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The Chlorhexidine:β-Cyclodextrin Inclusion Compound: Preparation, Characterization and Microbiological Evaluation

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Abstract

The 1 : 2 chlorhexidine : β-cyclodextrin (Cx : βCD) complex was prepared and characterised using X-ray crystallography, infrared spectroscopy, thermal analysis and nuclear magnetic resonance. The minimum inhibitory concentration (MIC_{50}) of the chlorhexidine : β-cyclodextrin inclusion compound against *Streptococcus mutans, Eubacterium Lentum, Fusobacterium nucleatum, Bacteroides fragilis* and *Actinomices actinomycetemcomitans* was determined. The Cx : βCD inclusion compound inhibited the bacterial growth at a low concentration.

Introduction

Knowledge about the role of microorganisms in the initiation of gingivitis and periodontitis progression has increased dramatically following the recognition of bacterial plaque as the major etiological factor in chronic gingivitis. In addition, the participation of specific organisms in chronic periodontal diseases has been demonstrated [1–3]. Some antibacterial agents had received attention in periodontal therapy basically as adjuncts to the physical methods. The antimicrobial agents were used to inhibit either plaque formation or active plaque [4]. Different antibiotics are used locally or systematically and they have been assessed for their effects in the treatment of dental diseases. Unfortunately, the potential risk of the development of bacterial resistance, hypersensitivity or other specific side effects must be considered in routine clinical plaque control [5, 6]

Chlorhexidine (Figure 1) mouthrinse has a markedly better clinical antiplaque effect than other antiseptic agents tested clinically. A local delivery system with this antiseptic has shown a reduction in some putative periodontal pathogens [6]. However, this antiseptic has some adverse effects such as alteration of taste perception, increased staining of teeth and other oral surfaces, and an increase in calcified deposits, transient parotitis, minor irritation and reversible desquamation in young children [7, 8].

Cyclodextrins are cyclic linked oligosaccharides (α-1,4) of α-D-glucopyranose units arranged in a torus-like configuration, the "lining" of the internal cavity leading to the formation of inclusion complexes. β-Cyclodextrin contains 7 cyclic glucopyranose units [9]. The potential use of natural cyclodextrins and their synthetic derivatives has been studied to improve certain properties of drugs, such as solubility, stability and or bioavailability [10, 11].

We evaluated the characterization and antimicrobial activity of the new inclusion compound chlorhexidine : β-cyclodextrin: against *Actinobacillus actinomycetemcomitans, Streptococcus mutans, Eubacterium lentum, Fusobacterium nucleatum*, and *Bacteroides fragilis*.

Experimental

Material and methods

β-Cyclodextrin hydrate (βCD) and chlorhexidine hydrochloride (Cx) were purchased from Aldrich and used without further purification. The inclusion compound was prepared by the freeze-drying method from Cx and βCD aqueous solution in a 1 : 2 molar ratio. The physical mixture Cx : βCD was prepared by mixing together in a mortar at the same molar ratio and used as a comparison group. The drug content in the freeze-dried sample was determined by UV spectroscopy.

Physical measurements

The compounds and free agents were characterized by the following methods:

X-ray crystallography: the X-ray powder diffraction patterns of the samples were recorded on a Rigaku X-ray diffractometer. The samples were irradiated with monochromatized Cu K_α radiation and analyzed with 2θ angles

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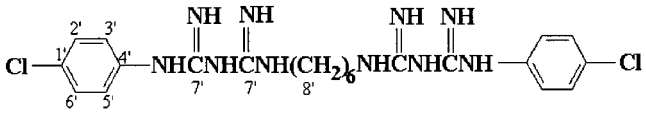

Figure 1. Chemical structure of chlorhexidine.

between 5 and 40°. The voltage, current, and time per step were 30 Kv, 5 mA, and 1 min, respectively.

Infrared (IR) spectra were obtained from KBr pellets in the 4000–500 cm^{-1} region and recorded on a Mattson FTIR-3000 spectrometer.

Ultraviolet spectra were recorded with a HP 8452A diode array spectrophotometer.

Thermogravimetric analysis (TG) curves were recorded on a Mettler TA 4000 thermogravimetric analyzer (TG 50) at a scan rate of 10 °C/min. The sample mass was 2 mg. Differential-scanning calorimetry curves (DSC) were recorded on a Shimadzu DSC-50. The sample mass was 3.5 mg in an aluminum pan with lid using a heating rate of 10 °C/min, under a nitrogen atmosphere.

Nuclear magnetic resonance NMR: ^{1}H-NMR and ^{13}C-NMR spectra of β-cyclodextrin, Cx and the Cx : βCD inclusion compounds were obtained on a Bruker NMR spectrometer at 400 MHz at room temperature, using DMSO-d_6 as solvent and TMS as internal reference.

Antimicrobial tests: the minimal inhibitory concentration (MIC) was determined using 27 isolates of *A. actinomycetemcomitans* recovered from 70 periodontitis subjects, and 7 reference strains: *A. actinomycetemcomitans* ATCC 29522, ATCC 29523 and FDCY, *S. mutans* ATCC 10556, *E. lentum* ATCC 25559, *F. nucleatum* ATCC 10953, and *B. fragilis* ATCC 233745. The MIC was determined by using the agar dilution method [12]. Fresh solutions of Cx : βCD (1 : 2), a physical mixture and βCD were prepared daily. Two fold serial dilutions of antimicrobial agents were prepared in brain heart infusion agar (BHI) supplemented with 0.5% yeast extract. The agent's concentrations ranged from 0.125 to 64 g/mL. Agar without any substance was used as control. After inoculation using a Steers replicator given a final inoculum of approximately 10^5 CFU/spot [13]. Plates inoculated with *S. mutans, A. actinomycetemcomitans, E. lentum, F. nucleatum* and *B. fragilis* were incubated in anaerobic conditions (90% N_2/10% CO_2), at 37 °C, for 48 hours. The MIC was defined as the lowest concentrations of antimicrobial agents capable of inhibiting macroscopic growth of the organism.

Results and discussion

Initially, the 1 : 2 stoichiometry of the Cx : βCD complex was suggested through ultraviolet visible spectroscopy at 260 nm.

X-ray Analysis: the diffraction patterns of chlorhexidine, β-cyclodextrin, the inclusion compound and physical mixture are shown in Figure 2. The XRD diffraction of pure β-cyclodextrin (A) and chlorhexidine (B) shows highly crystalline patterns. The inclusion compound XRD (D) shows

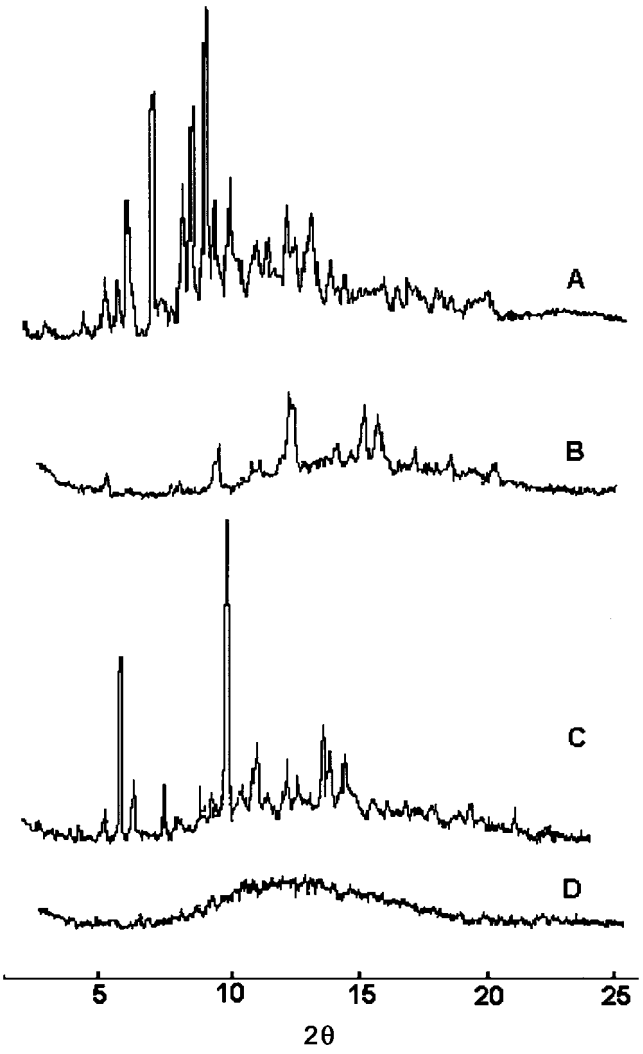

Figure 2. Powder X-Ray diffractograms: (A) β-cyclodextrin, (B) chlorhexidine, (C) physical mixture Cs : βCD and (D) inclusion compound Cx : βCD.

a more amorphous pattern when compared to the XRD of the free components and physical mixture. This suggests a disorder phenomenon upon inclusion. The physical mixture (C) has similar characteristics to the pure substances: β-cyclodextrin and chlorhexidine.

Infrared spectroscopy: the IR spectra of β-cyclodextrin, chlorhexidine, the physical mixture and inclusion compound are shown in Figure 3. Two peaks were observed in the chlorhexidine IR spectrum (B) in the 3400 cm^{-1} region that can be attributed to ν_{as} and ν_s − NH, respectively. This hydrogen bonding suggests the formation of highly associated chlorhexidine systems. On the other hand, bands were observed at 1650, 1600, 1550 and 1500 cm^{-1} that can be attributed to C=C stretching of the aromatic moiety of the chlorhexidine. The inclusion compound infrared spectrum (D) showed the sharpening of the IR bands at 3500–3300 cm^{-1} and 1100 cm^{-1}, OH and C—O—C, respectively, when compared to the same IR bands in the physical mixture and free chlorhexidine and β-cyclodextrin. These results confirm the breakdown of some hydrogen bonding upon inclusion. On the other hand,

Table 1. ^{1}H NMR chemical shifts, δ (ppm) of C—H protons in βCD, Cx, Cx : βCD and ($\Delta\delta$) of all protons in (DMSO-d_6), TMS

βCD	Cx	βCD δ	Cx δ	Cx-βCD $\Delta\delta$*	Cx-βCD $\Delta\delta$*
OH_2	OH_2, OH_6	5.73	7.71	−0.05[4]	+0.24[3]
OH_3	H_3, H_5	5.68	0.96	−0.05[1]	+0.21[3]
H_1	—CH_2	4.79	1.19	−0.017**	−0.05[2]
H_2	‖ —C—NH	3.31	10.14	−0.03	−0.31
H_3	‖ —C—NH	3.53	3.03	−0.05	0
H_4		3.29		−0.03	
H_5		3.51		−0.12	
H_6		3.33		−0.03	
—OH_6		4.52		−0.13	

* $\Delta\delta$ = (δ inclusion compound).
** Doublet after inclusion.
[1] Multiplet after inclusion.
[2] Broad peaks after inclusion.
[3] Multiplet after inclusion, new peaks appear.
[4] Deshielding (+); shielding (−).

lowering of the C=C stretching intensity of the aromatic moiety was observed when compared to the same modes in the physical mixture and free chlorhexidine. The host : guest interaction in the solid state was verified as the physical mixture spectrum (C) showed some mode modifications.

Thermogravimetric analysis: TG and DSC curves for the pure substances, the physical mixture, and the inclusion compounds are shown in Figures 4 and 5, respectively. The TG curve of chlorhexidine shows a high stability from 25 to 200 °C, after which a sequential thermal decomposition process was observed. The TG curve of the physical mixture and inclusion compound formed by freeze-drying (D) were very similar and showed a lower thermal stability when compared to Cx-di(HCl) and β-cyclodextrin. In the Cx : βCD inclusion compound the TG curve showed a 15% loss of mass between 25–90 °C. This is associated with to the release of water molecules from the βCD cavity. Moreover, a second mass loss in the range between 220–400 °C was observed, in this way changes in the thermal stability of the inclusion compound was verified when compared with βCD and free chlorhexidine. The Cx : βCD DSC curve shows abrogates of the chlorhexidine melting point peak at 139 °C when compared to the physical mixture and pure chlorhexidine. A new endothermic peak at 239 °C was also observed that could be associated with the new supramolecular compound formation. The physical mixture DSC is quite different from the inclusion compound DSC curve and free components DSC curves. This could suggest a host : guest interaction is present in the physical mixture.

Nuclear magnetic resonance

The results of ^{1}H and ^{13}C NMR of β-cyclodextrin, Cx and the Cx : βCD inclusion compound are shown in Tables 1 and 2, respectively. The ^{1}H NMR data clearly show a large change in the βCD and Cx spectrum after the drug inclusion.

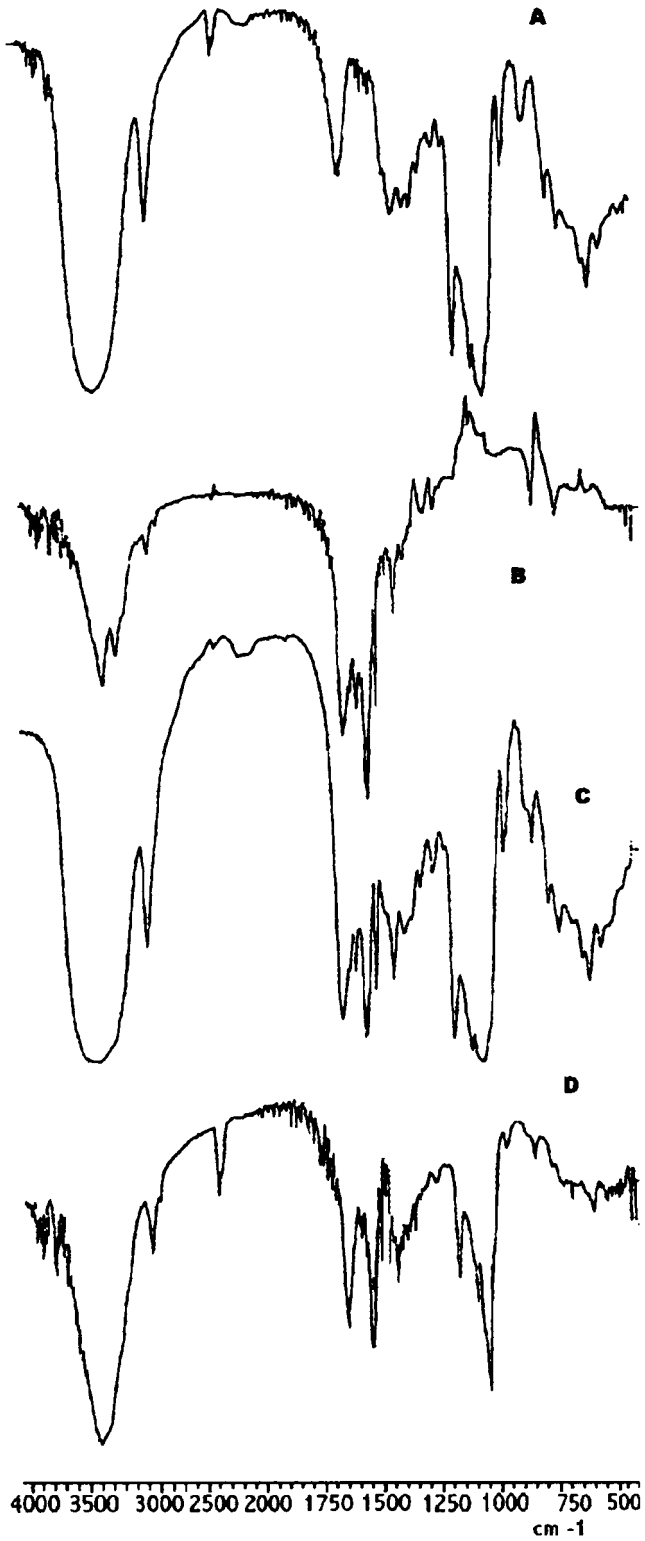

Figure 3. Infrared spectra of: (A) β-cyclodextrin, (B) chlorhexidine, (C) physical mixture Cx : βCD and (D) inclusion compound Cx : βCD.

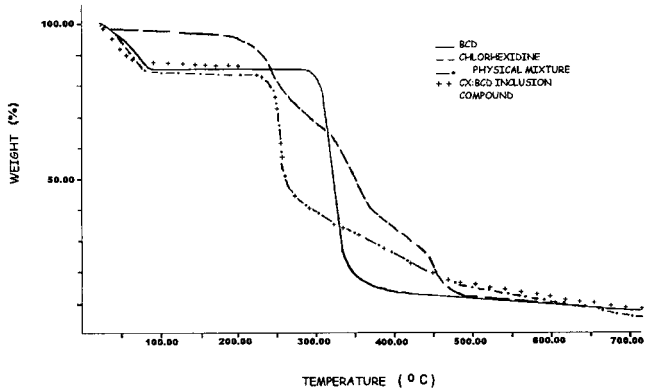

Figure 4. TG curves of: (—) β-cyclodextrin, (- - -) chlorhexidine, (— ● —) physical mixture (++) and inclusion compound Cx : βCD.

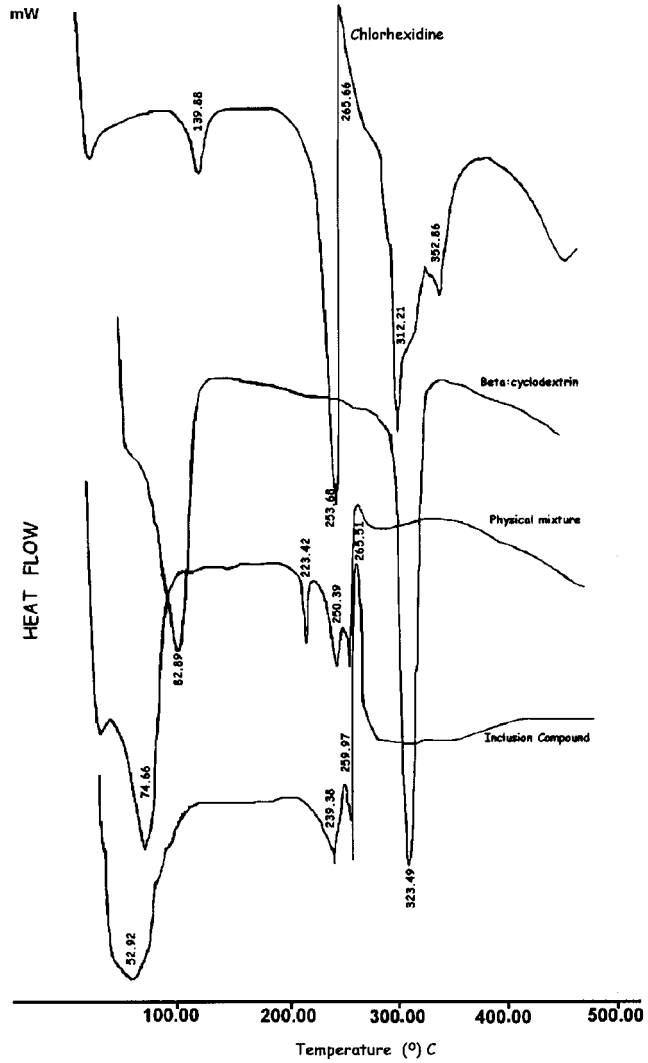

Figure 5. DSC curves of: β-cyclodextrin; chlorhexidine; physical mixture, and inclusion compound Cx : βCD.

Table 2. ^{13}C NMR chemical shifts, δ (ppm) of βCD, Cx, Cx : βCD and ($\Delta\delta$) of carbons in (DMSO-D_6), TMS

Carbon		βCD	Cx	Cx-βCD	Cx-βCD
βCD	Cx	δ	δ	$\Delta\delta^2$	$\Delta\delta^2$
1	1′	102.15	126.64	−0.22*	+0.144
2	2′, 6′	72.26	128.33	−0.21	+0.10[1]
3	3′, 5′	73.29	121.75	−0.23	+0.35[1]
4		81.77	1.37	−0.20	*
5		72.60	160.01	−0.15	
6		60.22	25.78	−0.25	

* The peak was not verified.
[1] Shielding.
[2] $\Delta\delta$ = inclusion compound.

Table 3. Range and minimum inhibitory concentration MIC and MIC_{50} values μg/mL of: chlorhexidine (Cx), physical mixture Cx : βCD (1 : 2), chlorhexidine : β-cyclodextrin (Cx : βCD) and β-cyclodextrin

Chemical compounds	Range MIC	MIC_{50}
Chlorhexidine(Cx-di(HCL))	<0.125-8	0.5
Cx : βCD physical mixture	<0.124-4	0.5
Cx : βCD inclusion compound	<0.125-4	0.25
β-cyclodextrin	<0.125–>64	0.5
Brain heart infusion agar	–	–

Initially a multiplet is observed at 5.66 ppm in the ^{1}H NMR spectrum of the Cx : βCD. This result could be explained as a breakdown of the flip-flop hydrogen bonding between OH_2—OH_3 of β-cyclodextrin after inclusion of Cx. Moreover, it is possible to verify the appearance of a doublet and triplet at 4.76 ppm and 4.44 ppm, associated with H_1 and the —OH_6, respectively. It is interesting however to observe a $\Delta\delta$ magnitude of −0.03 ppm and −0.12 of the H_3 and H_5, positions as also observed in reference 14. This may suggest a shielding effect from aromatic π electrons and therefore the presence of the aromatic moiety of the Cx molecule in the β-cyclodextrin cavity. In addition, the drastic changes in the ^{1}H NMR resonance profile corroborates this inclusion. Clearly one observes these changes in the aromatic protons H_2, H_6, H_3 and H_5 (Figure 1) of the phenyl moiety at 7.71 and 6.96 ppm of the Cx upon inclusion. In fact the multiplet peak is observed when compared to the same ^{1}H NMR protons of the free Cx but also the deshielding of these protons H_2, H_6, H_3 and H_5. Finally, one observes a $\Delta\delta$ of 0.31 ppm for the NH protons of Cx shielding effect after inclusion and this may suggest the formation of new hydrogen bonding of the drug with βCD.

The ^{13}C NMR spectra of βCD, Cx and the Cx : βCD and the $\Delta\delta$ magnitude allow verification of the following observations: first, a $\Delta\delta$ of 0.10–0.35 ppm for the aromatic carbons of Cx. These results are analogous to those observed in the ^{1}H NMR spectra. Interesting, however, there are deshielding effects from βCD upon inclusion in the cavity. Second, a $\Delta\delta$ from −0.15 to −0.25 ppm was observed for the shielding phenomena to all cyclodextrin carbons from

Table 4. MIC values (μg/mL) of the reference and periodontitis subjects strains tested against chlorhexidine, physical mixture (PM), chlorhexidine:β-cyclodextrin (Cx:βCD), and β-cyclodextrin (βCD)

Bacteria	Strains	Minimum inhibitory concentration μg/mL			
		Cx	PM	(Cx : βCD)	βCD
S. mutans	ATCC 10556	4	1	1	>64
E. lentum	ATCC 25559	8	0.5	4	>64
F. nucleatum	ATCC 10953	8	0.5	4	>64
B. fragilis	ATCC 23745	<0.125	<0.125	<0.125	<0.125
A. actinomycetemcomitans	ATCC 29522	4	2	2	>64
A. actinomycetemcomitans	Periodontitis subjects	0.5–4	0.5–4	0.25–2	0.25–>64
Brain heart infusion agar (BHI)		–	–	–	–

the phenyl moiety of Cx. These results are in accord with a previous study [14].

Microbiological study

The minimum inhibitory concentration MIC_{50} range of chlorhexidine, β-cyclodextrin, the inclusion compound and the physical mixture are presented in Table 3. The physical mixture and Cx : βCD MIC range was the lowest from <0.125 to 4 μg/mL when compared with βCD < 0.125–> 64 μg/mL and chlorhexidine < 0.125-8 μg/mL. The MIC_{50} of the inclusion compound determinated against *S. mutans, E. Lentum, F. nucleatum, B. fragilis* and *A. actinomycetemcomitans* showed higher efficacy of the Cx : βCD complex, that is two fold greater (0.25 μg/mL) than free chlorhexidine (0.5 μg/mL) and the physical mixture (0.5 μg/mL). In addition, UV measurements showed the fraction of chlorhexidine in the inclusion compound was 50% less due to the molar ratio 1 : 2, in this way the concentration was four fold less than pure chlorhexidine.

Table 4 shows the MIC values for *S. mutans, E. lentum, F. nucleatum, B. fragilis* and *A. actinomycetemcomitans. E. Lentum* and *F. nucleatum* show higher sensitivity towards the physical mixture (0.5 μg/mL) when compared to Cx : βCD (4 μg/mL). It was observed that the physical mixture and inclusion compound MIC values toward *S. mutans* were similar (1 μg/mL); *B. Fragilis* was susceptible to all tested substances (<0.125 μg/mL) in the same form. The *A. actinomycetemcomitans* reference strains (ATCC 29522) were susceptible to the inclusion compounds and physical mixture (Table 4). Finally, the *A. actinomycetemcomitans* strains isolated from periodontitis subject were more susceptible to the inclusion compound (0.25–2 μg/mL) when compared to the physical mixture and chlorhexidine (0.5–4 μg/mL).

The chlorhexidine : β-cyclodextrin MIC_{50} in this work was lower when compared to the MIC_{10} determined against Gram-positive (2.0 μg/mL) and against Gram-negative bacteria (0.5 μg/mL) found by other authors [15]. The bacteria group tested in the present study is very sensitive, especially to the inclusion compound. Thus, treatments with chlorhexidine formulations with β-cyclodextrin could reduce their numbers significantly. This is an important point, especially in high-risk individuals to dental caries and periodontal disease. The β-cyclodextrin presence could explain the Cx : βCD antimicrobial activity for two reasons: first, it has adherence to the cell wall due to the OH groups and therefore increases the compound inhibition degree and, second because the β-cyclodextrin is retarding the chlorhexidine delivery [16].

The low solubility of chlorhexidine hydrochloride can be the reason to limit its utilization in mouthrinse preparation [17]. At present, there are no data regarding the efficacy of chlorhexidine : β-cyclodextrin inclusion compounds as antiseptic agents. An inhibitor may be present at a relatively high concentration and delivery itself at sublethal levels and therefore it could interfere with bacterial metabolism [18]. Consequently, it would seem that substantially more evidence is required, especially about the minimum concentrations of the inclusion compound on the biofilm growth. This study verified the formation of the new supramolecular compound chlorhexidine : β-cyclodextrin and the modification of its antibacterial activity in vitro increasing its efficacy with low concentrations against pathogenic bacteria.

Acknowledgments

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