Biomedicine & Pharmacotherapy 104 (2018) 268–274

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

Positive effects of antitumor drugs in combination with propolis on canine osteosarcoma cells (spOS-2) and mesenchymal stem cells

Pedro Negri Bernardino[a], Paulo Ricardo Oliveira Bersano[b], João Ferreira Lima Neto[c], José Maurício Sforcin[a,*]

[a] São Paulo State University (UNESP), Institute of Biosciences, Campus Botucatu, Brazil
[b] Laboratory of Pathology and Legal Veterinary Medicine, Faculty of Veterinary Medicine, State University of Ceará, Fortaleza, CE, Brazil
[c] Pharmaceutical-Biochemistry Technology Department, School of Pharmaceutical Science, University of São Paulo, São Paulo, SP, Brazil

ARTICLE INFO

Keywords:
Propolis
Osteosarcoma
Cytotoxic action
Apoptosis

ABSTRACT

The combination of lower concentrations of antitumor drugs (carboplatin – CARB, doxorubicin – DOX, and methotrexate – MET) with propolis was investigated against canine osteosarcoma (spOS-2) and mesenchymal stem cells (MSC) *in vitro*. The mechanism of action in the combinations was analyzed. spOS-2 cells were incubated up to 72 h with propolis (50 μg/ml) alone or in combination with CARB (10–400 μmol/l), DOX (0.5–2 μmol/l) or MET (50–200 μmol/l). Cell viability was assessed by MTT assay, apoptosis/necrosis by flow cytometry, and MSC was incubated with the optimum combination. Propolis alone exerted no cytotoxic action against spOS-2 cells, whereas CARB (400, 200 and 100 μmol/l) exhibited the highest cytotoxic effects comparing to DOX and MET. The combination of propolis with the lowest concentrations of CARB led to better results comparing to CARB alone, which was not observed using DOX and MET. Apoptosis was involved in the action of propolis + CARB in spOS-2 cells. MSC were not affected by CARB/propolis, indicating that the cytotoxic action of the combination was specific to tumor cells but not to normal ones. Propolis improved the action of CARB against spOS-2 cells using lower concentrations of this drug, without affecting MSC. These findings are relevant and indicate a possible application of propolis in OSA treatment.

1. Introduction

Propolis has been used in folk medicine for centuries showing uncountable biological activities, such as antitumor, immunomodulatory, anti-inflammatory, antimicrobial, antioxidant and others [1]. The ancient Greeks, Romans, and Egyptians were aware of the healing properties of propolis and made extensive use of its medicinal properties. In the middle ages, propolis was not very popular and its use in mainstream medicine declined. However, the knowledge of its medicinal properties survived in traditional folk medicine. Interest in propolis rearose in Europe during the renaissance [2]. Several works have been carried out *in vitro* and *in vivo* demonstrating propolis potential for the development of new antitumoral drugs [1,3].

Canine cancers may occur spontaneously showing a similar clinical pathophysiology to equivalent human cancers. Osteosarcoma (OSA) or osteogenic sarcoma is a primary bone neoplasm frequently diagnosed in dogs [4]. The biological behavior of OSA is aggressive and initially restricted to the bone microenvironment, with metastatic progression [5]. Although relatively rare in humans, the rate of canine OSA is 75× higher than that of humans. High risk breeds for developing canine osteosarcoma tend to be some of the larger and giant dogs, including the Rottweiler, Great Pyrenees, mastiff, Dobermann pinscher, Irish wolfhound, Scottish deerhound. Working across species, researchers may combine scientific findings to understand the origins of cancer in order to develop novel therapies to benefit both human and animals [6].

Chemotherapeutic agents such as carboplatin (CARB), cisplatin, and doxorubicin (DOX) have been used as a therapy in veterinary medicine to induce remission of the tumor [7]. However, the outcome of chemotherapy in dogs is unpredictable and may result in failure to respond to cytotoxic drugs [8]. Therapeutic studies involving *in vitro* models have used primary cultures rather than monoclonal neoplastic cell strains, because the primary culture is more similar to what occurs in patients, by presenting different neoplastic clones. For this reason, the primary culture became an excellent model for research in oncology [9]. In the search of new anticancer drugs, propolis has been pointed out because of its cytotoxic action *in vitro* towards different tumor cells. The main mechanisms of its antitumor effects *in vitro* involve apoptosis,

* Corresponding author.
E-mail address: sforcin@ibb.unesp.br (J.M. Sforcin).

https://doi.org/10.1016/j.biopha.2018.05.027
Received 26 March 2018; Received in revised form 3 May 2018; Accepted 8 May 2018
0753-3322/ © 2018 Elsevier Masson SAS. All rights reserved.

cell cycle arrest and interference on metabolic pathways [10,11]. Besides, propolis exhibited an immunomodulatory action in tumor-bearing mice [12,13].

There are different types of propolis in Brazil such as green, red and brown propolis, and we used a sample collected in the Beekeeping Section of the University that was previously analyzed by chromatographic techniques. The main constituents of this green sample were phenolic compounds (flavonoids, aromatic acids and benzopyranes), diterpenes and triterpenes and essential oils, among others [1,3]. The effects of propolis were investigated in combination with lower concentrations of three different anticancer drugs (CARB, DOX and methotrexate – MET), in order to verify whether such combinations could reach the same efficiency of the drug alone. The mechanisms of action of propolis alone or in combination were also investigated. The effects using the best combination were assayed in mesenchymal stem cells (MSC).

2. Material and methods

2.1. Ethical aspects and spOS-2 primary cultures

Tumor fragments were taken from a dog in the Veterinary Hospital and FMVZ Veterinary Pathology Service – UNESP, *Campus* of Botucatu, Brazil. The dog's owner was informed about this study, permitting the publication of the data after signing a Free and Informed Consent Form.

Cells were transferred to 25 cm^2 flasks containing Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich Quimica, Madrid, Spain), supplemented with 10% fetal bovine serum (Gibco, Paisley, UK), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B. Primary cultures of canine osteosarcoma were categorized according to a biochemical panel by alizarin red and by target proteins such as vimentin, cytokeratin, osteocalcin, osteopontin, osterix, and cyclo-oxygenase-2 (Cox2) by flow cytometry. Cox2 was also evaluated by immunohistochemistry and separated into Cox2-positive or Cox2-negative cultures. Thus, spOS-2 refers to a culture with upregulated Cox2 expression.

This study was approved by the FMVZ Ethics Committee – UNESP (protocol 98/2008).

2.2. Mesenchymal stem cells

MSC were kindly provided by the Biobank of Cells of the Laboratory of Investigative and Comparative Pathology, School of Veterinary Medicine and Animal Science, UNESP.

2.3. Propolis, antitumor drugs and their combinations

Propolis was collected in the Beekeeping Section, UNESP, Campus of Botucatu. After grinding, ethanolic extracts of propolis were prepared (30 g of propolis, completing the volume to 100 ml with 70% ethanol) and left for a week in the absence of bright light at room temperature, under moderate shaking. Extracts were filtered and their dry weight was calculated (130 mg/ml).

Propolis was used in different concentrations: 50, 25 and 10 µg/ml, according to previous standardization in our laboratory [10]. Carboplatin, doxorubicin, and methotrexate were purchased from Vancel®-Darrow Laboratórios S/A and their concentrations were defined according to literature: CARB = 400, 200, 100, 50, 25 and 10 µmol/l; DOX = 2, 1 and 0.5 µmol/l [4,14–16], and MET = 200, 100 and 50 µmol/l [17–20].

Propolis and the drugs were diluted in DMEM as described above.

In order to verify the effects of propolis in combination with the drugs, propolis was added to CARB, DOX and MET using the concentrations that affected spOS-2 cell growth without leading to cytotoxic effects near 0%, comparing cell viability to the effects of the drugs alone.

In vitro assays were carried out in triplicate.

2.4. Viability assay

After detachment from the flasks using trypsin, the cells were cultivated in a 96 wells U-bottomed plate (Corning) at a final concentration of 2×10^4 cells/ml, adding 100 µl/well. After 24 h incubation for cells adherence, the supernatant of cell culture was discarded and the researched solution was added. The same procedures were performed with 70% ethanol (propolis solvent), using 0.385, 0.1925 and 0.077 µl 70% ethanol/ml, corresponding respectively to the concentration present in 50, 25 and 10 µg/ml of the ethanolic extract of propolis.

To evaluate the effects of the combinations, propolis (50 µg/ml) was added to CARB (50, 25 and 10 µmol/l), to DOX (2 and 1 µmol/l), and to MET (50 and 25 µmol/l). The stimuli (100 µl) were added to the 96-well plates and control cells only contained the medium. Assays were carried out in triplicate and cell viability was assessed after 6, 24, 48 and 72 h.

Cell viability was performed using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT – Sigma-Aldrich, USA) colorimetric assay. After each period of time, supernatants of cell cultures were discarded and 100 µl of MTT (1 mg/ml) was added to the culture cells for 3 h. Afterwards, MTT was taken out and 100 µl of dimethylsulfoxide (DMSO) was added to dissolve the formazan salt. Optical densities (O.D.) were read at 540 nm in an ELISA reader and the percentage of cell viability was calculated using the formula: [O.D. test / O.D. control] × 100. Assays were carried out in triplicate, comparing the effects to control in the respective time scale.

Cell viability was also used to evaluate cell growth after incubation with stimuli, comparing the effects over time to the control at 6 h, to verify a cytotoxic or cytostatic action.

Mesenchymal stem cells were cultivated similarly to OSA cells and MTT test was used to determine cell viability, in order to verify whether the cytotoxic action of the combination was specific to tumor cells but not to healthy ones.

2.5. Evaluation of necrosis/apoptosis by flow cytometry

In this assay, only the combinations that exhibited the highest activities were assessed. Thus, only propolis (50 µg/ml), CARB (10, 25 and 50 µmol/l) and their combinations were evaluated.

Cells (2×10^5 cells/ml) were cultured in 24 well plates adding 300 µl of DMEM in each well. After 24 h for cell adherence, supernatants were removed and stimuli were added in a final volume of 1 ml for 6, 24, 48 and 72 h. Afterwards, supernatants were collected and put in cytometry tubes without discarding dead cells, and trypsin (250 µl) was added to the wells for 1 min at 37 °C. Plates were shaken carefully, 250 µl of fetal bovine serum was added and the final volume transferred to the corresponding tubes.

Subsequently, tubes were centrifuged for 10 min at 200 × *g*, supernatants were discarded, and the pellets were resuspended in 200 µl PBS pH 7.4 containing Ca^{+2} and Cl–. This content was divided into two tubes: one tube was used as negative control and no substance was added after this procedure and, to another tube, 5 µl of annexin V (A13201 – combined with Alexa Fluor 488 – Invitrogen) was added. After 30 min, 1 µl of propidium iodide (PI) was added and the sample was homogenized. After 10 min, the tubes were transferred and read in the flow cytometer FACS Calibur BD TM.

The dot plot system identified living cells, apoptotic cells and necrotic cells and those with double reading. Data was based on a sample of 10,000 cells and expressed in percentages [21].

2.6. Statistic analysis

Profile analysis was employed to compare the groups and moments, and to evaluate a possible interaction moment x group. The significance level of 5% was adopted.

Table 1
Viability (%) of canine osteosarcoma cells (spOS-2) after incubation with different concentrations of propolis, doxorubicin, methotrexate and carboplatin comparing to control in the respective time scale.

Treatment	Period of time				
	Concentration	6 h	24 h	48 h	72 h
Propolis	10 µg/ml	96.0 ± 6.9	92.7 ± 11.0	93.7 ± 3.2	99.0 ± 1.7
	25 µg/ml	100.0 ± 0.0	90.3 ± 12.7	94.3 ± 5.1	100.0 ± 0.0
	50 µg/ml	92.3 ± 2.9	83.3 ± 2.7	92.0 ± 5.0	99.7 ± 0.6
Doxorubicin	0.5 µmol/l	96.7 ± 4.2 ^{A}	91.3 ± 5.0 ^{A}	92.7 ± 0.6 ^{A}	98.3 ± 1.5 ^{A}
	1 µmol/l	91.7 ± 6.5 ^{A}	85.7 ± 5.5 ^{A}	91.7 ± 6.4 ^{A}	84.0 ± 9.5 ^{A}
	2 µmol/l	94.7 ± 6.1 ^{A}	71.7 ± 11.5 ^{B}	79.0 ± 7.0 ^{B}	59.3 ± 3.5 ^{B}
Methotrexate	50 µmol/l	100 ± 0.0 ^{A}	68.7 ± 9.0 ^{B}	84.3 ± 3.2 ^{A}	38.7 ± 1.5 ^{C}
	100 µmol/l	87.0 ± 19.0 ^{A}	79.0 ± 19.0 ^{B}	81.7 ± 5.0 $^{A/B}$	39.7 ± 2.3 ^{C}
	200 µmol/l	90.0 ± 8.4 ^{A}	67.3 ± 8.4 ^{B}	81.0 ± 6.2 $^{A/B}$	40.0 ± 2.6 ^{C}
Carboplatin	10 µmol/l	100.0 ± 0.0 ^{A}	96.0 ± 4.0 ^{A}	97.7 ± 3.2 ^{A}	71.7 ± 4.2 ^{B}
	25 µmol/l	96.0 ± 6.9 ^{A}	100.0 ± 0.0 ^{A}	92.3 ± 11.6 ^{A}	43.7 ± 3.1 ^{C}
	50 µmol/l	100.0 ± 0.0 ^{A}	95.0 ± 8.7 ^{A}	92.7 ± 7.0 ^{A}	41.0 ± 2.6 ^{C}
	100 µmol/l	82.0 ± 4.4 $^{A/B}$	18.7 ± 4.0 $^{C/D}$	3.3 ± 0.5 ^{D}	2.7 ± 0.2 ^{D}
	200 µmol/l	78.0 ± 10.1 ^{B}	12.7 ± 3.1 $^{C/D}$	3.6 ± 0.2 ^{D}	2.9 ± 0.1 ^{D}
	400 µmol/l	67.3 ± 4.0 ^{B}	17.7 ± 1.2 $^{C/D}$	4.1 ± 0.6 ^{D}	2.7 ± 0.1 ^{D}

Data represent means ± standard deviation of 3 similar assays, in triplicate. Different letters indicate significant differences among the groups and moments in the same treatment ($P < 0.0001$).

3. Results

3.1. Effects of propolis and the drugs on OSA cell viability

Propolis alone did not exert a cytotoxic action towards spOS-2 cells irrespective of its concentration and time scale (Table 1). 70% ethanol, in the percentages equivalent to propolis concentrations, had no effect on OSA cell viability (data not shown).

CARB, DOX and MET exerted an inhibitory action on spOS-2 cells. DOX was less efficient and its action occurred after 24 h, reaching 59.3% of viable cells using 2 µmol/l after 72 h comparing to control in this time period. The other concentrations (0.5 and 1 µmol/l) did not affect cell viability. There was an effect of group, of moment and an interaction moment × group ($P < 0.0001$). MET was more effcient than DOX, exhibiting a cytostatic action till 48 h and a cytotoxic action after 72 h ($P < 0.0001$), when cell viability reached approximately 40% in all concentrations (Table 1).

CARB exhibited the best inhibitory action against spOS-2 cells. The highest concentrations (100, 200 and 400 µmol/l) exerted a potent cytotoxic activity over time: cell viability was around 67–82% after 6 h, lower than 20% after 24 h, and 2–3% after 48 and 72 h. Initially, only the concentrations 100, 200 and 400 µmol/l were analyzed; however, such concentrations affected cell viability so that lower concentrations (10, 25 and 50 µmol/l) were further investigated to observe from which concentration CARB could affect cell growth. CARB (10 µmol/l) affected cell viability only after 72 h. Using 25 and 50 µmol/l, there was a significant difference from 10 µmol/l at 72 h, and an interaction moment × group ($P < 0.0001$) (Table 1).

The percentage of control cells was compared in all time periods to control at 6 h to observe cell growth and to identify whether cell viability remained similar to the initial percentage (cytostatic action) or whether cells died (cytotoxic action). One may verify in Fig. 1 that cells grew over time after incubation with propolis. DOX (2 µmol/l) exerted only a cytostatic action after 72 h, and MET (all concentrations) after 24 and 72 h ($P < 0.0001$). CARB exhibited an inhibitory effect on cell growth using the 3 highest concentrations, and spOS-2 cell growth was similar to control up to 48 h using the 3 lowest concentrations ($P < 0.0001$).

3.2. Effects of propolis in combination with the drugs

The highest cytotoxic effects of DOX alone (2 µmol/l) occurred after 24 h. No synergistic effects were seen combining propolis (50 µg/ml) with DOX (1 and 2 µmol/l) comparing to DOX alone (Table 2).

Regarding methotrexate, similar results were obtained using all concentrations, and propolis was added to its lower and half concentration (50 and 25 µmol/l, respectively), to observer a possible cytotoxic action higher than its highest concentrations (100 and 200 µmol/l). No significant differences were seen combining propolis to MET (Table 2).

A positive interaction was seen combining propolis and carboplatin (25 and 50 µmol/l) after 24, 48 and 72 h. Even the lowest concentration of CARB (10 µmol/l) in combination with propolis was significantly different from CARB alone after 48 and 72 h (Table 2). There was an effect of moment, of group and an interaction moment × group.

Overall, comparing all treatments, the highest activities towards spOS-2 cells were: CARB (400, 200 and 100 µmol/l) and CARB (50 and 25 µmol/l) + propolis (50 µg/ml) > MET (200, 100 and 50 µmol/l), MET (50 and 25 µmol/l) + propolis (50 µg/ml), CARB (50 and 25 µmol/l) and CARB (10 µmol/l) + propolis (50 µg/ml) > DOX (2 µmol/l) and DOX (2 µmol/l) + propolis (50 µg/ml) > CARB (10 µmol/l), DOX (0.5 and 1 µmol/l), DOX (1 µmol/l) + propolis (50 µg/ml), and propolis (50, 25 and 10 µg/ml).

3.3. Evaluation of apoptosis/necrosis in spOS-2 cells

In order to understand the main mechanism of action of propolis in combination with CARB, assays were carried out to investigate apoptosis/necrosis after incubation with propolis (50 µg/ml), CARB (50, 25 and 10 µmol/l) and the combinations that exerted the highest cytotoxic effects.

In the isotypic control, there was a high percentage of viable cells (98.5%) and a very low percentage of cells in apoptosis and necrosis (0.25 and 0.3% respectively). One may observe that the main mechanism by which the combination affected spOS-2 cells was the induction of apoptosis (Fig. 2). There was a percentage (5–18%) of necrotic cells probably due to the manipulation of cells, and not due to the

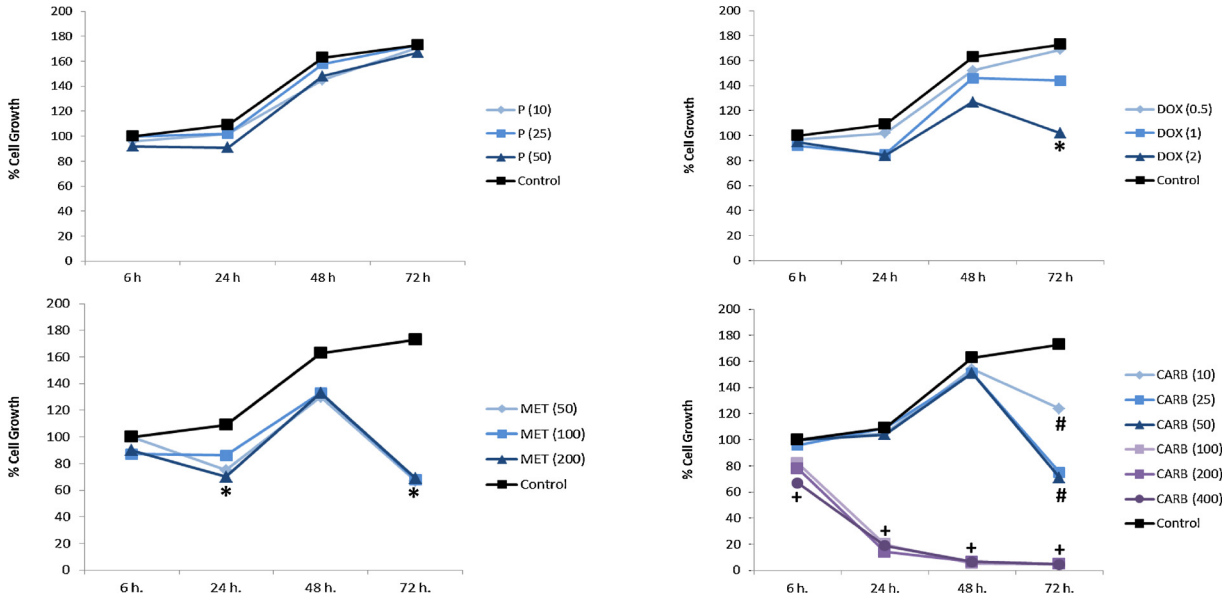

Fig. 1. Percentage (%) of canine osteosarcoma cells (spOS-2) growth after incubation with different concentration of propolis (P – 10, 25 and 50 μg/ml), doxorubicin (DOX – 0.5, 1 and 2 μmol/l), methotrexate (MET – 50, 100 and 200 μmol/l) and carboplatin (CARB – 10, 25, 50, 100, 200 and 400 μmol/l) comparing to control. Data represent means ± standard deviation of 3 similar assays, in triplicate. *significantly different from control at 6 h ($P < 0.0001$). $^{+}$significantly different from the 3 lowest concentrations ($P < 0.0001$); $^{\#}$significantly different from control only after 72 h ($P < 0.0001$).

Table 2
Viability (%) of canine osteosarcoma cells (spOS-2) after incubation with propolis (P – 50 μg/ml) in combination with doxorubicin (DOX – 1 and 2 μmol/l), methotrexate (MET – 25 and 50 μmol/l) and carboplatin (CARB – 10, 25 and 50 μmol/l) comparing to control in the respective time scale.

Treatments and concentrations	Period of time			
	6 h	24 h	48 h	72 h
DOX (1 μmol/l)	91.7 ± 6.5 ^{A}	85.7 ± 5.5 ^{A}	91.7 ± 6.4 ^{A}	84.0 ± 9.5 $^{A/B}$
DOX (2 μmol/l)	94.7 ± 6.1 ^{A}	71.7 ± 11.5 ^{B}	79.0 ± 7.0 ^{B}	59.3 ± 3.5 ^{B}
DOX (1 μmol/l) + P	87.7 ± 7.5 ^{A}	98.0 ± 3.5 ^{A}	87.0 ± 2.6 ^{A}	75.7 ± 5.0 ^{B}
DOX (2 μmol/l) + P	99.3 ± 1.2 ^{A}	99.0 ± 1.7 ^{A}	85.3 ± 3.8 $^{A/B}$	68.7 ± 4.0 ^{B}
MET (50 μmol/l)	100.0 ± 0.0 ^{A}	68.7 ± 9.0 ^{B}	84.3 ± 3.2 $^{A/B}$	38.7 ± 1.5 ^{C}
MET (25 μmol/l) + P	90.0 ± 10.5 ^{A}	78.3 ± 5.5 ^{B}	75.3 ± 6.5 ^{B}	53.3 ± 7.8 ^{C}
MET (50 μmol/l) + P	92.3 ± 8.0 ^{A}	77.3 ± 1.2 ^{B}	85.0 ± 2.0 $^{A/B}$	57.7 ± 7.2 ^{C}
CARB (10 μmol/l)	100.0 ± 0.0 ^{A}	96.0 ± 4.0 ^{A}	97.7 ± 3.2 ^{A}	71.7 ± 4.2 ^{B}
CARB (25 μmol/l)	96.0 ± 6.9 ^{A}	100.0 ± 0.0 ^{A}	92.3 ± 11.6 ^{A}	43.7 ± 3.1 ^{C}
CARB (50 μmol/l)	100.0 ± 0.0 ^{A}	95.0 ± 8.7 ^{A}	92.7 ± 7.0 ^{A}	41.0 ± 2.6 ^{C}
CARB (10 μmol/l) + P	100.0 ± 0.0 ^{A}	86.7 ± 11.6 ^{A}	73.7 ± 4.9 ^{B}	45.3 ± 11.0 ^{C}
CARB (25 μmol/l) + P	96.7 ± 3.1 ^{A}	60.7 ± 5.9 $^{B/C}$	5.7 ± 1.3 ^{D}	2.4 ± 0.1 ^{D}
CARB (50 μmol/l) + P	96.7 ± 4.2 ^{A}	51.7 ± 1.5 ^{C}	3.0 ± 3.0 ^{D}	2.4 ± 0.2 ^{D}

Data represent means ± standard deviation of 3 similar assays, in triplicate. Different letters indicate significant differences among groups and moments of the same drug ($P < 0.0001$).

stimuli, since the amount of necrotic cells is not correlated to the MTT assay, which analysed the cell viability.

On the other hand, Fig. 2 shows that apoptosis was the predominant mechanism for spOS-2 cell death. Initially, the percentage of apoptotic cells was low (3–12%), similar to the percentage of necrotic cells. However, this percentage increased significantly after incubation with propolis + CARB (50 μmol/l) for 48 h and with propolis + CARB (10, 25 and 50 μmol/l) for 72 h, and the values were similar to those achieved by MTT test. This finding corroborates the hypothesis that the main mechanism of action of propolis alone or in combination with CARB towards spOS-2 cells is the induction of apoptosis.

3.4. Effects of propolis + carboplatin on mesenchymal stem cells

Propolis and CARB alone did not affect MSC (Table 3). CARB alone (50, 25 and 10 μmol/l) decreased spOS-2 cell viability (Table 1) without affecting MSC viability (Table 3). Regarding the combinations, propolis + CARB (25 and 50 μmol/l) led to a mild decrease in the viability of MSC only after 48 h (~70%), while spOS-2 cells were severely affected by propolis in combination with all CARB concentrations (Fig. 3).

4. Discussion

Propolis constituents come from substances actively secreted by plants (lipophilic materials in leaves and leaf buds, resins, mucilage, gums, lattices, among others) and chromatographic techniques have been applied to the analysis and isolation of its components. The chemical analysis of our sample revealed that it contained benzoic acid, dihydrocinnamic acid, coumaric acid, caffeic acid, prenyl-*p*-coumaric acid, flavonoids, artepillin C, trihydroxymethoxy flavonon, tetrahydroxy flavonon, and triterpenes [22,23].

Although the antitumoral effects of propolis have been reported [1], here propolis alone did not affect spOS-2 cells viability. Propolis exerted

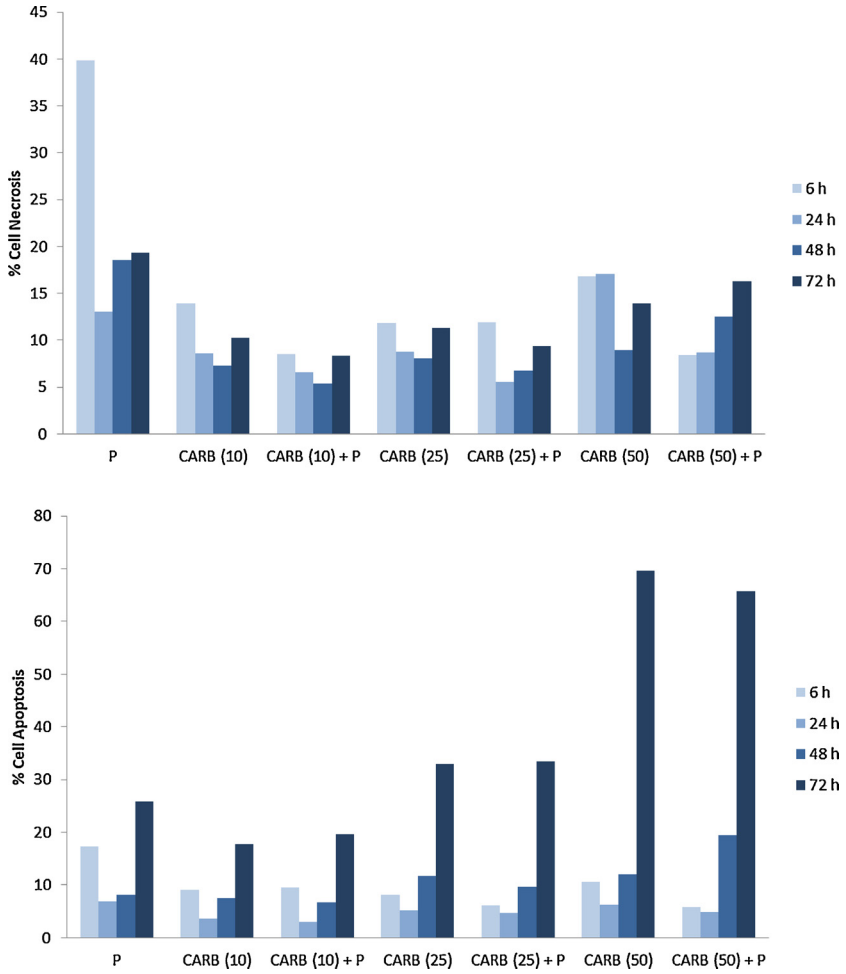

Fig. 2. Percentage (%) of necrotic and apoptotic cells after incubation with propolis (P – 50 µg/ml), carboplatin (CARB – 10, 25 and 50 µmol/l) and their combinations after 6, 24, 48 and 72 h. The figure shows a representative flow cytometry analysis data from the annexin V/PI assay.

Table 3
Viability (%) of mesenchymal stem cells after incubation with propolis (P – 50 µg/ml), carboplatin (CARB – 10, 25 and 50 µmol/l) and their combinations, comparing to control in the respective time scale.

Concentrations of propolis (P) and carboplatin (CARB)	Period of time			
	6 h	24 h	48 h	72 h
P (50 µg/ml)	100.0 ± 0.0	99.7 ± 3.2	83.1 ± 1.7	80.9 ± 1.1
CARB (10 µmol/l)	100 ± 0.0 ^{A}	89.7 ± 17.7 ^{A}	88.8 ± 10.0 ^{A}	83.6 ± 28.3 $^{A/B}$
CARB (25 µmol/l)	100 ± 0.0 ^{A}	100 ± 0.0 ^{A}	90.2 ± 13.3 ^{A}	93.2 ± 11.7 ^{A}
CARB (50 µmol/l)	100 ± 0.0 ^{A}	100 ± 0.0 ^{A}	96.9 ± 5.3 ^{A}	97 ± 5.1 ^{A}
CARB (10 µmol/l) + P	99.2 ± 1,3 ^{A}	100 ± 0.0 ^{A}	82.6 ± 10.8 $^{A/B}$	83,6 ± 14.5 ^{A}
CARB (25 µmol/l) + P	100 ± 0.0 ^{A}	100 ± 0.0 ^{A}	65.7 ± 18.0 ^{B}	88.4 ± 20.0 ^{A}
CARB (50 µmol/l) + P	86 ± 12.0 ^{A}	100 ± 0.0 ^{A}	75 ± 15.6 ^{B}	86 ± 23.2 ^{A}

Data represent means ± standard deviation of 3 similar assays, in triplicate. Different letters indicate significant differences among the groups and moments ($P < 0.0001$).

neither a cytostatic nor a cytotoxic activity when cell growth was compared to control at 6 h. Previous findings of our group revealed that the cytotoxic action of propolis on HEp-2 cells was exclusively due to its constituents, with the solvent having no effect [24]. Similarly, Cinegaglia et al. verified the action of geopropolis produced by *Melipona fasciculata* on canine osteosarcoma (spOS-2) with no effects using 70% ethanol [25].

CARB, DOX and MET have been used extensively in chemotherapy. The latter is not usually used for OSA treatment but its pharmacodynamics is different from the former drugs, antagonizing pholate and inhibiting the production of purines, pyrimidines, thymidylate and the methylation of DNA, that are necessary for tumor cells [26–28]. CARB, DOX and MET exerted an inhibitory action on spOS-2 cells (CARB > MET > DOX).

In an attempt to explore propolis potential in combination with drugs, it has been shown that it may increase the action of some antibiotics. A synergistic effect of propolis with ciprofloxacin to treat *Staphylococcus aureus*-induced keratitis has been reported [29]. Propolis diminished the resistance of bacterial wall to amoxycilin, ampicilin and cephalexin, and exhibited synergistic effects with antibiotics acting in the ribosome (chloramphenicol, tetracycline and neomycin) [30,31]. In order to investigate the effects of propolis in combination with

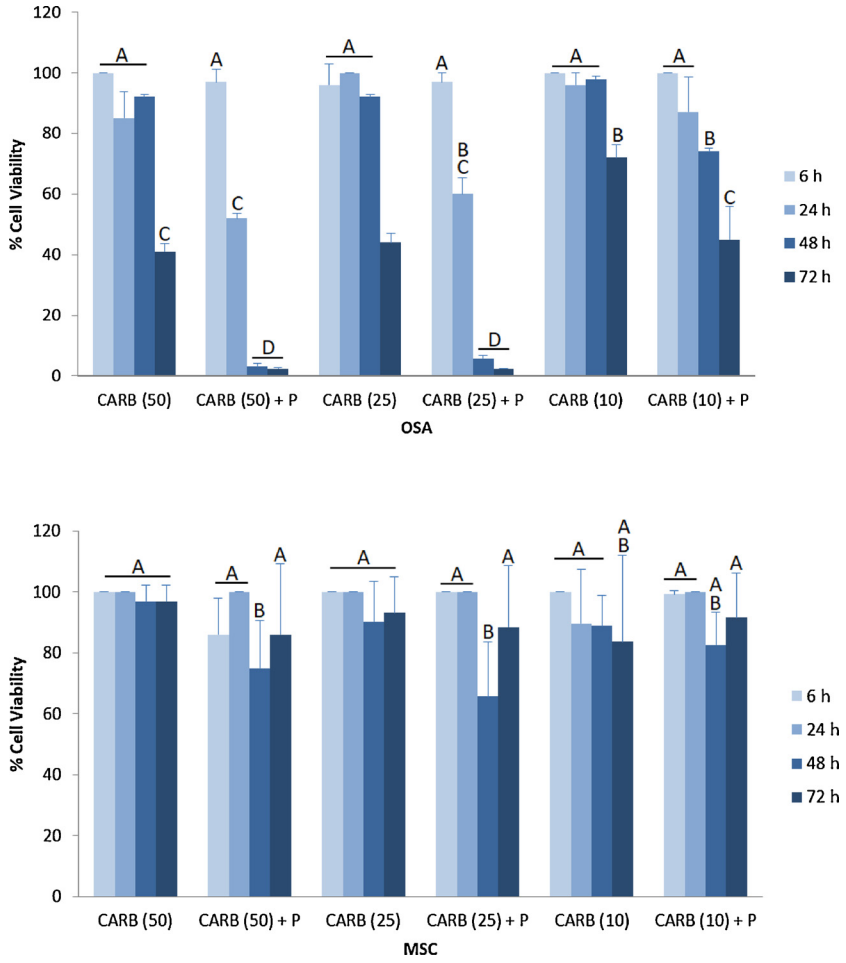

Fig. 3. Comparison of the viability (%) of canine osteosarcoma cells (OSA) and mesenchymal stem cells (MSC) after incubation with propolis (P – 50 µg/ml) + carboplatin (CARB – 10, 25 and 50 µmol/l) over time. Data represent means ± standard deviation of 3 similar assays, in triplicate. Different letters indicate significant differences among the groups and moments in both graphs (OSA and MSC) ($P < 0.01$).

antitumor drugs, spOS-2 cells were incubated with DOX, MET and CARB plus propolis.

The combination of propolis with DOX (2 µmol/l) and MET (25 µmol/l) exerted a cytostatic action towards spOS-2 cells, and cell viability was > 68.7% and > 53.3% after 72 h, respectively. Interestingly, the addition of propolis to lower concentrations of CARB (10, 25 and 50 µmol/l) was more efficient than the drug alone (> 2.4%). These findings are relevant and have pratical applications: although this work was an *in vitro* approach, CARB could be used in lower concentrations *in vivo* adding propolis to treat OSA-bearing dogs, what could reduce side effects.

The antitumor effects of propolis from different geographic regions have been reported, as well as the action of isolated constituents, such as flavonoids, terpenes and caffeic acid phenethyl ester (CAPE) [11]. The authors concluded that the main mechanisms of action by which propolis exerts a cytotoxic action towards tumor cells are apoptosis and cell cycle arrest. Although there was a percentage of necrotic cells, apoptosis was the predominant mechanism by which the combination propolis + CARB led to spOS-2 cell death.

Finally, to verify whether the cytotoxic action of the combination affected tumor cells but not regular ones, its effect was investigated in canine mesenchymal stem cells. Propolis and CARB alone or in combination did not affect MSC cells, demonstrating that this combination could be a promising candidate to treat canine osteosarcoma by inducing apoptosis in spOS-2 cells without affecting mesenchymal stem cells.

Conflicts of interest

The authors declare no conflict of interest in the present work.

Acknowledgements

We are grateful to Prof. Lídia Raquel de Carvalho, Department of Biostatistics, UNESP, for the protocol design and statistical analysis and to Prof. Christiaan Moora for English assistance. This study was supported by São Paulo Research Foundation (FAPESP – 2012/21158-9 and 2009/53493-9).

References

[1] J.M. Sforcin, Biological properties and therapeutic applications of propolis, Phytother. Res. 30 (2016) 894–905.
[2] A.K. Kuropatnicki, E. Szliszka, W. Krol, Historical aspects of propolis research in modern times, Evid. Based Complement. Altern. Med. 2013 (2013) 1–11.
[3] J.M. Sforcin, V. Bankova, Propolis: is there a potential for the development of new drugs? J. Ethnopharmacol. 133 (2011) 253–260.
[4] B. Wolfesberger, C. Hoelzl, I. Walter, G.A. Reider, J.G. Thalhammer, M. Skalicky, M. Egerbacher, *In vitro* effects of meloxicam with or without doxorubicin on canine osteosarcoma cells, J. Vet. Pharmacol. Ther. 29 (2006) 15–23.
[5] K.L. Wycislo, T.M. Fan, The immunotherapy of canine osteosarcoma: a historical and systematic review, J. Vet. Intern. Med. 29 (2015) 759–769.
[6] J.D. Schiffman, M. Breen, Comparative oncology: what dogs and other species can teach us about humans with cancer, Philos. Trans. R. Soc. Lond. B Biol. Sci. 370 (2015) 20140231.
[7] A.E. Lane, M.L. Black, K.M. Wyatt, Toxicity and efficacy of a novel doxorubicin and carboplatin chemotherapy protocol for the treatment of canine appendicular osteosarcoma following limb amputation, Aust. Vet. J. 90 (2012) 69–74.

[8] D. Larone, C. Delprat, Quimioterapia Anticancerígena, 1st ed., Roca, 2004.
[9] R.I. Freshney, Culture of Animal Cells, 6th ed., John Wiley & Sons, Hoboken, NJ, USA, 2010 732 p.
[10] N.C. Cinegaglia, P.R.O. Bersano, M.C. Búfalo, J.M. Sforcin, Anticancer effects of geopropolis produced by stingless bees on canine osteosarcoma cells *in vitro*, Evid. Based Complement. Altern. Med. 2013 (2013) 1–6.
[11] M.A.E. Watanabe, M.K. Amarante, B.J. Conti, J.M. Sforcin, Cytotoxic constituents of propolis inducing anticancer effects: a review, J. Pharm. Pharmacol. 63 (2011) 1378–1386.
[12] N. Orsolić, A.B. Saranović, I. Basić, Direct and indirect mechanism(s) of antitumour activity of propolis and its polyphenolic compounds, Planta Med. 72 (2006) 20–27.
[13] F. Missima, A.C. Pagliarone, C.L. Orsatti, J.P. Araújo Jr., J.M. Sforcin, The effect of propolis on Th1/Th2 cytokine expression and production by melanoma-bearing mice submitted to stress, Phytother. Res. 24 (2010) 1501–1507.
[14] K.L. Mealey, R. Barhoumi, K. Rogers, D.T. Kochevar, Doxorubicin induced expression of P-glycoprotein in canine osteosarcoma cell line, Cancer Lett. 126 (1998) 187–192.
[15] H.T. Ta, C.R. Dass, I. Larson, P.F. Choong, D.E. Dunstan, A chitosan-dipotassium orthophosphate hydrogel for the delivery of doxorrubicin in the treatment of osteosarcoma, Biomaterials 30 (2009) 3605–3613.
[16] L.A. Wittenburg, L. Bisson, B.J. Rose, C. Korch, D.H. Thamm, The histone deacetylase inhibitor valproic acid sensitizes human and canine osteosarcoma to doxorrubicin, Cancer Chermother. Pharmacol. 67 (2011) 83–92.
[17] J. Mordoh, R.D. Chacón, J. Filmus, Isolation of tumor cells from patients with osteosarcoma and analysis of their sensitivity to methotrexate, J. Cancer Res. 41 (1981) 3621–3628.
[18] H. Diddens, T. Teufel, D. Niethammer, High-dose methotrexate therapy with leucovorin rescue: *in vitro* investigation on human osteossarcoma cell lines, Cancer Chemother. Pharmacol. 20 (1987) 128–132.
[19] S. Decker, W. Winkelmann, B. Nies, F. Van Valen, Cytotoxic effect of methotrexate and its solvent on osteosarcoma cells *in vitro*, J. Bone Jt. Surg. 81 (1999) 545–551.
[20] R. Tomoda, M. Seto, Y. Hioki, J. Sonoda, A. Matsumine, K. Kusuzaki, A. Uchida, A low-dose methotrexate inhibits lung metastasis and lengthens survival in rat osteosarcoma, Clin. Exp. Metastasis 22 (2005) 559–564.
[21] J. Liu, J. Bradford, C. Langsdorf, A Simple, Fast and Quantitative Single-Step Dead-Cell Indicator for Flow Cytometry, Apoptosis & Viability Scientific Posters, website Life Technologies, 2009, http://www.lifetechnologies.com/br/en/home/life-science/cell-analysis/flow-cytometry/cell-health-and-viability-assays-for-flow-cytometry/apoptosis-assays-for-flow-cytometry.html.
[22] V. Bankova, G. Boudourova-Krasteva, S. Popov, J.M. Sforcin, S.R.C. Funari, Seasonal variations of the chemical composition of Brazilian propolis, Apidologie 29 (1998) 361–367.
[23] B.J. Conti, V. Bankova, J.M. Sforcin, Chemical composition of the same Brazilian propolis sample analysed in 1997 and in 2012: no freezing effect, Nat. Prod. Commun. 10 (2015) 1279–1280.
[24] M.C. Búfalo, J.M.G. Candeias, J.M. Sforcin, *In vitro* cytotoxic effect of Brazilian green propolis on human laryngeal epidermoid carcinoma (HEp-2) cells, Evid. Based Complement. Altern. Med. 6 (2009) 483–487.
[25] N.C. Cinegaglia, P.R.O. Bersano, M.C. Búfalo, J.M. Sforcin, Cytotoxic action of Brazilian propolis *in vitro* on canine osteosarcoma cells, Phytother. Res. 27 (2013) 1277–1281.
[26] K. Baluz, M.G.T. Carmo, G. Rosas, O papel do ácido fólico na prevenção e na terapêutica oncológica: revisão, Rev. Bras. Cancerol. 48 (2002) 597–607.
[27] T. Huang, S. Ding, T. Hsu, C. Kao, Effects of mineral trioxide aggregate (MTA) extracts on mitogen-activated protein kinase activity in human osteosarcoma cell lines (U2OS), Biomaterials 24 (2003) 3909–3913.
[28] L. Yi, F. Guo, A. Chen, The experiment study on LMWH inhibiting the expression of livin and inducing the apoptosis of osteosarcoma cells, Chin.-Ger. J. Clin. Oncol. 6 (2007) 587–590.
[29] H. Oksuz, N. Duran, C. Tamer, M. Cetin, S. Silici, Effect of propolis in the treatment of experimental *Staphylococcus aureus* keratitis in rabbits, Ophtalmic Res. 37 (2005) 328–334.
[30] R.O. Orsi, A. Fernandes Jr, V. Bankova, J.M. Sforcin, The effects of Brazilian and Bulgarian propolis *in vitro* against *Salmonella* Typhi and synergism with antibiotics acting on the ribosome, Nat. Prod. Res. 26 (2012) 430–437.
[31] R.O. Orsi, J.M. Sforcin, S.R.C. Funari, A. Fernandes Jr, V. Bankova, Synergistic effect of propolis and antibiotics on the *Salmonella* Typhi, Braz. J. Microbiol. 37 (2006) 108–112.