Journal of Clinical Toxicology

Hapon et al., J Clin Toxicol 2014, 4:6
DOI: 10.4172/2161-0495.1000222

Research Article Open Access

Aqueous Extract of *Prosopis strombulifera* (LAM) BENTH Induces Cytotoxic Effects against Tumor Cell Lines without Systemic Alterations in BALB/c Mice

Hapon MB[1,2], **Hapon MV**[3,4], **Persia FA**[1], **Pochettino A**[5], **Lucero GS**[3,4] **and Gamarra-Luques C**[1,6]

[1]*Laboratorio de Reproducción y Lactancia, IMBECU-CONICET, Mendoza, Argentina*

[2]*Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Cuyo, Mendoza, Argentina*

[3]*Laboratorio de Fitopatología, IBAM-CONICET, Mendoza, Argentina*

[4]*Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Mendoza, Argentina*

[5]*Laboratorio de Toxicología Experimental, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina*

[6]*Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina*

[*]**Corresponding author:** Carlos Gamarra Luques, Instituto de Medicina y Biología Experimental de Cuyo (IMBECU), CCT Mendoza. Av Ruiz Leal s/n. Casilla de Correo 0855. CP 5500, Mendoza Capital, Provincia de Mendoza, Argentina, Tel: (54+261) 5244198; E-mail: cgamarra@mendoza-conicet.gob.ar

Received date: Oct 06, 2014, **Accepted date:** Dec 29, 2014, **Published date:** Dec 31, 2014

Abstract

Prosopis strombulifera (Lam.) Benth. is a rhizomatous shrub native to the northern and central zones of Argentina. The analgesic and antibiotic properties of this plant had been demonstrated but there are no previous reports of its cytotoxic activity. The goal of this work was to analyze the cytotoxic activity of P. strombulifera against HCT-116 and MCF-7 cell lines, and to evaluate toxic systemic effects in BALB/c mice. Changes induced by the aqueous extract from leaves on tumoral cell lines were studied by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay, Trypan blue dye exclusion assay, optical microscopy, Western Blot analysis of PCNA and cPARP, LDH activity, Ames´ test and clonogenic survival. Oral sub-chronic toxicity was assessed on BALB/c mice at concentrations up to 150 mg/animal/day. Analyses included animal/organs weight; erythrocytes, leukocytes and platelets number; and serological determinations of glucose, ASAT, ALAT, urea and creatinine. Extract has induced cytotoxicity, affecting proliferation and viability in both cell lines in a dose and time-response manner. IC_{50} was 2.25 µg/ml and LC_{50} 5.05 µg/ml in HCT-116, while values were 3.01 µg/ml and 7.52 µg/ml, respectively, in MCF-7. In both cell lines, the antiproliferative effect was confirmed by reduction of PCNA protein expression. When LC_{50} concentrations were used, extract-induced necrosis (evidenced by the increase in extracellular LDH activity), apoptosis (increased cPARP protein expression) and clonogenic survival diminution. Mutagenic activity of extract was caused at concentrations of 500 µg/ml (99 and 64-fold higher than HCT-116 and MCF-7 LC_{50} concentrations). Animal studies demonstrated that no significant toxicity was induced. In conclusion, this is the first report of *P. strombulifera* cytotoxic activity against tumoral cell lines. Sub-chronic extract administration did not cause deleterious effects *in vivo*. Altogether, the presented results make *P. strombulifera* a promising natural product for cancer research and treatment.

Keywords: *Prosopis strombulifera*; Natural products; Cytotoxicity; Antiproliferative; Lethality; Mutagenicity

Introduction

At present, more than twelve million people worldwide are diagnosed annually with some type of cancer. At least one third of them will not survive the disease. The progressively aging population, late diagnosis, and poor response to current treatments make cancer the second cause of death around the world [1]. The search for new chemotherapeutic agents is therefore a priority in order to find new therapeutic approaches to improve cancer prognosis and treatment.

Many drugs used in oncology have been provided by nature. Antitumor agent research among natural sources has been a successful strategy. The contribution of plants to cancer treatment is evidenced by the success of drugs like vinblastine, vincristine, irinotecan, topotecan, paclitaxel and docetaxel, as well as a number of other compounds that are currently being evaluated in clinical trials [2,3].

In the Eastern hemisphere, ancestral medicine had been greatly developed and the description of natural bioactive compounds is considerable. In contrast, in Argentina, the study of compounds obtained from regional plants is emerging. There are more than five hundred species of plants in Mendoza province, central west Argentina for which "folkloric medicine" has described several uses to preserve and aid health [4]. Only a small number of them have recently been studied to confirm their phytopharmaceutical properties.

Prosopis strombulifera (Lam.) Benth. is a rhizomatous shrub up to 1.5 m in height that grows in the northern and central zones of Argentina [5]. In Mendoza, it is mainly native to the northern part of the province [6]. This species is locally known as "retortuño", "retortón" or "mastuerzo". The plant has been used by the Huarpe pre-Columbian tribe as astringent, anti-inflammatory, odontalgic and anti-diarrheic agent [4,7]. Recent scientific studies have confirmed part of its ethnopharmacological uses, describing the molecular mechanism involved in the analgesic effect of this plant [8] and its biological activity against microorganisms such as *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi* [9-11]. The chemical compounds described in the species include flavonoids, tannins, carbohydrates and a small amount of saponins and steroids [8]. The

chemical groups described more precisely are the polyamines (putrescine, spermidine and cadaverine) and jasmonic acid derivatives [12]. To our knowledge, there are no reports of cytotoxic actions of *P. strombulifera* related to antiproliferative or lethal activity against tumor cells.

The goal of this work was to analyze the cytotoxic activity of the crude aqueous extract obtained from leaves of *P. strombulifera*. More specifically, the present study was conducted to evaluate antiproliferative, lethal and mutagenic actions of the plant extract *in vitro* against human tumor cell lines HCT-116 and MCF-7 (colorectal cancer and breast adenocarcinoma, respectively), and to evaluate the toxic systemic effects on BALB/c mice.

Materials and Methods

Crude extracts preparation

Prosopis strombulifera was collected in December 2012 in Lavalle county, Mendoza, Argentina (33° 44'10" S, 68° 21' 30.5" W). The botanical identifications were performed by two of the authors (C Gamarra-Luques and MV Hapon). A voucher specimen (MERL 61824) was deposited in the Mendoza Ruiz Leal herbarium. The crude extract was obtained according to the protocol described by Widmer and Laurent [13]. Briefly, 20 g of leaves were autoclaved in 200 ml distilled water for 1 hour. The solids were then separated by paper filtration and the volume was boiled down to 20 ml. Final extract concentration was representative of 1 g of leaves by milliliter (1 g/ml). Before use, crude extract was sterilized by passing through a 0.22 µm pore size filter.

Drugs and reagents

DMEM, (Dulbecco´s Modified Eagle Medium), penicillin and streptomycin were obtained from GIBCO, USA. Fetal bovine serum was obtained from Internegocios, Argentina. Primary antibodies against for the following proteins were used at the designated dilutions: cleaved poly (ADP-ribose) polymerase (cPARP, 1:5,000, BD Signal Transduction); proliferating cell nuclear antigen (PCNA, 1:1,000 Novus Biologicals, USA). Alpha-tubulin (1:20,000) and Protease Inhibitor Cocktail were purchased from Sigma-Aldrich, USA. Secondary antibodies conjugated to horseradish peroxidase and rose in rabbit and mouse were obtained from Santa Cruz Biotechnology, USA and Cell Signaling Technology, USA, respectively.

Cell culture and *in vitro* treatment

The colorectal carcinoma (HCT-116) and mammary adenocarcinoma (MCF-7) cell lines were cultured in DMEM, supplemented with 10% fetal bovine serum, 100 IU of penicillin and 100 µg/ml streptomycin. Culture conditions were fixed at 37°C, in a humidified atmosphere enriched by 5% CO_2. 24 hs after cell plating, treatments were dissolved directly in the culture media. Treatment time was different in both cell lines: 48 hs in HCT-116 and 72 hs in MCF-7. The selected treatment times were based on each cell line doubling time (DT) to let the control cells complete, at least, two entire cell cycles. In our culture conditions the DT calculated for HCT-116 was 20.05 ± 0.1h and for MCF-7 was 34.53 ± 0.1h.

To culture *Salmonella typhimurium*, 2.5% Bacto-Difco nutrient broth was prepared in distilled water. Glucose minimal agar plate (MA plate) contained 1.5% agar, 0.02% $MgSO_4 \cdot 7H_2O$, 0.2% citric acid, 1% K_2HPO_4, 0.35% $NaHNH_4PO_4 \cdot 4H_2O$ and 2% glucose. Top agar contained 0.75% agar and 0.5% NaCl.

Cytotoxicity assay by MTT

A colorimetric assay using MTT was performed [14]. MCF-7 and HCT-116 cells were seeded in 96-well microplates (2,500 and 7,500 cells/well/100 µl, respectively). 24 hrs later, the medium was aspirated and replaced by medium containing *P. strombulifera* extract at concentrations ranging from 0 to 10 µg/ml in both cell lines. HCT-116 cells were then incubated for 48 hrs and MCF-7 for 72 hrs. After the indicated times, medium was replaced by 100 µl of MTT solution (0.5 mg/ml in DMEM, without phenol red or FBS). Cells were incubated for an additional 4 hrs. MTT solution was then removed and 100 µl of DMSO added; the plates were shaken for 10 min to dissolve the formazan crystals. The optical density was measured using a Thermo Scientific Multiscan Elisa reader at 570 nm. The optical density obtained in untreated control cells was taken as 100% viability. Percent cytotoxicity was calculated as 100% viability. The assay was performed three times in triplicate.

Dye exclusion assay

Dose and time-response experimental designs were performed to quantify cell number and viability by trypan blue exclusion assay. Briefly, 7.5×10^4 cells were seeded into 6-well plates. When dose-response was analyzed, extract concentration used was in a range of 0 to 14 µg/ml for 48 hrs in HCT-116 and 72 hrs in MCF-7. In a time-response approach, cells were harvested at 0, 12, 24, 36, 48 and 72 hrs after treatment started. Trypsinized cells were resuspended in 1ml of phosphate buffered saline (PBS). Equal volumes of cell suspension and trypan blue (0.3% in PBS) were mixed and incubated for 5 min. Cells were then counted in a Neubauer haemocytometer chamber using a clear-field microscopy. Concentration of extract needed to achieve 50% of growth inhibition is indicated as IC_{50}, while extract concentration where 50% cell death was observed is indicated as LC_{50} [15,16]. Assays were performed three times in triplicate.

Morphological assessment of cell changes

Cells were seeded on cover-slides placed on the bottom of 6 well plates. After 24 hrs, control cells were fixed and stained. Culture medium containing treatment was replaced in the other cell groups. After 48 hrs in HCT-116 and 72 hrs in MCF-7, the remaining cells were fixed with methanol and stained with GIEMSA solution. Cover-slides were then mounted, evaluated and photographed with a Nikon Eclipse 200 microscope.

Western Blot analysis

After the treatment cells were scraped, pelleted, washed with PBS and lysed by the addition of two volumes of lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% IGEPAL, 1X Protease Inhibitors Cocktail and 1 mM NaF). Cells were disrupted by passing them through 21 gauge needles and gently rocked on ice for 30 min. Lysates were centrifuged at 16,000 g for 15 min at 4°C, and the supernatant was considered the whole cell extract. Protein content was assessed in the supernatant by the bicinchonic acid method (BCA; Pierce, Rockford, IL). Protein aliquots of 30 µg were separated in a 12% (w/v) acrylamide gel by SDS-PAGE and transferred to PVDF membranes. The blots were blocked in 5% (w/v) non-fat milk in TBS containing 0.1% (v/v) Tween-20. Blots were probed overnight with the

appropriate dilution in 2.5% BSA of each of the primary antibodies, and incubated with 1:5,000 dilution of peroxidase conjugate secondary antibody for 1 h at room temperature. The blots were washed, developed by chemiluminescence, using a ChemiDoc XRS + System (Bio-Rad, Laboratories). Band densitometric analysis was performed using Image Lab Software version 4.0 from Bio-Rad Laboratories.

Lactate dehydrogenase activity determination

LDH enzyme activity was measured in the culture media using a diagnostic LDH-L kit (Wiener lab, Rosario, Argentina) according to the instructions provided by the manufacturer. In brief, the supernatant of each well was collected after treatment. A substrate provided by the kit was then incubated at 37°C for 5 min, followed by the addition of NAD to the mixture. Spectrophotometric absorbance at 340 nm was registered for 2 min. Finally, LDH concentration measured in units/liter (U/l) was obtained by the equation [LDH]=(ΔA/min) × factor, where ΔA indicates absorbance difference between second 120 and 1, and factor represents $\varepsilon_{NAD/NADH}$ = 6230 M^{-1} cm^{-1}.

Clonogenic survival assay

To determine whether there is an added long-term lethal imprinting, we subjected pretreated cells to a clonogenic survival assay as previously described [17-20]. Briefly, 500 control and treated viable cells were placed in 6-well plates and cultured in fresh media without treatment until colonies were large enough to be clearly discerned. At this point, medium was removed and dishes were washed with PBS. Colonies were fixed with methanol and stained with crystal violet. The wells were then washed with tap water and dried at room temperature. The colonies, defined as groups of ≥ 30 cells, were scored manually with the aid of an inverted microscope. Clonogenic survival was expressed as the percent number of colonies quantified in the different treatment concentrations, considering the colonies formed by control cells as 100%.

Mutagenicity assay (Ames' test)

Mutagenicity activity was evaluated in a bacterial reverse mutation assay by the standard plate incorporation assay (Ames´ test) [21]. The *S. typhimurium* histidine-requiring test strains TA98 and TA100 were used. Bacteria were aerobically grown at 37°C in Nutrient Broth Bacto-Difco. The test was carried out by adding 0.2 ml of sterile 0.5 mM histidine-biotin and 0.1 ml of the overnight bacterial culture (approximately 1 × 108 bacteria/ml) to 2.0 ml of molten top agar (45°C). Concentrations from 0.5 to 500 µl/ml of the *P. strombulifera* crude extract were added to top agar tubes, which were then gently vortexed and subsequently transferred to plates with minimal glucose agar (30 ml/plate). Duplicate plates were poured for each concentration of reaction mixture in at least two independent experiments. After incubation at 37°C for 48 h in darkness, the His+ revertant colonies were manually counted.

Animals and *in vivo* treatment

Adult male BALB/c mice bred in our laboratory, 6 weeks old at the onset of treatment, were used. They were kept in a light (lights on 6:00 AM to 10:00 PM) and temperature (22-24°C) room. Mice chow (Cargill, Córdoba, Argentina) and tap water were available *ad libitum*.

Treatments were performed by diluting aqueous extract in the drinking water. Aqueous extract concentrations were in a range of 0-150 mg/animal/day for 28 days. A toxicity study was performed in accordance to "repeated dose 28 day-oral toxicity study in rodents", TG 407-OECD Guidelines for the Testing of Chemicals [22]; when nontoxic compounds are tested, no effects would be expected at a concentration of 1000 mg/kg/day (30 mg for 30 g animal). We tested increased concentrations until extract concentration affected animal water consumption. Animal water consumption and body weight were determined three times a week.

All animals were cared in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health. All procedures were approved by the Institutional Animal Care and Use Committee of School of Medical Science, Universidad Nacional de Cuyo (Protocol approval N° 30/2014).

Organ weight, blood cells and serological determinations

After treatment, animals were sacrificed by decapitation and troncal blood was collected. One aliquot was obtained with 10% EDTA to avoid clotting, and used for blood cell quantification. The other aliquot was used to obtain serum after coagulation and centrifugation, and used for biochemical characterization. Animals were necropsied, and liver, kidneys and spleen dissected for macroscopical analysis and weight determination.

Blood cell count was performed according to the Manual Prático de Hematología [23]. To determine hematocrit percentage, blood was centrifuged in heparinized capillaries and the length of fractions measured with a caliper. Erythrocyte, leukocyte and platelet counts were obtained using a, Neubauer hemocytometer chamber, and final concentrations calculated.

Glucose, aspartate aminotransferase (ASAT/GOT), alanine aminotransferase (ALAT/GPT), urea and creatinine serum levels were determined using specific Wienner Lab kits (Wienner Lab, Argentina), according to the manufacturer´s instructions. Briefly, commercial standards and samples were incubated with specific enzymes/substrates and formed products quantified by spectrophotometric absorbance. Final concentrations of products were calculated using mathematical formulas provided with the kit.

Statistical analysis

Data are expressed as mean ± standard error (SEM). Data were analyzed using GraphPad Prism 5.0 software. To assess IC_{50} and LC_{50}, a sigmoidal dose-response analysis was performed and values were considered acceptable when goodness of fit showed $R^2 \geq 0.90$. Comparisons between two groups were done using Student's T test. When more than two groups were compared, one-way ANOVA followed by Dunnett's multiple comparison test was used. Curve slopes in the study of mice body weight change were compared using linear regressions. In all cases, statistical significance was considered when $p<0.05$.

Results

Cytotoxicity effects of *P. strombulifera* on cancer cell lines.

In this study, we evaluated the aqueous extract activity in a dose and time-response experimental design.

The standard MTT assay was performed to demonstrate cytotoxicity in HCT-116 and MCF-7 cell lines. In both cases, *P. strombulifera* was able to induce cell toxicity in a dose-response manner, and 100% cytotoxicity was reached at concentrations close to 10 µg/ml (Figure 1).

We then quantified proliferation and viability by dye exclusion assay with trypan blue. The IC_{50} and LC_{50} were calculated to determine extract potency (Figure 1). The extract showed the highest potency in HCT-116 cells, the calculated IC_{50} was 2.25 ± 0.1 µg/ml and LC_{50} was 5.05 ± 0.1 µg/ml (Figure 1). In MCF-7, IC_{50} was 3.01 ± 0.1 µg/ml, while LC_{50} was 7.52 ± 0.1 µg/ml. In both cell lines, IC_{50} concentrations were able to reduce proliferation with limited lethal effects. The notorious reduction in cell proliferation at this concentration, without a significant lethality induction, was considered a cytostatic effect. The remaining viable cells at 10 µg/ml were not metabolically active, as shown by MTT cytotoxicity at this concentration.

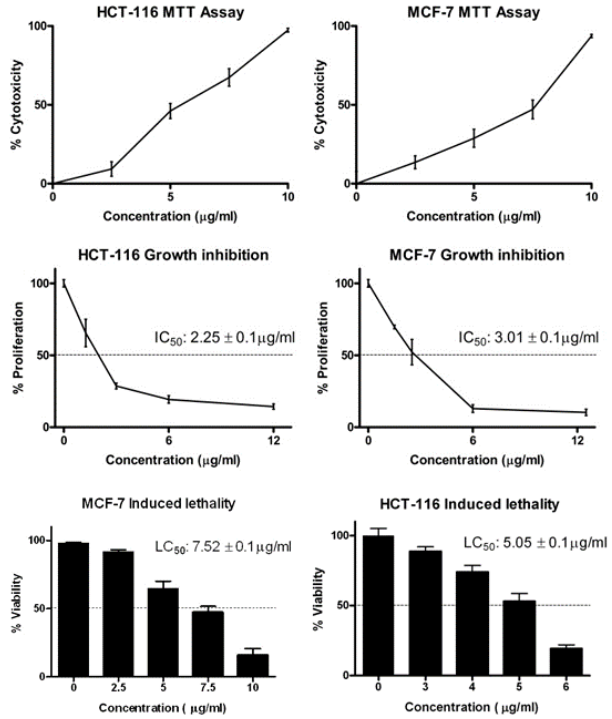

Figure 1: Dose-response effect of *P. strombulifera* crude extract on HCT-116 and MCF-7 cell lines. Cytotoxicity by MTT colorimetric assay, proliferation and viability by trypan blue dye exclusion assay are shown. Results are given as mean ± SEM of three different assays performed in triplicate.

Evaluation of the time-dependent proliferation and viability response revealed that cell damage increased along with the treatment time course (Figure 2). HCT-116 and MCF-7 (Figure 2) morphology changes were observed after treatment with IC_{50} and LC_{50} concentrations. Cell number, nuclear and cytoplasm alterations were more evident at higher concentrations than at the beginning of the treatment.

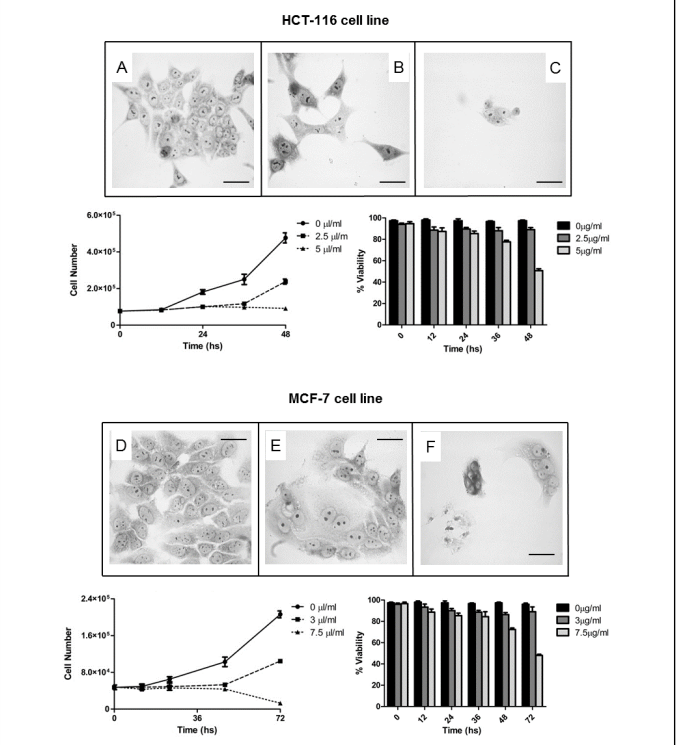

Figure 2: Time-response effect of *P. strombulifera* crude extract on HCT-116 and MCF-7 cell lines. A, B and C show optical microscopy of HCT-116 cells before and after treatment with the respective IC_{50} and LC_{50} concentrations of extract. D, E and F show the same conditions in MCF-7. Cell number and viability is presented at 0, 12, 24, 36 and 48 hrs in HCT-116; and 0, 12, 24, 36, 48 and 72 hrs in MCF-7. Time course of 0 µg/ml, IC_{50} and LC_{50} concentrations are presented for both cell lines. Proliferation and viability results are shown as mean ± SEM of three different assays performed in triplicate.

In both cell lines, cell number response to IC_{50} treatments showed an evident reduction in the proliferation curve slope. On the other hand, LC_{50} concentrations considerably affected cell number; final cell count showed no proliferation in HCT-116 and a lower cell number than at time 0 in MCF-7.

Time course viability was slightly reduced with IC_{50} treatments after 12 hrs in both cell lines. LC_{50} concentrations produced a similar reduction at 12 hrs, accompanied by a notorious reduction in viability during the last 24 hrs of treatment.

The molecular confirmation of cell cycle progression arrest was demonstrated by Western Blot analysis. Figure 3 shows a dose-dependent reduction in the expression of proliferating cell nuclear antigen (PCNA) in both cell lines.

Altogether, the cytotoxic assay, cell number and viability, morphological features, and molecular changes demonstrated a cytotoxic effect of *P. strombulifera* aqueous extract on human tumor cell lines in a dose and time-dependent manner.

Determination of mode of cell death

After having observed lethal activity of the extract, we performed a new set of assays in order to evidence the mode of cell death related to viability reduction. Western Blot analysis was carried out to reveal apoptosis by detection of cleavage in poly ADP ribose polymerase (PARP) protein. The biochemical determination of extracellular activity of lactactate deshidrogenase enzyme (LDH) was selected to determine necrosis (Figure 3).

Apoptosis, determined by cleaved PARP (cPARP) protein expression increase, was evident in both cell lines when the extract treatment reached LC_{50} concentration (5 µg/ml in HCT-116 and 7.5 µg/ml in MCF-7).

LDH is an intracellular enzyme that leaks into the medium when cells die in association with cytoplasmic membrane disruption. Consequently, determination of LDH activity in medium is a valid method to determine necrosis [24]. Enzyme activity determination showed increased values of LDH starting at IC_{50} concentrations in both cell lines. In HCT-116 cells, LDH values were significantly higher than in the untreated group at concentrations above IC_{50}. In both cell lines, LDH activity was statistically different between treatments and control cells at LC_{50}.

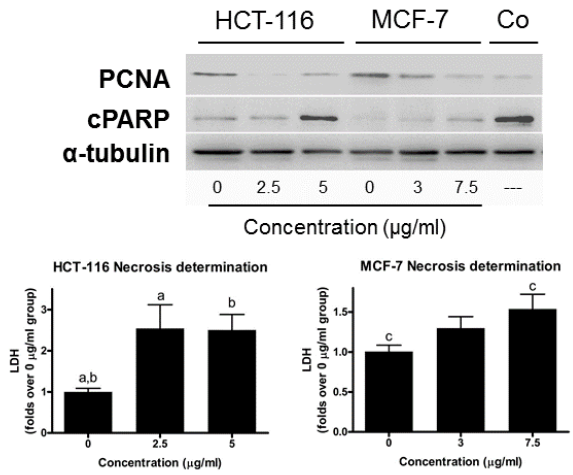

Figure 3: Proliferating cell nuclear antigen (PCNA) and cleaved poly ADP ribose polymerase (cPARP) protein expression, and extracellular lactate dehydrogenase activity (LDH) after *P. strombulifera* treatment on HCT-116 and MCF-7 cell lines. Treatment concentrations presented are 0 µg/ml, IC_{50} and LC_{50} in both cell lines. α-tubulin was used as protein loading control. Western Blot positive control group (Co) was obtained from proteins of harvested MCF-7 culture cells. LDH results are shown as mean ± SEM of three different assays performed in triplicate. Statistical difference between treatments and control group (a, b and c) was determined using one-way ANOVA and Dunnett's post-test for multiple comparisons, $p<0.05$.

Our results indicate that the lethality induced by *P. strombulifera* is not related to an exclusive mode of cell death. Increased expression of cPARP protein, together with an increase in extracellular LDH activity, indicated the coexistence of apoptosis and necrosis.

Long-term clonogenic survival

We sought to determine whether the cells that had not died in the experimental time period were able to recover from damage and regain the ability to form colonies. Both cell lines assayed were able to recover from the cytostatic effect induced by IC_{50} concentrations (2.25 µg/ml for HCT-116 and 3 µg/ml for MCF-7). When the treatment increased to LC_{50} concentrations (5 µg/ml for HCT-116 and 7.5 µg/ml for MCF-7), we observed a statistically significant reduction in the number of positive colonies, indicating that the cells were not able to recover their reproductive capacity once treatment was ended (Figure 4).

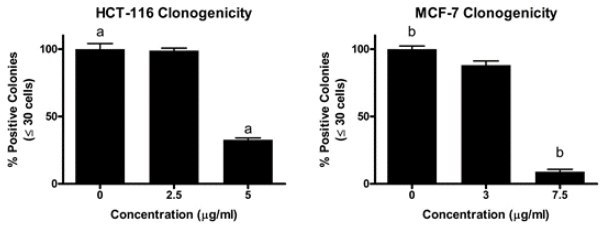

Figure 4: Clonogenic survival assay of *P. strombulifera* treated HCT-116 and MCF-7 cell lines. Positive colonies (≥ 30 cells) after 2 weeks are presented. HCT-116 and MCF-7 cells were previously treated at 0 µg/ml, IC_{50} and LC_{50}; for 48 and 72 hrs, respectively. Clonogenicity of the control cells was considered 100%. Results are shown as mean ± SEM of three different assays performed in triplicate. Statistical difference between treatments and control group (a and b) was determined by one-way ANOVA and Dunnett's post-test for multiple comparisons, $p<0.05$.

Changes observed in clonogenicity are interesting because they indicate a long-term reproductive damage when concentrations reach LC_{50} levels. Changes induced by the extract in cell homeostasis affect long-time survival even when the treatment does not kill the cells during initial exposure. In terms of compound efficacy, the reduction in long-term clonogenic survival, in combination with the acute cytotoxic action, suggest the presence of a very effective pharmacological compound.

Mutagenic activity of *P. strombulifera* crude extract

The *Salmonella* mutagenicity test has been extensively used to measure the mutagenic potential of many compounds [25-27]. The strains TA98 and TA100 have point mutations in the histidine biosynthetic operon that render them unable to grow in the absence of histidine. Cultures of bacterial strains in the presence of mutagenic compounds drive mutations that make microorganisms able to grow and form detectable colonies without adding histidine to the culture agar. Consequently, increased capability to grow and form colonies is indicative of mutagenic activity.

Addition of aqueous *Prosopis* extract to the bacteria culture media did not induce significant mutations until concentrations reached 500 µg/ml. The mutagenic value obtained represents 99 and 64 times the concentrations measured as LC_{50} in HCT-116 and MCF-7, respectively (Figure 5). In accordance with these results, DNA damage is not the mechanism that mediates biological activity of the crude extract at the concentrations that induce cytotoxicity in tumoral cell lines.

Study of *P. strombulifera* toxic effects on Balb/c mice

After *in vitro* determination of the *P. strombulifera* crude extract activity, we considered it relevant to establish how treatment could affect homeostasis *in vivo*. We performed anatomical, hematological and serological studies to examine possible alterations induced by treatment.

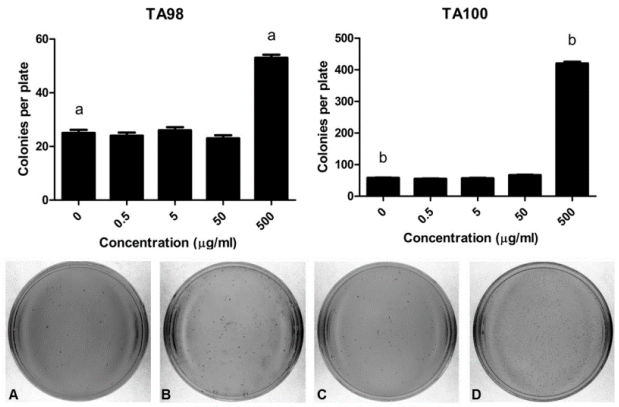

Figure 5: Mutagenicity of *P. strombulifera* crude extract. Revertant colonies of TA98 and TA100 strains of S. typhimurium at different concentrations of extract are presented. Mutagenic capability is evident by bacterial colony growth in agar. Results are shown as mean ± SEM of the absolute number of colonies per plate using one-way ANOVA and Dunnett's post-test for multiple comparisons, (*) $p<0.05$. Pictures A and B show positive colonies at 0 and 500 µg/ml extract concentration in TA98, while C and D show same concentrations in T100.

Water consumption was calculated as 4.5 ± 0.5 ml/mouse/day. Aqueous extract was then diluted at 30, 90 and 150 mg/mouse/day. Because there were no significant differences between treatments, only the results at the highest concentration used are shown.

Animal body weight change during treatment was recorded. Only a small, not statistically significant reduction in the final weight was induced by extract administration (Figure 6).

We studied the macroscopic appearance of organs and their weight, as well as the number of blood cells, to analyze whether treatment induced toxicity in detoxifying organs, such as liver or kidneys, or a rapidly proliferating fraction of cells, such as spleen and circulating blood cells (Table 1). Organs did not show differences in weight, color or structure. No macroscopical signs of ischemia, bleeding, fibrosis or degenerative proliferation were observed.

Hematocrit and the number of circulating blood cells were similar after treatment. Extract toxicity on rapidly proliferating cells or organs related to the hematological system can therefore be excluded.

Serum values of glucose and urea were determined to evaluate integrity in carbohydrate and protein metabolism, respectively. In addition, hepatic enzymes (ASAT/GOT and ALAT/GPT) and creatinine levels in serum were measured to study the functional status of liver and kidneys, respectively. None of the assessed biochemical parameters showed statistical differences between groups.

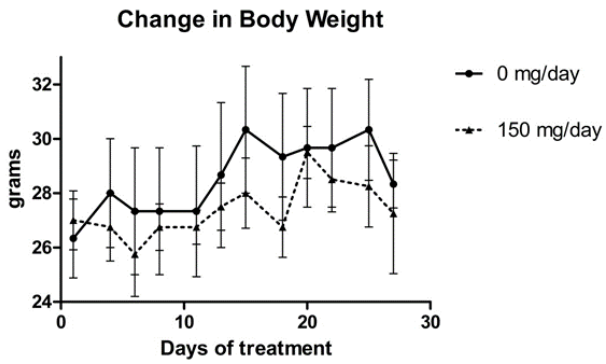

Figure 5: BALB/c change in body weight along sub-chronic toxicity assay (28 days of treatment). Curves of 0 mg/animal/day and 150 mg/animal/day are presented. Mean ± SEM of n=5 animals per group are used. Linear regression analysis ($p<0.05$) to compare curve slopes did not show significant differences.

Mouse and organ weight		
Detail	**0 mg/day**	**150 mg/day**
Body (g)	30.67 ± 1.76	27.25 ± 2.21
Liver (g)	1.59 ± 0.30	1.32 ± 0.17
Kidneys (g)	0.43 ± 0.11	0.45 ± 0.06
Spleen (g)	0.15 ± 0.01	0.11 ± 0.02
Blood cell determination		
Hematocrit (%)	30.11 ± 0.72	32.14 ± 2.85
Erythrocytes (10^6 cells/µl)	3.37 ± 0.67	3.32 ± 0.41
Leukocytes (10^3 cells/µl)	3.72 ± 0.41	3.41 ± 0.21
Platelets (10^5 cells/µl)	4.87 ± 0.27	5.29 ± 0.12
Serum biochemistry		
Glucose (mg/dl)	79.72 ± 12.10	99.56 ± 12.81
ASAT/GOT (U/l)	21.90 ± 3.77	30.14 ± 6.45
ALAT/GPT (U/l)	46.23 ± 16.67	33.47 ± 9.60
Urea (g/l)	0.67 ± 0.07	0.82 ± 0.16
Creatinine (mg/l)	4.93 ± 0.16	5.47 ± 0.16
Data presented are mean ± SEM of n=5 animals per group. Comparisons by Student´s T test ($p<0.05$) did not show significant differences between 0 mg/animal/day and 150 mg/animal/day.		

Table 1: BALB/c anatomical, hematological and serum biochemistry after 28 days of *P. strombulifera* treatment.

Discussion

Because of their widespread structural and biological diversity, natural products are an important source of new drugs and new

chemical entities. Plant-based drug discoveries have mainly led to the development of anticancer and anti-infectious agents, and continue to contribute to the new leads in clinical trials [28]. It is remarkable that approximately 50% of chemotherapeutic agents currently used are natural products, their analogs or derived compounds [29]. The discovery of new drug leads from medicinal plants may be aided by ethnopharmacology, which is a mode of scientific investigation related to the indigenous medicinal uses of a particular species [30]. The main goal of the present work was to describe the *in vitro* cytotoxic effects of *P. strombulifera* and its possible in vivo toxicity. The plant was selected from several native plants in Mendoza, Argentina for which folkloric medicine describes many uses in health preservation.

In the current study, antiproliferative, lethal and mutagenic effects of *P. strombulifera* crude extract was analyzed on colorectal (HCT-116) and breast adenocarcinoma (MCF-7) cell lines, and sub-chronic toxicity was studied in a BALB/c mouse model.

In vitro results demonstrate that *P. strombulifera* induces cytotoxicity in HCT-116 and MCF-7 cell lines. The experimental design used in the present work allows discriminating how proliferation and cell death contributes to cytotoxicity. Anti-proliferative and lethal actions increase in a dose and time-dependent manner. *P. strombulifera* activity on cell lines can be compared with 5-fluorouracil (5-FU), an antitumoral agent that is currently used worldwide. While aqueous extract of *P. strombulifera* has IC_{50} and LC_{50} values ranging between 2.5 -7.5 μg/ml, IC_{50} reported values for 5-FU are 18.92 μg/ml in HCT-116 [31] and 7.5 μg/ml in MCF-7 [32]. In terms of compound potency, *P. strombulifera* extract is similar to 5-FU.

Necrosis and apoptosis are the modes of cell death triggered by the extract in both cell lines. Coexistence of both mechanisms is not a new report, even when considering natural derivatives. The same has been reported for the action of the venom of Cuban scorpions *Rhupalurus junceous* on human tumor cell lines of epithelial and hematopoietic origin, as well as on normal cells [33]. The polyamine derivative putrescine-1,4-dicinnamide isolated from the mushroom *Pholiota spumosa* also shows this particularity [34]. It is possible that induction of necrosis and apoptosis by *P. strombulifera* treatment depends on extract concentration and cell line molecular targets. Nevertheless, the dual mechanisms of cell death activated by *P. strombulifera* aqueous extract represent an interesting attribute that increases its biological efficacy.

The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, preserving its reproductive capacity to form a large colony or a clone. A cell survival response denotes the relationship between the concentration of the agent used to produce an insult and the fraction of cells that retain their ability to reproduce [17]. The clonogenic survival reduction induced by *P. strombulifera* indicates a long-term reproductive damage. In an oncological context, diminished clonogenicity represents a powerful treatment trait related to the reduction in cell repopulation, a decrease in metastatic potential and a lower emergence of secondary drug resistance [18,35,36].

After having demonstrated *P. strombulifera* cytotoxicity, we considered it relevant to analyze the mutagenic potential of the extract. Actually, in oncology, there are compounds that act by inducing DNA damage. A typical example is the group of platinum derivatives, which mainly form different types of DNA adducts. Despite their confirmed efficacy, platinated drugs act as mutagenic agents by themselves, leading to many undesirable effects. Some of these effects collaborate to by-pass drug actions and allow the acquisition of harmful biological properties, such as collateral effects, secondary resistance and regrowth capacity. The achievement of these capabilities may increase post-treatment malignancy [37,38]. We performed mutagenicity studies in order to determine whether our extract induces DNA alterations. The results presented herein demonstrate that the extract concentrations used to induce cytotoxic effects did not lead to DNA mutations. Consequently, the extract can be used without risking the unexpected effects observed as a consequence of DNA injuries.

An important contribution of this work is related to the study of in vivo sub-chronic toxic effects. Even though there are several reports of human consumption [4,7,39], there are no detailed toxicological studies related to *P. strombulifera*. Using a healthy model of BALB/c mice, our study excludes any deleterious effects on animal homeostatic balance.

In conclusion, this is the first report of the cytotoxic effect of *P. strombulifera* aqueous extract on *in vitro* cell lines. The outcome of the treatment involves cytostasis at lower concentrations and lethal effects at higher concentrations. The mechanisms associated to cell death are apoptosis and necrosis. Regarding the long-term outcome of treatment, lethal concentrations were able to induce persistent cell damage, as was evidenced by a significant reduction in clonogenic survival assay. Mutagenic activity of the extract was not induced by effective concentrations used on cell lines. Lastly, sub-chronic administration of the extract did not induce toxicity *in vivo*. Altogether, these results make *P. strombulifera* aqueous extract a promising natural product for cancer research and treatment.

Acknowledgements

The authors are grateful to Dr. Mariella Superina and Tiffany Weidner for the editing of the manuscript. We thank Drs. Fanelli and Nadin for the generous gift of the cell lines and Dr. Ezquer for the reagents provided. This work was supported by 06/J372 and 06/J437 grants, from Secretaría de Ciencia, Técnica y Postgrado (SeCTyP) - Universidad Nacional of Cuyo - Argentina.

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, et al. (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893-2917.
2. Pereira DM, Valentao P, Correia-da-Silva G, Teixeira N, Andrade PB (2012) Plant secondary metabolites in cancer chemotherapy: where are we? Curr Pharm Biotechnol 13: 632-650.
3. Ruffa MJ, Ferraro G, Wagner ML, Calcagno ML, Campos RH, et al. (2002) Cytotoxic effect of Argentine medicinal plant extracts on human hepatocellular carcinoma cell line. J Ethnopharmacol 79: 335-339.
4. Roig F (2002) Flora medicinal mendocina. Las plantas medicinales y aromáticas de la provincia de Mendoza (Argentina) (1st edn), EDIUNC Serie Manuales Nº 33, Mendoza, Argentina.
5. Ariza Espinar L, Barboza GE, Bonzani NE, Cantero JJ, Filippa EM, et al. (2006) Flora medicinal de la provincia de Córdoba. Pteridófitas y antófitas silvestres o naturalizadas (1st edn) Museo Botánico, Córdoba, Argentina.
6. González Loyarte M, Gaviola S, Rodeghiero A, Buk E, Menenti M (2007) Methodological proposal for assessing peripheral lands to the Lavalle irrigated oasis, Mendoza (Argentina). Rev FCA UNCuyo 38: 109-126.
7. Hadad MA, Ribas YA (2010) Raíces Huarpes: Uso medicinal de plantas en la comunidad de Lagunas del Rosario, Mendoza, Argentina. (1stedn) Eds María Cecilia Montani y Cecilia Vega Riveros, San Juan, Argentina.

8. Saragusti AC, Bustos PS, Pierosan L, Cabrera JL, Chiabrando GA, et al. (2012) Involvement of the L-arginine-nitric oxide pathway in the antinociception caused by fruits of Prosopis strombulifera (Lam.) Benth. J Ethnopharmacol 140: 117-122.
9. Anesini C, Perez C (1993) Screening of plants used in Argentine folk medicine for antimicrobial activity. J Ethnopharmacol 39: 119-128.
10. Pérez C, Anesini C (1994) Antibacterial activity of alimentary plants against Staphylococcus aureus growth. Am J Chin Med 22: 169-174.
11. Pérez C, Anesini C (1994) In vitro antibacterial activity of Argentine folk medicinal plants against Salmonella typhi. J Ethnopharmacol 44: 41-46.
12. Reginato MA, Abdala GI, Miersch O, Ruiz OA, Moschetti E, et al. (2012) Changes in the levels of jasmonates and free polyamines induced by Na2SO4 and NaCl in roots and leaves of the halophyte Prosopis strombulifera. Biologia 67: 689-697.
13. Widmer TL, Laurent N (2006) Plant extracts containing caffeic acid and rosmarinic acid inhibit zoospore germination of Phytophthora spp. pathogenic to Theabroma cacao. European Journal of Plant Pathology 115: 377-388.
14. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63.
15. Freeburg EM, Goyeneche AA, Seidel EE, Telleria CM (2009) Resistance to cisplatin does not affect sensitivity of human ovarian cancer cell lines to mifepristone cytotoxicity. Cancer Cell Int 9: 4.
16. Musa MA, Badisa VL, Latinwo LM, Cooperwood J, Sinclair A, et al. (2011) Cytotoxic activity of new acetoxycoumarin derivatives in cancer cell lines. Anticancer Res 31: 2017-2022.
17. Munshi A, Hobbs M, Meyn RE (2005) Clonogenic cell survival assay. Methods Mol Med 110: 21-28.
18. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C (2006) Clonogenic assay of cells in vitro. Nat Protoc 1: 2315-2319.
19. Rafehi H, Orlowski C, Georgiadis GT, Ververis K, El-Osta A, et al. (2011) Clonogenic assay: adherent cells. J Vis Exp.
20. Gamarra-Luques CD, Goyeneche AA, Hapon MB, Telleria CM (2012) Mifepristone prevents repopulation of ovarian cancer cells escaping cisplatin-paclitaxel therapy. BMC Cancer 12: 200.
21. Maron DM, Ames BN (1983) Revised methods for the Salmonella mutagenicity test. Mutat Res 113: 173-215.
22. http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-thetesting-of-chemicals-section-4-health effects
23. http://www.slideshare.net/priscilaoliveira3975/manual-de-hematologia-18585125
24. Tang YQ, Jaganath IB, Sekaran SD (2010) Phyllanthus spp. induces selective growth inhibition of PC-3 and MeWo human cancer cells through modulation of cell cycle and induction of apoptosis. PLoS One 5: e12644.
25. McDaniels AE, Reyes AL, Wymer LJ, Rankin CC, Stelma GN Jr (1993) Genotoxic activity detected in soils from a hazardous waste site by the Ames test and an SOS colorimetric test. Environ Mol Mutagen 22: 115-122.
26. Vargas VM, Motta VE, Henriques JA (1993) Mutagenic activity detected by the Ames test in river water under the influence of petrochemical industries. Mutat Res 319: 31-45.
27. Negi PS, Jayaprakasha GK, Jena BS (2003) Antioxidant and antimutagenic activities of pomegranate peel extracts. Food Chemistry 80: 393–397.
28. Saklani A, Kutty SK (2008) Plant-derived compounds in clinical trials. Drug Discov Today 13: 161-171.
29. Pfisterer PH, Wolber G, Efferth T, Rollinger JM, Stuppner H (2010) Natural products in structure-assisted design of molecular cancer therapeutics. Curr Pharm Des 16: 1718-1741.
30. Heinrich M, Gibbons S (2001) Ethnopharmacology in drug discovery: an analysis of its role and potential contribution. J Pharm Pharmacol 53: 425-432.
31. Xu DB, Wang YL, Yue Y, Wu SC, Ding H (2013) [Inhibitory effect of a novel histone deacetylases inhibitor FK228 on human colon cancer HCT-116 cells in vitro and in vivo]. Zhonghua Zhong Liu Za Zhi 35: 814-818.
32. Gao J, Yan Q, Liu S, Yang X (2014) Knockdown of EpCAM enhances the chemosensitivity of breast cancer cells to 5-fluorouracil by downregulating the antiapoptotic factor Bcl-2. PLoS One 9: e102590.
33. Díaz-García A, Morier-Díaz L, Frión-Herrera Y, Rodríguez-Sánchez H, Caballero-Lorenzo Y (2013) In vitro anticancer effect of venom from Cuban scorpion Rhopalurus junceus against a panel of human cancer cell lines. J Venom Res 4: 5-12.
34. Russo A, Piovano M, Clericuzio M, Lombardo L, Tabasso S, et al. (2007) Putrescine-1,4-dicinnamide from Pholiota spumosa (Basidiomycetes) inhibits cell growth of human prostate cancer cells. Phytomedicine 14: 185-191.
35. Creton G, Benassi M, Di Staso M, Ingrosso G, Giubilei C, et al. (2006) The time factor in oncology: consequences on tumour volume and therapeutic planning. J Exp Clin Cancer Res 25: 557-573.
36. Facompre N, Nakagawa H, Herlyn M, Basu D (2012) Stem-like cells and therapy resistance in squamous cell carcinomas. Adv Pharmacol 65: 235-265.
37. Sedletska Y, Giraud-Panis MJ, Malinge JM (2005) Cisplatin is a DNA-damaging antitumour compound triggering multifactorial biochemical responses in cancer cells: importance of apoptotic pathways. Curr Med Chem Anticancer Agents 5: 251-265.
38. Chaney SG, Campbell SL, Bassett E, Wu Y (2005) Recognition and processing of cisplatin- and oxaliplatin-DNA adducts. Crit Rev Oncol Hematol 53: 3-11.
39. Hurrell JA, Ulibarri EA, Puentes JP, Buet Costantino F, Arenas PM, et al. (2011) Leguminosas medicinales y alimenticias utilizadas en la conurbación Buenos Aires-La Plata, Argentina. Boletín latinoamericano y del caribe de plantas medicinales y aromáticas 10: 443-455.