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Original Article

Bactericidal and anti-biofilm properties against *Streptococcus mutans* and *Streptococcus sobrinus* of rhinacanthin-C isolated from *Rhinacanthus nasutus*

Sirinthip Klaophimai[1], Phisit Pouyfung[2], Arthit Klaophimai[3*], and Kongthawat Chairatvit[1*]

[1] *Department of Oral Biology, Faculty of Dentistry, Mahidol University, Ratchathewi, Bangkok, 10400 Thailand*

[2] *Department of Occupational Health and Safety, School of Public Health, Walailak University, Tha Sala, Nakhon Si Thammarat, 80160 Thailand*

[3] *Department of Oral Microbiology, Faculty of Dentistry, Mahidol University, Ratchathewi, Bangkok, 10400 Thailand*

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Abstract

Although chlorhexidine (CHX) is commonly used as a standard antiseptic treatment in the oral cavity, its potent cytotoxicity on human periodontal tissues has been reported. Natural anti-biofilm medications could be therefore used as alternatives. In this study, the anti-bacterial and anti-biofilm activities of rhinacanthin-C (Rh-C) isolated from *Rhinacanthus nasutus* were determined against *Streptococcus* spp. (*S. mutans* and *S. sobrinus*), responsible for dental caries or tooth decay. Rh-C showed strong inhibitory and bactericidal effects against both tested species. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for *S. mutans* were 4 and 8 µM, respectively, and the MIC and MBC for *S. sobrinus* were 2 and 4 µM, respectively. As expected, 0.12 % (w/v) CHX, being a concentration present in commercial mouth rinses, showed a complete inhibition on biofilm formation and a decrease in the bacterial biomass in the formed biofilms, shown by biofilm formation assay and confocal laser scanning microscopy, respectively. Likewise, Rh-C showed concentration dependent (0-32 µM) anti-adherent property of both streptococci on biofilm formation and a decrease in the bacterial biomass in the formed biofilms. Interestingly, the effective anti-biofilm concentrations of Rh-C did not have cytotoxic effects on human gingival fibroblast (HGF) cell viability, whereas incubation with 0.12 % (w/v) CHX decreased HGF cell viability to approximately 25 %. The results suggest the potential use of natural-based Rh-C (up to 32 µM) as an effective anti-bacterial and anti-biofilm agent in oral topical rinses or toothpastes for prevention of caries.

Keywords: bacterial biofilm, dental caries, natural-based anti-bacterial, natural-based anti-biofilm agent

1. Introduction

Dental caries known as tooth decay is a serious public health problem worldwide, especially in children and elderly. Dental caries is defined as the localized destruction of susceptible dental hard tissues by acid production from bacterial fermentation of dietary carbohydrates (Selwitz, Ismail, & Pitts, 2007). The disease process is initiated by the formation of bacterial biofilm or dental plaque on a tooth surface. Biofilm formation can be described in three sequential stages: attachment of planktonic bacterial cells to a surface, maturation of 3-dimensional structure of biofilm, and dispersion or a release of planktonic bacteria from the biofilm

*Corresponding author
Email address: arthit.kla@mahidol.ac.th;
kongthawat.cha@mahidol.ac.th

to promote new biofilm formation at other sites (Rabin *et al*., 2015). Due to the complex 3-dimensional structure, bacteria in the biofilms become more resistant to anti-microbial treatment compared to planktonic bacteria (Krzyściak, Jurczak, Kościelniak, Bystrowska, & Skalniak, 2014).

Regarding the bacterial varieties in dental biofilms, the high acid-producing and acid tolerant *Streptococcus* spp. (*S. mutans* and *S. sobrinus*) in dental biofilms are responsible for the initiation of dental caries (Sbordone & Bortolaia, 2003). In general, chlorhexidine (CHX) is used as a standard antiseptic treatment in the oral cavity due to its effective bactericidal activity (Salem, Adams, Newman, & Rawle, 1987). CHX containing mouthwash formulations are therefore the general standard for preventing plaque formation and development of caries. However, potent cytotoxicity and changes in morphology of human periodontal tissues *e.g.* human gingival fibroblasts have been reported after exposure to CHX (Wyganowska-Swiatkowska *et al*., 2016). The effective concentrations of CHX in mouthwash formulations are 0.12% (w/v) or 0.2% (w/v); however, the lower concentration is recommended to minimize the side effects on fibroblasts (Najafi *et al*., 2012).

The use of natural plant-based anti-bacterial and anti-biofilm agents has notably increased due to their effectiveness in anti-bacterial function and low cytotoxicity against normal cells. *Rhinacanthus nasutus* KURZ. is an annual herb that is found commonly in Southeast Asia including Thailand (Figure 1a). Roots and leaves of *R. nasutus* have been used in Thai traditional medicine for treating diseases, such as skin disease. The active ingredients in the extract of *R. nasutus* possess several biological activities including anti-bacterial, anti-viral, and anti-proliferative activities (Gotoh *et al*., 2004; Ngoc *et al*., 2019; Puttarak, Charoonratana, & Panichayupakaranant, 2010). The main bioactive components of the plant are naphthoquinone compounds that exhibit anti-bacterial (Ravichandiran, Sheet, Premnath, Kim, & Yoo, 2019), anti-viral (Ngoc *et al*., 2019), anti-tumor (Gotoh *et al*., 2004), anti-allergenic (Tewtrakul, Tansakul, & Panichayupakaranant, 2009), and anti-inflammatory activities (Punturee, Wild, & Vinitketkumneun, 2004).

Rhinacanthin-C (Rh-C) (Figure 1b) is classified as a naphthoquinone ester that is found mainly in *R. nasutus* (Phisit Pouyfung, Prasopthum, Sarapusit, Srisook, & Rongnoparut, 2014). Rh-C has been shown to inhibit human topoisomerase II activity by binding to the ATP-binding pocket of the enzyme (Boonyalai, Sittikul, Pradidphol, & Kongkathip, 2013; Klaophimai, Pouyfung, Wongnoppavich, & Chairatvit, 2023). Rh-C is a strong anti-bacterial agent especially against Gram-positive bacteria (Puttarak *et al*., 2010). However, the anti-growth and anti-biofilm properties of Rh-C have not been studied, especially against *S. mutans* and *S. sobrinus* that are the major causes of dental caries.

In this study, the anti-growth and anti-biofilm activities of Rh-C isolated from *R. nasutus* were determined against *S. mutans* and *S. sobrinus*. Rh-C possesses bactericidal and anti-biofilm properties against *S. mutans* and *S. sobrinus* without cytotoxic effects on human gingival fibroblast (HGF) cell viability. The results reported herein support the use of Rh-C as an effective anti-bacterial and anti-biofilm agent in oral topical rinses or toothpastes for prevention of caries.

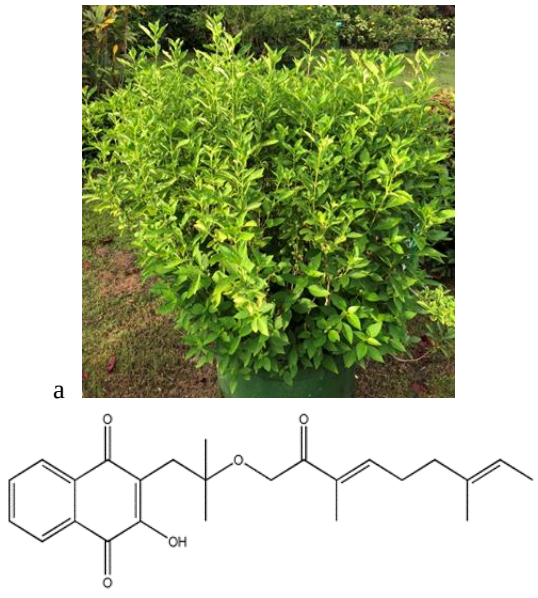

Figure 1. *Rhinacanthus nasutus* KURZ. (a) and chemical structure of rhinacanthin-C (Rh-C), MW = 410.5027 (b)

2. Materials and Methods

2.1 Materials

Streptococcus mutans (ATCC 25175) and *Streptococcus sobrinus* (ATCC 33478) were obtained from the American Type Culture Collection (ATCC), USA. Bacterial cultures were maintained in Nutrient Agar (NA) plates (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C and brain heart infusion (BHI) broth (Becton Dickinson, East Rutherford, NJ, USA) was used as culture medium in all assays. Chlorhexidine (CHX) gluconate was purchased from Polipharm (Samut Prakan, Thailand). Rh-C isolated from *R. nasutus* was provided by Dr. Phisit Pouyfung, Walailak University, (Phisit Pouyfung *et al*., 2014). The purity of isolated Rh-C was ≥ 95 %. Rh-C was dissolved and stored in dimethyl sulfoxide (DMSO).

2.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

MIC was determined using 2-fold serial broth dilution standard method based on the reference protocol of the CLSI (2017) Performance standards for antimicrobial susceptibility testing, 27th ed. Supplement M100, Wayne, PA: Clinical and Laboratory Standards Institute. Briefly, 100 µl of tested bacterial cultures (10^6 CFU/mL) was added to each well of a 96-well plate in the presence of various concentrations of Rh-C ranging from 0 to 128 µM diluted in BHI broth with the total final volume of 200 µl. The addition of the solvent (DMSO) was used as a control (0 µM). After 24 h incubation at 37 °C and 5 % CO_2, MIC was determined by observing the lowest concentration of Rh-C caused an end to visible growth. For MBC, 25 µl of mix bacterial culture with different Rh-C concentrations were transferred to BHI agar and incubated at

37 °C with 5 % CO_2 for 24 h. MBC was determined as the lowest concentration of Rh-C that kills 99.9 % of the bacterial population (leaving less than 5 colonies/plate). CHX was used as a positive control.

2.3 Effect of Rh-C on *S. mutans* and *S. sobrinus* adherence to saliva-coated plate assay

S. mutans or *S. sobrinus* (10^6 CFU/mL) cultured in 1 % sucrose BHI were incubated with various concentrations of Rh-C (0-32 μM) or 0.12 % w/v CHX in a 96-well plate pre-coated with 10 % human saliva in PBS (pH 7.2). The addition of the solvent (DMSO) was used as the control experiment (0 μM). After 24 h incubation at 37 °C and 5 % CO_2, viability of adherent cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA) as previously reported (He et al., 2012). The formazan product was measured using a microplate reader at 570 nm (Biotek instrument, USA).

2.4 Effect of Rh-C on bacterial biomass in biofilm

Determination of the bacterial biomass in biofilm was carried out using confocal laser scanning microscope (CLSM) analysis as previously described (He *et al*., 2012). *S. mutans* or *S. sobrinus* (10^8 CFU/mL) in 1 % sucrose BHI were cultured on sterile glass slides (13 mm diameter) pre-coated with 10 % human saliva. After 24 h incubation at 37 °C and 5 % CO_2, the glass slides were washed with PBS and then incubated with different concentrations of Rh-C (0-32 μM) or 0.12 % w/v CHX. The solvent (DMSO) was used as the negative control (0 μM). After 24 h of incubation, bacterial biofilm on the glass slides was stained with a mix of 10 μM SYTO™ green fluorescent nucleic acid stain and 60 μM propidium iodide (PI) (Thermo Fisher Scientific, Invitrogen, Waltham, MA, USA) for 15 min. The glass slides were then subjected to CLSM (Leica Microsystems, Switzerland). Optical images of 512 x 512 pixels were analyzed by COMSTAT 2 program (Heydorn *et al*., 2000).

2.5 Effect of Rh-C on HGF cell toxicity and cell viability

Human gingival fibroblast (HGF) cells (ATCC, PCS-201-018™) were grown in Dulbecco's modified Eagle's medium (DMEM) (Thermo Scientific Hyclone, Rockford, IL, USA), supplemented with 10 % (v/v) fetal bovine serum (Thermo Scientific Hyclone), and 1xAntibiotic-Antimycotic (Thermo Scientific Hyclone) at 37 °C and 5 % CO_2. Approximately 10^6 HGF cells were plated in each well of a 6-well culture plate. After 24 h, cells were incubated with indicated concentrations of Rh-C (0 to 40 μM) or 0.12 % (w/v) CHX. The solvent (DMSO) was used as the negative control (0 μM). Morphology of cells was examined under a light microscope after 15, 30, and 45 min incubation with Rh-C. For cell viability assay, approximately 7,500 cells were plated in each well of a 96-well culture plate. After 24 h, cells were treated with indicated concentrations of Rh-C (0 to 40 μM) or 0.12 % (w/v) CHX for 24 h. Total cell protein representing the cell growth and cell viability was subsequently determined by sulforhodamine B (SRB) colorimetric assay as previously described (P. Pouyfung, Choonate, Wongnoppavich, Rongnoparut, & Chairatvit, 2019).

2.6 Statistical analysis

All experiments were performed in triplicate. Differences in the mean values among the groups were determined by applying one-way analysis of variance using SPSS 18.0 software (SPSS Inc., Chicago, IL). Data are expressed as mean ± S.D. with p-value < 0.05 considered significant.

3. Results

3.1 Anti-microbial effect of Rh-C on *S. mutans* and *S. sobrinus*

Bacterial cultures were incubated with various concentrations of Rh-C ranging from 0-128 μM for 24 h and the MIC and MBC were determined. As a control, CHX effectively inhibited and killed all tested bacteria with comparable MIC and MBC values (Table 1). However, Rh-C showed strong inhibitory and bactericidal effects against both *S. mutans* and *S. sobrinus* (Table 1). The MIC and MBC for *S. mutans* were 4 and 8 μM, respectively, and the MIC and MBC for *S. sobrinus* were 2 and 4 μM, respectively (Table 1). The results indicate that Rh-C possesses anti-microbial activity against *S. mutans* and *S. sobrinus*.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Rh-C against *Streptococcus mutans* and *Streptococcus sobrinus*

Streptococcus spp.	CHX		Rh-C	
	MIC (% w/v)	MBC (% w/v)	MIC (μM)	MBC (μM)
S. mutans	0.0019	0.0019	4	8
S. sobrinus	0.0025	0.0025	2	4

3.2 Anti-adherent property of Rh-C on biofilm formation

Both *S. mutans* and *S. sobrinus* have been implicated as the primary causes of dental caries through an adherent property of the bacteria, enabling them to attach to the tooth surface and form biofilms known as dental plaque (Hamada & Slade, 1980). To test the Rh-C for an inhibitory effect on biofilm formation by both bacteria, biofilm formation assay was performed. Culture of bacteria was incubated with increasing concentrations of Rh-C (0, 1, 2, 4, 8, and 16 μM) in 96-well plate pre-coated with human saliva. After 24 h, viable bacterial adherent cells were determined using MTT assay. As a control, 0.12 % (w/v) CHX, this being a concentration present in commercial mouth rinses, almost completely inhibited bacterial cell adherence shown by the percentage of bacterial cell viability on saliva coated plates (Figure 2a and 2b). Incubation with Rh-C at 4, 8, and 16 μM significantly reduced the cell viability on saliva coated plates for bacterial cells of both species. Notably, the observed

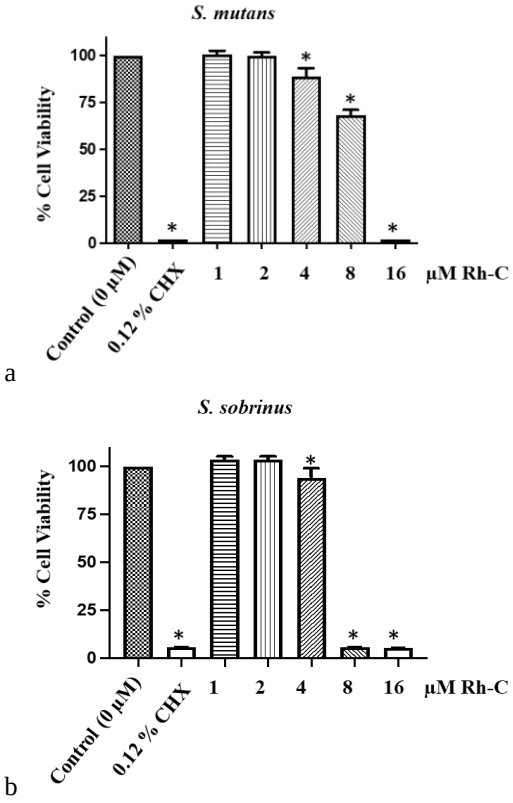

Figure 2. The anti-adherent effect of Rh-C on *S. mutans* and *S. sobrinus* biofilm formation. Culture of bacteria was incubated with indicated concentrations of Rh-C or 0.12% (w/v) CHX in 96-well plate pre-coated with human saliva. After 24 h incubation, viable *S. mutans* (a) or *S. sobrinus* (b) adherent cells were determined using MTT assay. Results are expressed as means from three separate experiments. Bar = SD, * $p < 0.05$ relative to the control without treatment.

effects of 16 µM Rh-C on *S. mutans* and of 8 and 16 µM Rh-C on *S. sobrinus* were comparable to the effect of 0.12 % (w/v) CHX. The results indicate that Rh-C could inhibit attachment of both *S. mutans* and *S. sobrinus* for biofilm formation.

3.3 Decreasing *S. mutans* and *S. sobrinus* biomass in the biofilms by Rh-C

To further study the anti-biofilm activity of Rh-C, the amounts of live and dead bacteria in the biofilms were determined by fluorescent dyes with CLSM. The biofilms of *S. mutans* or *S. sobrinus* were pre-formed on glass slides and then incubated with indicated concentrations of Rh-C (0, 8, 16, and 32 µM) for 24 h. The biofilms were stained with SYTO^{TM} (green) and PI (red) to stain live and dead bacteria, respectively, before subjecting to CLSM. Visualization of the biofilm structure in the control (without treatment) showed the majority of live cells and only a few dispersed dead bacterial cells (Figure 3a and 3b). As a positive control, incubation of the *S. mutans* (Figure 3a) or *S. sobrinus* (Figure 3b) biofilm with 0.12 % (w/v) CHX showed a decrease in live cell intensity (green) concomitantly with an increase in dead cell intensity (red). Likewise, incubation with Rh-C decreased the number of live cells (green) and increased the number of dead cells (red) in a dose-dependent manner for both *S. mutans* (Figure 3a) and *S. sobrinus* (Figure 3b). The overlaid green (live) and red (dead) signals further confirmed the higher ratio of dead/live bacterial cells in the biofilms when the cultures were incubated with increasing concentrations of Rh-C (Figure 3a and 3b). The calculated live and dead biomass in the biofilms by COMSTAT quantitative analysis of confocal images further indicated a decreased *S. mutans* (Figure 3c) and *S. sobrinus* (Figure 3d) biomass in the biofilms in a Rh-C dose dependent fashion.

3.4 Low cytotoxicity of Rh-C against human gingival fibroblast (HGF) cells

We next determined the cytotoxic effects of Rh-C on human gingival fibroblast (HGF) cells. First, HGF cells were treated with increasing concentrations of Rh-C up to 40 µM. After 15, 30, and 45 min incubation, morphology of HGF cells was observed under light microscope. As expected, the shrinkage of HGF cells was first observed after incubation with 0.12 % (w/v) CHX for 15 min (Figure 4a). However, no significant difference in morphology of HGF cells was observed between the untreated controls and the Rh-C treated groups even when the concentration of Rh-C was up to 40 µM at any tested time point (Figure 4a). The results indicate that the effective anti-biofilm concentrations of Rh-C did not affect HGF cell morphology.

To further confirm the safety of Rh-C, HGF cell viability was determined after incubation with increasing concentrations of Rh-C for 24 h using SRB assay. As shown in Figure 4b, incubation with 0.12 % (w/v) CHX decreased HGF cell viability to approximately 25 %. In contrast, all tested concentrations of Rh-C had almost no effect on HGF cell viability (Figure 4b). The results further confirm the safety of Rh-C to HGF, even when the cells are treated with the compound for 24 h.

4. Discussion

Biofilms are an oral health problem since they are associated with oral diseases including the development of dental caries. Certain bacterial varieties in the oral biofilms, especially high acid-producing and acid tolerant *Streptococcus* spp. (*S. mutans* and *S. sobrinus*) in dental biofilms are responsible for the initiation of dental caries (Sbordone & Bortolaia, 2003). In this study, we characterized the anti-bacterial and anti-biofilm properties of Rh-C isolated from *R. nasutus* on *S. mutans* and *S. sobrinus*. Rh-C showed a strong anti-growth effect on both planktonic *S. mutans* and *S. sobrinus* with MIC and MBC of 4 and 8 µM, respectively, for *S. mutans* and 2 and 4 µM, respectively, for *S. sobrinus*. The present study supports a previous report on the potent bactericidal activity of the *R. nasutus* crude extract against several species of Gram-positive bacteria including *S. mutans* (Puttarak *et al.*, 2010). In fact, the anti-microbial activity of Rh-C was also tested against Gram-negative *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* which are responsible for periodontal diseases. However, Rh-C was not active against these tested Gram-negative bacteria even at 128 µM concentration (data not shown) probably due to the distinctive envelope structure making Gram-negative bacteria

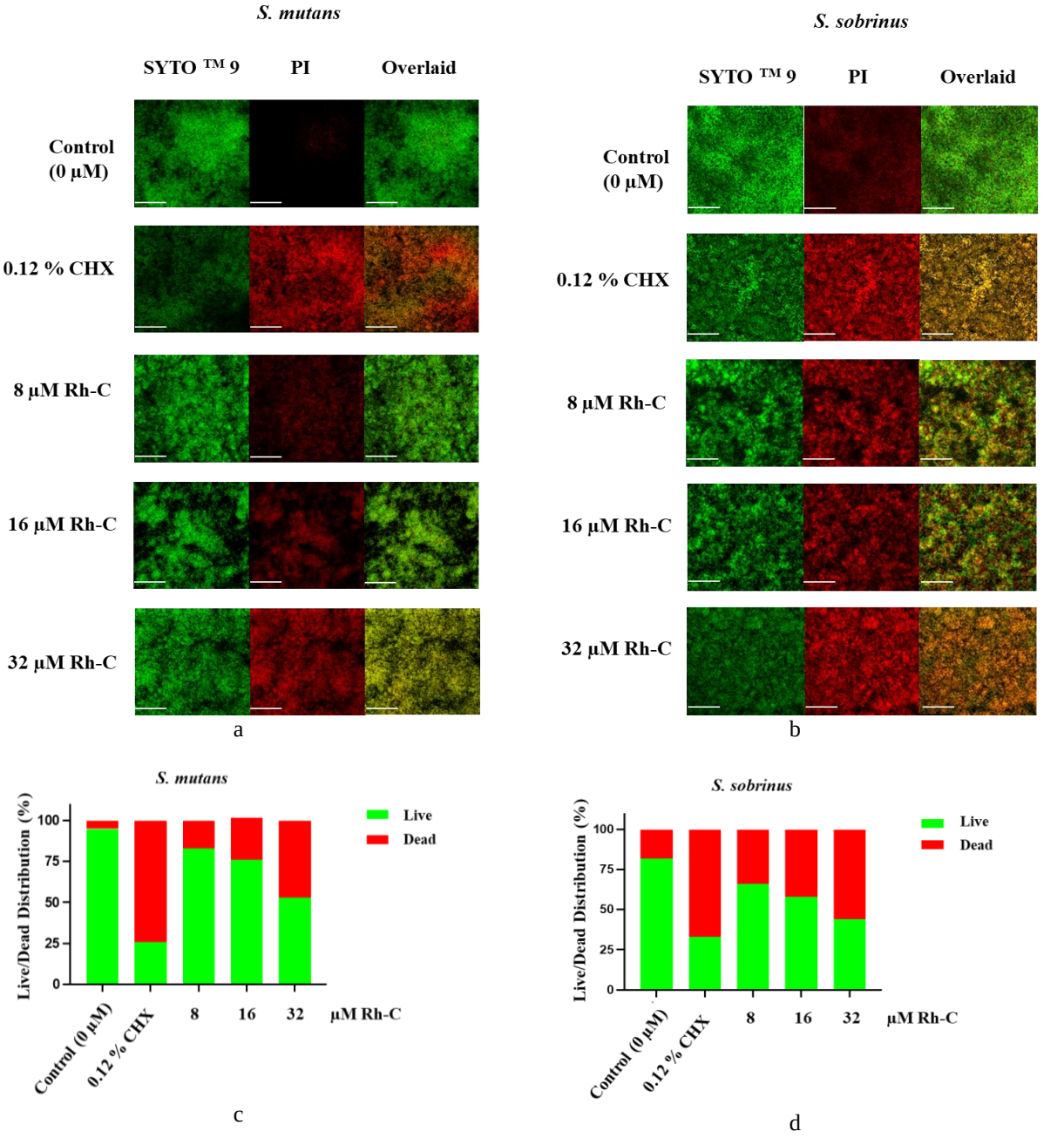

Figure 3. A decrease in the *S. mutans* and *S. sobrinus* biomass in the biofilms induced by Rh-C. The bacterial biofilm was pre-formed on glass slides and then incubated with indicated concentrations of Rh-C or 0.12% (w/v) CHX for 24 h. The *S. mutans* (a) and *S. sobrinus* (b) biofilms were stained with SYTO™ (green) and PI (red) to stain live and dead bacteria, respectively before subjecting to confocal laser scanning microscopy. Results shown are representative pictures from three separate experiments. Bar = 50 µm. Live and dead biomass in the *S. mutans* (c) and *S. sobrinus* (d) biofilms were calculated using COMSTAT for quantitative analysis of the obtained confocal images.

more resistant to antibiotic treatments than Gram-positive bacteria (Breijyeh, Jubeh, & Karaman, 2020). Nevertheless, Rh-C isolated from *R. nasutus* is a promising bactericidal compound against *S. mutans* and *S. sobrinus*.

The formation of biofilms is a complicated process comprising different stages which could be targeted to prevent biofilm development by natural anti-biofilm agents. Biofilms are initially formed by attachment of planktonic bacteria to form protective three-dimensional structures in which the bacterial cells become adaptively resistant to antibiotics and other disinfectants, more so than their planktonic cell counterparts (Krzyściak *et al*., 2014). The ideal strategy for inhibition of biofilm formation is targeting the first stage of biofilm development (Mishra *et al*., 2020). For the first time, our findings have demonstrated that Rh-C could inhibit attachment of both *S. mutans* and *S. sobrinus* to a saliva-

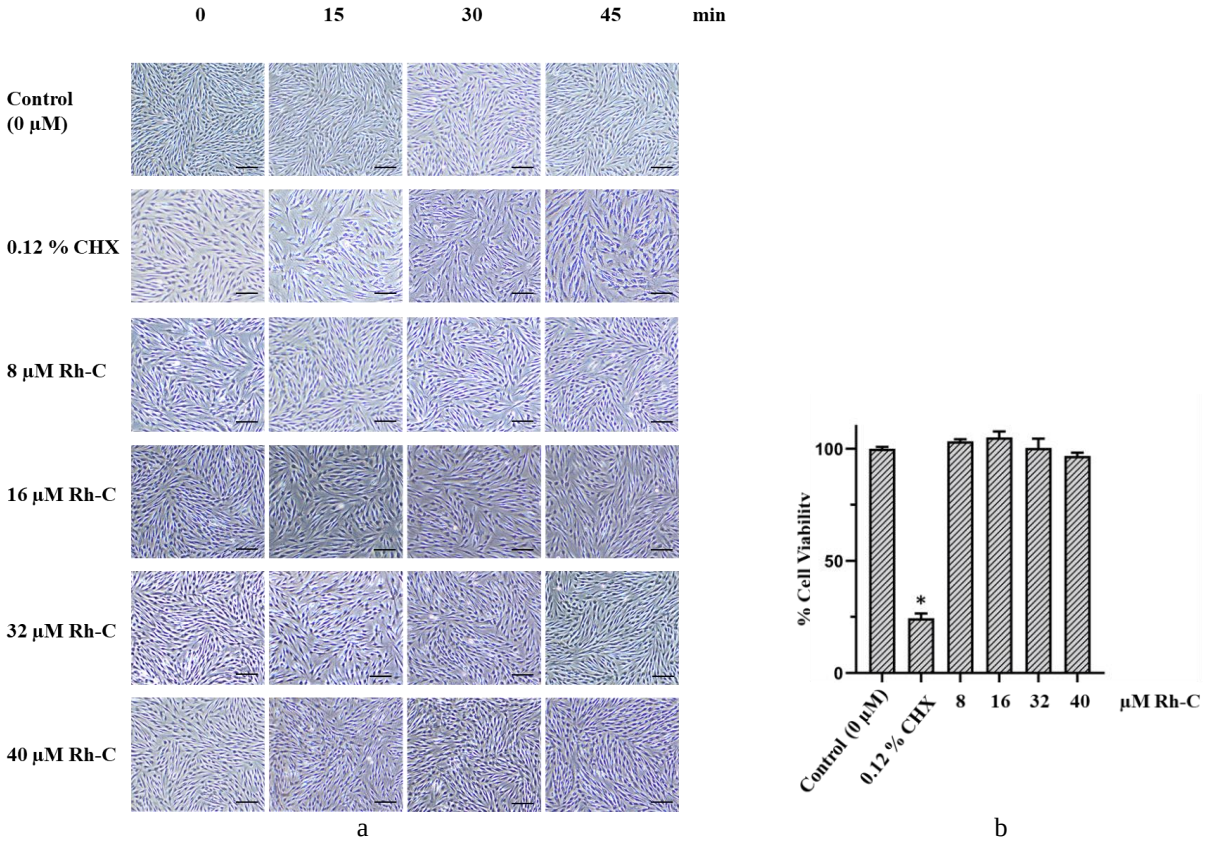

Figure 4. Safety of Rh-C to HGF cells. HGF cells were treated with Rh-C at indicated concentrations or 0.12% (w/v) CHX for 15, 30, and 45 min. Morphology of cells was observed under light microscope at each time point (a). Bar = 200 µm. HGF cells were treated Rh-C at the indicated concentrations or 0.12% (w/v) CHX for 24 h and cell growth was assayed by the SRB assay. Results are expressed as means from three separate experiments. Bar = SD, * $p < 0.05$ relative to the control without treatment.

coated surface. Moreover, the biomass in the pre-formed biofilms was significantly reduced by treatment with Rh-C, as shown by a decrease in the live/dead cells ratio in the biofilms, suggesting that Rh-C is an effective anti-biofilm agent for *S. mutans* and *S. sobrinus*. Due to the 3-dimensional complex biofilm, the effective concentrations of Rh-C required for anti-biofilm formation (16 µM) and for reduction of biomass in the biofilm (32 µM) were higher than the concentrations for bactericidal effects on planktonic bacteria. Although the underlying molecular mechanism of Rh-C anti-biofilm activity is currently unknown, Rh-C has been shown to inhibit human topoisomerase II (Klaophimai *et al.*, 2023). One strategy to combat bacterial biofilms is to inhibit bacterial cell division and survival (Roy, Tiwari, Donelli, & Tiwari, 2018). Rh-C could possibly inhibit DNA gyrase, bacterial type II topoisomerases, leading to bacterial cell cycle arrest followed by inhibition of bacterial biofilm formation.

Due to high bactericidal and anti-biofilm capability, CHX is a chemical commonly used in antiseptic mouthwashes. A range of 0.12 % - 0.2 % (w/v) CHX is commercially formulated in the mouthwashes. However, recent studies have shown cytotoxic effects of CHX on human periodontal tissues, such as gingival fibroblasts and other oral cells, even at a concentration of 0.04 % (w/v) CHX (Wyganowska-Swiatkowska *et al.*, 2016). One of the advantages of using natural products is having relatively few side effects, probably due to a low cytotoxicity against normal cells. Up to a concentration of 40 µM and up to 24 h incubation, Rh-C had little cytotoxic effect on HGF, making Rh-C a safe anti-plaque natural ingredient to be used in mouthwash or toothpaste. Based on our present study, the effective concentration of active substance Rh-C extracted from *R. nasutus* for anti-biofilm formation without toxicity to HGF was 32 µM. Further studies are required to gain more insights into molecular anti-biofilm mechanisms of Rh-C on *Streptococcus* spp. The results of our study suggest potential for use of natural-based Rh-C as an alternative anti-bacterial and anti-biofilm agent in oral topical rinses or toothpastes to prevent caries.

5. Conclusions

In summary, we reported herein on the bactericidal and anti-biofilm activities of Rh-C isolated from *R. nasutus* against *S. mutans* and *S. Sobrinus*, which are major causative factors initiating dental caries. Rh-C could inhibit the attachment of both these planktonic bacteria to a surface, which attachment is the first stage of biofilm development. Moreover, Rh-C could reduce the bacterial biomass in a formed biofilm without effecting on viability of HGF cells.

Acknowledgements

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