at 10, 100, and 1,000 μg/ml during 1 and 24 hr; then SOD (a) and (b) and Px (c) and (d) activities were determined. In (e) and (f) cells were incubated with aqueous extract for 15 and 60 min plus pure exogenous SOD. Bars represent the mean $\pm$ SEM of three experiments performed in triplicate. $^{*}p < .05$, $^{**}p < .01$ significantly different with respect to basal values according to ANOVA plus Dunnett's test.

It can be concluded that the inhibition of cell proliferation was related to the activation of ERK pathway and the subsequent translocation of NF-κB to the nucleus, leading to the induction of iNOS (inducible NO sintase) and increase in NO levels, which in turn activates P-38 pathway leading to apoptosis. Finally, it was observed that these tumor lymphocytes need to have low levels of H_2O_2 to allow the proliferation, which can be accomplished by maintaining a low Mn^{2+}–SOD and high Px activities. *L. divaricata* increased the activity of SOD, which leads to the increase of H_2O_2 levels. The development of new drugs thatproduce an increase in H_2O_2 levels can be a therapeutic strategy, clinically useful for the treatment of these type of cells.

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