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Dietary supplementation with procyanidin-rich *Pinus pinaster* extract is associated with attenuated Ehrlich tumor development in mice

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ABSTRACT

Inflammation and oxidative stress are related to cancer initiation and progression. We hypothesized that dietary supplementation with a procyanidin-rich *Pinus pinaster* extract (Pyc) with known antioxidant and anti-inflammatory effects could induce systemic protection, thereby attenuating tumor development. To test our hypothesis, mice were subjected to long-term supplementation (20 days, every 24 h) with saline, 25 mg/kg resveratrol or 100 mg/kg Pyc. Pyc was administered at a maximum tolerated oral dose, previously determined using toxicity indicators. Ten days after Ehrlich ascites tumor induction, weight gain and abdominal circumference increase were calculated. Ascitic fluid from six mice/group was evaluated by determining total volume; tumor packed cell volume; cell viability; tumor cell death type; inflammatory infiltrate; and levels of tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), carbonyl proteins, lipid peroxidation, cyclooxigenase-2 (COX-2) expression and Akt phosphorylation (p-Akt). Ten mice/group were monitored to evaluate survival. Pyc and resveratrol were associated with reduced weight gain (>30%), abdominal circumference and ascitic volume. Tumor packed cell volume was reduced in Pyc-supplemented mice (26%), which had the largest tumor cell count reduction (>35%), increased ascitic fluid apoptosis rates (20%) and the longest survival

Abbreviations: Akt, protein kinase B; AU, arbitrary units; COX, cyclooxygenase; HED, human equivalent dose; IL-1β, interleukin 1 beta; NFκB, nuclear factor kappa B; MTOD, maximum tolerated oral dose; p-Akt, phosphorylated Akt; Pyc, standardized procyanidin-rich *Pinus pinaster* extract (proanthocyanidins ≥95.6%); ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TNF-α, tumor necrosis factor alpha.

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(>2-fold). Pyc and resveratrol treatment both reduced inflammatory infiltrate and levels of TNF-α, IL-1β, carbonyl proteins, lipid peroxidation (~ 30%) and p-Akt (up to 4-fold). Only Pyc significantly inhibited COX-2. Pyc attenuated oxidative and inflammation mediators and impaired tumor development, supporting our hypothesis and suggesting Pyc as a candidate for future studies in multitargeted dietary-based cancer prevention approaches.

1. Introduction

A complex molecular network in which inflammation and cell proliferation mediators are connected has currently become evident. These mediators represent targets for chemoprevention, which is defined as the use of agents able to prevent, delay or suppress carcinogenesis [1]. In the context of cancer, dietary supplements with anti-inflammatory and antioxidant effects that can be consumed for prolonged periods with minimal risk of side effects have been considered a valid strategy for chemoprevention [2]. The mechanism of action of such compounds deserves to be further evaluated for improved results in cancer prevention and treatment.

French maritime pine bark extract (*Pinus pinaster* Aiton) is described in the United States pharmacopeia as a dietary supplement. This extract, which is conventionally standardized in procyanidins (65-75%), also contains taxifolin and phenolic acids. Procyanidins are members of the proanthocyanidin (or condensed tannins) class of flavonoids. The glycosides of these compounds are substrates for hydrolyzing and conjugating enzymes in the small intestine, liver and colon, being conjugated to *O*-glucuronides, sulfate esters and *O*-methyl esters absorbed in the plasma. This biotransformation involves phase II hepatic metabolism and facilitates the excretion via urine and bile, which is partially performed by colonic microflora in the case of compounds not absorbed in the small intestine. Upon reaching the colon, aglycones are further catabolized to low molecular weight compounds that can readily be absorbed [3-5]. Previously, a study suggested that maritime pine bark extract displays enhanced biologic activities as a mixture compared to its purified components individually [6]. Approved for clinical use, it has low toxicity with eventual mild, unwanted effects [3]. Its anti-inflammatory and antioxidant effects are well known, along with other activities [7].

Antigenotoxic effects detected *in vitro* have revealed this extract's potential to prevent the first stages of carcinogenesis [8]. Studies with tumor cell lines have demonstrated its pro-apoptotic effects [6,8]. This extract has proven effective in alleviating adverse effects of oncologic treatments [9]. Its topical administration was able to protect against ultraviolet-induced tumorigenesis in mouse skin [10]. Despite the promising results of these previous studies, whether this extract is capable of inducing systemic protection against tumorigenesis is unknown. This activity would presumably allow consideration of protection against other types of tumors. However, this has currently been poorly investigated.

This work aimed to evaluate whether dietary supplementation with a standardized procyanidin-rich *Pinus pinaster* extract (Pyc) impairs Ehrlich ascites tumor development in mice. This tumor model establishment and progression *in vivo* involves markedly inflammation [11]. Therefore, it was hypothesized that this extract, endowed with known anti-inflammatory effects, induces systemic protection in animals, thereby a tumor development would be more difficult. To test our hypothesis, healthy mice were orally supplemented with the extract, followed by tumor inoculation. Biomarkers of tumor progression, inflammation and oxidative stress were evaluated. This study's contribution is related to the identification and mechanism of a health protectant potentially useful for human cancer chemoprevention.

2. Methods and materials

Pyc (proanthocyanidins ≥95.6%) and resveratrol (99.51%), both with their respective physico-chemical and microbiological quality certificates, were purchased from Galena Química Farmacêutica Ltda, SP Brazil (Batch n. 1412007202 and 15105981C).

2.1. Animals

Male Balb-c mice (~2 months old, weighing 20 ± 1 g) were used in accordance with ethical guidelines (NIH Publication No. 80-23, revised 1978) and the approval of the ethics committee of Universidade do Sul de Santa Catarina, Palhoça, Brazil (15.025.4.01.IV). Mice were housed under controlled conditions (12 h light/dark cycle, 22 ± 2°C, 60% air humidity) and had access to standard food and water *ad libitum*. They were allowed to acclimatize for at least 5 days.

2.2. Determination of maximum tolerated oral dose of Pyc

Fig. 1 summarizes the study design, showing its workflow. The study was carried out in two main phases: an initial phase, in which the Pyc maximum tolerated oral dose (MTOD) was determined, and a second chemoprevention study phase. Pyc and control compounds were always administered orally. MTOD had to be determined because previous studies evaluating the activities of French maritime pine bark extract in mice used heterogeneous doses. Furthermore, MTOD was determined because at this time, a procyanidin-rich extract was used. Therefore, a short-term experiment was first performed (Fig. 1, 1st step) in which Pyc was administered every 24 h for a week to healthy mice allocated into: a control saline-treated group; Pyc was tested at a wide range of doses (25, 50, 75, 100, 200 and 300 mg/kg), each of which was examined in a group of six mice. Mice were monitored on a daily basis for two hours following Pyc administration. Toxicity indicators included mortality, eye responses, piloerection, behavioral and other apparent changes [12]. Given the anti-inflammatory activity of the maritime pine bark extract [7], a mechanical hyperalgesia indicator (von Frey hair test) was included in screening. One hour after the last Pyc administration (day 7), complete Freund's adjuvant 70% (20 μL) was injected into the right hind paws of mice, and the effect was evaluated 60 min later. Paws were stimulated with a pressure of

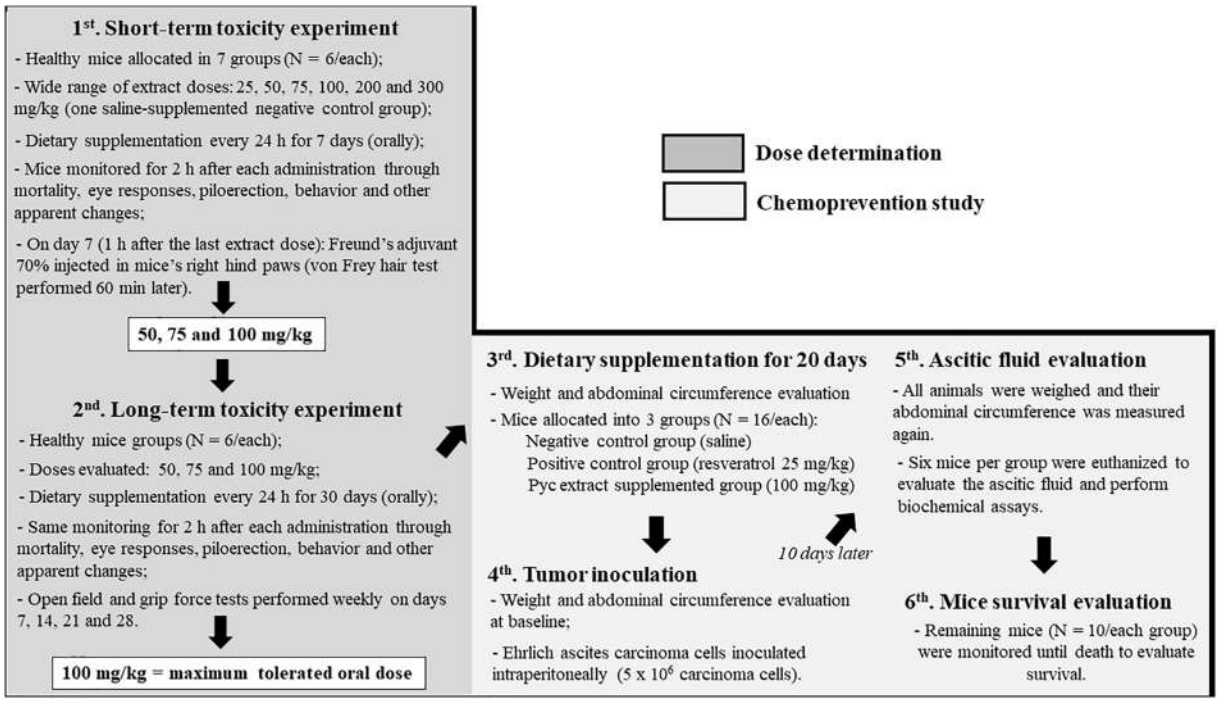

Fig. 1 – Study workflow for evaluating the potential of dietary supplementation with *Pinus pinaster* extract (Pyc) (proanthocyanidins ≥ 95.6%) to impair development of Ehrlich ascites tumors in Balb-c mice. N corresponds to the mouse number in each group.

0.6 g von Frey filaments (Stoelting, Kiel WI USA). A withdrawal response frequency to 10 applications of von Frey filaments is presented as a percentage response [13].

Second, a narrower range of doses (50, 75 and 100 mg/kg) was screened according to initially obtained results (Fig. 1, 2nd step). These doses were administered every 24 h, as before, but now for a prolonged time (1 month). Another set of mice were allocated into groups (n=6/each), and the same signs of toxicity evaluated previously [12], including influence on locomotor activity and grip force, were monitored during this time. Open field and grip force tests were performed 1 h after Pyc administration on days 7, 14, 21 and 28. The open field arena floor (Insight, SP Brazil) used to evaluate toxic effects on locomotor activity was divided into 12 equal squares, and the number of squares crossed by the animal (all paws) in a 6-min session was quantified [14]. Grip strength was used to evaluate whether muscle force was affected by taking advantage of tendency of mice to grasp a grid while suspended by their tails. A trapeze was attached to a force transducer (Columbus Instruments, OH USA), and the force produced during the pull on the bar was measured at intervals with 5 min of rest. Animals were moved in a rostrocaudal direction until the grip was broken. Data were recorded as the highest values obtained from three consecutive trials [13].

2.3. *Chemoprevention study*

Another set of mice was used for chemoprevention analysis through a study design similar to those performed previously [15,16]. Mice were subjected to administration (gavage) every 24 h for 20 days. Mice were allocated into groups (n = 16/each): one negative control group supplemented with saline (50 μL); one positive control group supplemented with resveratrol (25 mg/kg) (resveratrol dose complied with the literature [17]); and a group administered Pyc at MTOD, 100 mg/kg (Fig. 1, 3rd step). With Pyc's MTOD, a human equivalent dose (HED) was calculated using the formula: HED (mg/kg) = animal dose (mg/kg) × (animal km)/(human km), where animal km was equal to 3, and human km was equal to 37 [18].

Twenty-four hours after the last doses, all mice were weighed, and their abdominal circumferences were measured at baseline (Fig. 1, 4th step). Ehrlich carcinoma cells (5 x 10^6) were intraperitoneally inoculated [19]. Ten days later, weight and abdominal circumference were measured again, and six mice per group were euthanized by cervical dislocation to collect ascitic fluid (Fig. 1, 5th step). The remaining mice (n=10/each group) were monitored until death to evaluate survival (Fig. 1, 6th step) using the Kaplan-Meier method [20].

2.4. *Histopathological assays*

All ascitic fluid was collected in graduated tubes that were centrifuged (5 min, 5,000 g) to measure packed tumor cell volume. Tumor cell viability was assessed by trypan blue exclusion [21]. Aliquots of ascitic fluid were smeared onto glass slides and stained (Romanowsky method). Inflammatory infiltrates were counted under an Olympus CX41 microscope (Japan). For each sample, 100 cells were counted [11].

2.5. *Type of tumor cell death*

Aliquots of ascitic fluid (25 μL) were stained with ethidium bromide (100 μg/mL) and acridine orange (100 μg/mL) (5 μL) to visualize nuclear changes characteristic of necrosis and apoptosis. Viable cells appeared green. Early apoptotic cells

stained green and contained bright spots in the nuclei. Late apoptotic cells incorporated ethidium bromide and become orange. In contrast to necrotic cells, these cells exhibit condensed and fragmented nuclei. Morphology was examined using a fluorescent BX41 Olympus microscope (Japan). For each sample, 100 cells were evaluated. Results are expressed as percentages of viable, apoptotic and necrotic cells [22].

2.6. Cytokines quantification

Ascitic fluid was homogenized in PBS (1:5 v/v) and centrifuged (10 min, 3000 g, 4°C). The supernatant (100 μL) was used to measure tumor necrosis factor alpha (TNF-α) and interleukin (IL) 1 beta (IL-1β) using immunoassay kits (ELISA) according to the manufacturer's instructions (Biolegend, San Diego CA USA). Cytokine concentrations were estimated by interpolation from a standard curve and measured at 450 nm (correction wavelength 540 nm) in a plate reader (Perlong DNM-9602, Nanjing Perlove Medical Equipment Co, China). The results are expressed as picogram/milligram of protein [13].

2.7. Western blot

Whole cell lysates were prepared with ascitic fluid using Ripa buffer (150 mM NaCl, 1% Igepal® CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Trizma®, pH 8.0, 1% protease and 3% phosphatase inhibitor cocktails from Sigma-Aldrich Cat. P8340 and P5726) and Laemmli buffer (60 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulphate, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue). Equal amounts of protein (50 μg) were subjected to SDS-PAGE, followed by electroblotting to polyvinylidene difluoride membranes that were incubated overnight with primary antibodies (1:500). After washing, membranes were incubated with secondary antibodies for 1 h (1:5000). Antibodies used were as follows: rabbit polyclonal antibodies anti-COX-2 (AB5118 Merck Millipore MA USA), anti-Akt (# 9272 Cell Signaling Technology MA USA), anti- p-Akt, anti-actin (sc-33437 and sc-7210 Santa Cruz Biotechnology TX USA, respectively), and polyclonal goat anti-rabbit IgG (AP132P Merck Millipore). Immunodetection was performed using a chemiluminescence detection kit for horseradish peroxidase-coupled antibodies (Cyanagen, Srl Italy). Actin was used as the loading control [11]. Blots were quantified using ImageJ freeware (US National Institute of Health).

2.8. Oxidative stress biomarkers

Ascitic fluid was centrifuged (5 min, 5000 g), and the supernatants were diluted (1:5 v/v) in PBS containing butylated hydroxy toluene (0.1%). Lipid peroxidation was measured using a thiobarbituric acid reactive species reaction [23]. Carbonyl proteins were quantified using 2,4-dinitrophenylhydrazine [24]. Data are normalized by protein content.

2.9. Statistical analyses

The number of animals per group was determined taking into consideration data of a previous study with similar design [15] and our team's previous experiences [19,25]. Power analysis indicated that at least six mice per group were needed to detect differences in tumor growth in the setting of a power of .80 and an α probability of 0.05 [26]. Data are shown as the means ± standard deviation. Normal distribution of data was analyzed by the Shapiro-Wilk test. Data were subsequently analyzed by analysis of variance (ANOVA) followed by Bonferroni test. Survival data were evaluated as area under curves [19]. Comparisons were performed using GraphPad Prism software (San Diego, USA). Values of $P \leq .05$ were considered statistically significant.

3. Results

Table 1 shows data for the short-term experiment to identify Pyc's MTOD. The dose of 25 mg/kg was excluded due to inefficacy in the mechanical hyperalgesia test ($P > .05$). Doses of 200 and 300 mg/kg were excluded due to toxicity. Doses 50, 75 and 100 mg/kg were retested in the long-term MTOD experiment. Fig. 2A and B show data on ambulation and grip force. Data related to all doses tested in this phase revealed no toxicity. Therefore, 100 mg/kg was assumed as Pyc's MTOD in Balb-c mice. The chemoprevention study was subsequently performed using Pyc's MTOD. Calculated HED was 8.1 mg/kg.

Table 2 summarizes mouse morphological data obtained ten days after tumor inoculation (n = 16/group) and data on the initial analysis performed with ascitic fluid from six mice per group. In mice from the negative control group, weight and abdominal circumferences were increased by 50% and 30%, respectively, compared to initial measurements. Pyc and resveratrol restrained weight gain and abdominal circumference increase compared to negative controls ($P \leq .05$). Mice supplemented with Pyc or resveratrol had reduced volumes of ascitic fluid (close to 30%); however, only Pyc caused

Table 1 – Determination of maximum tolerated oral dose of standardized *Pinus pinaster* extract (Pyc) (proanthocyanidins ≥95.6%) in Balb-c mice. Pyc was administered every 24 h for one week. The control group received saline. Data shows the von Frey's filament test response, toxicity signs and mortality percentages.

Dose (mg/kg)	Frequency of paw withdrawal (%)	Signs of toxicity	Mortality (%)
0 (control)	80 ± 20	None	0
25	85 ± 15	None	0
50	65 ± 15*	None	0
75	63 ± 12*	None	0
100	45 ± 13*	None	0
200	40 ± 21*	Glassy eyes Piloerection Aggressiveness	33.3
300	35 ± 20*	Glassy eyes Piloerection Aggressiveness	50

Each dose was tested in a group of six mice. Frequency of paw withdrawal was expressed as the means ± standard deviation. Data were analyzed by analysis of variance (ANOVA) and Bonferroni test. *Statistical difference compared to control group ($P \leq .05$).

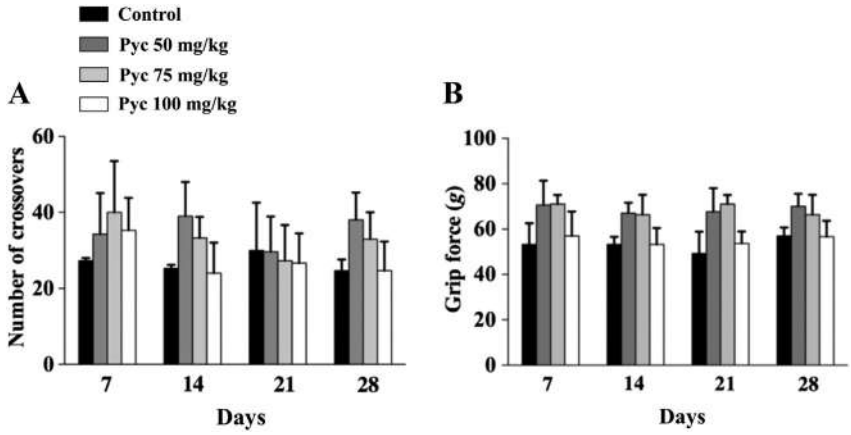

Fig. 2 – Ambulation (A) and grip force (B) in healthy Balb-c mice receiving standardized *Pinus pinaster* extract (Pyc) (proanthocyanidins ≥ 95.6%) for 30 days (gavage every 24 h). The control group received only saline. Assessments performed weekly 1 h after Pyc administration. Each dose was tested in a group of six mice. Values are shown as the means ± standard deviation. Data were analyzed by analysis of variance (ANOVA). All Pyc doses caused no changes compared to controls ($P > .05$).

significant reduction in the volume of tumor packed cells (nearly 25%) ($P \leq .05$).

Data in Fig. 3A illustrate cell counts in ascitic fluid through trypan blue exclusion. Ascitic fluid of mice supplemented with either Pyc or resveratrol presented increased rates of cell death, and data confirmed that Pyc was the one most able to cause reduction in total nonviable plus viable tumor cells ($P \leq .05$). Fig. 3B shows data on differentiating viable cells from cells undergoing apoptosis or necrosis. Data revealed that cells from mice supplemented with Pyc and resveratrol showed increased rates of apoptosis, and again, ascitic fluid from mice supplemented with Pyc had reduced numbers of viable cells ($P \leq .05$). Fig. 3C shows data on mouse survival. The percentage of survivors is shown along with days after tumor inoculation. Increases in the area under the curves indicate increased survival. The smallest area in arbitrary units (AU) corresponded to mice from the negative control (120 AU). The area became larger (270 AU) when mice were supplemented with resveratrol, whereas Pyc produced the largest area under the curve (310 AU).

Figs. 4 and 5 (A and B) show data on inflammatory biomarkers measured in ascitic fluid. Fig. 4A shows that Pyc and resveratrol reduced the percentage of infiltrating inflammatory cells in ascitic fluid, whereas Pyc exerted increased efficacy ($P \leq .05$). Fig. 4B and C show that ascitic fluid from mice supplemented with Pyc or resveratrol exhibited reduced concentrations of both TNF-α and IL-1β ($P \leq .05$).

Fig. 5A and B show that supplementation with Pyc and resveratrol inhibited COX-2 expression in cells of ascitic fluid compared to the negative controls; however, Pyc was the agent that reached significance ($P \leq .05$). Fig. 5A and C contain data showing that supplementations with Pyc and resveratrol are associated with reduced phosphorylated Akt, (p-Akt)/Akt ratio, compared to negative controls ($P \leq .05$). No difference was observed between Pyc and resveratrol in this respect. Fig. 5D and E show data regarding lipid peroxidation (concentration of malondialdehyde) and carbonyl proteins in ascitic fluid from six mice per group. Supplementation with either Pyc or resveratrol caused similar reduction in both parameters compared to negative controls ($P \leq .05$).

4. Discussion

Both efficacy and safety of administered compounds are dependent upon the dose. Conversely, the risk of toxicity increases as doses are enhanced under prolonged use. Therefore, the initial phase in the current study was to

Table 2 – Body weight gain and abdominal circumference increase in Ehrlich ascites tumor-bearing mice orally supplemented with 100 mg/kg of standardized *Pinus pinaster* extract (Pyc) (proanthocyanidins ≥95.6%) for 20 days every 24 h. Ascitic fluid volume and tumor packed cell volume. Resveratrol (25 mg/kg) was used to supplement positive control mice, whereas saline was used in negative control mice.

Parameters	Negative control	Pyc	Resveratrol
Body weight gain (g)	11.2 ± 0.9	7.7* ± 0.3	8.0* ± 1.0
Abdominal circumference increase (cm)	1.6 ± 0.7	1.1* ± 0.7	1.0* ± 0.6
Total ascitic fluid volume (mL)	9.2 ± 2.2	6.2* ± 1.4	6.0* ± 1.1
Tumor packed cell volume (mL)	5.0 ± 0.7	3.7* ± 0.8	4.4 ± 0.6

Body weight gain and abdominal circumference increase determined in 16 mice per group. Ascitic fluid volume and tumor packed cell volume evaluated in six mice per group. Data obtained ten days after tumor induction. Data were expressed as the means ± standard deviation and were analyzed by analysis of variance (ANOVA) and Bonferroni test. *Statistical difference compared to negative control group ($P \leq .05$).

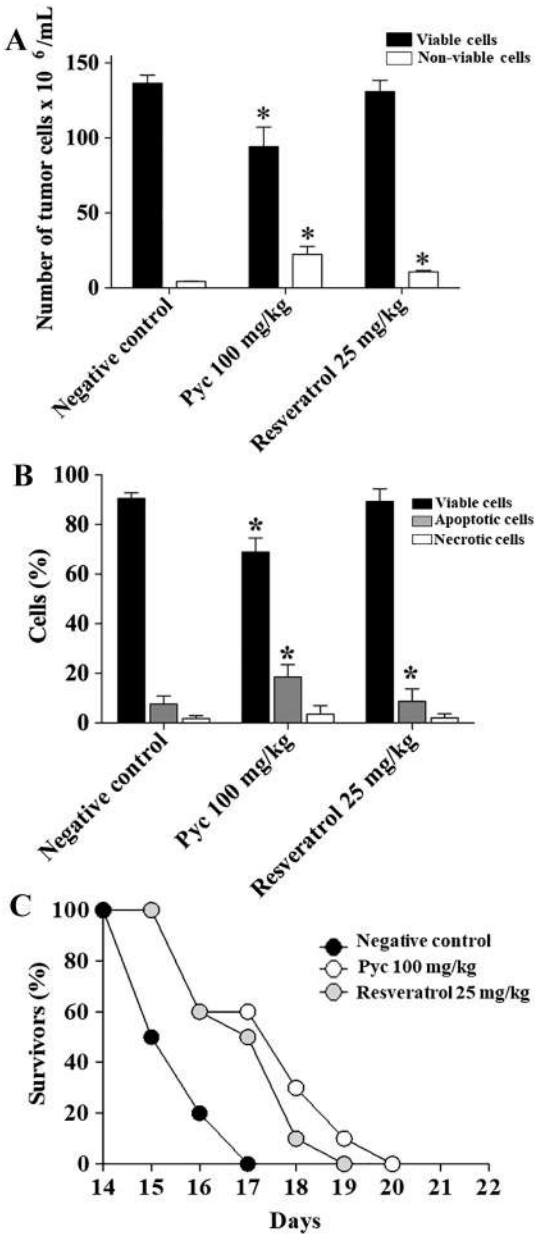

Fig. 3 – Cell viability (A) and type of tumor cell death (B) in ascitic fluid from Ehrlich ascites tumor-bearing mice orally supplemented with 100 mg/kg of standardized *Pinus pinaster* extract (Pyc) (proanthocyanidins ≥ 95.6%) every 24 h for 20 days. Mouse survival (C). Resveratrol (25 mg/kg) was used to supplement positive control mice, whereas saline was used in negative control mice. Cell viability (A) and tumor cell death (B) are shown as the means ± standard deviation of six mice per group. These data were obtained ten days after tumor induction and were analyzed by analysis of variance (ANOVA) and Bonferroni test. *Statistical difference compared to negative controls ($P \leq .05$). Survival data (C) correspond to 10 mice per group and were analyzed as area under curves.

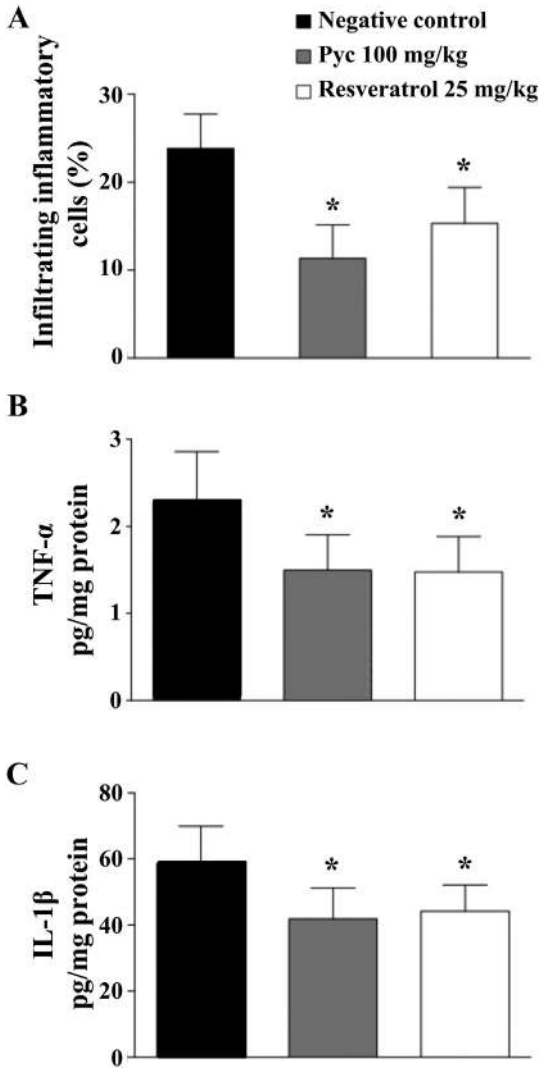

Fig. 4 – Percentage of infiltrating inflammatory cells (A) and concentration of tumor necrosis factor (TNF)-α (B) and interleukin (IL)-1β (C) in ascitic fluid from Ehrlich tumor-bearing mice orally supplemented with 100 mg/kg of standardized *Pinus pinaster* extract (Pyc) (proanthocyanidins ≥ 95.6%) every 24 h for 20 days. Resveratrol (25 mg/kg) was used to supplement positive control mice, whereas saline was used in negative control mice. Data are shown as the means ± standard deviation of six mice per group. Data were obtained ten days after tumor induction and were analyzed by analysis of variance (ANOVA) and Bonferroni test. *Statistical difference compared to negative controls ($P \leq .05$).

determine Pyc's MTOD in Balb-c mice, which was 100 mg/kg a day. The same dose of conventional maritime pine bark extract was used previously by Lee and collaborators [27], who worked with a mouse model of ozone exposure (oral administration for 5 days), and Liu and collaborators [28], who used a single intragastric dose to treat asthmatic airway injury in infant Balb-c mice. The latter study showed that the extract at 100 mg/kg markedly inhibited the number of inflammatory cells, as well as levels of IL-4, IL-5, IL-9, and IL-13 in bronchoalveolar lavage fluid of ovalbumin-induced mice [28]. Notwithstanding, it is necessary to remember that the conventional extract is standardized in procyanidins (65-75%), containing other less abundant constituents such

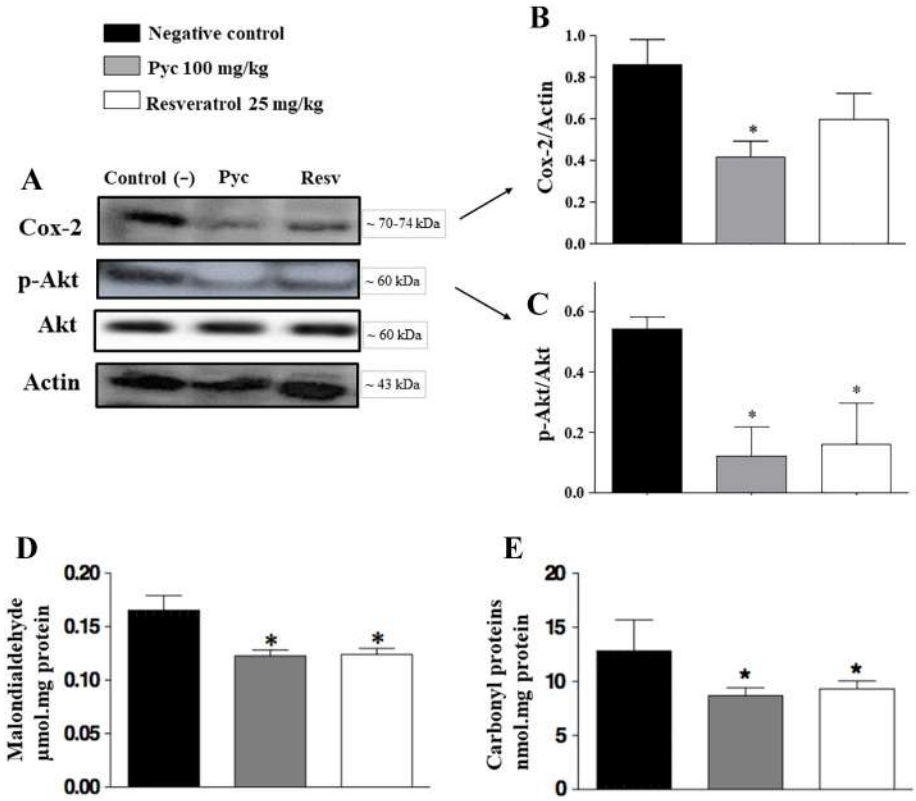

Fig. 5 – Cyclooxygenase 2 (Cox-2) expression and protein kinase B phosphorylation (p-Akt) in ascitic fluid cells from Ehrlich tumor-bearing mice orally supplemented with 100 mg/kg of standardized *Pinus pinaster* extract (Pyc) (proanthocyanidins ≥ 95.6%) every 24 h for 20 days (A). Ratio of Cox-2/Actin and p-Akt/Akt (B and C). Lipid peroxidation (malondialdehyde concentration) (D) and carbonyl protein concentration (E) in ascitic fluid. Resveratrol (25 mg/kg) was used to supplement positive control mice, whereas saline was used in negative control mice. Data are shown as the means ± standard deviation of six mice per group. Data were obtained ten days after tumor induction and were analyzed by analysis of variance (ANOVA) and Bonferroni test. *Statistical difference compared to negative controls ($P \leq .05$).

as caffeic acid, ferulic acid and taxifolin [3]. Pyc used in the present study contained proanthocyanidins (≥95.6%) and water (≤5.0%).

An effort to translate our experimentally determined Pyc MTOD to the human context was done using the HED calculation. Deductively, the result suggested a dose closer to 650 mg in an 80 kg human. The French maritime pine bark extract is usually commercially available in the form of oral tablets or capsules containing between 50-100 mg, which are generally accepted as safe daily doses. The safety of higher doses evaluated in light of the literature suggests that the dose estimated in the current work may be feasible; however, more studies evaluating safety are necessary.

A previous study estimated that the daily intake of proanthocyanidins found in various foods by the US population (>2 years old) was approximately 57.7 mg/person [29]. However, the data of some previous studies support the safety of higher doses. One recent study suggested the safety of a 4-week oral intake of an 80% proanthocyanidin-rich grape seed extract in healthy subjects at doses as high as 2500 mg [30]. In fact, there are doubts concerning proanthocyanidin absorption because it is known that highly polymerized versions are very difficult to absorb. The maritime pine bark extracts contain mostly oligomeric procyanidins, which are dimers and trimers of catechin and epicatechin [3,4].

A literature search for studies that tested maritime pine bark extract's procyanidins at high doses revealed that previously, single extract oral doses up to 300 mg/day or multiple-doses (200 mg/day for 5 days) were tested in healthy volunteers in a study assessing pharmacokinetic parameters. The authors did not mention toxicity. Instead, they showed that after a single ingestion, compounds derived from the extract were rapidly absorbed, and the majority of them were detectable throughout an experimental period of 14 h. Rapidly absorbed catechins exhibited peak plasma concentration 4 h after oral ingestion. From this point, catechin concentrations began to decrease, whereas their derived metabolite concentration (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone) began to rise, exhibiting a peak at 8 h that was maintained up to 14 h. Analysis of steady state plasma samples revealed significant phase II metabolism [4]. In another study, the conventional extract was used at oral doses up to 360 mg/day in chronic venous insufficiency patients (n = 40) for 4 weeks with good tolerability [31].

In the current study, resveratrol was chosen as the positive control because its chemopreventive effects were previously demonstrated in Ehrlich tumor-bearing mice. In this case,

resveratrol acted by reducing inflammatory cytokines, leukocytes and oxidative stress [32]. Previously, a study with mice had shown that oral administration of resveratrol at doses close to 25 mg/kg daily for prolonged periods caused no toxicity [17].

The initial indicators of Pyc's chemoprotective activity were attenuation of body weight gain and increased abdominal circumference. Ascitic volume was reduced in mice supplemented with Pyc. In Ehrlich ascites tumor-bearing mice, a rapid increase in ascitic volume was expected due to the inflammatory reaction following intraperitoneal inoculation [11]. Ascitic fluid volume is associated with tumor progression, and it gives information about tumor nutritional status and availability of nutrients. Supplementation with Pyc was responsible for the highest restraint on tumor growth. In mice supplemented with Pyc, tumors were smaller, as evidenced by a reduced volume of tumor packed cells and smaller numbers of tumor cells. These data are indicators of Pyc's antiproliferative action. Furthermore, a reduced number of inflammatory cells were found in ascitic fluid from Pyc supplemented mice. Data also revealed increased rates of apoptosis in ascitic samples of Pyc supplemented mice, indicating a reason that tumors in these mice were smaller and, consequently, why these mice lived longer.

Afterwards, experiments were performed to evaluate Pyc's influence on inflammation and cell proliferation pathways. These are essential attributes in a multitargeted approach, which is currently among the best strategies for cancer chemoprevention [1]. Inflammation is related to Ehrlich tumor progression. Fernandes and collaborators [11] reported that following Ehrlich tumor peritoneal inoculation, as tumors grow, blood leukocytes and inflammatory infiltrate into the tumor increase, along with expression of COX-1 and IL-1β. Evaluations presumed a role for nuclear factor kappa B (NF-κB) in Pyc's mechanism of action. NF-κB inhibition by conventional standardized French maritime pine bark extract was already previously shown in a study that suggested it may be a potential compound for the future development of anti-atherosclerotic therapy in cardiovascular disease [33]. Therefore, some up- and downstream mediators of NF-κB pathways were also evaluated. One of these mediators is TNF-α, a proinflammatory cytokine known to induce apoptosis. However, in tumor cells, apoptosis tends not to occur when NF-κB is activated in response to TNF signaling. This downregulates the apoptotic signal by enhancing specific anti-apoptotic gene expression [34]. Likewise, binding of IL-1β to its receptor can activate NF-κB as well [35]. The first evidence suggesting that impaired NF-κB signaling was involved in chemoprevention caused by Pyc were due to reduced concentrations of TNFα and IL-1β found in ascitic fluid from mice supplemented with this extract. Ascitic fluid from these mice exhibited increased rates of apoptosis.

Further evidence of NF-κB inhibition during Pyc's activity was indicated by reduced expression of COX-2, a downstream NF-κB mediator [36]. A previous study had already demonstrated that the French maritime pine bark extract caused COX-1 and COX-2 inhibition in healthy human volunteers when administered orally [7]. Our data show that COX-2 was inhibited in ascitic fluid cells from mice supplemented with Pyc (compared to negative controls). This was another finding associated with longer survival in response to Pyc. Meanwhile, COX-2 inhibition in response to resveratrol was not as apparent.

Thereafter, the Akt pathway was investigated. This pathway plays a central role in cell survival, proliferation and malignancies. IL-1β binding to its receptor may activate Akt. Akt is then activated through phosphorylation. Our data suggest that Akt phosphorylation was restrained, since total Akt levels remained unchanged. Such a change could be a consequence of an inhibition in Akt expression; however, that did not appear to be the case. Some studies suggest that Akt regulates NF-κB activity by inducing phosphorylation and degradation of the IκB inhibitor, which is a hallmark in Akt-transformed tumor cells. NF-κB activity has been considered essential for Akt-induced oncogenicity [37]. Data in this study suggest an association between reduced proliferation and tumor size together with reduced Akt-phosphorylation in ascites from mice supplemented with Pyc.

The last evaluated biomarkers were related to reactive oxygen species (ROS) and oxidative stress. ROS are implicated in NFκB pathways, and ROS interactions with NF-κB are characterized by a high degree of complexity due to ROS capability to act in several cell compartments simultaneously [38]. ROS can either activate or inhibit NF-κB. Notwithstanding, ROS are associated with increased IL-1β production and activation of Akt pathway [39], both of which were reduced in response to Pyc. Pyc's antioxidant activity in ascitic fluid was demonstrated through reduced lipid peroxidation and carbonyl proteins. This action may have contributed to NF-κB inhibition, decreased IL-1β production and reduced Akt phosphorylation, culminating in the effects mediated by Pyc on inflammation and tumor progression.

In conclusion, this study demonstrated that Pyc supplementation is related to attenuated oxidative and inflammatory mediators and impaired tumor development, leading to acceptance of the initial hypothesis. Impairment of NF-κB activation and NF-κB-regulated products, including the Akt pathway, seems to play a role in the involved mechanisms of action. These data suggest Pyc as a candidate for future studies in multitargeted dietary-based cancer prevention approaches. These findings also contribute to justifying study continuity of this compound to identify more precisely the active constituent(s), or even the active metabolite(s), since δ-(3,4-dihydroxy-phenyl)-γ-valerolactone's available half-life data [4] favor the idea that it can eventually have activity as well. This work makes progress in that it adds to knowledge about the chemoprotective actions of *Pinus pinaster* procyanidins.

This study does have limitations. These findings were only able to partially reproduce the clinical scenario. Three broad approaches to the clinical use of chemopreventive agents have been described. 'Primary chemoprevention' involves administration of agents to the general 'healthy' population or to those without overt disease but with particular risk factors. 'Secondary chemoprevention' involves identification of individuals with premalignant lesions and administration of agents to prevent progression to invasive cancer. 'Tertiary chemoprevention' is defined as administration of agents to prevent recurrence or additional

primary cancers in individuals who have undergone successful treatment of early disease [1,40]. These last types of chemoprevention imply the need for selectivity. After all, the last thing anyone wants is to "protect cancer cells".

The present study is more related to primary chemoprevention and is still limited regarding the presence of risk factors. Using an animal model, we had to distribute the procedures across a timeline. One may accept that to study primary chemoprevention, one researcher might conceivably design a clinical trial in the form of a prospective cohort, following up healthy subjects using supplementation until a participant eventually developed the outcome (cancer). This design is one of the primary limitations due to it being so time consuming. Many previous studies have attempted to replicate this scenario using animals and chemically induced tumor models. However, the conversion rate in animals is very low and fluctuates in severity. These models are also tremendously time consuming. For these reasons, the Ehrlich tumor model was chosen in this work, as it possesses classical chemoprevention targets: inflammation and oxidative stress. In fact, this model is most important for examining tumor establishment and expansion (progression), all of which are accompanied by remarkable inflammation. With this awareness, we decided to initially investigate evidence of efficacy rather than selectivity. Some of our analyses were performed 10 days after discontinuation of mouse supplementation and tumor inoculation. This does not necessarily mean that the effects of Pyc extend to this point. This time point was chosen to assess impairment caused at the time of establishment and early processes related to tumor progression close to tumor induction, which at the tenth day was identified as attenuated tumor development in mice receiving Pyc supplementation. This report carries the expectation of supporting further studies, primarily clinical trials, to examine *Pinus pinaster* extract. Our team is currently interested in the selectivity of its chemoprotective effects.

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