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Ethanolic extract of *Caryocar brasiliense* fruit peel promotes death and cell cycle control in canine osteosarcoma cells

Extrato etanólico da casca de fruta de *Caryocar brasiliense* promove a morte e o controle do ciclo celular em células osteossarcoma caninas

El extracto de etanol de la cáscara de la fruta *Caryocar brasiliense* promueve la muerte y el control del ciclo celular en las células del osteosarcoma canino

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Leandro Lopes Nepomuceno
ORCID: https://orcid.org/0000-0001-5839-0046
Federal University of Goiás, Brazil
E-mail: leandro_lopes795@hotmail.com
Nayane Peixoto Soares
ORCID: https://orcid.org/0000-0001-9031-174X
Federal University of Goiás, Brazil
E-mail: nayane.peixoto@gmail.com
Juliana Carvalho de Almeida Borges
ORCID: https://orcid.org/0000-0001-5938-1993
Federal University of Goiás, Brazil
E-mail: julianacarvalhovet@ufg.br
Vanessa de Souza Vieira
ORCID: https://orcid.org/0000-0003-3023-5612
Federal University of Goiás, Brazil
E-mail: biovanessa23@hotmail.com
Dayane Kelly Sabec Pereira
ORCID: https://orcid.org/0000-0002-8886-4668
University Center Assis Gurgacz, Brazil
E-mail: daya_ks@hotmail.com
Kleber Fernando Pereira
ORCID: https://orcid.org/0000-0002-5102-6273
Federal University of Parana, Brazil
E-mail: kleber.ufpr@gmail.com

Vanessa de Sousa Cruz
ORCID: https://orcid.org/0000-0002-8914-5964
Federal University of Goiás, Brazil
E-mail: desousacruzvanessa@gmail.com
Jorge Luís Ferreira
ORCID: https://orcid.org/0000-0001-7111-4847
Federal University of Tocantins, Brazil
E-mail: jlferreira@mail.uft.edu.br
Emmanuel Arnhold
ORCID: https://orcid.org/0000-0003-0922-146X
Federal University of Goiás, Brazil
E-mail: emmanuelarnhold@yahoo.com.br
Eugênio Gonçalves de Araújo
ORCID: https://orcid.org/0000-0002-0242-2827
Federal University of Goiás, Brazil
E-mail: earaujo65@live.com

Abstract

The Cerrado biome is a source of chemical molecules with great medicinal potential. The substances extracted from pequi, the fruit of *Caryocar brasiliense*, have antiparasitic, antioxidant, and antineoplastic properties. Canine osteosarcoma is a highly aggressive and metastatic bone tumor, not very responsive to current chemotherapy modalities. This study aimed to show the effects of the ethanol extract of pequi peel (EEPP) on canine osteosarcoma cells, in addition to suggesting a metabolic route that explains the action of the extract. D-17 cells were seeded and exposed to EEPP at concentrations of 0, 10, and 100 µg/mL. After treatment, the cell nuclei were marked with DAPI and quantified using fluorescence microscopy. The expression of proteins p53, Ki-67, Bcl-2, Akt, AMPK, and mTOR was analyzed using immunocytochemistry. By using DAPI, we found a reduction in the number of quantified nuclei, time, and dose-dependent. The labeling of p53, Ki-67, and Bcl-2, antibodies decreased in groups exposed to EEPP. The opposite was observed with Akt, AMPK, and mTOR antibodies, where the protein was not found in the control group but was expressed in groups exposed to EEPP. We suggest a possible metabolic route where EEPP promotes cell death and cell cycle control in D-17 cells.

Keywords: Apoptosis; Autophagy; Cycle block; Cellular metabolism; Cytoprotection.

Resumo

O bioma Cerrado é fonte de moléculas químicas com grande potencial medicinal. As substâncias extraídas do pequi, fruto do *Caryocar brasiliense*, têm propriedades antiparasitárias, antioxidantes e antineoplásicas. O osteossarcoma canino é um tumor ósseo altamente agressivo e metastático, pouco responsivo às modalidades atuais de quimioterapia. Este estudo teve como objetivo mostrar os efeitos do extrato etanólico da casca de pequi (EEPP) sobre as células do osteossarcoma canino, além de sugerir uma rota metabólica que explique a ação do extrato. As células D-17 foram semeadas e expostas a EEPP nas concentrações de 0, 10 e 100 µg / mL. Após o tratamento, os núcleos celulares foram marcados com DAPI e quantificados por microscopia de fluorescência. A expressão das proteínas p53, Ki-67, Bcl-2, Akt, AMPK e mTOR foi analisada por imunocitoquímica. Ao usar o DAPI, encontramos uma redução no número de núcleos quantificados, tempo e dose-dependente. A marcação de anticorpos p53, Ki-67 e Bcl-2 diminuiu nos grupos expostos a EEPP. O oposto foi observado com os anticorpos Akt, AMPK e mTOR, onde a proteína não foi encontrada no grupo controle, mas foi expressa nos grupos expostos à EEPP. Nós sugerimos uma possível rota metabólica onde EEPP promove a morte celular e o controle do ciclo celular em células D-17.

Palavras-chave: Apoptose; Autofagia; Ciclo de bloqueio; Metabolismo celular; Citoproteção.

Resumem

El bioma del Cerrado es una fuente de moléculas químicas con gran potencial medicinal. Las sustancias extraídas del pequi, fruto del *Caryocar brasiliense*, tienen propiedades antiparasitarias, antioxidantes y antineoplásicas. El osteosarcoma canino es un tumor óseo metastásico y altamente agresivo, que no responde mucho a las modalidades de quimioterapia actuales. Este estudio tuvo como objetivo mostrar los efectos del extracto etanólico de piel de pequi (EEPP) sobre las células del osteosarcoma canino, además de sugerir una ruta metabólica que explica la acción del extracto. Se sembraron células D-17 y se expusieron a EEPP a concentraciones de 0, 10 y 100 µg / ml. Después del tratamiento, los núcleos celulares se marcaron con DAPI y se cuantificaron mediante microscopía de fluorescencia. La expresión de las proteínas p53, Ki-67, Bcl-2, Akt, AMPK y mTOR se analizó mediante inmunocitoquímica. Al usar DAPI, encontramos una reducción en el número de núcleos

cuantificados, dependiente del tiempo y de la dosis. El marcaje de los anticuerpos p53, Ki-67 y Bcl-2 disminuyó en los grupos expuestos a EEPP. Lo contrario se observó con los anticuerpos Akt, AMPK y mTOR, donde la proteína no se encontró en el grupo control, sino que se expresó en los grupos expuestos a EEPP. Sugerimos una posible vía metabólica en la que EEPP promueve la muerte celular y el control del ciclo celular en las células D-17.

Palabra clave: Apoptosis; Autofagia; Ciclo de bloqueo; Metabolismo celular; Citoprotección.

1. Introduction

The Cerrado biome is a source of molecules with great medicinal potential. The molecules produced by the secondary metabolism of plants, in response to adverse environmental conditions, have always been suppliers or inspirers of medicinally active ingredients. Highlighting the pequi tree, a plant native to the Brazilian Cerrado, which has shown pharmaceutical and medicinal potential for various diseases such as injuries, gastritis, with antiparasitic and antineoplastic properties (Espindola, 2007; Miranda et al. 2008; Miranda et al. 2014).

Osteosarcoma is the most common type of bone tumor, occurring more frequently in animals of giant breeds, such as the Rottweiler, German Shepherd, Boxer, Doberman Pinscher, and Irish Setter, usually aged 6 to 10 years. Males and animals weighing more than 40 kg are more predisposed to this cancer (Szewczyk et al. 2015). However, its prognosis is usually unfavorable since it presents an insufficient response to the treatments currently used.

The p53 is an important modulator of cell death and survival, and its regulation (inhibition of mutant p53 and activation of inactive p53) can be a therapeutic approach to cancer cells. The regulatory capacity of gene transcription involved in apoptosis can result in the activation of the Bax gene whose transcription product, the Bax protein, will inhibit the anti-apoptotic action of the Bcl-2 protein (Bouaoun et al. 2016). Negative regulation, induced by p53 in Bcl-2, together with positive Bax regulation, promote the release of cytochrome c by the mitochondrion, while activation of CASPASE-3 causes cell death (Moya et al. 2016; Peixoto et al. 2017).

The mTOR, a signaling molecule extensively studied and related to therapy in the last 10 years, plays a critical role in oncogenesis. This 239 kDa serine/threonine kinase protein downstream at the PI3K/AKT pathway (Laplante & Sabatini, 2009; Ching & Hansel, 2010). The mTOR promotes cellular effect through two subunits, mTORC1 and mTORC2, and

relates to survival, cell cycle control, and autophagy (García & Alessi, 2008; Luo et al. 2018). Its main function is related to the growth and proliferation of cells, the regulation of autophagy by phosphorylation, and consequent inactivation of the ULK complex (Unc-51-like kinase), inhibiting the autophagy signaling cascade. By inhibiting mTORC1, it fails to express the negative control in the ULK complex, which in turn promotes autophagy (Jung et al. 2010).

Autophagy is the process by which cell components are degraded by a lysosomal enzyme pathway, providing cells with essential amino acids, nucleotides, and fatty acids. This process allows cells to survive by producing energy and macromolecules under unfavorable energy and nutritional conditions. On the other hand, it allows the elimination of dysfunctional organelles and proteins. However, if the autophagic process is prolonged and compromises the normal functioning of cells, it can culminate in the activation of cell death pathways by autophagy (Banerji & Gibson, 2012).

Ki-67 was first identified in nucleated Hodgkin cells (Lymphoma) (Gerdes et al. 1983). It is highly expressed in the cell cycle, but absent and/or reduced in the resting/G0 phase (Gerdes et al. 1984). This characteristic caused Ki-67 to become a clinically important proliferation marker of various types of cancers (Gerdes et al. 1987; Dowsett et al. 2011).

In this study, we aim to show the effects of the ethanol extract of pequi peel compared to the results obtained from the nuclei count by DAPI and immunocytochemistry, in addition to suggesting a possible metabolic route that explains the action of ethanol extract of pequi peel in D-17 cells.

2. Material and Methods

The experiment was developed at the Multi-User Laboratory for the Evaluation of Molecules, Cells, and Tissues, of the Veterinary and Animal School of the Federal University of Goiás, through quantitative analysis and methodological studies to evaluate the toxicity of the ethanol extract of pequi peel in canine osteosarcoma cells. The methodology used follows a universal standard, which allows the reproducibility of the experiments Pereira et al. (2018).

Extract production

The extract was processed using peels of the pequi fruit, collected in Nova América, Goiás, Brazil (15.032232º S and 49.942103º W at 730.5 m altitude) concerted and processed

by Souza (Souza et al. 2019), the exsiccate was deposited in the herbarium of the Federal University of Goiás, number UFG-43-833. The samples were collected in the wild in authorized orchards, the fruit was collected without any damage to the producing species and/or impacts on the regional fauna, according to the above and following the guidelines of the Conselho Nacional do Meio Ambiente (CONAMA) is currently published resolutions between September 1984 and January 2012, in its guideline, Section V - On Intervention or Eventual Suppression and Low Environmental Impact of Vegetation in APP. VIII - scientific research, as long as it does not interfere with the ecological conditions of the area, nor does it give rise to any type of direct economic exploitation, subject to other requirements provided for in the applicable legislation, through this, it was approved the collection and use of pequi peel.

Chemical analyzes performed by high-performance liquid chromatography coupled with high-resolution mass spectrometry of the extract of *Caryocar brasiliense* were performed by Ferreira, 2019 belonging to the same research group.

The extract was processed through fragmentation and drying of exocarp (peel) of *Caryocar brasiliense*, in a forced-air oven at 40 °C. The samples were milled in razor mills, with a 20 mm granulometry. After homogenizing and weighing, the sample macerated using a mechanical stirrer for 4 hours.

Ethanol was removed from the solution using a rotary evaporator with a water bath at 40 °C. The crude extract was diluted in DMSO (Dimethyl sulfoxide) in a ratio of 1g/10ml. The ethanol extract of pequi peel (hereafter called EEPP) solution was kept in an amber container at -20 ºC. Treatments were prepared by dilution of EEPP in Dulbecco's modified Eagle's medium (DMEM) at concentrations established in the experimental design.

Cell cultivation

Osteosarcoma cells (D-17, BCRJ 0276, Lot 000573, Passage 239, Species *Canis familiaris*), originating from ATCC (American Type Culture Collection - Manassas, VA, USA), were purchased from the Rio de Janeiro Cell Bank (BCRJ - Rio de Janeiro, Brazil). They were kept in a humidified incubator at 37 ºC and an atmosphere of 5% CO_2. Cultivation was performed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, plus 1% L-glutamine and 0.1% amphotericin B.

Experimental design

Canine osteosarcoma cells (OSC) were seeded in microscope slides modified for cell culture (Lab Tek II Chamber Slides), of four wells, at concentrations of $1x10^4$/well and exposed to EEPP extract treatments, according to the concentrations previously established of 0, 10, and 100 µg/mL. For the analysis of immunofluorescence and immunocytochemistry, we used six (06) independent fields with photo documentation of each treatment well.

Nuclei counting assay stained with DAPI

The cells seeded in the culture slides were pre-sensitized with the treatment based on the ethanolic extract of pequi peel. After the experimental phase, the cells were fixed with 4% paraformaldehyde for 30 minutes. Then, the slides were kept in a freezer at -80 ºC. For the assay, the slides were previously conditioned until they returned to room temperature. Soon after, 10 µl of DAPI (H1200 VECTASHIELD) was added for 30 minutes. The slides were evaluated in Leica DM4 B upright microscopes with fluorescence at EX: 325 - 375, EM: 435 – 485, and DC: 400.

For the nuclei counting method, the images were analyzed using the ImageJ software. The cell counter plugin was used to count the number of positive nuclei for DAPI marking. These data were used for experimental quantification and submitted to the aesthetic test.

Immunocytochemistry assay

All steps in this assay were performed by the automated immunohistochemistry/immunocytochemistry Bond-Max (Leica), following the protocol suggested by the manufacturer, with a standard dilution of 1:500 for all highlighter. The expression of p53, Bcl-2, mTOR, Ki-67, AMPK and Akt receptors was analyzed by performing immunocytochemistry using primary anti-P-p53 5G76 (monoclonal/mouse, SC717, Santa Cruz Biotechnology, USA); anti-Bcl-2 C21 (polyclonal/rabbit, SC783, Santa Cruz Biotechnology, USA); anti-Phospho-mTOR Ser2481 (polyclonal/rabbit, Ser473,); anti-Ki-67 (polyclonal/ rabbit, SC15402, Santa Cruz Biotechnology, USA); anti-Phospho-AMPKα (monoclonal/Rabbit, Thr172 - 40H9); Anti-phospho-Akt (polyclonal/Rabbit, pSer473, Sigma-Aldrich). The negative control was done by replacing the primary antibody with 3% BSA. The data obtained by photomicrography was evaluated on a qualitative basis.

The evaluation of the results of immunocytochemistry was performed using the Allred-score technique, as described by Fedchenko & Reifenrath, 2014. The semi-quantitative scoring system was carried out in stages, where the percentage of cells with positive marking was initially established and the SPPC (Sum of Percentage of Positive Cells) was established in categories from 0 to 5. Then, the intensity of the expression was made with the use of QS (Qualitative Score) in scores from 0 to 3. The product of the sum of these two categories SPPC and QS, by combined score, in a final value between 0 and 8, establishes the SSS (Semiquantitative Scoring System).

Statistical analysis

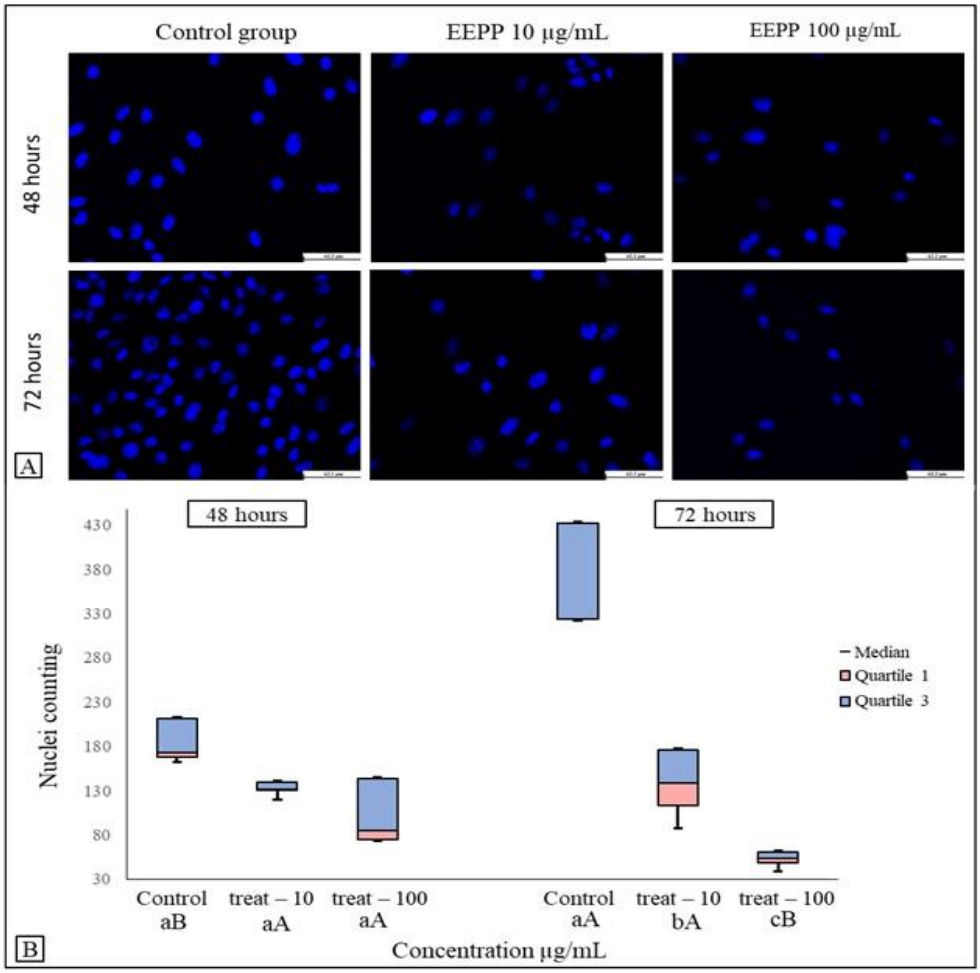
To normalize the data and remove possible outliers, we used a rigorous mathematical model, in which values higher and lower than these intervals were removed from the dataset. Thus, to obtain the threshold for the upper interval, we summed the standard deviation and mean of the dataset, and for the lower interval, we subtracted the mean with the standard deviation. This model was used independently for each experimental group.

We used the randomized block design (RBD), using an Analysis of Variance (ANOVA) with Tukey's posthoc test to compare the treatments, considering a 5% significance. To assess the immunocytochemistry of the SSS values, they were subjected to analysis of variance, and the treatment groups were compared, using the t-test at 5% probability. We performed the analysis using the package easyanova of R software (Arnhold, 2013; Team, 2013).

3. Results and Discussion

The nuclei counting in the groups analyzed presented the following results: in 48 hours of treatment (Control - 201.38; 10 µg/mL- 121.90; 100 µg/mL - 138) and in 72 hours of treatment (Control - 423.98; 10 µg/mL-141.18; 100 µg/mL - 29.16). In Figure 1, when comparing the number of nuclei, within the treated groups, to the concentration of the administered dose (10 and 100 µg/mL), we found no significant difference in the 10 µg/mL dose (48 hours - 121.90; 72 hours - 141.18) between treatments. However, we found a statistically significant difference between treatments for the 100 µg/mL dose (48 hours - 138; 72 hours - 29.16). Our results indicate a time-dependent reduction in nuclei counting as the extract concentration increases.

Figure 1. Result obtained in the technique of nucleus staining by DAPI with Boxplot-style graphical presentation.



A - DAPI marking for D-17 cell nuclei (40x magnification, with a 62.2 µm scale); B - Boxplot graphs of the quantification of the nucleus stained by DAPI, with control, treatment 10 µg / mL, treatment 100 µg / mL, treated with EECP with exposure time of 48 and 72 hours; Analysis of variance compared using the Tukey test at 5% significance; Lowercase - analyze within the same time, Uppercase - analyze between treatments.
source: Own authorship.

As suggested by Netten et. al. 1994, the fully automated enumeration of nuclei in stained cells is a technique of analytical precision. The system contains all components common to image processing and analysis: automated focus, acquisition, restoration, segmentation, measurement, and classification. Exclusive to this application, which accurately reports the spatial perspective, associated with the number of cells counted. Through this report, the extract may have had a precise quantified nuclei reduction effect, which shows the decrease in viable cells, depending on the dose and time.

To explain the effects shown by the nuclei counting method, immunocytochemistry shows the perspective of an effect associated with activation and expression of metabolic

pathways. The results demonstrated the presence of signaling proteins to block and develop the cell cycle (Table 1).

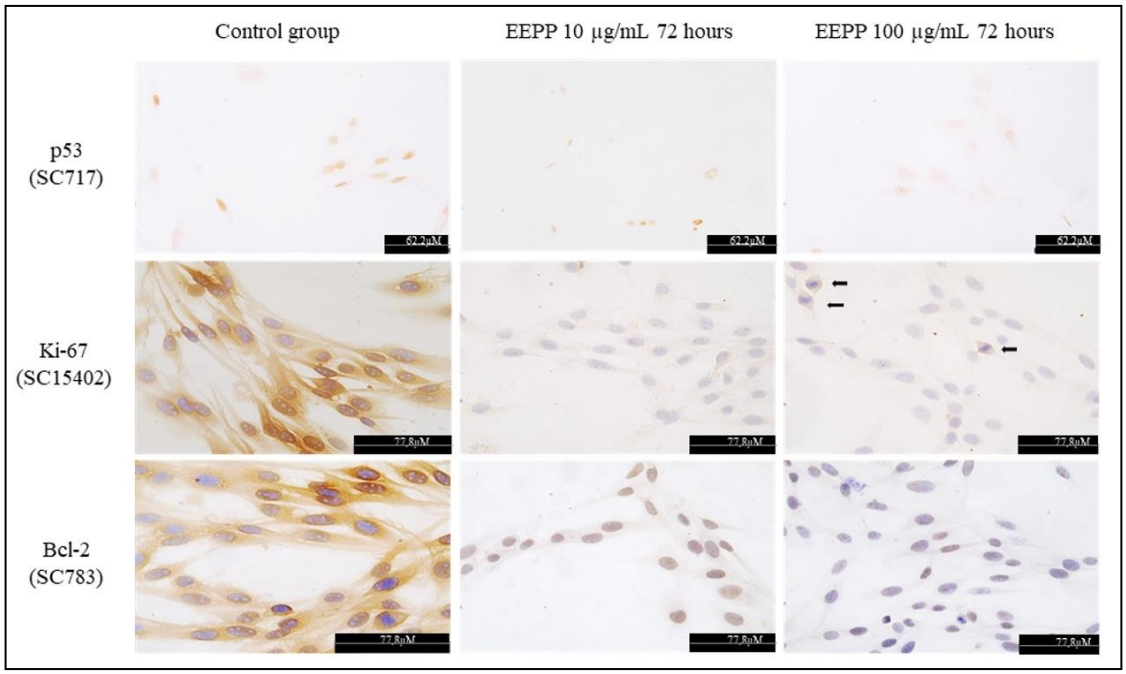
Table 1. Mean results from the combined semi-quantitative scoring system for the different antibodies tested on Osteosarcoma cells in vitro, in response to treatment with EEPP, at concentrations of 10 and 100 µg / mL, at 48 hours and 72 hours.

		Control 48 hours	10 µg/mL 48 hours	100µg/mL 48 hours	Control 72 hours	10µg/mL 72 hours	100µg/mL 72 hours
AKT	mSPPC	5	5	5	5	5	5
	mQS	1	2	2.75	1,2	2	3
	mSSS	6.00^{c}	7.00^{b}	7.75^{a}	6.20^{c}	7.00^{b}	8.00^{a}
AMP	mSPPC	1.5	5	5	1.5	5	5
	mQS	0	1	1	0	1	3
	mSSS	1.50^{c}	6.00^{b}	6.00^{b}	1.50^{c}	6.00^{b}	8.00^{a}
Bcl2	mSPPC	5	5	5	5	5	5
	mQS	2	1.75	1.75	2	1	1
	mSSS	7.00^{a}	6.75^{a}	6.75^{a}	7.00^{a}	6.00^{b}	6.00^{b}
Ki67	mSPPC	5	5	5	5	5	5
	mQS	1	1	2	3	1	1
	mSSS	6.00^{c}	6.00^{c}	7.00^{b}	8.00^{a}	6.00^{c}	6.00^{c}
Mtor	mSPPC	0	3.33	5	0	5	5
	mQS	0	1	2.75	0	2	3
	mSSS	0.00^{d}	4.33^{c}	7.75^{a}	0.00^{d}	7.00^{b}	8.00^{a}
p53	mSPPC	5	5	5	5	5	5
	mQS	3	2	2.67	3	1	1.33
	mSSS	8.00^{a}	7.00^{ab}	7.67^{a}	8.00^{a}	6.00^{b}	6.33^{b}

mSPPC = mean of the sum of the percentage of positive cells; mQS = mean of qualitative scores; mSSS = mean of the semi-quantitative scoring system. The letters a, b, c and d in superscript, refer to the statistical result of the t test such that mean values within a row that share the same letter are not statistically different at a level of significance of $P<0.05$.
source: Own authorship.

The p53 protein was more expressed in the control group. However, in the groups treated with EEPP, the protein labeling was subtle according to the concentration and exposure time (Figure 2). Where SSS decrease was observed in the groups treated with 10 µg / mL, in the two exposure times. There was an increase in SSS in the groups treated with 100 µg / mL, but it remains even lower statistically than the control group.

Figure 2. Photomicrographs of slides containing canine osteosarcoma cells, line D-17, after the immunocytochemistry test by the automated Bond-Max processor (Leica).



DAB, hematoxylin in contrast and staining of antibodies p53, Ki-67 and Bcl-2. Note that the control group shows marking by p53, Ki-67 (arrows show cells that are not in G0), Bcl-2 marking with these decreased in the groups exposed to the ethanolic extract of the peel. Source: Own authorship.

We observed a reduction in the mutant p53 expression after treatment with the ethanolic extract of pequi peel, which may imply the resumption of cell cycle arrest and induction of apoptosis by alternative routes. One possibility would be the activation and promotion of the BIM protein, which acts independently of p53.

The anti-apoptotic protein Bcl-2 was also highly expressed in the control group, where the SSS of the Bcl-2 protein decreased in the groups that received treatment with the extract, about the control group, which also demonstrates the degree of tumor malignancy of that cell. In the treated groups, we found a reduction in the production and activity of this protein, however, in the two exposure times, there was no statistical difference between the dosages of 10 µg / mL and 100 µg / mL.

The p53 is a crucial protein for cell cycle control because, as a guardian of the genome, it is responsible for detecting changes in DNA and interrupting the cell cycle at the first checkpoint. In addition to triggering processes of repair and death of damaged cells, by commanding several other proteins. The mutation of this protein interrupts the action of the checkpoint, provides the survival and multiplication of neoplastic cells, worse prognosis, and resistance to the action of chemotherapeutic agents. The mutant p53 was expressed in the

control groups of this experiment, which demonstrates the potential for malignancy of D-17 cells. According to the literature (Happo et al. 2010 and Santidrián et al. 2010), independent and intermediate BIM protein routes have been demonstrated, efficient in triggering the apoptosis process in cancer cells. After using chemotherapy, Santidrián et al. 2010 demonstrated that acadesine (AICAR) induces apoptosis by a mechanism independent of p53 and AMPK, through the positive regulation of BIM and NOXA in chronic lymphocytic leukemia cells.

Proteins of the Bcl-2 family are probably the most obvious therapeutic target to modulate apoptosis, as they are effector proteins in this cellular process. It has been shown that certain substances are capable of inducing apoptosis and this activity has been related to the activation of CASPASE 3 (Chakrabarty et al. 2002). CASPASE 3 has a broad spectrum of activity, being modulated by proteins Bcl-2 and Bcl-xL (Leung et al. 2005).

Likewise, we found intense Ki67 labeling in the control group cells and a reduction in its expression in the groups treated with the pequi extract. In the statistical analysis of the Ki67 protein, it was possible to observe a decrease in SSS in the groups that received treatment with the extract, within 72 hours, the same did not occur within 48 hours. According to the results, Ki-67 reduced expression due to the extract effect as the concentration increased.

Ki-67 is a protein that acts in the cell cycle regulation, promoting cell division. The Ki-67 protein is present in all phases of the cell cycle, except during the resting or G0 phase. D-17 cells have high mitotic power and are in a constant division, a fact that was evidenced by intense Ki-67 labeling in the control group. However, after treatment with EEPP, the expression of Ki-67 was reduced, which is possibly related to the cell cycle arrest, keeping the cell in a resting/G0 phase. These data, together with the results observed in the mutant p53 expression, suggest that the ethanolic extract of pequi peel acts during this phase of the cell cycle, thus explaining the reduction in this protein's expression and, consequently, reducing cell division.

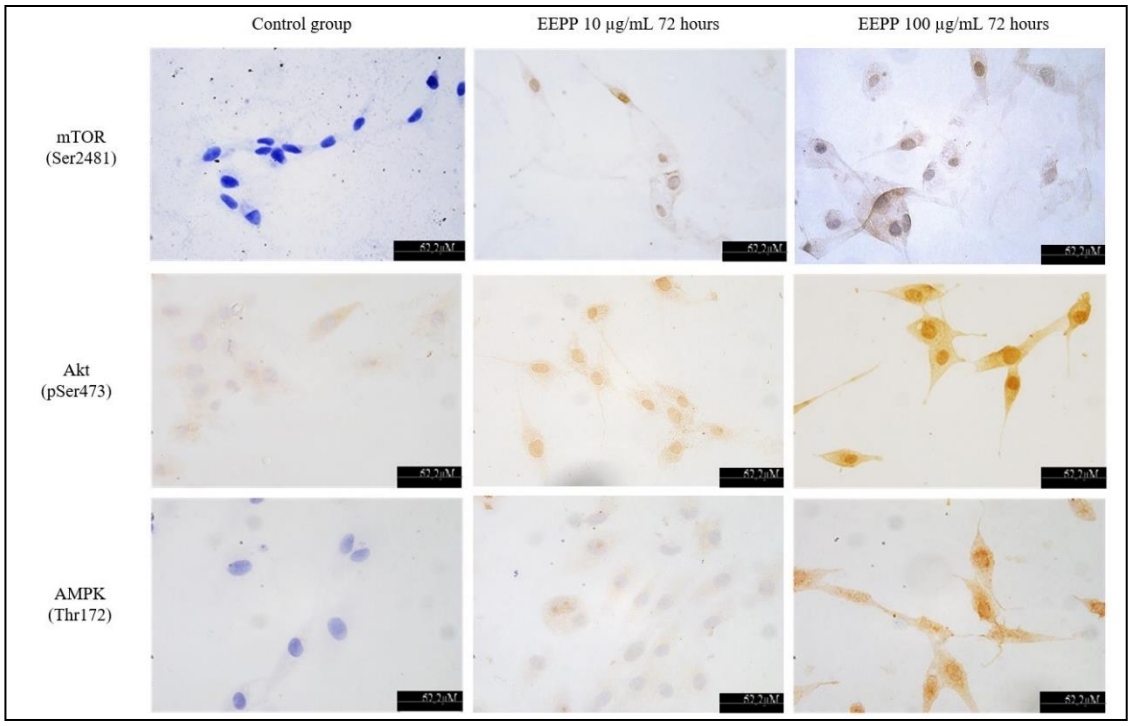
The marking intensity for proteins Bcl2, Ki67, and p53 decreased in a dose-dependent manner, showing lowest intensity at 72 hours. However, an increase in staining was evident for the Ki67 and p53 markers in the 100 µg / mL group at 48 hours. This fact was also observed in the quantification of cells by DAPI, which showed a non-significant increase in the number of cells for the group treated with 100 µg / mL. These two specific markers are directly related to the arrest of the cell cycle and the induction of cell division (Bouaoun et al. 2016; Dowsett et al. 2011), pointing to more effective anti-proliferative control of

Osteosarcoma cells at the 72-hour time.

Sun & Kaufman, 2018 e Sobecki et al. 2017 demonstrated that the reduction in cell counting with Ki-67 labeling is possibly related to the initial stage of the cell cycle since low levels of Ki-67 can lead the cell to return to control of the cell cycle. These results corroborate the findings of the present study, which found a reduction in the number of nuclei, in the intensity of p53 and Ki-67 labeling, after treatment with EEPP.

On the other hand, unlike that observed with the other markers in this study, the mTORC2 protein was not found in the control group (Figure 3), which indicates that D-17 cells are mTORC1-dependent.

Figure 3. Photomicrographs of slides containing canine osteosarcoma cells, line D-17, after the immunocytochemistry test by the automated Bond-Max processor (Leica).



DAB, hematoxylin in contrast and mTOR, Akt and AMPK antibodies staining. Intense staining was observed for the group treated with EECP where the antibodies mTOR, Akt and AMPK were well expressed. In the control group, the label for the same antibodies was absent for the mTOR and AMPK antibody, and a discrete label for the Akt antibody. Source: Own authorship.

Thus, it is possible that the EEPP promoted the activation of mTORC2, Akt, and AMPK in the treated group. The measurement data demonstrates that SSS was higher than 3 in AKT and mTOR protein, showed a dose-dependent increase in both exposure times to the extract, with a significant difference. There was difference in the SSS of the AMP protein between the groups that received treatment and the control group, within 48 hours of

exposure, but this difference was dose-dependent over 72 hours.

Furthermore, the results suggest a complex mechanism that involves a set of pathways (Figure 4), where EEPP possibly promotes cell death and control of the cell cycle by apoptosis and autophagy, between G0 and G1 phases, in D-17 cells.

Figure 4. Proposed model for the action of the ethanol extract of the pequi peel.

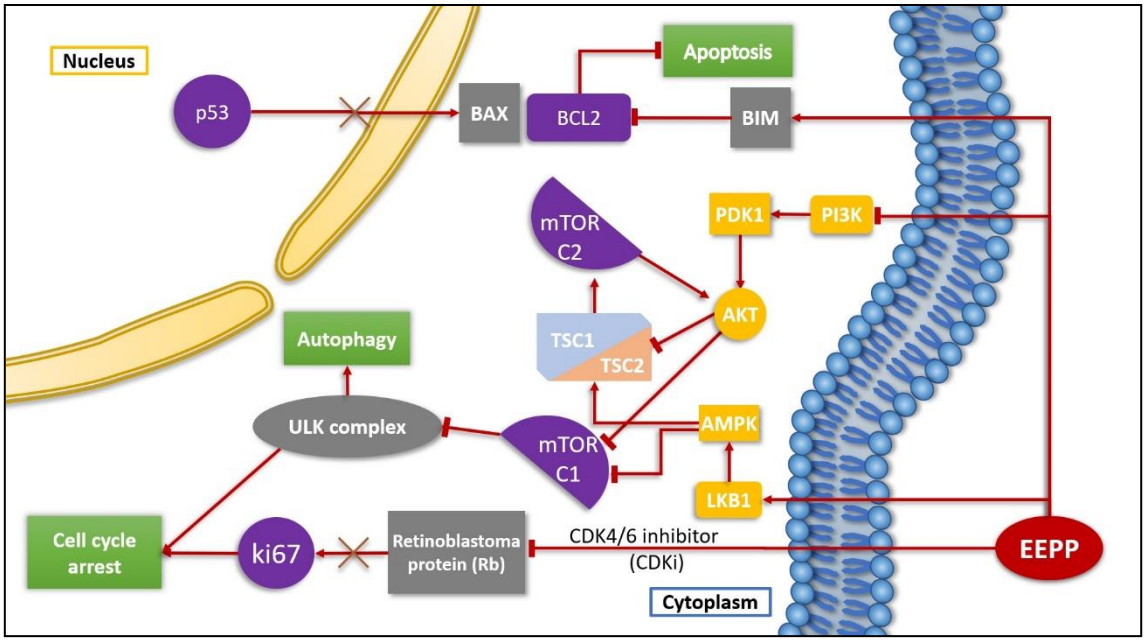


Diagram of the associations of the ethanolic extract of the pequi bark, proposed model and their respective involvement in the metabolic pathways of canine osteosarcoma cells, line D-17; after key expression, taken by immunocytochemistry. Source: Own authorship.

We suggest an alternative activation route with the promotion of cell control, the PI3K/AKT, and (tuberous sclerosis complex) TSC1/TSC2 pathway, which has mTORC2 phosphorylation as a key protein. These data are supported by other studies, such as Zhao et al. (2018) in which TSSC3 was shown for the first time to induce autophagy by inhibiting the Src-dependent PI3K/Akt/mTOR pathway in human osteosarcoma cells (33 patients). Moreover, the same authors suggested a TSSC3-induced autophagy process, contributing to suppress tumorigenesis and metastases in the referred cells, both in vitro and in vivo. Possibly, based on the results found in this research, this mechanism has occurred in canine osteosarcoma cells treated with EEPP, when we found expressive labeling for Akt, as seen in Figure 3.

According to Kan et al. (2016), the autophagy mechanism promoted by mTORC1, via (serine/threonine kinase) LKB1/AMPK, where it promotes the activation of the TSC1/TSC2 and consequently the activation and expression of mTORC2, is well understood. Thus, the

imbalance between the mTOR proteins (mTORC1 and mTORC2), promoted by the action of EEPP, may have contributed to the autophagy occurring in D-17 cells through the control of neoplastic cells. Studies are reporting that the LKB1 pathway can induce cell apoptosis through activation of the JNK pathway. Gan et al. (2014) found that LKB1 is critical for TRAIL-induced cell apoptosis and death-associated protein-3 (DAP3) by apoptosis in osteosarcoma cells (HEK293T and 143B). These studies suggest similar mechanisms of action for the extract studied, where it can express the AMPK phosphorylation in the treated group, closing the LKB1/AMPK and TSC1/TSC2 activation pathways.

The ethanolic extract of pequi peel (EEPP) showed efficiency in the control of canine osteosarcoma cells by inducing the cell cycle arrest and promoting cell death in-vitro study, possibly due to autophagy and/or apoptosis. These facts can be suggested due to the expression of key proteins analyzed in this study: p53, Bcl-2, mTOR, Ki-67, Akt, and AMPK. The extract action can be promoted by the joint and synergistic action of the multiple active principles contained in it. Furthermore, these results were compared with data from other cancerous cell lines of mesenchymal origin, relating the effects of the ethanol extract from the peel of the pequi to other cells of the same embrionic origin as Osteosarcoma.

4. Conclusion

The ethanolic extract of pequi peel showed a reduction in the number of nuclei quantified by the DAPI technique and in the expression of p53, Ki-67, and Bcl-2, in addition to an increase in the expression of mTOR, Akt, and AMPK proteins. Thus, we suggest a metabolic route where the EEPP promotes cell death and control of the cell cycle, possibly by apoptosis and autophagy, between phases G0 and G1, in D-17 cells.

For future perspectives, this research will analyze the toxic effect in non-cancerous cells of mesenchymal origin and the effects of the extract in association with other chemotherapeutic agents, aiming to improve the adverse effects and enhance the treatment of neoplasms.

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Percentage of contribution of each author in the manuscript

Leandro Lopes Nepomuceno – 15%

Nayane Peixoto Soares – 7%

Juliana Carvalho de Almeida Borges – 7%

Vanessa de Souza Vieira – 7%

Dayane Kelly Sabec Pereira – 7%

Kleber Fernando Pereira – 7%

Vanessa de Sousa Cruz – 10%

Emmanuel Arnhold – 10%

Jorge Luís Ferreira – 10%

Eugênio Gonçalves de Araújo – 20%