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Cytotoxicity and residual effect of ethanol extract of pequi peel on canine osteosarcoma cells

Citotoxicidade e efeito residual do extrato etanólico da casca do pequi sobre células de osteossarcoma canino

Citotoxicidad y efecto residual del extracto etanólico de la piel de pequi en las células del osteosarcoma canino

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Abstract

The development of new chemotherapy drugs is a premise for the scientific community in recent years, mainly through the use of plants and other natural sources. In this context, analyses of cytotoxicity and residual effect have become essential for the establishment of new therapies. Therefore, this study aims to evaluate the cytotoxic and residual effects of ethanol extract of pequi peel (EEPP) on canine osteosarcoma cells. For this, we used analyses of cell viability, cytotoxicity, IC_{50}, and cell survival after therapy in treatments of 24, 48, and 72 hours. We used the design of the randomized block, using an Analysis of Variance with Tukey's posthoc test to compare the treatments, considering a 5% significance. The extract showed no cytotoxic effect within 24 hours of treatment, but a protective effect, or possibly a mitotic-inducing effect on osteosarcoma cells. However, at 48 and 72 hours, we found gradual cytotoxicity favoring the effective control of neoplastic cells, obtaining, on average, 55% of cytotoxicities at concentrations of 10 to 100 µg/mL. IC_{50} values were 4.05 µg/mL for the 48-hour group and 4.53 µg/mL for the 72-hour group. The treatment residual effect reduced cell growth after the exposure to the extract. The survival factor was lower (18.81%) in the 72-hour group, with a concentration of 10 µg/mL and higher (81.33%) in the 48-hour group, with a concentration of 100 µg/mL. These results suggest that the ethanol extract of pequi peel

promotes the increase in cell viability within 24 hours, cytotoxic action in exposures over 48 hours, and time-dependent residual effect on canine osteosarcoma cells.

Keywords: Dog; Chemotherapeutic; Survival; Tumor; Viability.

Resumo

O desenvolvimento de novos quimioterápicos é premissa da comunidade científica nos últimos anos, principalmente por meio do uso de plantas e outras fontes naturais. Nesse contexto, análises de citotoxicidade e efeito residual têm se tornado essenciais para o estabelecimento de novas terapias. Portanto, este estudo tem como objetivo avaliar os efeitos citotóxicos e residuais do extrato etanólico da casca do pequi (EEPP) sobre as células do osteossarcoma canino. Para isso, foram utilizadas análises de viabilidade celular, citotoxicidade, IC_{50} e sobrevida celular após terapia nos tratamentos de 24, 48 e 72 horas. Utilizou-se o delineamento de blocos ao acaso, utilizando-se a Análise de Variância com teste posthoc de Tukey para comparação dos tratamentos, considerando significância de 5%. O extrato não mostrou nenhum efeito citotóxico em 24 horas de tratamento, mas um efeito protetor, ou possivelmente um efeito indutor de mitose nas células de osteossarcoma. Porém, em 48 e 72 horas, encontramos citotoxicidade gradativa favorecendo o controle efetivo das células neoplásicas, obtendo-se, em média, 55% de citotoxicidades nas concentrações de 10 a 100 µg / mL. Os valores de IC_{50} foram 4,05 µg / mL para o grupo de 48 horas e 4,53 µg / mL para o grupo de 72 horas. O efeito residual do tratamento reduziu o crescimento celular após a exposição ao extrato. O fator de sobrevivência foi menor (18,81%) no grupo de 72 horas, com concentração de 10 µg / mL e maior (81,33%) no grupo de 48 horas, com concentração de 100 µg / mL. Esses resultados sugerem que o extrato etanólico da casca do pequi promove aumento da viabilidade celular em 24 horas, ação citotóxica em exposições acima de 48 horas e efeito residual dependente do tempo nas células do osteossarcoma canino.

Palavras-chave: Cão; Quimioterápico; Sobrevivência; Tumor; Viabilidade.

Resumen

El desarrollo de nuevos fármacos quimioterápicos es una premisa para la comunidad científica en los últimos años, principalmente mediante el uso de plantas y otras fuentes naturales. En este contexto, los análisis de citotoxicidad y efecto residual se han convertido en fundamentales para el establecimiento de nuevas terapias. Por lo tanto, este estudio tiene como objetivo evaluar los efectos citotóxicos y residuales del extracto etanólico de piel de pequi (EEPP) en células de osteosarcoma canino. Para ello, utilizamos análisis de viabilidad

celular, citotoxicidad, IC_{50} y supervivencia celular tras la terapia en tratamientos de 24, 48 y 72 horas. Utilizamos el diseño del bloque al azar, utilizando un Análisis de Varianza con la prueba posthoc de Tukey para comparar los tratamientos, considerando una significancia del 5%. El extracto no mostró ningún efecto citotóxico en las 24 horas posteriores al tratamiento, pero sí un efecto protector, o posiblemente un efecto inductor de la mitosis en las células del osteosarcoma. Sin embargo, a las 48 y 72 horas, encontramos una citotoxicidad gradual que favorece el control efectivo de las células neoplásicas, obteniendo, en promedio, un 55% de citotoxicidades a concentraciones de 10 a 100 µg / mL. Los valores de CI50 fueron 4.05 µg / mL para el grupo de 48 horas y 4.53 µg / mL para el grupo de 72 horas. El efecto residual del tratamiento redujo el crecimiento celular después de la exposición al extracto. El factor de supervivencia fue menor (18,81%) en el grupo de 72 horas, con una concentración de 10 µg / mL y mayor (81,33%) en el grupo de 48 horas, con una concentración de 100 µg / mL. Estos resultados sugieren que el extracto etanólico de la piel de pequi promueve el aumento de la viabilidad celular en 24 horas, la acción citotóxica en exposiciones superiores a 48 horas y el efecto residual dependiente del tiempo sobre las células del osteosarcoma canino.

Palabras clave: Perro; Quimioterápico; Supervivência; Tumor; Viabilidad.

1. Introduction

Brazil has a flora rich in biodiversity, and particularly the Cerrado biome contains medicinal tropical plants with potential for the development of chemotherapeutic agents. The knowledge about these plants and their potential is still poor and mostly traditional. The scientific community has intensified the discovery of new chemotherapeutic agents in recent years through the use of plants and other natural sources (Ozi et al. 2011).

Caryocar brasiliense has a therapeutic potential already observed by traditional medicine, being widely used for its anti-inflammatory and healing power in the treatment of respiratory diseases, gastric ulcers, muscle, and rheumatic pain. The *Caryocar brasiliense* oil, rich in unsaturated fatty acids, has been used in the food and cosmetic industry, and the plant's stem bark and leaves have antiparasitic effects (de Oliveira et al. 2015).

The pequi peel is rich in phenols that act as antioxidants that inhibit and reduce the damage caused by the deleterious activities of free radicals or non-radical reactive species. Such situations can be exerted by different mechanisms of action, preventing the formation of free radicals, favoring the repair and reconstitution of damaged biological structures (Roesler et al. 2008).

Currently, therapies for osteosarcoma in humans and animals consist of the use of cisplatin, ifosfamide, doxorubicin, and methotrexate. However, these drugs for control and treatment of this disease may promote severe adverse effects (cardiotoxicity, nephrotoxicity, ototoxicity, and hepatotoxicity). The ideal drug is the agent that in small doses promotes therapeutic effect and few side effects. Plant extracts can promote efficient pharmacological action and, in some cases, prevent or avoid the adverse effects caused by chemotherapy (Gabriel et al. 2017; Suárez et al. 2017).

Osteosarcoma is a malignant bone tumor of mesenchymal origin, highly aggressive, with high mortality. Moreover, it has high metastatic potential, especially in the lungs. Diagnosis is based on clinical history, physical exams, and complementary laboratory tests such as radiography and histopathology (Evola et al. 2017; Xie et al. 2017; Brown et al. 2018).

The dog is the most suitable experimental model for osteosarcoma control since the neoplasm has biology, complications, and clinical results similar to human osteosarcoma. In addition to the histological characteristics of the primary tumor and the metastasis pattern, there is an analogy in the responsiveness of drugs such as cisplatin and anthracyclines (Cruz et al. 2018).

Therefore, this study aims to evaluate the in vitro cytotoxic and residual effects of ethanol extract of pequi peel on canine osteosarcoma cells. For this purpose, cell viability, cytotoxicity, IC_{50}, and cell survival factor after therapy were calculated using the exposure times of 24, 48, and 72 hours.

2. 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 extraction

We used pequi fruit peels collected in the municipality of Nova América, Goiás, Brazil (15.032232º S, and 49.942103º W at 730.5 m altitude) processed by Souza et al, 2019

the exsiccatae was deposited in the herbarium of the Universidade Federal de Goiás under the 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. Being approved for the collection and use of pequi peel in the current research.

Chemical analyses performed using high-performance liquid chromatography (HPLC) coupled with high-resolution mass spectrometry (HRMS) of *Caryocar brasiliense* extract were performed by Ferreira, 2019 from the same research group.

The extract was obtained through the fragmentation and drying of exocarp (peel) of Caryocar brasiliense, in a forced-air oven at 40 °C. The samples were milled in razor mills to a size of 20 mm. Then, the samples were weighed and macerated using a mechanical stirrer for 4 hours.

Ethanol was evaporated off on an Ika-Werke 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 culture

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 5% CO_2 atmosphere. 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 (CSC) were seeded in 96 wells plates, at concentrations of $1x10^4$/well and exposed to EEPP extract treatments, according to the concentrations

previously established of 0, 1, 10, 100 and 1000 µg/mL. We used three (03) independent experiments (repetitions) in quintuplicate to verify the treatment effect regarding the dose and exposure time.

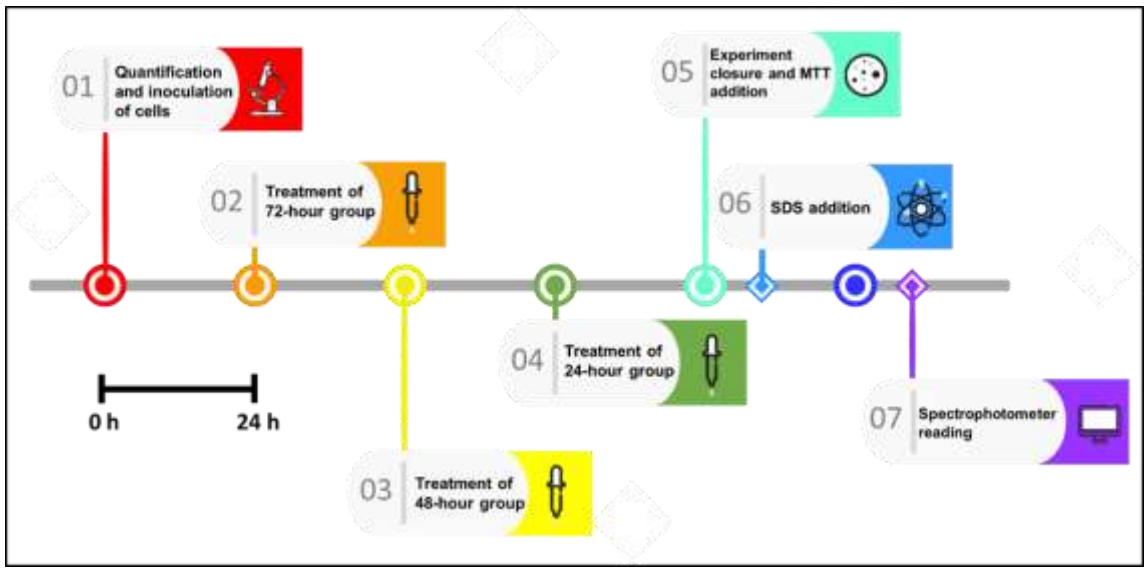
Cell viability and IC_{50} assay

We used 96 well plates with cells at concentrations of $1x10^4$ cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% of bovine fetal serum to develop the tetrazolium salt reduction assay (MTT) by the pyruvate dehydrogenase complex present in the mitochondrial matrix. The treatments were randomly distributed, following the homoscedasticity criteria.

At the end of each treatment, we added 10 µl of tetrazolium (MTT - 3- (4,5-Dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide - Sigma-Aldrich) to each well. After incubation for three hours, we added 50 µl of 10% sodium dodecyl sulfate (SDS - Vivantis Biochemical) diluted in 0.01N HCL per well. Cells were incubated for 24 hours at room temperature in a light protected incubation chamber.

The optical density of each plate was quantified in a spectrophotometer with a 532nm wavelength. To better elucidate the MTT assay, a simplified scheme of the main processing steps is shown in Figure 1.

Figure 1 - Timeline chart of protocol main steps suggested for the MTT colorimetric assay in 24-hours intervals.



Source: Author's compilation

To calculate the cell viability (CV) of CSC culture, data obtained through spectrophotometry were converted into formulas (equation 1), processed, and evaluated statistically. The values obtained for cytotoxicity (CC) originated from the percentage difference of the viability equation (equation 2). Finally, the absorbance values were used in the GraphPad Prism program to obtain the IC_{50} for the ethanol extract of pequi peel.

Cell survival assay

We tested a new independent experiment with three repetitions in quintuplicate to obtain the cell survival factor. We inoculated 5x102 canine osteosarcoma cells into 24-well plates, submitted to EEPP treatment at concentrations of 10 µg/mL and 100 µg/mL. The treatment time was kept only for the periods of 48 hours and 72 hours. To remove the treatment in the cell survival technique, we continuously washed the plates with PBS and added a DMEM culture medium with 1% fetal bovine serum. The plate maintenance lasted for 10 days with media changes and recurrent washing, according to the standard cell culture.

At the end of the tenth day of each experimental time, we discarded the medium in each well and then performed the cells suspension with 200µl of trypsin. Thereafter, we centrifuged them at 140 rcf (g) for 10 minutes, discarding the supernatant afterward. The cells were then resuspended in 1000 µl of fresh medium (DMEM) and quantified in a Neubauer chamber. We analyzed the result means using the survival factor formula (equation 3).

$$\%CV = (\text{mean TA}/\text{mean CG})*100 \quad \text{(equation 1)}$$

$$\%CC = 100- [(\text{mean TA}/\text{mean CG})*100] \quad \text{(equation 2)}$$

$$\%\ SF = (\text{number of CCAT}/\text{number of CCCG}) \times 100 \quad \text{(equation 3)}$$

* SF = surviving fraction; CCAT = cells counted after treatment; and CCCG = cells counted in control group; CV = cell viability; TA = tested absorbance; CG = control group; and CC = cell cytotoxicity.

Statistical analysis

To normalize the data and remove possible outliers, we used a rigorous mathematical criterion, in which values higher and lower than these intervals were removed from the quintuplicate dataset. Thus, to obtain the threshold for the upper interval, we summed the

standard deviation and mean, and for the lower interval, we subtracted the mean with the standard deviation, this criterion was used independently for each experimental group.

We used the design of the randomized block (RBD), using an Analysis of Variance (ANOVA) with Tukey's posthoc test to compare the treatments, considering a 5% significance. We performed the analysis using the package easyanova of R software (Arnhold, 2013; Team, 2013).

To calculate the IC_{50}, the results of optical density from the spectrophotometer were analyzed using the GraphPad Prism statistical program (GraphPad Software, San Diego, CA, USA). A simple linear regression model was adopted to relate dependent variables and independent variables in the 24, 48- and 72-hour group, which demonstrate the mathematical model with R2, which explains an association between these two variables.

3. Results and Discussion

The cell viability and cytotoxicity data from the effect of ethanol extract of pequi peel on canine osteosarcoma cells were found through the MTT assay. Initially, we did not find a cytotoxic effect of EEPP in the 24-hour treatment, in which there was a growth of 6.7% of cells in comparison to the control group (Table 1).

Table 1 - Cell viability and cytotoxicity values obtained through absorbance from spectrophotometry in 532 nm wavelength for samples with EEPP treatment after the exposition of 24, 48, and 72 hours.

TIME	TREATMENT	VC (%)	CC (%)	Tukey
24h	CON	100,00	0,00	a
	1 µg/mL	106,70	0,00	a
	10 µg/mL	100,91	0,00	a
	100 µg/mL	105,43	0,00	a
	1000 µg/mL	26,27	73,73	b
48h	CON	100,00	0,00	a
	1 µg/mL	99,05	0,95	a
	10 µg/mL	72,43	27,57	b
	100 µg/mL	56,37	43,63	c
	1000 µg/mL	16,68	83,32	d
72h	CON µg/mL	100,00	0,00	a
	1 µg/mL	72,12	27,88	b
	10 µg/mL	63,16	36,84	c
	100 µg/mL	49,70	50,30	c
	1000 µg/mL	10,97	89,03	d

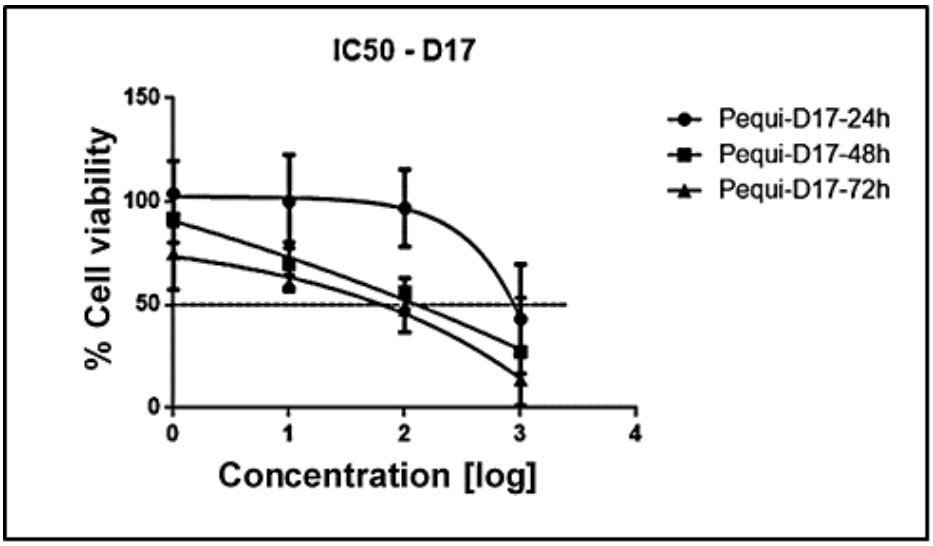
* VC = cell viability; CC = cell cytotoxicity; and CON = control.
Source: Author's compilation

The increase in cell viability at concentrations below 100 µg/mL in the 24-hour exposure time suggests that the EEPP might have had a cell protection effect. This can be explained by the antioxidant action of substances found in the pequi peel, such as the phenolic compounds discussed by Ferreira, 2019. Another possibility would be the induction of mitosis, which would not be interesting in terms of prognosis and survival time for patients with this type of neoplasia. In this case, other tests would be necessary to prove this effect and its possible consequences.

We evidenced a cytotoxic effect in the 48-hour and 72-hour treatments, promoting an effective control of neoplastic cells. We obtained 60 to 50% of cytotoxicity at concentrations of 10 to 100 µg/mL, being the optimum concentrations in the present study.

We found a progressive increase in the IC_{50} with the exposure time to the extract. However, the best interval for the cytotoxicity effects was observed in the 48-hour group (Figure 2).

Figure 2 - IC_{50} values obtained for the 24-, 48- and 72-hour groups respectively, using the GraphPad Prism statistical program.



Source: Author's compilation.

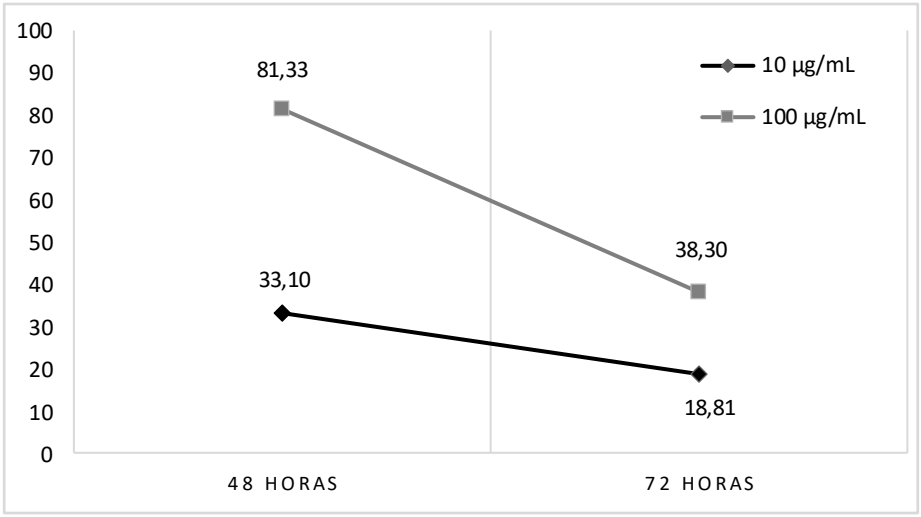
In the present study, the IC_{50} values obtained were 4.05 µg/mL for the 48-hour group and 4.53 µg/mL for the 72-hour group. Thus, we evidenced cytotoxicity for the D-17 cell line from EEPP treatments for the 48- and 72-hour groups at concentrations of 10 µg/mL and 100 µg/mL.

On the other hand, it is evident that, at higher concentrations, the EEPP has a cytotoxic effect, reducing the neoplasia. Comparative studies provided by Silva, 2018 when evaluating

the in vitro cytotoxicity effect of the ethanol extract of gabirobeira leaves (crude) on osteosarcoma cells, showed IC_{50} values greater than 9.49 µg/mL and 35.73 µg/mL in groups treated for 48 hours and 72 hours, respectively. Thus, the data suggest that EEPP can promote cytotoxicity in 50% of treated cells, at lower concentrations of 4.05 µg/mL in 48 hours and 4.53 µg/mL in 72 hours, which demonstrates a chemotherapeutic potential. Moreover, the results suggest the need for new trials, establishing comparative models with substances already used for the treatment of osteosarcoma, to propose potential alternative therapies, as demonstrated by Stokol et al. 2020. In that study, they evaluated the cytotoxicity of aldoxorubicin and doxorubicin in canine osteosarcoma cells (D-17), with IC_{50} of 16.2 µM and 26.2 µM, respectively, suggesting that the compounds would act in neoplastic and non-neoplastic cells. In addition to knowing the adverse effects of using those drugs, it is possible that the EEPP, due to its lower IC_{50}, promotes fewer consequences for patients.

As a complement to validate the data, we performed a cell survival assay to evaluate the residual effect in the 48- and 72-hour groups. The results show that the cell growth after the treatment was lower (18.81%) in the 72-hour group at a concentration of 10 µg/mL and greater (81.33%) in the 48-hour group at a concentration of 100 µg/mL (Figure 3).

Figure 3 - Surviving fraction of each group regarding concentration and exposure time to EEPP in the 48- and 72-hour groups.



Source: Author's compilation.

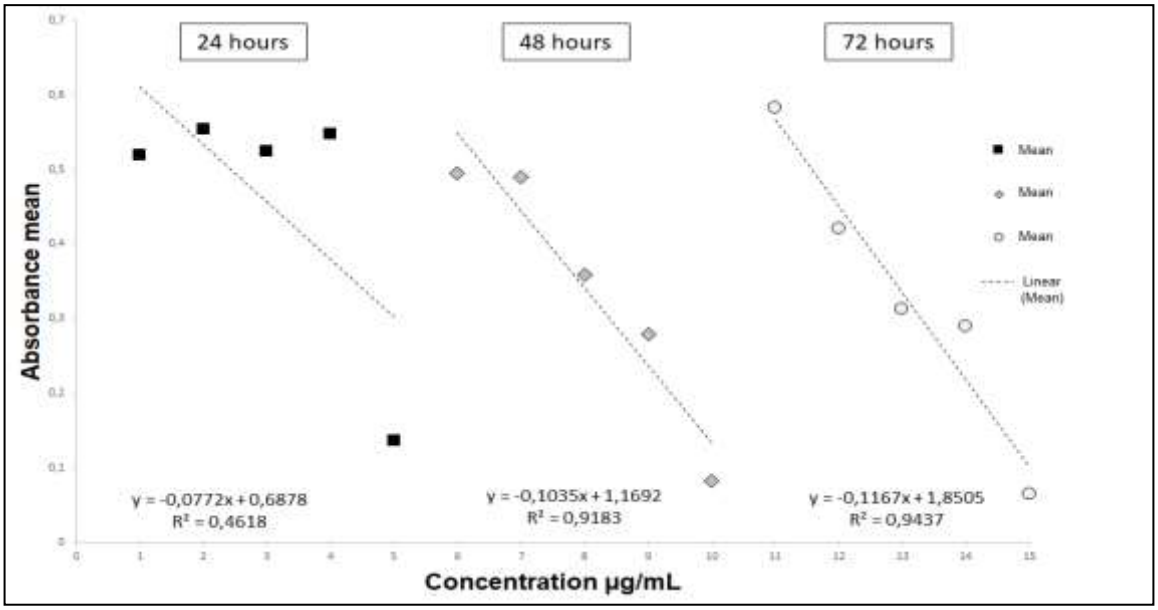
In addition to the reported cytotoxic effect, the residual effect of EEPP can be evaluated using the cell survival assay through the extract action after treatment, inhibiting the multiplication of cancer cells. The results suggested that EEPP has a residual effect on

osteosarcoma cells, thus favoring its use as a possible treatment of neoplasms, as reported by Miranda-Vilela et al. 2014. We also emphasize that, when subjected to the 48-hour treatment, it reduced the cell proliferation due to the residual cytotoxic effect of the extract, concomitant with a desired therapeutic effect at small concentrations (10 µg/mL), which may suggest lower adverse effect. Another advantage of this residual effect is the possible action to potentiate the effects of 48- and 72-hour groups, in addition to minimizing the proliferative action of the 24-hour group (Plumb, 2004).

Cruz et al. 2018 found lower values than those in our results when analyzing the action of Beta Lapachona on canine osteosarcoma cells. They found lower cell growth after treatment (0.50%) in the 72-hour group with a concentration of 1.0 µM and higher (13.93%) in the 24-hour group with a concentration of 0.3 µM. These values show little significant cell growth resumption, which are results similar to the findings of the present study.

We proposed a simple linear regression from the MTT assay data using the treatments absorbance tested in three analysis times, to verify the metabolic activity of the D-17 cell line. The models explain the variability of the biological activity studied, in which the cell viability and cytotoxicity effect of the ethanol extract of pequi peel on canine osteosarcoma cells (Figure 04).

Figure 4 - Metabolic activity values of the D-17 cell line obtained from the absorbance of the treatments tested in three analysis times of 24, 48 and 72 hours after exposure to the ethanolic extract of pequi peel.



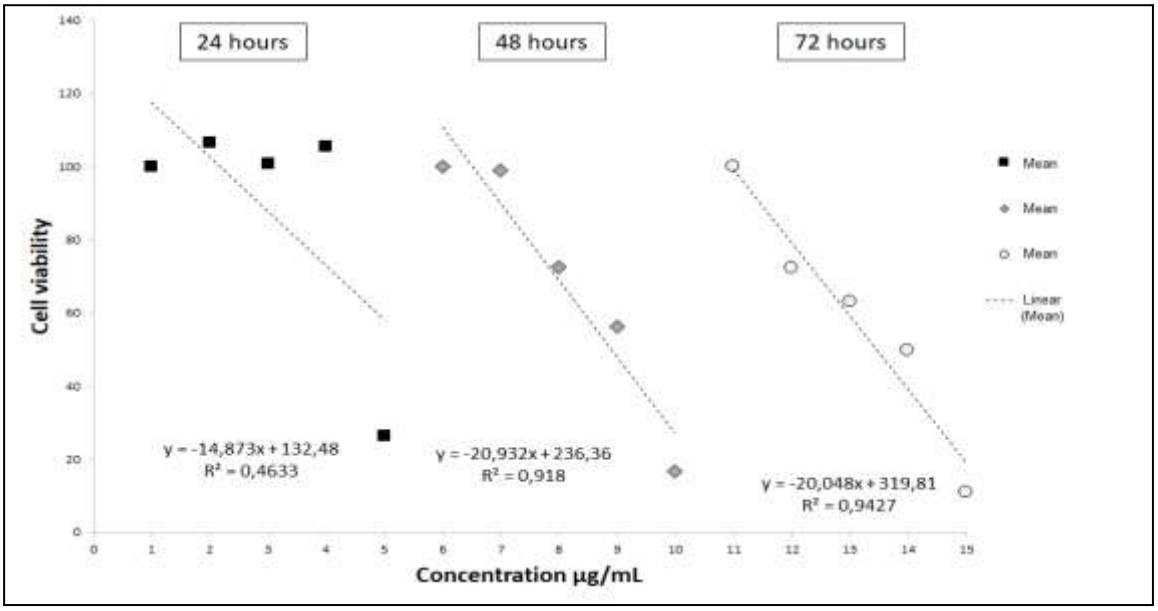
* Concentration values of 1 - 5, correspond to the values of the control treatment, 1 µg/mL, 10 µg/mL, 100 µg/mL, 1000 µg/mL, respectively, for the 24-hour exposure time; Concentration

values of 6 - 10, correspond to the values of the control treatment, 1 µg/mL, 10 µg/mL, 100 µg/mL, 1000 µg/mL, respectively, for the 48-hour exposure time; Concentration values of 11 - 15, correspond to the values of the control treatment, 1 µg/mL, 10 µg/mL, 100 µg/mL, 1000 µg/mL, respectively, for the 72-hour exposure time.
Source: Author's compilation

We found a dose-dependent cytotoxic effect, i.e., as the exposure time increases, the greater is the cytotoxic effect. The same occurs for the model fit, with coefficients of determination (R^2) of 0.46, 0.91, and 0.94 for the exposure times of 24, 48, and 72 hours, respectively.

In the same way, these results were corroborated by the regression models from the cell viability (Figure 5), with R^2 values of 0.46, 0.91, and 0.94 for the exposure times of 24, 48, and 72 hours, respectively, showing a dose-dependent effect for both models.

Figure 5 - Cell viability values of the D-17 cell line obtained from the absorbance of the treatments tested in three analysis times of 24, 48 and 72 hours after exposure to the ethanolic extract of pequi peel.



* Concentration values of 1 - 5, correspond to the values of the control treatment, 1 µg/mL, 10 µg/mL, 100 µg/mL, 1000 µg/mL, respectively, for the 24-hour exposure time; Concentration values of 6 - 10, correspond to the values of the control treatment, 1 µg/mL, 10 µg/mL, 100 µg/mL, 1000 µg/mL, respectively, for the 48-hour exposure time; Concentration values of 11 - 15, correspond to the values of the control treatment, 1 µg/mL, 10 µg/mL, 100 µg/mL, 1000 µg/mL, respectively, for the 72-hour exposure time.
Source: Author's compilation

The regression models demonstrated robustness and fidelity in comparison to the tetrazolium salt conversion parameter (absorbance) and the cell viability, showing important

associations for understanding the MTT. Thus, suggesting the effect of treatment (concentration) about the time of exposure, which explains the quantification of MTT to viable cells. The generated equations can provide punctual evaluations using cell viability or even absorbance data to assess other untested concentrations and still obtain a prediction of possible expected effects.

There are clear reasons for carrying out the experimental test of the ethanolic extract of pequi peel in understanding the therapeutic and cytotoxic action in vitro test, the effective dose is between 10 and 100 µg/mL. This result provides only a guide for initial dosage, which must be adjusted to the factors studied such as the cell type evaluated, exposure time, and desired effect. The efficacy and toxicity of the extract are directly related to the association with several active principles that act in synergism, which promotes the result. Although the model does not accurately assume the clinical outcome, the extract may still be useful as a possible anti-tumorigenic agent.

4. Conclusion

The ethanol extract of pequi peel promotes the increase in cell viability within the 24-hour exposure time. It also shows cytotoxic effects at concentrations equal or over 10 µg/mL, with exposure time equal to or greater than 48 hours. Finally, the extract has a time-dependent residual effect on canine osteosarcoma 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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