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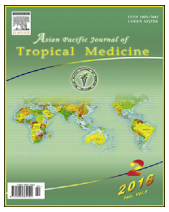

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Effect of roselle calyx extract on *in vitro* viability and biofilm formation ability of oral pathogenic bacteria

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ABSTRACT

Objective: To investigate the effect of the roselle calyx extract (RCE) (*Hibiscus sabdariffa* L.) on the *in vitro* viability and biofilm formation ability of oral pathogenic bacteria.

Methods: RCE was prepared by soaking roselle calyx powder with ethyl alcohol for 24 h at room temperature. After centrifugation, the extract was lyophilized. Then, the extract was dissolved in phosphate-buffered saline, the pH was adjusted, and the extract was aseptically filtered. We used *Streptococcus mutans*, *Streptococcus sanguinis*, *Lactobacillus casei*, *Actinomyces naeslundii*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Prevotella intermedia* in this study. The antibacterial activity of the RCE was determined by treating the cells of these bacteria with the extract for 10 or 20 min at room temperature. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration was determined using the microdilution method, and the effect of the RCE on the ability to form biofilm was determined using a polystyrene micro plate assay. In addition, we used the WST-1 assay to determine the cytotoxicity of the RCE on HGF, Ca9-22 and KB cells.

Results: The RCE had antibacterial activity against oral bacteria used in this study. In particular, most significant antibacterial activity was observed against *Fusobacterium nucleatum*, *Prevotella intermedia* and *Porphyromonas gingivalis*. The MIC and minimum bactericidal concentration were 7.2 mg/mL–28.8 mg/mL and 14.4 to >57.6 mg/mL. The RCE had an inhibitory effect on biofilm formation at the MIC and sub-MIC levels. In addition, the RCE had low cytotoxic effects on HGF, Ca9-22 and KB cells.

Conclusions: Thus, our results indicate that the RCE may be used for preventing oral diseases.

1. Introduction

Dental caries and periodontitis have become a global health problem. *Streptococcus mutans* (*S. mutans*), a gram-positive coccus, is the causative agent of dental caries; however, other bacteria, including *Lactobacillus* and *Actinomyces* may be involved in human dental caries. These species adhere and accumulate on the tooth surface by producing extracellular polysaccharides from sucrose in the oral cavity. This specific characteristic of the bacterial species is essential for the formation and development of the biofilm [1,2]. Specific bacteria isolated from human dental biofilm such as *Porphyromonas gingivalis* (*P. gingivalis*), *Prevotella intermedia* (*P. intermedia*), *Fusobacterium nucleatum* (*F. nucleatum*), and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) induce periodontitis [3]. These infections can be prevented by mechanical removal of the oral biofilm by brushing the teeth and flossing. However, some individuals, particularly children and elderly, may not be able to achieve mechanical removal of the biofilm [4].

Chlorhexidine (CHX) is generally accepted as the standard antibiofilm agent in the field of dentistry. However, the use CHX not only remains controversial but also has adverse effects, including staining of teeth, detrimental effect on vital tissues and development of hypersensitivity reactions [2,5,6]. Therefore,

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development of novel agents for inhibiting the growth and ability of biofilm formation of bacteria is required as one of the strategies for the prevention of dental caries and periodontitis.

The use of plant extracts as alternative medical treatments has become popular in the recent years. The term 'plant products' usually refers to secondary metabolites produced by plants. Typically, these substances serve as the defense mechanism for the plant against predation by microorganisms, insects, and herbivores [7]. *Hibiscus sabdariffa* L (family Malvaceae), commonly known as roselle or red sorrel in English, is widely grown in Central and West Africa, Southeast Asia, and other regions. Roselle is an annual, erect, bushy, 2.4 m tall herbaceous subshrub. It grows widely in the tropical and subtropical areas. The thick, red and fleshy, cup-shaped part of the flower is known as calyx; the calyx has been used worldwide in cold and hot beverages, puddings and jellies, *etc* [8–10].

Roselle calyx is rich in secondary metabolites, which have medicinal properties. Previous studies have shown that the calyces contain flavonoids such as gossypetin, hibiscetin, sabdaretine, alkaloids, and saponins [11,12]. In addition, roselle extract contains hibiscus acid, hydroxybenzoic acids, flavonols, anthocyanins and other polyphenolic compounds [13]. Roselle extract has been used in folk medicine. The extract has antihypertensive [14], hepatoprotective [15], antihyperlipidemic [16], antioxidant [17], anticancer [18], anti-inflammatory [19], antimicrobial properties [12,20]. Although there were studies reporting the effects of roselle as an herbal medicine, to date, only a few studies have examined the effects of roselle calyx extract (RCE) as an antibacterial agent, particularly in the field of dentistry. Thus, the purpose of our study was to investigate the effects of RCE on oral pathogenic bacteria, particularly the antibacterial effect on target organisms, inhibition of biofilm formation, and cytotoxic effect on human oral cells.

2. Materials and methods

2.1. Preparation of RCE

Plant material was collected and identified by the Central Research and Development of Medicinal Plant and Traditional Medicine, Tawangmangu, Central Java, Indonesia. A total of 16 g roselle calyx powder was soaked in 160 mL ethyl alcohol (Wako Pure Chemical Industries Ltd) with shaking for 24 h at room temperature. After centrifugation, the extract was lyophilized. Then, the extract was dissolved in phosphate-buffered saline (PBS), the pH was adjusted to 7.0, and the extract was aseptically filtered through a disposable membrane filter unit with a 0.45 μm pore size. The extract was stored in the freezer at −20 °C for the further experiments.

2.2. Bacterial strains and culture conditions

The bacteria used in this study were *S. mutans* Ingbritt, *Streptococcus sanguinis* (*S. sanguinis)* ATCC 10556T, *Lactobacillus casei* (*L. casei*) ATCC 4646, *Actinomyces naeslundii* (*A. naeslundii*) ATCC 12104T, *A. actinomycetemcomitans* ATCC 29522, *F. nucleatum* JCM 6328, *P. gingivalis* ATCC 33277T and *P. intermedia* ATCC 25611T. *S. mutans*, *S. sanguinis*, and *L. casei* were cultured in trypticase soy (TY; Difco, Detroit, MI, USA) agar supplemented with yeast extract (1 mg/mL). Brain-heart infusion-blood agar supplemented with hemin (BHI-HM) (10 μg/mL; Sigma, St Louis, MO, USA), and menadione (5 μg/mL; Sigma) was used to culture *A. naeslundii*, *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, and *P. intermedia*. All strains were cultured under anaerobic conditions (85% N_2, 10% H_2, and 5% CO_2) at 37 °C for 72 h.

2.3. Antibacterial activity test

The colonies of bacteria cultured under anaerobic conditions for 3 d in TY/BHI-HM agar were suspended in PBS. The suspension was centrifuged (15 000 rpm, 4 °C, 5 min), washed, and diluted in PBS to obtain 107 cfu/mL. Then, a 500 μL aliquot of the bacterial suspension was centrifuged, and the pellets were treated with 250 μL of RCE at a concentration of 144 mg/mL or PBS as a control for 10 or 20 min. Subsequently, the pellets were washed 3 times and were suspended in PBS. We inoculated 100 μL of the bacterial suspension on an agar plate and incubated it under anaerobic conditions at 37 °C for 3 d–5 d. The numbers of viable bacteria were counted after the incubation period. The percentage of viable bacteria was determined as a proportion of control (100%). The experiments were performed in triplicate for each bacterial species.

2.4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC were determined using microdilution methods [21]. Bacteria from overnight culture were adjusted to an optical density (OD) of 1.0 at 600 nm, and then diluted in appropriate growth medium. Bacterial suspensions of each bacterium were plated on 96-well flat-bottom microplates (Sigma–Aldrich, USA) and were treated with different concentrations of the RCE to obtain final bacterial concentration of 1×10^5 cfu/mL to 5×10^6 cfu/mL. Then, the plates were incubated at 37 °C in anaerobic conditions for 24 h. The MIC was defined as the lowest concentration of the extract that completely inhibits the growth of the bacteria as detected by the unaided eye. For the determination of the MBC, a 100 μL aliquot of the bacterial suspension was inoculated on agar plate from those wells that contained the RCE at the MIC and at concentrations higher than the MIC. The MBC was defined as the lowest concentration at which bacteria did not grow on the agar plate after the incubation period.

2.5. Effect on biofilm formation

The inhibitory effect of different concentrations of the RCE on the ability to form biofilm was examined by using a polystyrene micro plate assay. The bacterial suspensions from overnight broth culture were adjusted to an OD of 1.0 at 600 nm, and then diluted in BHI-broth supplemented with sucrose 0.5%–2% (*S. mutans*, *S. sanguinis*, *A. naeslundii* and *L. casei*), BHI-broth (*A. actinomycetemcomitans*) or GAM-broth (*P. gingivalis*, *P. intermedia*, and *F. nucleatum*). Thereafter, the bacterial suspensions were treated with different concentrations of extract or PBS as a control in 96-well flat-bottom micro plate. The final concentration of bacteria was 1×10^5 cfu/mL to 5×10^6 cfu/mL. The plates were then incubated in anaerobic condition for designated times appropriate for each bacterium (16 h–72 h).

After incubation, the medium was removed, and then, the wells were gently washed with PBS and air-dried. The biofilm formed on the bottom of the plate was stained with 50 µL of 0.1% crystal violet for 15 min at room temperature, and the wells were gently washed and bound dye was extracted by adding 200 µL of ethyl alcohol. The amount of biofilm formed was quantified by measuring the resulting ethyl alcohol solution at 595 nm on a microtiter plate reader TECAN Infinite TM 200 (Tecan Deutschland GmbH, Crailsheim, Germany). The inhibitory effect of RCE on biofilm formation was determined as a proportion of control (100%). All experiments were performed in triplicate.

2.6. Cytotoxicity

Human gingival fibroblast and human mouth epithelial cells (Ca9-22 and KB cells) were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (100 µg/mL). The cells were cultured at 37 °C in a 5% CO_2 incubator to obtain 90%–100% confluence and used at 3 passages–10 passages. After washing with PBS, the cells were detached from the culture plate by treatment with 0.05% trypsin-EDTA (GIBCO). The cells were counted using a haemocytometer (EKDS, Tokyo), and then diluted to obtain 1×10^5 mL. A 100 µL of the cell suspension was cultured in a 96-well plate and incubated it for 24 h at 37 °C in a CO_2 incubator. Then, the cells were treated with 100 µLRCE, CHX, or PBS for 20 min. For measurement of viable cells, 10 µL of water-soluble tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2//-5-tetrazolio]-1, 3-benzene disulfonate (WST-1, Roche, Germany) was added to the cells. They were incubated for 30–60 min at 37 °C in a CO_2 incubator, and then the absorbance of each well was measured by using microtiter plate reader at a wavelength of 450 nm. Viable cells were evaluated as follows: [(OD450 of treated cells and reagent – OD450 of reagent without cell)/(OD450 control cells and reagent – OD450 of reagent without cell)] × 100. The decrease in viable cells was expressed as a percentage of control. All experiments were performed in triplicate.

2.7. Statistical analysis

Statistical analysis was performed using SPSS 21 software. Results were obtained in triplicates and were expressed as mean ± standard deviation. The significance of the differences between groups was determined using independent *t*-test with a value of $P < 0.05$–0.01.

3. Results

3.1. Antibacterial activity

The results of the effects of the RCE on bacterial viability were shown in Figure 1. The numbers of viable bacteria in the RCE group were significantly lower than those in the control group. The numbers of almost all the bacteria, except those of *S. sanguinis*, *L. casei*, and *A. naeslundii*, decreased by more than 50% after treatment with the RCE for 10 min. Treatment with the RCE for 20 min decreased the numbers of all bacteria by more than 50%. The RCE had the strongest activity on *F. nucleatum*, *P. intermedia*, and *P. gingivalis*. The number of viable bacteria decreased by more than 90% ($P < 0.05$) at treatment times of 10 and 20 min.

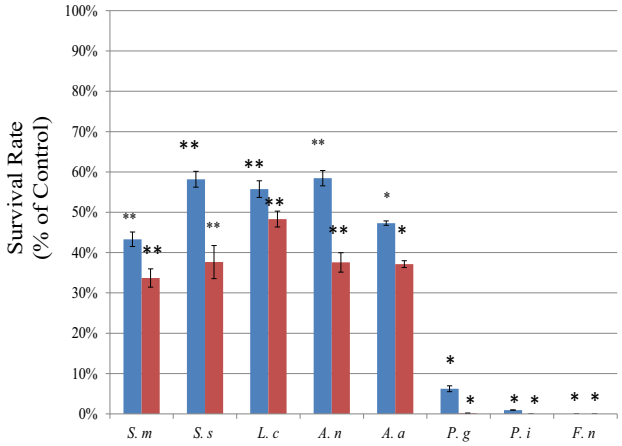

Figure 1. Effect of RCE on the viability of oral pathogenic bacteria. *S. m*: *S. mutans*, *S. s*: *S. sanguinis*, *L. c*: *L. casei*, *A. n*: *A. naeslundii*, *A. a*: *A. acetemcomitans*, *P. g*: *P. gingivalis*, *P. i*: *P. intermedia*, *F. n*: *F. nucleatum*. The viable bacteria were expressed as percent of control. $^{*}P < 0.05$, $^{**}P < 0.01$: significantly different from the control.

3.2. MIC and MBC

The RCE (pH 7.0) showed bacteriostatic and bactericidal effects against eight oral pathogenic bacteria. The MIC and MBC values were shown in Table 1. The MIC ranged from 7.2 mg/mL to 28.8 mg/mL and the MBC ranged from 14.4 mg/mL to more than 57.6 mg/mL. *S. mutans, F. nucleatum*, and *P. gingivalis* were the most sensitive bacteria (MIC = 7.2 mg/mL). The least sensitive bacteria were *S. sanguinis*, *L. casei*, and *A. actinomycetemcomitans* (MIC = 28.8 mg/mL).

3.3. Effect on biofilm formation

The effect of the RCE on the formation of biofilm by *S. mutans*, *S. sanguinis*, *L. casei*, *A. naeslundii*, *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia* and *F. nucleatum* was shown in Figure 2. RCE at the MIC and sub-MIC levels were used in these experiments. RCE inhibited biofilm formation by *S. mutans*, *A. naeslundii*, *P. gingivalis*, *P. intermedia* and *F. nucleatum* at concentration of 0.9 mg/mL ($P < 0.01$), that by *L. casei* at 1.8 mg/mL ($P < 0.01$), and that by *S. sanguinis* and *A. actinomycetemcomitans* at 7.2 mg/mL ($P < 0.01$–0.05). The 50% inhibitory concentration of RCE on biofilm formation was 0.9 mg/mL for *S. mutans*, *A. naeslundii*, *P. intermedia*, *P. gingivalis* and *F. nucleatum*; 7.2 mg/mL for

Table 1
MIC and MBC of RCE against eight oral pathogenic bacteria.

Bacterial species	MIC (mg/mL)	MBC (mg/mL)
S. mutans	7.2	57.6
S. sanguinis	28.8	57.6
L. casei	28.8	>57.6
A. naeslundii	14.4	>57.6
A. actinomycetemcomitans	28.8	57.6
F. nucleatum	7.2	14.4
P. gingivalis	7.2	28.8
P. intermedia	14.4	28.8

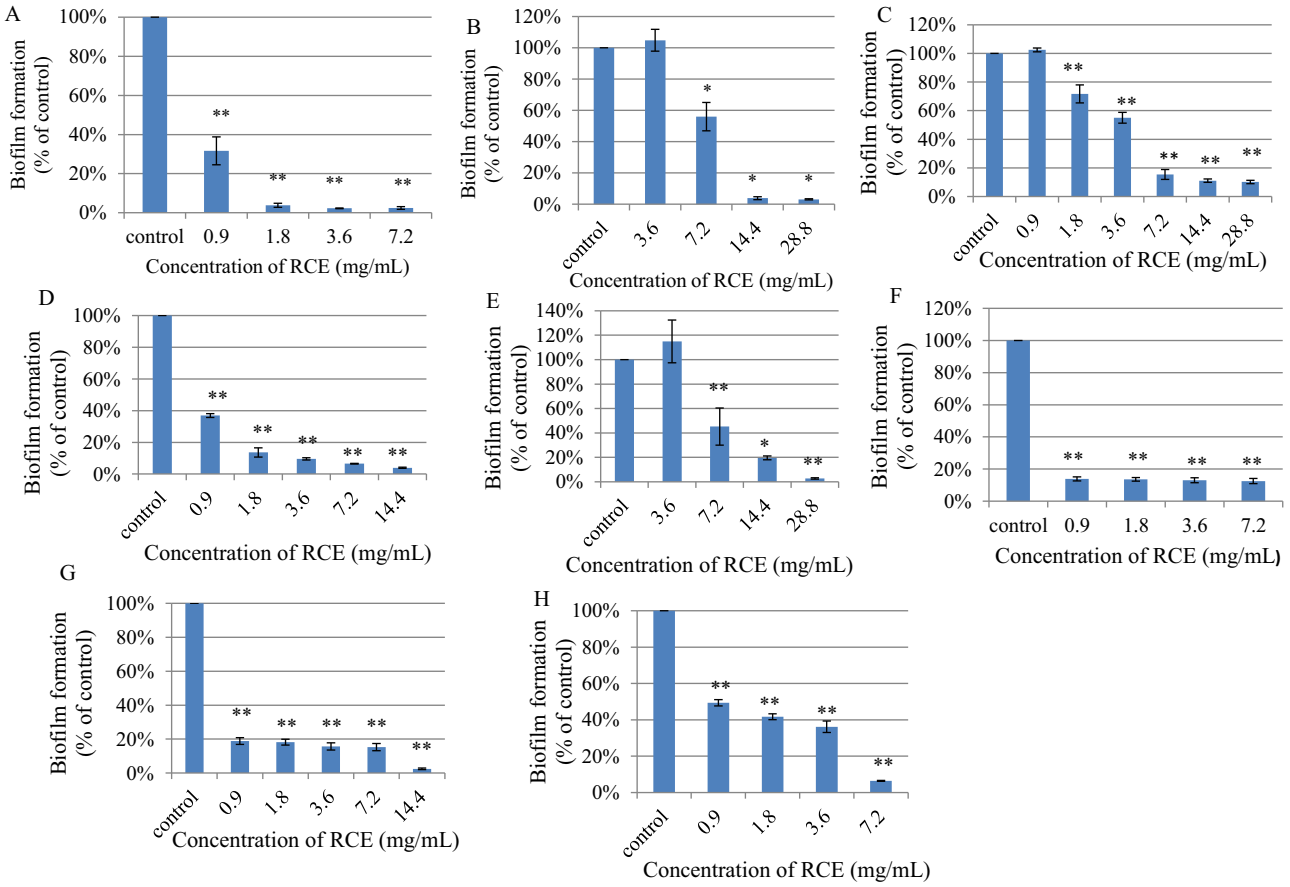

Figure 2. Inhibitory effect of RCE on biofilm formation.
A: *S. mutans*, B: *S. sanguinis*, C: *L. casei*, D: *A. naeslundii*, E: *A. acetemcomitans*, F: *P. gingivalis*, G: *P. intermedia*, and H: *F. nucleatum*. Experiment used RCE at MIC and sub-MIC levels in triplicate. The biofilm formation was performed as percent of control. *$P < 0.05$, **$P < 0.01$: significantly different from the control.

L. casei and *A. actinomycetemcomitans*; and 14.4 mg/mL for *S. sanguinis*.

3.4. Cytotoxicity

To determine the cytotoxic effects caused by the RCE, we determined the viability of cells from the oral cavity by using WST-1 assay. Cell viability of all cell lines used in this study was ≥60% after treatment with the RCE for 20 min (Figure 3). HGF cells showed the highest viability (82.9%), whereas Ca9-22 cells showed the lowest viability (60.9%). Our results showed that the RCE was less cytotoxic to oral cells. No statistically significant differences were observed between the viability of HGF, Ca9-22, and KB cells and the control cells ($P > 0.05$). Thus, the percentage of viable cells in the RCE-treated group was not significantly different from that of control group. However, the viability of cells treated with CHX was significantly different from that of the control cells ($P < 0.05$).

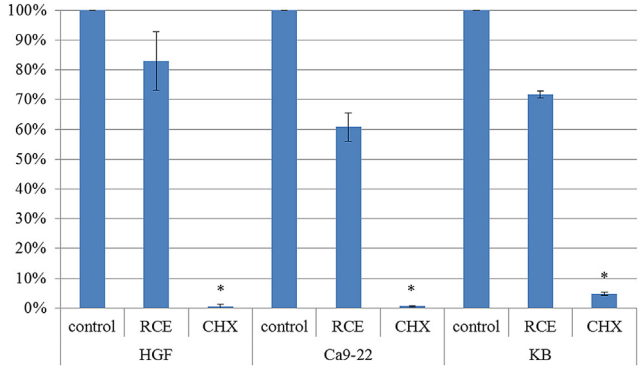

Figure 3. Cytotoxicity of RCE on human oral cells.
Control: PBS, CHX: 0.05%, RCE: 144 mg/mL. The viable cells were performed as percent of control. *$P < 0.05$: significantly different from the control.

4. Discussion

CHX is widely used in mouthwashes for the prevention and treatment of oral diseases because it can inhibit the growth of oral pathogenic bacteria. However, CHX is cytotoxic to human periodontal cells, inhibits protein synthesis, affects mitochondrial activity, and thus, has adverse effects on vital tissues [5]. Therefore, it is important to find the alternative agents that are less cytotoxic and can be used for prevention of oral diseases. In this study, we used the extract of roselle calyx, a plant that is known to have many medicinal properties.

We found that RCE had bactericidal activity against both cariogenic and periodontopathic bacteria. RCE showed strongest inhibitory activity against *F. nucleatum*, *P. intermedia* and *P. gingivalis*, which indicated that RCE was more effective against gram-negative bacteria than gram-positive bacteria. The difference in the effect of RCE on gram-negative bacteria and gram-positive bacteria may be because of the differences in the bacterial cell wall structure. The peptidoglycan layer in the cell

wall of gram-positive bacteria is thicker than that in the gram-negative bacteria, which inhibits the RCE from entering the cell membrane of gram-positive bacteria [21]. The MBC of RCE was different for gram-negative and gram-positive bacteria; the MBC was 14.4 mg/mL–57.6 mg/mL and 57.6 mg/mL to >57.6 mg/mL, respectively. Our results are consistent with those of a previous study, which showed that the roselle extract had the highest zone of inhibition for *Escherichia coli*, a non-oral gram-negative bacterium [22]. The antibacterial activity observed in our study may be because of the main compound in the RCE, such as flavonoids. Flavonoids have the ability to bind with bacterial cell walls. In addition, with the number of hydroxyl groups present on the phenolic ring increase because of hydroxylation, which in turn leads to increase in the antimicrobial activity [7].

Among the gram-negative bacteria, *A. actinomycetemcomitans* was the least sensitive against RCE; the MIC of *A. actinomycetemcomitans* approached to the MIC of the gram-positive bacteria. This finding was also reported in a previous study. *A. actinomycetemcomitans* was less sensitive than *P. intermedia*, *P. gingivalis* and *F. nucleatum* to garlic extract [23]. Moreover, *A. actinomycetemcomitans* was less sensitive to a combination of metronidazole and amoxicillin [24]. These bacteria may modulate the aspect of virulence factor and control cellular adaption to growth under limiting conditions [25].

Oral biofilm plays in important role in the pathogenesis of oral diseases, and the inhibiting the biofilm formation is one of the approaches for preventing oral diseases. For determining the effect of RCE on biofilm formation, the extract at sub-MIC level is used. Thus, the decrease of the amount of biofilm formed is not because of the inhibition of bacterial growth, but because of the inability of the bacteria to form biofilm. Our results showed that RCE at sub-MICs level could inhibit the formation of biofilm by eight bacteria in a dose-dependent manner. Although the mechanisms underlying the inhibitory effects of RCE on the ability to form biofilm are still unknown, the inhibitory effects may be due to flavonoid and tannins present in the RCE. The inhibitory effects of the extract on biofilm formation depend on the phenolic compounds present in the extract, because these compounds bind strongly to proteins and the enzymes, thus the bacteria are unable attached to the tooth surface. Adhesion and colonization are very important steps for biofilm formation [2]. We showed that the RCE at sub-MIC levels significantly inhibited biofilm formation by gram-positive and gram-negative bacteria. The effects of RCE on biofilm produced by a mixed culture of bacteria should be examined in future studies.

During the development of novel agents as oral care products, their toxic effects on human oral cells should be carefully examined. An ideal oral care product should be an efficient antimicrobial agent but should not be toxic to human oral cells. Our results showed that the viability of HGF, Ca9-22 and KB cells treated with RCE was not significantly different from that of control cells. Thus, RCE is safe to be used as an oral care product. Our findings are consistent with those reported previous study, which showed that the RCE was safe in brine shrimps lethality assay [12].

CHX is chemical substance with excellent antimicrobial action. It is active against a wide range of microorganisms. However, a previous study showed that CHX at a concentration of more than 0.05% completely inhibits protein synthesis in human periodontal ligament cells. Thus, CHX may cause detrimental effects on vital tissues [5]. In addition, our study showed that CHX has cytotoxic effect against HGF, Ca9-22 and KB cells. These results indicate that RCE is safer than CHX as an oral care product.

Our results indicate that RCE exerts antibacterial activity against gram-positive and gram-negative bacteria, with a strong activity against gram-negative bacteria. Moreover, at sub-MIC levels, RCE inhibits the formation of biofilm by gram-positive and gram-negative bacteria. In particular, RCE has lower cytotoxicity than CHX, a product widely used as mouthwashes. Therefore, because of the favorable bioactivity and a simple process involved in producing the extract from the plant, RCE has a high potential to be used as a novel agent for preventing oral diseases. Further studies are required to investigate the effects of RCE in clinical practice and to examine the complexity of life span of oral bacteria in natural environments.

Conflict of interest statement

The authors report no declaration of interest.

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