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Turmeric ethanol extract (*Curcuma longa* L.) reduces apoptosis and promotes canine osteosarcoma cell proliferation

Extrato etanólico de açafrão (Curcuma longa L.) reduz apoptose e promove proliferação de células de osteossarcoma canino

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Abstract
Curcuma longa L., also known as turmeric, has been widely studied for its various therapeutic properties, including antineoplastic action. The ethanolic extract of the plant contains several phenolic compounds, especially curcumin. Osteosarcoma is a predominant bone tumor in dogs and humans, characterized by high metastatic potential and an unfavorable prognosis. The aim of this study was to investigate the effects of turmeric ethanol extract on canine osteosarcoma cells from established culture. The cells were cultured and treated with different curcumin concentrations (0, 10 µM, 20 µM, 50 µM, 100 µM, and 1000 µM) and exposure times (24h, 48h, and 72h). We first performed tetrazolium reduction technique (MTT) assay and calculated IC_{50}. An immunocytochemistry assay was performed after extract treatment to verify the expression of mutated p53 and therefore study the proliferative potential of malignant cells; Bcl-2 and Ki-67 were used to assess apoptosis and the degree of malignancy, respectively. The extract enhanced the proliferation of canine osteosarcoma cells, reaching 3,819.74% at 50 µM of curcumin. The extract also significantly altered the expression of mutated p53 and Ki-67 proteins but not that of Bcl-2, suggesting that it did not induce this antiapoptotic pathway. Overall, these results are prerequisite to better understanding how natural compounds such as turmeric ethanolic extract affect cell proliferation and could be used to treat various diseases.
Keywords: bone neoplasms; D-17; natural compounds; therapeutic safety

Resumo
A *Curcuma longa* L., planta conhecida popularmente como açafrão, tem sido amplamente estudada por suas diversas propriedades terapêuticas, incluindo a ação antineoplásica. O extrato etanólico da planta contém diversos compostos fenólicos, com destaque para a curcumina. O osteossarcoma é um tumor ósseo predominante em cães e humanos, caracterizado por apresentar alto potencial metastático e prognóstico desfavorável. Procurou-se investigar os efeitos de diferentes concentrações de curcumina do extrato etanólico de açafrão sobre células de osteossarcoma canino de cultura estabelecida. As células foram cultivadas e submetidas ao tratamento com extrato com diferentes concentrações de curcumina (0, 10 µM, 20 µM, 50 µM, 100 µM e 1000 µM) e tempos de exposição (24h, 48h e 72h) pelo EEA. Inicialmente, foram realizados: técnica de redução do tetrazólio (MTT) e cálculo da IC_{50}. Posteriormente, após o tratamento com o extrato, realizou-se o ensaio de imunocitoquímica para verificar a expressão de p53 mutada e estudar o potencial proliferativo das células malignas; Bcl-2, com intuito de averiguar o estímulo de via antiapoptótica; e o marcador Ki-67, que sinaliza aumento no grau de malignidade. O extrato promoveu proliferação de células de osteossarcoma canino, com incremento de até 3819,74% na concentração de 50µM de curcumina. O composto também alterou a expressão das proteínas p53 mutante e Ki-67 significativamente, mas não alterou a expressão de Bcl-2, mostrando que não induziu a via antiapoptótica mediada por esta. Estes resultados demonstram que o extrato etanólico do açafrão apresenta potencial proliferativo sobre células de osteossarcoma canino, sugerindo a necessidade de conscientização e conhecimento dos reais efeitos de determinados compostos naturais, considerados seguros ao serem utilizados como tratamento de diversas enfermidades.
Palavras-chave: compostos naturais; D-17; neoplasias ósseas; segurança terapêutica

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Introduction

Plant-derived products are among the most promising sources for medicine production [1,2]. Indeed, 25% of medications prescribed worldwide are plant-derived; among the 252 medicines that make up the essential drugs list of the World Health Organization (WHO), 11% are entirely of plant origin [3,4]. Cerrado, the second largest biome in Brazil, offers many plants featured in popular medicines despite habitat destruction [5]. Its widespread biodiversity includes natural products that are often used as therapeutics [6].

Although secondary plant compounds harbor therapeutic potential, some of their substances can harm human health. More studies should explore the pharmacological mechanisms and their possible side effects [7] in addition to identifying which compound is responsible for each therapeutic action [8,9]. The final product may have undergone acquisition, preparation, or conservation that do not render its phytochemical composition identical to that of the native plant [10].

Curcuma longa L., popularly known as turmeric, belongs to the family Zingiberaceae of genus Curcuma; it is a food spice appreciated worldwide to color and flavor foods. Although the plant originates from the tropical forests of India, the biggest supplier worldwide [11,12], it is also widely consumed and produced in Mara Rosa, a central municipality in Brazil. Turmeric contains a large fraction (6.8%) of curcumin, a highly therapeutic molecule [13]. Curcumin reduces lipogenesis and exerts antipruritic, anti-inflammatory, antineoplastic, analgesic, antimicrobial, antifungal, antiviral, and antioxidant effects [14–18]. In practice curcumin has been widely sold as a phytotherapeutic agent to treat inflammatory bone diseases in canine [19] and human patients [20]. However, its mechanisms and related cell signaling pathways remain poorly characterized.

A previous study showed that pure curcumin inhibits tumor development in canine osteosarcoma (CO) cells by activating extrinsic apoptosis through the JNK kinase and the caspase-3 pathways, which are mediated by the cAMP/AMPK signal transduction pathway. Furthermore, curcumin can reduce the expression of the p53 mutant protein without interfering in the autophagic pathway of AKT/mTOR [18]. However, other compounds found in turmeric extract such as phenols are antioxidants that neutralize free radicals [21] and could interfere with the antiapoptotic capabilities of curcumin.

CO is an aggressive neoplasm that represents up to 85% of all bone tumors in dogs. Most tumors originate in the metaphyseal region of the bones in the appendicular skeleton. The risk factors for CO include sex, breed, history of trauma, and whether the dog is neutered. Female dogs have a lower risk, while larger dogs (>25 kg) are at greater risk. Affected animals usually exhibit lameness and swelling and may experience pathological fracture, especially in the femur. CO prognosis is generally poor due to the high global rate of metastasis. Common treatment includes amputation and follow-up chemotherapy (adjuvant); the disease-free interval and the global survival time are typically 291 and 284 days, respectively. Few therapeutic alternatives have emerged in recent decades, warranting new advances to treat CO [22]. Mutated tumor suppressor genes are potentially related to the proliferation and migration of neoplastic cells, leading to metastasis [23]. Ideal therapeutic agents would reduce such proliferation and induce apoptosis in tumor cells to improve chances of survival [18].

The present study investigated how the ethanolic extract of *Curcuma longa* L. (TEE) from the region of Mara Rosa(Goiás, Brazil) affected canine osteosarcoma cells and analyzed the expression of biomarkers relevant to the progression of this neoplasm.

Material and methods

The experiment was developed at the Multiuser Laboratory for the Evaluation of Molecules, Cells, and Tissues in the Veterinary and Zootechny School at the Federal University of Goiás.

Preparation of the turmeric ethanolic extract (Curcuma longa L.)

A turmeric ethanolic extract was obtained and prepared as previously described [24]. The rhizomes of *Curcuma longa* L. were acquired from the Turmeric Products Cooperative of Mara Rosa (Cooperaçafrão), located in the town of Mara Rosa, state of Goiás, Brazil. The rhizomes were first removed from the soil, then washed and dried on a kiln to be processed. Afterwards, 100 g of the dry and ground material were added to 1000 ml of absolute ethanol, which underwent cold percolation. The supernatant was filtered out, and the solvent was evaporated on a rotary evaporator (IKA® RV 05 Basic) [25].

The composition of the extract was previously characterized [24]. According to TEE, the extract contains on average 16.7% curcumin, as measured by fluorescent high pressure liquid chromatography. The total content of phenolic compounds was determined from the standard curve of gallic acid (gallic acid equivalent: GAE) as 691.49 GAE/100 g. The extract's ability to scavenge free radicals was assessed from its discoloration of DPPH (2,2 diphenyl-1-picrylhydrazyl) and a standard reagent (Trolox) equivalent to vitamin E. The TEE has an antioxidant activity of approximately 18.45 µM Trolox/g of sample; the dislocation percentage of DPPH is 55% at IC_{50} of 18.1 µg/mL. The crude extract was stored in a refrigerator and protected from photodegradation at every step.

Cell culture

Canine metastatic osteosarcoma cells (D-17, BCRJ 0276, batch 000573, pass 239, species Canis familiaris) originated from ATCC (American Type Culture Collection -

Manassas, VA, USA) were acquired from the Cell Bank of Rio de Janeiro (UFRJ–Rio de Janeiro, Brazil). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM Thermo Fisher, Waltham, MA, USA) enriched with 10% fetal bovine serum, penicillin, and streptomycin (10,000 I.U./mL), amphotericin B, and L-glutamine (all reagents from Cultilab, Campinas, Brazil) and maintained in a humidified incubator at 37°C and 5% CO_2.

Cell viability and cytotoxicity assay

After culture, the cells were quantified on a Neubauer chamber, and 1×10^4 cells in 200 µL of DMEM per well were seeded in 96-well plates. The plates were maintained in a humidified incubator at 37°C with 5% CO_2 for 24 hours. Then the medium was discarded, and the wells were treated with TEE diluted in 1% of DMSO (dimethyl sulfoxide) at various concentrations of curcumin calculated from its molecular weight (368.38 g/mol) and its concentration in TEE (16.7%): 0 µM, 10 µM, 20 µM, 50 µM, 100 µM, and 1000 µM (Table 1). The cells were exposed to TEE at 24, 48, or 72 hours. The control group was treated only with DMSO. The wells were treated randomly, and each treatment was performed in quintuplicate for three independent experiments.

Table 1. Dilutions of the turmeric ethanolic extract (Curcuma longa, L.) at different molar concentrations of curcumin (molecular weight 368,38 g/mol), whose concentration in the extract is 16.7% [24]. DMSO: dimethyl sulfoxide, diluent of the crude extract. DMEM: Dulbecco's Modified Eagle Medium

Curcumin Concentration (µM)	Semi-solid crude extract (mg)	Solution dilution	
		DMSO (µL)	DMEM (µL)
0	0	4000	6000
10	6	4000	6000
20	12	4000	6000
50	31	4000	6000
100	59	4000	6000
1000	590	4000	6000

A cell viability assay - the reduction of tetrazolium salt (MTT assay) - was performed after the treatment period. The culture medium was discarded, and 10 µL of tetrazolium (MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) were added to each well. The plates were incubated for three hours. Fifty µL of sodium dodecyl sulfate (SDS–Vivantis Biochemical) were added to each well to terminate the reaction, and the plates remained incubated for 24 hours at room temperature. Optical density was measured with a spectrophotometer (Awareness Technology Stat Fax 2100, 425–540 nm, Palm City, FL, USA). Three wavelengths were used to discern the absorbances measured by the spectrophotometer because the size of the molecules, density, dilution, and other factors can influence the physical properties of the samples and thus interfere with the ideal wavelength [26].

Cell viability (CV) was determined by the equation

$$CV = \frac{A_{treatment}}{A_{control}} \text{ x } 100$$

where A indicates absorbance. Data were collected from three independent studies. One-way analysis of variance (ANOVA) and Tukey's post-hoc test were performed in R using the package *easyanova*® to compare groups as previously described [18]. A p-value <0.05 was considered statistically significant.

Immunocytochemistry

The D-17 cells were seeded on culture slides with a Falcon™ (Corning, Glendale, EUA) chamber at a concentration of 1×10^4; they were cultured and exposed to TEE for 48 hours at the studied concentrations (0, 10, 20, 50, 100, or 1000 µM). After this period, the cells were fixed with 4% paraformaldehyde for 30 minutes.

All steps were executed using the automatic processor BOND-MAX (Leica Biosystems, Nussloch, Germany) with the following reagents: BOND Polymer Refine Detection, BOND Wash Solution, BOND Epitope Retrieval 1, BOND Dewax Solution, BOND Epitope Retrieval 2, BOND DAB (3,3'-diaminobenzidine chromogen), and hematoxylin. The antibodies used were anti-Bcl-2, anti-p53 (SC71785 monoclonal of mouse), and anti-Ki-67, which were diluted 1:500 in 1.5% bovine serum albumin. A group that was not treated with primary antibody served as a control.

Immunostaining was evaluated by counting the total number of cells in five distinct fields and determining the percentage of antibody-stained cells, a procedure adapted from Fedchenko & Reifenrath [27]. To establish the expression intensity score (EIS), a blind semi-quantitative analysis was performed to establish an expression intensity score (EIS) whose values ranged from 0 to 3 according to the shades on a colorimetric scale [18] (Figure 1). The positively stained cells ratio (PSCR) was calculated as the ratio of the number of stained cells to the total number of cells multiplied by 100. From this value several categories were established: 0 for 0%, 1 for <1%, 2 for 1%–10%, 3 for 11%–33%, 4 for 34%–66%, and 5 for >67%. The PSCR was added to the EIS to define a semi-quantitative combined scoring system (SCSS) whose average and standard deviation were calculated and outliers excluded. ANOVA and the Kruskal–Wallis test were performed with R software and package easyanova®, where $p < 0.05$ indicated statistical significance [18].

Figure 1. Reference shades used to score the different stain intensities of the antibodies in the immunocytochemistry analysis. Adapted from Soares et al.[18].

Results

Cell viability and cytotoxicity assay

Table 2. Mean cell viability values obtained by the averages of the spectrophotometry in the wavelengths 496, 540, and 570 nm for the canine osteosarcoma cell lineage D-17, which were treated with various concentrations of turmeric (*Curcuma longa*, L.) ethanolic extract for different exposure times

Exposure (h)	Concentration (µM)	Cell Viability (%)	Tukey
24 [AB]	0	100	d
	10	321,48	d
	20	625,28	cd
	50	3654,62	a
	100	2097,53	b
	1000	1262,41	c
48 [A]	0	100	d
	10	313,56	d
	20	297,94	d
	50	3945,09	a
	100	3000,44	b
	1000	1831,8	c
72 [B]	0	100	c
	10	197,88	c
	20	267,97	c
	50	3859,52	a
	100	2672,73	b
	1000	542,51	c

The experiment was performed with different concentrations and exposure times to determine if the TEE affected viability of the D-17 cells. The averages of the treatments and the exposure times were compared. The compound exponentially and significantly affected proliferation until the concentration of TEE approached 50 µM. Cell proliferation decreased at higher concentrations. Extract exposure times did not significantly affect cell proliferation (Table 2).

This effect can be better understood when an average of the cell proliferation values was taken on the three experimental times in relation to the used concentrations, as demonstrated on Table 3.

Table 3. Average of the Cell Viability values obtained through the absorbance generated from the averages of the spectrophotometry in the wavelengths 496, 540 and 570 nm for the canine osteosarcoma cells of the lineage D-17, of the control and treated groups with 0, 10 µM, 20 µM, 50 µM, 100 µM or 1000 µM of the turmeric (*Curcuma longa*, L.) ethanolic extract

Curcumin Concentration (µM)	Cell Viability (%)	Tukey
0	100	d
10	277,65	d
20	397,07	d
50	3819,74	a
100	2590,23	b
1000	1212,24	c

Table 4. Assigned scores and semi-quantitative combined scoring system (SCSS) from Fedchenko & Reifenrath[12] to assess slides with canine osteosarcoma cells of the lineage D-17 treated with the turmeric (*Curcuma longa*, L.) ethanolic extract. The cells were stained with anti-Bcl-2, -KI-67 or -mutant p53 antibody and stained with hematoxylin. Independent statistical analysis (ANOVA and Kruskal-Wallis test, $p < 0.05$) was performed for each antibody

Antibody	Curcumin Concentration(µM)	Posetively Labeled Cell Relation	Expression Intensity Score	Semiquantitative Combined Punctuation System
Bcl-2	0	5	2.1029[a]	7.1029[a]
	10	5	1.8750[a]	6.8750[a]
	20	5	1.4444[a]	6.4444[a]
	50	5	2.7500[a]	7.7500[a]
	100	5	2.5000[a]	7.5000[a]
	1000	5	2.2250[a]	7.1000[a]
p53	0	5	1.0441[b]	6.0441[b]
	10	5	1.4500[ab]	6.4500[ab]
	20	5	1.1111[b]	6.1111[b]
	50	5	1.6750[ab]	6.6750[ab]
	100	5	1.9000[ab]	6.9000[ab]
	1000	5	2.9000[a]	7.9000[a]
Ki-67	0	5	1.5588[ab]	6.5588[ab]
	10	5	0.8250[b]	5.8250[b]
	20	5	1.5000[ab]	6.5000[ab]
	50	5	2.2500[a]	7.2500[a]
	100	5	2.1500[ab]	7.1500[ab]
	1000	5	2.6000[a]	7.6000[a]

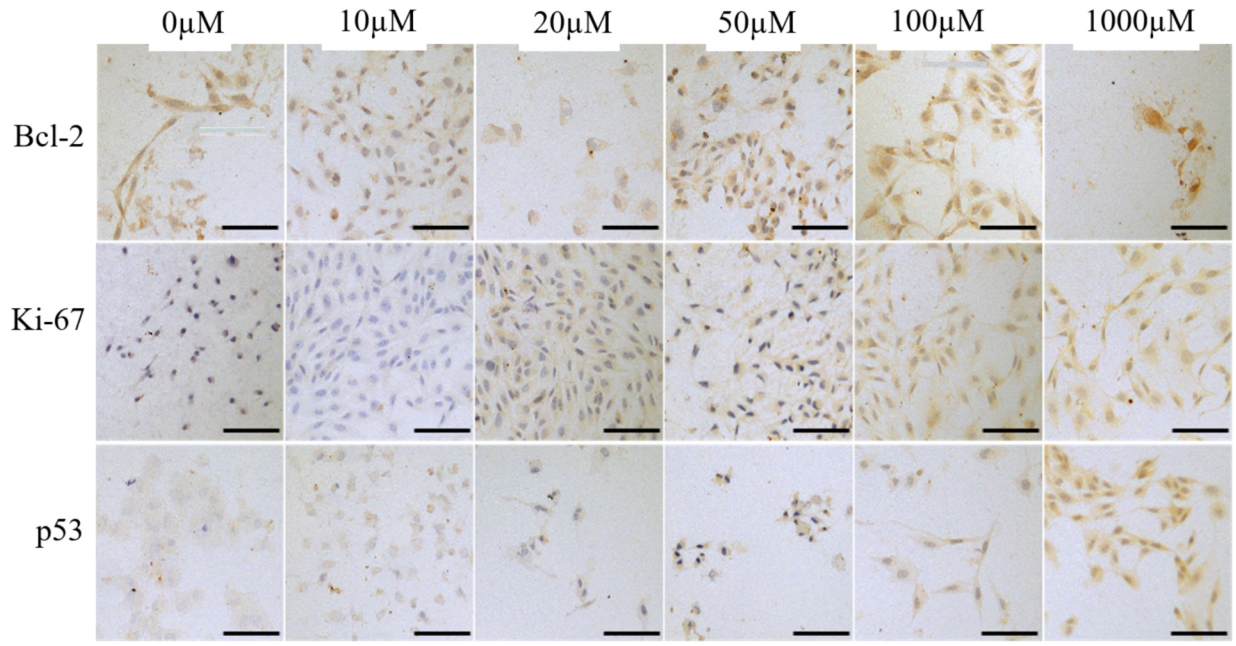

Figure 2. Images of the canine osteossarcoma D-17cells treated with the turmeric (Curcuma longa, L.) ethanolic extract with different curcumin concentrations and labeled with anti-Bcl-2, -KI-67 or -mutated p53 antibody and counterstained with hematoxylin. Labeling intensities of Ki-67 and mutated p53 protein increased with extract concentration. Scale bars indicate 100 μm. assigned scores underwent statistical analysis as described in Table 3.

Discussion

This work explored how turmeric (*Curcuma longa* L.) affects proliferation of canine CO cells. To date no studies have examined the effect of this extract on proliferation of cancer cell lineages let alone that of canine CO cells: reports focus solely on the benefits of C. longa L. This is unsurprising given the low toxicity of the plant and its isolated compounds; a retrospective study on the toxicity of turmeric and its main component, curcumin, found that doses as high as 3.6 g of daily curcumin for six months or 4 g of fermented turmeric extract for 12 weeks was safe in humans [28].

Contrary to this study's findings, many have concluded that the constituents of turmeric, especially curcumin, are highly effective against cancer. Their anti-proliferative and anti-cancer activities have proven promising in pre-clinical studies, though rare side effects have been described in normal cells [28–30].Yet we have found that TEE boosts cell proliferation of D-17 cells according to MTT assay (Figure 2). The cells exposed to 50 μM of curcumin showed the highest percentage of cell viability, which declined at higher TEE concentrations. The exposure time to the extract did not significantly influence cell proliferation.

We expected that curcumin would cause extrinsic apoptosis of D-17 cells given previous findings in cells of the same lineage [18]. Curcumin can induce intrinsic and extrinsic apoptosis in different tumor cell lineages in addition to inducing the production of reactive oxygen species, which positively regulate apoptosis receptors on the tumor cell membrane. The compound also regulates the expression and activity of native p53 to inhibit the proliferation of tumor cells and increase apoptosis while inhibiting NF-κB and COX-2, which are involved in the superexpression of antiapoptotic genes such as Bcl-2 [31]. Curcumin previously inhibited the growth of MG-63 CO cells, inducing apoptosis by activating caspase-3 and reducing expression levels of Bcl-2 [32]. Here, on the contrary, TEE did not kill canine CO cells.

Importantly, this study used turmeric ethanolic extract, which comprises various compounds. Although the mechanisms of its major compounds are defined, its remaining phytochemical products are poorly characterized and therefore may exert unknown side effects or exponential toxicity. The metabolites of the extract may also interact with metabolic pathways to activate genes that cultivate neoplasms [33,34] or protect tumor cells.

The pharmacological effects and mechanisms of the isolated compounds of turmeric are predominantly

deemed beneficial, but this does not indicate that the extract studied here is safe under the same conditions. The cancer cells studied here increasingly proliferated when exposed to greater concentrations of TE and thus curcumin, which in isolation has proven efficacious against cancer cell lineages through the STAT3 apoptotic pathway. Curcumin also inhibited proliferation of thyroid papillary adenocarcinoma cells in a concentration-dependent manner [35].

The total amount of phenols in TEE is reportedly 691.49 mg of EAG/100 g. Recently, the ethanolic extract of the *pequi* shell (Caryocar brasiliense, Camb.), whose total phenol content (696.91 mg of EAG/100 g) is similar to that of the TEE used in this study, exerted protective effects in human coronary endothelial cells [36]. Most phenols in turmeric are curcuminoids, but other phenolic compounds such as caffeic acid, coumaric acid, and quercetin [21] are antioxidants found in the *pequi* extract.

We qualitatively and semi-quantitatively evaluated the expression levels of mutated p53 protein, Ki-67, and Bcl-2 using automated immunocytochemistry [27,37]. The data revealed relatively strong expression levels of mutated p53 protein and Ki-67, especially in the group treated with 1000 µM of the extract. The mutated p53 protein was found in and thus affected the cellular proliferation of every experimental group; its expression is a reliable marker of proliferation and tumor progression because the mutation in the *TP53* gene changes the structure of p53 protein, which loses its ability to repair DNA or induce tumor cell death [38][39]. Ki-67 also indicates potential malignancy potential in various types of neoplasms, including canine OS cells [37,40–42].

The expression level of Bcl-2 in canine CO cells did not significantly change here (Table 4), unlike previous studies that have found higher expression levels of Bcl-2 protein in cells treated with 50 µM. Although the mechanisms here remain unknown, its unchanging expression here could be related to other constituents in the extract that have antiapoptotic effects in our cells. Bcl-2 regulates cell apoptosis, as it encodes a protein in the mitochondrial membrane that inhibits cell death by apoptosis and activating cell proliferation [43,44].

The outcome of this study disagrees with similar studies that evaluated the cytotoxicity of other plant extracts on cancer cells by measuring cell proliferation and expression of oncogenes [37,40,45,46]. This warrants precaution when administering plants and their derivatives as phytotherapeutics, whose efficacy and safety are assured almost instantaneously because they are native and organic. However, plant metabolites can cause unknown and potentially deleterious effects such as subacute chronic toxicity, carcinogenesis [34,47], or the proliferation of pre-existent atypical cell clones, such as reported here.

This study is the first to show the proliferative properties of turmeric extract on canine OS cells. Our findings highlight the importance of validating every potentially therapeutic compound even if it is naturally derived; the mechanisms of action and related signaling pahways should be revealed to confirm safety and efficacy of prescribed doses.

Conclusion

Turmeric ethanolic extract increased the *in vitro* proliferation of D-17 canine OS cells and the expression of proteins related to oncogenesis and tumor malignancy. More studies should explore the apoptotic mechanisms of turmeric before using it to treat CO in dogs or humans.

Conflict of interests

The authors declare no conflict of interest.

Author contributions

Conceptualization: NM Alves, VS Cruz, E Arnhold, LL Nepomuceno, NP Soares and EG Araújo. *Formal Analysis*: VS Cruz, LL Nepomuceno, D Graziani and E Arnhold. *Funding acquisition*: EG Araújo. *Investigation*: NM Alves, VS Cruz, NP Soares, D Graziani, PAM Gonçalves, GHS Badan and AM Santos. *Methodology*: NM Alves, VS Cruz, E Arnhold, LL Nepomuceno, NP Soares, D Graziani, PAM Gonçalves, GHS Badan and AM Santos. *Project Administration*: VS Cruz and EG Araújo. *Resources*: EG Araújo. *Supervision*: EG Araújo. *Validation*: VS Cruz and E Arnhold; *Writing (original draft)*: NM Alves. *Writing (review & editing)*: NM Alves, VS Cruz, E Arnhold, GHS Badan and EG Araújo.

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