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Original Article

Evaluation of the Effect of Five Colombian Propolis Extracts on the Expression of Genes Associated with Cell Cycle and Inflammation in a Canine Osteosarcoma Cell Line

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ABSTRACT

Introduction: Propolis has anti-inflammatory, antitumor, antibacterial and immunomodulatory properties, which is why it is suggested that it could be used as an alternative or complementary drug therapy in the treatment of various pathologies such as cancer, diseases chronic-degenerative and infectious. **Materials and Methods:** In this study, canine bone fibroblasts were used as control cells, and the canine Osteosarcoma cell line (OSCA-8) was acquired to evaluate the effects of ethanol extracts of propolis from five Colombian regions on these cells. The cytotoxic effect was evaluated by examining changes in cell viability and proliferation. Furthermore, the expression of some relevant and characteristic genes related to the tumor phenotype, related to the proinflammatory process and cell cycle, was assessed. **Results:** Thus, the evaluation of the relative expression of some genes associated with the cell cycle and inflammation could improve the understanding of the cytotoxic effect of propolis extracts on OSCA-8 cell lines. **Conclusion:** The first time in Colombia, the biological activity of ethanol extracts of propolis was evaluated regarding the inflammation and cell cycle pathways. After 48 hr, the Colombian EEP had an effect on the increase in both OSCA-8 cells and fibroblasts.

Keywords: Biological activity, Cell cycle, Gene expression, Inflammation, Osteosarcoma, Propolis.

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INTRODUCTION

According to data from the International Agency for Research on Cancer (IARC), 19.3 million new cases of different types of cancer were presented in the year 2020, and 10 million deaths were caused by this cause. By the year 2030, this number is expected to increase to 10.[1] Thus, given the high mortality and incidence of cases that occur annually, multiple strategies have been developed to treat cancer, including chemotherapy, radiotherapy, immunotherapy, and surgery. Despite their efficacy and longer survival, pharmacological agents such as doxorubicin, carboplatin, mitomycin, and methotrexate also present multiple adverse effects associated with non-specific toxicity. In most cases, acute toxicity and its consequences are treated with complementary and specific drugs. However, the associated complications result in a lack of adherence to treatment, among other problems. Conversely, chronic toxicity usually results in irreversible damage that can increase the morbidity and mortality of patients with indications for treatment.[2] Another obstacle that is associated with chemotherapy is resistance to treatment. Even though it is true that tumor cells are sensitive at the start of treatment, they often develop resistance mechanisms based on functional and structural events that limit the usefulness of drugs.[3]

The World Health Organization estimates that 25% of all medicines and 60% of antitumor drugs are derived from plants, implying a flow of more than 83 billion dollars.[4] A number of active principles are made from molecules that come from nature or are changed to make them more effective.[5] So, natural products are important in the development of drugs to treat some human diseases, and cancer is no exception.[6,7]

On the other hand, natural compounds have become more popular for treating various diseases because they are cheaper, more effective, and don't have as many side effects as traditional drugs.[8] Another advantage of using natural products and their metabolites is their proven biological effect on human health. Some compounds exert their pharmacological activity by imitating endogenous metabolites as molecules that participate in signal transduction, for which it has been proven its high efficiency, rapid effect, and low toxicity.[9] The effect on health of natural products could be due to a synergy between the components of the product, to their chemical structures that allow them to bind

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to a wide variety of target molecules involved in human diseases, and to the reduction of toxicity.[10] And finally, consumers are more confident in natural products than in industrially produced pharmaceuticals.

Although alternative therapeutic tools do not replace traditional treatments, they could reduce their dosage due to the above. Propolis comes from various botanical sources from which bees collect their exudates and process them to obtain this natural mixture, so its characteristics and composition depend on these plant sources. Both *in vitro* studies and *in vivo*, Propolis has demonstrated cytotoxic capacity through the induction of apoptosis in different tumor cell lines.[11] It has been demonstrated that the effect on the inhibition of cell growth in tumor lines is dose-dependent. Likewise, polyphenolic compounds, such as some derivatives of caffeine acid, interrupt the cell cycle, specifically in the G2/M phases.[12] On the other hand, propolis has been shown to reduce tumor nodules by increasing the activity of Natural Killer (NK) cells against Murine lymphomas.[13] The polyphenolic compounds in propolis are antioxidants, but they can be a pro-oxidant agent in high doses and cause mitochondrial dysfunction and apoptosis. This mechanism has been shown to be especially important in diseases such as cancer.[14,15] However, the immunomodulatory effect has also been proven, and ethanol extracts of propolis contribute to improve the response of the immune system, specifically the innate[16] and decrease the synthesis of pro-inflammatory cytokines such as IL-1β and IL-6, demonstrating that it can be an alternative in the management of chronic-inflammatory diseases.[17]

With the development of this work, cell cultures were established and characterized to determine the effect experimentally on the expression of some genes related to the tumor and inflammatory process of Ethanol Extracts of Colombian Propolis (EEP) in order to understand and approach the molecular mechanisms by which these could cause cytotoxicity.

MATERIALS AND METHODS

Obtaining propolis samples

Five samples of Colombian propolis were taken from different regions of the country: in the town of Usme, Bogotá (USM), at an altitude close to 2,460 MSL; in the municipality of Fusagasugá, department of Cundinamarca (FUS), at an altitude of 1720 MSL; in the municipality of Puerto López, department of Meta (MET), at an altitude close to 365 MSL; in the municipality of Silvia, department of Cauca (SIL), at an approximate height of 2,454 MSL and finally in the municipality of Cajibío, in the department of Cauca (CAJ), at a height of close to 1,750 MSL with characteristics in terms of the temperature ranged between 8 and 33°C. The propolis samples were collected in the form of pastes between the months of July and December using plastic nets and scraping techniques. As previously mentioned, the chemical composition of propolis will depend on the flora and its specific situation due to the thermal floor and the time of year in which they are collected. The samples were crushed, their weight was calculated, and they were homogenized into 4.5 g portions, each of which was dissolved in 15 mL of 70% ethanol. The ethanol extracts of propolis were obtained at room temperature in amber-type bottles in order to prevent the entry of light.[18]

The establishment and characterization of control fibroblast cells

Bone fibroblasts obtained from a canine specimen that did not have osteosarcoma were used as controls. The sample was taken from the acetabular bone tissue and adjacent to it, through the open incisional biopsy procedure.[19] The tissue was then fragmented into pieces of approximately 2 mm in diameter. The fragments were placed in a 25 cm^2 culture flask with an adherent internal surface without adding any component or culture medium, and they were incubated for 48 hr at 37°C and 5% CO_2. The cells were transferred to 25 cm^2 flasks with DMEM culture medium (Santa Cruz Biotechnology®), supplemented with 10% fetal bovine serum (LABG&M) and a streptomycin cocktail with amphotericin B. When the culture reached 80%, the cells were transferred to a new flask. They were trypsinized and then passed into wells at a concentration of $1x10^5$ cells per well. These cells were evaluated using an inverted microscope and counted in a Neubauer chamber stained with Trypan blue. The proliferation curve was elaborated using data taken every 24 hr over four consecutive days. The averages were compared. The culture was characterized by several tests, initially a karyotype was performed to confirm that they were indeed fibroblasts obtained from the species *Canis lupus* familiaris. For this cell density, 80 µL of colchicine (16 mg/mL) were added to a 75 cm^2 culture flask, and the exposure was for a period of 2.5 hr. Baglole described a standard protocol for culturing and obtaining fibroblasts, with washings, trypsinization, obtaining metaphases by hypotonic shock, dripping, and fixation on slides.[20] The corresponding metaphases were banded C[21] and banded G.[22] For morphological characterization by histochemistry, hematoxylin and eosin staining techniques were used, as well as trichrome staining techniques were used for connective tissue, specifically type I collagen fibers.[23,24] Lastly, immunohistochemistry was performed using the antibody against vimentin.[25] A commercial canine osteosarcoma cell line (OSCA-8) was purchased from the Kerafast commercial house, and it was thawed using standard methods.[26] Cell populations were monitored for 18 hr and the change in confluence and characteristics of the culture medium were observed. The culture continued under standard conditions.

Evaluation of the cytotoxic effect of Colombian EEPs

For the cytotoxicity tests, MTT assays were used to measure cell proliferation following the protocol of the CytoselectTM MTT Cell Proliferation Assay kit, from the commercial house CELL BIOLABS. To evaluate cell death, the LDH assay was

performed, which colorimetrically detects the activity of the lactate dehydrogenase enzyme, which acts as a marker of cell lysis and/or cell death. The protocol of the CytoselectTM LDH cell proliferation assay kit from the commercial company CELL BIOLABS was followed. Both OSCA-8 cells and control fibroblasts were used to carry out the different assays, considering that they were not found in a passage greater than number 5. For this and the other assays that will be described, it was used as a positive control for cytotoxicity on OSCA-8 cells and fibroblasts after treatment with the chemotherapeutic agent doxorubicin.

Gene characterization of cells by microarray

For the assay with the microarray, canine fibroblasts were taken from the primary culture in passage 3 and seeded in a 25 cm^2 culture flask at a density of $1x10^5$ cells/mL. The cells were not exposed to any treatment, only in culture medium without serum for 48 hr. Total RNA extraction was performed as described below. The 260/280 and 260/230 ratios were determined after RNA quantification. As part of the microarray assay, a test was done to check the integrity of the RNA by electrophoresis. The samples were then hybridized and analyzed using Affymetrix GeneChip Canine Genome 1.0 arrays (Affymetrix Santa Clara, CA). This chip enabled us to analyze the simultaneous transcription of nearly 18,000 genes from the *Canis lupus* familiaris species.

Data from the microarray correspond to relative expression, and for characterization purposes, a list of genes was obtained as a result of the comparison of relative expression between untreated fibroblasts and untreated OSCA-8 cells. It can be seen from the electrophoresis run that there was no degradation and the two corresponding expected bands are observed, the 28S and 18S rRNA. The RNA integrity test indicates that the sample extraction and maintenance process was carried out properly.

RNA extraction

After applying the EEP treatments to the cells, which were seeded in 25 cm^2 culture flasks at a density of $1x10^5$ cells/mL, the total RNA extraction assay from OSCA-8 cells and fibroblasts was carried out with the commercial kit (InviTrap® Spin Universal RNA Mini Kit, Molecular Stratec), following the protocol recommended by the manufacturer.

The optimal purity index for values greater than 1.8 was quantified by nanodrop spectrophotometry, 'Thermo Scientific.' The quality of the extracted RNA was then evaluated by electrophoresis in a 1.2% agarose gel in the presence of urea, to verify the integrity of this nucleic acid. 18S and 28S bands were identified. Each test was done three times.

Reverse transcription assay

The ProtoScript® M-MulV First Strand cDNA Synthesis Kit (Biolabs, Inc.) was used to carry out the retrotransscription process to obtain cDNA. Initial dilutions were made to equalize the total RNA concentrations. 2 µL of nuclease-free deionized water from the kit was mixed with 2 µL of oligo T and 4 µL of total RNA. The mixture was then denaturated at 70ºC for 5 min. Then, 10 µL of the kit reaction mixture was added to each tube, and finally, 2 µL of the reverse transcriptase was added. After that, the tubes were taken to the thermocycler, which was programmed as follows: 1 cycle at 42ºC, 1 cycle for an hour at 80ºC, and 1 cycle for 5 min at 4ºC. Following this, dilutions were made and a cDNA concentration of approximately 8 ng/L was obtained from each sample.

Quantitative real-time PCR (qPCR)

The relative expression of the selected genes was determined using the real-time quantitative polymerase chain reaction (qPCR) technique, with which the quantification cycle (Cq or Ct) was determined.[27] The relative expression values for each of the genes of interest were calculated using the Roche Lightcycler 96 thermocycler software. The expression of the genes of interest was calculated by the equation:

$$\text{Expression rate} = ET(CqTCal - CqT) \div ER(CqRCal - CqR),$$

where ET and ER correspond to the amplification efficiencies of each evaluated gene and the reference gene, respectively; CqT and CqR represent the quantification cycle of each of the genes of interest and the reference gene, respectively. The CqT and CqR terms refer to the Quantification Cycle (Cq) for each of the genes of interest and the reference gene.[28] The primers for the qPCR used in this work are listed in Table 1.

After that, the optimal hybridization temperature of the primers was determined by a temperature gradient PCR in the Roche Lightcycler 96 thermocycler, accompanied by the respective melting temperature curve. The thermocycler setup was as follows: first step at 95ºC for 15 sec, a single cycle, and the next step, the PCR itself with 45 cycles: 95ºC for 10 sec, temperature gradient from 60 to 50ºC for 60 sec, extension at 68ºC for 30 sec, a final extension cycle of 5 min at 68ºC, and a final melting cycle: 9ºC for 10 s, 65ºC for 60 sec, and 97ºC for 1 sec. Finally, cooling at 37ºC for 30 sec.

An electrophoresis of agarose gels confirmed the presence of amplified samples. Once the primers were set at the best temperatures for hybridization, qPCR runs were done in real time with the FastStart Essential DNA Green Master 2X kit (Roche). A pre-incubation at 95ºC for 300 sec was performed in the Roche Lightcycler 96 thermal cycler, followed by two amplification steps: one at 95ºC for 15 sec, and the other at the specific melting temperature of each pair of primers. The specificity of the qPCR products was assessed both by agarose gel electrophoresis and by melting temperature curves. In all cases, the efficiencies of the qPCR reaction were established to be between 1.8 and 2.2, which is considered favorable for the respective gene expression

Table 1: The specificities of the primers used for the qPCR assay.

Gene	ID (NCBI)	Primer Sequence	Amp Size (pb)	Hybridization temperature (°C)
GADPH[28]	403755	F: TGTCCCCACCCCCAATGTATC R: CTCCGATGCCTGCTTCACTACCTT	100	60
CCND1[29]	449028	F: GCCTCGAAGATGAAGGAGAC R: CAGTTTGTTCACCAGGAGCA	117	58
TP53[30]	403869	F: CGCAAAAGAAGAAGCCACTA R: TCCACTCTGGGCATCCTT	119	58
CDKN1A Own Elaboration	474890	F: CAGACCAGCATGACAGATTTC R: GCTCTCATTTCTAGGAGCTTG	105	59
CCNA2[31]	483845	F: TCATGGTGTTTCTCTCCTCAAC R: AGGCAGTCTTTCGCTGTTAGA	62	59
IL6[32]	403985	F: GCACTGAGAAAGGAGATGTGTGACAAG R: CCTGATTGAACCCAGATTGGAAGC	130	57
IL1R2[32]	611453	F:CCCATAACAAAATGGACATGAAGATTCAG R:GTAAGCGAGAGGTTCCCTTCACATTTAGA	102	57

quantification assays, in addition to establishing an adequate dynamic range of these assays.

Statistic analysis

The mean and standard deviations were calculated for the statistical analysis of both cytotoxicity and qPCR results. One and two-way analyses for the Analysis of Variance (ANOVA) were used, followed by Newman Keuls tests for multiple comparison analysis and the Bonferroni test for determining the differences between the values. It was obtained between the treatment and control groups. The results were expressed as means and standard deviations. Only results with p values less than 0.05 were considered significant.

The qPCR results were analyzed using the comparative method of Ct (or Cq) values, determining the $2^{-\Delta\Delta Ct}$ value, and the "fold change" to calculate the differential relative expression.[33] This method allowed us to determine the quantification of the relative expression compared to the normalized value Ct of the samples with and without treatment. Three independent experiments were performed with duplicate assays. The Kruskall Wallis statistic was used to determine how different the genes of the treated cells were from the untreated cells.

RESULTS

Characterization of primary cultured fibroblasts

Healthy dog bone fibroblasts were obtained that served as control cells for the tests, which in primary culture showed morphological characteristics that agreed with expectations, that is, cells with elongated cytoplasmic processes, star-shaped and with ramifications that allow them to interact physically. Hematoxylin and eosin (H&E) staining and Masson's trichrome staining revealed that the cells were fusiform with cytoplasmic extensions. The nucleus was oval and located in the center of the cell. Similarly, its interaction with adjacent fibroblasts could be observed in a cross-linked manner (Figure 1-a and 1-b).

After 4 consecutive days of monitoring of the fibroblasts, the presence of cells on the surface of the wells was observed, showing a progressive increase in confluence. The proliferation of canine fibroblasts derived from primary culture shows that between 24 and 48 hr there was no significant change in cell number, but between 72 and 96 hr exponential proliferation is observed. For an established period of time, it was impossible to observe a stationary phase of the proliferation of these fibroblasts (Figure 1-e).

In immunocytochemistry, the antibody against vimentin was used, and it showed that there were fibroblasts. The cytoskeletal protein vimentin acts as an anchor to cell organelles such as fibroblasts. There is more detail in the photographs of the cytoplasm and the interaction between the fibroblasts. The oval-shaped nucleus can be observed with a different tone in some cells (Figures 1-c and 1-d).

The chromosomes of canine fibroblasts were visualized during the second phase of mitosis, metaphase. G banding and C banding allowed us to identify the number of chromosomes, 2n=78, comprising 76 autosomal and 2 sex chromosomes and some of their structural features. Chromosome counting was performed in 10 different metaphases, and the results showed in all cases that the cells were diploid and corresponded to the species *Canis lupus*.

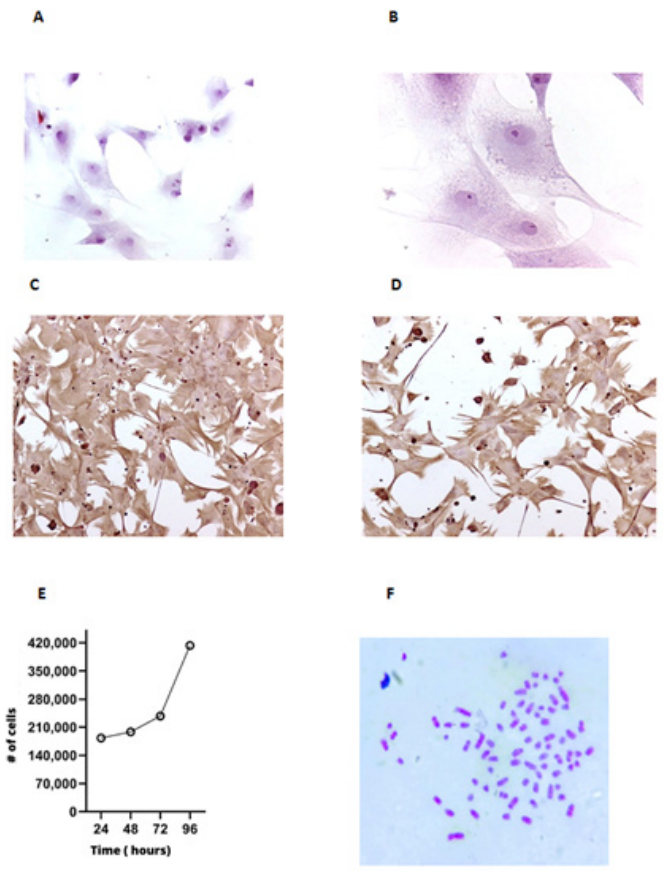

Figure 1: The morphology of canine fibroblasts derived from primary culture stained with Masson's trichrome: (A) 4X; (B) 40X. The morphological characterization of fibroblasts was performed using the antibody against vimentin with a magnification of (C) 10X; (D) 40X. The photos were taken using the ACT-1 Nikon software in a 1 mm field at different magnifications. Cell proliferation curve of control fibroblasts (E) Cytogenetic analysis of canine fibroblasts with C (F) banding.

Cytotoxicity Assays

According to the evaluation of the effect of ethanol, which was used as a solvent to obtain the propolis extracts, it was possible to demonstrate with the cell viability test measured by the MTT method in different periods of time that, both in OSCA cells-8 as control fibroblasts, this slightly decreases the viability. In general, cytotoxicity is dependent on both the exposure time and the concentration of the extracts and the relationship of cytotoxicity with these two variables is proportional. After 48 hr of exposure, it can be observed that the EEP of the USM sample is the one that most affects the viability with respect to the other treatments. In all EEP treatments, the highest concentrations, 50 and 100 µg/mL, at 72 hr, did not significantly differ in their cytotoxic effect on OSCA-8 cells. Likewise, the FUS extract at 24 and 48 hr, at the highest concentration, caused the least cytotoxicity compared to the other treatments (Figure 2).

The cytotoxic effect of the five EEPs on fibroblasts is similar to that observed in osteosarcoma cells. The extracts of MET and USM reduce the viability of fibroblasts, except for the MET extract, which, even with the highest concentrations, does not reduce the viability of the fibroblasts after 48 hr. In this same period of time, the USM extract at 50 µg/mL is the most cytotoxic. At 72 hr, the CAJ and SIL extracts, at a concentration of 25 µg/mL, decrease the viability of OSCA-8 cells more than fibroblasts, whereas the USM extract at 25 µg/mL increases the viability of OSCA-8 cells more than fibroblasts. It is also observed that the effect of EEP FUS is similar in both types of cells at 72 hr, in the four concentrations. However, it is observed that there is significant variability between the data in the treatments with this extract.

Cell death assay-LDH

The cytotoxicity of the five EEPs on OSCA-8 cells was evaluated using the LDH method, which measures cell death by the presence of this enzyme in the extracellular medium. The cytotoxicity is closely related to concentration and exposure time in all cases. It is noted that the least cytotoxic extract, compared to the others, was MET at the lowest concentrations, 25 and 50 µg/mL. USM extract at a concentration of 50 µg/mL is more cytotoxic than the other extracts at the same concentration, but the effect is more evident at 72 hr. The FUS extract was the extract that caused the least cell death between 48 and 72 hr, with the exception of 100 µg/mL concentrations (Figure 3).

It is observed that cell death measured by the LDH assay in canine fibroblasts from primary culture is dependent on the concentration of propolis and the time of action, being greater at 72 hr and at concentrations of 50 and 100 µg/mL. The USM and FUS samples were the most likely to cause cell death in fibroblasts at 48 hr, at all concentrations tested. After 72 hr and at concentrations of 25 and 50 µg/mL, the USM extract also turned out to be the most cytotoxic; however, at the highest doses of 50 and 100 µg/mL, the MET extract was the one that caused the most damage. According to the LDH test, the cytotoxic effect of the five Colombian EEPs is lower on the OSCA-8 cells, especially at 48 hr and more by the MET, CAJ and MET. At 72 hr, the CAJ and SIL extracts are still less toxic than the four EEP concentrations tested. All concentrations except 50 µg/mL show considerable variation in the FUS extract data (Figure 3).

The expression of genes related to cell cycle and inflammatory response

In the OSCA-8 cells, relative expression of the genes associated with inflammation, IL-6, and IL1-R2, was found to be higher in EEP extracts from the USM, CAJ, and SIL samples after 48 hr of incubation. The increase in expression was particularly significant for the interleukin 1 receptor type 2 (IL1-R2) gene. The CAJ and SIL samples had the biggest effect on the expression of genes related to the inflammatory process. The EEP MET did not significantly modify the expression of either of the two genes (Figure 4). However, the expression of the IL-6 gene in the fibroblasts by the EEP USM did not change significantly; otherwise, it occurred with the IL1-R2 gene, which significantly increased its expression after exposure to EEP USM.

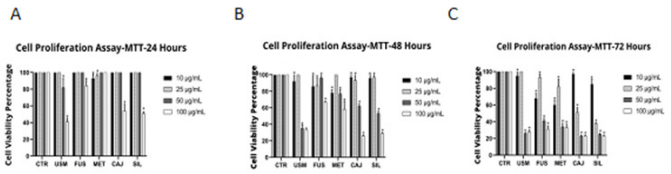

Figure 2: The cytotoxic activity of the five Colombian EEPs was evaluated at 10, 25, 50 and 100 µg/mL on OSCA-8 cells at 24 (A), 48 (B) and 72 (C) hr, measured using the MTT. The negative control was considered to be 100% viability. The results were expressed as the mean±standard deviation of quadruplicates (*n*=4), * indicates *p*<0.0001, compared to negative control

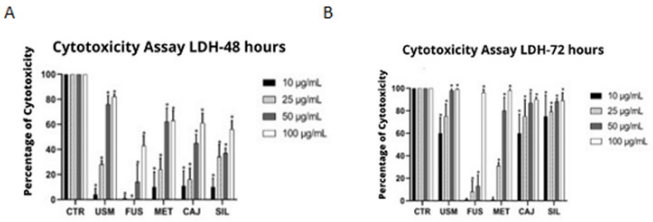

Figure 3: The effect of five Colombian EEPs on cell death in OSCA-8 cells was measured by the LDH assay in two periods of time 48 (A) and 72 hr (B). The positive control was considered to be cells that had died 100% of the time. Results are expressed as the mean standard deviation of quadruplicates (*n*=4). * Indicates *p*=0.0001 compared to the positive control.

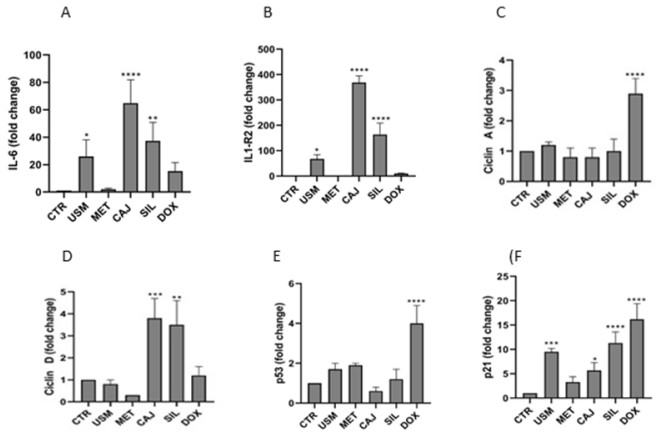

Figure 4: Four Colombian EEPs (USM, CAJ and SIL: 25 µg/mL; MET: 50 µg/mL) were evaluated after 48 hr of exposure, on the relative expression of the (A) IL-6 and (B) IL-1R2. (C) CCNA2; (D) CCND1; (E) TP53; (F) CDKN1A The concentration of doxorubicin was 0.25 µM. Results were expressed as the mean±standard deviation of triplicates (*n*=3), **** indicates *p<0.0001*; ** indicates *p<0.001*; * indicates *p<0.05*, compared to the control whose relative expression was considered to be a value of 1, which were the cells not exposed to any treatment. The GADPH gene was used as a normalizing gene.

The four extracts did not significantly affect the expression of the gene encoding cyclin A (CCNA2), whereas the positive control, that is, doxorubicin, increased significantly. The relative expression of the CCND1 gene was enhanced by the action of extracts from the CAJ and SIL samples. In contrast, EEP USM and MET do not significantly alter the expression of this gene regarding the negative control. The EEP, USM, MET and SIL extracts increase the expression of the TP53 gene in OSCA-8 cells, although not significantly, while doxorubicin significantly increases the expression of this gene. The only treatment that downregulated the relative expression of the gene encoding the p53 protein was CAJ. Finally, the SIL, USM and CAJ extracts were treatments that increased the amount of the gene that makes p21 (CDKN1A). MET did not cause big changes. The positive control treatment (DOX) was the one that significantly increased the expression of this gene (Figure 4).

The relative expression of genes related to the cell cycle in fibroblasts after treatment with the Colombian EEP USM indicates that the only one that was down-regulated was the mRNA of the cyclin A gene. On the other hand, the genes that make cyclin D, p53, and p21 increased their expression after 48 hr of exposure.

Gene characterization of fibroblasts by microarray

Based on the literature, a review of genes that are characteristically expressed in fibroblasts of the Homo sapiens species was carried out; some of them are used as specific markers of these cells, such as P4HA1 and the genes that code for collagen (different isoforms), including COL1A2. All the equivalents of these genes were found in the microarray performed on canine fibroblasts from primary culture (Table 2). These results deepen the different procedures carried out to characterize the cells obtained and used as control in this study. However, in some cases different isoforms were found, possibly because they were genes belonging to a different species.

DISCUSSION OF RESULTS

The biological activity of propolis is diverse and directly related to its chemical composition and the interaction between its components, which results in a synergistic action.[40] There is no information available on the ethnopharmacological activity of Colombian propolis associated with the antitumor effect, except for our own previous reports in which it was shown that EEP induced early and late apoptosis, related to the alteration of mitochondrial membrane potential, likewise, a significant decrease in the invasion, in canine osteosarcoma cells.[41,42]

In the first place, the characterization consisted in evaluating proliferation, the exponential phase was established between 72 and 96 hr, a period of time in which the cell confluence was close to 70%, which could suggest that contact stimulates cell growth. These results coincide with tests carried out in the characterization of primary culture fibroblasts cultured with 10% SFB by other authors.[43] The findings were consistent with what has been reported in the literature, which describes a morphology that is not homogeneous among the same population of cells, but in general, they are predominantly elongated cells with short, thin, or irregular morphology.[39] Finally, we determined vimentin with immunocytochemistry, since it is used as a mesodermal biological marker, useful for the identification of fibroblasts. Our control cultures were positive for this biomarker.[44] Fibroblasts also have different morphological characteristics depending

Table 2: Characteristic genes of the expression profile of dog fibroblasts obtained by microarray.

Gene	Protein
P4HA1	Prolyl-4-hydroxylase[34,35]
COL1A2*	Collagen, type 1, alpha 2[36]
PCOLCE2	Procollagen C-endopeptidase enhancer[36]
MME	Membrane metallo-endopeptidase[37]
ALDH7A1**	Aldehyde dehydrogenase 7 family, member A1[37,38]
SLC2A3	Solute carrier family 2 (facilitated glucose transporter], member 3[37,38]
MATN2	Matrilin 2[37,38]
MXI1	MAX interactor 1, dimerization protein[37,38]
ANGPTL4***	Angiopoietin-like 4[37,38]
FGFR1	Fibroblast growth factor receptor 1[37]
FGF1	Fibroblast growth factor 1 [37,38]
DST	Dystonin[37]
TPM1****	Tropomyosin 1[37]
MAP2K6	Mitogen-activated protein kinase kinase 6[37]
FBN1	Fibrilin 1[37,39]
FBN2	Fibrilin 2[37,39]
EFEMP2	Fibulin 4[37,39]
LAMA4	Laminin, Alpha 4 [36-38]
GOLGA8B*****	Golgin A8 family, member B[37,38]

* In the microarray used for the characterization of the fibroblasts, other isoforms of the gene that codes for collagen were found: COL11A1, COL12A1, COL14A1 and COL15A1.** The genes: ALDH9A1 and ALDH2 were found in the microarray.*** The ANGPTL2 and ANGPTL6 isoforms were found in the microarray.**** The TPM2 isoform was found in the microarray.***** The GOLGA4 and GOLGA3 isoforms were found in the microarray.

on whether they are active or inactive. In the latter case, active fibroblasts have a bigger size and more organelles, such as those in the extracellular matrix.

With the characterization of the gene expression profile through the microarray, the expression of genes characteristic of fibroblasts was confirmed, comparing the profile of cells without being exposed to any treatment with a review in the literature of fibroblasts of the human species. This extrapolation was made.[45] Several cell lines from the species *Canis lupus* familiaris have served as alternative models for the study of human diseases, including various types of cancer, such as osteosarcoma.[46]

Five Colombian EEP samples (USM, FUS, MET, CAJ and SIL), taken from regions with different flora characteristics, were previously characterized regarding chemical composition using GC-MS.[18] Ethanol, tested in three different periods of time, did not significantly affect cell viability, compared to controls (doxorubicin), which suggests that the solvent used to prepare the extracts is not the cause of the cytotoxic effect. The effect of the EEP on OSCA-8 cells was antiproliferative, reducing cell viability in a dose- and time-dependent manner. This indicates that the stoichiometric relationship is proportional to cytotoxicity. These results are consistent with previous reports describing the anti-porliferative activity of EEP in different cancer cell lines. Red propolis extracts from Brazil were found to be toxic to pancreatic cancer cells at a concentration of 10 μg/mL,[47] and to human cervical adenocarcinoma cells at a concentration of 7.5 μg/mL.[48] The extract also showed cytotoxic activity against human bladder carcinoma cells at a concentration of 95 μg/mL,[49] to human cervical adenocarcinoma (HeLa) cells at a concentration of 7.5 μg/mL[48] and to human leukemia cell lines: K562, HL60, NB4, RS4, B15 and REH. Also have a cytostatic and apoptosis-inducing effect at concentrations between 10 and 70 μg/mL.[50]

Green, brown, and red propolis from Brazil also killed cancer cells at concentrations ranging from 50 to 100 μg/mL.[51] The cytotoxic effect of ethanolic extracts of propolis has also been tested in other *in vitro* models of canine osteosarcoma, specifically in the spOS-2 cell line.[52] Results of decreased cell viability were observed in this model. It was possible to identify that the combination of drugs such as doxorubicin, carboplatin and cisplatin with EEP could be a more effective treatment of canine osteosarcom in these same types of cells. Study of cytotoxic activity *in vitro* in culture of U2OS and Saos-2 osteosarcoma cells has shown that CAPE has anti-proliferative activity in concentrations similar to complete propolis extracts, 50-60 and 30-40 μg/mL, respectively.[41,53]

The USM extract was the one that generated the most cytotoxicity in OSCA-8 cells at concentrations of 50 µg/mL and 100 µg/mL at 48 hr, which is consistent with the cell death assay. This extract was characterized by its composition of flavonoids and diterpenes. It has been considered that flavonoids are anticancer agents since they have proapoptotic, antiproliferative, and antioxidant effects.[54] Flavonoids are the major components of propolis, and a large part of its pharmacological activity is attributed to them.[55] The MET extract caused a big cytotoxic effect on OSCA-8 cells, as shown by both tests (MTT and LDH). This sample was found to have a high content of benzophenones, especially nemorosone. This compound has been linked to an anticancer effect in different cell lines, such as breast cancer (MCF-7), which involves molecular mechanisms such as blocking the cell cycle in the G0/G1 phase.[56] In prostate cancer cells, it has also been shown to cause proapoptotic events. However, prenylated benzophenones such as nemorosone have had less cytotoxic effect on non-cancerous cells. It has been shown that the inhibition of proliferation in pancreatic cancer cells is due to the presence of prenylated benzophenones. These results match what we found in our study. The effect of the Colombian EEP on canine fibroblasts and OSCA-8 cells was determined by the MTT method at 48 hr. Only at a concentration of 100 µg/mL decreased cell viability in fibroblasts, while in OSCA-8 cells at 10, 50 and 100 µg/mL. Cell death in OSCA-8 cells oscillates approximately between 25–65% at concentrations from 25 to 100 µg/ml, while cell death in fibroblasts does not exceed 50%.[57]

The two EEPs identified as USM and MET, which contain a significant amount of diterpenes in their composition, compared to the other samples, also showed a greater cytotoxic effect on OSCA-8 cells than on fibroblasts, measured by both MTT and LDH. Diterpenes have been attributed to biological effects such as antioxidant, anti-inflammatory, and anticancer, among others. This is why they have been considered promising for obtaining drugs. Some extracts of green propolis from Brazil, rich in ethyl acetate, have shown cytotoxic activity against different lines of lung (A549), liver (HepG2) and cervical (SW756) carcinoma. The concentrations at which they present said effect vary between 1.8 and 6.3 µg/mL. In HeLa cells, the tumoricidal action was at a mean concentration of 87 µg/mL.[58]

All the extracts caused more cell death in OSCA-8 cells than in fibroblasts at 48 hr. At 72 hr, USM, CAJ and SIL induced greater cell death. With the MTT method, the decrease in cell viability is not as apparent at 48 and 72 hr, but EEP is also observed to be more cytotoxic in OSCA-8 cells than in fibroblasts. This result could suggest that there is a selective and differential effect between cancerous and non-cancer cells. Moreover, the results suggest that the IC_{50} of EEP in fibroblasts is higher than in OSCA-8 cells.

The anti-inflammatory effect of propolis and some of its constituents is reported in the literature. Polyphenols, especially, are attributed to antioxidant action due to the presence of hydroxyl groups that neutralize the activity of free radicals. This is one of the mechanisms by which participates in the regulation of the inflammatory process.[59] The inflammatory process involves a series of events that involve the participation of cytokines such as TNF-α, IL-6, and IL-1β, as well as chemokines and lipid mediators such as leukotrienes.[60] When the chronic inflammatory process is present, it can be an activating factor for tumor initiation and subsequent uncontrolled cell proliferation and even metastasis. The role of IL-6 is dual, and evidence suggests that it induces signaling cascades associated with NFkB and the MAPK family that favor proliferation in cancer cells in the tumor microenvironment. Moreover, the pleiotropic activity of this cytokine allows it to behave as a tumor immunomodulator, where it stimulates the activation, mobilization, and infiltration of immune system cells such as $CD8^+$.[61] The expression of the genes encoding IL-6 and IL1-R2 was evaluated by exposure to Colombian EEP in OSCA-8 cells. The extracts of CAJ, SIL, and USM significantly increased the expression of these genes. These results match those of studies that show that propolis in human dendritic cells activates the signaling pathway mediated by NFkB that makes IL-6, TNF-α, and IL-10.[59] It has been shown that an increase in the number of surface receptors for interleukins, such as IL1-R2, increases the production of IL-6 and VEGF.[62]

The di and triterpenes, important constituents of the CAJ and SIL samples, might affect the cell expression profile by raising the expression levels of Toll-Like Receptors (TLR), in addition, it has been seen that some propolis stimulate the chemotactic activity of neutrophils. The Brazilian propolis has anticancer activity associated with this mechanism against different prostate cell lines, including LNCaP, at concentrations of 6.2 uM.

These findings could suggest that an increase in inflammatory mediators such as IL-6 and IL1-R2 would have an effect on antitumor immunity with OSCA-8 cells. Furthermore, it is unlikely that the cytotoxic activity of the EEPs of CAJ and SIL would be associated with inflammation mechanisms. Further trials are required to test this hypothesis with cells of the immune system such as lymphocytes and macrophages. The effect of the USM sample on the expression of the IL-6 and IL1-R2 genes was not the same in fibroblasts as in OSCA-8 cells. They suggest that the expression profiles of at least these two genes are modified according to cell type. Likewise, the expression patterns of cancer and non-cancer cells behave differently and therefore the effect of compounds such as propolis is different.

Another mechanism associated with the antiproliferative action of propolis in cancer cells is the blocking of the cell cycle. After exposure to the 4 treatments, the CDKN1A gene changed the most in OSCA-8 cells. The effects of the SIL, USM, and CAJ extracts were the most significant. Exposure to EEP USM, MET, and to a lesser extent SIL increased the expression levels of the TP53 gene. Expression levels of cyclin A did not change in OSCA-8 cells with any of the treatments, and those of cyclin D were significantly

increased with the CAJ and SIL extracts. These results suggest that cell cycle progression in canine osteosarcoma cells could be interfered with in the G1/S phase, which is dependent on the inhibitory action of p53, but to a greater extent on p21, and independent of cyclin D.

In fibroblasts, as in OSCA-8 cells, the USM treatment increased the expression of p53 and p21. However, in both cases, the expression of p21 was higher. This result suggests that the mechanism of action of Colombian USM EEP in both cancer and non-cancer cells is probably the same, inducing cell cycle arrest in G1/S. It also suggests that the increase of p21 to some extent could be independent of p53. Proteins other than p53 regulate p21, for example, BRCA1, is a human tumor suppressor gene that regulates the cell cycle, induces an increase in intracellular p21 levels, FOX proteins bind to the region promoter of the CDKN1A gene, in HCT-116 cells and increase its expression.[63] In HCT-116 (p53-/-) colorectal cancer cells, the expression of the CDKN1A gene was increased by exposure to doxorubicin via NFκB, specifically by the p65 subunit.[64]

Propolis and some flavonoids, such as CAPE, have been shown to induce the arrest of the cell cycle. CAPE, for example, managed to increase the levels of p53, p21 and p27 proteins in human colorectal cancer cells, HT-29, HCT-116 and SW480, at 48 hr with a dose-dependent effect.[65] In other studies, it was found that CAPE induces apoptosis through the activation of Bax, p53 and p21, mediated by kinases of the MAPK family (p38, ERK and JNK) in C6 glioma cells.[66] Other compounds derived from propolis, such as propolin G, were able to reduce the proliferation of human breast cancer cells (MCF-7 and MDA-MB-231) and rat glioma cells (C6), at a dose between 8.5 and 10.3 μM, the proposed mechanism was based on the increase in the expression of the TP53 gene and CDKN1A.[67] Propolis extracts from Taiwan have been associated with an increase in the expression of genes such as CDKN1A and TP53. These genes are related to a potent antitumor activity and therefore to the inhibition of tumor growth. According to the expression assay, the positive control, doxorubicin, is observed in OSCA-8 cells. It seems to slow down the cell cycle in the G1/S phase dependent on the action of p53/p21The increase in cyclin A induced by DOX suggests that the transition in the G2/M phase is not altered. These results are consistent with the mechanism of action of doxorubicin reported in U2OS and MG-63 human OSA cells.[68]

Overall, the five EEPs presented a greater cytotoxic effect on OSCA-8 cells, compared to control fibroblasts. This was further evidenced by the cell death assay (LDH), which could indicate a more potent effect. The first time in Colombia, the biological activity of ethanol extracts of propolis was evaluated regarding the inflammation and cell cycle pathways, using the qPCR technique for the quantification of relative gene expression. After 48 hr, the Colombian EEP had an effect on the increase in both OSCA-8 cells and fibroblasts. The effect was greater in the cell line, which generates a perspective towards the evaluation of more genes involved in the inflammatory pathway to establish if the effect could be pro or anti-inflammatory. The cytotoxic effect induced by Colombian EEPs after 48 hr of exposure in OSCA-8 cells and fibroblasts could be related to a block in the G1/S phase transition mediated by the expression of the CDKN1A gene.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

OSCA: Osteosarcoma; **NK:** Natural Killer; **EEP:** Ethanol extracts of Colombian propolis; **LDH:** Lactate dehydrogenase; **RNA:** Ribonucleic acid; **DNA:** Deoxyribonucleic acid; **PCR:** Polymerase chain reaction; **USM:** Usme; **FUS:** Fusagasugá; **MET:** Meta; **CAJ:** Cajibío; **Sil:** Sivia; **IC_{50}:** Half maximal inhibitory concentration.

SUMMARY

Propolis has anti-inflammatory, antitumor, antibacterial and immunomodulatory properties, which is why it is suggested that it could be used as an alternative or complementary drug therapy in the treatment of cancer, diseases. The evaluation of the relative expression of some genes associated with the cell cycle and inflammation could improve the understanding of the cytotoxic effect of propolis extracts on OSCA-8 cell lines. The first time in Colombia, the biological activity of ethanol extracts of propolis was evaluated regarding the inflammation and cell cycle pathways.

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