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## **INHIBITION OF MARINE ALGAE EXTRACTS ON *PORPHYROMONAS GINGIVALIS* ORAL BIOFILM FORMATION**

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### **ABSTRACT**

Oral biofilm of *Porphyromonas gingivalis* as main etiological factor of chronic periodontitis may be addressed as target of periodontotherapy. Marine algae synthesize various secondary metabolites with potential inhibitory and reducing activities toward bacterial biofilm formation. In this research, red algae (*Gracilaria* sp. and *Botryocladia* sp.) and green algae (*Enteromorpha* sp., *Halimeda opuntia*, *Caulerpa sertularioides*, *C. racemosa*, and *Codium* sp.) were investigated for their antibiofilm potentials through removing *P. gingivalis* biofilm *in vitro*. Algae were dried and extracted in ethanol, followed by evaporation and freeze-drying to produce algae crude powder. Algae extracts at various concentrations (5-250 µg/mL) were tested for their antibiofilm activity by performing *in vitro* *P. gingivalis* biofilm on the 96-well microtiter plate and quantifying the remaining biofilm cells using crystal violet assay. Three of 7 ethanolic algae extracts, i.e. *Botryocladia* sp., *H. opuntia*, and *C. sertularioides*, showed significant reducing activity against the existing *P. gingivalis* biofilm. The highest antibiofilm activity was reached by green algae extract of *C. sertularioides*. At 5 µg/mL, *C. sertularioides* reduced up to 50% *P. gingivalis* biofilm after 15 min exposure time. Our results suggest that selected marine algae may be potentially applied for periodontotherapy via reducing the existing oral bacterial biofilm *in vitro*.

**Key Words:** Marine algae, *Porphyromonas gingivalis*, Oral biofilm, Antibiofilm activity.

### **INTRODUCTION**

Algae are a very diverse group of marine organism, high morphological variation, mostly photoautotrophic ranging from unicellular to multicellular form. Multicellular algae are divided into three groups, red algae (Rhodophyta), green algae (Chlorophyta) and brown algae (Phaeophyta) (Diaz-Pulido and McCook, 2008). Algae are vulnerable to microbes ability to occupy every conceivable habitat for life, they will grow and form an adherent structure called biofilm. The formation of biofilm can have detrimental effects on the host organism (Rao *et al.*, 2006).

Naturally, algae have used chemical defense

mechanism against biofilm by producing bioactive compounds either a primary metabolites or secondary metabolites, including carotenoids, terpenoids, xanthophylls, chlorophyll, vitamins, saturated and polyunsaturated fatty acids, amino acids, acetogenins, polyphenols, alkaloids, halogenated compounds and polysaccharides such as agar, carrageenan, proteoglycans, alginate, laminaran, rhamnan sulfate, galactosyl glycerol and fucoidan (de Almeida *et al.*, 2011). Based on this defense mechanism, algae could have high potential for medical treatments which involve biofilm formation as the major cause of disease.

Periodontal disease is a disorder of teeth supporting structures, including the gingiva, periodontal ligament and alveolar bone (Kesic *et al.*, 2008). Healthy gingival sulcus has a flora dominated by equal proportions of Gram-positive cocci, especially *Streptococcus* spp, and

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*Actinomyces* sp. However, the proportions of strict anaerobic, Gram-negative, and motile organisms increase significantly in accordance with increasing severity of diseases. Major Gram-negative periodontopathogens are identified, including *Porphyromonas gingivalis*, *Prevotella intermedia*, *Bacteroides forsythus*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Capnocytophaga* sp., and *Campylobacter rectus* (Periasamy and Kolenbrander, 2009). Also, it is reported that the main etiological factor in periodontal disease is biofilm of anaerobic bacteria in particular periodontopathogens (Wolff *et al.*, 1994).

Chemical synthetic agent such chlorhexidine is commonly used for treatment of periodontal disease. However, prolong treatment by this agent stimulates tooth staining (Addy and Hunter, 2005). Therefore, exploration of marine organisms for their properties on inhibition and elimination of oral biofilm may offer potential strategic for management of periodontal diseases. This study was aimed to investigate antibiofilm effect of marine red and green algae on reduction of *P. gingivalis* biofilm *in vitro*.

## MATERIALS AND METHODS

### Collection and extraction of marine algae

Seven marine algae were collected from Binuangen (Banten province) and Garut (West Java province) on April 2012. Red algae (*Gracilaria* sp. and *Botryocladia* sp.) and green algae (*Enteromorpha* sp., *Halimeda opuntia*, *Caulerpa sertulaioides*, *C. racemosa*, and *Codium* sp.) were identified by Dr. Rory A. Hutagalung (Faculty of Biotechnology, Atma Jaya Catholic University, Jakarta). Samples were freeze-dried and stored in vacuum bag at 4°C prior to extraction.

Marine algae were extracted using ethanol and incubated overnight. The supernatant were separated and the extraction procedures were repeated twice. Then, evaporation at 55°C with vacuum oven was used to separate the ethanol from the crude extract. The crude extract powder was stored at room temperature.

### Preparation of sample and artificial saliva

Marine crude powder was diluted in 100% dimethyl sulfoxide (DMSO) for preparation of the stock solutions with final concentrations at  $10^5$  and  $10^4$  µg/mL. Then, the stock solutions were diluted again with 25% DMSO until final concentrations at  $10^3$  and  $10^2$  µg/mL. The working solutions used for the antioxidant assay were arranged with concentration range of 0-250 µg/mL. For artificial saliva, a 1% type III mucin from porcine stomach (Sigma-Aldrich) was dissolved with adherence buffer and autoclaved at 12°C for 15 min.

### Preparation of bacterial strain

*Porphyromonas gingivalis* (ATCC 33277; American Type Culture Collection, Rockville, MD, USA) was a gift from Prof. Jae-Kwan Hwang (Department of

Biotechnology, Yonsei University, Korea). Brain heart infusion (BHI) agar was used for growth and maintenance of microorganism, while BHI broth supplemented with 3% (w/v) sucrose (BHIS) was used for the growth of bacterial biofilm. *P. gingivalis* was cultured and incubated anaerobically by using anaerobic gas pack (Anaerogen, Oxoid) in an anaerobic jar at 37°C for 24 h. Bacterial suspension used for biofilm assay was prepared by harvesting overnight cultures and the turbidity was adjusted by 0.5 McFarland standard with final concentration of  $10^5$  CFU/mL.

### In vitro biofilm formation

Biofilm formation was performed in 96-well polystyrene microtiter plate *in vitro* by modification method of (Yanti *et al.*, 2009). First, 50 µL of sterile mucin was pipetted into the plate, gently shaken for 3 h. Then, the excess mucin was removed followed by overnight air-drying in the laminar flow cabinet. For positive control (untreatment) and treatment (algae extracts), a 200 µL of *P. gingivalis* inoculum at  $10^5$  CFU/mL cell density was inoculated to each well, while for negative control, inoculum was substituted with 200 µL BHIS. The plates were anaerobically incubated for 24 h at 37°C.

Biofilm reduction and quantification. After 24 h incubation, bacterial inoculum was disposed, then biofilm in each well was treated with algae extracts at range concentrations of 5-250 µg/mL and 5-60 min exposure time. Furthermore, the extracts and unbound cells were discarded, while remaining biofilm was washed twice with 200 µL of 50 mM PBS (pH 7.2) and air-dried for 1 h in the laminar flow cabinet. The biofilm was quantified by crystal violet assay. Remaining biofilm in each well was stained with 110 µL of 0.4% crystal violet solution for 30 min, then was washed with 300 µL of sterile distilled water till the negative control wells turning colorless. Afterward, the biofilm cell was diluted with 200 µL of ethanol. A 100 µL of the solution was transferred to new plates. The absorbance of remaining biofilm cell in each plate was measured at 595 nm using microplate reader (Bio-Rad). The percentage of remaining biofilm was calculated using the equation as follow:  $(OD_{595} \text{ of the extract} - OD_{595} \text{ of the negative control}) / (OD_{595} \text{ of the positive control} - OD_{595} \text{ of the negative control}) \times 100\%$ . All experiments were performed quadruplet with triplicate independent repetition.

### Statistical analysis

Data were expressed as mean ( $n = 3$ ) and standard deviation (SD) by computational analysis from triplicate experiments. Statistical analysis of untreated and algae extract-treated *P. gingivalis* biofilms was performed by analysis of variance (SPSS 11.0 for Windows).

## RESULTS AND DISCUSSION

The results of *P. gingivalis* biofilm reduction

assay by treatment of algae extracts at range concentrations of 5-250 µg/mL with 5-60 min exposure time were shown in Table 1. Among all algae, the ethanolic extracts of *Botryocladia* sp., *H. opuntia*, and *C. sertularioides* extracts demonstrated potential reducing activity toward *P. gingivalis* biofilm *in vitro*. At concentration up to 25 µg/mL and exposure time of 30 min, all algae extracts removed approximately 50% of the existing *P. gingivalis* biofilm *in vitro*. In addition, *Botryocladia* sp. and *H. opuntia* extracts at lowest concentration (5µg/mL) reduced 67 % and 58 % of the existing *P. gingivalis* biofilm after 30 min exposure time. Interestingly, *C. sertularioides* extract at 5 µg/mL showed the highest reducing activity up to 50% within 15 min of exposure time in comparison with other extracts.

*P. gingivalis* biofilm is associated with periodontitis. Various treatments of the elimination of *P. gingivalis* cells, the inhibition of *P. gingivalis* biofilm formation, and reduction of the existing *P. gingivalis* biofilm could prevent and manage periodontitis (Gurenlian, 2007). In this study, we focused on investigation of antibiofilm effect of several marine green and red algae through reducing the existing *P. gingivalis* biofilm *in vitro*. Researches of searching for antibiofilm agents are still limited to the used of synthetic chemical agents and few plant natural products. However the exploration of marine natural products for antibiofilm candidates is not yet reported.

Our study demonstrated that algae extracts of *Botryocladia* sp., *H. opuntia*, and *C. sertularioides* exerted antibiofilm activity toward *P. gingivalis* biofilm *in vitro* (Table 1). A clinical study using *Enteromorpha linza* marine extract in mouthrinse showed that the extract significantly reduced plaque, improved the condition of gingival tissues, and reduced the bleeding after 6 weeks of treatment. Moreover, *E. linza* extract also inhibited the bacterial growth of *P. gingivalis* and *P. intermedia* (Cho *et al.*, 2011). Recent study on furanone, a secondary metabolite derived from red marine algae, demonstrated that it was able to reduce the biofilm thickness of *Bacillus*

*subtilis* up to 25% (Ren *et al.*, 2002).

Previous studies described that synthetic agents such as chlorhexidine gluconate, oxantel and azithromycin (AZM) were also reported for their potential effects as antibiofilm agents against *P. gingivalis* biofilm *in vitro*. Chlorhexidine gluconate at 12 mg/L for 24 h exposure time reduced approximately 65% of the existence of *P. gingivalis* biofilm (Solmaz *et al.*, 2012). *P. gingivalis* biofilm treated with AZM at 0.125 µg/mL for 3 days exerted up to 80% reducing activity compared to that of control group (Maezono *et al.*, 2011). Oxantel treatment at 125 µM for 30 min also effectively reduced up to 8 µm of *P. gingivalis* biofilm thickness (Dashper *et al.*, 2010). These synthetic agents seemed very effective in reducing *P. gingivalis* biofilms, but prolonged treatment may lead to side effects. The usage of chlorhexidine gluconate could cause toothstaining, while other synthetic antibiotic such metronidazole and minocycline might stimulate bacterial resistance (Noiri *et al.*, 2003; Addy and Hunter, 2005).

Nowadays, natural product research targeting periodontotherapy with potential reducing activity, low side effects, and safe efficacy has become the focus of the investigation. Hosainzadegan & Delfan (2007) reported that the exposure of dentol 10% (w/w), an essential oil derived from *Satureja khuzestanica*, to *P. aeruginosa* and *S. aureus* biofilms significantly reduced the existing biofilms up to 67% reducing activity (Hosainzadegan and Delfan, 2007). In other study, after a 30 min exposure with macelignan isolated from *Myristica fragrans* at 10 µg/mL, the entire primary colonizer bacteria biofilm *in vitro* was significantly removed up to 50% (Yanti *et al.*, 2008).

Mechanisms of the reduction of *P. gingivalis* biofilm in this research are still not certainly known. Nevertheless, they can be predicted based on other previous studies. The marine algae extracts may contain primary or secondary metabolites which have antibiofilm activity, such as bacteria growth inhibitor, quorum sensing inhibitor (quorum quenching), and disruption of biofilm (Ren *et al.*, 2002; Long *et al.*, 2003; Aciades and Riquelme, 2008; Molobela *et al.*, 2010).

**Table 1. Effect of algae extracts at various concentrations and exposure times on reduction of the existing *P. gingivalis* biofilm *in vitro***

Percentage of biofilm remaining after crude extract treatment (% ± SD)*							
Sample	Exposure time (min)	Concentration (µg/mL)					
		5	10	25	50	100	250
<i>Gracilaria</i> sp. (Rhodophyta)	5	64 ± 3	68 ± 8	73 ± 5	61 ± 5	55 ± 7	53 ± 7
	10	70 ± 9	80 ± 9	91 ± 1	64 ± 12	80 ± 2	69 ± 1
	15	70 ± 4	82 ± 7	80 ± 6	67 ± 5	70 ± 9	75 ± 5
	30	41 ± 7	71 ± 8	68 ± 5	46 ± 7	49 ± 5	69 ± 9
	60	50 ± 7	32 ± 7	32 ± 9	62 ± 0	61 ± 7	38 ± 7
<i>Botryocladia</i> sp. (Rhodophyta)	5	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	10	88 ± 2	78 ± 8	75 ± 6	81 ± 7	82 ± 7	85 ± 8
	15	96 ± 8	59 ± 6	55 ± 4	70 ± 5	46 ± 2	94 ± 8
	30	33 ± 3	40 ± 6	51 ± 4	50 ± 4	51 ± 4	39 ± 9
	60	46 ± 6	42 ± 5	37 ± 7	29 ± 8	28 ± 6	27 ± 7

<i>Halimeda opuntia</i> (Chlorophyta)	5	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	10	97 ± 4	82 ± 2	86 ± 1	82 ± 9	94 ± 2	78 ± 6
	15	71 ± 7	55 ± 3	56 ± 5	74 ± 3	85 ± 8	98 ± 5
	30	42 ± 3	60 ± 9	52 ± 5	56 ± 3	64 ± 5	52 ± 8
	60	36 ± 7	34 ± 3	35 ± 6	34 ± 9	36 ± 6	43 ± 4
<i>Caulerpa sertularioides</i> (Chlorophyta)	5	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	10	100 ± 0	94 ± 8	89 ± 11	95 ± 3	92 ± 9	92 ± 7
	15	52 ± 9	52 ± 6	53 ± 5	55 ± 3	65 ± 7	51 ± 1
	30	64 ± 8	49 ± 7	54 ± 0	45 ± 1	76 ± 9	54 ± 6
	60	35 ± 7	42 ± 9	31 ± 8	27 ± 5	33 ± 2	50 ± 6
<i>Enteromorpha</i> sp. (Chlorophyta)	5	36 ± 3	57 ± 2	44 ± 6	51 ± 5	52 ± 9	74 ± 2
	10	48 ± 8	42 ± 4	49 ± 3	45 ± 7	48 ± 8	55 ± 7
	15	51 ± 6	43 ± 5	48 ± 8	42 ± 4	45 ± 4	44 ± 6
	30	57 ± 7	57 ± 9	54 ± 4	51 ± 6	45 ± 5	51 ± 6
	60	49 ± 3	49 ± 8	55 ± 7	49 ± 7	45 ± 6	40 ± 1
<i>Caulerpa racemosa</i> (Chlorophyta)	5	67 ± 0	68 ± 6	49 ± 6	39 ± 3	51 ± 3	79 ± 3
	10	42 ± 9	49 ± 3	48 ± 8	46 ± 2	54 ± 4	49 ± 8
	15	44 ± 9	69 ± 5	50 ± 6	49 ± 5	46 ± 2	55 ± 2
	30	53 ± 7	58 ± 6	45 ± 6	50 ± 6	47 ± 9	52 ± 5
	60	52 ± 11	46 ± 9	44 ± 7	51 ± 7	44 ± 3	47 ± 7
<i>Codium</i> sp. (Chlorophyta)	5	74 ± 7	70 ± 7	81 ± 6	43 ± 9	64 ± 7	68 ± 3
	10	62 ± 8	68 ± 8	73 ± 3	59 ± 6	64 ± 3	70 ± 6
	15	79 ± 7	71 ± 2	78 ± 13	80 ± 4	50 ± 9	54 ± 2
	30	53 ± 6	57 ± 8	62 ± 7	53 ± 7	47 ± 8	56 ± 4
	60	46 ± 5	58 ± 4	48 ± 3	50 ± 9	53 ± 5	56 ± 4

\*values are expressed as the percentage of absorbance (595 nm) of cells in treated well compared with that in untreated wells (considered 100%), SD is standard deviation of the mean percentage of absorbance (595 nm) of cells derived from three times of independent experiment and quadruplet (wells) per experiment.

## CONCLUSION

The algae extract of *C. sertularioides* possessed the highest antibiofilm activity through reduction of the existing *P. gingivalis* biofilm *in vitro*. Further research is needed to get more understanding of the reducing molecular mechanisms, while acquiring purified bioactive compounds that exert antibiofilm activity towards *P.*

*gingivalis* oral biofilm.

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