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Effects of natural antimicrobial compounds propolis and copaiba on periodontal ligament fibroblasts, molecular docking, and in vivo study *in Galleria mellonella*

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ABSTRACT

Root canal treatment addresses infectious processes that require control. Occasionally, the radicular pulp is vital and inflamed, presenting a superficial infection. To preserve pulpal remnants, conservative procedures have gained favor, employing anti-inflammatory medications. This study investigated the effects of propolis (PRO), and copaiba oil-resin (COR) associated with hydrocortisone (H) and compared their impact to that of Otosporin® concerning cytotoxic and genotoxic activity, cytokine detection, and toxicity in the *Galleria mellonella* model. Human periodontal ligament fibroblasts (PDLFs) were exposed to drug concentrations and evaluated by the MTT assay. Associations were tested from concentrations that did not compromise cell density. Genotoxicity was evaluated through micronucleus counting, while cytokines IL-6 and TGF-β1 were detected in the cell supernatant using ELISA. Molecular docking simulations were conducted, considering the major compounds identified in PRO, COR, and H. Increasing concentrations of PRO and COR were assessed for acute toxicity in *Galleria mellonella* model. Cellular assays were analyzed using one-way ANOVA followed by Tukey tests, while larval survivals were evaluated using the Log-rank (Mantel-Cox) test ($\alpha = 0.05$). PRO and COR promoted PDLFs proliferation, even in conjunction with H. No changes in cell metabolism were observed concerning cytokine

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levels. The tested materials induce the release of AT_1R, proliferating the PDFLs through interactions. PRO and COR had low toxicity in larvae, suggesting safety at tested levels. These findings endorse the potential of PRO and COR in endodontics and present promising applications across medical domains, such as preventive strategies in inflammation, shedding light on their potential development into commercially available drugs.

1. Introduction

The current meaning of vital pulp treatment (VPT) encompasses all strategies aimed at preserving dental pulp vitality [1]. Pulpitis consists in a dental pulp inflammation as a response to an aggressive stimulus which can be produced by carious process, unsatisfactory restorations, pulp exposure, trauma, and other pulpal irritants [2–4]. While some cases of pulpitis can be managed conservatively, others necessitate a radical treatment [1–4]. According to the clinical signs and symptoms indicative of pulpitis, the Endodontic American Association established some subcategories, being reversible and irreversible pulpitis widely accepted terms for classifying teeth affected by pulpitis [3].

Reversible pulpitis suggests inflammation within the pulp that has the potential to revert to normal or fibrous tissue post-treatment [2–4]. Conversely, severe symptoms often signify irreversible pulpitis, requiring pulp removal [3,4]. Irreversible pulpitis advances through tissue compartments, gradually moving towards the apex, possibly resulting in pulp necrosis if left untreated [5–7]. It is also known that more research is needed on the threshold of irreversible damage, often limited to a portion of the coronal pulp [1,2,5].

Nevertheless, recent research delving into conservative approaches for VPT has exhibited encouraging clinical outcomes. These minimally invasive, biologically based treatments aim to preserve parts, or all pulp tissue, through an improved understanding of pulp repair mechanisms and the advancement of bioactive pulpal biomaterials [8–11].

In cases involving VPTs, endodontists encounter a root canal system that is not entirely contaminated. This is because microorganisms and their byproducts are confined to the superficial pulp tissue, in contact with the microbial contamination source [6,12]. Therefore, the affected teeth require a procedure involving the removal of the affected portion of the pulp followed by the application of a biomaterial or medication with anti-inflammatory properties [4,5].

In cases considered to be irreversible pulpitis, this procedure is performed on an emergency basis for temporary relief of painful symptoms. For these conditions, a commercially available and otologic drug, Otosporin® (FQM, Rio de Janeiro, RJ, Brazil), is used between endodontic treatment sessions. Its composition consists of two antibiotics, polymyxin B sulfate and neomycin sulfate, associated with a corticosteroid, hydrocortisone (H) in an aqueous vehicle, with anti-inflammatory function [13,14].

However, the utilization of this medication raises questions, given that the infectious condition is confined to a limited superficial area, whereas the root section of the pulp is solely inflamed, devoid of microorganisms and maintaining its vitality. Moreover, the use of antibiotics may foster the selection of resistant bacteria, which can acquire the capability to exchange resistance genes, rendering them impervious to the prescribed antibiotics [15,16]. Consequently, the concept of combining natural antimicrobial compounds with a corticosteroid presents a promising and viable alternative in these cases.

Propolis (PRO), also known as bee glue, is a resinous substance gathered by honeybees from diverse plants and utilized within their hives. This natural material has been employed as a medicinal remedy in traditional folk medicine since ancient times [17,18]. PRO is a rich natural compound exhibiting antibacterial efficacy similarly to antibiotic medications. Notably, it possesses substantial anti-inflammatory and antioxidant properties and demonstrates the ability to stimulate stem cells [19]. These beneficial attributes are linked to the presence of flavonoids and other phenolic compounds in its composition [17,18]. In the field of endodontics, PRO emerges as a valuable substance for intracanal medication owing to its previously demonstrated anti-inflammatory and antibacterial activities [20–22].

Another noteworthy substance is copaiba oil-resin (COR), derived from the Brazilian Amazon and extracted from trees of the genus *Copaifera* (known as copaibeiras) [23]. This oil-resin is particularly emphasized in alternative medicine for its notable anti-inflammatory and curative attributes [24]. Within Amazonian populations, COR stands out as one of the most crucial renewable natural remedies [23]. Its constituents, diterpenes, and sesquiterpenes, have demonstrated antimicrobial properties [25–27].

To our knowledge, no studies have explored the effectiveness of these natural antimicrobial compounds combined with a corticosteroid or compared their individual effects with a commercially available product like Otosporin®. This product, occasionally used in cases of vital pulp, was designed for otological use. Therefore, the necessity arises to introduce a distinct medication specifically crafted for Endodontics, rather than repurposing an otological medication, adding to a great current trend of research prioritizing conservative treatments [4,5,8–11].

In this context, our study conducted experiments across various scientific levels, encompassing cytotoxicity, genotoxicity, cytokine detection, and in vivo toxicity assessments. The objective of this work was to explore the potential of natural antimicrobial compounds as viable endodontic medications.

2. Materials and methods

2.1. Characterization of PRO and COR

The solid green propolis was obtained from the state of Minas Gerais (Brazil) and typified as Brazilian Green Propolis (BRPX). The crude resin was extracted with ethanol, dried, to obtain the “dry extract of propolis” (PRO) and analyzed by high-performance liquid chromatography (HPLC) (Merck-Hitachi, Darmstadt, Germany) according to previous studies with L-7100 pumps and an L-7200 auto-sampler [21,28].

A reverse phase column, specifically the Lichrochart 100 RP-18 (12.5 ×0.4 cm, particle diameter of 5 μm; Merck), was utilized for chromatography. The mobile phase consisted of water/formic acid (95:5, v/v) (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min, employing a linear gradient. The analysis time was set at 50 min, and detection occurred at 280 and 340 nm using a diode array (detector L-7450; Merck-Hitachi). Data analysis employed the manufacturer-provided software (DAD Manager, Darmstadt, Germany), with compound identification based on comparison with authentic standards exhibiting identical retention times and UV spectra, evaluated using diode array detection.

The quantification of polyphenols in PRO was performed using standards (Fig. 1). To quantify p-coumaric and caffeic acids, prenylated p-coumaric acids, such as 3,5-diprenyl-4-hydroxycinnamic acid (**12**), and cinnamic acid derivatives, we prepared individual calibration curves, p-coumaric acid (**3**) (Sigma-Aldrich Chemistry, USA), while caffeic and caffeoylquinic acids were quantified using caffeic acid (**2**) (Sigma-Aldrich). Standards for the quantification of kaempferol (**5**), kaempferide (**10**), and 2,2-dimethyl-2 H-1-benzopyran-6-propenoic acid (**6**) were not available [29,30].

COR was collected in the experimental field of Brazilian Agricultural Research Corporation (EMBRAPA) Eastern Amazon, in the city of Belterra, State of Pará, Brazil, between the geographic coordinates 02°38001100 S of latitude and 54°56001400 W of longitude. *Copaifera*

reticulata Ducke was the tree species used for the oil-resin extraction, which was performed through artificial exudation from the trunk, being at least a 30-year-old native tree. A voucher specimen of this plant is deposited in the IAN EMBRAPA Herbarium (Belém, Pará, Brazil), under the Exsiccate register: 183.939. The oil-resin presented the following characteristics: light yellow color (gold), dense, clear, and translucent.

To analyze the chemical composition of COR, a gas chromatographic mass spectrometry (GCMS) analysis was conducted following established procedures [31,32]. The sample was diluted to 0.1% in dichloromethane, and 1.0 μL of the solution was injected using an Agilent 7693 autosampler into an Agilent 7890B chromatograph. The chromatograph utilized a fused silica capillary column coated with a phase 5%-phenyl-95%-methylsilicone (DB5-MS, 30 m x 0.25 mm × 0.25 μm). The oven temperature ranged from 60 to 240 ºC, increasing at a rate of 3 ºC/min. Hydrogen served as the carrier gas at a flow rate of 1.5 mL/min. A flame ionization detector, maintained at 280 ºC, was employed as the chromatographic detector. The injector operated at 250 °C, utilizing a split ratio of 1:20. The peak areas (triplicate) were obtained from the detector signal and divided by the total area for expressing the result (area %).

For component identification, the same sample was injected into an Agilent 7890 A chromatograph coupled to an Agilent 5975 C mass selective detector, operated in electronic ionization mode, with ionization energy of 70 eV. The monitored mass ranged from 40 to 550 u, at a rate of 3.15 sweeps/s. The transfer line was operated at 260 °C, the ion source at 220 °C and the analyzer (quadrupole) at 150 °C. The chromatographic and column conditions were the same as those described above, except for the carrier gas, using helium (1.0 mL/min). To calculate the linear retention indexes, a solution of alkanes (C7 to C26) was injected under the same conditions as above described.

The components were identified by comparing their mass spectra and retention indices (RIs) with those of standard substances found in literature data [33] and existing system libraries, such as the Wiley Registry of Mass Spectral Data and the National Institute of Standards and Technology (NIST) Mass Spectral Database This procedure enabled the acquisition of COR samples with high purity (Fig. 2).

2.2. Cellular assays

2.2.1. Primary culture of PDLFs

Ethical approval was obtained from the local Ethics Committee for Human Research at Bauru School of Dentistry, University of São Paulo (number: 4.631.872). The obtention of the PDLFs occurred according to previous studies [28–30]. PDLFs were cultured for growth in Dulbecco's modified Eagle medium (DMEM, Gibco, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen Corporation) and antibiotics (100 mg/mL penicillin, 100 mg/mL streptomycin, 0.5 mg/mL amphotericin B; Invitrogen Corporation). Cultures were maintained at a temperature of 37ºC in a humidified atmosphere containing 5% CO2 and 95% air. Cells utilized in experiments were at passage 4.

2.2.2. Phenotypic characterization of PDLFs

The phenotypic characterization of fibroblasts followed previously described protocols [34,35]. PDLFs were identified as fibroblasts based on their morphology and positive expression for procollagen I and fibroblast-specific protein-1 (FSP-1) [33,34].

Immunostaining was performed by using goat anti-human procollagen type I 1:100 (catalog #sc-25973; Santa Cruz Biotechnology, Santa Cruz, USA) or mouse anti-human FSP-1 (1B10) (Abcam, Cambridge, UK), followed by fluorescein conjugated anti-mouse immunoglobulin G (Abcam). Slides were then mounted with mounting medium containing DAPI (40,6-diamidino-2-phenylindole dihydrochloride hydrate) for nuclear staining. Images were captured by an inverted confocal microscope (Leica TCS-SPE; Leica, Manheim, Germany), which showed positive cells for procollagen I and FSP-1, indicating their mesenchymal origin.

2.2.3. Preparation of medications

Prior to cell experiments, macrodilution assays were conducted in screw-capped tubes containing media broth and standardized inoculums like described previously [20]. These assays aimed to evaluate the antimicrobial efficacy of Otosporin®, H, and natural compounds PRO and COR against various endodontic pathogens (data not shown). The tubes were incubated at 37ºC to determine the minimum inhibitory concentration (MIC). Subsequent subcultures were plated on agar-based media and incubated at 37ºC to ascertain the minimum bactericidal concentration (MBC). Both MIC and MBC were evaluated using the two-fold-serial dilution technique.

Based on the obtained results, stock solutions of 10% PRO and 5% COR were prepared using DMSO as the vehicle. These solutions were further diluted and utilized in subsequent cell assays as detailed hereafter.

A microtube (1.5 mL, Eppendorf, Hamburg, Germany) was initially weighed on a 0.0001 g precision analytical digital weight scale (Adventurer Ohaus AR2140 class I, São Bernardo do Campo, Brazil). After tare the scale, 0.13 g of the pasty green PRO extract was weighted in the microtube. To obtain a 10% concentrated stock solution of PRO (w/v), 1300 μL of DMSO was added to the extract, while 50 μL of COR was added to 950 μL of dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to obtain a 5% concentrated stock solution.

Considering that Otosporin® comprises a blend of two antibiotics with a hydrocortisone (H) concentration of 10 mg/mL, 250 μL of Otosporin® were combined with 1750 μL of alpha-modified Eagle's medium (α-MEM) (Termo Fisher Scientific, Waltham, USA) supplemented with 10% FBS, resulting in a solution containing 1.25 mg/mL of H concentration.

For the isolated evaluation of H performance, an appropriate hydrocortisone for cell culture analysis was procured from a commercial source (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, 0.02 g of the hydrocortisone was dissolved in 2 mL of a 1:1 solution of ethanol and chloroform (Synth, Diadema, São Paulo, Brazil), as per the manufacturer's guidelines. The stock solutions were filtered using a 0.22 μm pore size membrane filter (Merck Millipore, Burlington, Massachusetts, USA) and incubated in a chamber at 37 ºC overnight.

2.2.4. Dilutions in DMSO and α-MEM

To assess the effectiveness of various concentrations using two-fold serial dilutions in the MTT [3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide] assay, the 10% PRO and 5% COR solutions were diluted utilizing DMSO [36,37].

The 10% PRO and 5% COR solutions underwent two-fold serial dilution in eight microtubes (200 μL, Eppendorf, Hamburg, Germany) containing DMSO, resulting in eight different dilutions (1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128). Subsequently, 10 μL from each dilution was transferred to separate microtubes containing α-MEM to assess the concentration that did not compromise cell density.

Similarly, with Otosporin® and H, 10 μL from each stock solution were added to microtubes containing α-MEM to create eight different concentrations using the same dilution series as described earlier. The final concentration of DMSO during the experiments remained below 1%.

2.2.5. Cell density

The Periodontal Ligament Fibroblasts (PDLFs) were detached and seeded into 96-well plates at a concentration of 1.25×10^4 cells per well. After 18 h to allow for cellular attachment, 100 μL of different solutions were added to the wells, including medium alone, medium containing 1% DMSO, and eight concentrations of each evaluated medication: PRO, COR, Otosporin®, and H ($n = 8$ for each).

The MTT assay was conducted after a 24-hour incubation period. The cell supernatant was replaced with 20 mL of MTT solution (Sigma-

Aldrich) at a concentration of 5 mg/mL in phosphate-buffered saline (PBS), followed by the addition of 180 μL of 10% FBS α-MEM. The cells were then incubated for 4 h. Afterward, the MTT solution was replaced with 100 mL of DMSO. The optical density was measured using a plate reader (FLUOstar Optima; BMG Labtech, Ortenberg, Germany) at a wavelength of 570 nm. These tests were performed in triplicate for each concentration of the medications evaluated.

After identifying dilutions that supported substantial cell density, combinations of medications were initiated to assess the cell density when the natural compounds PRO or COR were combined with H. Furthermore, a combination involving all three medications, PRO + COR + H, was also examined. Specifically, the following concentrations were chosen PRO 1:8, COR 1:16, and H 1:2.

For the combinations of PRO + H or COR + H, a 1:1 proportion of each diluted medication was added to the wells ($n = 8$). Meanwhile, for the PRO + COR + H combination, a 1:1:1 proportion of each diluted medication was added to the wells ($n = 8$). Additionally, control wells containing each medication dilution were assessed ($n = 8$). These evaluations were conducted alongside α-MEM with or without DMSO. The MTT assay was performed after a 24-hour incubation period, with triplicate measurements taken as previously described for optical density assessment.

2.2.6. Genotoxic assay

To conduct the genotoxic assay, fibroblasts were seeded onto sterile circular glass coverslips in 24-well plates at a density of 5×10^4 cells per well and allowed to adhere for 24 h at 37 ºC. The first dilution that enabled more than 70% cell density for each medication was chosen. Cells were then exposed to 500 μL of each test medication. Following a 15-minute incubation period, the drug-containing medium was discarded, and the cells were fixed in acetone. Subsequently, the fixed cells were mounted in a mounting medium containing DAPI.

The glass coverslips with cells were observed using a Nikon Eclipse Ti fluorescence microscope at 400 × magnification. The micronucleus count was determined by examining cells with micronuclei among 100 cells within each quadrant of the circular coverslip, totaling four microscopic fields. Untreated cell cultures and hydrogen peroxide (H_2O_2 – 1 mM) were employed as negative and positive controls, respectively. The experiment was conducted in triplicate to ensure accuracy and reproducibility of results.

2.2.7. Cytokine Detection in PDLFs Supernatants

Cytokine production in cell supernatant was assessed using the enzyme-linked immunosorbent assay (ELISA) kit obtained from R&D Systems (Minneapolis, MN, USA), following the manufacturer's protocol.

Cells were seeded into 24-well plates at a concentration of 5×10^4 cells per well and allowed to adhere for 24 h in 10% FBS α-MEM at 37 ºC and 5% CO_2 in triplicate. Subsequently, the cells were exposed to drug-containing medium (500 μL) using the initial dilution from each medication that exhibited substantial cell density. Control groups consisted of medium alone or with DMSO ($n = 6$). After 24 h of incubation, the supernatants were collected, centrifuged at 1000g for 5 min at 4 ºC, and then stored at − 20 ºC for subsequent analysis.

For the detection of cytokines in the cell supernatant, the DuoSet ELISA kits for Interleukin-6 (IL-6) (DY206–05) and Transforming Growth Factor Beta-1 (TGF-β1) (DY240–05) (R&D Systems) were used. 96-well plates were covered and incubated with capture antibodies diluted in PBS for 15–18 h at room temperature (RT). After this incubation, the plates were washed with PBS Tween 20 0.5% (PBS-T) and incubated with blocking solution which consists in PBS with 1% bovine serum albumin (BSA) for 2 h / RT. Plates were washed and incubated with cell supernatants or with known amounts of recombinant proteins for 1 h / RT. After this period, the plates were washed and incubated with the detection antibodies for 2 h / RT. A new wash was performed followed by incubation with peroxidase in PBS/BSA for 30 min. The plates were washed again, and the substrate added. The reading was performed in a wavelength of 490 nm in a spectrophotometer for plates (Bio-Rad, Hercules, USA). These steps were performed also in triplicate according to previously described methodologies [34,35,38,39].

2.2.8. Molecular docking simulation

The 3D atomic coordinates of main compounds of PRO and COR, including H were projected using the ChemDraw Ultra 8.0. software. The geometry optimization was performed by molecular mechanics (UFF) force field using Avogadro 1.2.0 v software after download (https://avogadro.cc/releases/avogadro_120/). Partial atomic charges were calculated employing Austin Model 1 (AM1) semi-empirical methodology, implemented in MOAPC2016 (http://openmopac.net/MOPAC2016.html), and subsequently the geometry optimization and electrostatic partial atomic charges (CHELPG) were computed using the ab initio method HF/6–31 G* by ORCA 5.0.3 (https://orcaforum.kofo.mpg.de/app.php/portal).

Molecular docking was performed from the energy-optimized compounds. The three-dimensional geometry models of Angiotensin II 1 Receptor (AT_1R) was retrieved from the Protein Data Bank (PDB) (https://www.rcsb.org/structure/4zud) (PDB ID: 4ZUD, resolution 2.80 Å) and used as geometry reference to perform the redocking validation test. The target was prepared by the removal of heteroatoms (water, ions, ligands), and by checking for potential structural errors, mainly those located at the recognition sites explored.

Molecular docking was performed employing AutoDock Tools-1.5.6 software (https://autodock.scripps.edu/). The compounds were then redocked represented by a grid box with dimensions 40 × 40 × 40 centered at the coordinates X = − 42.915, Y= 63.980, and Z = 26.840. The redocking protocol validation test was performed in the same software and target but using the Inverse Agonist Olmesartan. Fifty runs with ten resulting poses each were generated to be analyzed. The parameters of Genetic Algorithm were kept as default in both studies, and the docking resulting poses were ordered according to their score values and analyzed using the freeware visualization program Discovery Studio Visualizer (https://discover.3ds.com/discovery-studio-visualizer-download). All possible interactions and the steric complementarity obtained were analyzed considering a maximum distance of 3.0 Å for the hydrogen bonds and a minimum angle of the hydrogen donor of 120 ºC and hydrogen acceptor of 90 ºC. Bindings involving electronic π systems such as π -cation, π -stacking dipole-dipole and ion-dipole interactions were also considered, according to pre-settled criteria in Discovery Studio Visualizer program.

For PRO, the complexes were numbered as follows: p-Coumaric acid (1); Drupanin (2); Artepillin C (3); and Culifolin (4). Regarding COR, the complexes were numbered as follows: β-bisabolene (5); (*E*)-α-bergamotene (6); (*E*)-caryophyllene (7); (*Z*)-α-bisabolene (8); and finally, hydrocortisone (9).

2.3. In vivo essay in Galleria mellonella

2.3.1. Evaluation of toxic activity

Galleria mellonella (*G. mellonella*) larvae were used as an invertebrate model from the laboratory of the Microbiology and Immunology of the School of Dentistry, Institute of Science and Technology, São Paulo State University (ICT-UNESP, São José dos Campos, São Paulo, Brazil). To evaluate the acute toxicity of PRO and COR in vivo, increasing concentrations for each natural compound were selected considering a range between the experiments on PDLFs and previously performed macrodilution essays. Each experimental group consisted with 10 randomly selected larvae in the final larval phase with a body weight between 250 and 300 mg. The insects were maintained at 37 °C in an incubator until use and inclusion criteria were applied as indicated previously [40]. Selected larvae presented light-colored and free of dark spots and/or pigments on their cuticle, which could indicate the larvae's involvement in an infectious process, influencing the outcome of the

experiment. All analyses were performed in triplicate.

Three increasing concentrations of PRO (1, 2, and 3 mg/mL) and COR (0.3, 0.6, and 0.9 mg/mL) were tested. The natural antimicrobial compounds were prepared in DMSO and subsequently diluted in PBS to achieve each specified concentration. Each inoculum (10 μL) was administered into the last right proleg of individual larvae using a 25 μL Hamilton syringe (Hamilton, Reno, NV, USA). As a comparative reference, PBS was injected (10 μL) into the last left proleg of every larva. Control groups included PBS, PBS + DMSO, and an additional group with no injection administered. The final concentration of DMSO did not exceed 1%.

Subsequently, the larvae were kept in Petri dishes at 37 °C in the dark, without nutrition. After 24 h of inoculations, the number of dead *G. mellonella* larvae was recorded daily until 120 h (5 days) for analysis of the survival curve. Larvae were considered dead when they presented no movement upon touch [40–42].

2.4. Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). The Shapiro–Wilk normality test was employed to assess data distribution. Following the assessment of normality, data obtained from cellular assays underwent one-way ANOVA followed by Tukey's test for further analysis. For comparing survival curves between treated and non-treated larvae, the Log-rank (Mantel-Cox) test was employed. A significance level of 5% was adopted.

3. Results

3.1. PRO and COR contents

The dried soft extract of propolis BRPX (PRO) presented a high content of phenolic compounds and HPLC analysis revealed its composition: monocaffeoylquinic acid (**1**), caffeic acid (**2**), p-coumaric acid (**3**), 3,5-di-*O*-caffeoylquinic acid (**4**), kaempferol (**5**), 2,2-dimethyl-2 H-1-benzopyranpropenoic acid, culifolin (**6**), 3-prenyl-4-hydroxycinnamic acid, drupanin (**7**), 4-hydroxy-3(*E*)-(4-hydroxy-3-methyl-2-buthenyl)− 5-prenylcinnamic acid (**8**), 3-prenyl-4-(2-methylproprionyloxy)-cinnamic acid (**9**), kaempferide (**10**), 3-hydroxy-2,2-dimethyl-8-prenyl-2 H-1-benzopiran-6-propenoic acid (**11**), 3,5-diprenyl-4-hydroxycinnamic acid, Artepillin-C, 2,2-dimethyl-8-prenyl-2 H-1-benzopyran-6-propenoic acid (**12**) and 3-prenyl-4-dihydrocinnamoyloxy cinnamic acid (**13**) were detected (Table 1; Fig. 1). The Artepillin-C (**12**) corresponds to the main marker in Brazilian green propolis BRPX type.

GCMS analysis revealed the main presence of the following compounds in COR: β-bisabolene (27.9%), (*E*)-α-bergamotene (19.2), (*Z*)-α-bisabolene (15.3%), and (*E*)-caryophyllene (11.4%) as main components (Table 2; Fig. 2).

Table 1
Chemical characterization and quantification of some compounds in PRO according to HPLC method.

RT (min)	Name	g/ 100 g
1.77	Monocaffeoylquinic acid (**1**)	0.158
2.32	Caffeic acid (**2**)	0.130
3.45	p-Coumaric acid (**3**)	3.180
4.84	3,5-di-*O*-Caffeoylquinic acid (**4**)	2.055
17.66	3-Prenyl-4-hydroxycinnamic acid, Drupanin (**7**)	1.210
20.12	3-Prenyl-4-(2-methylproprionyloxy)-cinnamic acid (**9**)	0.490
24.8	3-Hydroxy-2,2-dimethyl-8-prenyl-2 H-1-benzopiran-6-propenoic acid (**11**)	0.248
26.72	3,5-Diprenyl-4-hydroxycinnamic acid, Artepillin-C (**12**)	5.950

RT: Retention time; Eight compounds were quantified since the analytical standards for them were obtained with calibration curves.

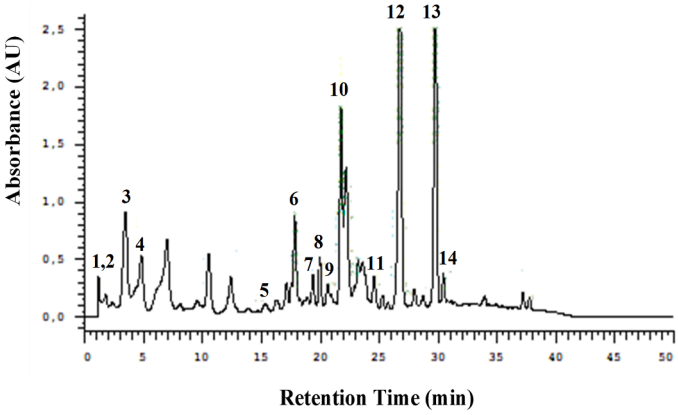

Fig. 1. : Chemical characterization of PRO (from BRPX propolis) dried ethanolic extract analyzed by High-performance liquid chromatography (HPLC) and monitored at wavelengths of 280 and 320 nm. Peaks are: monocaffeoylquinic acid (1), caffeic acid (2), p-coumaric acid (3), 3,5-di-O-caffeoylquinic acid (4), kaempferol (5), 2,2-dimethyl-2 H-1-benzopyranpropenoic acid (culifolin) (6), 3-prenyl-4-hydroxycinnamic acid (drupanin) (7), 4-hydroxy-3(*E*)-(4-hydroxy-3-methyl-2-buthenyl)− 5-prenylcinnamic acid (8), 3-prenyl-4-(2-methylproprionyloxy)-cinnamic acid (9), kaempferide (10), 3-hydroxy-2,2-dimethyl-8-prenyl-2 H-1-benzopiran-6-propenoic acid (11), 3,5-diprenyl-4-hydroxycinnamic acid, Artepillin-C (12), 2,2-dimethyl-8-prenyl-2 H-1-benzopyran-6-propenoic acid (13) and 3-prenyl-4-dihydrocinnamoyloxy cinnamic acid (14). AU corresponds to Absorbance Units.

Table 2
Chemical composition of the Copaiba oil-resin used in the present study revealed by gas chromatography.

Component	RI	Percentage (%)
α-copaene	1372	0.2
β-elemene	1389	1.5
cyperene	1395	0.2
(*Z*)-α-bergamotene	1412	0.2
(*E*)-caryophyllene	1416	11.4
(*E*)-α-bergamotene	1433	19.2
(*Z*)-β-farnesene	1440	0.6
epi-β-santalene	1444	0.9
α-humulen	1450	2.3
(*E*)-β-farnesene	1455	1.9
(*E*)-β-bergamotene	1477	2.6
β-selinene	1482	2.5
α-selinene	1491	1.9
(*Z*)-α-bisabolene	1500	15.3
β-bisabolene	1507	27.9
β-sesquiphelandrene	1521	2.5
(*E*)-γ-bisabolene	1529	2.4
caryophyllene oxide	1540	1.7
Kaurene	2032	0.7

RI: retention index.

3.2. Cell density, genotoxic effect, and cytokine detections

To determine whether PRO and COR could promote cytotoxic effects on PDLFs after 24 h, a cell density assay testing different concentrations of each natural compound was performed. Moreover, different concentrations from the otologic drug Otosporin® and the corticosteroid H were evaluated to select viable dilutions to perform associations and to provide a global comparison.

Fig. 3A shows PRO presenting a cytotoxic effect on PDLFs at 1, 1:2, and 1:4 dilutions ($p < 0.05$). From the 1:8 to 1:128 dilutions, a cell density higher than 70% was detected. Fig. 1B presents COR with higher cell density from the 1:16 to 1/128 dilutions ($p < 0.05$). Fig. 1C and D corresponding to the H and Otosporin® respectively, showed high cell density in all dilutions ($p > 0.05$). Therefore, PRO 1:8, COR 1:16, and H 1 were used in all combined groups. Fig. 4 shows the global comparison

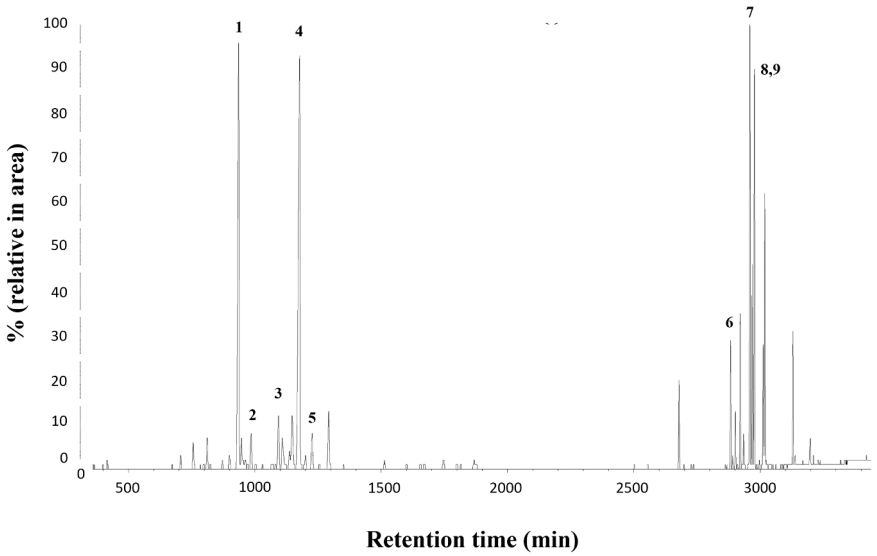

Fig. 2. Fig. 2**:** Cromatographic profile of the *Copaifera reticulata* by GCMS. Peaks are: (*E*)-caryophyllene (1), (*E*)-α-bergamotene (2), (*Z*)-α-bisabolene (3), β-bisabolene (4), β-sesquiphellandrene (5), kauren-16-ene (6), kaur-16-en-18-oic acid, methyl ester (7), copalic acid, methyl ester (8), and kauran-19-oic acid, methyl ester (9).

of cell density between medications as well as associations. All of them showed high cell density. However, PRO, PRO + H, PRO + COR + H, and Otosporin® promoted cell density higher than the controls (control and only DMSO) with significant differences ($p < 0.05$).

Dilutions that provided cell density above than 70% were selected from MTT assay and did not promote genotoxic effects on PDLFs. All the experimental groups were unable to increase the number of micronuclei formation after treatment with different medications and associations. In this case, only the positive control group (H_2O_2) showed genotoxic activity.

Regarding cytokine detections, the comparison was performed according to the first dilution which provided cell density above than 70% from each tested medication and all of them presented similar TGF-β1 and IL-6 values compared to the control group ($p > 0.05$), detected at the cell supernatants after 24 h of contact between media containing drugs on PDLFs (Fig. 5).

3.3. Molecular docking simulation

The AT_1R crystalline structure retrieved from the Protein Data Bank (PDB ID: 4ZUD) is in complex with Olmesartan, a drug capable of interacting at the AT1R agonist site. The binding pocket for Olmesartan comprises residues from all seven transmembrane helices and two extracellular loops. Residues critical for interacting with Olmesartan include Tyr35, Trp84, and Arg167 [37]. Docking simulations were summarized based on the criterion of selecting conformations with the lowest docking energy (Table 3).

The most potent compounds, leading to the formation of complexes 3 and 4 (Fig. 6), were Artepillin C and Culifolin. These compounds exhibit a higher number of hydrogen bonds with the critical amino acids at the binding site, indicating their superior binding mode with the target.

The results revealed several bindings, including hydrogen bonds, π-π stacking (aromatic interactions), hydrophobic and Van Der Waals bonds, according to the classification of ligand-protein contacts of the software employed. Artepillin C interacts via hydrogen bonds with two ($TYR35^{1.78}$ and $ARG167^{2.10}$) of the three critical amino acid residues with ligands at agonist active site. The same phytochemical additionally demonstrated three hydrophobic bindings with TYR35, and two with TYR84. The results obtained for Culifolin in complex 4 also allowed identifying hydrogen bonds with two ($TYR35^{1.95}$ and $ARG167^{2.20}$) of the three critical amino acids, and additional six hydrophobic bindings with TRY84 and three with TRY35.

Complex 9, formed with H, is within an intermediate score between complexes 3 and 4, and is the complex with the highest number of hydrogen bonds, including one of the critical amino acids, $ARG167^{1.98}$. Regarding COR, presenting an oleo-resinous nature, its compounds promote hydrophobic bindings that do not have the necessary strength to overcome the numerous bindings presented by the other phytochemicals analyzed. However, these bindings are possible to happen. Fig. 7 shows general bindings about the main compounds in PRO, COR, and H with the AT_1R.

3.4. In vivo toxicity

The invertebrate model of *G. mellonella* was employed to investigate the acute systemic toxicity of PRO and COR in vivo. Increasing concentrations of these substances were injected into the larvae, and their survival was monitored for 120 h. Fig. 8 illustrates that both PRO and COR did not induce toxic effects on the larvae when administered at concentrations determined based on PDLF performances, respectively. Despite the range of concentrations assessed, determining the lethal concentration of PRO or COR capable of causing mortality in 50% of the larvae was not feasible. This observation suggests a potentially safe application of these extracts in clinical settings. Additionally, no significant differences were detected between the tested concentrations for each natural compound ($p > 0.05$).

4. Discussion

In conservative endodontics, due to the lack of alternative substances in situations such as vital pulp inflammation, natural substances were tested to fulfill an anti-inflammatory function. Cell assays addressed PRO and COR in comparison with a usual medication, Otosporin®, and the in vivo essay checked PRO and COR in a toxicologic view. In this set of experiments, the results suggested that the natural antimicrobial compounds can be considered feasible and promising alternative medications.

Cell density was accessed through MTT assay which is a method able to infer possibilities when an absorbance increases or decreases due to mitochondrial activity, being an important strategy to investigate substances and new materials in endodontics [43,44]. Moreover, a detection of these effects is important to delineate in vivo strategies as well as to infer if these materials could act in different ways depending on the distance which they are applied [45]. For cell tests, PDLFs were selected,

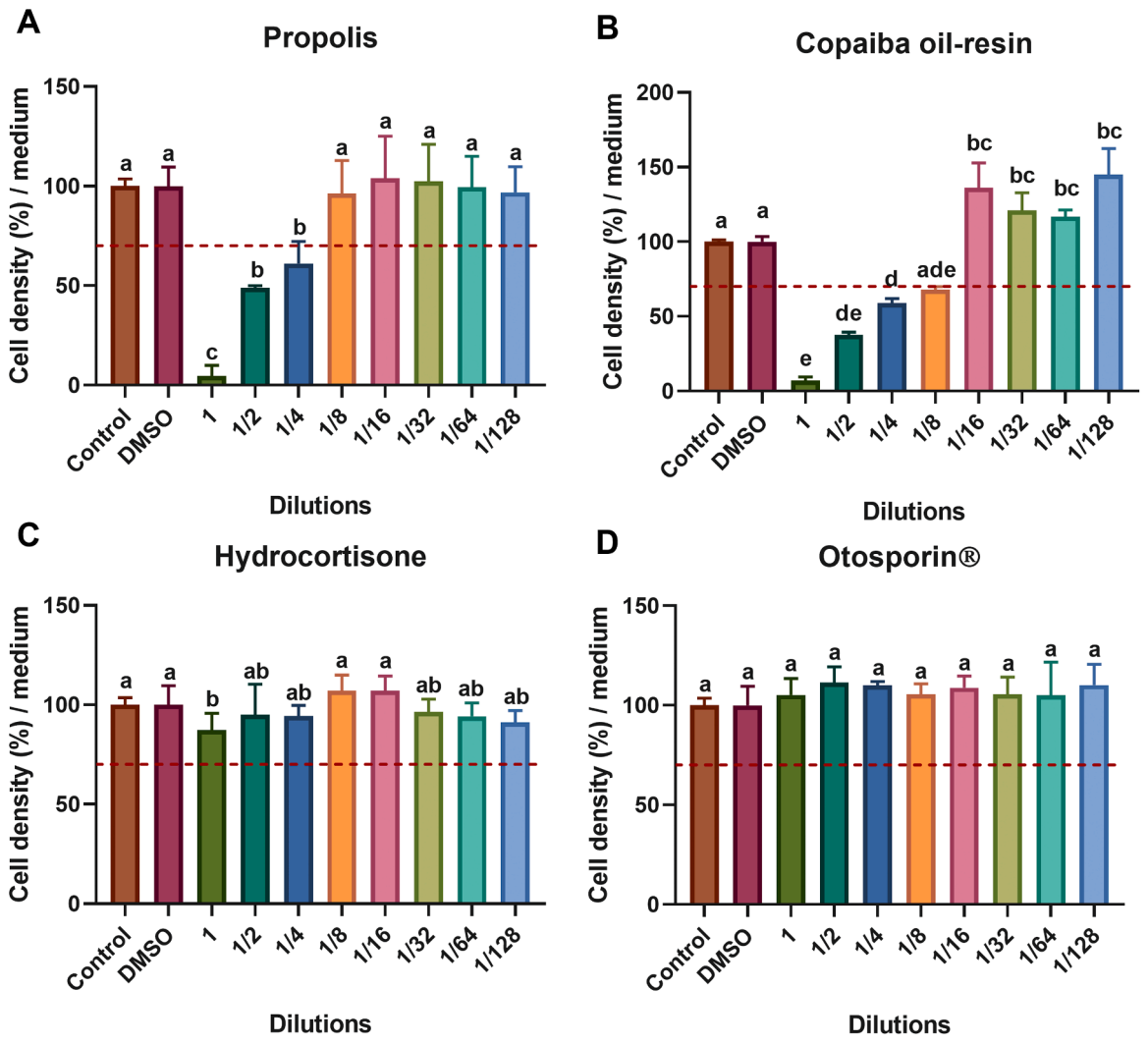

Fig. 3. : Cell density percentage (%) according to MTT analysis in PDLFs after 24 h of exposure to different dilutions of medications evaluated: (A) Propolis; (B) Copaiba oil-resin; (C) Hydrocortisone; (D) and Otosporin®. Cells were also exposed to medium alone or dimethylsulfoxide (controls). The results show mean and standard deviation of the experiments. Different letters represent significant differences between groups. One-way ANOVA and Tukey's tests ($p < 0.05$). The horizontal dashed line indicates 70% of cell density.

since in a vital pulp root canal treatment, which a dental pulp tissue is removed at the site of exposure, living periodontal tissue is exposed to the root canal system and substances applied [46]. In these cases, PDLFs are the cells present around the apical area and are involved in the tissue response generated by endodontic materials [47].

Otosporin® is a commercially available otologic medication containing hydrocortisone (H) in its formulation [13,14]. Hydrocortisone, a topical steroidal anti-inflammatory agent, is utilized to alleviate inflammatory responses, alleviate postoperative discomfort, and promot tissue repair [48,49]. Dilutions of Otosporin® and H exhibited significant cell densities from the first and second dilutions, respectively, indicating their clinical relevance. These medications were investigated considering a 1.25 mg/mL concentration for H in the own Otosporin® as start point according to a previous experiment performed which selected the minimal bactericidal concentration against oral bacteria (data not shown).

The effect of COR exhibited a dose-dependent increase in cell proliferation, surpassing the control group from the 1:16 dilution ($p < 0.05$). To date, there is limited research on the impact of COR on human PDLFs. In a prior study within restorative dentistry, COR was combined with pulp capping materials to investigate dentine bridge formation by dental pulp stem cells in non-contaminated exposed dental pulp tissue. This study revealed that COR enhanced cell response to the materials, notably increasing cell proliferation [50]. Additionally, various *Copaifera* oleoresins have demonstrated an activator profile in human monocytes without compromising cell viability [51]. In experimental models of periodontitis in rats, the oil-resin derived from *Copaifera reticulata Ducke* exhibited anti-inflammatory effects, alleviating periodontal damage [52]. Interestingly, this specific oil-resin derives from the identical tree species examined in our current research.

In the meantime, PRO achieved cell densities like the control starting from a 1:8 dilution ($p > 0.05$). Notably, when PRO and COR were combined with H, they elicited higher cell densities. The interplay of PRO with other medications significantly impacted cell densities, surpassing the control in the PRO+H and PRO+COR+H associations. The proliferative effects of PRO have been documented in the literature, where the green PRO extract was found to be non-toxic to bone marrow-derived mesenchymal stromal cells, supporting their proliferation, migration in vitro, and tissue regeneration [53]. Additionally, it demonstrated a proliferation effect on periodontal ligament fibroblasts [54]. Al-Shaher *et al.* also emphasized the non-toxic nature of PRO diluted in DMSO, reinforcing the importance of introducing PRO into the endodontic arsenal [55].

The genotoxicity test was conducted to assess the potential risks

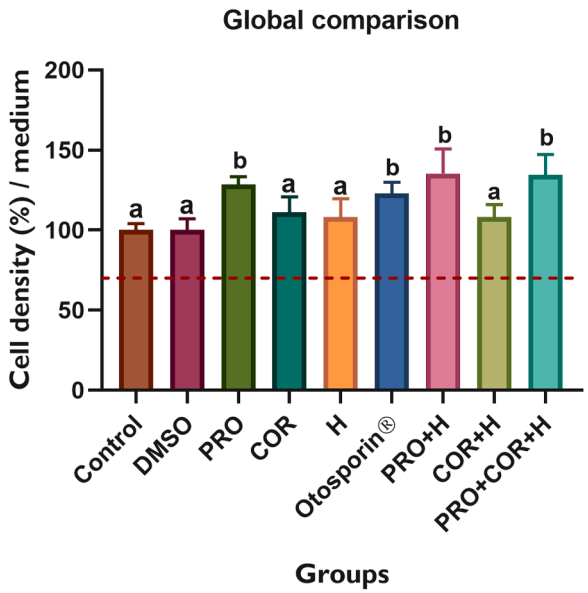

Fig. 4. : Global comparison of cell density percentage (%) according to MTT analysis in PDLFs after 24 h of exposure to different medications including Propolis, Copaiba oil-resin, Hydrocortisone, Otosporin®, and the associations: PRO+H, COR+H, and PRO+COR+H. Cells were also exposed to medium alone or dimethylsulfoxide (controls). Dilutions whose promoted higher cell densities were used to evaluate the medications and their associations. Medications and associations tested did not affect cell viability. The results show mean and standard deviation of the experiments. Different letters represent significant differences between groups. One-way ANOVA and Tukey's tests ($p < 0.05$). The horizontal dashed line indicates 70% of cell density.

posed by toxic materials on the DNA of human cells by observing micronuclei formation [56]. Micronuclei arise due to the loss of chromosomal material or its fragments during cell mitosis, resulting in the formation of smaller nuclei or micronuclei [57]. In this study, H_2O_2 served as a positive control due to its highly genotoxic nature [58]. The tested dilutions were selected based on the results of the MTT test, considering only the initial dilutions that demonstrated 70% viability of PDLFs. It's important to note that exposing PDLFs to stimuli for 24 h might limit our understanding compared to a longer exposure period, which could yield different outcomes.

Comparing the present findings with prior studies poses challenges due to variations in methodologies used. For instance, a distinct component from a different species of COR was examined in gastric cancer cells, resulting in an increased frequency of micronuclei in a dose-dependent manner [59]. However, it's important to note that our study analyzed a spectrum of components within a pure oil-resin rather than a single isolated component. Similarly, in a study involving the green extract of PRO, like the one utilized here, genotoxic activity was investigated using a *Drosophila melanogaster* model, revealing an absence of mutagenic and recombinogenic actions [60]. Nevertheless, it's crucial to acknowledge that higher concentrations of PRO exhibited toxic effects [60,61].

In this study, the administration of natural medications and their combinations did not result in an increased release of TGF-β1 and IL-6 by PDLFs after 24 h. This suggests that there was no significant alteration in cellular metabolism, and the dilutions applied did not induce any harmful stimuli. In the context of pulpitis, dental pulp tissue triggers a repair response when faced with microbial invasion, and growth factors, including TGF-β1, become integrated into the organic dentin matrix [62,63]. TGF-β1 plays a crucial role in mediating the signaling for odontoblast differentiation and mineralization [64]. On the other hand, IL-6 is a cytokine produced during inflammation and actively participates in immune responses. It is synthesized by various cells, including fibroblasts, in response to stimuli such as infection and trauma [60]. Additionally, IL-6 has been linked to the development of periapical lesions [65,66].

COR from different species have exhibited varying effects on cytokine modulation, showing inhibition or increase [37,44]. For instance, an increase in TGF-β was observed, indicating an anti-inflammatory response in macrophages infected by *Trypanosoma cruzi* forms [37]. PRO has demonstrated anti-inflammatory properties and a protective role against post-surgical adhesive complications. In studies involving rats, PRO was found to reduce TGF-β1 and IL-6 levels in peritoneal adhesions [67]. Isolated compounds present in PRO were observed to modulate TGF-β1 expression in rats with cardiorenal dysfunction and hypertrophy [68]. Furthermore, a systematic scoping review highlighted the anti-inflammatory action of PRO attributed to the inhibition

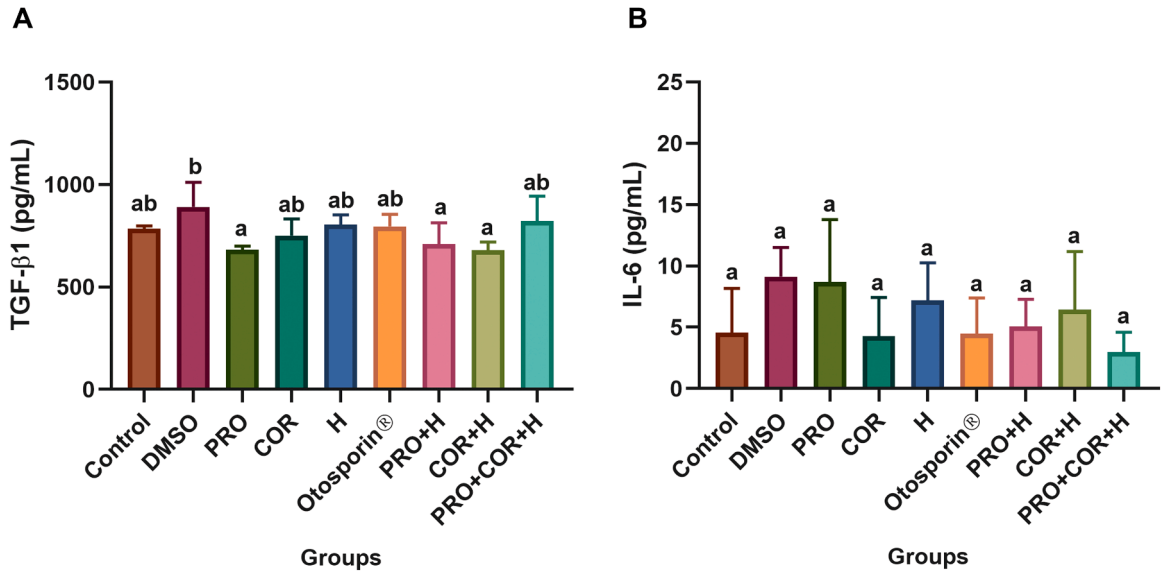

Fig. 5. : Cytokine concentrations for (A) TGF-β1 and (B) IL-6 according to ELISA assay in PDLFs after 24 h of exposure to medications including Propolis, Copaiba oil-resin, Hydrocortisone, Otosporin®, and the associations: PRO+H, COR+H, and PRO+COR+H. Cells were also exposed to medium alone or dimethylsulfoxide (controls). Dilutions whose promoted higher cell densities were used to evaluate the medications and their associations. The results show mean and standard deviation of the experiments. Different letters represent significant differences between groups. One-way ANOVA and Tukey's tests ($p < 0.05$).

Table 3
Interactions of AT_1R (PDB ID: 4ZUD) amino acid residues with ligands at agonist active site.

Complex	Binding affinity, DG (Kcal/mol)	Amino acids involved and distance (Å) in hydrogen binding interactions	Amino acids involved and distance (Å) in hydrophobic interactions
1	-4.85	ARG167 (1.84), TYR35 (1.94), TYR87 (1.95)	PRO285 (4.99), ILE288 (5.29)
2	-6.23	TYR92 (1.92), THR88 (1.83)	TYR292 (4.58), TRP84 (3.77), TRP84 (4.46), TRP84 (3.80), LEU81 (4.51), VAL108 (5.09), LEU81 (5.36), ILE288 (5.13), TYR35 (4.24), TYR35 (4.46), PHE77 (5.43), PHE77 (5.15), TRP84 (5.38)
3	-7.66	TYR35 (1.78), ARG167 (2.10), SER105 (1.97)	TYR292 (3.55), TRP84 (4.54), TRP84 (4.18), ILE31 (4.01), PRO285 (4.01), PRO285 (4.70), ILE288 (5.04), VAL108 (3.89), ILE288 (4.64), TYR35 (4.57), TYR35 (4.76), PHE77 (5.11), TYR92 (4.49), TYR292 (4.17), VAL108 (5.05), ILE288 (5.26)
4	-8.46	TYR35 (1.95), ARG167 (2.28), TYR87 (1.89), ALA181 (3.57)	TRP84 (3.48), TRP84 (4.57), TRP84 (4.01), VAL108 (4.39), ILE288 (4.72), ILE288 (4.85), LEU81 (5.35), VAL108 (3.81), ILE31 (4.54), PRO285 (3.6), PRO285 (4.33), ILE288 (3.86), TYR35 (4.71), TYR35 (5.48), PHE77 (5.38), PHE77 (5.22), TRP84 (4.63), TRP84 (4.95), TRP84 (4.88), TYR92 (4.68), TYR292 (4.61), ILE288 (5.41)
5	-7.11	—	TYR35 (3.99), TRP84 (3.73), VAL108 (4.19), ILE288 (5.43), LEU81 (4.65), ILE288 (5.19), ILE31 (4.13), PRO285 (4.20), PRO285 (4.07), ILE288 (4.18), TYR35 (5.22), TYR35 (5.44), TYR35 (5.03), PHE77 (5.19), PHE77 (5.01), TRP84 (4.84), TRP84 (4.23), TYR292 (4.87), TYR292 (5.05)
6	-7.44	—	TRP84 (3.98), TYR35 (3.60), VAL108 (3.81), VAL108 (5.02), ILE288 (4.08), LEU81 (4.55), VAL108 (3.97), VAL108 (4.29), ILE288 (4.21), LEU81 (5.15), ILE288 (5.33), ILE38 (3.97), CYS289 (3.47), ILE38 (4.01), LEU81 (3.50), TYR35 (4.20), TYR35 (4.38), PHE77 (4.40), PHE77 (5.16), TRP84 (3.77), TRP84 (5.16), TRP84 (4.59), TRP84 (5.35), TYR292 (4.62), TYR292 (4.73)
7	-6.97	—	TRP84 (3.79), TRP84 (3.71), VAL108 (4.60), ILE288 (5.07), VAL108 (3.90), ILE288 (4.52), TYR35 (5.04), TYR35 (5.07), TRP84 (4.94), TRP84 (4.70), TRP84 (3.74), TRP84 (4.65), TYR87 (5.07), TYR292 (5.15)
8	-7.09	—	TYR292 (3.70), ILE31 (4.11), PRO285 (3.71), ILE288 (4.14), ILE288 (5.21), LEU81 (4.92), VAL108 (3.56), ILE288 (5.21), TYR35 (5.08), TYR35 (4.51), PHE77 (4.69), PHE77 (5.02), TRP84 (3.94), TRP84 (5.44), TRP84 (4.65), TYR92 (5.06), TYR292 (4.48)
9	-7.72	ARG167 (1.98), TYR87 (2.03), TYR87 (1.96)	TYR92 (3.45), ILE288 (4.50), ILE288 (4.31), TRP84 (4.43)

1: Coumaric acid; 2: Drupanin; 3: Artepillin C; 4: Culiofolin; 5: β-bisabolene; 6: (*E*)-α-bergamotene; 7: (*E*)-caryophyllene; 8: (*Z*)-α-bisabolene; 9: Hydrocortisone.

of cytokines, including IL-6 [69].

Combining our results with previous findings in literature, AT_1R is an interesting biological target for a molecular docking simulation study. In inflammatory conditions such as provoked by microorganism invasion, the Renin-angiotensin system (RAS) performs an important function modulating cells behavior [70,71]. RAS is an endocrine system that promotes the formation of angiotensin (Ang) II, which is related with periodontal diseases and exerts its effects through the interaction with specific Ang II receptors (AT_1 and AT_2) [70,71]. Ang II is also responsible for the upregulation of TGF-β1 in various cell types, all of which are related to pro-fibrotic mediation [72]. Ang II have been detected in periodontal ligament tissues as well as an increase in TGF-β1 expression, suggesting that Ang II mediates the loading signal in stretched cells to induce TGF-β1 expressions [73]. Pizzatto *et al.* demonstrated the role of AngII on stem cells of apical papilla [71]. The presence of a local RAS in cells was detected and showed that AngII modulates cell function by affecting proliferation, cytokine production, and cell mineralization potential [71].

It is plausible to propose that the administration of PRO and COR in combination with H could potentially induce the synthesis of AngII. Subsequently, AngII might interact with the agonist active site of AT_1R, potentially instigating a stimulatory effect on this receptor, which may have contributed to the proliferation of PDLFs in the present study. Considering this hypothesis, we employed a molecular docking approach to delve deeper into the molecular interactions occurring between the tested materials and the active site of AT_1R.

The present results showed the occurrence of these bindings, specially from PRO compounds, mainly Artepillin C and Culifolin. These events are important because weak intermolecular interactions, such as hydrogen and hydrophobic bonds, play pivotal roles in stabilizing ligands that are energetically favored [74,75].

Hydrogen bonds significantly influence the specificity of ligand binding [74,75]. Their substantial contribution is exemplified through a computational technique known as the grid, designed to identify energetically favorable binding sites for ligands on specific target molecules with known structures. Wolfenden *et al.* conducted extensive studies on the role of the hydroxyl group in drug-receptor interactions using transition state analogs. Their findings suggested that the presence of multiple hydrogen bonds concurrently can notably enhance the strength

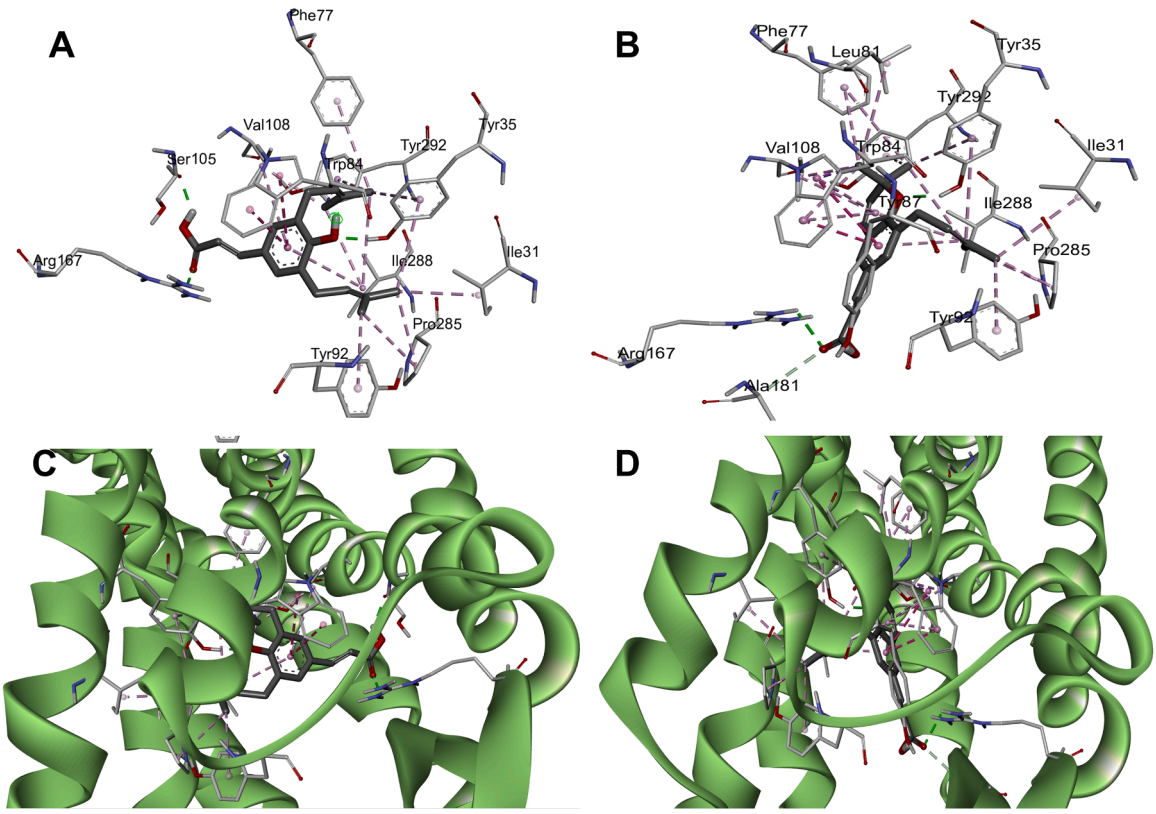

Fig. 6. : (A) 3D projection of the phytochemical Artepillin C and its network of interactions with active site amino acids. (B) 3D projection of the phytochemical Culifolin and its network of bindings with active site amino acids. (C) Solid-strip representation for AT_1R, showing the orientation profile of the best solution found for the phytochemical Artepillin C. (D) Solid-strip representation for AT_1R, showing the orientation profile of the best solution found for the phytochemical Culifolin.

and stability of interactions between a drug and its receptor [74].

Studies have investigated not only natural antimicrobial compounds for novel medication development but also synthesized molecules, demonstrating benefits beyond antimicrobial activities through cellular assays and molecular dockings. For example, benzoxazole derivatives like 2-(p-methylphenyl)− 5-(2-substitutedacetamide)benzoxazole share a structural resemblance with adenine and guanine bases, facilitating diverse receptor interactions as hydrogen bond acceptors and engaging in hydrophobic interactions due to their lipophilic nature [76]. Additionally, methyl α-D-glucopyranoside (α-MGP) esters derived from modified carbohydrates exhibit potential as synthetic compounds with mild anticancer activity and lower toxicity, showing binding affinity in ligand-protein interactions [77]. Meanwhile, nucleosides such as cytidine, crucial RNA components and uridine precursors, demonstrate enhanced binding properties in modified analogues compared to the original drug [78].

Distinguishing between synthesized molecules and natural antimicrobial compounds reveals differences in origin, structure, and mechanisms. Synthesized molecules typically target specific functionalities for optimized efficacy, featuring more defined chemical compositions conducive to easier standardization [76–78]. In contrast, natural antimicrobial compounds, originating from diverse biological sources, possess inherently complex structures that contribute to their greater variability [23,28,29,79]. Future research endeavors could focus on exploring potential synergistic effects or conducting comparative analyses between synthesized molecules and natural antimicrobial compounds.

Galleria mellonella serves as an established invertebrate model for assessing the toxicity of natural compounds due to its simplicity and cost-effectiveness [41,42]. This study marks the first investigation into the toxicity of COR in *G. mellonella* larvae, comparing its effects with PRO. Pure compounds were diluted in DMSO, resulting in three escalating concentrations for PRO (1, 2, and 3 mg/mL) and COR (0.3, 0.6, and 0.9 mg/mL), considering a ratio derived from the cell (PDLF) dilution outcomes and the minimum bactericidal concentrations of each natural compound. Larval survival was monitored for up to 120 h (5 days), following the established protocol [42]. Despite observing an 80% survival rate for each higher concentration, no significant differences were noted compared to controls and other concentrations. A prior study assessed the acute toxicity in *G. mellonella* by inoculating cinnamoyloxy-mammeisin, an anti-inflammatory compound isolated from geopropolis (stingless bee propolis), revealing no notable harmful effects [80]. Additionally, another component isolated from PRO, caffeic acid phenethyl ester, was found to prolong the survival of larvae infected with *Candida albicans* [81].

Given the established status of Otosporin® and H as drugs, our initial objective was to ascertain if PRO and COR might exhibit any potential toxicity in vivo. The observed protective action of the pure compositions of PRO and COR, possibly related to the MTT results indicating PDLFs proliferation, could stem from their varied organic compounds. It's important to note that the choice of vehicle for compound dilution can influence toxicity outcomes [41]. In this study, DMSO was selected as the vehicle due to its miscibility in various organic solvents. Moreover, its wide application as a solvent or vehicle in biological studies is attributed to its polar characteristics, allowing the dissolution of a wide array of both polar and nonpolar substances [36,37].

This study underscores the significance of utilizing typified samples in experiments. The PRO type utilized, BRPX, is recognized for its high concentration of bioactive compounds [28]. As for COR, it is a typified sample obtained from *Copaifera reticulata Ducke*. In the Brazilian Amazon region, this type of oil-resin is extensively employed in folk medicine by people from diverse backgrounds and age groups [79]. However, obtaining pure oil-resin can pose challenges, often resulting in the availability of bark infusions sold in bottles in urban areas [23]. The

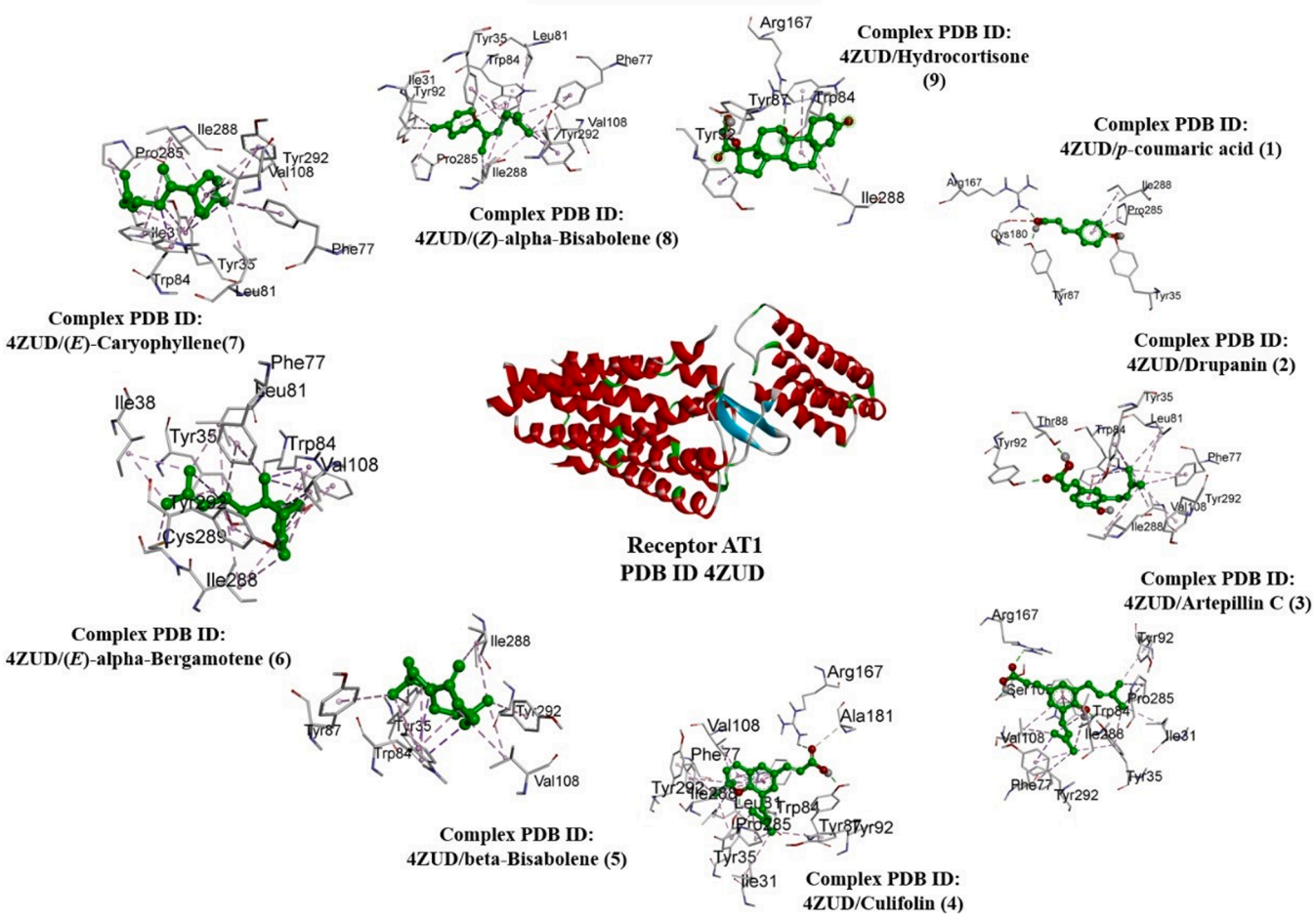

Fig. 7. : General bindings about the main compounds founded in PRO, COR, and H with the Receptor AT_1 (AT_1R) in Protein Data Bank (PDB) ID 4ZUD. Complexes: (1) Coumaric acid; (2) Drupanin; (3) Artepillin C; and (4) Culiofolin; (5) β-bisabolene; (6) (*E*)-α-bergamotene; (7) (*E*)-caryophyllene; (8) (*Z*)-α-bisabolene; and (9) Hydrocortisone.

choice to procure a typified COR sample aims to confirm the presence of essential oils, predominantly composed of sesquiterpenoids such as caryophyllenes. Furthermore, the inclusion of diterpene acids is crucial, ensuring significant biological activities [23,25–27].

Distinct responses exhibited by natural products can be attributed to various factors, including their origin and extraction methods. These factors contribute to diverse chemical compositions and subsequently influence their biological properties [42,82,83]. Therefore, to attain reliable insights into the performance of a specific natural compound, it is crucial to compare compounds derived from the same origin and extracted using similar methods whenever feasible. This approach not only ensures better reproducibility of experiments but also facilitates more accurate comparisons.

This study holds significance for potential future clinical applications, particularly in the context of vital pulp root canal treatments. After the removal of infected pulp segments and the infected dentine surrounding it, the primary objective is to fill the cavity with a medication possessing antimicrobial and anti-inflammatory properties. This is crucial for eliminating postoperative pain and safeguarding against recontamination between treatment sessions [1–4,84]. Other medications in Endodontics are intended to eliminate residual microorganisms after the root canal preparation. These microorganisms might be partially dislodged from their biofilms located in concealed ramifications within the root canal system or found deep within the dentine tubules [85]. While Otosporin® may not be the optimal choice due to its antibiotic composition, which could potentially lead to microbial resistance, the investigated natural antimicrobial compounds, PRO and COR, have shown promising results, especially when combined with the corticosteroid. However, additional evidence from randomized clinical studies involving these medications, either in isolation or in combination, is imperative.

The potential of these promising natural products extends beyond endodontic applications, offering possibilities for preventive approaches across dental, pharmaceutical, and medical domains. This prompts the need for further investigations into natural antimicrobial compounds in varied contexts. Additionally, the innovative nature of this research, which holds the promise of developing new drugs or medicines that could potentially become commercially available in the future, paves the way for extensive research into natural compounds.

In conclusion, the present study was designed to explore the potential of natural antimicrobial compounds, PRO and COR, as prospective candidates for endodontic medications. Through a series of comprehensive experiments, including cytotoxicity assessments, genotoxicity evaluations, cytokine detection, and in vivo toxicity studies, we aimed to unravel the therapeutic efficacy of these compounds. The determined dilutions of PRO and COR promoted proliferative activity on PDLFs especially when used in conjunction with hydrocortisone and exhibited non-genotoxic effects. Interestingly, the application of these compounds did not significantly alter the levels of TGF-β1 and IL-6, aligning closely with the impact observed with Otosporin®, a conventional medication in clinical use. Notably, our findings suggest that PRO, COR, and their combinations with H might trigger AngII release, implicating potential effects on inflammatory responses and PDLF proliferation. Moreover, in an invertebrate model using *G. mellonella* larvae, PRO and COR

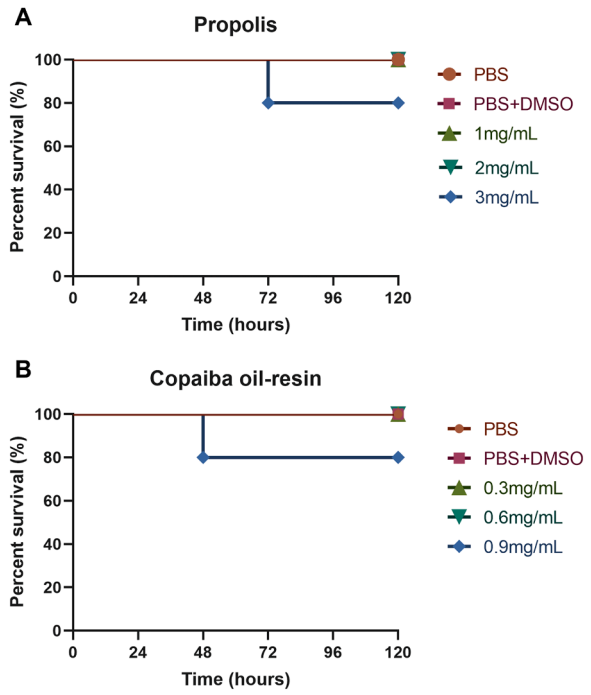

Fig. 8. : Toxicity of PRO and COR in *Galleria mellonella* systemic model. The larvae were treated with PRO at 1, 2, and 3 mg/mL; COR at 0.3, 0.6, and 0.9 mg/mL; phosphate-buffered saline (PBS); and PBS with dimethylsulfoxide (DMSO) as controls. The larvae survival was monitored for up to 120 h. Data were evaluated by Log-rank (Mantel-Cox) test ($p > 0.05$).

displayed no apparent toxic effects at the tested concentrations, highlighting their promising safety profiles for potential future clinical applications in endodontics.

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CRediT authorship contribution statement

Sharma Prasahnt Kumar: Funding acquisition, Investigation, Resources, Visualization, Writing – original draft. **Junqueira Juliana Campos:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Validation. **Shahbazi Mohammad-Ali:** Funding acquisition, Investigation, Resources, Supervision, Validation, Visualization, Writing – review & editing. **Santos Letícia Martins:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization. **Gonçalves Carolina Passarelli:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing – original draft. **Sipert Carla Renata:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft. **Pedrinha Victor Feliz:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **de Andrade Flaviana Bombarda:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. **Queiroga Carmem Lucia:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation. **Marcucci Maria Cristina:** Conceptualization, Data curation, Formal analysis, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft. **Garcia Maíra Terra:** Investigation, Methodology, Resources, Validation, Visualization. **Lameira Osmar Alves:** Data curation, Investigation, Resources, Supervision, Validation, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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