ANTIMICROBIAL ACTIVITY OF VIOLACEIN AGAINST ORAL BACTERIA ASSOCIATED WITH HALITOSIS: AN *IN VITRO* STUDY

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Palavras-chave: Halitose. Violaceína. Compostos Voláteis de Enxofre. Patógenos Orais. Língua Saburrosa.

RESUMO

Introdução: a violaceína é um pigmento roxo natural produzido por bactérias ambientais que apresenta ação antimicrobiana, particularmente contra bactérias Gram-positivas. A halitose intraoral (HIO) é uma condição definida pelo odor desagradável que emana da boca, cuja principal fonte são os compostos sulfurados voláteis produzidos por bactérias Gram-negativas da saburra lingual. No tratamento da HIO, antimicrobianos têm sido indicados como adjuvantes, incluindo produtos naturais. **Objetivo**: assim, este estudo avaliou o potencial antimicrobiano de um extrato de violaceína em patógenos-chave da HIO (*Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Fusobacterium nucleatum*, *Prevotella intermedia, Solobacterium moorei*). **Materiais e Métodos**: bactérias foram cultivadas em meio ágar sangue para fastidiosos, em anaerobiose, e suspensões de 10^9 células/ml foram semeadas. O extrato bruto de violaceína obtido de *Chromobacterium violaceum* foi diluído em solução aquosa com 25% de etanol nas concentrações de 8, 4, 2, 1, 0,5 e 0,25 mg/ml. Através do método de disco difusão, 10 µl de cada diluição foram depositados nas placas semeadas. A clorexidina (0,1%) e a solução etanólica a 25% foram usadas como controles. As placas foram incubadas em anaerobiose a 37°C por 72h, e os halos de inibição foram registrados. **Resultados**: embora a clorexidina tenha apresentado os maiores halos de inibição do do que o extrato, a maioria das espécies foi inibida nas concentrações de 4 e 8 mg/ml (p<0,05). *P. gingivalis* e *F. nucleatum* foram as espécies mais afetadas em relação às outras bactérias, porém só foi observada significância estatística para *P. gingivalis* (p<0,05). **Conclusão**: o extrato bruto de violaceína de *C. violaceum* demonstrou atividade antimicrobiana contra bactérias orais associadas a HIO, sendo um potencial antimicrobiano a ser estudado como adjuvante no controle da HIO.

Keywords: Halitosis. Violacein. Volatile Sulfur Compounds. Oral Pathogens. Coated Tongue.

ABSTRACT

Introduction: violacein is a natural purple pigment produced by environmental bacteria that presents antimicrobial activity, particularly against Gram-positive bacteria. Intraoral halitosis (IOH) is a condition defined by the unpleasant odor emanating from the mouth, whose main source are volatile sulfur compounds, produced by Gram-negative oral bacteria on the tongue coating. In IOH treatment, antimicrobials have been indicated as chemical adjuncts, including natural products. **Objective**: thus, this study tested the antimicrobial activity of a violacein extract on key IOH-related bacteria (*Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Solobacterium moorei*). **Materials and Methods**: bacteria were cultured in fastidious anaerobe blood agar in anaerobiosis, and 10^9 cells/ml suspensions were plated. Crude extract of violacein obtained from *Chromobacterium violaceum* was diluted in a 25% ethanol aqueous solution to 8, 4, 2, 1, 0.5 and 0.25 mg/ml. Using the disk agar diffusion method, 10 µl aliquots of each dilution were deposited on the seeded plates. Chlorohexidine (0.1%) and 25% ethanol solution were used as controls. Plates were incubated in anaerobiosis at 37°C for 72h, and the inhibition halos were recorded. **Results**: although chlorhexidine showed higher inhibition halos than the violacein extract, most species were inhibited at 4 and 8 mg/ml concentrations (p<0.05). *P. gingivalis* followed by *F. nucleatum* were the most affected species in relation to the other bacteria, although statistical significance was only reached for *P. gingivalis* (p<0.05). **Conclusion**: crude violacein extract from *C. violaceum* demonstrated antimicrobial activity against IOH-associated oral bacteria, being a potential antimicrobial to be studied as an adjunct in the control of IOH.

Submitted: February 27, 2023
Modification: April 8, 2023
Accepted: April 27, 2023

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INTRODUCTION

Halitosis is an oral health condition that describes an unpleasant odor emanating from the mouth, of an oral or non-oral source, that leads to discomfort, embarrassment, and/or psychosocial disadvantages.[1-4] Currently, halitosis is the third most common reason for referral to dentists, with a reported prevalence of up to 50%.[5,6] Intraoral halitosis (IOH) is responsible for 90% of the cases and it is associated with the production of volatile sulfur compounds (VSCs), such as hydrogen sulfide, methyl mercaptan and dimethyl sulfide.[7,8]

These compounds result from protein degradation by certain oral bacteria. Bacterial substrates include sulfur-containing amino acids such as cysteine, cystine, and methionine present in saliva or gingival fluid.[9] Among the major IOH causing bacteria are the periodontal putative pathogens *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Porphyromonas endodontalis* and *Eikenella corrodens*, as well as the species *Centipeda periodontii* and *Solobacterium moorei*.[10-16] These microorganisms are usually found in the gingival sulcus, periodontal pockets and interdental spaces, as well as in the deep crypts of fissures and grooves of the posterior dorsum of the tongue, where the low redox potential favors their growth.[10-16]

The causative and/or promoting factors of halitosis include specific foods, poor oral hygiene, tobacco products, deep carious lesions, periodontal disease, peri-implant disease, pericoronitis, mucosal ulcerations, impacted food or debris, decreased salivary flow and especially tongue coating.[4-6,17]

Based on an accurate diagnosis, the treatment plan of IOH typically involves a mechanical and/or chemical approach. Mechanical treatment is primarily achieved by adequate tooth brushing, tongue cleaning, interdental cleaning, and/or professional periodontal therapy. The chemical approach involves chemical reduction of halitosis-causing bacteria and/or neutralization of their odorous products through the use of mouthwashes containing distinct substances such as triclosan, zinc salts, chlorine dioxide, essential oils, cetylpyridinium chloride and/or chlorhexidine (CHX).[4,18-20]

However, the prolonged use of these substances may cause side effects, including pigmentation of teeth, restorations and oral mucosa, altered taste, mucosal erosion, increased formation of supragingival calculus, burning sensation, among others.[21,22] In addition, some studies have shown the emergence of certain isolates exhibiting greater CHX tolerance and low-level exposure to CHX can result in the development of cross-resistance to antibiotics. Although more research is needed to investigate this cross-resistance, the consequences of this can be dire.[23,24] In response to these challenges, new alternatives are being explored as possible treatment options, specifically the use of natural substances with antimicrobial properties.[25]

A promising substance is violacein,[26-30] a natural purple/blue pigment produced by certain strains of the *Janthinobacterium* and *Chromobacterium* genera, *Pseudomonas luteoviolacea*, *Duganella* sp., *Collimonas* sp., among others.[31-33] This indole-like secondary metabolite formed by a 5-hydroxy indole, an oxindole and a 2-pyrrolidone has shown a wide range of biological properties, such as antioxidant, antiparasitic, antifungal, antitumor, antiviral, antiulcerogenic, anti-inflammatory, antileukemic, antiprotozoal and broad-spectrum antimicrobial activities.[29,30,34-37] Of the latter, there is limited data on its antimicrobial properties against Gram-negative pathogenic bacteria,[33,34] in particular oral pathogens. Thus, the present investigation aimed to test *in vitro* the potential antimicrobial activity of violacein against key IOH-associated bacteria.

MATERIALS AND METHODS

Cultivation and violacein extraction from strain *Chromobacterium violaceum*_DSM 30191 (figure 1) were carried out according to Caldas *et al.*[38] Violacein crystals were then solubilized in sterile distilled water containing 25% ethanol PA (Isofar, Duque de Caxias, RJ, Brazil) to make a 40 mg/ml stock solution. The solution was thoroughly mixed, and serially diluted to final concentrations of 8, 4, 2, 1, 0.5 and 0.25 mg/ml. A commercial solution of 1% CHX (1% Chlorhexidine Gluconate, Needs, Droga Raia, RJ, Brazil) was diluted in sterile distilled water to a concentration of 0.1% to be used as a positive control. Negative controls included the phosphate buffer solution (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4 pH 7.4), distilled water, and distilled water + ethanol used in the violacein dilutions.

The strains *Porphyromonas gingivalis* (ATCC BAA-308 strain W83), *Porphyromonas endodontalis* (ATCC 35406), *Fusobacterium nucleatum* (ATCC 25586), *Prevotella intermedia* (ATCC 25611) and *Solobacterium moorei* (FO 678) were cultivated on Fastidious Anaerobe Agar (FAA) with 5% sterile defibrinated sheep blood, anaerobically at 37ºC for approximately 5 days. Colonies of each species were suspended in PBS and their concentrations adjusted to an $OD_{600nm} = 1$ (~10^9 cells/ml). Then, 100 µl of each bacterial suspension were plated on FAA + blood media in triplicate. Aliquots of 10 µl of each violacein concentration (8, 4, 2, 1, 0.5, 0.25 mg/ml), 0.1% CHX and negative controls were deposited on the inoculated FAA + blood plates which were incubated at 37ºC in anaerobic conditions for 72 h. For all species tested, 3 independent experiments were performed in triplicate.

Then, the plates containing each species were analyzed for the presence of areas of bacterial growth inhibition around each violacein concentration deposited on the plate. The degree of inhibition (size and translucency of the halo) was defined as: 0, no inhibition (complete growth); 1, growth in the halo, although some inhibition occurred; 2, no growth. The scores were dichotomized into presence (scores 1 and 2) and absence (score 0) of inhibition, and the frequency calculated for each concentration and each species evaluated in triplicate. Comparisons between different violacein concentrations regarding the presence or absence of inhibition for all species were examined by the McNemar test. Comparisons between species in each concentration were tested by the Chi-square test. The significance level was 5%.

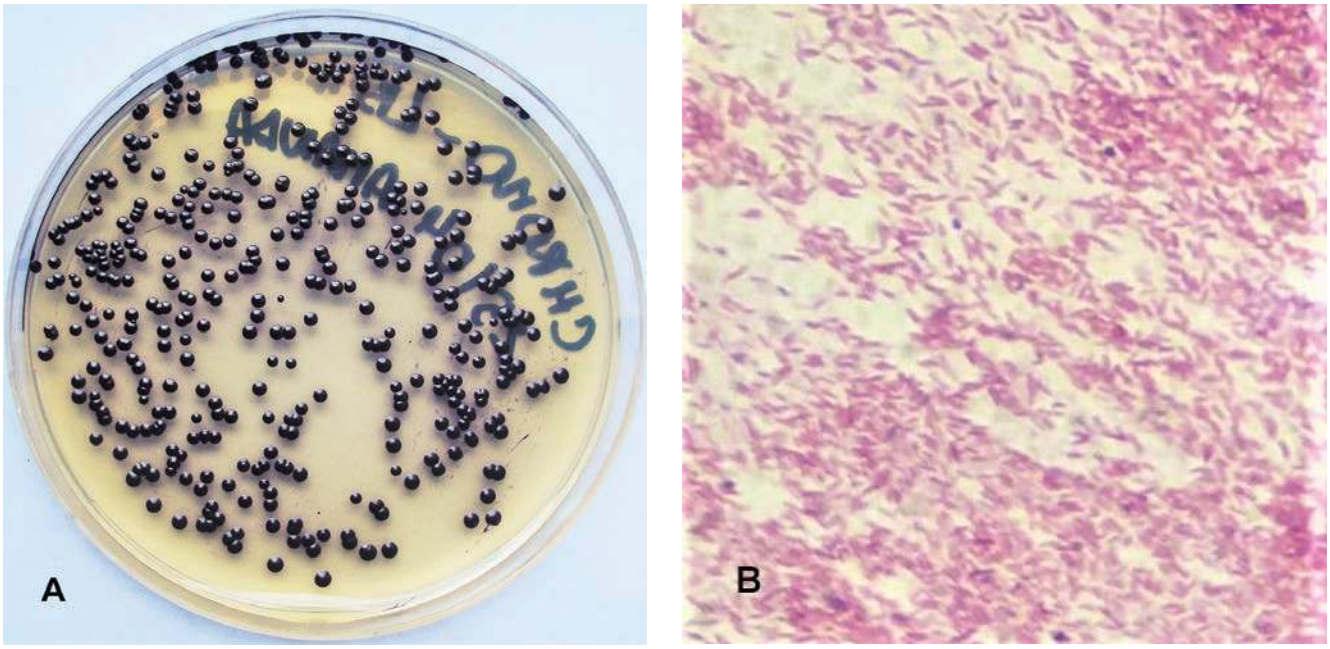

Figure 1: *(A) Chromobacterium violaceum cultivated on Tripticase soy agar plates in aerobiosis. Colony morphology after 48 hours of cultivation, showing a dark blue/purple pigment (left image). (B) Cell morphology shown by light microscopy (1000x) after staining, revealing the Gram-negative coccobacilli appearance (right image).*

RESULTS

The frequency of inhibition of all species tested in three independent experiments in triplicate is shown in Figure 2. Overall, violacein extract at a concentration of 8 mg/ml was able to inhibit the growth of all species tested, similarly to the positive control of CHX at 0.1%. Nevertheless, the diameter of the halos (mm) were smaller for the 8 mg/ml of violacein in relation to CHX (data not shown). Due to the hydrophobicity of the extract, these measurements (in mm) were not considered, but only presence or absence of inhibitory halo.

The 4 mg/ml concentration was significantly effective in comparison to the other concentrations, inhibiting 4 of the 5 oral species tested ($p<0.01$, McNemar Chi-Square test). No significant effects were observed among the lower concentrations (2 mg/ml to 0.250 mg/ml). The antimicrobial effect of the violacein extract on each species is shown in Figure 3. *P. gingivalis* was the only one that showed relative sensitivity to all violacein concentrations, whereas *F. nucleatum* was the second most sensitive pathogen to the compound. *S. moorei* and *P. intermedia* were only inhibited at the highest concentrations. Despite the sensitivity of *P.*

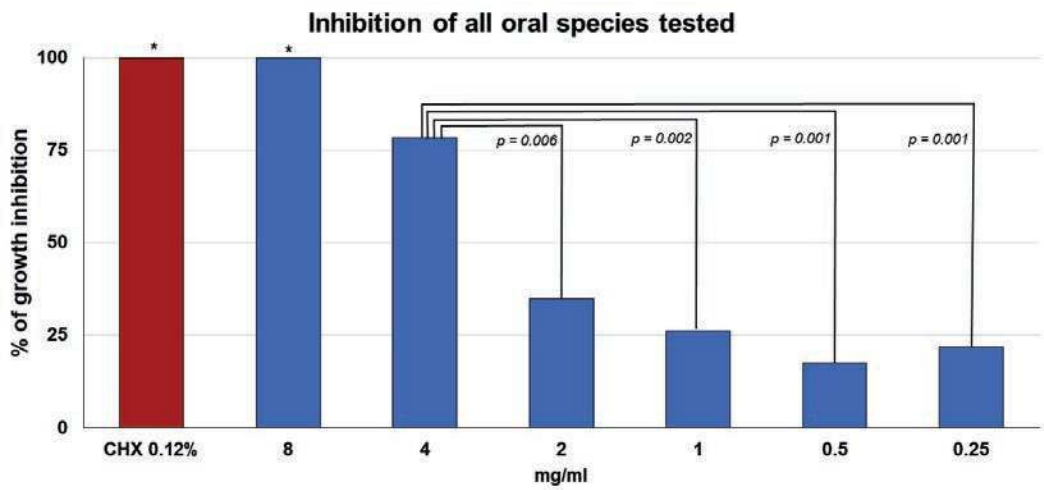

Figure 2: *Frequency of growth inhibition (%) of all oral species tested at different violacein extract concentrations. A total of 3 independent experiments in triplicate were performed. Significant higher rate of inhibition was observed at 4 mg/ml in comparison to the other concentrations ($p<0.01$, McNemar Chi-Square test). No significant differences were observed between concentrations ≤ 2mg/ml. *Due to 100 % growth inhibition, McNemar test was not computed.*

gingivalis to all concentrations, the other species of the genus *Porphyromonas*, *P. endodontalis*, was only sensitive to the concentration of 8 mg/ml, suggesting a potential specificity of the violacein extract to species of the same genus. Comparisons in the frequency of inhibition among oral species at each concentration showed that *P. gingivalis* was significantly more inhibited than the other four pathogens at 1 and 0.250 mg/ml concentrations ($p<0.05$, Pearson's Chi-Square test). At 2 mg/ml, significant differences were seen between *P. gingivalis* and *P. endodontalis*, *S. moorei* or *P. intermedia* ($p>0.05$), but not *F. nucleatum* ($p>0.05$, Pearson's Chi-Square test).

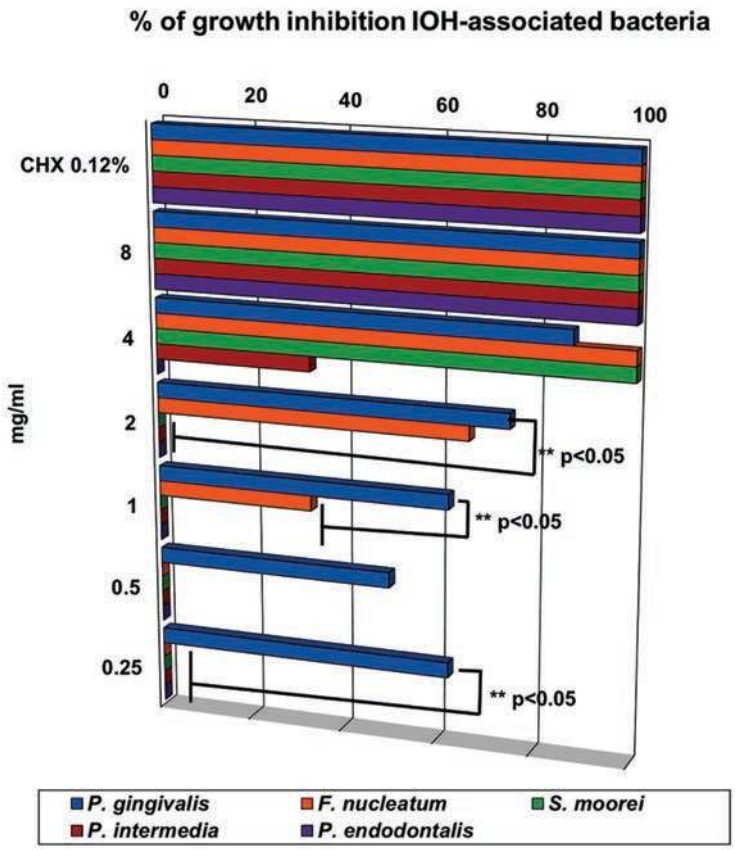

Figure 3: *Inhibition (%) of each oral species associated with halitosis at different violacein extract concentrations. For each species, three independent experiments were performed in triplicate. Significant differences in the rate of inhibition was observed between P. gingivalis and the other pathogens at 1 and 0.250 mg/ml (**p<0.05). At 2 mg/ml, P. gingivalis differed from P. intermedia, S. moorei and P. endodontalis (**p<0.05), but not from F. nucleatum (p>0.05, Pearson's Chi-Square test).*

DISCUSSION

The application of natural antimicrobials as adjunctive chemical therapy in the control of IOH has been widely explored, with many potential candidate compounds.[25] Among those, the microbial pigment violacein presents various pharmacological and chemical properties that make this metabolite an attractive pharma product.[26-30] Although violacein was shown to be effective against several bacterial pathogens of medical relevance, no data regarding its antimicrobial efficacy over Gram-negative oral pathogens are available. In particular, IOH-associated pathogens are predominantly Gram-negative, anaerobic and proteolytic species that could be targets for natural products with antimicrobial properties. Therefore, this study evaluated the *in vitro* antimicrobial activity of crude violacein extract from *C. violaceum* on key oral species associated with IOH. The *in vitro* method used here was the diffusion in agar, modified and adapted according to several previous pilot experiments to evaluate the concentrations of the bacterial inoculum and the compound. Due to its low solubility in water, violacein crystals were dissolved in 25% of ethanol. The 25% alcoholic solvent was used as controls in all experiments, showing none antimicrobial activity on the oral species tested. Moreover, diffusion of violacein through the agar mesh of the solid media was limited, and therefore, the diameter of the inhibition halo (in mm) was not considered in the evaluation of its antimicrobial efficacy, but only the presence or absence of inhibition halo. Despite the limitations, this is a fast and easy to perform screening test to select target species and

ideal concentrations. Overall, the data showed that most species were inhibited at high concentrations (8 and 4 mg/ml), with *P. gingivalis* being the most susceptible bacteria, followed by *F. nucleatum*, an also important periodontal pathogen. These findings are promising, as violacein is usually effective against Gram-positive bacteria and not Gram-negative pathogens, as we found.[27,29-33,36] Moreover, *P. gingivalis* and *F. nucleatum* are major producers of VSCs, which causes IOH.[10,12,13,16] The Gram-positive species *S. moorei* was also relatively inhibited by violacein. This microorganism is strongly associated with halitosis, as it is capable of converting cysteine directly into hydrogen sulfide. It also produces VSCs from mucin in the presence of an exogenous protease present in the dorsal region of the tongue, and from salivary glycoproteins. Consequently, this may explain the high levels of *S. moorei* normally found in the tongue coating and saliva of patients with halitosis.[11,14,16]

The effects of this violacein extract at different concentrations and against different species are certainly influenced by several factors such as the extraction method, the bacterial source of the compound, the solubilizing vehicle and the substantivity of the solution employed. Violacein is produced by a number of bacteria of different genera found in various marine and soil environments.[27,29,30] In particular, the saprophytic species *C. violaceum* is very abundant in tropical and subtropical regions of the world, allowing the extraction of large amounts of violacein.[31,32] Therefore, for this initial stage of our study we used a crude violacein extract from *C. violaceum* in order to evaluate whether this compound had any antimicrobial property on halitosis-causing bacteria. Further investigation to characterize the specific substances that make up and confer antimicrobial efficacy to this extract are now justified. Consequently, components other than violacein may have been responsible for the antibacterial activity on these species.

Although the mechanisms associated with the antimicrobial activity of violacein are not fully known, for Gram-positive microorganisms, it acts by disrupting the cell membrane integrity, causing leakage of intracellular constituents. In Gram-negative bacteria, this action is hampered by the outer cell wall that acts as a barrier that absorbs violacein and prevents it from breaking through the cytoplasmic membrane.[30,31,33,36] This bacterial pigment has been shown to present antibacterial activity against several bacteria; however, there are no data on the literature regarding its activity against Gram-negative IOH-related bacteria. In the current study, our preliminary findings demonstrated some antibacterial activity, however at relatively high concentrations, which may require cytotoxicity testing for its use *in vivo* as a therapeutic approach for halitosis. Variations in violacein cytotoxicity are observed depending on the cell type, indicating the occurrence of cell type-specific mechanisms of violacein. The cytotoxicity of purified violacein to non-tumor cells is in the range of 5–12 ìM, whereas no *in vivo* toxicity of major organs is observed, even with very high doses (up to 7.5 mg/Kg).[26-28,30] Here, crude violacein extract, and not purified violacein, was used, which may explain in part the antibacterial efficacy at high concentrations. Thus, further assessment of cytotoxicity of the crude violacein extract will be required.[34]

Another limitation of this study was that the antimicrobial effect of violacein on halitosis-bacteria grown in a biofilm structure was not investigated. This fact is very important because the tongue accumulates biofilm and is the main site for the production of VSCs in the oral cavity.[39,40] Therefore, the effect of violacein in preventing the formation of tongue biofilm or disorganization of the already formed biofilm needs to be evaluated. In addition to the antibacterial activity, the future application of violacein in IOH therapy may be directed to neutralizing VSCs,[41] as observed for many other natural and synthesized components.[19,20,42,43] Finally, the *in vivo* high efficacy of violacein for IOH management may be achieved by incorporating other substances to the compound, resulting in greater substantivity, a key feature of the antimicrobial efficacy of CHX in the oral cavity.[21,22] Within the current global context of the search for natural products that are ecological and as effective as their synthetic counterparts are,[25] the results of this study are promising, as violacein is a potential candidate as an adjuvant for the mechanical treatment of IOH. However, more studies are needed to properly explore their properties and/or improve them for use in the clinical routine of oral health care.

CONCLUSION

Crude violacein extract from *Chromobacterium violaceum* at concentrations between 4 – 8 mg/ml showed antimicrobial activity against most oral pathogens associated with halitosis, in particular *P. gingivalis*, *F. nucleatum* and *S. moorei*. Additional adjustments regarding concentrations, combination with vehicles to increase substantivity, evaluation of its action on other oral pathogens and biofilms, as well as on the production of VSCs are necessary to validate its real effectiveness in the control of intraoral halitosis.

ACKOWLEDGMENTS

This study was supported in part by the National Council for Scientific and Technological Development (CNPq), Brasilia; and Foundation for Research Financial Support in the State of Rio de Janeiro (FAPERJ) Grant/Award Number: E-26/201.162/2014, Brazil.

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