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ORIGINAL ARTICLE

Pyrostegia venusta (Ker Gawl.) Miers Crude Extract and Fractions: Prevention of Dental Biofilm Formation and Immunomodulatory Capacity

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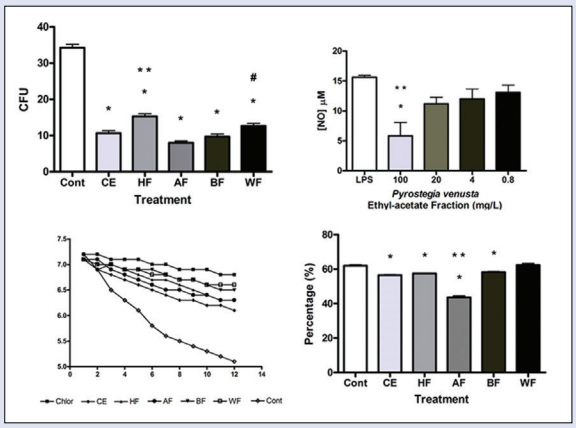
ABSTRACT

Background: Caries and periodontal diseases remain as important diseases in the Brazilian population. One important pathogen associated with this situation is *Streptococcus mutans* and other important factor is this pathogen's ability to adhere firmly to the tooth surface leading to dental biofilm formation and caries development. **Objectives:** Determine the antibacterial and other biological activities of *P. venusta* related to its potential to be used in the treatment of caries and periodontal disease. **Methods:** The growth inhibition by *P. venusta* of *Streptococcus mutans*, *S. mitis, S. oralis* and *Candida albicans* was determined using the broth microdilution method. In addition, the effect of the samples in adherence and reducing production of acids by *S. mutans*, and germ-tube formation of *C. albicans* was analysed. The Nitric Oxide (NO) production and cytotoxicity of *P. venusta* to peripheral blood mononuclear cells (PBMC) and RAW 264.7 Cell Line Murine Macrophage from Blood were assessed. **Results:** The crude extract (CE) and ethyl-acetate (AF) and n-butanol (BF) fractions showed antibacterial activity. The ethyl-acetate (AF) fraction showed the highest inhibition percentage against the adherence of *S. mutans* and *C. albicans* cells without budding, beyond NO production inhibition. There was not any cytotoxicity in the murine macrophages RAW 264.7 cells. **Conclusion:** Our results suggest that *P. venusta* presents potential to be used as a preliminary source of compounds that can provide helpful activity when used in prophylaxis or treatment of caries or periodontal disease.

Key words: Anti-bacterial agents, *Candida* spp., caries, periodontal disease, *Streptococcus* spp

SUMMARY

- Biological activities of Pyrostegia venusta and its potential for use in formulations for the prevention of oral diseases.



Abbreviations used: NO: Nitric oxide, PBMC: Peripheral blood mononuclear cells, CE: Crude extract, AF: Ethyl-acetate fraction, BF: n-butanol fraction, HF: Hexane fraction, WF: Water fraction, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, ATCC: American Type Culture Collection, CFU: Colony-forming units, BHI: Brain heart infusion, RPMI: Roswell Park Memorial Institute, MOPS: 3-(N-morpholino)propanesulfonic acid, DMEM: Dulbecco's modified Eagle's médium, LPS: Lipopolysacharide, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, OD: Optical density, AC: Acteoside, VB: β -OH-Verbascoside, TB: Trypan blue

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INTRODUCTION

Caries and periodontal disease are the most prevalent oral diseases in Brazilian population, with dental biofilm being considered the primary etiological factor in the establishment of these pathologies.[1] *Streptococcus mutans* is one of the most important etiological agents of dental caries in humans, and it is considered co-responsible for the initial phase of

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cariogenic lesions.[2] The adherence has a well-established role in the virulence of *S. mutans* since it is capable of synthesizing extracellular glucans through sucrose using glucosyltransferase enzyme. The glucans molecules have the ability to grip to various solid surfaces, which gives them the ability to adhere firmly and irreversibly to the tooth surface and can lead to dental biofilm formation and caries development.[3,4] *Streptococcus oralis* and *Streptococcus mitis* are also commonly found in dental biofilm, however, due to the absence of acidogenic or aciduric properties in these microorganisms, they are associated only with the early stages of dental biofilm formation, not acting directly on the demineralization of tooth enamel, but only making it more suitable for colonization by *S. mutans*.[5,6]

Another fairly common oral disease is oral candidiasis that occurs due to infection by *Candida* spp.[7] *Candida albicans* is the yeast, most frequently isolated from the oral cavity and, in some situations, can behave as an opportunistic pathogen, for example, in situations of low immunity, poor oral hygiene, low salivary flow, and use of implants.[7,8] The biology of *C. albicans* presents different aspects, including the ability to show distinct morphologies. The unicellular yeast phase can generate a bud and form hyphae. The formation of hyphae or filaments enables the cell to exert mechanical strength helping the microorganism to penetrate epithelial surfaces of the host, and once in the bloodstream, to act on the endothelium. These mechanisms allow *C. albicans* to insert tissues deeper into the host.[9]

It is well established that pathogenic microorganisms are the primary causative agents of periodontal diseases.[10] However, it is known that cytokines and inflammatory mediators can cause local tissue destruction reflecting clinically in periodontal injuries and alveolar bone loss.[10,11] The antimicrobial and immunomodulatory activities of medicinal plants have been widely investigated in several experimental models to seek auxiliary or alternative strategies in the treatment of infectious and inflammatory processes in the oral cavity.[12] The species *Pyrostegia venusta* (Ker Gawl) Miers, belonging to the family Bignoniaceae, is popularly known as "flor-de-São-João" or "cipó-de-São-João" and in folk medicine, it is used for the treatment of cough, bronchitis, colds, diarrhea, vitiligo, erysipelas, jaundice, and in the treatment of uterine and genital tract infections in women and female newborn.[13-15] Consequently, due to the important biological activities attributed to *P. venusta* in studies at the literature not only as the melanogenic, antitumor, anthelmintic, antinociceptive, and antioxidant but also as antimicrobial and immunomodulatory agent,[16-21] the aim of this work was to evaluate the potential of crude extract (CE) and fractions from *P. venusta* in the prevention of major oral diseases.

MATERIALS AND METHODS

Plant material

P. venusta flowers were collected in Naviraí, Mato Grosso do Sul, Brazil, in June 2006. A voucher herbarium specimen was identified by Doctor Maria Auxiliadora Milaneze Gutierre and was deposited under number HUEM 11708 at the Universidade Estadual de Maringá, Paraná, Brazil. The fresh flowers were washed, dried at room temperature, and then pulverized. CE was obtained by turbo-extraction (Skymsen) of 200 g of the flower with 30% ethanol in water for 15 min. The organic solvent was eliminated by rotavapor under reduced pressure and lyophilized to yield a CE. Next, the CE (30 g) was suspended in water (300 mL) and partitioned with hexane (300 mL; HF), ethyl-acetate (AF) (300 mL; AF), and *n*-butanol (300 mL; *n*-butanol [BF]) to obtain water fraction (WF).

Determination of the minimum inhibitory concentration, minimum bactericidal concentration, and minimum fungicidal concentration

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined according to the methods of Clinical and Laboratory Standards Institute, using samples of *S. mutans* (ATCC) American Type Culture Collection 25175, *S. mitis* ATCC 49456, *S. oralis* ATCC 10557, and *C. albicans* ATCC 0175 provided by the Instituto Nacional de Controle de Qualidade em Saúde, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. The concentrations of extracts and fractions were 1000 μg/mL, 500 μg/mL, 250 μg/mL, 125 μg/mL, 62.5 μg/mL, 31.2 μg/mL, and 15.6 μg/mL. MIC corresponded to the lowest concentration of CE and fractions able to inhibit the visible growth of microorganisms on the broth liquid medium (turbidity) with no more than 10 colony-forming units (CFU) of bacteria or fungus in the agar solid medium. The minimum bactericidal and fungicidal concentrations corresponded to the lowest concentration of CE and fractions able to inhibit the visible growth of microorganisms on the broth liquid medium associated with absence of growth on agar solid medium.[22-24]

Glass cover slip assay

The method of Hamada and collaborators was used, partially modified. *S. mutans* ATCC cultured for 24 h in Brain-Heart Infusion (BHI) broth at 37°C and in microaerophilia (10% CO_2) were centrifuged for 20 min and resuspended with buffer (3.0 mL) adjusted by the McFarland scale (1.5×10^8 cells/mL). From this bacterial suspension, a sample (1.5 mL) was added to buffer (control, 1.5 mL) or to CE/fractions (1.5 mL) in the MIC, previously determined. These were incubated at 37°C in microaerophilia for 4 h, centrifuged and resuspended with BHI + sucrose (3.0 mL). Serial dilutions were made in BHI medium: 10^{-1} and 10^{-2}. The 10^{-2} dilution (500 μL) was placed on a 24 well plate with a glass cover slip. The plate was incubated at 37°C in microaerophilia for 2 h. Next, the suspension was removed from each well, and the glass coverslip was removed and rinsed with buffer to be placed into another 24 well plate. Hence, 10% sheep-blood agar in BHI (500 μL) was added on each cover slip, and the plate was incubated. CFU were counted after 24 h of incubation at 37°C in microaerophilia in triplicate. Viable colonies in dishes with 10% sheep-blood agar were also counted.[3,25]

Effect of the crude extract and fractions on the production of acids

S. mutans (1 mL of the inoculum, consisting of the equivalent to 1.5×10^8 CFU/mL – 0.5 MacFarland scale tube, was seeded in 100 mL red phenol broth containing 1% glucose and the CE/fractions) then incubated at 37°C in microaerophilia for at least 12 h and, at regular intervals of 1 h, a sample (4 mL) of the culture was removed and its pH measured with a pH meter.[3,26]

Effect on germ-tube formation and budding of *Candida albicans*

The previously determined MIC of extract crude and fractions from *P. venusta*, diluted in RPMI 1640 medium with MOPS, were tested to assess the effect on budding of *C. albicans*. The plates were incubated at 37°C for 48 h and observed by a negative-staining technique using 7% aqueous nigrosin in each smear by light microscopy, and the percentage of budding cells was calculated.[27]

Cell culture

Murine macrophages RAW 264.7 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen Corporation, New York, USA) and supplemented with 2 mM L-glutamine, heat-inactivated 10% fetal bovine serum and 50 μg/ml gentamicin and buffered with sodium bicarbonate. The cultures were maintained at 37°C in 5% of CO_2/air mixture. RAW 264.7 cells (2×10^5 cells/mL) were plated in 96-well microplates and kept in culture for 3 h for adhesion and stability of the macrophage culture. After this period, the culture supernatants were removed carefully in order

to remove nonadherent cells and replaced in DMEM with or without lipopolysacharide (LPS) (1 μg/ml) (*Escherichia coli* 055:B5; Sigma-Aldrich, USA) in the presence or absence of CE and fractions of *P. venusta* at the follow concentrations 100 μg/ml, 20 μg/ml, 4 μg/ml, and 0.8 μg/ml. After 24 h, the culture supernatant was collected to evaluate the ability of CE and fractions to inhibit nitric oxide (NO) or it potential of cytotoxicity.

Nitric oxide production

NO production was indirectly estimated by measuring nitrite concentration in the supernatant through a reference curve with sodium nitrite. The supernatants (50 μL) were transferred to a new microplate and 50 μL of Griess reagent (1% sulfanilamide + 0.1 naphthylethylenediaminedihydrochloride in 5% phosphoric acid, Sigma Chemical Co.,) added, freshly prepared. After 10 min, the absorbance was measured at a wavelength of 570 nm. As a negative control, macrophages were stimulated with LPS at a concentration of 1 μg/ml and treated with nonspecific inhibitor of NO synthase NG-Monomethyl-L-Arginine (L-NMMA at 20 μg/ml) and as positive control macrophages stimulated with LPS to 1 μg/ml and untreated were used.

Cell viability

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by density centrifugation on a Ficoll-Paque Plus (GE Healthcare Biosciences, Uppsala, Sweden). PBMC (5×10^4) were incubated with CE and fractions (1000 μg/ml) in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/ml streptomycin, 10 mL/L nonessential aminoacid and 5% heat-inactivated fetal calf serum, for 1 h before the addition of 1 μg/ml LPS. After 24 h of culture, cell viability was determined by Trypan blue exclusion. In the murine macrophages, RAW 264.7 cell cultures cell viability was determined by the diphenyl tetrazolium assay-(MTT).[28] Briefly, MTT (5 mg/mL) was dissolved in DMEM, sterilized through 0.22 μm membranes and added to the plate containing CEs or fractions plus LPS, 10 μL/well, for 4 h at 37°C in a 5% de CO_2/air mixture. RAW 264.7 cells were incubated without compounds and used as viability control. Cell viability was directly proportional to OD value. The number of viable cells was expressed as a percentage relative to control cells, measured as 100% OD_{570}, treated/OD_{570}, control.

Statistical analysis

Data were analyzed using GraphPad Prism 4 software (GraphPad Prism Software® San Diego, California, GraphPad Software, Inc., 7825 Fay Avenue, Suite 230, La Jolla, CA 92037 USA) by analysis of one-way variance followed by multiple comparison tests (Tukey test). Results that showed $P < 0.05$ were considered statistically significant.

Table 1: Antimicrobial activity of Pyrostegia venusta crude extract, fractions and constituents

	S. mutans		*S. mitis*		*S. oralis*		*C. albicans*	
	MIC[a,c]	MBC[c]	MIC	MBC	MIC	MBC	MIC	MFC[c]
CE[b]	500	1000	500	1000	500	1000	1000	>1000
WF	1000	>1000	500	1000	500	1000	1000	>1000
HF	1000	>1000	1000	>1000	1000	>1000	1000	>1000
AF	500	1000	500	1000	500	1000	1000	>1000
BF	500	1000	500	1000	500	1000	1000	>1000
AC	100	>100	100	> 100	100	>100	100	>100
VB	100	>100	100	> 100	100	>100	100	>100

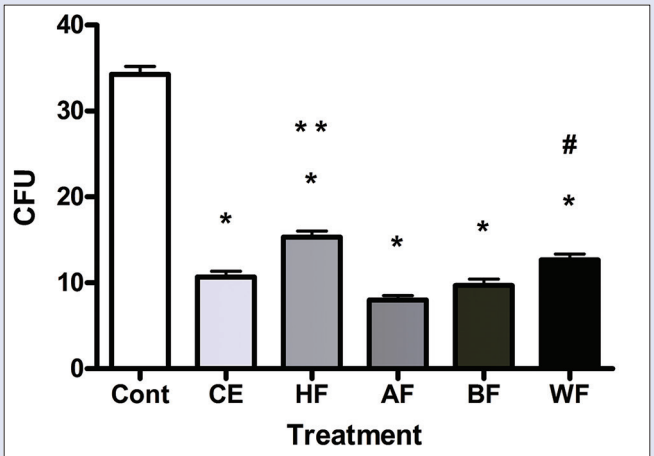
a - MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; MFC: Minimum Fungicidal Concentration; b - CE: Crude extract; WF: Water fraction; HF: Hexane fraction; AF: Ethyl acetate fraction; BF: *n*-butanol fraction; AC: Acteoside; VB: β-OH-verbascoside; c - mg/L

RESULTS AND DISCUSSION

CE, AF, and BF fractions showed 1000 μg/ml MBC against all the bacterial tested but were not able to inhibit *C. albicans* growth on higher concentration tested (CFM > 1000 μg/ml). WF showed 1000 μg/ml MBC against *S. mitis* and *S. oralis* but was not effective against *S. mutans* and *C. albicans*. HF showed not to be effective against all the microorganisms tested, with a MBC/minimum fungicidal concentration >1000 μg/ml [Table 1].

Some authors proposed a classification for the antimicrobial activity of plant extracts according to MIC, considering strong inhibitors (MIC down or equal to 0.5 mg/ml), moderated inhibitors (MIC between 0.6 and 1.5 mg/ml), and weak inhibitors (MIC up to 1.6 mg/ml).[29] However, there are other authors who recommended another classification according to MIC: If the extract shows MIC lower than 100 μg/ml, the activity is considered good; if the extract shows MIC between 101 and 500 μg/ml, the activity is considered moderated; if the extract shows MIC between 501 and 1000 μg/ml, the activity is considered weak, and if the extract shows MIC greater than 1000 μg/ml, the extract is considered inactive.[24] Our results demonstrated that CE and fractions from *P. venusta* flowers showed a moderate to strong activity against pathogenic microorganisms associated with caries and periodontal disease, according to the Aligiannins group classification, and they showed a weak to a moderate activity against these same pathogens when the Holetz group classification is considered, with an inhibition MIC from 500 to 1000 μg/ml when tested against the microorganisms. Taking into account that all extracts showing MIC down to 2 mg/ml could be considered having antimicrobial potential against *C. albicans*, our results corroborate to assume *P. venusta* as a good plant in the study of new molecules to treat oral diseases, and it is according with many authors who observed moderate activity in fractions of the flowers and leaves from *P. venusta* against Gram-Positive and Gram-Negative bacteria.[18,21,29,30]

Caries prevention depends on the control of microbial dental biofilm. Thus, current research aims at finding agents which have direct action



Treatment	*S. mutans* Control	CE	HF	AF	BF	WF
CFU Number	34.3 ± 2.8	10.7 ± 1.6	15.3 ± 1.6	8.0 ± 1.3	9.7 ± 1.9	12.7 ± 1.6
Inhibition %	Zero	68.9	55.3	76.7	71.8	63.1

Figure 1: Adherence inhibition of *Streptococcus mutans* when using crude extract (CE) and fractions of *Pyrostegia venusta*. The data represent the mean of triplicate from one of three experiments. *$P < 0.0001$ when compared to contril; **$P < 0.01$ when compared CE, AF and BF; #$P < 0.01$ when compared to AF

Table 2: Cell viability in Peripheral blood mononuclear cells (PBMC) and murine macrophages RAW 264.7

Treatment	Cell viability[a] by TB (%)	Cell viability[b] by MTT (%)
CE	41.6±1.05	100
HF	52.1±1.97	100
AF	76.1±1.74	100
BF	70.0±1.33	100
WF	44.8±1.76	100
Control	87.5±1.08	100

a - Cell viability measured by Trypan blue exclusion. Cytotoxic effects on LPS-treated PBMC after 24 h culture are indicated as percentage of surviving cells. b - Cell viability measured by the MTT. The viable cells number was expressed as a percentage relative to control cells, measured as 100%XOD570, treated/OD570, control

on cariogenic microorganisms or that can interfere with factors involved in biofilm formation. The adhesion test result shows that all *P. venusta* compounds used here were able to inhibit the adherence of *S. mutans* to the smooth surface in the presence of sucrose, with the ethyl-acetate fraction showing the best percentage (76.7%) [Figure 1].

In association with these results, the production of acid was investigated because it is a good indicator of the fermentation of carbohydrate by bacteria and can lead to a reduction in dental plaque pH triggering demineralization of teeth and the formation of cariogenic lesions. In addition, low pH conditions promote the proliferation of aciduric and acidogenic species, including *S. mutans* and *Lactobacillus* spp., important microorganisms associated with oral diseases. Thus, one strategy for preventing caries is the use of an agent able to inhibit acid production in dental plaque.[31,32] *P. venusta* compounds were effective in reducing acid production by *S. mutans,* showing an effect comparable to that observed with the control chlorhexidine. The decrease of pH was significantly inhibited by CE and all fractions studied [Figure 2].

Despite being saprophytic yeast, *C. albicans* can behave as an opportunistic pathogen in some situations.[9,33] CE and fractions from *P. venusta* were able to inhibit the budding formation of *C. albicans*, with AF showing the highest percentage inhibition of cells from bud formation (56.3%). In contrast, WF did not show inhibition, considering its percentage was comparable to the negative control, with only 37.6% of cells without budding [Figure 3].

Bacteria causing periodontal diseases and inflammatory cytokines can induce a higher synthesis of NO by macrophages in the periodontal tissues. The local consequence of high NO production is not only involved in the defense mechanisms of the host but also contributes to tissue damage, causing destruction in tooth supporting tissues. NO production was inhibited by AF and BF when the concentration of 100 µg/ml was used. BF was able to inhibit NO production at 20 µg/ml too. However, CE, HF, and WF were not able to inhibit NO production in the concentrations used [Figure 4]. This is promising in the research for new anti-inflammatory agents in addition to having an associated ability to control the growth of pathogenic bacteria, representing an emerging concept in the treatment of periodontitis.[31,34,35]

Viability of PBMC cells was lower than murine macrophage RAW264.7 cells according with the percentage of viable cells against total cells observed after culture with CE and all fractions from *P. venusta* stimulated with LPS. CE was the most cytotoxic, with 58.3% of PBMC cells being not viable. On the other hand, AF was the less cytotoxic, showing 76.1% of viable PBMC cells. In the murine macrophage RAW 264.7 cells, MTT assay showed a result quite different with no one of the treatments and concentrations used resulting in any cytotoxicity. This can be explained by the concentrations of the compounds used in the two assays, since in the assay by Trypan blue exclusion we used a higher concentration (1000 µg/ml) while in the MTT assay we used the 100 µg/ml concentration. In addition, our research group demonstrated previously that the cytotoxic assay by Trypan blue might present a higher number of unviable cells when compared to the MTT assay as observed in our study [Table 2].[36]

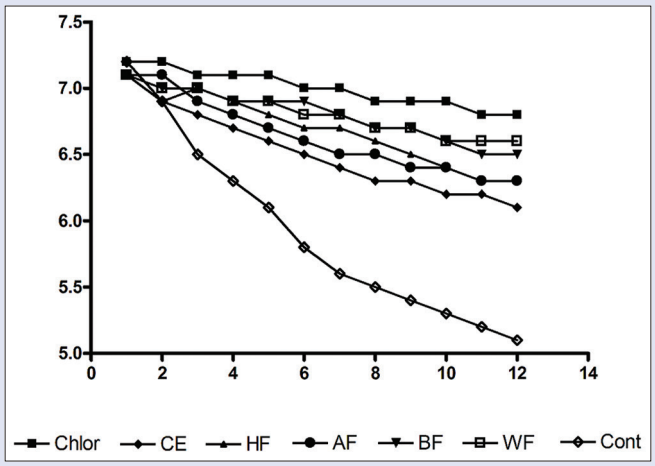


Figure 2: pH values observed in *Streptococcus mutans* culture in red phenol broth treated with crude extract (CE) and fractions of *P. venusta*

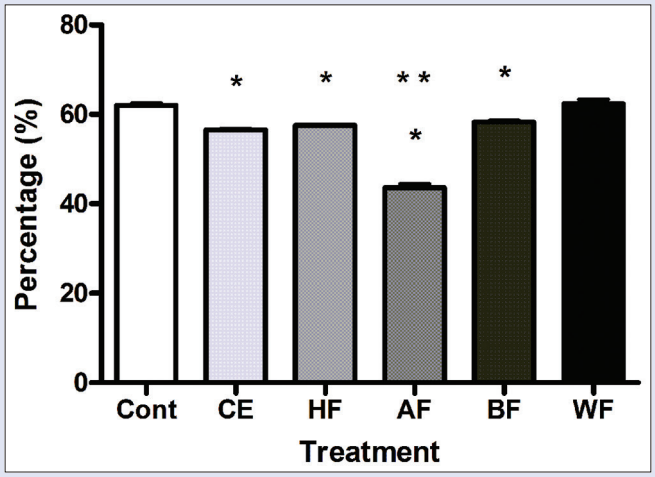


Figure 3: Budding of *Candida albicans* was inhibited by crude extract and fractions of *Pyrostegia venusta*. The data represent the mean of triplicate from one of three experiments. *P < 0.0001 when compared to control; **P < 0.001 when compared to CE, HF and BF

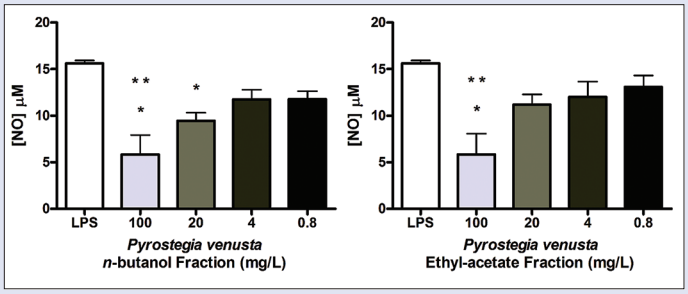


Figure 4: Nitric oxide production inhibition by n-butanol and ethyl-acetate fractions. The data represent the mean of triplicate from one of three experiments. *P < 0.001 when compated to control; **P < 0.001 when compared to the other concentrtions used

Our results suggest that *P. venusta* presents potential to be used as a preliminary source of compounds that can provide helpful activity when used in prophylaxis or treatment of caries or periodontal disease. The ethyl-acetate fraction showed promising results justifying further studies to investigate the mechanisms of action and the possible development of a new oral antiseptic agent.

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Conflicts of interest

There are no conflicts of interest.

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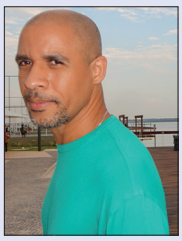

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