

Effect of *Stryphnodendron adstringens* (Mart.) Coville extract in aerobic and anaerobic microorganisms and mammalian cells

Efeito do extrato de *Stryphnodendron adstringens* (Mart.) Coville em microrganismos aeróbios e anaeróbios e células de mamíferos

Efecto del extracto de *Stryphnodendron adstringens* (Mart.) Coville en microorganismos aeróbicos y anaeróbicos y células de mamíferos

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Abstract

Stryphnodendron adstringens (Mart.) Coville is a medicinal plant known for its anti-inflammatory and antimicrobial properties. This study evaluated some biological activities of extract from *S. adstringens*. Antimicrobial activity was checked in planktonic cultures and monomicrobial biofilms on aerobic, and anaerobic dental microorganisms. Analyzes of cytotoxicity using MTT assay, and genotoxicity by micronucleus test were performed in human keratinocytes (HACAT), murine macrophages (RAW 264.7), and murine fibroblasts (L929). The anti-inflammatory

effect was checked in RAW 264.7 stimulated by lipopolysaccharide (LPS) from *Escherichia coli*, and treated with the plant extract. The levels of cytokines, and nitric oxide (NO) were measured by ELISA, and Griess method, respectively. Data were analyzed by ANOVA, followed by Tukey's, or Kruskal-Wallis, and Dunns tests ($P \leq 0.05$). Biofilms of anaerobic bacteria were very susceptible to the plant extract. Effective concentrations showed cell viability $> 50\%$, except 25 mg/mL for HACAT after 24 h of exposure. The extract of *S. adstringens* was not genotoxic for RAW 264.7. LPS associated with extract increased the production of all cytokines, except TNF- α . However, the plant extract decreased the production of NO. In conclusion, the extract of *S. adstringens* affected biofilm of anaerobic bacteria using non-cytotoxic concentrations for RAW 264.7, L929, and HACAT cells.

Keywords: Antimicrobial; Anti-inflammatory; Bacteria anaerobic; Biofilms; Medicinal plant; Phytotherapy.

Resumo

Stryphnodendron adstringens (Mart.) Coville é uma planta medicinal conhecida por suas propriedades anti-inflamatórias e antimicrobianas. Este estudo avaliou algumas atividades biológicas do extrato de *S. adstringens*. A atividade antimicrobiana foi verificada em culturas planctônicas e biofilmes monomicrobianos em microrganismos aeróbios e anaeróbios de interesse odontológico. Análises de citotoxicidade utilizando ensaio de MTT e genotoxicidade por meio do teste de micronúcleo foram realizadas em queratinócitos humanos (HACAT), macrófagos murinos (RAW 264.7) e fibroblastos murinos (L929). O efeito anti-inflamatório foi verificado em RAW 264.7 estimulado por lipopolissacarídeo (LPS) de *Escherichia coli* e tratado com o extrato da planta. Os níveis de citocinas e óxido nítrico (NO) foram medidos por ELISA e método de Griess, respectivamente. Os dados foram analisados por ANOVA e teste de Tukey ou Kruskal-Wallis e teste de Dunns ($P \leq 0,05$). Os biofilmes de bactérias anaeróbias foram muito suscetíveis ao extrato da planta. As concentrações efetivas mostraram viabilidade celular $> 50\%$, exceto 25 mg/mL para HACAT após 24 h de exposição. O extrato de *S. adstringens* não foi genotóxico para RAW 264.7. O LPS associado ao extrato aumentou a produção de todas as citocinas, exceto TNF- α . No entanto, o extrato da planta diminuiu a produção de NO. Em conclusão, o extrato de *S. adstringens* afetou o biofilme de bactérias anaeróbias usando concentrações não citotóxicas para células RAW 264.7, L929, e HACAT.

Palavras-chave: Antimicrobiano; Anti-inflamatório; Bactéria anaeróbia; Biofilme; Fitoterapia; Planta medicinal.

Resumen

Stryphnodendron adstringens (Mart.) Coville es una planta medicinal conocida por sus propiedades antiinflamatorias y antimicrobianas. Este estudio evaluó algunas actividades biológicas del extracto de *S. adstringens*. La actividad antimicrobiana se verificó en cultivos planctónicos y biopelícula monomicrobianos en microorganismos aerobios y anaerobios de interés dental. Se realizaron análisis de citotoxicidad mediante ensayo MTT y genotoxicidad mediante ensayo de micronúcleos en queratinocitos humanos (HACAT), macrófagos murinos (RAW 264.7) y fibroblastos murinos (L929). El efecto antiinflamatorio se verificó en RAW 264.7 estimulado por lipopolisacárido (LPS) de *Escherichia coli* y tratado con el extracto vegetal. Los niveles de citocinas y óxido nítrico (NO) se midieron mediante ELISA y el método de Griess, respectivamente. Los datos se analizaron mediante ANOVA y prueba de Tukey o prueba de Kruskal-Wallis y Dunns ($P \leq 0,05$). Las biopelículas de bacterias anaerobias fueron muy susceptibles al extracto de la planta. Las concentraciones efectivas mostraron viabilidad celular $> 50\%$, excepto 25 mg/mL para HACAT después de 24 h de exposición. El extracto de *S. adstringens* no fue genotóxico para RAW 264.7. El LPS asociado con el extracto aumentó la producción de todas las citocinas, excepto TNF- α . Sin embargo, el extracto de la planta disminuyó la producción de NO. En conclusión, el extracto de *S. adstringens* afectó el biopelícula de bacterias anaerobias utilizando concentraciones no citotóxicas para RAW 264.7, L929 y HACAT.

Palabras clave: Antiinflamatorio; Antimicrobiana; Bacterias anaerobias; Biopelícula; Fitoterapia; Planta medicinal.

1. Introduction

The search for new antimicrobial drugs has become increasingly important due, mainly, to the resistance that microorganisms develop after a while under the action of these drugs. The study of medicinal plants can be an excellent alternative because they have a rich constitution of active biomolecules. *Stryphnodendron adstringens* (Mart.) Coville is a medicinal plant rich in tannins belonging to the Fabaceae family. Native to southeastern and central-western Brazil, it produces fleshy, light-brown fruits with many seeds (Lorenzi H & Matos FJA, 2002; Maroni BC, 2006). Empirically, *S. adstringens* has been used as an antiseptic and antimicrobial as well as for treatments of diabetes, inflammation, gastritis, liver disease, pain, diarrhea, and bleeding (Costa et al., 2013; De Santana et al., 2016). Scientifically, some studies demonstrate that the aqueous extract of *S. adstringens* has antioxidant and anticancer properties (da Silva Baldivia et al., 2018), antifungal, including action against fluconazole-resistant isolates (de Freitas et al., 2018), pronounced anti-inflammatory activity (Henriques et al., 2016), and antimicrobial action (Costa et al., 2010; Ferreira, Palmeira, Souza, Almeida, Figueiredo, Pequeno, Arruda, 2010).

This fact is important because several oral diseases have a dysbiotic microbial biofilm as the main etiological factor. Biofilms are three-dimensional structures in which several species of microorganisms are surrounded by an extracellular matrix that hinders the action of the immune system and antimicrobial agents. The species that make up the oral biofilm change as the maturity of the structure increases and may also vary between individuals. (Karched et al., 2015; Pathan, 2017).

There are many species capable of forming biofilms, such as *Candida albicans*, a yeast that can also form germ tubes and has adhesion proteins (Ganguly & Mitchell, 2011). It is the main species responsible for causing oral candidosis. It can grow in anaerobiosis, being able to contribute to periodontal and endodontic infections (Bernal-Treviño et al., 2018; Razina et al., 2017). *Enterococcus faecalis* is an optional anaerobic bacterium that can invade dentinal tubules, initiate biofilm, and is associated with secondary endodontic infections (Tennert, Fuhrmann, Wittmer, Karygianni, Altenburger, Pelz, 2014; Wilson et al., 2015). *Pseudomonas aeruginosa* is an opportunistic aerobic pathogen that causes different acute and chronic infections. One of the main causes of nosocomial infections, and also associated with persistent root canal infections (Crone et al., 2020; Pourhajibagher et al., 2017). *Staphylococcus aureus*, considered an important opportunistic nosocomial pathogen is an optional anaerobic bacterium that frequently colonizes the nostrils without causing problems (Mehraj J, Witte W, Akmatov MK, Layer F, 2016). *Streptococcus mutans* is an optional anaerobic microorganism prevalent in the caries process. Important in the biofilm initiation process, it is capable of synthesizing extracellular polymers (Krzyściak, Jurczak, Kościelniak, Bystrowska B, 2014).

Obligate anaerobic bacteria can also form a biofilm, such as *Fusobacterium nucleatum*, an important bacterium for the maturation of a biofilm, and also is correlated with cases of gingivitis and chronic periodontitis (Karched et al., 2015; Okuda et al., 2012). *Parvimonas micra* is an anaerobic and proteolytic bacterium found in the gingival sulcus. Currently, the frequency of case reports in the literature correlating *P. micra* with spondylodiscitis (spinal infection) has been extremely high and some patients had a dental problem before spinal infection (Durovic et al., 2020; van Duijvenbode et al., 2018). *Porphyromonas endodontalis* is a pathogen isolated from periodontal pockets, periapical lesions, endodontic infections and biofilm on the back of the tongue of patients with chronic periodontitis. (Pérez-Chaparro et al., 2018; Rôças & Siqueira, 2018). *Porphyromonas gingivalis* is considered the main etiologic agent of periodontal diseases (Bostanci & Belibasakis, 2012; Tamai et al., 2011).

In addition, microorganisms can also generate significant inflammatory processes in their hosts, due to their presence in the organism or only their constituents, such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), for example. Oral infections caused by the microorganisms described above can cause acute or chronic inflammatory processes requiring pharmacological intervention to minimize additional tissue damage (Headland & Norling, 2015). Also, to an active substance being effective, it needs to be safe. There are many tests to show how much an organism tolerates a substance. Among them, *in vitro* tests using cell, and tissue cultures preferably mammals. The cytotoxicity tests allow evaluating the biological response of cells to a particular substance, through specific aspects of metabolism, morphological, or even cell growth and viability. Genotoxicity tests demonstrate effects on DNA, gene mutations, and chromosomal aberrations. Some studies have shown that *S. adstringens* maintains cell viability when analyzed on mammalian cell culture (de Oliveira et al., 2013; Henriques et al., 2016; Ishida et al., 2006), and has a protective effect on cellular genetic material (Costa et al., 2010; Pellenz et al., 2018).

There is a lack of studies in the literature that evaluated the antimicrobial activity of *S. adstringens* extract on such microorganisms of dental interest, particularly on anaerobic bacteria. It is also scarce studies with analysis of cytotoxicity to find a concentration that is effective and without toxic effects and that can be applied in the dental clinic in the future.

The purpose of this study was evaluated the biological activities of extract from *S. adstringens* (Mart.) Coville, such as antimicrobial effect on planktonic culture, and biofilms of *C. albicans*, *E. faecalis*, *P. aeruginosa*, *S. aureus*, *S. mutans*, *F. nucleatum*, *P. endodontalis*, *P. gingivalis*, and *P. micra*; cytotoxicity and genotoxicity in mammalian cells (murine macrophages, murine fibroblasts, and human keratinocytes); and anti-inflammatory action in LPS-stimulated murine

macrophages.

2. Methodology

2.1 Extract

The glycolic extract of *S. adstringens* (Mart.) Coville was prepared from the stem bark of the plant and obtained commercially at 200 mg/mL of propylene glycol (Mapric, São Paulo, Brazil).

2.2 Microorganisms

Reference strains from American Type Culture Collection (ATCC) were used such as *C. albicans* (ATCC 18804), *E. faecalis* (ATCC 4083), *P. aeruginosa* (ATCC 15442), *S. aureus* (ATCC 6538), *S. mutans* (ATCC 35688), *F. nucleatum* (ATCC 25586), *P. endodontalis* (ATCC 35406), *P. gingivalis* (ATCC 33277), and *P. micra* (ATCC 23195). The microorganisms were frozen at -80 °C, and activated in a specific culture medium according to the metabolic needs, being Yeast Nitrogen Base broth (YNB - Sigma-Aldrich, St. Louis, USA), for *C. albicans*, and Brain Heart Infusion broth (BHI - Himedia), for aerobic bacteria. They were incubated at 37 °C for 24 h, with 5% CO₂ for *S. mutans*. For anaerobic bacteria, Brucella broth (Acumedia, Michigan, USA) supplemented with 5% sterile defibrinated sheep blood (Newprov, Pinhais-PR, Brazil), 1% hemin (Sigma-Aldrich), and 1% menadione (Sigma-Aldrich) was used. These bacteria were incubated in anaerobiosis with nitrogen 80.01%, carbon dioxide 10.02%, and hydrogen 9.97%; (Whitley DG250 Workstation, West Yorkshire, UK) at 37 °C for 48 h.

2.3 Antimicrobial activity in planktonic cultures

The Minimum Inhibitory Concentration (MIC) was determined by the broth microdilution method, according to the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2002, 2003, 2007), as described in documents M27-A2 for yeasts, M7-A6 for aerobic microorganisms, and M11-A7 for anaerobic microorganisms.

The microorganisms were prepared in sterile saline solution (NaCl 0.9%) with turbidity adjusted to 0.5 of the McFarland scale, from a 24 h culture for aerobic microorganisms at 37 °C. The anaerobic bacteria were grown in Brucella broth supplemented at 37 °C for 48 h, before preparation in saline solution. In 96-well plates, 100 µL of broth was added in 10 wells, and 100 µL of the extract was added only in the first well of the microplate, from which a two-fold serial dilution was performed, totaling 10 different concentrations. For this, Mueller Hinton broth (Himedia) for bacteria, and RPMI 1640 medium (with glutamine, without bicarbonate and with red phenol indicator) (Himedia) buffered with 3-(N-morpholino) propane sulfonicpropane sulfonic acid (MOPS – Sigma-Aldrich) at pH 7.0, for *C. albicans* were used. For aerobic bacteria and yeast, 100 µL of inoculum was added to the wells and the microplates were incubated at 37 °C for 24 h. For anaerobic bacteria, 10 µL of standardized inoculum was added to the wells, and the microplates were incubated in anaerobiosis for 48 h. The MIC was determined as the lowest concentration of extract capable of inhibiting microbial growth was observed with the naked eye.

To determine the Minimal Microbicide Concentration (MMC) of the plant extract on aerobic bacteria and yeast, the contents of the wells (100 µL) of the MIC, as well as the adjacent wells were seeded on BHI agar for the bacteria, and Sabouraud-dextrose (Himedia) for yeast. After 48 h of incubation, MMC was determined on the plate with no colonial growth. Regarding the anaerobic bacteria, the well contents were homogenized and 20 µL of each concentration were seeded in supplemented Brucella agar, and incubated under anaerobic condition for 48 h.

2.4 Antimicrobial activity in monomicrobial biofilms

Initially, the action of the plant extract was verified in monomicrobial biofilms of *C. albicans*, *E. faecalis*, *P. aeruginosa*, *S. aureus*, and *S. mutans* (De Oliveira et al., 2014). For biofilm formation, each microbial suspension was standardized at 10^7 Colony-Forming Units per milliliter (CFU/mL) in saline and added to microtiter plates (200 μ L/well). The microplates were incubated (37 °C) under agitation (75 rpm) for 90 min. The supernatant was discarded and culture medium was added, being YNB broth for *C. albicans*, and BHI broth for bacteria. Incubation (37 °C) was performed for 48 h, with the replacement of the culture medium after 24 h. The biofilms were exposed to the plant extract for 5 min or 24 h, using effective concentrations. Sterile saline was used as a negative control. Ten replicates were performed in each experimental group.

Posteriorly, 200 μ L of MTT solution (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the wells and the microplates were incubated at 37 °C for 1 h in the dark. Afterward, the MTT solution was removed and 200 μ L of dimethylsulfoxide (DMSO – Sigma-Aldrich) was added to the wells. After incubation of 10 min, the microplates were stirred for another 10 min, and proceeded to read on a spectrophotometer (Biotek ELx808) at 570 nm. The Optical Densities (OD) were converted into the viability percentage of the biofilm.

To evaluate the action of the plant extract on biofilms of *F. nucleatum*, *P. micra*, *P. endodontalis*, and *P. gingivalis*, 100 μ L/well of microbial suspension (1.5×10^8 CFU/mL), and 100 μ L/well of supplemented Brucella broth were added in microplates. The plates were then incubated at 37 °C in anaerobiosis for seven days with medium replacement every 48 h. After, the biofilms were exposed to the plant extract for 5 min or 24 h. Supplemented Brucella broth and 0.1% chlorhexidine were used as controls. These solutions were discarded, the biofilms were washed with sterile saline and disintegrated with an ultrasonic homogenizer (Sonoplus HD 2200 - Bandelin Electronic GmbH & Co. KG, Berlin, Germany), with 25% energy for 30 s. The suspension was diluted, and 20 μ L seeded on Brucella agar. After 48 h of incubation, colonies were counted and given their concentration in CFU/mL.

2.5 Cell culture

Murine macrophages (RAW 264.7) and human keratinocytes (HACAT) from the Cell Bank of Rio de Janeiro - Paul Ehrlich Scientific-Technical Association (APABCAM, Rio de Janeiro, Brazil), and murine fibroblasts (L929) from the Laboratory of Dynamics of Cellular Compartments from the Vale do Paraíba University (UNIVAP, São José dos Campos, São Paulo, Brazil) were cultured in Dulbecco's modified Eagle's medium (DMEM-LGC Biotechnology, Cotia, Brazil) supplemented with 10% fetal bovine serum (Invitrogen, New York, USA), 1% penicillin-streptomycin (Gibco, Grand Island, New York, United States), and kept in cell culture flasks (TPP, Switzerland) at 37 °C with 5% CO₂ and atmospheric humidity. The culture medium was changed every 48 h. After subconfluency, the cells were used in the tests. The Trypan blue exclusion test (0.4%, Sigma-Aldrich) was performed to count the cells.

2.6 Cytotoxicity analysis

In 96-well microplates (Kasvi), 200 μ L of DMEM containing 4×10^4 viable cells were added to the wells. After 24 h of incubation, 200 μ L/well of the extract were added on the cells at different concentrations, pure DMEM was used as control ($n = 8$ /group). The supernatants were discarded and cell viability checked by MTT assay (as mentioned before), after 4 h of incubation.

2.7 Genotoxicity analysis

The micronucleus (MN) test was performed according to the Organization for Economic Co-operation and Development (Organization for Economic Co-operation and Development (OECD), 2014).

For the test, 5×10^5 viable cells/well were grown in 24-well plates for 24 h. The cells were then exposed at different extract concentrations diluted in DMEM, 5 μ M ethyl methanesulfonate (EMS), was used as a positive control, or pure DMEM was used as a negative control for 24 h. Next, the cells were incubated with cytochalasin B (Sigma-Aldrich) at 6 μ g/mL for 24 h. Then, the cells were fixed with methanol for 10 min and stained with 300 nM DAPI diluted in PBS for 10 min. Cells were washed with PBS and observed under fluorescence microscopy, where 2000 cells/well per concentration ($n = 3$) were evaluated. MN were identified as DNA structures contained in the cytoplasm separated from the main nucleus, surrounded by a nuclear membrane, and including an area smaller than 1/3 of the area of the main nucleus. Cells with less than 5 MN were counted.

2.8 Anti-inflammatory activity

In 24-well plates, RAW 264.7 were cultured at 5×10^5 cells/well for 24 h. After incubation, culture medium with different concentrations of extract from *S. adstringens* was added. At the same time, LPS from *E. coli* (Sigma) at 1 μ g/mL was added to the wells. Groups without LPS were used as controls. After 24 h of incubation, the supernatants were collected and stored at -20 °C for quantification of pro-inflammatory (TNF- α , IL-1 β , IL-6, and IL-17), and anti-inflammatory (IL-10) cytokines by ELISA (R & D Systems, Minneapolis, United States of America) according to the manufacturer's instructions.

2.9 Production of nitric oxide (NO)

The production of NO by RAW 264.7 was determined by the concentration of nitrite detected by the Griess reagent. For this, 100 μ L of cell supernatant, and 100 μ L of Griess reagent were added in 96-well plates. After 10 min, the absorbance of the wells was read on a microplate spectrophotometer at 570 nm. To calculate the nitrite concentration, a standard curve of nitrite from 23 to 0.72 μ g/mL was used.

2.10 Statistical analysis

The results were presented in mean values (\pm standard deviation) and data distribution was assessed by Shapiro-Wilk. The data that presented normal distribution were analyzed by ANOVA complemented by Tukey's test, with significance of 5% ($P \leq 0.05$). The results that did not present normal distribution were analyzed by Kruskal-Wallis and Dunns tests ($P \leq 0.05$).

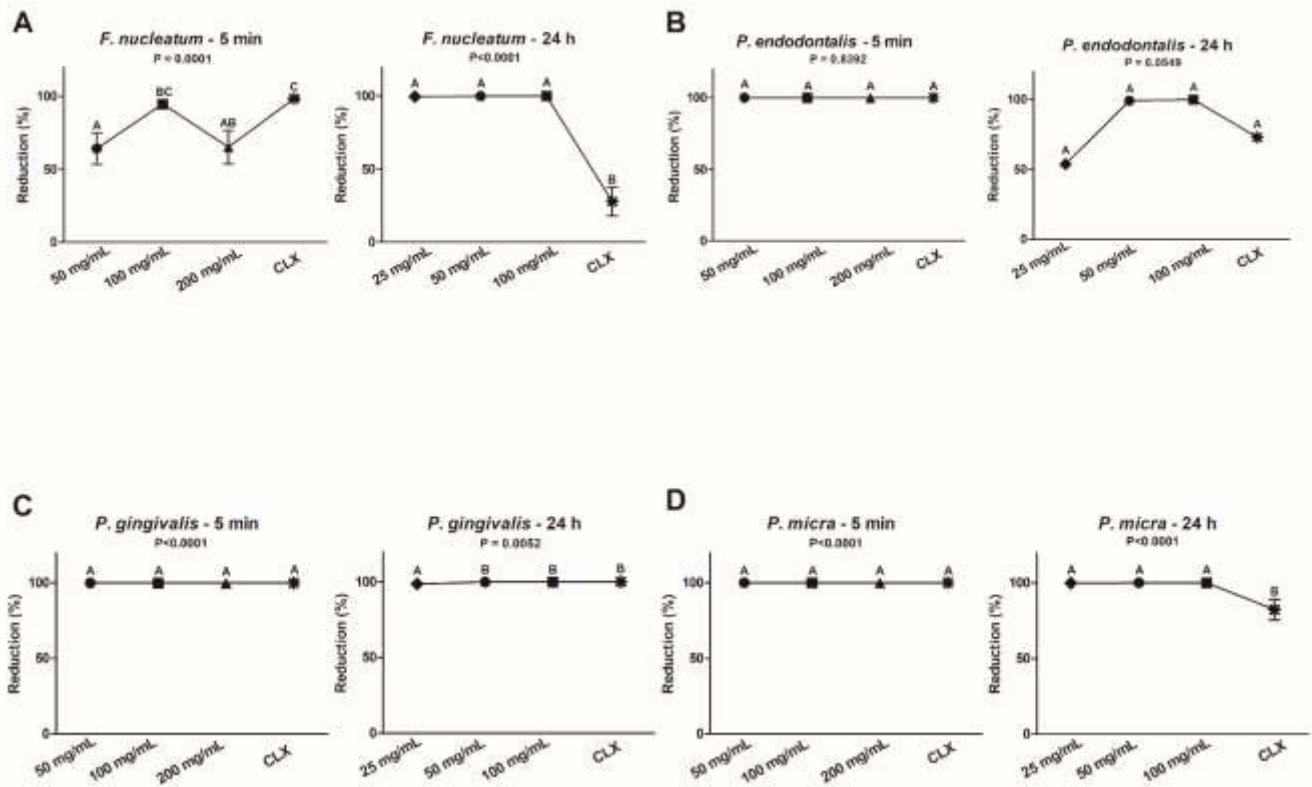
3. Results

3.1 Antimicrobial activity

Regarding the antimicrobial activity of extract from *S. adstringens* on aerobic microorganisms, it was possible to observe action only on *P. aeruginosa*. There was no growth inhibition for the other microorganisms. In the microdilution tests, the highest concentration analyzed was 50 mg/mL and in the tests of the antibiofilm action of the extract, higher concentrations (50, 100, and 200 mg/mL) were used, and only the biofilm of *P. aeruginosa* suffered a significative reduction, however, no statistical difference ($p > 0.05$) was found.

Regarding anaerobic microorganisms, it was not possible to determine the MIC due to the turbidity of extract from *S. adstringens*, and the color of the medium (data not shown). Our study showed an MMC of 50 mg/mL for *F. nucleatum*, and > 100 mg/mL for other species. Concerning monomicrobial biofilms from anaerobic bacteria, a significant susceptibility to the plant extract was verified, with a minimum reduction of 50% (*F. nucleatum*, 5 min), as shown in Figure 1.

Figure 1. Action of extract from *S. adstringens* (Mart.) Coville on anaerobic biofilms. Different letters indicate statistically different groups. Mean values \pm standard deviation of the reduction of biofilm mono species of A) *Fusobacterium nucleatum*, B) *Porphyromonas endodontalis* C) *Porphyromonas gingivalis*, and D) *Parvimonas micra*. CLX: chlorhexidine. ($n = 8$. Kruskal-Wallis, Dunns test, $P \leq 0.05$).

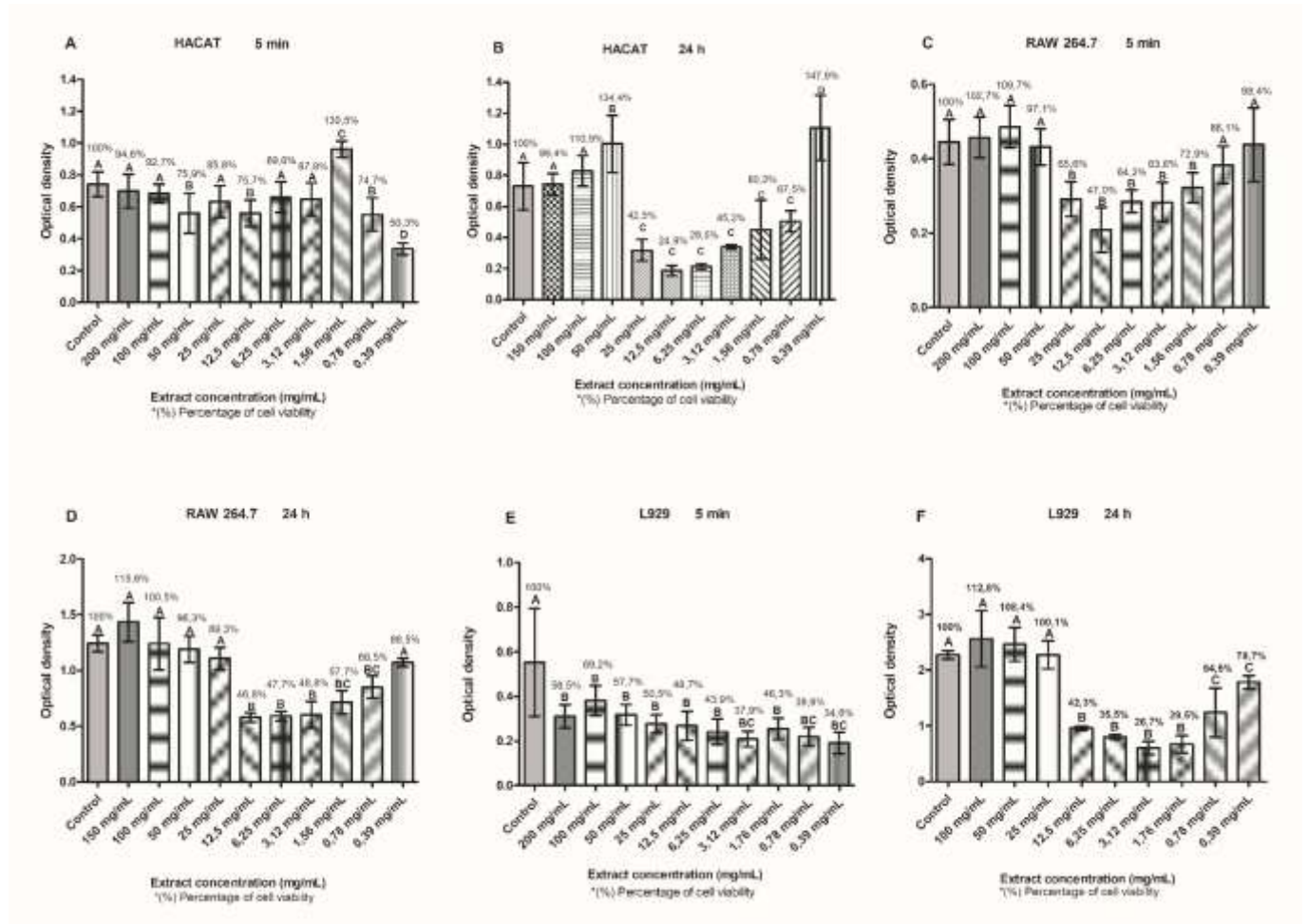


Source: Authors.

3.2 Cytotoxicity

Ten concentrations were evaluated of bark extract from *S. adstringens* in RAW 264.7, L929, and HACAT. Different responses were observed (Figure 2); however, the effective concentrations for anaerobic bacteria showed cell viability $>50\%$ in mammalian cells, generally.

Figure 2. Cytotoxicity of extract from *S. adstringens* (Mart.) Coville. Mean values \pm standard deviation of the extract cytotoxicity on A) Human keratinocytes (HACAT) for 5 min, B) Keratinocytes (HACAT) for 24 h, C) Murine macrophages (RAW 264.7) for 5 min, D) Murine macrophages (RAW 264.7) for 24 h, E) Murine fibroblasts (L929) for 5 min, and F) murine fibroblasts (L929) for 24 h. ($n = 8$. ANOVA, Tukey's test, $P \leq 0.05$).

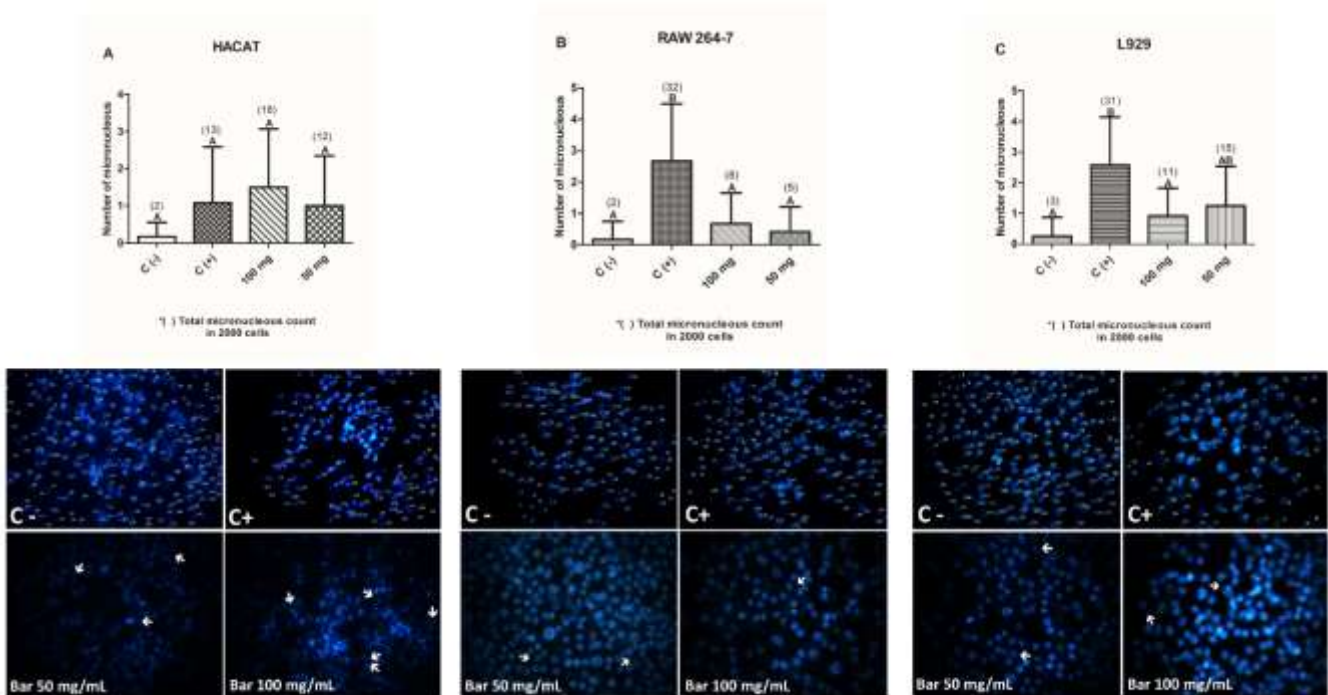


Source: Authors.

3.3 Genotoxicity

The genotoxicity of two concentrations (50 and 100 mg/mL) of extract from *S. adstringens* was tested in HACAT, RAW 264.7, and L929 (Figure 3). These concentrations were not genotoxic for murine macrophages. At the concentration of 100 mg/mL, the extract was not cytotoxic to murine fibroblasts. Human keratinocytes were less sensitive to the genotoxic agent used (EMS) and all treatments were statistically similar making the results inconclusive.

Figure 3. Genotoxicity of *S. adstringens* (Mart.) Coville extract. Different letters indicate statistically different groups. Mean values \pm standard deviation of the extract genotoxicity on A) human keratinocytes (HACAT), B) murine macrophages (RAW 264.7) and C) murine fibroblasts (L929). C (-) = pure DMEM, C (+) = EMS. Extract concentration (mg/mL). ($n = 8$. ANOVA, Tukey's test, $P \leq 0.05$). In the photos, micronuclei (MN) are DNA fragments located around and close to the cell nucleus (indicated by white arrows), viewed by fluorescence microscopy and may have variable size but always smaller than the cell nucleus and varied amount, as shown in the photos aligned with each graph. Its presence characterizes DNA damage. The left, sequence of photos of MN in HACAT in the control groups, and with Barbatimão extract (Bar 50 mg/mL and 100 mg/mL). In the middle, sequence of photos of MN in RAW 264.7 and to the right, sequence of photos of MN in L929.

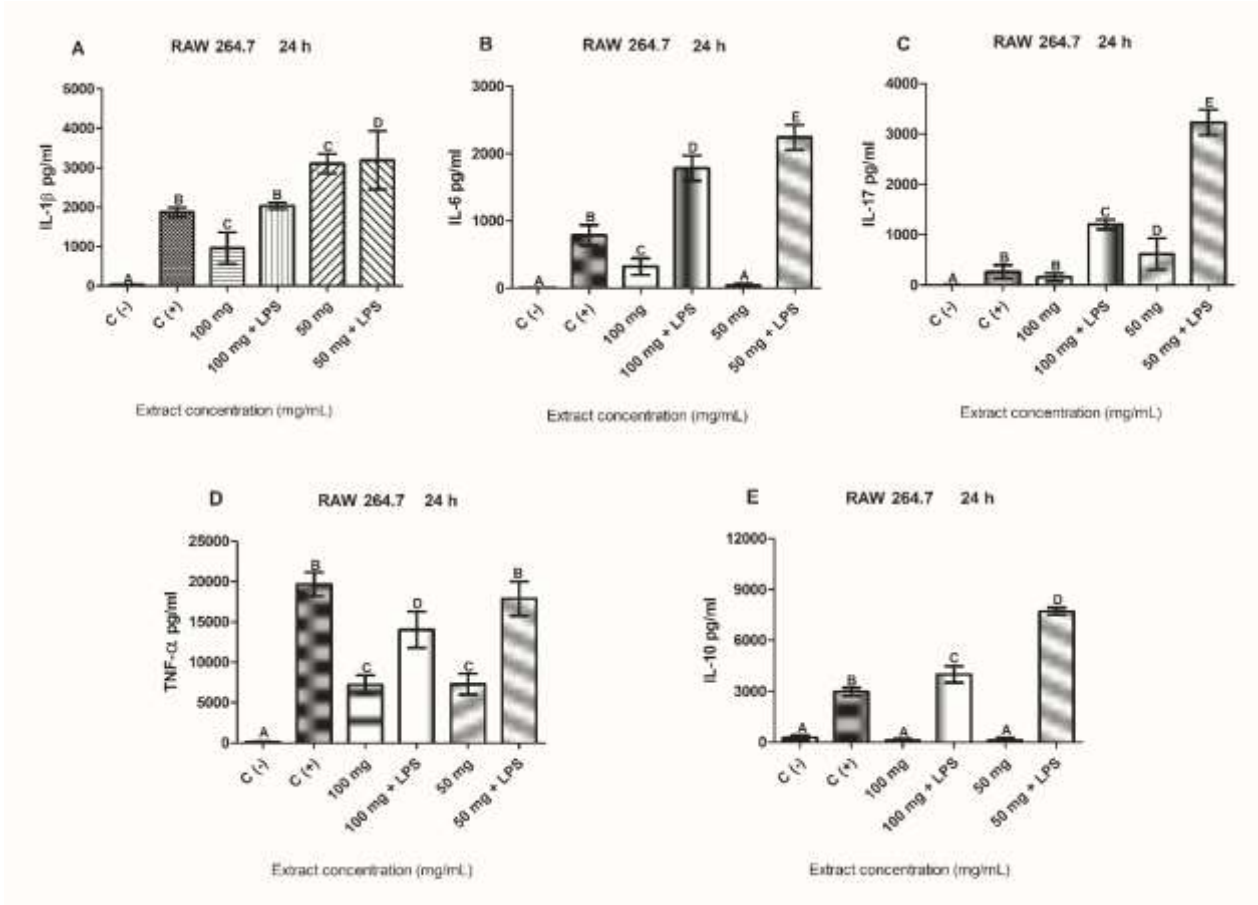


Source: Authors.

3.4 Anti-inflammatory activity

In the evaluation of anti-inflammatory activity, it was possible to observe that the extract only increased the release of IL-1 β and IL-17 at 50 mg/mL in non-LPS-stimulated macrophages. On the other hand, when the extract was associated with LPS stimulation, all the cytokines were increased, except TNF- α , which was reduced in the concentration of 100 mg/mL but was similar to the positive control with 50 mg/mL (Figure 4).

Figure 4. Action of *Stryphnodendron adstringens* (Mart.) Coville extract on the production of cytokines in murine macrophages (RAW 264.7). Different letters indicate statistically different groups. Mean values \pm standard deviation (pg/mL) of: A) IL-1 β , B) IL-6, C) IL-17, D) TNF- α e E) IL-10. C (-) = DMEM, C (+) = DMEM + LPS. (n = 8. ANOVA, Tukey's test, $P \leq 0.05$).



Source: Authors.

3.5 Production of NO

The ability to inhibit the production of nitric oxide (NO) can contribute to the performance of the extract's anti-inflammatory activity. The average values of nitrite (pg/mL), referring to the production of nitric oxide, obtained in each experimental group, in comparison with the control groups, are shown in Table 1. It was observed that in the groups treated with *S. adstringens* extract at 25 mg/mL (+ LPS), and 50 mg/mL (+ LPS) there was a significant reduction in the production of NO in relation to the group with LPS and without extract ($P < 0.05$). The groups treated with *S. adstringens* extract at 100 mg/mL group (+ LPS) promoted higher levels of nitric oxide, which was similar to the positive control (LPS without extract). The negative control group (without LPS stimulation) had the lowest level of nitrite, being statistically different from the other groups ($P < 0.05$).

Table 1. Level of nitrite (pg/ml) obtained in experimental and control groups

Experimental groups	Level of nitrite (pg/mL)
Negative control	0.124±0.082 ^A
Positive control (LPS)	2.351±0.806 ^B
<i>S. adstringens</i> 100 mg/mL + LPS	2.872±0.000 ^B
<i>S. adstringens</i> 50 mg/mL + LPS	1.227±0.659 ^C
<i>S. adstringens</i> 25 mg/mL + LPS	0.962±0.274 ^C

Different letters indicate statistically different groups. (ANOVA, Tukey's test, $P \leq 0.05$).
Source: Authors.

4. Discussion

In the present study, the plant extract was not able to inhibit the growth of the aerobic microorganisms analyzed, except *P. aeruginosa*. Likewise, only the monomicrobial biofilm of *P. aeruginosa* was significantly decreased. There are no reports in the literature regarding the antimicrobial activity of this plant extract on *E. faecalis*, and *P. aeruginosa* regarding the antibiofilm action on the other strains tested in the present study.

To check the antimicrobial action of some fractions from extract of *S. adstringens*, the agar diffusion test, and microdilution were used on ATCC strains of *C. albicans*, *S. mutans*, *S. aureus*, and *Aggregatibacter actinomycetemcomitans*. The vegetable compound showed antimicrobial activity at different levels for all strains tested (Pereira et al., 2011). A gel formulation produced from fractions of samples of the stem and bark of *S. adstringens* (Mart.) Coville was able to promote antifungal effect on clinical strains of *Candida* in addition to presenting anti-inflammatory action. The antimicrobial effect of the formulation was evaluated on planktonic cultures and biofilm. It was identified that the tannins present in the fractions of the *S. adstringens* extract were responsible for the antibiofilm action (de Freitas et al., 2018).

Regarding the antimicrobial action of other plant extracts of such species studied, *Rosmarinus officinalis* L., and *Thymus vulgaris* L. extracts acted efficiently on planktonic cultures, and monomicrobial biofilms of *C. albicans*, *S. aureus*, *E. faecalis*, *S. mutans*, and *P. aeruginosa* (de Oliveira et al., 2017; Oliveira et al., 2017).

With respect to anaerobic microorganisms, there are not yet studies in the literature reporting the antimicrobial activity of *S. adstringens* extract on anaerobic microorganisms here evaluated. On the other hand, different extracts from Mediterranean plants, such as *Cistus creticus* L., *Cistus monspeliensis* L., *Origanum vulgare* L., *Rosmarinus officinalis* L., *Thymus longicaulis* C., and *Salvia sclarea* L. have shown antimicrobial potential for several microorganisms, including *C. albicans*, *S. aureus*, *S. mutans*, *E. faecalis*, *P. gingivalis*, *F. nucleatum*, and *P. micra* among clinical isolates and ATCC strains. All the evaluated extracts effectively inhibited the obligate anaerobic bacteria (Hickl et al., 2018).

Also, the inhibitory potential of *Olea europaea* extract has been found in periodontal pathogens, such as *P. gingivalis*, *F. nucleatum*, and *Prevotella intermedia* (Karygianni et al., 2014). Some compounds from *O. europaea*, and *Pistacia lentiscus* have also demonstrated antimicrobial action on several oral pathogens. These molecules were highly effective against *Streptococci* spp., *P. gingivalis*, *F. nucleatum*, and *P. micra*. However, the extracts were ineffective for *P. intermedia* (Karygianni et al., 2019). *Melaleuca alternifolia* oil has also shown antimicrobial activity against *P. gingivalis* and *P. endodontalis*, using subinhibitory concentrations (Graziano et al., 2016). The potential to reduce substances related to halitosis has also been shown by several plant extracts, such as *Caesalpinia ferrea* Mart., *Cinnamomum cassia* B., *Mallow sylvestris* L., *Punica granatum* L., *Rosmarinus officinalis* L., *Aeolanthus suaveolens* (Als.) Spreng., *Syzygium aromaticum* L., and

Tamarindus indica L., since these natural products have acted against microorganisms that produce volatile sulfur compounds, such as *F. nucleatum*, *P. gingivalis*, *P. intermedia*, and *P. micra* (Veloso et al., 2020).

Concerning anaerobic biofilm studies, 24 h contact to the *S. adstringens* extract on biofilms of *F. nucleatum*, and *P. micra* promoted a reduction of 100% at all analyzed concentrations, differing statistically from the chlorhexidine 0.12% that promoted a percentage low reduction. This substance is considered a gold standard anti-plaque agent and in the present study, the extract of *S. adstringens* acted better than chlorhexidine. Regarding 5 min contact to the *S. adstringens* extract, all evaluated concentrations acted similarly to 0.12% chlorhexidine in the reduction *P. gingivalis*, *P. endodontalis*, and *P. micra* biofilms, presenting a high percentage of reduction. The results presented here indicate that *S. adstringens* has strong antibiofilm activity in all tested anaerobic strains. There are not yet studies in the literature that have reported the effect of *S. adstringens* extract in the anaerobic pathogens evaluated in this study. However, chemical constituents of *Pinus elliottii* oleoresin have presented antimicrobial action on some anaerobic oral pathogens including *P. gingivalis* and *F. nucleatum*, both planktonic culture and biofilms. *P. elliottii* oleoresin and its main compound exhibited antibacterial activity and the acid prevented all the evaluated bacteria from forming biofilm (Caetano da Silva et al., 2014). Plant oils from *Leptospermum scoparium* and *Melaleuca alternifolia* inhibited the adhesion of *P. gingivalis* and *S. mutans* suggesting that phytochemical oils suppress biofilm formation (Takarada et al., 2004). There are no studies in the literature on the antibiofilm action of natural compounds on *P. micra* and *P. endodontalis*. Currently, there has been a considerable increase in the studies about systemic infections related to *P. micra* such as spondylodiscitis (infection in the spine). A case series reported in 2020 showed that of the six cases, five *P. micra* is the only pathogen isolated from spine infection (Durovic et al., 2020). Analyzing alternative therapeutic agents that are effective against infections caused by these microorganisms is very important and in the present study, the plant extract was very efficient against this pathogen.

Regarding cytotoxicity analyzes, in the present study, the effective concentrations for anaerobic bacteria showed cell viability >50%. tannins from the *S. adstringens* (Mart.) Coville bark were not very cytotoxic for murine macrophage (J774G8) cells and green monkey kidney cells (Vero), but the cutoff point of this study was 50% (Ishida et al., 2006). The extract of the bark of *S. adstringens* (Mart.) Coville was tested on RAW 264.7, and was found cell viability of 65% using a concentration of 12.5 mg/mL and > 70% for concentrations of 50, 25, and 3.13 mg/mL (de Oliveira et al., 2013). Another study with an extract from the *S. adstringens* bark demonstrated cell viability > 90% for the human monocytes (THP-1) cells (ATCC TIB-202) (Henriques et al., 2016). The cytotoxic effect of the extract from leaves *S. adstringens* (Mart.) Coville was tested in two human breast cancer cell lines (MCF-7 and MDA-MB-435), and also on the primary culture of rat marrow cells. The study concluded that the extract was cytotoxic with a 50% cutoff point (Sabino et al., 2018).

Concerning genotoxicity analyzes, in the present study, the highest concentrations tested were not genotoxic for RAW 264.7 and L929. The effect of the *S. adstringens* hydroalcoholic extract has also been evaluated for cell viability, DNA damage, and apoptosis induction in keratinocytes (HaCaT), and fibroblast (HFF-1) cell lines. The results have suggested that the extract may have a genoprotective and anti-apoptotic effects for human keratinocytes and fibroblasts (Pellenz et al., 2018). The *S. adstringens* ethanolic extract was used at 50, 100, and 200 mg/kg, and was found that the two higher concentrations reduced the genotoxicity caused by mitomycin C (Silva-de-Andrade et al., 2006). Another study demonstrated that the proanthocyanidins rich fraction of the stem bark of *S. adstringens* was not genotoxic at 750, 1500, and 2000 mg/kg and also conferred protection to the cellular genetic material of the cyclophosphamide-treated groups at 750 mg/mL (Costa et al., 2010). Besides, was verified the antigenotoxic activity of aqueous and ethanolic extracts of *S. adstringens* leaves on genotoxicity caused by cyclophosphamide (Filho et al., 2011). On the other hand, the aqueous solution of *S. adstringens* was genotoxic at 30 mg/mL for erythrocytes of mice treated with mitomycin C (Chaves DS, Lemes SR, Montes de Sousa MA, da Rocha Souza

EGA, Alves de Araújo L, Mrué F, 2017). The plant product showed relative genotoxicity when evaluated by SOS-Inductest (Vilar et al., 2010).

About anti-inflammatory activity, it has already been reported that there is the increased IL-1 β in RAW 264.7 treated with 50 mg/mL of extract from *S. adstringens*, but in this study, the same concentration reduced the release of TNF- α which did not occur in the present study (de Oliveira et al., 2013). Another study demonstrated that there is a reduction of TNF- α using human monocytes (THP-1) and the extract of *S. adstringens* (Mart.) Coville at concentrations of 250, 125, and 62.5 μ g/mL (Henriques et al., 2016). It was also observed that the extracts did not increase the production of TNF- α in cells not stimulated with LPS. No studies on the action of *S. adstringens* on other cytokines have been found in the literature.

To the production of NO, there are no reports in the literature on the action of *S. adstringens* for the production of NO. This shows the importance of the current study because we found that *S. adstringens* may be a potential NO inhibitor. Some natural extracts and their phytochemicals, such as *Apios americana* (Seo et al., 2018), *Inula Japonica* (Jin et al., 2016), and the propolis ethanolic extract (Onbas et al., 2016) have demonstrated potent NO inhibitor effect when evaluated on LPS-stimulated RAW 264.7 cells.

5. Conclusion

In conclusion, extract of *S. adstringens* (Mart.) Coville was effective for monomicrobial biofilms of anaerobic bacteria at low non-cytotoxic concentrations for RAW 264.7, L929, and HACAT cells. This study has elucidated important biological activities of *S. adstringens* extract however, for further analysis, the authors believe to be necessary additional *in vitro* trials and the near future, start *in vivo* analyzes in invertebrate models that allow to obtain preliminary results such as the toxicity of the plant extract *in vivo*.

List of abbreviations

- ATCC: American Type Culture Collection
- BHI: Brain Heart Infusion broth
- CFU/mL: Colony-forming units per milliliter
- DMEM: Dulbecco's modified Eagle's medium
- DMSO: Dimethylsulfoxide
- EMS: Ethyl methanesulfanate
- HACAT: Human keratinocytes cells
- IL-10: Interleukin 10
- IL-1 β : Interleukin 1 beta
- IL-6: Interleukin 6
- J774G8: Murine macrophage cells
- L929: Murine fibroblasts cells
- LPS: Lipopolysaccharide
- LTA: Lipoteichoic acid
- MCF-7: Breast adenocarcinoma
- MDA-MB-435: Melanoma cell line
- MIC: Minimum Inhibitory Concentration
- MMC: Minimal Microbicide Concentration

MN: Micronucleus

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

NaCl: 0.9% Sterile saline solution

NO: Nitric Oxide

OD: Optical Densities

RAW 264.7: Murine macrophages cells

THP-1: Human monocytes cells

TNF- α : Tumor necrosis factor alpha

Vero: Green monkey kidney cells

YNB: Yeast Nitrogen Base broth

Declarations

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

FLS: conception, design of the experiments, interpretation of data and drafting of the manuscript. IA: design of the experiments and interpretation of data. LPR: analysis and interpretation of data. JGS: analysis and interpretation of data. ETF: design of the experiments. VMM: drafting of the manuscript. LFFF: design of the experiments, interpretation of data and drafting of the manuscript. JRO: review of the manuscript and prepared the linguistic correction of the manuscript. MPS: critical analysis, review of the manuscript and prepared the linguistic correction of the manuscript. MCMR: critical analysis and review of the manuscript. LDO: conception, analysis and interpretation of data, critical analysis and review of the manuscript. All authors read and approved the final manuscript.

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