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Antimicrobial susceptibility of anaerobic bacteria

Strong antimicrobial activity of collinin and isocollinin against periodontal and superinfectant pathogens *in vitro*

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ABSTRACT

Periodontitis pathogenesis involves activation of host immune responses triggered by microbial dysbiosis. Therefore, controlling periodontal pathogens *in-vivo* is a main goal of periodontal therapy. New antimicrobials might help to control periodontal infection and improve treatment outcomes at "the dark times" of increasing antibiotic resistance. Here, we determined the biological activity of collinin and isocollinin against 8 bacterial strains. Antimicrobial activity of collinin and isocollinin, chlorhexidine digluconate (CHX) and sodium hypochlorite (NaClO) was evaluated against clinically relevant periodontal bacteria, like *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella intermedia*, *Dialister pneumosintes* strains and superinfectants like *Escherichia coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa* strains. A broth microdilution test was carried out to determine the minimum inhibitory concentration of collinin and isocollinin against those strains, and bacterial viability was determined by resazurin assay at diverse concentration and exposure times. *P. gingivalis* was the most susceptible strain to collinin and isocollinin (MIC 2.1 μg/mL and 4.2 μg/mL respectively). Other periodontal pathogens showed MICs <17 μg/mL for collinin and MICs between 20 and 42 μg/mL for isocollinin, whereas CHX and NaClO showed MICs of 62 and 326 μg/mL, respectively. Collinin and isocollinin also exhibited antimicrobial activity against superinfectant bacteria (MIC < 21 and < 42 μg/mL, respectively). Overall, collinin and isocollinin showed a remarkable antibacterial activity against relevant periodontal and superinfective bacteria, especially against *P. gingivalis* (MIC 2.1 μg/mL and 4.2 μg/mL respectively) and the highly virulent *P. aeruginosa* (MIC 5.2 and 20.8 μg/mL, respectively).

1. Introduction

Worldwide preventable oral diseases, such as caries and periodontitis, affect four billion people, generating eating problems and pain, reducing quality of life, increasing Disability-Adjusted Life Year (DALYs) and increasing health systems costs related to disease treatment [1,2].

These prevalent diseases are mainly produced by microbial dysbiosis, caused by proliferation of pathogenic microbiota and concomitant suppression of beneficial species, which could cause severe damage in oral tissues homeostasis [3]. Gingival inflammation, increased bleeding on probing, apical migration of junctional epithelium, detach of gingival tissues from root surfaces, pocket formation, gingival recession, alveolar bone loss, and increased tooth mobility are typical features of periodontal disease, which are caused by the activation of immune response, triggered by periodontal pathogens and virulence factors [3,4]. Possibly, these well described signs and symptoms are result of undetermined genetic susceptibility, early microbial dysbiosis and immune system dysregulation [5].

Significant increase in the proportion of important pathogenic microorganisms of periodontal lesions have been reported by cultivation and molecular identification methods [3,6,7]. The most important periodontal pathogens are *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Treponema denticola, Fusobacterium nucleatum, Tannerella forsythia, Eikenella corrodens, Dialister pneumosintes* and new species such as *Filifactor alocis* among other unculturable species as well as opportunistic species from family Enterobacteriaceae [6]. In 1988 Slots and collaborator's first reported enteric rods such as *Pseudomonas* and *Candida* species in severe periodontitis and then, detected *Staphylococcus aureus* in periodontitis demonstrating the importance of opportunistic

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pathogens in oral disease [8,9] which carry important virulence factors and are recalcitrant to elimination/control by mechanical therapy.

It has been shown that routine mechanical removal of bacterial biofilm by brushing and flossing should promote oral health and prevent caries and periodontal disease and is the most valuable antimicrobial approach when rigorously practiced [7]. However, most people do not exercise proper and meticulous biofilm control due to diverse reasons, including poverty, limited oral health care access, lack of access to oral care technology, lack of conscience and motivation and poor oral education [10].

Chemical control of oral biofilm is a promising tool to prevent and to treat oral disease [11,12]. Currently, several chemical compounds are been used to control pathogenic members of the oral microbiota. However, oral microbes are showing increased resistance against antibiotics and medicated rinses. Thus, new promising products from natural sources are being tested for more effective oral biofilm control [12].

A natural coumarin derivative so-called collinin has shown important activity against a key periodontal pathogen such as *P. gingivalis* [13], and has been successfully tested against several other pathogenic and prevalent diseases [14–20]. However, there is still scarce evidence to support its antimicrobial activity in periodontal disease and against superinfectant bacteria.

In this work, collinin and isocollinin were synthesized, purified and characterized following reported methods [21]. The antimicrobial activity of both isomers was tested against pathogens of medical and oral clinical interest, in addition bacterial susceptibility, cell viability and time-kill assays were evaluated.

2. Materials and methods

2.1. Preparations of collinin isomers and reference biocides

Briefly, collinin and isocollinin (Fig. 1) were synthesized by first producing the chemicals daphnetin and geranyl bromide, which were obtained by two parallel reactions. First, pyrogallol (Alfa Aesar, Tewksbury, MA, USA), propiolic acid (Alfa Aesar, Tewksbury, MA, USA) and sulfuric acid (Merck, Darmstadt, Germany) were stirred at 125 °C for 50 min. Daphnetin was isolated from the reaction product as a brown color solid after several purification steps, which are presented in Ref. [21]. On the other hand, geraniol (Alfa Aesar, Tewksbury, MA, USA), phosphorus tribromide (Alfa Aesar, Tewksbury, MA, USA), and diethyl ether (Merck, Darmstadt, Germany) were stirred at 0 °C for 3 h, in a light-isolated flask under N_2 (Cryogas INDURA group, Cali, Colombia) atmosphere. Geranyl bromide was obtained as a golden oil once purified from the reaction crude according to Ref. [21].

Geranyl bromide was then added to daphnetin in acetone (Alfa Aesar, Tewksbury, MA, USA) and DBU (Merck, Darmstadt, Germany), and stirred at 28 °C for 5 h. The resulting crude was purified according to Ref. [21]. Thus, three main products were obtained: one dialkylated compound, which was separated as an oil using solvent extraction, and two alkylated compounds separated by using column chromatography, to obtain the chemical precursors of collinin and isocollinin as white solids. The isocollinin precursor, 7-hidroxy 8-geranyloxy coumarin, was added to a suspension of sodium hydride (Alfa Aesar, Tewksbury, MA, USA) in THF (Merck, Darmstadt, Germany) at 0 °C, under N_2 (Cryogas INDURA group, Cali, Colombia) atmosphere, and the resulting solution was stirred at 28 °C for 2 h. Iodomethane was then added and the solution was continuously stirred until the precursor was totally consumed (*ca.* 16 h). Isocollinin was obtained as a white solid after several purification steps described in Ref. [21], A similar procedure was also performed to obtain Collinin as a white solid. Collinin required an additional recrystallization procedure (hexane/ethanol 96% v/v) due to the presence of impurities, including the raw material that was not consumed. A stock solution of isocollinin and collinin dissolved in ethanol (100 μg/mL both) were stored at 4 °C in amber vials.

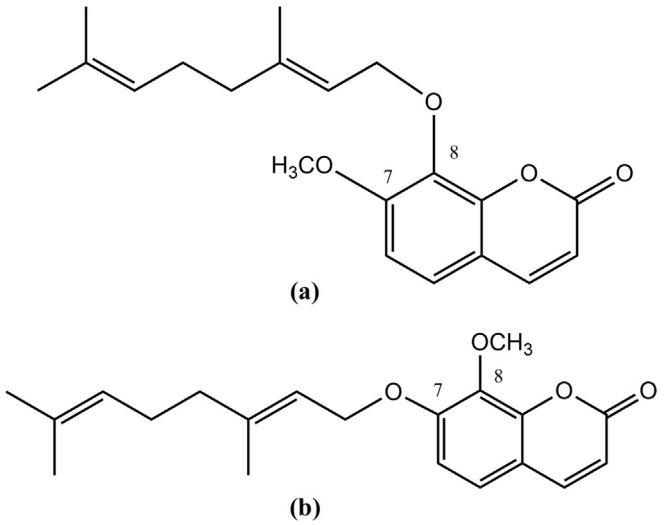

Fig. 1. Chemical structures of (a) 7-methoxy 8-geranyloxy coumarin (Isocollinin), and (b) 7-geranyloxy 8-methoxy coumarin (Collinin).

Two commonly used biocides, chlorhexidine digluconate (CHX) (PerioGard, Colgate, DF, Mexico) and sodium hypochlorite (NaClO) (Blancox, Bogota, Colombia), were tested to compare the performance of both collinin isomers. Stock solutions of CHX and NaClO were prepared at 1,200 μg/mL and 50,000 μg/mL, respectively, in sterile water.

2.2. Bacterial strains and culture conditions

Reference strains of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, and strains of *A. actinomycetemcomitans* ATCC 29522, *P. gingivalis* ATCC 33277, *F. nucleatum* ATCC 25586, *P. intermedia* ATCC 25611, and *Dialister pneumosintes* ATCC 33048, were cultivated in BHI broth medium (Sharlau, Barcelona, Spain) with 0.0005% hemin (Sigma Aldrich, St. Louis, MO, USA). Anaerobic bacteria were cultured in anaerobic jars (Oxoid, AnaeroJar 2.5L, Hampshire, UK) (1% O_2, 9% CO_2) and *A. actinomycetemcomitans* was grown under microaerophilic conditions (5% CO_2), at 37 °C for 48 h, whilst aerobic strains were cultured at 37 °C during 24 h. The initial inoculum for the susceptibility assays was prepared in sterile PBS (Thermo Fisher Scientific, Gibco, Paisley, Scotland, UK) and adjusted to reach optical density (OD) of 0.08 at 600 nm (Biochrom, Ultrospec-10, Cambridge, UK) equivalent to 0.05 on the McFarland standard (1.5×10^8 CFU/mL). This standard inoculum was diluted 1:10 in BHI broth for all assays.

2.3. Bacterial susceptibility assay

The MIC of each agent was determined following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) with some modifications according to the nutritional characteristics of each specie [22]. Serial two-fold dilutions of biocides (CHX 1,200 to 2.3 μg/mL), (NaClO 50,000–97 μg/mL) and (collinin and isocollinin, 100 to 0.2 μg/mL) were prepared in culture media supplemented with BHI and mixed in wells of 96-well plates. Positive control wells were used, containing bacteria but not the biocide and also,

negative control wells with CHX at 1,200 μg/mL without bacterial inoculum.

Cultures were incubated at 37 °C for 48 h under conditions previously described. Bacterial growth was monitored measuring the OD at 600 nm in a spectrophotometer (NanoDrop One, Thermo Fisher, Madison, WI, USA). The MIC was taken as the lowest concentration of biocide that prevents visible growth and reduces absorbance up to 0.04 or less.

2.4. Cell viability

The nonviable cells lose rapidly the metabolic capacity to reduce resazurin into resorufin (fluorescent dye). An assay based on this biological activity was used to assess cell viability at different times (CellTiter-Blue Cell Viability Assay, Promega, Madison, WI, USA). Briefly, the procedure consisted in adding the single reagent directly to cells cultured in 100 μl of BHI broth and placed in a 96-well plate (Costar, Corning, NY, USA), the intensity of the color produced is directly proportional to the number of viable cells. After a 3-h incubation under anaerobic or aerobic conditions depending on the specie, absorbance was measured using a plate-reading fluorometer (Stat Fax 2100 Microplate Reader, Palm City, FL. USA).

Minimal Bactericidal Concentration (MBC) was the lowest biocide concentration at which the well showed no capacity to reduce resazurin, that is, no color change was observed. The absence of bacterial growth was confirmed in new agar cultures with the optimal growth conditions. At MBC, no bacterial growth was detected.

2.5. Statistical analyses

All experiments were performed in triplicate. Quantitative data is presented as means with standard deviations (SD), and qualitative data is presented as proportions or percentages. The means and SD were compared using a two-way ANOVA followed by Sidak's multiple comparison post hoc test using statistics software (Prism, version 7.0, GraphPad, San Diego, CA, USA). Values with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Synthesis and identification of collinin isomers

From 1 g of pyrogallol, 140 mg of collinin and 550 mg of isocollinin were obtained, which correspond to overall molar yields of 2.6 and 13.9%, respectively. Both isomers were characterized by nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) and differential scanning calorimetry (DSC). Additional 1D NOESY NMR experiments were performed with the precursors of collinin and isocollinin, to verify the identity of each isomer. Purities of 98.7 and 98.0 ± 0.2% for isocollinin and collinin were estimated by DSC, according to the methodology described in the ASTM E928 standard (ASTM, 2014) [23]. Further information about the synthesis and characterization procedures of these compounds are reported elsewhere [21].

3.2. Bacterial susceptibility and cell viability assays

Table 1 presents the antimicrobial activity of different chemical agents against bacteria of clinical importance. Clorhexidine digluconate (CHX) and sodium hypochlorite (NaClO) were used to compare the antimicrobial effect of the collinin isomers. Overall, and considering the standard deviation, periodontophatogens were more susceptible to collinin and isocollinin than to CHX and NaClO. NaClO presented the lowest antimicrobial activity against all bacterial strains tested (MIC ≥ 300 μg/mL) (data not shown).

P. gingivalis was the most resistant strain to CHX (MIC 62.9 μg/mL), while it showed significantly more sensitivity to collinin and isocollinin (MIC 2.1 μg/ml and 4.2 μg/ml, respectively). In addition, the other periodontal bacteria showed high sensitivity to collinin (MIC < 17 μg/mL), while it was lower with isocollinin and CHX (20 < MIC < 42 and MIC = 31.0 μg/mL, respectively). Whereas, superinfectant strains showed a similar sensitivity to collinin and CHX (MIC < 20 μg/mL), while these were less sensitive to isocollinin (MIC > 20 μg/mL).

Fig. 2 presents the antimicrobial effect of collinin isomers and CHX against periodontopathic and superinfective pathogens. Overall, the differences between the effect of collinin and CHX were statistically significant, considering both the effect of those two biocides on all bacteria strains ($p < 0.0001$) and between strains ($p < 0.001$). In the case of isocollinin and CHX, the differences between the effect of biocides were not statistically significant ($p = 0.9852$), while between strains were significant ($p < 0.1$). There were differences in the antimicrobial activity of both isomers.

Collinin showed better antimicrobial activity against periodontal pathogens than CHX, and differences in their performance were statistically significant ($p < 0.04$), except against *P. intermedia* ($p = 0.2683$). On the other hand, the effect of collinin and CHX over superinfectant strains did not showed significant differences. Moreover, isocollinin showed a similar antimicrobial activity to that performed by CHX, except against *P. gingivalis* and *S. aureus,* which were higher and statistically significant for isocollinin ($p < 0.0001$) and CHX ($p < 0.001$), respectively. Finally, comparing the performance of both isomers, they showed a similar antimicrobial activity against all tested bacteria, except for *D. pneumosintes, A. actinomycetemcomitans* and *S. aureus,* which exhibited more susceptibility to collinin ($p < 0.001$).

3.3. Cell viability assay

The MBC values obtained in the cell viability assay were similar to the MIC values presented in Table 1. However, these results showed that collinin isomers were more bactericidal over periodontal pathogens than CHX. By contrast, CHX was more bactericidal over superinfective strains than the collinin isomers. Particularly, *P. gingivalis* was the most susceptible strain against both isomers (MBC 5.2 and 10.4 μg/mL), and the most resistant to CHX (MBC 62.7 μg/mL). While *E. coli* was more resistant to either collinin isomers (MBC 33.3 μg/mL). *E. coli* resulted more susceptible to CHX (MBC 15.7 μg/mL) (Fig. 3).

The time-kill assay used *E. coli* as a reference strain to perform all tests, given that it showed more resistance against both collinin isomers. With concentrations of 50 μg/mL of isocollinin, *E. coli* was killed even after a 24-h follow-up; and at any lower concentration of isocollinin, *E. coli* remained viable (Fig. 4). Collinin showed a better performance to kill *E. coli* since 25 μg/mL were enough to control bacterial viability. At lower concentrations, it was observed a significant reduction in the bacterial growth; however, after 8-h follow-up, an exponential rising in bacterial population was detected, and *E. coli* was viable, confirmed by the resazurin assay and agar sub-cultivation.

4. Discussion

In this study, antimicrobial activity *in vitro* of collinin and isocollinin was tested against individual microbial strains, such as five important periodontal pathogens - ATCC strains and another three important bacteria of medical interest. Collinin and isocollinin demonstrated remarkable antibacterial activity against

Table 1
Evaluation of the antimicrobial activity of different chemical agents against bacteria of clinical importance after 24-48 h of exposure.

Strains	Isocollinin (μg/mL)		Collinin (μg/mL)		CHX (μg/mL)		NaClO (μg/mL)	
	MIC (mean)	(SD)	MIC (mean)	(SD)	MIC (mean)	(SD)	MIC (mean)	(SD)
Periodontophatics								
A.actinomycetemcomitans	41.7	11.8	5.2	1.4	31.7	9.0	325.8	92.3
P. gingivalis	4.2	1.4	2.1	0.8	62.9	17.4	651.0	183.8
F.nucleatum	20.8	5.9	10.4	2.9	31.7	9.0	651.0	183.8
P.intermedia	33.3	11.8	16.7	5.9	31.7	9.0	651.0	183.8
D.pneumosintes	41.7	11.8	10.4	2.9	31.7	9.0	325.8	92.3
Superinfectants								
E.coli	20.8	5.9	20.8	5.9	15.7	4.7	651.0	183.8
S.aureus	41.7	11.8	10.4	2.9	3.9	1.1	651.0	183.8
P.aeruginosa	20.8	5.9	5.2	1.4	15.7	4.7	1302.0	368.6

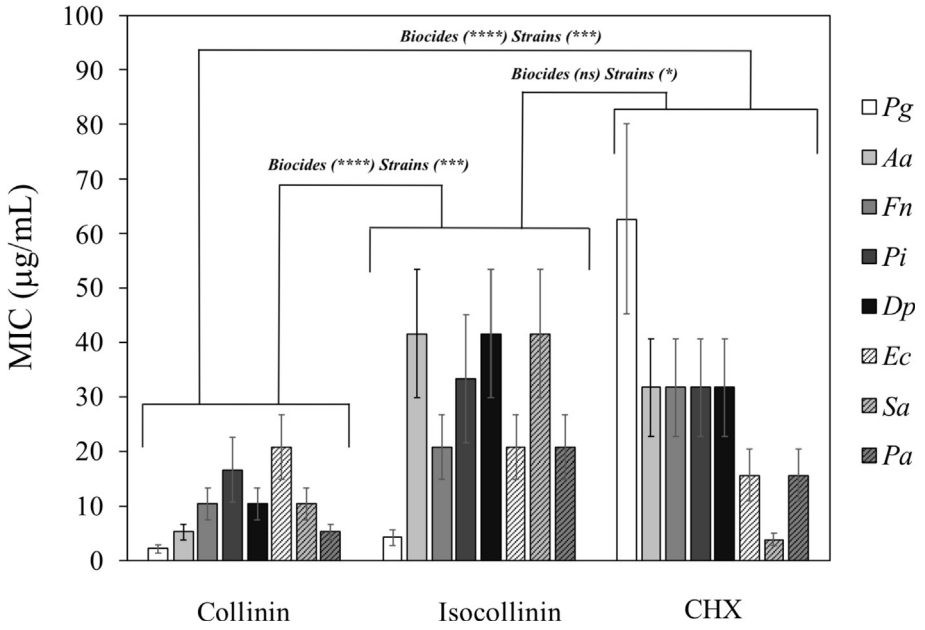

Fig. 2. Minimum Inhibitory Concentration (MIC) of collinin isomers and CHX against periodontopathic and superinfective strains, in a multivariable analysis. Bars represent the mean values and standard deviation of the MIC, obtained in the bacterial susceptibility assays, made by triplicate. *: $p < 0.1$, ***: $p < 0.001$, ns: not significant.

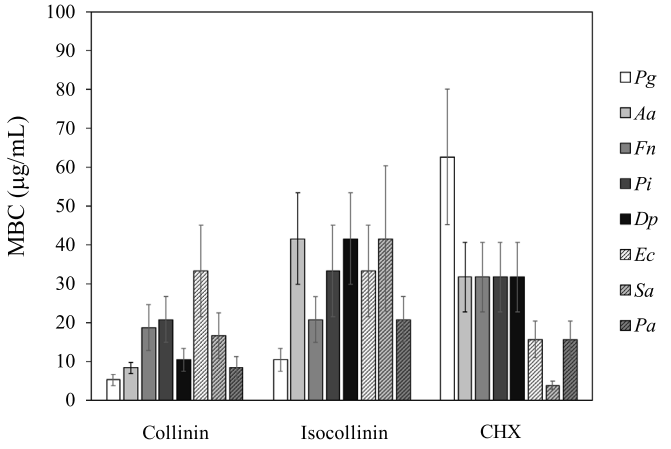

Fig. 3. Minimum Bactericidal Concentration (MBC) of collinin isomers and CHX against periodontopathic and superinfective strains. Bars represent the mean values and standard deviation of the MBC, obtained in the bacterial susceptibility assays and resarzurin assay, made by triplicate.

A. actinomycetemcomitans ATCC 29522, *P. gingivalis* ATCC 33277, *P. intermedia* ATCC 25611, *D. pneumosintes* ATCC 33048 and *F. nucleatum* ATCC 25586. Collinin isomers also presented high antimicrobial activity against superinfectant ATCC strains, particularly *P. aeruginosa*. These results are promising for developing further research directed to control oral and medical pathogenic biofilms, where microbes have shown increasing resistance against antimicrobials [3,6,7].

Caries and periodontal diseases affect a large proportion of the world population and are considered a public health problem [5]. In the case of periodontitis, the presence and overgrowth of pathogenic microorganisms are related to disease progression and disease aggravation [6,24,25]. A variety of active agents have been tested during the last 40 years to prevention, treatment and control of periodontitis.

The keystone pathogen *P. gingivalis* has been reported as the most frequent microorganism in patients with chronic and aggressive periodontitis, with a prevalence of approximately 72% in Colombia [26], and in Latin America, Missailidis et al. [27] affirm that on average this microorganism can be found in 89% of patients with periodontitis, 30% with gingivitis and 8% in periodontally healthy subjects. Other periodontal pathogens are also frequently reported in patients with periodontitis at diverse countries in Latin

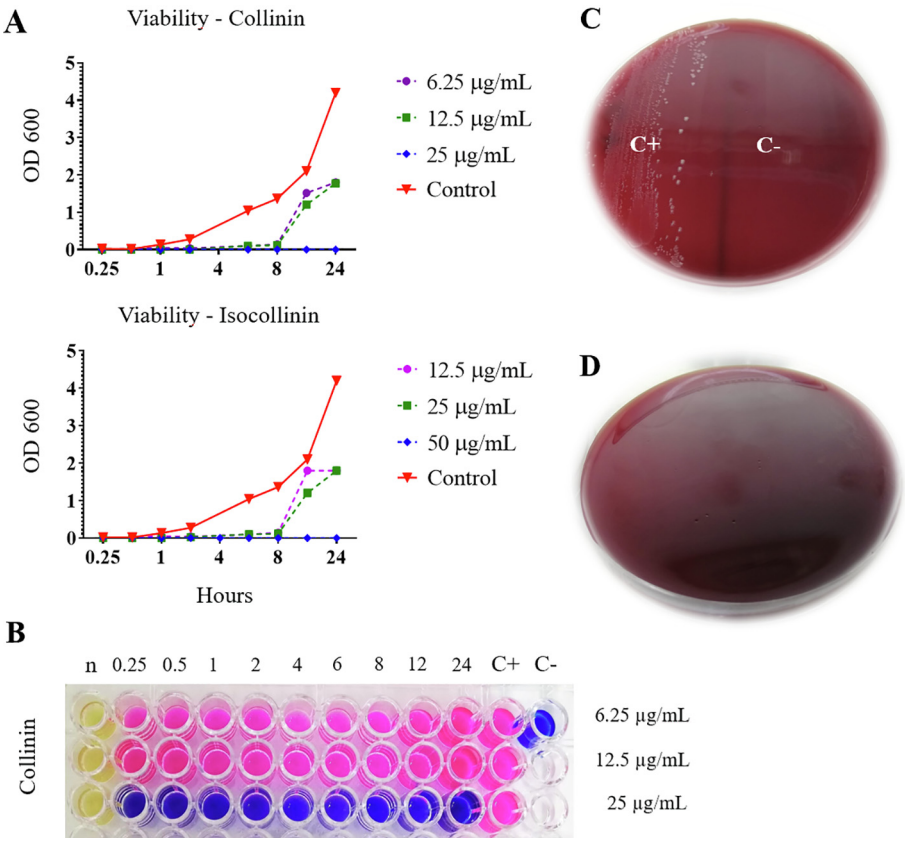

Fig. 4. Time-Kill and Cell Viability assays with 3 critical concentrations of Collinin and Isocollinin in *E. coli* ATCC 25922. A. Line graphs showing the change in optical density at 600 nm (OD 600 equivalent 1.5×10^8 CFU/ml) in the different time points of exposition in hours respect to control. B. Example of resarzurin assay with collinin to evaluate cell viability in different times, the pink dots represent the viable cells, the blue dots represent the non-viable cells, the positive control (C+) had no biocide, so the bacteria were viable, and the negative control (C-) had CHX at high concentration (0.12%), so the bacteria were not viable. C. Example of negative and positive controls used in the viability confirmation assay by blood agar culture. D. Example of MBC confirmation with blood agar culture, this result corresponds to the blue dots observed in the resarzurin assay (non-viable cell).

America [28]. In Chile for example, found *A. actinomycetemcomitans* (19.4%), *P. gingivalis* (83.4%) and superinfective bacteria (17.6%) in patients with chronic periodontitis [29]. In Argentina, *P. gingivalis, Treponema denticola, Tannerella forsythia* (red-complex) [30,31]. In Venezuela, *P. intermedia*, *P. gingivalis* are the most frequently recovered microorganism [32]. Finally, in Mexico, reported patients with periodontitis and rheumatoid arthritis, *P. intermedia* in 89% and *P. gingivalis* in 58% of the subjects [33].

This work showed that both collinin isomers have a remarkable antimicrobial effect against all periodontal pathogens tested, especially against *P. gingivalis*. Previous studies have shown that collinin significantly inhibits the growth of *P. gingivalis* at concentrations $\geq$12.5 µg/mL [13], whereas we find that even at lower concentrations $\geq$ 2.1 and $\geq$ 4.2 µg/mL of collinin and isocollinin, respectively, are enough to inhibit *P. gingivalis*, a result even better compared with the gold standard chlorhexidine (CHX), the antimicrobial used most frequently in the clinical practice. Differences on the MIC values for collinin of that reported by Santos et al., in 2013, can be due to a difference in the purity of the compound, which is not reported in the publication. Furthermore, the characterization of collinin performed by Santos et al., follow the work done by Curini et al. in Ref. [34]. As Pardo-Castaño et al. argued [21], Curini et al. probably obtained the position isomer isocollinin instead of collinin, which according to our study exhibited a higher MIC (4.2 µg/mL) than collinin (2.1 µg/mL).

Collinin and isocollinin also exhibited the capacity to reduce the growth of other important periodontal pathogens, such as *A. actinomycetemcomitans* and *F. nucleatum*, at concentrations between (5.2–41.7 µg/mL) and (10.4–20.8 µg/mL), respectively. Hence, the antimicrobial effect demonstrated herein by both collinin isomers against main periodontal pathogens, confirm and improve previous findings [13] that might result in alternatives for antimicrobial periodontal therapy.

We also observed that both molecules at the right concentrations are also able to kill the test microbes even up to 24 h after exposure. This suggests that the antimicrobial activity is persistent, a very useful feature to control recalcitrant oral biofilms that harbor in deep periodontal lesions where there is a greater density of pathogens [28].

On the other hand, superinfectants are considered transient colonizing pathogens that can impair oral health status in some patients. A multicenter study conducted in 5 cities of Colombia [26], showed that 38% periodontitis patients harbored superinfective bacteria. In this work, compared to CHX, collinin and isocollinin showed similar antimicrobial activity against the superinfecting strains. In addition, Betancourt and collaborators [35], also showed that 36% periodontitis patients harbored superinfectant bacteria, among them, *E. coli* and *P. aeruginosa* were the most frequent pathogens. These results suggest that pathogens, considered as superinfectants, are a problem in periodontitis patients mainly in Latin America and possibly other developing countries.

It was shown that both collinin isomers could have a broad spectrum of action against other microorganisms of medical importance that not only participate in periodontal disease, but also in important nosocomial and life-threatening infections. Collinin activity against resistant strains of *Mycobacterium tuberculosis*

has been reported [18] and a similar inhibition pattern for *S. aureus*, *E. coli* and *P. aeruginosa*, at concentrations >24 μg/mL, was found in this work.

Currently, the gold standard for the treatment of periodontal disease is scaling and root planing and some studies have suggested that better results are obtained if antibiotic therapy with amoxicillin + metronidazole is included [36]. However, the generalized increase in bacterial resistance against many antimicrobials suggests that new therapeutic alternatives to control pathogenic microorganisms are needed. Studies conducted in Colombia [37,38] found a decrease in the susceptibility of periodontal pathogens to amoxicillin and metronidazole; among them, *P. gingivalis* and *A. actinomycetemcomitans* were highlighted. This suggests that the development of new antimicrobial agents against these pathogens could be a good therapeutic alternative. Additionally, chlorhexidine gluconate is the antimicrobial agent most frequently used in clinical practice for its accepted effect [39]. However, the collinin isomers showed a greater antimicrobial effect, which suggests that studies with these compounds need to be expanded to determine their clinical application in medicine and dentistry.

5. Conclusions

Collinin and isocollinin demonstrated a remarkable antibacterial activity against relevant periodontal pathogens, such as *A. actinomycetemcomitans*, *F. nucleatum*, and especially against *P. gingivalis* (MIC of 2.1 and 4.2, μg/mL, respectively). Whereas chlorhexidine digluconate (CHX) and sodium hypochlorite (NaClO) showed MIC against *P. gingivalis* of 62.7 and 651 μg/mL, respectively. Both collinin isomers also presented antimicrobial activity against superinfectant strains, particularly *P. aeruginosa* (MIC 5.2 of collinin and MIC 20.8 μg/mL of isocollinin), while CHX 15.7 μg/mL to inhibit its growth.

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Declaration of competing interest

The authors declare no competing financial interest.

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