

Roselle flower petals extract inhibits periodontal pathogenic biofilms



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Abstract

Objectives: The present study aimed to determine the effect of roselle flower petals extract on *Fusobacterium nucleatum* and *Porphyromonas gingivalis* biofilms in vitro.

Material and Methods: Crystal violet biofilm assays were carried out to evaluate the effect of roselle flower petals extract on *F. nucleatum* ATCC-25586 and *P. gingivalis* ATCC-3327 biofilms. Each bacterium was cultured in Brain Heart Infusion broth for 48 h at 37°C under anaerobic conditions. Subsequently, 200 µL (10^7 CFU/mL) of bacterial suspension was distributed in a 96-well plate for 48 h to form both mono- and dual-species biofilms. Different concentrations of the roselle flower extract and 0.2% chlorhexidine-gluconate (positive control) were added to the biofilms in the wells and incubated for 1 h, 6 h, and 24 h.

Results: There was a significant biofilm reduction after treatment with roselle flower petals extract at all concentrations and incubation times compared to the negative control ($p < 0.05$). The most effective concentrations for inhibiting the monospecies biofilms of *F. nucleatum* and *P. gingivalis* and the dual species biofilm were 100%, leading to a 99.8%, 100%, and 90.6% reduction of the biofilm compared to the control, respectively.

Conclusion: Roselle flower petals extract is effective at inhibiting *F. nucleatum* and *P. gingivalis* biofilms. This anti-biofilm agent may be developed as an alternative therapy to prevent periodontal disease. Future studies are needed to explore the mechanisms of action of the active compounds.

Keyword: Biofilm, *Fusobacterium nucleatum*, *Hibiscus sabdariffa* L., *Porphyromonas gingivalis*, Roselle flower

Cite this Article: Sebastian J, Widyarman AS. 2021. Roselle Flower Petals Extract Inhibits Periodontal Pathogenic Biofilms. *Journal of Dentomaxillofacial Science* 6(2): 102-105. DOI: [10.15562/jdmfs.v6i2.1122](https://doi.org/10.15562/jdmfs.v6i2.1122)

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Introduction

Periodontal disease is one of the most common diseases of the oral cavity and is the major cause of tooth loss in adults. According to the 2018 Indonesian Basic Health Research survey, the prevalence of periodontal disease in Indonesia reached 57.6% for all age groups.¹ Periodontitis is defined as an inflammatory disease of the supporting tissues such as periodontal ligaments and alveolar bones caused by microorganisms that leads to recession of the teeth or progressive destruction of teeth.² The etiology of periodontal diseases has focused on bacterial plaque, microbial by-products, and the host immune response. Notably, bacteria accumulate to form plaques on teeth, commonly known as oral biofilms.³

Oral biofilm is defined as a diverse community of microorganisms found on the surface of teeth embedded in a matrix of extracellular polymeric substances (EPS).⁴ Specific microorganisms in biofilms involved in periodontitis include the dominant anaerobic Gram-negative rods such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*.⁵

According to the World Health Organization (WHO), herbal plants are the best source of compounds for antimicrobial medicine.⁶ Many herbal plants originating from Indonesia have been

studied for their effectiveness in inhibiting oral pathogens biofilms.⁷⁻⁹ One of them is the roselle flower (*Hibiscus sabdariffa* L.), originating from Indonesia. *Hibiscus sabdariffa* L. has reportedly been used as an anti-inflammatory, antiseptic, antibacterial, analgesic, and antipyretic agent. This plant has been used to treat abscesses, fever, and cough, to reduce blood pressure and viscosity, to reduce cholesterol, uric acid, and triglycerides in urine, as a diuretic, and for its anti-cancerous, antitumor, and anti-leukemia properties.¹⁰

Hibiscus sabdariffa L. has different active ingredients such as flavonoids, citric acid, saponins, vitamins A, B, and C, calcium, iron, phosphorus, niacin, and 18 amino acids, one of which is arginine, which is beneficial for cell rejuvenation.¹¹ Kirdpon et al.¹² studied its antibacterial properties and found that roselle extract had a lethal effect against *Mycobacterium tuberculosis*.¹² Another study showed the antibacterial effect of *Hibiscus sabdariffa* L. against *Aeromonas hydrophila* (Gram-negative bacteria) and *Streptococcus agalactiae* (Gram-positive bacteria).¹³ Suwandi et al.¹⁴ showed that *Hibiscus sabdariffa* L. has an antibacterial effect against *Streptococcus sanguinis*, the causative bacteria of gingivitis.¹⁴ However, the antibacterial effect of *Hibiscus sabdariffa* L.

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Received: 8 December 2020
Revised: 19 July 2021
Accepted: 25 July 2021
Available Online: 1 August 2021

against biofilms of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, the causative bacteria of periodontitis, has not yet been studied. The aim of this study was to determine the effect of Roselle flower petals (*Hibiscus sabdariffa* L.) extract on *Fusobacterium nucleatum* and *Porphyromonas gingivalis* biofilms in vitro.

Material and Methods

Study design and extract preparation

The study was conducted in the laboratory using a post-test only control group design. First, to make the roselle flower petals extract, flower petals were picked from a roselle (*Hibiscus sabdariffa* L.) plant, and the seeds were removed. The petals were weighed and dried in an open area at room temperature for 14-21 days until completely dry and hard. The dried petals were then weighed (1000g) and ground using a grinder. The extraction was obtained by the maceration method using ethanol absolute (Merck, Darmstadt).

Phytochemical assay

The ethanol extracts of the plants were tested for phytochemical assay to confirm the presence of alkaloids, saponins, tannins, phenolics, flavonoids, triterpenoids, steroids, and glycosides in roselle (*Hibiscus sabdariffa* L.) plant. The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

Bacterial culture

Fusobacterium nucleatum and *Porphyromonas gingivalis* were cultured on agar media by the stroking method, placed in an anaerobic jar at 37°C, and incubated for 24 h. Once the colonies were formed, they were placed in 25 mL of sterile Brain Heart Infusion (BHI) (Oxoid, Hampshire) broth. The tubes were closed and incubated in an incubator for 3×24 h at 37°C. The incubated bacterial suspensions were measured for colonial growth

using a microplate reader (SAFAS MP96, SAFAS, Monaco) at a wavelength of 490 nm until reaching a concentration of 10⁷ CFU/mL.

Biofilm assay

The biofilm assay was performed by first diluting *Fusobacterium nucleatum* ATCC 25586 and *Porphyromonas gingivalis* ATCC 33277. When the cultures reached 0.25-0.3 (1×10⁷ CFU/mL), they were distributed in a 96-well flat-bottom microplate (Biologix, Shandong). These cultures were then inoculated in BHI broth for 2×24 h at 37°C under an anaerobic atmosphere. At following, they were added with roselle flower petals extract (*Hibiscus sabdariffa* L.) at different concentrations (100%, 50%, 25%, 12.5%, 6.25%, 3.12%, and 1.5%), and the inhibitory effect was observed after 1, 6 and 24 h. The bacteria were stained with violet crystal (0.5% w/v). The biofilms were counted using a microplate reader (SAFAS MP96, SAFAS, Monaco) at a 490 nm wavelength, adding 200 µL ethanol absolute (Merck, Darmstadt) for 15 min. An additional biofilm was tested without roselle flower petals extract as a negative control and another with 0.2% chlorhexidine as a positive control.

Statistical analysis

The data were analyzed by the Shapiro-Wilk normality test and homogeneity test. If the data were normally distributed (p>0.05), one-way ANOVA tests were performed at a significant difference of p<0.05 along with the post-hoc least significant difference (LSD) tests at p<0.05 using SPSS for Macintosh, version 25 (IBM, New York).

Results

Based on [table 1](#) the phytochemical analysis at the *Balai Penelitian Tanaman Rempah dan Obat* (Balitro) Laboratory, the roselle flower petals extract contained alkaloids, saponins, flavonoids, and glycosides. Also, the results showed that the

Table 1 phytochemical assay result

No.	Example	Type of Testing	Test Result	Test Method
1.	Roselle Flower Petals Extract	Phytochemical Test:		Qualitative
		- Alkaloid	+	
		- Saponin	+	
		- Tannin	-	
		- Phenolic	-	
		- Flavonoid	+	
		- Triterpenoid	-	
		- Steroid	-	
		- Glycoside	+	

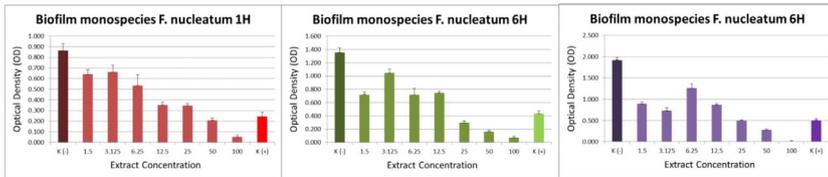


Figure 1 Result of concentration of roselle flower petals extract effect on the monospecies biofilm of *F. nucleatum* at an incubation period of 1, 6 and 24 h

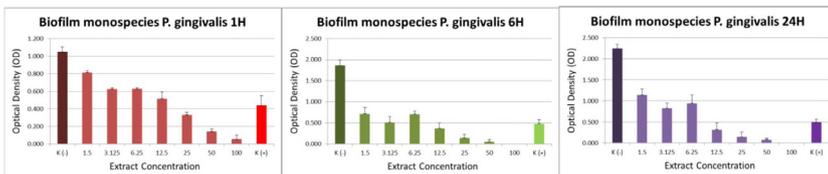


Figure 2 Result of concentration of roselle flower petals extract effect on the monospecies biofilm of *P. gingivalis* at an incubation period 1, 6 and 24 h

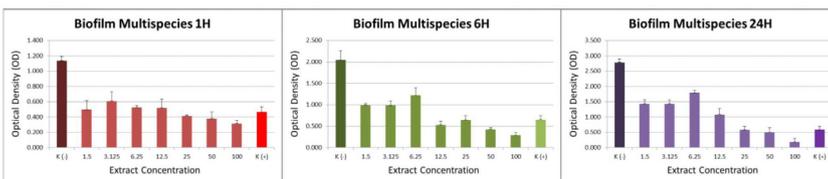


Figure 3 Result of concentration of roselle flower petals extract effect on multispecies biofilm at an incubation period of 1, 6 and 24 h

extract inhibited the formation of *F. nucleatum* and *P. gingivalis* biofilms. The best concentration for inhibiting the biofilm formation of *F. nucleatum* and *P. gingivalis* was 100% (w/v) for 24 h ($p < 0.05$). The highest concentration of 100% (w/v) is expected to have the highest amount of antibacterial substances for inhibiting the biofilms.

Discussion

In this study, roselle flower petals extract was found to be effective at inhibiting *F. nucleatum* and *P. gingivalis* biofilms. This research is also supported by a previous study that found that roselle flower extract could reduce *P. gingivalis* growth, with higher concentrations producing greater inhibition of biofilm formation.¹⁵ There are compounds in roselle flower petals that exercise an antibacterial effect through different mechanisms of action. For example, alkaloids exert an antibacterial effect by interrupting the formation of peptidoglycan bacterial cells, affecting their formation and causing cell death through the rupture of cell walls (lysis).¹⁶ Alkaloids also inhibit DNA synthesis by inhibition of topoisomerase.¹⁷

The glycosides in the roselle flower petals extract analyzed herein were saponin and flavonoid. Saponin destroys the cytoplasm membrane

and affects the membrane permeability of bacterial cells, causing the exchange of intra- and extracellular substances to be uncontrolled. In this case, substances such as enzymes, amino acids, nutrients, and water can also exit the cell, inhibit the bacterial growth.¹⁸ This mechanism of action can also lead to protein and enzyme leakage from the cell.¹⁹ In addition, the antibacterial activity of saponin is due to the active compounds on its surface that act like detergents, decreasing the surface tension of bacterial cells.²⁰ It can then pass through the outer membrane and vulnerable cell walls and bind to the cytoplasm membrane, decreasing the stability of bacterial cells and causing the cytoplasm to eventually exit the cell, resulting in cell death.²¹

Flavonoids has three different mechanisms that work synergistically as an antimicrobial agent. They inhibit nucleic acid synthesis, cell membrane functioning, and energy metabolism.²² Specifically, in inhibits nucleic acid synthesis, ring A and compound B from flavonoids play an important role in the process of interrelation or hydrogen bonding by accumulating nucleic acid bases thus inhibiting DNA and RNA. Flavonoids exhibiting this activity were robinetin, myricetin and (-)-epigallocatechin. Protein and lipid synthesis were also affected but to a lesser extent. The authors suggested that the B ring of the flavonoids may play a role in intercalation or hydrogen bonding with the stacking of nucleic acid bases and that this may explain the inhibitory action on DNA and RNA synthesis.²³ Flavonoids can also damage the permeability of bacterial cells, microsomes, and lysosomes as the interaction of flavonoid and bacterial DNA.²⁴

Meanwhile, the mechanism of action by which flavonoids inhibit membrane cell functioning is through forming complex compounds with dissolved extracellular proteins and damaging bacterial membrane cells, causing intracellular compounds to discharge.²⁵ Furthermore, interrupted membrane permeability inhibits binding to enzymes such as ATPase and phospholipase.²⁶ Finally, energy is required by bacteria for macromolecule biosynthesis. However, flavonoids obstruct energy metabolism by inhibiting the use of oxygen by bacteria and by inhibiting cytochrome C reductase, which in turn inhibits cell metabolism.²⁴

Conclusion

Roselle flower petals extract is effective at inhibiting *F. nucleatum* and *P. gingivalis* biofilms. This antibiofilm agent may be developed as an alternative therapy for preventing periodontal disease. Further studies are needed to explore the mechanism of actions of the compounds contained within.

Acknowledgment

Authors would like to thank Faculty of Dentistry and Microbiology Center of Research and Education (MiCORE) laboratory for the support for this study.

Conflict of Interest

The authors report no conflict of interest.

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