

Recycled pequi (*Caryocar brasiliense*, Camb.) shell ethanolic extract induces apoptosis in canine osteosarcoma cells

Extrato etanólico de cascas recicladas de pequi (Caryocar brasiliense, camb.) induz apoptose em células de osteossarcoma canino

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Abstract

Osteosarcoma is a mesenchymal cancer associated with high mortality in dogs and in humans. The biodiversity-rich Cerrado, the predominant biome in the Midwest region of Brazil, is native to locally appreciated fruits such as pequi (*Caryocar brasiliense*, Camb). Although this plant has been frequently used in folk medicine, the pharmacological properties of pequi fruit shells have not been fully evaluated. Thus, this study aimed to determine the cytotoxic activity of ethanol extract of recycled pequi shells on canine osteosarcoma cells *in vitro*. Cells were cultured and treated with final extract concentrations of 0, 0.029 $\mu\text{g}/\mu\text{L}$, 0.29 $\mu\text{g}/\mu\text{L}$, and 2.91 $\mu\text{g}/\mu\text{L}$ for 24, 48, or 72 hours. Cell viability assay using trypan blue exclusion method and tetrazolium reduction method, cell survival assay, and double labeling with annexin V and propidium iodide were performed in the treated osteosarcoma cells. These allowed the determination of IC_{50} , survival fraction, and type of cell death, respectively. Pequi shell ethanol extract at a concentration of 2.91 $\mu\text{g}/\mu\text{L}$ showed the greatest inhibition of osteosarcoma cell growth *in vitro*, resulting in a 71.80% decrease in growth compared to the control. The mean IC_{50} was 155.2 $\mu\text{g}/\text{mL}$ at 72 hours. The calculated survival fractions showed that cell growth at 72 hours was 3.33% lower in cells treated with 2.91 $\mu\text{g}/\mu\text{L}$ extract. Results from the double labeling experiment suggest that apoptosis was the predominant type of cell death in cells treated with 2.91 $\mu\text{g}/\mu\text{L}$ extract. These results demonstrate that ethanol extract of recycled pequi shells promotes apoptosis in canine osteosarcoma cells.

Keywords: Antineoplastic; canine osteosarcoma; Cerrado biome; D-17; pequi; sustainability; tannins.

Resumo

O osteossarcoma é uma neoplasia mesenquimal associada à alta mortalidade em cães e humanos. O pequi (*Caryocar brasiliense*, Camb.) é nativo do Cerrado, bioma rico em biodiversidade predominante na região Centro-Oeste do Brasil. Embora esta planta seja frequentemente utilizada na medicina popular, as propriedades farmacológicas da casca do pequi ainda são pouco conhecidas. Este estudo teve como objetivo determinar a atividade citotóxica do extrato etanólico de cascas de pequi recicladas em células de osteossarcoma canino. As células foram cultivadas e tratadas com concentrações finais de extrato de zero, 0,029 $\mu\text{g}/\mu\text{L}$, 0,29 $\mu\text{g}/\mu\text{L}$ e 2,91 $\mu\text{g}/\mu\text{L}$ por 24, 48 ou 72 horas. As células foram submetidas a ensaio de viabilidade celular pelos métodos de exclusão de azul de tripiano da redução de tetrazólio para determinar a IC_{50} , ao ensaio de sobrevivência celular para calcular a fração de sobrevivência e à dupla marcação com anexina V e iodeto de propídio para determinar o tipo de morte celular. O extrato etanólico de casca de pequi na concentração de 2,91 $\mu\text{g}/\mu\text{L}$ apresentou a maior inibição do crescimento de células de osteossarcoma, com redução de 71,80% no crescimento em relação ao controle. A IC_{50} média foi de 155,2 $\mu\text{g}/\text{mL}$ em 72 horas. O crescimento celular em 72 horas foi 3,33% menor nas células tratadas com 2,91 $\mu\text{g}/\mu\text{L}$ de extrato. A apoptose foi o tipo predominante de morte celular em células tratadas com 2,91 $\mu\text{g}/\mu\text{L}$ de extrato. Esses resultados demonstram que o extrato etanólico de cascas de pequi recicladas promove apoptose em células de osteossarcoma canino.

Palavras-chave: Antineoplásico; Cerrado; D-17; osteossarcoma canino; pequi; sustentabilidade; taninos.

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Introduction

Canine osteosarcoma is a malignant and aggressive primary bone tumor, which shows similar characteristics with human osteosarcoma and frequently results in pulmonary metastasis⁽¹⁾. The high mortality associated with this disease correlates with its metastatic capacity, leading to death in up to 80% of animals. Studying and developing treatments for canine osteosarcoma are not only important

because numerous cases can be frequently seen in veterinary practice, but also because this can provide better insight for human osteosarcoma due to the similarity of the disease in both species⁽²⁾.

Multiple studies used plants as sources of potential bioactive and medicinal compounds, including natural products with inhibitory potential against neoplastic cells. *In vitro* studies using tumor cell lines have been utilized to

identify bioactive components in plants and to provide better insight into the mechanism of action these compounds in neoplastic cells⁽³⁾.

The Brazilian “Cerrado” is a considerable and valuable source of biomolecules, due to its characteristic savannah-like climate and vegetation. The antitumor potential of Cerrado plants, particularly of the symbolic plant, pequi (*Caryocar brasiliense*, Camb.), may reveal new alternatives in antitumor therapy. These alternative treatments may offer better efficacy in both humans and animals, while having minimal side effects compared to conventional antineoplastic treatments⁽³⁾⁽⁴⁾. Furthermore, the molecular diversity of natural products is far superior to that derived from synthetic processes, making it possible for chemical compounds present in plants to become potential drugs for various diseases⁽⁵⁾.

Assays evaluating the efficacy of secondary metabolites from the pequi fruit on cancer cells are valuable to verify the preventive, therapeutic, or even curative potential of this extract in neoplasms, such as osteosarcoma. Furthermore, recycling pequi shells, which are usually discarded, contributes to the sustainability of this approach. This investigation aimed to determine the cytotoxic activity of the ethanol extract of recycled pequi shells on canine osteosarcoma cells *in vitro*.

Materials and methods

This study was done at the Multiuser Laboratory for the Evaluation of Molecules, Cells, and Tissues, Graduate Program in Animal Science of the School of Veterinary and Animal Science, Federal University of Goiás (UFG), Brazil. Pequi shell extracts were prepared at the Animal Nutrition Laboratory (EVZ/UFG), and physicochemical analysis of the extract composition was performed at the Faculty of Pharmacy (UFG).

Cell Culture

Osteosarcoma cells (D-17, BCRJ 0276, Lot 000573, Passage 239) from ATCC (American Type Culture Collection - Manassas, VA, USA) were purchased from the Rio de Janeiro Cell Bank (BCRJ - Rio de Janeiro, Brazil) and maintained in a humidified incubator at 37 °C and 5% CO₂ atmosphere.

Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, penicillin, streptomycin (10.000 U.I./ml - 10 mg/ml), amphotericin B, and L-glutamine.

Preparation of Ethanol Extract of Recycled Pequi Shells

Pequi shells were obtained from the Supply Center of Goiás (Ceasa-GO), with shells selected from a freshly discarded pool which are in perfect morphological conditions (voucher UFG - 43-833, UFG Herbarium, Brazil) and within optimal ripeness. Shells were chopped to

a thickness of approximately 4 mm and placed in a forced-air oven at 60°C for 72 hours. Dried shells were crushed in an analytical mill to obtain a standardized particle size of 60 mesh (0.25 mm). Pequi shell bran was stored at -20°C, protected from light.

Cold percolation extraction was performed by percolating 1000 g of pequi shell bran in 1000 mL 95% ethanol (95:5 ethanol:water) for eight hours and stirred for one hour. The product was centrifuged at 4000 rpm and the supernatant was filtered. The solvent was evaporated in a rotary evaporator under reduced pressure to obtain the crude ethanol extract (EEPS). The extract was then stored at -4°C, protected from light for later use. EEPS samples were submitted for physicochemical analysis.

Experimental Design

D-17 canine osteosarcoma cells were seeded into 96-well plates at 1×10^4 cells/well with 100 µL of media and treated with EEPS stock solution (concentration 29.2 µg/µL) to obtain the following final dosages: 0.029 µg/µL (0.1 µL per well), 0.29 µg/µL (1 µL per well) and 2.91 µg/µL (10 µL per well). Cells untreated with EEPS (0 µg/µL) was used as the control group (CG). Cells were exposed for 24, 48, and 72 hours. All tests were carried out in triplicate in three independent experiments. The extracts were diluted in DMSO (Dimethyl sulfoxide, Cultilab, Campinas, Brazil) and stored at -20°C.

Cell Viability Assays

Trypan Blue Exclusion Method

D-17 cells were cultivated in 96-well plates with 100 µL per well at 1×10^4 cells/well for 24 hours, inside a humidified incubator at 37°C with 5% CO₂ atmosphere. Treatments were carried out using dosages of 0.029, 0.29, and 2.91 µg/µL EEPS for 24 h (G24), 48 h (G48), and 72 h (G72). The medium from each well was discarded and the cells were suspended in 100 µL of trypsin (Cultilab, Campinas, Brazil) after washing with Dulbecco's phosphate-buffered saline (DPBS, Life technologies, USA). Cells were dissociated with trypsin-EDTA (0.05%), which was later inactivated by adding 100 µL DMEM. The resulting solution was centrifuged at 130 RCF(g) for 10 minutes and decanted. Cells were resuspended in 100 µL of fresh culture medium and 10 µL of the suspension was transferred to Eppendorf-type microtubes containing 40 µL of trypan blue (Trypan Blue - Sigma-Aldrich, St Louis, USA). Cell viability was assessed by instilling 10 µL of cell-trypan blue mixture onto a slide and reading the slide in a Neubauer chamber under an optical microscope.

Cell viability was calculated by applying the following equation: % CV = (NC of treatment/NC of CG) × 100, where CV is cell viability and NC is the number of cells. Three independent experiments were performed in triplicate. Results were analyzed with analysis of variance (F-test) using the Microsoft Excel statistical tool.

Significance level was set at 0.05.

Tetrazolium Salt Reduction Method (MTT)

D-17 cells were cultivated and treated with EEPS as described above in the previous experiments. After treatment, 10 μ L of tetrazolium (MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide - Sigma-Aldrich) was added to each well. After incubation for three hours, 50 μ L of 10% sodium dodecyl sulfate (SDS - Vivantis Biochemical, CA, USA) diluted in 0.01 N HCL was added per well to stop the reduction reaction of MTT to formazan. Cells were incubated for 24 hours at room temperature. The optical density of each plate was quantified using a spectrophotometer (Awareness Technology Inc/Stat Fax 2100, 425 nm–540 nm, Palm City, FL, USA). The concentration at 50% inhibition of cell viability (Inhibitory concentration - IC_{50} , in μ M) was determined using the GraphPad Prism statistical program (GraphPad Software, San Diego, CA, USA). For this test, three independent experiments were performed in triplicate.

Cell Survival Assay

D-17 cells were cultivated and treated with EEPS as described above in previous experiments. After treatment, the medium from each well was discarded, and cells were suspended with 500 μ L of trypsin. Before centrifugation at 130 rcf (g) for 10 minutes, the medium was discarded, and cells were resuspended in 1 ml of DMEM. Cells were then cultured in six-well plates in a humidified incubator at 37°C with 5% CO_2 atmosphere for 15 days.

After incubation, the medium was discarded. The wells were rinsed with DPBS, dyed with trypan blue, placed inside a Neubauer chamber, and counted. The survival fractions were calculated using the following equation: %SF = (CCAT number/CCGC number) \times 100, where SF is the survival fraction, CCAT is the number of cells counted after treatment, and CCGC is the number of cells counted in the CG. Survival curves were plotted and data was analyzed using analysis of variance (F-test). Three independent experiments were carried out in triplicate.

Double Labeling Assay

Cells were cultivated in 12-well plates at 5×10^5 cells/well, inside a humidified incubator at 37 °C with 5% CO_2 atmosphere. Cells were treated with 2.91 μ g/ μ L EEPS for 24, 48, and 72 hours. At the end of the treatment, the medium from each well was stored in a properly labeled test tube, while viable cells were discarded. Double labeling was performed by adding 400 μ L of binding buffer, 5.0 μ L of annexin V-FITC, and 1.0 μ L of propidium iodide (Annexin V Apoptosis Detection Kit I - BD Biosciences, San Diego, USA) to each test tube. The resulting solution was incubated at room temperature for 15 minutes and then analyzed in a flow cytometer (FACSVerse, BD Biosciences). Early apoptosis was

identified in cells labeled only with annexin V, late apoptosis in cells labeled with both annexin V and propidium iodide, and necrosis in cells labeled only with propidium iodide. Three independent experiments were carried out in triplicate. Analysis of variance with post-hoc Tukey's test was used to compare the mean percentage of positive cells to each combination (annexin V only, propidium iodide only or both dyes simultaneously) using the eaysanova⁽⁶⁾ package of the R software. Significance level was set at 0.05.

Results

For this experiment, we recycled 180 kg of pequi shells, which was obtained from 300 kg of fruits and destined for disposal. This yielded approximately 9 kg of pequi shell bran. Extraction of 1 kg of pequi shell bran in 1000 mL 95% ethanol and subsequent cold percolation and rotary vaporization resulted in 450 mL EEPS, a 45% yield. The results obtained from the physicochemical analysis of the crude, undiluted EEPS are found in Table 1.

Table 1: Physicochemical parameters of the crude ethanolic extract of pequi shells (*Caryocar brasiliense*) used in this experiment. *GAE= Gallic acid equivalent.

PARAMETER	VALUE
Mean density	23.9015 g/L
Mean concentration	29.19 g/L
Mean pH	5.7
Density of Active Raw Extract	275.72 mg GAE*/mL
Total phenol concentration	83.10 mg GAE/mL (30.14% of active extract)
Tannin concentration	18.47 mg GAE/mL (22.23% of phenols in the active extract)
Flavonoid concentration	4.78 mg GAE/mL (5.75% of phenols in the active extract)
Other phenols	59.85 mg/mL (72.02% of phenols in the active extract)

The results obtained in the cell viability assay using the trypan blue exclusion method and the tetrazolium reduction method (MTT) to calculate the IC_{50} (55.2 μ g/mL) are shown in Table 2. Treatment with 2.91 μ g/ μ L of EEPS for 72 hours resulted in inhibition of growth by 71.80% relative to the control, the highest cytotoxic activity observed among the treatment groups.

The cell survival assay was used to assess cell proliferation capacity after exposure to EEPS. The calculated survival fractions showed that cell proliferation after treatment was lower (3.33%) in the group treated for 72 hours with 2.91 μ g/ μ L EEPS. On the other hand, proliferation reached 87.5% in the group treated for 48 hours with 0.29 μ g/ μ L EEPS. At 24 hours of exposure, we

found no statistically significant difference among the different concentrations of EEPS ($F = 2.92$, $P > 0.05$). On the other hand, at 48 and 72 hours of exposure, survival of cells treated with 2.91 $\mu\text{g}/\mu\text{L}$ EEPS concentration were significantly different from the other doses. However, for cells treated with 2.91 $\mu\text{g}/\mu\text{L}$ EEPS, we found no statistically significant difference with regard to exposure times.

Table 2: Cell viability assay with the Trypan Blue method and concentration values that inhibit 50% of cell viability (IC_{50}) in canine osteosarcoma cells treated with ethanolic extract of pequi shells (*Caryocar brasiliense*, Camb.) - EEPS. Results are expressed as mean percentages relative to the control group in $\mu\text{g}/\mu\text{L}$, along with the standard deviation (SD) and converted values according to the international standard units ($\mu\text{g}/\text{mL}$).

Exposure times	EEPS dosage and concentrations used			IC_{50}		
	0.1 μL	1.0 μL	10 μL	$\mu\text{g}/\mu\text{L}$	SD	$\mu\text{g}/\text{mL}$
	(0.029 $\mu\text{g}/\mu\text{L}$)	(0.29 $\mu\text{g}/\mu\text{L}$)	(2.91 $\mu\text{g}/\mu\text{L}$)			
24h	92.70 ^{a*}	97.49 ^{a*}	44.76 ^{b*}	0.1798	0.108	179.8
48h	99.19 ^{a*}	98.35 ^{a*}	34.22 ^{b*}	0.1625	0.740	162.5
72h	44.76 ^{a†}	34.22 ^{a,b†}	28.20 ^{b,c†}	0.1552	0.63	155.2

Different letters in the same line express significantly different results (Test F, $p < 0.05$). Different symbols in the same column express significantly different results (Test F, $p < 0.05$).

The data obtained in cell viability and cell survival assays showed that the greatest cytotoxic and antiproliferative activities were observed in cells treated with 2.91 $\mu\text{g}/\mu\text{L}$ for different exposure durations. For this reason, this dosage was used in further investigating the mechanism of cell death promoted by EEPS in D-17 cells of canine osteosarcoma using the double labeling assay with annexin V-FITC and propidium iodide. Mean values of cell percentages from samples of three replicates from the flow cytometry are shown in Figure 1. There was no necrotic cell death in cells exposed to EEPS for 24 hours, while cells exposed for 72 hours showed increased apoptosis induction at an early stage. However, the late phase of apoptosis was predominant at all exposure times ($97.07 \pm 0.25\%$ at 24 hours, $98.80 \pm 0.05\%$ at 48 hours and $91.29 \pm 0.42\%$ at 72 hours).

Discussion

The highest inhibitory effect on cell growth was observed in D-17 cells treated with 2.91 $\mu\text{g}/\mu\text{L}$ EEPS for 72 hours. In a previous study, a concentration-dependent inhibitory effect of phenolic compounds from carqueja (*Baccharis trimera*) extract has been reported in human uterine cervix tumor cell cultures. However, the inhibition was more accentuated (86%) and faster (24 h) than in the present study. This is probably due to the concentrated phenolic compounds extracted from the plant used in that

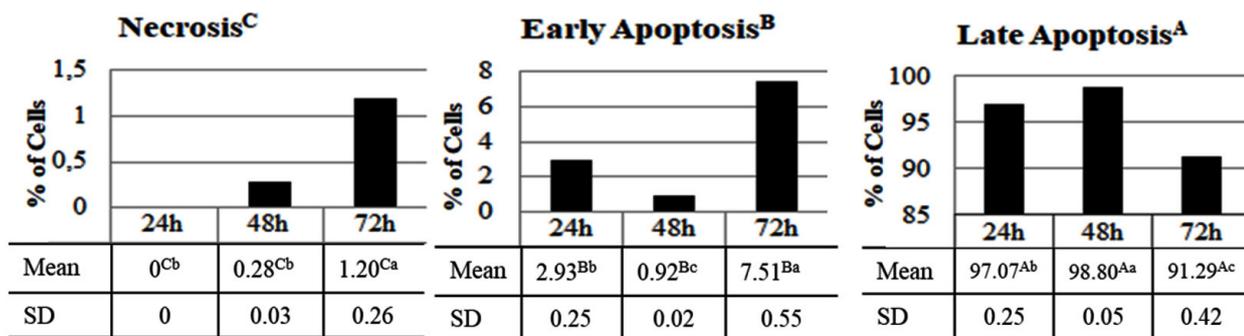


Figure 1: Mean and standard deviation (SD) values from the analysis of cell death mechanism promoted by the concentration of 2.91 $\mu\text{g}/\mu\text{L}$ of 3% ethanol extract of recycled pequi (*Caryocar brasiliense*, Camb.) shells in D-17 cells of canine osteosarcoma, using the double label assay with annexin V-FITC and propidium iodide. The x-axis represents the exposure time to the extract, and the y-axis, the percentage of cells. Flow cytometer (FACSVerse, BD Biosciences). Uppercase letters indicate significant difference between types of cell death, and lowercase letters indicate significant difference between exposure times (Tukey's Test, 5%).

trial, unlike the crude extract used in the present experiment⁽⁷⁾.

It is noteworthy that at all doses tested, the inhibitory effects of EEPS manifested only after 72 hours. This could be due to the presence of phenols, including the antioxidant flavonoids⁽⁸⁾, in the extract (Table 1), which conferred a protective effect on the viability of cells treated with up to a maximum concentration of 0.29 $\mu\text{g}/\mu\text{L}$ or for 24 hours of exposure.

The total concentration of the crude EEPS used in this experiment shown in Table 1 was 29.19 g/L, which is equivalent to 29.19 mg/mL or 29.2 $\mu\text{g}/\mu\text{L}$. Thus, doses of 0.1, 1, and 10 μL contained 2.92, 29.2, and 291.2 μg total solutes, respectively. This is comparable to the dose used for *Casearia sylvestris* ("Guaçatonga") extract, which inhibited the growth of MCF-7 human breast cancer cells by 20% using 2 $\mu\text{g}/\text{mL}$ of extract, and by up to 80% when treating cells with 18 $\mu\text{g}/\text{mL}$ extract for 24 hours⁽⁹⁾. For

comparison, in our study, we reached 55.24% growth inhibition with 2.91 $\mu\text{g}/\mu\text{L}$. In another study, treatment of cutaneous melanoma cells with mangosteen extract, *Garcinia mangostana*, significantly reduced the number of neoplastic cells at dosages between 1.0 and 5.0 mg/ml⁽¹⁰⁾, comparable to our most favorable result. Comparing the efficacy and potency of natural products is important, although one must consider the intrinsic response of the different cell lines, which certainly varies with each neoplastic type.

It should be emphasized that pequi, an essential plant in the Brazilian Cerrado, contains compounds that possess inhibitory effects on cell viability, that is, antiproliferative effects. Most research on active antineoplastic principles from the Brazilian flora have focused and contemplated the use vegetation of other biomes, while work with Cerrado plants, despite their biodiversity, are rare in the scientific literature. For instance, human squamous cell carcinoma cells were tested with 72 different extracts from plants found in the Amazon and Atlantic forests⁽¹¹⁾.

Although the 72-hour treatment showed a significant difference regarding inhibition of cell proliferation, there was no significant difference in IC_{50} values. A possible explanation is that the extract, in addition to components that cause cell damage, contained antioxidants⁽⁸⁾ that could maintain cell integrity, at least for a certain period. Thus, the action of EEPS could possibly be more dependent on exposure time than on dosage.

The mean IC_{50} values found in this experiment may seem high compared to those reported for other plant extracts to promote cytotoxicity in neoplastic cells. *Artemisia annua* (annual wormwood) commercial extract showed an IC_{50} of 65 μM in D-17 cells⁽¹²⁾, a value much lower than those found in the present study. However, this could be related to the artemisinin concentration found in *A. annua*. Considering the artemisinin concentration in the extract of 2 mg/ml or 7 mM, and the maximum amount of artemisinin found in the plant (0.45%), one may infer that the IC_{50} value was 185 $\mu\text{g}/\text{ml}$ as calculated from the concentration of total solutes. The IC_{50} value of pequi extract, therefore, could be lower than that obtained for *A. annua*.

A significant consideration determining the cytotoxicity of EEPS used in this research is the extraction method. The EEPS was prepared to ensure maximum extraction of phenols, which possess antioxidant properties⁽¹³⁾. Had other extraction methods been used, the IC_{50} could have been lower than that measured for all exposure times. This argument is supported by a previous report, in which the anthraquinone-rich dichloromethane fraction displayed the highest anticancer activity when evaluated in the human hepatoma cancer cell line HepG2, as compared to

extracts obtained using n-hexane, ethyl acetate, n-butanol, or water as solvents⁽¹⁴⁾.

Another crucial factor to take into consideration is the cell type used. It is likely that future studies using different tumor cells will report significantly different IC_{50} values for EEPS, compared to D-17 canine osteosarcoma cells used in this study. This is supported by the reported IC_{50} values ($\mu\text{g}/\text{mL}$) from the use of crude stem extract of African-native *Paullinia pinnata* L. (Sapindaceae) to inhibit the growth of neoplastic cells of different lineages: HepG2 (liver cancer - 42.8), MCF-7 (breast cancer - 43.1), MDA-MB231 (breast cancer - 73.8), A431 (skin cancer - >100), AGS (stomach cancer - 47.6), and LNCap (prostate cancer - 47.2)⁽¹⁵⁾.

Paradoxically, the EEPS metabolites which may possibly have cytotoxic effects are the phenols themselves. The extract of the popular carqueja (*Baccharis trimera*) had an antiproliferative effect against human cervical cancer cells after 24 hours of treatment. Moreover, the IC_{50} was calculated for the phenolic fraction of the extract at 482 $\mu\text{g}/\text{mL}$ ⁽⁷⁾. Considering the EEPS analysis shown in Table 1, the fraction corresponding to tannins may be the phenolic group responsible for the cytotoxic effects of EEPS.

As observed in this study, the tannin content of EEPS was 22.23% of the total phenols in the extract. Comparatively, a study carried out with breast cancer cells 4T1-luc2 tested the antitumor activity of weimaining (WMN). WMN is a condensed tannin compound extracted from the roots of the buckwheat *Fagopyrum cymosum* (Trevir.) Meisn., a type of Chinese herbal medicine⁽¹⁶⁾. The results showed time-dependent increase in tumor cell apoptosis, similar to the decrease in cell viability caused by 72 hours of EEPS treatment, suggesting that tannins play a crucial role in reducing cell viability.

Regarding the cell survival assay results, cell growth was reduced after treatment with 2.91 $\mu\text{g}/\mu\text{L}$ at different exposure times, further supporting the antitumor potential of EEPS. Similarly, proliferation of D-17 cells exposed to 2 μM of a complex of platinum with tris (2-carboxyethyl) phosphine decreased 53.79% \pm 5.15%, after two weeks⁽¹⁷⁾.

The antineoplastic activities of bioactive compounds found in fruits has constantly captured the attention of the scientific community, especially regarding the type of cell death that these substances can promote. In the present study, the promising concentration of EEPS, 2.91 $\mu\text{g}/\mu\text{L}$, promoted a high percentage of apoptosis in D-17 cells. The same type of cell death was observed in HepG-2 (liver), Caco-2 (colon), and MCF-7 (breast) neoplastic cell lines exposed to *Annona squamosa* fruit extract⁽¹⁸⁾, whose phenolic content ranged from 70.14 \pm 3.89 to 284 \pm 2.12 μg GAE/g and fruit peel flavonoid was 81.27 \pm 1.74 μg EQ equivalent/g. The

tannin fraction from *Terminalia bellirica* induced apoptosis in hepatocellular carcinoma xenografts in mice⁽¹⁹⁾, reinforcing our hypothesis that the tannin fraction may be the phenolic group responsible for the apoptotic effects of EEPS.

Apoptosis induction by chemicals or biological substances in any cancer type is a pivotal aim of current antitumoral studies⁽²⁰⁾, as this cell death process occurs without secondary inflammation⁽²¹⁾ or possible harm to adjacent normal cells. Previous works have shown that apoptosis was also observed in D-17 cells treated with bioactive compounds with antioxidant capacity. These include luteolin (a flavonoid molecule found in fruits and vegetables)⁽²²⁾, myricetin (an antioxidant found in berries)⁽²³⁾, saffron extract (rich in curcuminoids), and rosemary leaf extract (rich in carnosic acid)⁽²⁴⁾.

There was no necrotic cell death in cells exposed to EEPS for 24 h, while cells exposed for 72 h showed increased early-stage apoptosis induction. However, the late phase of apoptosis was predominant at all exposure times. Apoptosis occurs in response to various stimuli, including those that can activate the intrinsic apoptotic pathway by releasing cytochrome c from the mitochondria. Cytochrome c binds to proteins in the cytosol and activates caspase to promote cell death⁽²⁵⁾. In another study with D-17 cells, β -lapachone, a compound isolated from the sawdust of the “Ipê” wood (*Tabebuia* spp.) native to the Brazilian Cerrado, promoted early-stage intrinsic apoptotic pathway, which was related to the rupture of mitochondrial membranes⁽²⁶⁾. Notably, the concentration with the best results (1.0 μ M of β -lapachone or 2.42 μ g/ μ L) was lower than we used for pequi (2.91 μ g/ μ L). On the other hand, curcumin, a substance isolated from *Curcuma longa*, which is widely found in the Cerrado biome, induced extrinsic apoptosis in D-17 cells by activating caspase at a concentration of 50 μ M or 0.20 μ g/ μ L⁽²⁷⁾. Considering that β -lapachone and curcumin were used in their pure forms, the positive results we obtained with crude pequi extract in the present study can be considered remarkable.

The main finding of this research is that the ethanol extract of recycled pequi shells inhibited the proliferation of canine osteosarcoma cells through apoptosis. This is an unprecedented report and solidifies the importance of identifying EEPS components for the potential development of antitumor drugs. Throughout the history of biomedical research, other compounds from fruits and vegetables have been identified as valuable sources of antitumor drugs. Classic examples include the vinca alkaloids, taxanes, and camptothecins which were originally extracted from *Catharanthus roseus* (Madagascar), *Taxus brevifolia* (the Pacific), and the *Camptotheca acuminata* (China), respectively⁽²⁸⁾.

The Cerrado biome, a natural heritage for mankind, can be part of this success story in anticancer

therapeutics. Future studies for developing antitumor drugs can use the by-product of pequi, an approach guided by the principles of sustainability.

Conclusion

Treatment with 2.91 μ g/ μ L of recycled pequi (*Caryocar brasiliense*, Camb.) shell ethanolic extract reduces the viability and survival in D-17 canine osteosarcoma cells *in vitro* by promoting apoptosis. These results suggest that EEPS can potentially be an effective and sustainable alternative for the development of anticancer therapy in both canine and human osteosarcoma.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization: K.M.S. Braga, V.S. Cruz, E. Arnhold, E.G. Araújo; *Formal Analysis*: V.S. Cruz, E. Arnhold; *Funding acquisition*: E.G. Araújo; *Investigação*: K.M.S. Braga, V.S. Cruz; *Methodology*: K.M.S. Braga, V.S. Cruz, E. Arnhold, E.G. Araújo; *Project administration*: V.S. Cruz, E.G. Araújo; *Resources*: E.G. Araújo; *Supervision*: E.G. Araújo; *Validation*: V.S. Cruz, E. Arnhold; *Writing – original draft*: K.M.S. Braga; *Writing – review & editing*: K.M.S. Braga, V.S. Cruz, E. Arnhold, E.G. Araújo.

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