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研究題目：Effect of roselle calyx extract on in vitro viability and biofilm formation ability of oral pathogenic bacteria

目　的：

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

対象および方法：

Roselle calyx powder was soaked in ethyl alcohol for 24 h at room temperature. After centrifugation, the extract was lyophilized and dissolved in phosphate-buffered saline（PBS）. The pH was adjusted, and the extract was aseptically filtered. The bacteria used in this study were *Streptococcus mutans, Streptococcus sanguinis, Lactobacillus casei, Actinomyces naeslundii, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Porphyromonas gingivalis, and Prevotela intermedia*. The antibacterial activity of the RCE was determined by treating the bacterial cells with the extract for 10–20 min at room temperature. The minimum inhibitory concentration（MIC）and minimum bactericidal concentration（MBC）were determined using the micro-dilution method, and the effect of RCE on biofilm formation 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, Ca 9-22, and KB cells.

および考察：

Chlorhexidine（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 by inhibiting protein synthesis and affecting mitochondrial activity, and thus, has adverse effects on oral cells. Therefore, it is important to find 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（Fig. 1）. RCE showed the 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 MIC ranged from 7.2 to 28.8 mg/mL, while the MBC ranged from 14.4 to ＞57.6 mg/mL（Table 1）. The antibacterial activity observed in our study may be because of the main compound in RCE, such as flavonoids, which have the ability to bind to bacterial cell walls, disrupts the membrane integrity, resulting in death.

In addition, our results showed that RCE at sub-MIC levels could inhibit the formation of biofilm by eight bacteria in a dose-dependent manner（Fig. 2 and 3）. The inhibitory effects of the extract on biofilm formation may be depend on the phenolic compounds present in the extract, because these compounds bind strongly to proteins and the enzymes, thus bacteria are unable to attach to the tooth surface.

When considering the development of novel agents as oral care products, their toxic effects on human oral mucosal 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. Results from our study indicated that RCE is safe to be used as an oral care product（Fig. 4）.

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

Bacterial strains	MIC (mg/mL)	MBC (mg/mL)
S. mutans Ingbritt	7.2	57.6
S. sanguinis ATCC 10556^{T}	28.8	57.6
L. casei ATCC 4646	28.8	＞57.6
A. naeslundii ATCC 12104^{T}	14.4	＞57.6
A. actinomycetemcomitans ATCC 29522	28.8	57.6
F. nucleatum JCM 6328	7.2	14.4
P. gingivalis ATCC 33277^{T}	7.2	28.8
P. intermedia ATCC 25611^{T}	14.4	28.8

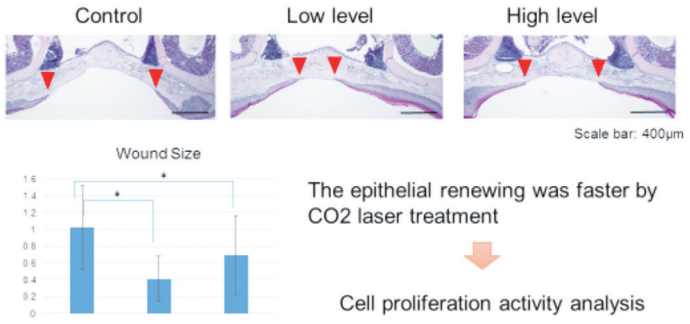

Figure 1. Effect of roselle calyx extract on the viability of oral bacteria. *S. m: S. mutans, S. s: S. sanguinis, L. c: L. casei, A. n: A. naeslundii, A. a: A. acetemcomitans, P. g: P. gingivalis, P. i: P. intermedia, F. n: F. nucleatum.* The viable bacteria were expressed as percent of control. $^{*}p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

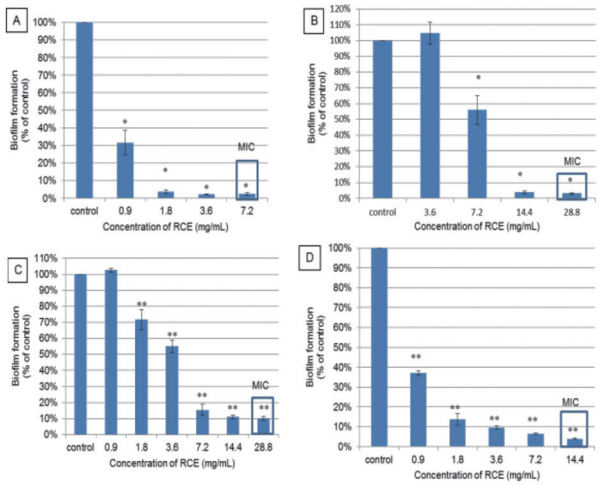

Figure 2. Inhibitory effect of roselle calyx extract on biofilm formation ability by gram-positive bacteria. A: *S. mutans*, B: *S. sanguinis*, C: *L. casei*, D: *A. naeslundii*, RCE: roselle calyx extract. Experiment used RCE at MIC and sub-MIC levels in triplicate. The biofilm formation was shown as percent of control. $^{*}p < 0.05$, $^{**}p < 0.01$: significantly different from the control.

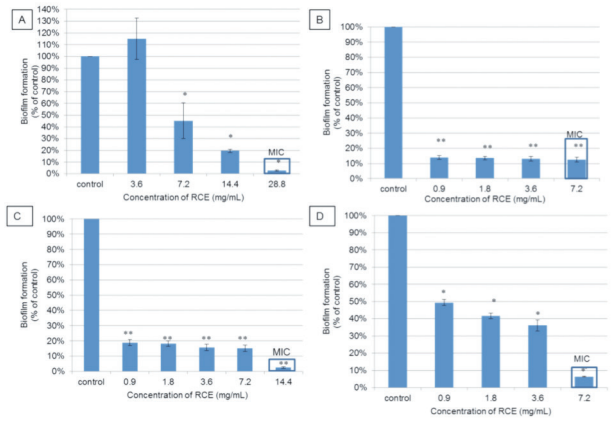

Figure 3. Inhibitory effect of roselle calyx extract on biofilm formation ability of gram-negative bacteria.
A: *A. acetemcomitans*, B: *P. gingivalis*, C: *P. intermedia*, D: *F. nucleatum*, RCE: roselle calyx extract. Experiment used RCE at MIC and sub-MIC levels in triplicate. The biofilm formation was shown as percent of control. $^{*}p < 0.05$, $^{**}p < 0.01$: significantly different from the control.

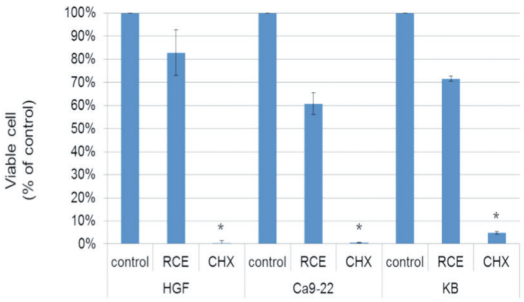

Figure 4. Cytotoxicity of roselle calyx extract in human oral cells.
Control: PBS, CHX: chlorhexidine 0.05%, RCE: roselle calyx extract 144 mg/mL. The viable cells were shown as percent of control. $^{*}p < 0.05$: significantly different from the control.

成果発表：（予定を含めて口頭発表，学術雑誌など）

Presentation

1. 2012, Oral Presentation（Student Research Grant）. Title：Antimicrobial effect of *Hibiscus sabdariffa* tea on *Streptococcus mutans*. Japanese Society of Bacteriology in Scientific Branch Meeting of Hokkaido, Obihiro, Japan.
2. 2012, Poster Presentation, Title：Antibacterial activity of Roselle tea（*Hibiscus sabdariffa*）against *Streptococcus mutans* in vitro. Japanese Association for Oral Biology in the 54th Annual Meeting, Koriyama, Japan.
3.. 2013, Oral Presentation（Student Research Grant）, Title：Roselle extract against biofilm formation of *Streptococcus mutans* and *Porphyromonas gingivalis*. Japanese Society of Bacteriology in Scientific Branch Meeting of Hokkaido, Abashiri, Japan.
4. 2013, Poster Presentation, Title：The cytotoxicity and antibacterial activity of Roselle ethanol extract on oral bacteria *in vitro*. Japanese Association for Oral Biology in the 55th Annual Meeting, Okayama, Japan.
5. 2014, Poster Presentation, Title：Roselle extract：The new agent for prevention of oral diseases. Japanese Society of Oral Therapeutics and Pharmacology, Osaka, Japan.
6. 2014, Poster Presentation, Title：Roselle extract as alternative agent for prevention of oral diseases. Health Sciences University of Hokkaido, Dental Alumni Association in Clinical Summer Meeting, Sapporo, Japan.
7. 2015, Poster Presentation, Title：Effect of Roselle calyx extract on biofilm formation, cytokine production and gingipain activity. Japanese Association for Oral Biology in the 57th Annual Meeting, Niigata, Japan.

8. 2015, Proceeding Conference, Title : Effect of roselle calyx extract on oral pathogenic bacteria and biofilm formation in vitro. The 2nd International Conference on Health Science, Yogyakarta, Indonesia.

Publication

1. Herastuti Sulistyani, Mari Fujita, Hiroshi Miyakawa, Futoshi Nakazawa. Effect of roselle calyx extract on in *vitro* viability and biofilm formation ability of oral pathogenic bacteria. *Asian Pacific Journal of Tropical Medicine*（inpress）http://dx.doi.org/10.1016/j.apjtm.2016.01.020
2. Herastuti Sulistyani, Mari Fujita, Futoshi Nakazawa. Effect of roselle calyx extract on gingipains activity, production of inflammatory cytokines, and oral bacterial morphology. *Journal of Microbiology, Biotechnology and Food Science*（under review）.