Antibacterial Activity of Sea Cucumber Extract to *Porphyromonas gingivalis* in vitro

Dian Mulawarmanti
Kristanti Parisihni
Yoifah Rizka Wedarti

Abstract

Background : Periodontitis is the disease of periodontal tissue which is the second common oral disease worldwide and affected the systemic health in general. *Porphyromonas gingivalis* is one of main periodontopathogenic bacteria related to periodontitis. The recent local adjunctive therapy in periodontitis is antibiotic treatment. Sea cucumber is a marine vertebratae used as traditional medicine and has been known to have antibacterial properties so it's potentially be explored as antibacterial agent in dentistry. **Aim** : to examine the antibacterial activity of sea cucumber extract *Sticopus hermanii and Holothuria atra* to *Porphyromonas gingivalis*. **Methods :** The study is an experimental laboratory research with post test only control group design. The samples were divided into 5 groups each consisted of 6 samples i.e : positive control was given tetracycline, negative control was given DMSO 1%, treatment group were given each of *Sticophus hermanii* and *Holothuria atra* extract with concentration of 20%, 40% and 80%. Antibacterial activity test was performed by disk diffusion method on Mueller Hinton agar. Diameter of inhibition zone was measured with digital caliper. **Result** : All treatment groups in all concentration showed inhibition zones but less than tetracycline as positive control. Anova and LSD statistical analysis showed the significant difference on *Sticophus hermanii* extract 40% and 80% and *Holothuria atra* extract 80% compared to negative and positive control group ($p<0,05$). **Conclusion :** *Sticophus hermanii* extract on the concentration of 40%, 80% and *Holothuria atra* extract on the concentration of 80% have antibacterial activity against *Porphyromonas gingivalis.*

Key words : Sticopus hermanii , Holothuria atra, Porphyromonas gingivalis

Correspondence: Dian Mulawarmanti, Department of Oral Biology, Faculty of Dentistry Hang Tuah University, Surabaya – Indonesia, Jl. Arif Rahman Hakim 150, Surabaya 60111 Indonesia, Telp 031-5912191, e-mail : dianmulawarmanti@yahoo.com

Background

Millions of people worldwide suffered from periodontitis, the second common oral disease which affected the systemic health in general[1] Periodontitis is an inflammation condition in periodontal tissue when continued untreated could cause futher progressive destruction to alveolar bone resulted in tooth mobility and tooth loss[2,3]. Periodontitis is one on he risk factor for cardiovascular disease[4], diabetes mellitus5, rheumatoid arthritis[6]. The proper treatment of periodontitis will not only restore oral health but also reduce the risk of systemic disease.

The primer cause bacterial etiology of periodontal disease are *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans,* which *P. gingivalis* is related

to chronic periodontitis while *A. actinomycetemcomitans* have its role on aggressive periodontitis [3,7].

Therapy of periodontitis consisted of mechanical treatment i.e : scaling and rootplaning and also adjunctive therapy of local antimicrobial agent[3]. Tetracycline is the common antimicrobial agent used to be combined with scaling in chronic periodontitis therapy and proved to increase the efficacy of treatment by inhibiting extracellular collagenase activity of mammalian osteoblast and neutrophil. Tetracycline could be given as systemic or local treatment, but local treatment is preferable regarding to the possibility to obtain the higher concentration and less the risk of bacterial resistance[8]. As for numerous other microorganisms, increasing antibiotic resistance among oral bacteria may also be a potential major public health problem. *P. gingivalis* has been know to be susceptible to tetracycline but some repost stated the resistance in sub gingival *P. gingivalis* isolates and the ones from odontogenic abcess. The level of resistance varies between countries, which can be attributed to the different use of antibiotics[9]. The potential candidate for antibacterial agent from natural resources will be beneficial and could be the alternative antibacterial agent regarding to this matter.

Indonesia is a maritime country that rich with the sea natural source. Some marine biota has been explored mainly for food industry, but it has been known that some species has been consumed as traditional medicine regarding to its medically content[10-14].

Research in medical potential of marine biota has been developed recently in marine pharmacology, it has been known that some marine biota has it potential medical use regarding its content[16]. Some sea cucumber species has been stated to have antibacterial effect. Alcoholic extract of *H. scabra* dan *H. miliaris* showed high potential antimicrobial against *Aeromonas hydrophila, Escherichia coli, Enterococcus sp, Klebsiella pneumonia, Pseudomonas aeruginosa, Salmonella typi, Staphylococcus aureus, Vibrio harveyi*[10]. Another sea cucumber *Sticophus variegatus* could inhibit *Escherichia coli, Pseudomonas* sp., *V. vionovica,* while *Sticophus cholronatus* and *Sticophus hermanii* could inhibit *Staphylococcus aureus, Escherichia coli, Vibrio anguila, V. vionovica*[15] .

Our previous study showed that sea cucumber extract of *Sticopus hermanii* had antibacterial activity against mixed periodontopathogenic bacteria of periodontitis[17], and decreased the mandibular bone resorpstion on wistar rat that have been induced by mixed periopathogenic bacteria[17,18].

The content of saponin and triterpene glycoside in sea cucumber extract assumed to be have the important role in its antibacterial bioactivity. Sea cucumber is natural source agent so, in the certain safe concentration is expected to be compatible to fibroblast cell of oral cavity mucosa. Regarding to its antibacterial potency, it is promising candidate to be explored as topical antimicrobial use in adjunctive treatment of periodontitis. In this study, the extract of two sea cucumber species: *Sticopus hermanii* and Holothuria *atra* will be tested its antibacterial activity against *P. gingivalis.* Considering that the two species of sea cucumber are plenty found in Jawa coastal, the exploration of the antibacterial activity will add more value as the potential therapy of oral disease.

Material and Methods

The study is an experimental laboratory research with the design of post test only control group design. Methanolic extract of *Sticopus hermani* and *Holothuria atra*

will be tested its antibacterial activity to *Porphyromonas gingivalis* ATCC 33277 by disk diffusion method.

Extract preparation

Adult sea cucumber weight 100-250 gr were collected from Karimunjawa coastal, washed by running water to clean the dirt, immersed in fresh water for one night to remove the salt and adherent parasite. Sea cucumber then splitted, the inner abdomen were removed then cleaned and washed, so only the flesh of the body proceed to next process.

Thirty five (35) sea cucumber sample were cut into small pieces of 3-10 cm, the wet weight then measured then dried up in solar dryer for 3-4 days to reduce the water content. The dried sea cucumber then cut into smaller pieces of 1 cm, mashed by blender the the weight were measured and ready for the maceration process. Two hundred and fifty (250) gram mashed dry sea cucumber sample immersed until soaked in 500 mL methanol solvent for 24 hour at room temperature, then filtered with filter paper to separate filtrate and residue.

Residue then reimmersed in 500 mL methanol solvent for 24 hour, again filtered with filter paper to separate filtrate and residue, resulted in maceration filtrate with the ratio of 250 gram sample / 1000 mL solvent (1:4 w/v).

Methanol (polar) filtrate got homogenized with 1000 mL hexane solvent (non polar) then performed partition with separatory funnel the each of the filtrate layer of methanol and hexane solvent were separated.

Methanol (polar) filtrate then got re-homogenized with 1000 mL chloroform solvent (semi polar), performed partition with separatory funnel the each of the filtrate layer of methanol and chloroform solvent were separated. Each filtrate were separated by its solvent with rotary evaporator until extract produced. The evaporated extract then placed in the vial and stored in -70^{0}C.

Bacterial suspension preparation

Stock of *Porphyromonas gingivalis* ATCC 33277 was obtained from Balai Laboratorium Kesehatan Daerah DIY. Pure culture were prepared by inoculating one single loop of bacterial colony to blood agar médium, incubated anarobically with CO2 in 37^{0}C for 2x24 hour. Bacteria then innoculated in BHI broth, adusted its turbidity to stándard McFarland 0,5 by nephelometer Phoenix.

Antibacterial activity testing by disk diffusion method

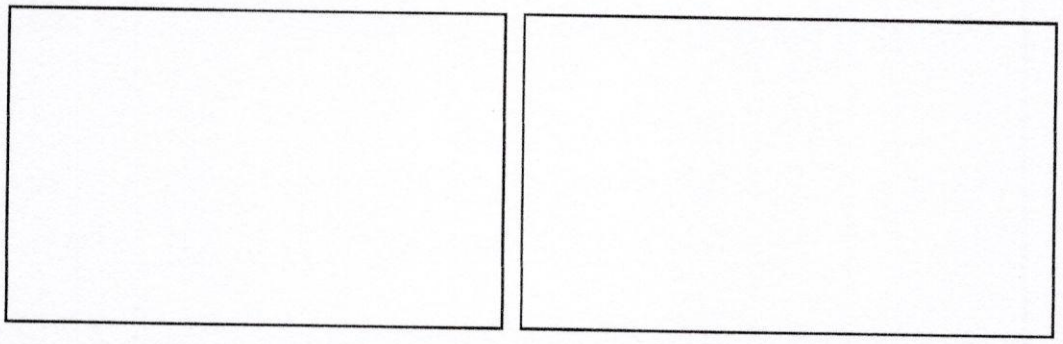
The samples were divided into 5 groups each consisted of 6 samples i.e : positive control was given tetracycline, negative control was given DMSO 1%, treatment group were given each of *Sticophus hermanii* and *Holothuria atra* extract with concentration of 20%, 40% and 80%. Antibacterial activity test was performed by disk diffusion method on Mueller Hinton agar.

Bacterial supension of *Porphyromonas gingivalis* equal to 0,5 McFarland was swabbed inoculated onto Muller Hinton agar plate. Sterile paper disks were immersed into each contrentation of extracts for treatment groups, for control negative groups in DMSO 1%, each for 15 second, and for the positive control group tetracycline disk were applied, then put on to Muller Hinton agar, gently pressed for a while and leave, incubated anarobically in 37^{0}C for 2x24 hour.

The clear zone around the disk showed inhibition effect to the growth of *Porphyromonas gingivalis*. Diameter of inhibition zone was measured with digital caliper.

Result

All the treatment groups and positive control showed the inhibition zones around the disk , but not the negative control as shown in fig 1.



a. b.

Fig 1. The inhibition zone on disk diffusion method of antibacterial activity of *Sticopus hermanii* extract (a) and *Holoturia atra* extract (b) in serial concentrations of 20%, 40%, 80, negative control (DMSO1%), positive control tetracycyline to *P. gingivalis*

The mean diameter of inhibition zone of the two extracts in all concentration vary from 6,38 – 8,82 mm on *Holothuria atra* , and 6,31 – 9,92 mm on Sticophus *hermanii*, less than the diameter of tetracycline on the positive control group which is 50,97-56.74 mm.

Table 1 The mean diameter of inhibition zone of *Sticophus hermanii* and *Holothuria atra* extracts on serial concentration extracts to *Porphyromonas gingivalis* compared to control groups (stated in mm)

GROUP		*Stichopus hermanii*	*Holoturia atra*
Positive control group	Mean	56,0933	53,4850
	Std. Deviation	1,04280	1,50078
Negative control group	Mean	5,5000	5,5000
	Std. Deviation	,00000	,00000
Consentration 20%	Mean	6,5500	6,4550
	Std. Deviation	,61511	,09182
Consentration 40%	Mean	6,9950	6,6433
	Std. Deviation	,30231	,11742
Consentration 80%	Mean	8,9333	7,7067
	Std. Deviation	,78429	,57812

Fig 2 The graphic of inhibition zone of *Sticophus hermanii* and *Holothuria atra* extracts on serial concentration extracts to *Porphyromonas gingivalis* compared to control groups

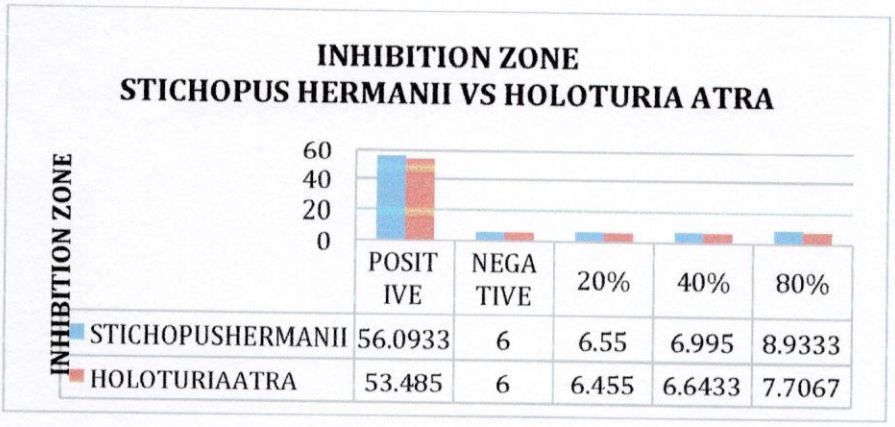


Table 2. ANOVA and LSD test summary of inhibition zone of *Sticophus hermanii* and *Holothuria atra* extracts on serial concentration extracts to *Porphyromonas gingivalis* compared to control groups

Dependent Variable	(I) GROUP	(J) GROUP	Mean Difference (I-J)	Sig.
STICHOPUS HERMANII	POSITIVE CONTROL GROUP	NEGATIVE CONTROL GROUP	50,0933*	,000
		CONSENTRATION 20%	49,5433*	,000
		CONSENTRATION 40%	49,0983*	,000
		CONSENTRATION 80%	47,1600*	,000
	NEGATIVE CONTROL GROUP	POSITIVE CONTROL GROUP	-50,0933*	,000
		CONSENTRATION 20%	-,5500	,161
		CONSENTRATION 40%	-,9950*	,015
		CONSENTRATION 80%	-2,9333*	,000
	CONSENTRATION 20%	POSITIVE CONTROL GROUP	-49,5433*	,000
		NEGATIVE CONTROL GROUP	,5500	,161
		CONSENTRATION 40%	-,4450	,253
		CONSENTRATION 80%	-2,3833*	,000
	CONSENTRATION 40%	POSITIVE CONTROL GROUP	-49,0983*	,000
		NEGATIVE CONTROL GROUP	,9950*	,015
		CONSENTRATION 20%	,4450	,253
		CONSENTRATION 80%	-1,9383*	,000
	CONSENTRATION 80%	POSITIVE CONTROL GROUP	-47,1600*	,000
		NEGATIVE CONTROL GROUP	2,9333*	,000
		CONSENTRATION 20%	2,3833*	,000
		CONSENTRATION 40%	1,9383*	,000
HOLOTURIA ATRA	POSITIVE CONTROL GROUP	NEGATIVE CONTROL GROUP	47,4850*	,000
		CONSENTRATION 20%	47,0300*	,000
		CONSENTRATION 40%	46,8417*	,000
		CONSENTRATION 80%	45,7783*	,000
	NEGATIVE	POSITIVE CONTROL GROUP	-47,4850*	,000

	CONTROL GROUP	CONSENTRATION 20%	-,4550	,286
		CONSENTRATION 40%	-,6433	,135
		CONSENTRATION 80%	-1,7067*	,000
	CONSENTRATION 20%	POSITIVE CONTROL GROUP	-47,0300*	,000
		NEGATIVE CONTROL GROUP	,4550	,286
		CONSENTRATION 40%	-,1883	,655
		CONSENTRATION 80%	-1,2517*	,006
	CONSENTRATION 40%	POSITIVE CONTROL GROUP	-46,8417*	,000
		NEGATIVE CONTROL GROUP	,6433	,135
		CONSENTRATION 20%	,1883	,655
		CONSENTRATION 80%	-1,0633*	,017
	CONSENTRATION 80%	POSITIVE CONTROL GROUP	-45,7783*	,000
		NEGATIVE CONTROL GROUP	1,7067*	,000
		CONSENTRATION 20%	1,2517*	,006
		CONSENTRATION 40%	1,0633*	,017

All treatment groups in all concentrations showed inhibition zones but less than tetracycline as positive control. Further statistical analysis by two-way ANOVA test and LSD multiple comparison test at 5% significance level showed the significant difference on *Sticophus hermanii* extract 40% and 80% and *Holothuria atra* extract 80% compared to negative and positive control group ($p<0,05$).

Discussion

The result of disk diffusion sensitivity test of the two sea cucumber extract *Sticophus hermanii* and *Holothuria atra* showed the inhibition zone on the growth of *P. gingivalis,* mean that these extracts had the potential of antibacterial activity. The more concentration yield the more inhibition zone in both extract, lower concentration of *Sticophus hermanii* of 40% resulted in significant antibacterial activity more than *Holothuria atra* 80% ($p<0,05$). Even so, the potency of antibanterial activity is less than tetracycline as the positive control.

In this study, tetracycline has been used as the positive control considering that it commonly used as the drug of choice against periodontopathogenic bacteria. Tetracycline antibiotics are protein synthesis inhibitors, inhibit bacterial protein synthesis by preventing the association of aminoacylt RNA with the bacterial ribosome. Therefore, to interact with their targets these molecules need to traverse one or more membrane systems depending on whether the susceptible organism is gram positive or gram negative. Tetracyclines traverse the outer membrane of gram-negative enteric bacteria through the OmpF and OmpC porin channels, as positively charged cation (probably magnesium)-tetracycline coordination complexes. The cationic metal ion-antibiotic complex is attracted by the Donnan potential across the outer membrane, leading to accumulation in the periplasm, where the metal ion-tetracycline complex probably dissociates to liberate uncharged tetracycline, a weakly lipophilic molecule able to diffuse through the lipid bilayer regions of the inner (cytoplasmic) membrane. Within the cytoplasm, tetracycline molecules are likely to become chelated since the internal pH and divalent metal ion concentrations are higher than those outside the cell. Indeed, it is probable that the active drug species

which binds to the ribosome is a magnesium tetracycline complex. Association of tetracyclines with the ribosome is reversible, providing an explanation of the bacteriostatic effects of these antibiotics[19]. Tetracyclines also have been found to inhibit matrix metalloproteinases. This mechanism does not add to their antibiotic effects, but has increase the efficacy of treatment by inhibiting extracellular collagenase activity[8].

Some sea cucumber extract has been known to have antibacterial properties regarding its content of saponin and triterpene glycoside[17]. Another class of compounds is saponins, commonly identified as holothurins, from sea cucumber. The structural features of these compounds are quite comparable to those of the bioactives from ganoderma, ginseng, and other medicinally popular tonic herbs. They have displayed a wide spectrum of biological effects such as hemolytic, cytostatic, antineoplastic, anticancer and antitumor activities. Sea cucumbers are rich in glycosides, particularly triterpene glycosides which are proven to have antibacterial and antitumor activities[21].

Mechanism of terpenoid or triterpene as antibacterial agent is to interact with porin in the bacterial outer membrane to form the strong polimer bond that make destruction to the porin that will reduce the cell wall permeability then caused the lost of nutrition which lead to the inhibition in bacterial growth.

The result of inhibitory zone in tetracycline id much higher than those of the *Sticophus hermanii* dan *Holothuria* atra extracts, probably influenced by the distinctive antibacterial mechanism of both, or the less potency of the sea cucumber extract that influenced by another factors.

Both *Sticophus hermanii* dan *Holothuria* atra have been extracted by methanolic extract[22]. The antibacterial compound of sea cucumber assumed to be polar for it is dissolved in methanol solvent and have been proven to have the antibacterial, antifungal and cytotoxic agent on some studies[10,12,21,22]. The sea cucumber extract studied in this research is the whole extract, which probably the optimum content of have not been explored and performed its optimal antibacterial activity. Saponin as triterpene glycoside has been known to have the immunomodulatory properties to induce macrophage as the response of infection[21] thus enhance the antibacterial activity, this property cannot be showed in in vitro by disk diffusion method, so further in vivo study is needed to be performed.

The antibacterial activity of *Sticophus hermanii* dan *Holothuria atra* to the

Conclusion

Sticophus hermanii extract on the concentration of 40%, 80% and *Holothuria atra* extract on the concentration of 80% have antibacterial activity against *Porphyromonas gingivalis*

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