Physalis angulata reduces the progression of chronic experimental periodontitis by immunomodulatory mechanisms

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ABSTRACT

Ethnopharmacological relevance: Physalis angulata is an herb found in tropical and subtropical regions of the world; it is widely applied in popular medicine due to the therapeutic properties of the whole plant and its parts. Extracts and infusions of this plant have been extensively applied in folk medicine worldwide to treat inflammatory and immune-mediated diseases, including oral inflammatory conditions such as sore throat and gingivitis.

Aim of the study: The present study was designed to investigate the protective effects of the ethanolic extract of *P. angulata* (EEPA) in a murine model of chronic periodontitis, aiming to corroborate its traditional use as an anti-inflammatory and immunomodulatory agent, and to point out possible mechanisms involved in these effects.

Materials and methods: EEPA was obtained from the stems of *P. angulata* collected in Belém (PA, Brazil). Chronic periodontitis was induced in male C57BL/6 mice by 12 administrations of lipopolysaccharide (LPS; 20 μg/1μL) into the gingival papilla in the course of 28 days. Starting from the 15th day after the first LPS injection, mice were daily treated with EEPA (50 or 100 mg/kg), nimesulide (25 mg/kg, reference drug), or vehicle by oral route for 14 days. At the end of the experimental period, alveolar bone loss was evaluated along with the gingival expression of biomarkers of periodontitis and cytokines by RT-q-PCR and ELISA. Hematological and biochemical parameters suggestive of systemic toxicity were also evaluated. The transcriptional activity of NF-κB was investigated using the luciferase assay in macrophages.

Results: Mice with chronic experimental periodontitis suffered alveolar bone loss that was prevented by the treatment with EEPA (50 or 100 mg/kg) or nimesulide (25 mg/kg). EEPA (50 and 100 mg/kg) and nimesulide (25 mg/kg) reduced mRNA levels of MMP-9 mRNA, but not of TIMP-1 in gingival tissue of periodontitis-induced mice. Both treatments also reduced the production of the pro-inflammatory cytokines IL-1β and IL-6. The treatment with EEPA (100 mg/kg) increased the production of the anti-inflammatory cytokine TGF-β. No hematological or biochemical alterations were caused by the daily treatment with EEPA. *In vitro* luciferase assay suggested that a putative mechanism of EEPA is reducing the transcriptional activity of NF-κB.

Conclusions: EEPA exhibited a disease-modifying effect in the chronic experimental periodontitis, along with unidentifiable systemic toxicity. This work corroborates the traditional use of *P. angulata* in oral inflammatory conditions and provides mechanistic hypotheses to explain its therapeutic effects.

1. Introduction

The genus *Physalis* (Solanaceae) encompasses around 120 herbaceous annual or perennial species distributed worldwide, mostly in temperate zones. Its name comes from the Greek "physa", meaning "bubble", an allusion to the shape of the fruiting calyx surrounding its

fruit like a dome (Tomassini et al., 2000). *Physalis angulata* L. is an herb found in some regions of Africa, Asia, and the Americas. This plant reaches up to 1 m in height, has a short stem, small cream-colored flowers, and yellow edible fruit (Bastos et al., 2008). *P. angulata* is widely applied in popular medicine due to the therapeutic properties of the whole plant and its parts. Extracts and infusions of this plant have been traditionally used in many countries for treating several illnesses, such as malaria, asthma, and dermatitis (Alves dos Santos et al., 2008; Rivera et al., 2015). In Brazil, some of the oldest reports on the use of *P. angulata* in folk medicine date back to 1874, as described in the book *Formulary and Medical Guide* (Ricardo et al., 2017). The traditional use of the plant in Brazilian communities has persisted through the decades, and is still relevant in the modern times (Branch and Silva, 1983).

Among the many therapeutic applications of *P. angulata*, ethnopharmacological studies have shown a broad use of this species in the treatment of inflammatory and immune-mediated diseases, such as rheumatism, hepatitis, cervicitis, and inflammatory conditions of the mouth and throat (Alves dos Santos et al., 2008; Bastos et al., 2008; Brustolim et al., 2010; Lin et al., 1992; Rengifo and Vargas-Arana, 2013; Rivera et al., 2015). This species is used for treating sore throat in Indonesia (Luliana et al., 2017), China (Lin et al., 1992), and India (Ramanpreet and Gupta, 2015). Moreover, *P. angulata* is used in Indonesia as a folk medicine in the treatment of gingivitis (Dalimartha, 2006; Luliana et al., 2017). Therefore, the traditional use of *P. angulata* strongly suggests that this plant is useful in the treatment of inflammatory diseases of the mouth.

Periodontitis is a multifactorial inflammatory disorder associated with an infectious component that leads to progressive destruction of the attachment apparatus of teeth (Tonetti et al., 2018). It affects more than 700 million people worldwide, which represents a prevalence of around 11% (Eke et al., 2015), making periodontitis an important public health issue. The disease is marked by dysbiosis, i.e., microbiota imbalance with development of complex communities of pathogenic bacteria (Abusleme et al., 2013; Hajishengallis et al., 2011; Paster et al., 2001). This triggers the activation of resident immune cells, ultimately leading to immune-mediated tissue damage (Tonetti et al., 2018) and alveolar bone loss (Walsh et al., 2006). The recognition of pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), via toll-like receptors results in: (1) production of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α) and the interleukins (IL)-1β and IL-6; (2) expression of the receptor activator of RANKL in osteoblasts (Wu et al., 2017), favoring bone-resorbing osteoclasts and the consequent alveolar bone loss (Pan et al., 2019; Soud et al., 2018; Walsh et al., 2006); and (3) degradation of periodontal tissue, mainly induced by inflammatory enzymes called extracellular matrix metalloproteinases (MMP), which play a key role in the development and maintenance of periodontitis (De Morais et al., 2018). Therefore, controlling the local immune and inflammatory response is fundamental for reducing tissue damage and preventing bone loss during periodontitis (Racz et al., 2014). In fact, several studies have shown that anti-inflammatory mediators attenuate the progression of bone loss in this condition (Claudino et al., 2008; Garlet, 2010; Gemmell and Seymour, 2004; Pan et al., 2019).

Considering the widespread traditional use of *P. angulata* in inflammatory and immune-mediated diseases, the present study was designed to investigate the protective effects of the ethanolic extract of *P. angulata* (EEPA) against the progression of chronic periodontitis in a preclinical setting, pointing out possible mechanisms involved in these effects.

2. Material and methods

2.1. Animals

A total of sixty male C57BL/6 mice with mean weight of 22–25 g were obtained from the animal facilities of the Gonçalo Moniz Institute, Oswaldo Cruz Foundation (FIOCRUZ). These animals were bred and kept in a controlled environment, in a room with light/dark cycles of 12

h, at a controlled temperature of 22–24 °C, with water and food *ad libitum*. All procedures were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals (NIH, 8023) and the FIOCRUZ Institutional Animal Care and Use Committee. The FIOCRUZ Ethics Committee for Animal Use approved all the procedures from this study (permit number: CEUA 022/2015).

2.2. Botanical material and production of the EEPA

Specimens of *Physalis angulata* ("fisális" or "camapu", in Portuguese; "cutleaf groundcherry", in English) were collected during the dry season (from June to November) in Belém (PA, Brazil). The correct botanical identification was made by Dr. Lúcia Carvalho; a voucher specimen was deposited in the Herbarium of the Federal University of Rio de Janeiro (voucher number RFA23907/08). The acceptance of the taxonomic nomenclature was confirmed at The Plant List (http://www.theplantlist.org/). For obtaining EEPA, stems of *P. angulata* (1 kg) were dried and crushed, followed by extraction with ethanol at 50–60 °C during 6 h. The ethanolic extract was concentrated under reduced pressure, yielding 100 g (10%) of crude extract. The concentrated ethanolic extract was maintained in a desiccator under vacuum until weight stabilized. This same extract has been previously characterized by Nogueira et al. (2013). The HPLC-UV analysis revealed that the major components of EEPA are physalins (56.3%); more specifically, physalin F (27.9%), physalin D (15.7%), physalin B (6.7%), and physalin G (6.0%). Moreover, minor components of the extract probably encompass other physalins and withasteroids, as suggested by the retention time of these molecules. In all *in vivo* experiments, EEPA was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), at a final concentration of 5%, which is considered adequate for pharmacological assays in mice (Colucci et al., 2008; Ueda et al., 2019).

2.3. Animal model of chronic periodontitis and experimental design

Periodontitis was induced by repeated injections of lipopolysaccharide (LPS) from *Escherichia coli*, as proposed by Trombetta-e-Silva et al. (2011), with minor modifications. To induce periodontal inflammation and bone loss, 20μg of LPS diluted in 1μL of sterile water were injected into the gingival papilla, located between the first and second upper left molars. This procedure was performed three times per week for four weeks. Considering that one of the objectives of this study was to evaluate the potential of EEPA for controlling established periodontitis, daily oral administrations of the extract were made for two weeks, starting at the 15th day of LPS injections.

A total of sixty male C57BL/6 mice were randomly assigned to five experimental groups: *naïve* (untreated mice without periodontitis) (n = 12); vehicle (periodontitis-induced mice treated with saline + 5% DMSO) (n = 12); EEPA50 (periodontitis-induced mice treated with EEPA at 50 mg/kg) (n = 12); EEPA100 (periodontitis-induced mice treated with EEPA at 100 mg/kg) (n = 12); and nimesulide (periodontitis-induced mice treated with nimesulide at 25 mg/kg) (n = 12). The doses of EEPA were selected based on a previous study that has shown the antinociceptive and anti-inflammatory effects of this same extract in different experimental models in mice (Espírito-Santo et al., 2019). Nimesulide (Sigma-Aldrich), a nonsteroidal anti-inflammatory drug (NSAID) widely used in dentistry for treating inflammatory conditions (Krasniqi and Daci, 2017), was used as the gold standard drug. Twenty-four hours after the last treatment, mice were euthanized by means of an anesthetic overdose, and tissues were collected for the analyses.

2.4. Analysis of the alveolar bone structure

After euthanasia, the maxillae of mice were removed and fixed in 10% v/v formaldehyde for 24 h, for morphometric analyses. Maxillae were then separated in two hemiarches (right and left), dissected and

stained with 1% w/v methylene blue, allowing the distinction between bone tissue and teeth. The hemiarches were photographed using a Sony DSC-W530 camera attached to a Leica EZ4 magnifying glass (25 × magnification). The greatest distance between the cementum-enamel junction and the alveolar bone crest was measured using the ImagePro software (Media Cybernetics, Inc.) and used to indicate alveolar bone loss. Two independent analyses were performed.

2.5. Real-time qPCR

The transcription of the *tnf-α, il-1β, il-6, il-10, tgf-β, mmp9,* and *timp-1* genes was evaluated by means of the real-time quantitative polymerase chain reaction (RT-qPCR), from the gingival tissue of mice at the end of the experimental period, as previously described (Evangelista et al., 2018). Total RNA was extracted from the gingival tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the concentration was determined by photometric measurements (Nanodrop, 2000 machine). A high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize cDNA from 1 μg of RNA, in accordance with the manufacturer's recommendations. Synthesis of cDNA and RNA expression analysis were performed by means of real-time PCR using the TaqMan gene expression assay for *tnf-α* (*Mm_00443258_m1*), *il-1β* (*Mm_0043228_m1*), *il-6* (*Mm_00446190_m1*), *il-10* (*Mm00439616_m1*), *mmp9* (*Mm_0044429_m1*), *tgf-β* (*Mm_00441724_m1*), and *timp-1*(Mm_00441818_m1). A no-template control (NTC) and a no-reverse transcription control (No-RT) were also included. All reactions were run in duplicate in an ABI7500 sequence detection system (Applied Biosystems) under standard thermal cycling conditions. The mean cycle threshold (Ct) values from the duplicate measurements were used to calculate the expression of the target gene, with normalization to an internal control *gapdh* (*Mm99999915_g1*), using the 2-DCt formula. Experiments with co-efficients of variation greater than 5% were excluded. The mean Ct values were used to calculate the expression of genes normalized to *gapdh*, using the Ct method of comparative PCR.

2.6. ELISA

Cytokines in the gingival tissue were quantified by means of the sandwich ELISA technique, as previously described (Gama et al., 2018). After euthanasia, the gingival tissue was removed and immediately stored at -80 °C. The samples were homogenized in phosphate-buffered saline (PBS; 100 mg tissue/mL), and the following were added: 0.4 M NaCl, 0.05% Tween 20, and protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KI aprotinin A/100 mL) (Sigma). The samples were then centrifuged for 10 min at 3000 g and aliquots of the supernatant were frozen at -80 °C for further quantification. Analyses on IL-1β, IL-6, and TGF-β were performed using the Duoset ELISA development system kit (R&D Systems), in accordance with the manufacturer's recommendations. The assays were performed in duplicate and the plates were read in a spectrophotometer (Spectra Max 190; Molecular Devices) at a wavelength of 450 nm. The results were expressed in picograms of cytokine per milligram of protein.

2.7. Hemogram and serum biochemistry

After cardiac puncture, blood samples were stored in vials containing EDTA (Wolforth, 2000). The following blood counts were determined: white blood cell count (WBC), neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), platelets (PLAT), and mean platelet volume (MPV). The values were determined using an automated method in the BC-2800 VET/Mindray equipment. Differential counts were obtained by means of a blood smear stained with Panopticus. Serum biochemical analyses included alanine

transaminase (ALT), aspartate transaminase (AST), and urea. For these, standard commercial reagents (Labtest®) with kinetic, enzymatic, or colorimetric methodologies were used at 37 °C, and readings were made in a semi-automatic spectrophotometer (Bio-Plus® biochemical analyzer).

2.8. Macroscopic analysis of organs and body mass variation

Kidneys, hearts, and livers were collected, photographed, and weighed to assess whether there were any significant changes in size, shape, weight, and consistency among the experimental groups. Additionally, daily weighing of the animals was done throughout the experimental period. The weight of each organ was normalized according to the body weight and represented as the weight per 10 g of body weight.

2.9. NF-κB luciferase assay

Macrophages cells line Raw 264.7 Luc bearing the pBIIX-luciferase (pBIIX-luc) targeting vector containing the firefly luciferase gene (luc) driven by two NF-kB binding sites from the kappa light chain enhancer in front of a minimal fos promoter were used. Cells were cultured in RPMI medium (Sigma) supplemented with 20% FBS and gentamycin (50 μg/mL) at 37 °C and 5% CO_2. For the luciferase reporter assay, 5 × 10^5 cells/mL were pretreated with vehicle (saline + 5% DMSO), EEPA (0.25, 0.5, or 1 mg/mL), or dexamethasone (15 μg/mL); after 1 h, cells were stimulated with LPS (500 ng/mL) and IFN-γ (5 ng/mL) for 3 h. The wells were washed with cold PBS and the cells were incubated with TNT lysis buffer (200 mM Tris, pH 8.0, 200 mM NaCl, 1% Triton X-100) for 20 min at 4 °C. The luciferase activity in cell lysates was measured using the Luciferase Assay System (Pro-mega, Madison, WI, USA). Samples were analyzed in a Globomax 20/20 luminometer (Promega) and data were expressed as relative light units (RLU) (Espirito-Santo et al., 2017).

2.10. Statistical analysis

The results were presented as the mean ± standard deviation (SD) from the analyses done on groups of mice. Comparisons among three or more treatments were made using one-way analysis of variance (ANOVA) with the Tukey's post-hoc test. The factors analyzed were treatments, time and treatment–time interaction. The results were analyzed using the Prism 7 software (GraphPad, San Diego, CA, USA). Statistical differences with $p < 0.05$ were considered significant.

3. Results

3.1. EEPA reduces the progression of alveolar bone loss

The distance between the cement-enamel junction and the alveolar bone crest in the vehicle group (periodontitis-induced mice treated with saline + 5% DMSO) was greater than that in the *naïve* group (untreated mice without periodontitis) ($p < 0.001$). This result indicates the occurrence of alveolar bone loss, confirming that the chronic periodontitis-induction protocol was effective (Fig. 1). In order to evaluate whether EEPA could inhibit the disease progression, evidenced by the alveolar bone loss, oral administrations of the extract started on the 15th day of LPS injections, and continued for the next two weeks. The results show that daily oral administrations of EEPA at 50 or 100 mg/kg inhibited the progression of alveolar bone loss, in comparison with the vehicle group ($p < 0.05$). This effect was similar to that of nimesulide, a NSAID widely used in dentistry for treating inflammatory conditions. Daily treatment with nimesulide (reference drug; 25 mg/kg) also inhibited the bone loss progression in comparison with the vehicle group ($p < 0.05$).
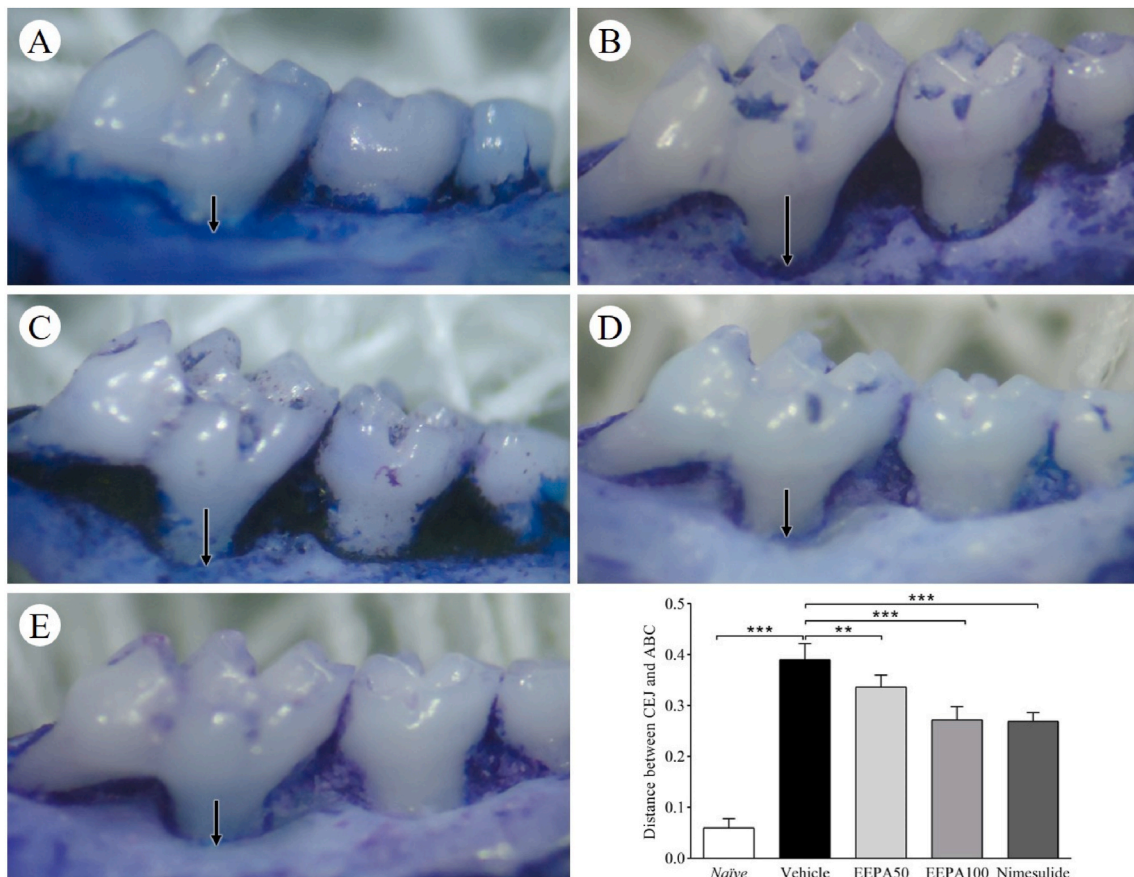
Fig. 1. Effect of daily oral treatment with EEPA on alveolar bone resorption in chronic experimental periodontitis. Panels A to E show, respectively, representative images of *naïve*, vehicle, EEPA at 50 mg/kg, EEPA at 100 mg/kg, and nimesulide at 25 mg/kg groups. Arrows indicate the distance between the cementum-enamel junction and the alveolar bone crest. Data were represented as mean $\pm$ standard deviation. Statistical significance **$p < 0.01$; ***$p < 0.001$, as determined by one-way ANOVA, followed by Tukey's multiple comparison test.

### 3.2. EEPA reduces the mRNA levels of MMP-9, but not of TIMP-1, in the gingival tissue

The next step of the study was the assessment of gene expression of periodontitis biomarkers in the gingival tissue. As shown in Fig. 2, the chronic experimental periodontitis (vehicle group) was associated with local increases in MMP-9 (Fig. 2A) and TIMP-1 (Fig. 2B) mRNA in comparison with the *naïve* group ($p < 0.05$). Treatment with EEPA (50 or 100 mg/kg) or nimesulide (reference drug; 25 mg/kg) significantly decreased the levels of MMP-9 mRNA in the gingival tissue of mice during the chronic experimental periodontitis, compared with the vehicle group ($p < 0.001$). On the other hand, TIMP-1 mRNA levels in the gingival tissue of mice with periodontitis were not altered by the treatments with EEPA or nimesulide.

3.3. EEPA promotes a local anti-inflammatory status by reducing the gingival levels of IL-1β and IL-6 and increasing those of TGF-β

Because alveolar bone resorption and tissue damage are associated with a local inflammatory response, the levels of pro-inflammatory cytokines were next evaluated (Fig. 3). Local increases in IL-1β, IL-6, and TNF-α mRNA were evidenced in the mice groups with chronic experimental periodontitis, in comparison with the *naïve* group. Oral treatment with EEPA at 50 or 100 mg/kg significantly decreased the IL-1β mRNA (Fig. 3A; $p < 0.001$), whereas, only at the 100 mg/kg dose, the extract reduced the IL-6 mRNA levels (Fig. 3B; $p < 0.01$). The TNF-α
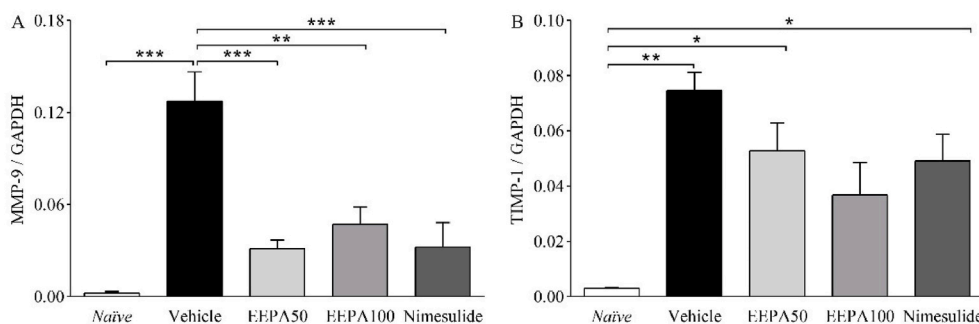
Fig. 2. Effect of daily oral treatment with EEPA on the expression of MMP-9 and TIMP-1 in the gingival tissue of mice with chronic experimental periodontitis. The gingival levels of MMP-9 (panel A) and TIMP-1 (panel B) mRNA were measured by RT-qPCR 24 h after the last daily treatment. The y-axis shows the mRNA expression of the target gene normalized by the constitutive *gapdh* gene. Data are represented as mean $\pm$ standard deviation; Statistical significance *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as determined by one-way ANOVA, followed by Tukey's multiple comparison test.
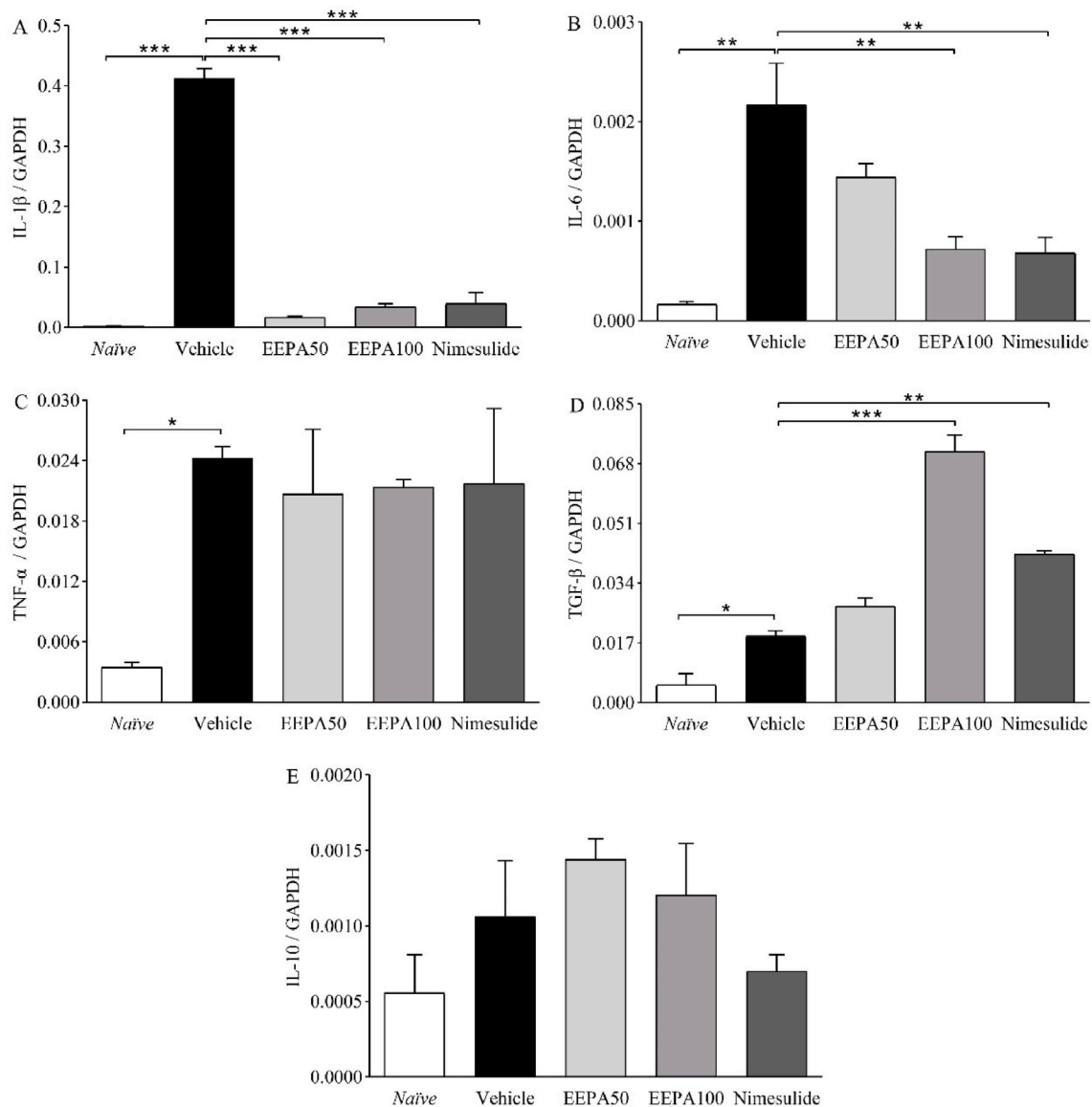
Fig. 3. Effect of daily oral treatment with EEPA on the expression of pro-inflammatory and anti-inflammatory cytokines in the gingival tissue of mice with chronic experimental periodontitis. The gingival levels of IL-1β mRNA (panel A), IL-6 mRNA (panel B), TNF-α mRNA (panel C), TGF-β mRNA (panel D), and IL-10 mRNA (panel E) were measured by RT-qPCR 24 h after the last daily treatment. The y-axis shows the mRNA expression of the target gene normalized by the constitutive *gapdh* gene. Data are represented as mean ± standard deviation; Statistical significance $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, as determined by one-way ANOVA, followed by Tukey's multiple comparison test.

mRNA in the gingival tissue of mice with periodontitis was not modified by the treatments (Fig. 3C). Oral treatment with nimesulide (reference drug; 25 mg/kg) reduced the mRNA levels of IL-1β and IL-6, but not of TNF-α, in the gingival tissue ($p < 0.01$).

The next step was to study the gingival tissue looking for a putative anti-inflammatory status. The chronic experimental periodontitis was associated with a slight local increase in TGF-β mRNA (Fig. 3D; $p < 0.05$), but not in IL-10 mRNA (Fig. 3E). Oral treatment with EEPA at 100 mg/kg ($p < 0.001$) or nimesulide at 25 mg/kg ($p < 0.01$) increased the local levels of TGF-β mRNA, in comparison with the vehicle group. The IL-10 mRNA in the gingival tissue was not modulated by any of the performed treatments.

In agreement with the PCR data, there was a local increase ($p < 0.001$) in the protein levels of the pro-inflammatory cytokines IL-1β (Fig. 4A) and IL-6 (Fig. 4B) in the vehicle group (experimental disease), as evidenced by the ELISA assay. Treatment with EEPA (50 or 100 mg/kg) or nimesulide (25 mg/kg) reduced the levels of both IL-1β and IL-6 ($p < 0.001$), in comparison with the vehicle group. On the other hand,

only the treatment with EEPA at 100 mg/kg increased the gingival levels of the anti-inflammatory cytokine TGF-β (Fig. 4C; $p < 0.05$).

3.4. Evaluation of the systemic alterations promoted by EEPA

To assess whether the treatments were associated with hematological and biochemical alterations, blood samples were analyzed using standard commercial reagents (Labtest®) with kinetic, enzymatic or colorimetric methodologies. Additionally, macroscopic changes in organs and body mass variation were also assessed. As shown in Table 1, mice treated with vehicle (saline + 5% DMSO) presented reduced white blood cell (WBC) counts and lymphocyte (LYM) values, compared with the *naïve* group ($p < 0.05$), thus indicating that the presence of chronic experimental periodontitis was associated with hematological changes. In mice with periodontitis that were daily treated with EEPA (50 or 100 mg/kg), no statistically significant hematological changes were observed, suggesting that this treatment reduced the systemic manifestations of the disease. Importantly, the daily treatment with nimesulide
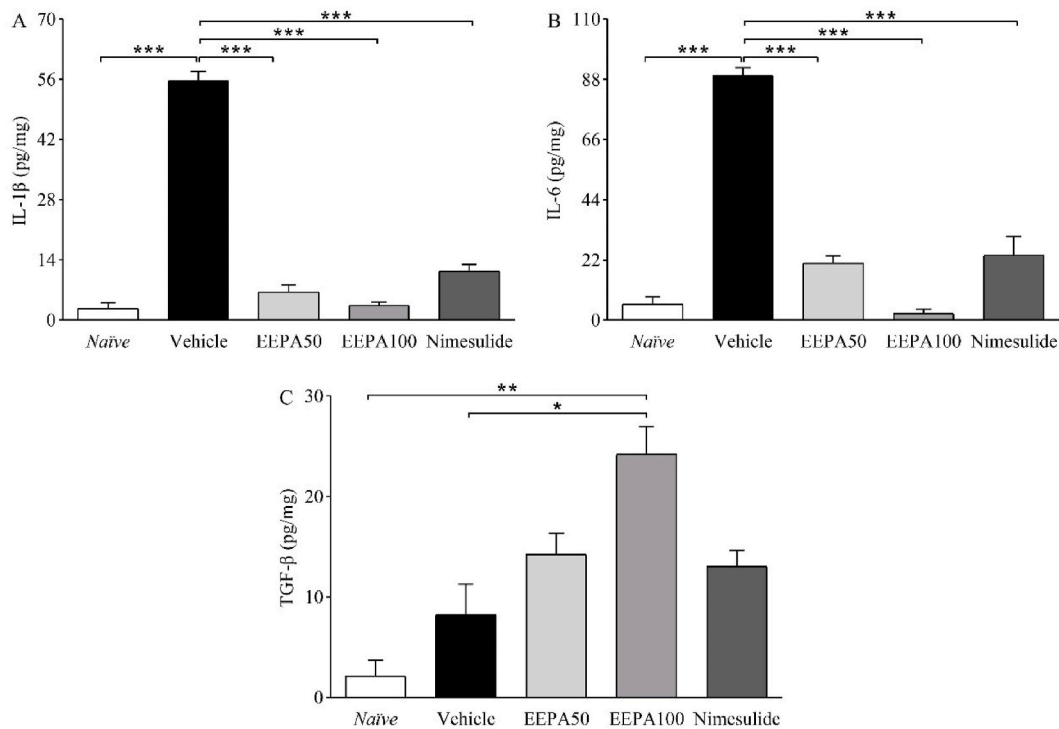
Fig. 4. Effect of daily oral treatment with EEPA on cytokine levels in the gingival tissue of mice with chronic experimental periodontitis. IL-1β (panel A), IL-6 (panel B), and TGF-β (panel C) levels were measured by ELISA 24 h after the last daily treatment. Panels show the gingival levels of IL-1β (A), IL-6 (B), and TGF-β (C) expressed as picograms of cytokine per milligram of protein. Data are represented as mean ± standard deviation. Statistical significance *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as determined by one-way ANOVA, followed by Tukey's multiple comparison test.

Table 1
Effects of daily oral treatment with EEPA or nimesulide on murine hematological parameters.

Hematological parameters	*Naïve*	Vehicle	EEPA 50 mg/kg	EEPA 100 mg/kg	Nimesulide
RBC ($10/mm^3$)	9.51 ± 0.31	9.05 ± 0.61	10.48 ± 0.90	9.33 ± 0.85	2.75 ± 0.25#*
HGB (g/dL)	13.55 ± 0.55	9.05 ± 0.61	14.64 ± 1.08	13.18 ± 1.33	3.80 ± 0.41#*
HCT (%)	46.06 ± 1.41	44.06 ± 2.06	51.60 ± 3.72	45.03 ± 3.84	13.78 ± 1.54#*
MCHC (g/dL)	29.65 ± 0.29	29.16 ± 0.81	28.38 ± 0.22	29.25 ± 0.52	27.56 ± 0.47#*
PLAT (mm^3)	837.83 ± 209.97	795.20 ± 117.05	760.80 ± 147.00	795.00 ± 124.21	288.00 ±89.76#*
WBC ($10^3/mm^3$)	7.05 ± 2.75	2.99 ± 1.98#	4.26 ± 2.28	3.70 ± 1.47	1.04 ± 0.54#
NEU (%)	0.90 ± 0.32	0.66 ± 0.38	0.53 ± 0.31	0.74 ± 0.19	0.18 ± 0.11#
LYM (%)	6.08 ± 2.54	2.27 ± 1.69#	3.68 ± 1.96	2.93 ± 1.30	0.78 ± 0.46#

RBC (red blood cells), HGB (hemoglobin), HCT (hematocrit), MCHC (mean corpuscular hemoglobin), PLAT (platelets), WBC (white blood cell count), NEU (neutrophils), and LYM (lymphocyte). Groups: *Naïve*; Vehicle (control group); EEPA 50 mg/kg; EEPA 100 mg/kg; Nimesulide (25 mg/kg). Data are represented as mean ± standard deviation. # Significance against the *naïve* group ($p < 0.05$; Tukey); * Significance against the vehicle group ($p < 0.05$; Tukey).

(25 mg/kg) not only was ineffective in preventing reductions in WBC and LYM associated with periodontitis, but also decreased all the other hematological parameters evaluated ($p < 0.05$), including red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular hemoglobin (MCHC), platelets (PLAT), and neutrophils (NEU).

Biochemical analyses showed no alterations in the serum levels of alanine transaminase (ALT), aspartate transaminase (AST), and urea of mice with chronic experimental periodontitis (Table 2). Neither of the performed treatments altered the evaluated parameters. Signs of systemic toxicity were also searched by analyzing the macroscopic appearance and weight of the liver, heart, and kidneys (Table 2). All the evaluated organs exhibited normal appearance in all the experimental groups, with absence of edema or other macroscopic changes. No statistically significant differences in the relative weights of organs were detected among the experimental groups.

Table 2
Effects of daily oral treatment with EEPA or nimesulide on murine serum biochemical markers and weight of organs (g/10 g of body weight).

Parameters	*Naïve*	Vehicle	EEPA 50 mg/kg	EEPA 100 mg/kg	Nimesulide
ALT (UI/L)	32,80 ± 4,65	21,40 ± 5,50	22,80 ± 3,19	25,00 ± 9,02	26,40 ± 5,50
AST (UI/L)	95,00 ± 21,22	100,60 ± 22,59	77,00 ± 11,29	88,00 ± 12,10	92,00 ± 15,50
UREA (mg/dL)	62,05 ± 7,76	54,73 ± 2,29	56,15 ± 1,62	53,82 ± 2,88	57,31 ± 4,30
Livers	0,45 ± 0,02	0,43 ± 0,02	0,44 ± 0,02	0,45 ± 0,04	0,43 ± 0,08
Kidneys	0,12 ± 0,00	0,10 ± 0,01	0,10 ± 0,00	0,11 ± 0,01	0,12 ± 0,01
Hearts	0,04 ± 0,00	0,04 ± 0,01	0,04 ± 0,00	0,04 ± 0,00	0,04 ± 0,00

Alanine transaminase (ALT), Aspartate transaminase (AST), Urea, Weight of organs (g/10 g of body weight). Groups: *Naïve*; Vehicle (control group); EEPA 50 mg/kg; EEPA 100 mg/kg; Nimesulide (25 mg/kg). Data are represented as mean ± standard deviation. No statistically significant differences were detected among the experimental groups.

3.5. EEPA reduces NF-κB-dependent transcriptional activity in macrophages

The NF-κB luciferase assay was performed aiming to identify a possible mechanism of action of EEPA. Firstly, a cell viability assay demonstrated that EEPA at 1 mg/mL or lower concentrations was not cytotoxic to Raw 264.7 Luc cells 72 h after incubation (data not shown); the effect of EEPA on NF-κB activation was then evaluated within the non-cytotoxic concentration range (Fig. 5). Macrophages stimulated with LPS and IFN-γ showed increased levels of NF-κB-dependent transcriptional activity ($p < 0.01$). The pretreatment with EEPA at 0.5 or 1 mg/mL significantly reduced NF-κB-dependent transcription when compared to the vehicle treated group ($p < 0.01$). A similar effect was observed for the reference drug dexamethasone ($p < 0.01$), which was not statistically different from EEPA.

4. Discussion

In this study, the therapeutic potential of EEPA was evaluated in an animal model of chronic periodontitis. The results demonstrated that daily oral treatment with EEPA inhibited alveolar bone resorption, reduced a major periodontitis marker in the gingival tissue, and modulated the local balance between pro- and anti-inflammatory cytokines. Additionally, daily oral treatment with EEPA did not induce hematological or biochemical changes, nor any apparent systemic toxicity, thus indicating that its toxicological profile is favorable. The luciferase assay performed on macrophages indicate that a potential mechanism of action of EEPA is the inhibition of NF-κB transcription. Altogether these data provide evidence that EEPA has therapeutic potential for the treatment of immuno-inflammatory conditions like periodontitis, corroborating the reports of traditional use of *P. angulata* as an anti-inflammatory and immunomodulatory agent.
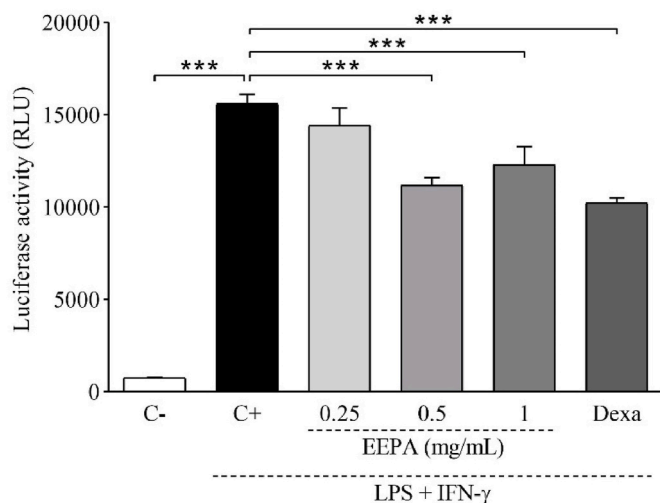
Repeated injections of LPS into the gingival papilla of mice induced mRNA transcription of MMP-9, a key protease involved in periodontitis. MMP-9 is a gelatinase B (collagenase) that acts by degrading the basal membrane, elastin, and collagens of types V, VII, and X, the main components of periodontal support tissues (Kim et al., 2013; Smith et al.,

Fig. 5. Effect of EEPA on NF-κB-dependent transcriptional activity. Raw 264.7 Luc cells were pretreated with vehicle (saline + 5% DMSO; C+), EEPA (0.25, 0.5, or 1 mg/mL), or the reference drug dexamethasone (Dexa; 15 μg/mL) and then stimulated with LPS (500 ng/mL) and IFN-γ (5 ng/mL). Negative control (C-) shows luciferase activity in unstimulated cells. The luciferase activity was measured in a luminometer and expressed as relative light units (RLU). Data are represented as mean ± standard deviation. Statistical significance ***$p < 0.001$, as determined by one-way ANOVA, followed by Tukey's multiple comparison test.

2004). Moreover, this enzyme enables the inflow of leukocytes to the damaged tissue and the release of cytokines and chemokines (Vandooren et al., 2013), favoring a pro-inflammatory microenvironment. The protective role of EEPA against bone resorption in experimental conditions was associated with a decrease in MMP-9 mRNA levels in the gingival tissue of mice with periodontitis. Considering the pivotal role of MMP-9 in periodontal tissue damage, targeting this enzyme could be an important strategy in periodontal therapy, allowing the prevention of alveolar bone resorption, teeth loss, and other comorbidities that affect patients' health.

The activity of MMP-9 is blocked by a specific tissue inhibitor of metalloproteinase (TIMP-1) (Duda et al., 2020). The balance between MMP-9 and TIMP-1 regulates the extension of periodontal tissue degradation, and hence the development and severity of periodontitis (De Morais et al., 2018). The chronic experimental periodontitis resulted in increased TIMP-1 mRNA levels in the gingival tissue of mice. This can be attributed to positive feedback mechanisms of protection against excessive damage. Hseu et al. (2011) have shown that the ethyl acetate extract of *P. angulata* suppresses the activity of MMP-9 by increasing the expression of endogenous inhibitors of the enzyme, including TIMP-1. In contrast, the treatment with EEPA at the tested doses did not alter the mRNA levels of TIMP-1 in mice with chronic experimental periodontitis. The discrepancy between the studies can be explained by differences in geographical origin, parts of the plants, and solvents used in the obtention of the extracts. Since the effect of EEPA was independent of TIMP-1 modulation, it is possible to propose that the protective action against bone resorption derives from the inhibition of MMP-9 expression.

The synthesis of MMP-9 is highly influenced by the pro-inflammatory environment established during periodontitis, which includes cytokines such as TNF-α and interleukins. This is corroborated by the present data, as shown by the significant increase in IL-1β, IL-6, and TNF-α mRNA in the gingival tissue of mice with chronic experimental periodontitis. IL-1β acts as a potent inducer of MMP-9 expression; the levels of this cytokine are typically increased in the gingival tissue and crevicular fluid during periodontitis (Xing et al., 2016). The release of IL-1β by mononuclear cells amplifies the inflammatory response and the bone loss via production of MMP-9 by fibroblasts of the periodontal ligament as well as by osteoblasts (Abe et al., 2001; Ramamurthy et al., 2002). This cytokine also enhances osteoclast formation and activity, and stimulates apoptosis of matrix-producing cells, resulting in inflammation, connective tissue breakdown, bone loss, and limited repair of the periodontium (Graves and Cochran, 2003). The oral treatment with EEPA strongly reduced both mRNA and protein levels of IL-1β in the gingival tissue of mice with chronic experimental periodontitis. Considering the relevance of IL-1β for the pathophysiology of periodontitis, its inhibition by EEPA may be a crucial event for the prevention of alveolar bone loss.

In addition to IL-1β, the cytokine IL-6 also plays an important role in bone resorption (Takagi et al., 2020). Although IL-6 can be detected at low levels in clinically healthy gingival tissues, its secretion is intensified during periodontal inflammation; the presence of this cytokine is closely associated with active periodontitis (Lins et al., 2007). In fact, IL-6 has been associated with both the acute phase of periodontitis and the progression of the disease (Ebersole et al., 2014). This cytokine stimulates the expression of the receptor activator of RANKL and reduces the expression of osteoprotegerin, thereby leading to osteoclastogenesis and bone resorption (Nagy and Penninger, 2015). In the present study, the animal model of periodontitis induced by LPS was marked by the increase in the levels of inflammatory cytokines, including IL-6. This agrees with reports that LPS has a notable effect on most types of inflammatory cells found in the periodontal tissues (Hiroshima et al., 2018; Ohno et al., 2017). The oral treatment with EEPA strongly reduced the levels of IL-6 in the gingival tissue of mice with periodontitis, suggesting that its inhibitory effects on bone resorption are not due to a direct action on osteoclasts, but rather a consequence of the

modulation of pro-inflammatory cytokines.

The ability of physalins isolated from *P. angulata* to reduce the production of pro-inflammatory cytokines has already been demonstrated under different experimental *in vitro* and *in vivo* conditions (Jacobo-Herrera et al., 2006; Lima et al., 2014; Pinto et al., 2016; Soares et al., 2003, 2006; Vieira et al., 2005). Nevertheless, it has been proposed that the ideal cytokine-based treatment consists of shifting the balance towards anti-inflammatory cytokines, rather than just inhibiting pro-inflammatory cytokines (Schäfers and Sommer, 2007). In the present study, the oral treatment with EEPA promoted modulatory effects on the production of cytokines, stimulating the synthesis of TGF-β, while reducing the levels of IL-1β and IL-6. TGF-β plays a protective role in periodontitis by suppressing the production of MMP-9, thereby reducing collagen degradation and the consequent loss of periodontal support tissues (Page, 1991). Moreover, TGF-β is one of the most important factors that counteract the effects of IL-1β, contributing to tissue regeneration during periodontitis (Andriamanalijaona et al., 2006). The modulatory effect on pro- and anti-inflammatory cytokines balance during periodontitis has been achieved by the use of mesenchymal stem cells therapy (Racz et al., 2014), but there are few reports of natural products with this therapeutic profile. The results described here suggest an ideal mechanism of action of EEPA for controlling periodontitis, although its effects still need to be confirmed in a clinical setting.

A large number of stimuli lead to activation of NF-κB signaling pathway, which acts as a pivotal regulator of inflammatory and immune response, controlling the expression of pro-inflammatory genes (Liu et al., 2017). To better understand the EEPA mechanisms of action, a possible modulatory effect of this extract on NF-κB was investigated. The *in vitro* luciferase assay has shown the reduction of NF-κB-dependent transcriptional activity in macrophages treated with EEPA. Macrophages have a key role in the modulation of immune and inflammatory responses; many of its effects are determined by the NF-κB signaling pathway. This important transcription factor is responsible for the synthesis of major pro-inflammatory molecules, such as TNF-α, IL-1β and IL-6 (Fujiwara and Kobayashi, 2005). Moreover, macrophage activation is deeply related to the development of periodontitis (Yang et al., 2018). Overall, our results support the hypothesis that EEPA acts by inhibiting the NF-κB signaling pathway and hence the production of pro-inflammatory mediators by macrophages, thereby promoting immunomodulatory effects in the chronic experimental periodontitis.

Considering that EEPA is mostly composed by physalins (Nogueira et al., 2013), and that the anti-inflammatory and immunomodulatory properties of these molecules have already been demonstrated by many authors (Jacobo-Herrera et al., 2006; Pinto et al., 2016; Soares et al., 2003, 2006; Vieira et al., 2005), these compounds are likely accountable for the pharmacological activities of EEPA in the chronic experimental periodontitis. Moreover, the minor withasteroid components can also contribute to the global effect of EEPA, since the anti-inflammatory and immunomodulatory properties of withasteroids have already been experimentally demonstrated (Tomassini et al., 2000). Further studies are needed to determine which compounds are accountable for the effects reported herein.

To investigate the safety of EEPA, an important assessment for the validation of traditional use, some parameters of systemic toxicity were evaluated. Daily oral treatment with EEPA did not cause behavioral changes, macroscopic organ alterations, nor did it alter any indicative parameters of hepatic, renal, or hematological toxicity, thus suggesting a favorable preclinical toxicological profile. Our results agree with previous *in vivo* studies that have demonstrated the low toxicity of systemic administrations of extracts and isolated compounds of *P. angulata* (Bastos et al., 2008; Espírito-Santo et al., 2019; Lima et al., 2014; Meira et al., 2015; Soares et al., 2006; Vieira et al., 2005). Importantly, daily oral treatment with the reference drug nimesulide reduced the hematological counts of RBC, HGB, MCHC, PLAT, and HCT. A significant reduction in RBC can be explained by a possible gastric mucosal hemorrhagic process and/or a decrease in erythropoietin due to renal

damage caused by the continuous use of nimesulide (Manocha et al., 2016), and it is indicative of absolute anemia. The reduction in mean hemoglobin concentration indicates possible hemorrhage and, consequently, corroborates the absolute anemia suggested by RBC and HGB in the nimesulide-treated mice.

5. Conclusions

The ethanolic extract of *P. angulata* (EEPA) effectively reduced the chronic periodontitis progression under experimental conditions. The effects of EEPA were mediated by the reduction of gingival levels of MMP-9, probably as a consequence of the cytokines balance towards an anti-inflammatory profile, as indicated by the reduction in IL-1β and IL-6 with concomitant increase in TGF-β levels. The immunomodulatory effects of EEPA could be associated with inhibition of the NF-κB pathway in inflammatory cells. The daily oral treatment with EEPA did not induce hematological or biochemical changes, nor any apparent systemic toxicity, revealing a better safety profile than that of the reference drug nimesulide. These results corroborate the traditional use of *P. angulata* in the treatment of oral inflammatory conditions, demonstrating possible mechanisms by which this plant promotes its anti-inflammatory and immunomodulatory effects. Nevertheless, it is important to bear in mind the limitations of animal models for representing human pathobiology, particularly in the case of periodontitis, which has a multifactorial and complex etiology that involves interactions among genes, lifestyles, and oral microbiome composition. The precise mechanisms of the protective effects of EEPA against alveolar bone loss as well as its applicability in the human periodontitis remain objects of further investigation.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Credit author statement

Paula Schons Vieceli: data curation, investigation, roles/Writing - original draft; *Paulo José Lima Juiz*: conceptualization, investigation, formal analysis, roles/Writing - original draft; *Pedro Santana Sales Lauria*: formal analysis, writing - review & editing; *Ricardo David Couto*: formal analysis, writing - review & editing; *Therezinha Coelho Barbosa Tomassini*: methodology, resources; *Ivone Maria Ribeiro*: methodology, resources; *Milena Botelho Pereira Soares*: funding acquisition, resources, writing - review & editing; *Cristiane Flora Villarreal*: conceptualization, supervision, formal analysis, writing - review & editing, project administration.

Declaration of competing interest

The authors report no conflicts of interest related to this study.

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