Research article

UHPLC–Q/Orbitrap/MS/MS fingerprinting and antitumoral effects of Prosopis strombulifera (LAM.) BENTH. aqueous extract on allograft colorectal and melanoma cancer models

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ABSTRACT

The aqueous extract of the Argentinean native plant, Prosopis strombulifera (PsAE), presents cytotoxicity against human cancer cell lines by inducing cytostasis, necrosis and apoptosis; with diminution of clonogenic survival; without genotoxic effects nor oral animal toxicity. Until now, the chemical extract composition and its in vivo antitumoral properties remain unknown; these studies are the aim of the current work. The PsAE was characterized by chemical fingerprinting and the metabolome was identified by tandem UHPLC-PDA-HESI-Q-orbitrap® mass spectrometry. Colorectal tumors were induced by DMH administration and melanomas resulted from B16-F0 S.C. cells injection; then, animals were treated orally with PsEA. To correlate in vivo results with in vitro cytotoxicity, B16-F0 cell were cultured to determine: cell proliferation and viability by dye exclusion assays, MTT and CFSE dilution; cell cycle distribution by flow cytometry; and immunoblotting of p21cip1, PCNA, cleaved caspase 3, cleaved PARP and TUBA1A. Based on UHPLC-OT-MS and PDA analysis, twenty-six compounds were identified, including: 5 simple organic acids, 4 phenolic acids, 4 procyanidins, 11 flavonoids, and 2 oxylipins. On C57BL6 mice, PsAE significantly increases the median survival on colorectal cancer and reduces the final volume and weight of melanomas. Over cultured cells, the treatment induce over-expression of p21, cytostasis by G2/M cell cycle arrest and apoptosis; while, on in vivo melanomas, treatment up-regulates p21 and slightly decreases PCNA. In conclusion, PsAE is composed by phenolic compounds which demonstrate cytotoxic and antitumoral properties when is orally administrated. Presented results support future research of PsAE as a potential phytomedicine for cancer treatment.

1. Introduction

Cancer consist of a group of illness with a common feature of uncontrolled cellular proliferation determined by several genetic, epigenetic and biochemical causes (Moreno-Sánchez et al., 2014). Despite of the many improvements obtained in cancer treatment, additional answers need to be found to overlap the remained deficiencies. Chemotherapy, as well as conventional treatments against cancer, usually determine unfavorable collateral effects and fails to control cancer progression. The utilization of medicinal plants were proposed as an option...
for the toxic effects determined by synthetic drugs (Rashid et al., 2002). A significant reduction in the undesired effects present in cancer treatment, may be achieved by the use of plant-derived products, among others natural therapies. If well, many anticancer agents were originally described from plants, there are many sources which remain not completely explored (Sultana et al., 2014). In a wide range of climate, Argentina has an abundant and diverse flora. Despite of this, the medicinal properties of native plants were not sufficiently explored; particularly, in relation to cancer. Until now, there are only a few scientific reports about plant antitumoral activities (Mamone et al., 2011).

*Prosopis strombulifera* (Lam.) Benth, from the Fabaceae family, is a rhizomatous shrub which grows up to 1.5 m height in Argentina, northern and central zones. Locally known as “retortuino”, the plant has been ethnopharmacologically used as an astringent, odontalgic, to treat inflammation and diarrhea; scientifically, its anti-bacterial and anti-nociceptive properties have been described. Beneficial biomedical properties of genus *Prosopis* were summarized by Persia et al. (2016).

In relation to cancer, *P. strombulifera* aqueous extract (PsAE) has demonstrated in vitro cytotoxic activity against colorectal and mammary adenocarcinoma cancer cell lines (HCT-116 and MCF-7, respectively) by induction of cytostasis, necrosis and apoptosis; with significant diminution in clonogenic survival at LC50 doses. By the Ames’ test, genotoxic effects of treatment were discarded at the cytotoxic doses used. Moreover, in a BALB/c mice model, no toxicity was evidenced at doses up to 150 mg/animal/day when orally administrated (Hapon et al., 2014).

We hypothesized that PsAE is composed of potential cytotoxic compounds that are able to induce antitumoral effects when are orally administrated. To promote PsAE as a plant-derived compound related to cancer, the objective of this work is to characterize the aqueous extract composition by UHPLC-Q-OT-HESI-MS/MS and to demonstrate its in vivo antitumoral properties in mice allograft models of colorectal and melanoma cancers. Additionally, the molecular intermediates of cytotoxic and antitumoral effects were also evaluated.

2. Results and discussion

2.1. UHPLC-Q-OT-HESI-MS/MS analysis of PsAE

The hyphenated chromatographic-spectrometric study of PsAE allowed the detection of 29 peaks (Figures 1A and 1B), where identification of 26 compounds was possible, including: 5 simple organic acids, 4 phenolic acids, 4 procyanidins, 11 flavonoids, and 2 oxylipins (Table 1). The full metabolome identification is explained below:

Simple organic acids: xylonic, citric, isocitric, grisenilosidic and tuberic acid glucoside; peaks 2-4, 12 and 14, respectively.

Phenolic acids: protocatechuic, piscidic, salicylic and marmesin; peaks 6, 7, 24 and 29, respectively.

Procyanidins: flavonols gallocatechin and epigallocatechin, catchin and procyanidin B1; peaks 8, 11, 13 and 15, respectively.

Flavonoids: daidzein 7-O-glucuronide, neoeriocitrin, kaempferol 3-O-neohesperidosid, myricitin 3-O-glucoside, rhoifolin, rutin, kaempferol glucoside, kaempferol rhamnoside, naringoside, formononetin 7-O-6/C19 acetylglucoside, kaempferol; peaks 5, 9, 10, 16-22 and 25, respectively.

Some compounds were identified as flavanones (l max 281 nm), other as isoflavones (l max 281 nm) and other as flavonols (l max 254–354 nm).

Oxylipins: unsaturated fatty acids trihydroxylinoleic acid and trihydroxyoctaenoic acid; peaks 26 and 28, respectively.

The chemical characterization of PsAE, identified 26 compounds; at least, 12 of them had been previously reported as cytotoxic. While, protocatechuic acid (Tsao et al., 2014), marmesin (Dong et al., 2018), rutin (Asfour and Mohsen, 2017), kaempferol, kaempferol glucoside and kaempferol rhamnoside (Wang et al., 2018) were described as isolate active compounds against cancer cells; citric acid, gallocatechin, epigallocatechin, procyanidin B and rhoifolin were reported as component of natural extracts with cytotoxic activity (Koyuncu, 2018; Navarro et al., 2017; Radan et al., 2017; Tremocoldi et al., 2018). The concurrence of the mentioned compounds into PsAE ensures its anticancer effects and promotes new research concerning its biological properties.

Figure 1. UHPLC-Q-OT-HESI-MS/MS fingerprints of PsAE. A. The total Ion Current (TIC) chromatogram. B. The UV-vis chromatogram at 330 nm.
Table 1. High resolution UHPLC PDA-Q orbitrap identification of metabolites in the PsAE.

| Peak# | Retention time (min.) | UV max | Tentative identification | Elemental composition [M-H] | Theoretical mass (m/z) | Measured mass (m/z) | Accuracy (ppm) | MSn ions (ppm) |
|-------|-----------------------|--------|--------------------------|-----------------------------|-----------------------|-------------------|----------------|---------------|
| 1     | 1.87                  | -      | Unknown                  | C27H31O14-                  | 579.17083             | 579.17083         | 2.96           | 285.04019 (kaempferol) |
| 10    | 10.03                 | 255-365| Kaempferol 3-O-neohesperidoside | C27H29O15-                  | 593.12952             | 593.12897         | 0.92           | 285.0610 (kaempferol) |
| 22    | 15.25                 | 255-365| Kaempferol               | C27H29O15-                  | 285.04046             | 289.07169         | 2.82           | 289.07169 (catechin) |
| 27    | 16.45                 | 220    | Unknown                  | C18H33O5-                   | 305.06671             | 305.06558         | 3.71           | 289.07169 (catechin) |
| 28    | 17.98                 | 220    | Trihydroxyoctaenoic acid | C18H33O5-                   | 329.23346             | 329.23225         | 3.57           | 289.07169 (catechin) |
| 29    | 19.38                 | 254-354| Marmesin                 | C14H19O4-                   | 245.08173             | 245.08084         | 3.63           | 289.07169 (catechin) |

Many natural derivatives are actually used to treat chronic diseases like cancer; these products are selected mainly for safety, availability, tolerance and its biological activity (Singh, 2007). In general, previous reports indicate that phytochemicals bring a higher protection acting combined than isolated (Malongane et al., 2017). Therefore, described constituents of PsAE support its cytotoxicity, potency and efficiency against a wide range of cancer cell; and promote their use as phyto-medical agents.

2.2. Evaluation of antitumoral properties of PsAE on mice

Because in vivo antitumoral properties of PsAE were not previously reported, to prove the extract capability of interfere with in vivo tumor progression, the present work shows the extract activity on two different models of cancer. One of them, are the colorectal tumors induced by the procarcinogen compound DMH; and the other, are the melanomas induced by subcutaneous injection of B16-F0 cells. Both are considered as allograft models of cancer in mice and, in the two cases, treatment was administrated at the major no toxic doses reported of 150 mg/animal/day (Hapon et al., 2014).

2.2.1. PsAE antitumoral activity: median survival determination on DMH induced colorectal cancer

To study the in vivo activity of PsAE against colorectal cancer, tumors were developed by DMH administration in BALB/c mice. A control group of animals, without DMH tumor induction, were used. These animals presented 100% survival during the experimental time (52 weeks), without any signs of tumor development. On the other hand, all animals DMH treated evidenced humane endpoints criteria related to colorectal cancer growth and were euthanized in consequence. Animals weight loss during experimental time was always between 5 to 8 %. The euthanasia criteria presented were anal tumor protrusion or anal bleeding. Necropsy evidenced colorectal polyps in all DMH treated animals, with no differences in the number of polyps between control and treated groups (18-24 polyps/animal). The diagnosis of colorectal adenocarcinoma was confirmed by histology. Kaplan-Meier analysis indicates that PsAE treated animals show the highest median survival, 34.5 weeks, whereas values of the not treated group and 5Fu treated were 24 and 27 weeks, respectively (Figure 2A). Survival differences between PsAE treated animals were significant in relation to the control group (Mantel Cox test, p < 0.0001) and 5Fu group (Mantel Cox test, p = 0.0030). The presented data demonstrate that PsAE, orally administrated, interferes with colorectal cancer progression. However, by these experiments, it is not possible to determine if plant derived active compounds act by hematological (systemic) or intraluminal (topic) ways.

2.2.2. PsAE antitumoral activity: systemic antimelanoma activity in allograft B16-F0 induced tumors

To investigate whether PsAE acts systemically to prevent melanoma progression, the extract was administrated orally in drinking water from day 1–22, after subcutaneous implantation of B16-F0 melanoma cells in C57BL/6wt mice. Two additional groups were added; a control group without any treatment; and the 5Fu treated group. All animals developed tumors, which were histologically confirmed. At the end of the experiment, day 22 after cells inoculation, the mean weight and volume of tumors excised from PsAE treated mice were significantly lower than the control group (Figure 2B). While control tumors weight was 2.389 ± 0.605 g, in PsAE group was significantly lower, 0.736 ± 0.190 g (p =
0.0204). When the final volume was analyzed, controls reach final volumes of 219 ± 47.9 mm³ whereas PsAE treated tumors reached a significantly diminished volumes of 101.7 ± 20.7 mm³ (p = 0.0306). The 5Fu treated group showed a final weight of 0.612 ± 0.50 g and a volume of 99 ± 27.8 mm³; no significant differences with PsAE values were found. Altogether, the presented results indicate the capability of PsAE to interfere with melanoma growth in vivo and demonstrate the systemic bioavailability of the active plant derived compounds.

2.3. In vitro evaluation of PsAE cytotoxic effects

To determine how PsAE exerts its antitumoral activity, B16-F0 murine melanoma cancer cells were cultured and studied in a dose-response experimental design.

2.3.1. PsAE induce cytotoxic effects by affection of proliferation and viability

Cell number and viability were quantified by dye exclusion assay with trypan blue, after 48 h of treatment. In vitro activity was quantified by IC₅₀ and LC₅₀ determination, the chemotherapeutic agent 5Fu was used as positive control. For PsAE, the estimated IC₅₀ was 2.44 ± 0.18 μg/ml and the LC₅₀ was 9.01 ± 0.22 μg/ml; whereas the calculated values for 5Fu were 0.12 ± 0.01 and 18.40 ± 1.04 μg/ml, respectively (Figures 3A and 3B). The obtained results evidence the capability of the PsAE to induce cytotoxicity in a dose-response manner by affection of cells proliferation and viability.

Next, we examined the morphological features in fixed and stained B16-F0 cells, 48 h treated, with IC₅₀ and LC₅₀ PsAE concentrations (2.4 and 9 μg/ml, respectively), in comparison with not-treated controls. The extract reduced cell density, caused rounding nucleus and enlarged cell cytoplasms; all changes were evident in a dose-response manner (Figure 3C).

To confirm the effects induced by PsAE treatment in cell proliferation two additional techniques were applied. By assessing cell metabolic activity with the MTT assay, we demonstrate how increased doses of PsAE induce growth inhibition in a dose-response manner (Figure 4A). In fact, the changes determined by quantification of the mitochondrial activity with PsAE and 5Fu, were able to induce the same proliferative changes to those calculated by the dye exclusion assay.

In addition, cell proliferation was studied by CFSE dilution in control and treated cells. Figures 4B, 4C and 4D show how CFSE fluorescence in control no treated cells (CoNT) results diminished in successive mitotic divisions. Similarly as was observed in dye exclusion and MTT assays, PsAE and 5Fu, used at IC₅₀ and LC₅₀ concentrations, interfere with normal cell division evidenced by increased CFSE fluorescence after 48 h of treatment (Figures 4B, 4C and 4D).

2.3.2. PsAE induce G2/M cell cycle arrest

To elucidate how PsAE affects the cell cycle progression, we assessed cell cycle distribution by flow cytometry analysis in B16-F0 cells (Figures 5A and 5B). Cells were incubated in the presence of PsAE for 24 h at IC₅₀ and LC₅₀ concentrations (2.4 and 9 μg/ml, respectively), untreated control cells were also assayed. As shown in Figure 5, treatment with PsAE induced a marked G2/M arrest with a concurrent reduction of cell population on G1 in both treated groups. Also, at the IC₅₀ concentration was observed an augmentation of >4N fraction, which represents cells with higher DNA content than tetraploid cells. Moreover, a slight increase in hypo-diploid DNA content (SubG1 phase) was observed at LC₅₀ concentration; which indicates apoptosis as a cell death mechanism.
involved (Gamarra-Luques et al., 2012). Presented results demonstrated that PsAE interferes with cell cycle progression mainly by the arrest in G2/M phase and augmentation of >4N cells DNA content; these mentioned cell cycle distribution changes are similar to compounds which exert cytotoxicity by mitosis interference (Dolecková et al., 2012; Liu et al., 2016; Yu et al., 2017; Zhao et al., 2019).

2.3.3. PsAE induce changes in protein expression related to the presence of cytostasis and apoptosis

Protein expression was determined to study the cytostatic and lethal effects of PsAE on B16-F0 cultured cells (Figs. 6A and 6B). Three independent assays, after 24 h of treatment at 0 μg/ml, IC50 and LC50 PsAE concentrations (2.4 and 9 μg/ml, respectively), were done. In IC50 treated cells, in respect to control not-treated cells (CoNT), the cyclin-dependent kinase inhibitor p21cip1 (p21) significantly increases (p = 0.0273), and cleaved caspase 3 increases significantly (p = 0.0500); these results are consistent with cell cycle arrest associated with growth inhibition. At lethal doses, compared with controls, the expression of p21 was increased (p = 0.0489), cleaved caspase 3 was significantly increased (0.0007); also, detection of cleavage in poly ADP ribose polymerase (cPARP) revealed the presence of apoptosis and its overexpression showed a significant increase (p = 0.0081); while PCNA was significantly reduced (p = 0.0395). Altogether, molecular markers indicate that cytostasis and lethality are induced in vitro by PsAE treatment mediated by a p21 related mechanism.

2.4. Molecular confirmation of PsAE antitumoral systemic effects

To correlate the molecular changes evidenced in vitro by PsAE treatment, with the antitumoral effects demonstrated in allograft melanomas after subcutaneous B16-F0 cells inoculation, the expression of cytostasis and apoptosis related proteins were analyzed. After 22 days of the melanoma induction, tumors of the animals orally treated with 150 mg/day of PsAE were compared to the tumors resulting from untreated animals (Figs. 7A and 7B). When the cell cycle arrest related proteins were studied, p21 significantly increases (p = 0.0133) and PCNA did not diminish significantly in the PsAE treated group. The apoptosis related protein cleaved PARP, was not detected in PsAE, nor in...
the control group (data not shown). In accordance to the explored molecular markers expression, it is possible to affirm that in vivo treatment with oral PsAE exerts antitumoral actions by cytostasis, without apoptosis mediated cell death. Moreover, in the used animal model in which the treatment was orally administrated, the systemic PsAE should reach bioactivity levels that are comparable with the IC50 concentration assayed in vitro.

As has been described in other natural compounds with systemic antimelanoma actions, PsAE treatment may activate multiple target pathways. Diverse compounds that induced G2/M cell cycle arrest

Figure 4. Proliferation changes in B16-F0 cell line. A: Growth inhibition percentage (%) in a dose response MTT proliferation assay. B, C and D: Flow cytometric analysis of cell division by dilution of CFSE. In B, CFSE representative histograms of control and treated cells (black line), pink solid histograms show undivided cells (generation 0, G0) and purple histograms represent the following generations (G1 and G2). Panel C display Percentage (%) of cells distribution by generation in different treatment paradigms. In D, tabular results and statistical comparison of cells distribution percentage (%) by generation, and mean/median fluorescence intensity of total cells. CoNT = Control No Treated; PsAE IC50 = 2.44 μg/ml; PsAE LC50 = 9.01; 5Fu IC50 = 0.12 μg/ml and 5Fu LC50 = 18.40 μg/ml. Treatments were performed for 48 h. Results are expressed as mean ± SEM, of three independent assays by triplicated and were compared by one-way ANOVA followed by Fisher LSD test. *: indicate significant differences (p < 0.0001) respect to control group.
accompanied by p21 up-regulation such as: genistein (obtained from soybeans), α-santalol (from Santalum album) and allyl-sulphides (obtained from garlic), among others (Ravindranath et al., 2004; Wang et al., 2012; Zhang and Dwivedi, 2011), have been reported. As PsAE, the mentioned compounds were described to act by the control of proliferative signals, altering cell cycle regulatory proteins and complexes, determining its proved efficacy accompanied by null collateral effects. Moreover, the multiple molecular targets involved, make able to explore and exploit the synergistic combinations with other current available chemotherapeutic agents, to promote the use of natural derived compounds as valuable complementary treatments against cancer.

3. Conclusions

The present study demonstrates that the PsAE UHPLC fingerprint is composed by phenolic compounds; which, at least 12 of them, were previously reported as cytotoxic against cancer cells; supporting, at least in part, the potential antitumoral activity of this plant. When PsAE is orally administrated, prevents tumor progression in vivo by a significant increase of the mice median survival with allograft colorectal tumors and cause diminution of the tumor growth in subcutaneous B16-F0 induced melanomas. The in vitro dose-response assays performed in this work show that PsAE induces G2/M cell cycle arrest and apoptosis in B16-F0 cells, determining a significant over expression of the cyclin-dependent kinase inhibitor p21<sup>CDK4</sup>. Finally, the molecular study of the mice induced melanomas indicates that systemic available plant derived compounds cause cytostasis with over expression of p21<sup>CDK4</sup> but not apoptosis. Altogether, the findings of the current work demonstrated the antitumoral activity of PsAE when is orally administrated. Further research are needed in order to explore its effect on the regulation of related signal pathways to support the plant extract use as complementary medicine for cancer treatment.

4. Materials and methods

4.1. Plant material and aqueous extract preparation

*P. strombulifera* was collected in February 2016 in 33° 44’ 01” S, 68° 21’ 30.5” W (Lavalle County, Mendoza, Argentina). Identified as MERL 61824 a voucher specimen is deposited in the Mendoza Ruiz Leal herbarium. To obtain PsAE, leaves were autoclaved in distilled water, 50 g/500 ml, for 1 h. After, solids separation by paper filtration, decoction was boiled until volume reach 50 ml. Before use, crude extract was sterilized by passing through a 0.22 μm pore size filter. The concentration (w/v) was estimated by the ratio between the weight of the leaves, expressed in grams, and the final volume obtained at the end of the preparation: 50 g/50 ml = 1 g/ml.

4.2. Detection and identification of PsAE compounds by UHPLC-Q-OT-HESI-MS/MS

By UHPLC-Q-OT-HESI-MS/MS PsAE compounds were identified and quantified. Collision and damping gas was performed by a Thermo Scientific Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Germany), controlled by the Chromeleon 7.2 Software; hyphenated with a Thermo high resolution Q Exactive focus mass spectrometer (Thermo, Bremen, Germany). A Zefiro nitrogen generator (Clantecnologica, Sevilla, Spain) was used to obtain pure nitrogen (>99.999%). Equipment calibration and parameters settings were performed as was previously reported by Simirgiotis et al. (2016). The chromatography parameters

![Figure 5. Cell cycle analysis of B16-F0 cells. A. Flow cytometric histograms show the DNA content and the corresponding percentages of cell cycle distribution in control, IC<sub>50</sub> and LC<sub>50</sub> (0, 2.4 and 9 μg/ml, respectively) PsAE concentrations. B. Cell cycle distribution in bars. Cytometric analysis was performed after 24 h treatment. Mean ± SEM of 3 independent assays by triplicate are presented.](image-url)
were: UHPLC C18 column (Acclaim, 150 mm × 4.6 mm ID, 5 μm, Restek Corporation, Bellefonte, PA, USA), at 25 °C. Detection settings were at 254, 280, 320, and 440 nm, and the PDA record was from 200 to 800 nm. Mobile phases used were: (A) 1% formic acid aqueous solution and (B) acetonitrile with 1% formic aqueous solution. The gradient program and time (min, % of B) was: (0.00, 5); (5.00, 5); (10.00, 30); (15.00, 30); (20.00, 70); (25.00, 70); (35.00, 5), and column equilibration for 12 min. The injection volume was 10 μl and the flow rate fixed at 1 ml/min. Samples and standards were dissolved in methanol and kept in the autosampler at 10 °C. The MS parameters were as follows: the HESI II and other parameters for the Q-orbitrap instrument were optimized as previously reported (Jiménez-González et al., 2018).

4.3. Animals and in vivo treatments

All animals were maintained in a controlled light (6:00 AM to 10:00 PM) and room temperature (22–24 °C). Drinking liquids and mice chow (Cargill, Argentina) were available ad libitum. In vivo treatment was performed by diluting PsAE (150 mg/animal/day) in drinking water. Intraperitoneal 5Fu was the chemotherapeutic control treatment administrated, at 30 mg/kg/week, until animal sacrifice.

To study the animal median survival after DMH colorectal tumor induction were used BALB/c males, 6 weeks old at the onset of treatment, bred in our Institute. Four groups were separated of 10 animals each. Group 1 was considered as survival control group and did not receive treatments. In the other 3 groups was performed the colorectal tumor induction by S.C. administration of DMM, 20 mg/kg/week, for 22 weeks (Persia et al., 2017). In animals of group 2 (DMH), only tumoral induction was performed and mice were considered as no treated tumor survival group. In group 3 (DMH-PsAE), 8 weeks after tumor inductions started, by dilution of PsAE in the drinking water, animals were treated at the indicated dose. In group 4 (DMH-5Fu), 8 weeks after tumor inductions started, animals receive 5Fu as described before. In the current experiment, the human endpoints to decide the animals’ euthanasia, were: anal bleeding, anal tumor protrusion, reduction of spontaneous activity, not eating/drinking, diarrhea, weight change more than 20 %, no micturition and dyspnea.

To study PsAE effects in allograft melanoma tumors, 3 groups of 10 C57BL6wt males, bred in our Institute, 6 weeks old, were used. In all groups, melanoma tumors were induced by subcutaneous inoculation of 1 × 10^6 B16-F0 (murine melanoma cancer cell line) in the right flank. Group 1, did not receive any treatment after tumor cells inoculation; groups 2 and 3, simultaneously with the tumor induction, animals received PsAE and 5Fu as was described previously. The animals were sacrificed at day 22 after cells inoculation. Then, developed tumors were dissected, measured, weighted, stored at -80 °C for immunoblotting and fixed for histology.

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 and N° 103/2017, respectively). All animals in the protocols were cared in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health.

4.4. Cell culture and in vitro treatments

Because, there is not available commercial mice colorectal cancer cell lines, the B16-F0 (ATCC, Virginia, EEUU) mice melanoma cell line was selected to perform the in vitro studies and to correlate with the in vivo findings. The cells were cultured as a monolayer in DMEM (Gibco, Grand Island, NY, USA), containing 10% v/v fetal bovine serum (HyClone, Canada), penicillin (100 IU/ml), streptomycin (100 μg/ml) and 3.7 mg/ml NaHCO_3. All cells were grown in a humidified atmosphere, containing 5%CO_2 at 37 °C. The treatments were dissolved in the culture media 24 h after cell plating. The chemotherapeutic agent 5Fu (Filaxis®, Argentina) was used as positive control at the indicated doses.
4.5. Dye exclusion assay

To quantify proliferation and viability, trypan blue exclusion assay was used in a dose-response experimental design. Briefly, 3 x 10^4 cells were seeded into 6-well plates. After 24 h, media was replaced with fresh culture media containing treatments at the indicated doses. After 48 h, total cells were collected by trypsinization, incubated with trypan blue and counted in a Neubauer haemocytometer chamber using a clear-field microscopy. To assess the IC50 (concentration of treatment that induces 50% of growth inhibition) and the LC50, (concentration of treatment that induces 50% of lethality), the total cell number and death cells were used to statistically calculate the indexes (see below, point 2.11).

4.6. MTT cell proliferation assay

The MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide)] is a colorimetric assay for assessing cell metabolic activity. It is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial enzymes, and the amount of formazan produced is directly proportional to the number of viable cells present. In 96-well microplates, 7 x 10^3/100 μl B16-F0 cells were seeded; after 24 h, the medium was changed by fresh medium containing PsAE or 5Fu (Filaxis®, Argentina) at indicated doses. 48 h later, medium was replaced by MTT solution (0.5 mg/ml in DMEM, without phenol red nor FBS, 100 μl/well). Then, plates were incubated for an additional 4 h. After MTT solution was removed, 100 μl of DMSO were added to dissolve the formazan crystals and shaken for 10 min. To measure the optical density a Thermo Scientific Multiscan Elisa reader was used at 570 nm. The optical density obtained in untreated control cells (treatment concentration: 0 μg/ml) was considered as 100% viability; and the other values were calculated in accordance. The assay was performed three times in triplicate.

4.7. Proliferative responses by CFSE dilution

For examining cells proliferative activity, a cell tracking dye carboxyfluorescein diacetate succinimidyl ester (CFDASE) was used. In the labeled cells, the marker diffuses in the cytoplasm were present acetate groups are cleaved to a CFSE, a fluorescent derivative which remains into the cell. After mitosis, CFSE fluorescence intensity is divided in a half with each generation and the number of rounds of cell division can be visualized and quantified. Changes in CFSE fluorescence profile allows to determine changes in cell division induced by any substance. Decreased fluorescent intensity indicates higher proliferation rate; conversely, an increased in fluorescence intensity indicates lower/null proliferation. In the current assay, B16-F0 cells were stained with 5 μM of CFSE Cell Tracer Kit (Invitrogen) for 5 min at 37 °C. Then, labeled cells were washed, quantified and seeded at 5 x 10^4 well into a 6-well plates. Treatments at the indicated doses were performed for 48 h. Later, cells were scraped and acquired in a FACS Aria III flow cytometer (BD Biosciences, Mountain View, CA) and FlowJo.10 analysis software (Three star, US); FlowJo proliferation tool was setting as follows: #peaks 4.00; Fixed ratio 0.66; fixed CV: 10.1; fixed backgd: 1087.0. All experiments were performed three times by triplicated.

4.8. Cell morphological analysis

Cells were seeded on cover-slides placed on the bottom of 6 well plates at a density of 3 x 10^4 cells/well and grown overnight. The next day, culture medium containing PsAE treatment was replaced at final concentrations of 0, 2.4 and 9 μg/ml; which represent control, IC50 and
LC\textsubscript{50} respectively. After incubation for 48 h, the remaining cells were fixed with methanol and stained with crystal violet solution. Coverslides were then mounted, evaluated and photographed with a Nikon Eclipse 200 microscope.

4.9. Cell cycle assay

Into a 6 well/plate, 5 \times 10^4 cells/well were seeded and grown overnight. Then, cells were treated with PsAE at 0 \mu g/ml, IC\textsubscript{50} and LC\textsubscript{50} concentrations, for 24 h. Later, cells were trypsinized, washed with PBS and fixed in 4% paraformaldehyde overnight. Then, fixed cells were washed with PBS, incubated with 0.3 ml PBS containing 20 \mu g/ml RNase, 0.3 % IGEPAL, 1 mg/ml sodium citrate and 50 \mu g/ml of propidium iodide for 30 min at 37 \degree C. The population at each stage of the cell cycle was analyzed by flow cytometry, using a BD FACSDiaIII\textsuperscript{TM} cytometer (BD Biosciences, Mountain View, CA) and FlowJo.10 analysis software (Three star, US).

4.10. Protein expression by western blot analyses

To determine protein expression, cultured B16-F0 cells were analyzed after 24 h of in vitro treatment. Melanomas generated in vivo by B16-F0 subcutaneous inoculation were also studied. Immunoblotting was performed as described previously (Hapon et al., 2014). To quantify and statistically compares the protein expression, cultures were performed by triplicated and three melanomas group were studied. Primary antibodies used were: cyclin-dependent kinase inhibitor p21\textsuperscript{kip1} (Termofisher AHZ0422), proliferating cell nuclear antigen (PCNA, Termofisher MA511358), caspase 3 (Casp3, Invitrogen, 74T2), cleaved poly (TUBA1A, Termo laboratories. Statistical analysis was performed using Image Lab Software version 4.0 from Bio-Rad Laboratories. The XRS 115-035-003 and 711-065-152). Blots were developed using a ChemiDoc XRS+ System (Bio-Rad, Laboratories) and band densitometric analysis was performed using Image Lab Software version 4.0 from Bio-Rad Laboratories.

4.11. Statistical analysis

All data are expressed as mean \pm standard error (SEM) and analyzed using GraphPad Prism 6.0 software. The animal survival curves were performed by the Kaplan Meier analysis and compared using a Mantel Cox test. To assess IC\textsubscript{50} and LC\textsubscript{50}, was performed a sigmoidal dose–response analysis and, when the goodness of fit showed R\textsuperscript{2} \geq 0.90, values were considered acceptable. When two groups were compared, the Student’s T test was used; whereas, when more than two groups were compared, one-way ANOVA followed by Fisher LSD test was used. In all cases, statistical significance was considered when p \leq 0.05.

Declarations

Author contribution statement

Fabio Andrés Persia, Mariana Elizabeth Troncoso, Jorge Bóquez: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Estefanía Rinaldini: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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