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Short Communication: Evaluation of antimicrobial activity of hydroxyapatites from *Anadara granosa* and *Achatina fulica* against *Porphyromonas gingivalis*

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Abstract. *Anitasari S, Yuniati Y, Agustin S. 2019. Short Communication: Evaluation of antimicrobial activity of* Anadara granosa *and* Achatina fulica *hydroxyapatites against* Porphyromonas gingivalis. *Bioteknologi 16: 1-4.* Hydroxyapatite bioceramic (HAp) plays a key role in reconstructive surgeries in both medicine and dentistry. However, its use is often saddled with the occurrence of post-surgery infection, thereby creating the need for antimicrobial therapy. The antibiotics used should support the body's defense mechanisms. Applying antimicrobials locally after surgery is promising, but the concentration must be lethal to pathogens and must be safe for normal human flora, as most oral antibiotic therapies are not effective. This study aimed to create HAp with good antimicrobial activity without causing harm to normal human flora. Antimicrobial activity of various combinations of hydroxyapatites from *A. granosa* and *A. fulica* against *P. gingivalis* were determined by the *Kirby-Bauer* method. The results showed that the inhibitory zone of HAp *A. granosa* (8.3±0) was similar to positive control Chlorhexidine (8.5± 0.1), but no activity was found in HAp of *A. fulica.* The HAp combination of *A. granosa* and *A. fulica*, at the ratio of 6: 4, had the smallest inhibitory zone (3.5±0 mm). It can be concluded that the differences in antibacterial activity of HAp from *A. granosa*'s and *A. fulica*'s might be influenced by concentration and mineral of HAp. Based on the results, it can be concluded that the inhibitory concentration was obtained at the ratio of 6: 4.

Keywords: Antibacterial, hydroxyapatite, *Porphyromonas gingivalis*

INTRODUCTION

In this new era of biomaterials, the ability for the human body to accept materials used for producing medical devices should be considered. This can include several factors, i.e., (i) the chemical and biological properties of the materials, (ii) biomaterials biocompatibility, and (iii) recipient's health condition (Nathanael et al. 2018).

In the field of dentistry, biocompatibility is the material capability to be compatible with dentition, oral mucosa, and the entire body systems without causing injury or damage to the cells and tissues. Biomaterials are used to replace a part of the body in dentistry, but there have been many cases of graft failure due to bacterial infections in the tooth area. Therefore, biomaterials must have antimicrobial activity so that they can reduce the failure of the grafting process (Pandey et al. 2016). Antibacterial agents are in high demand for sanitary materials.

Therefore, new materials with antibacterial activities are widely studied (Pandey et al. 2016; Nathanael et al. 2018). Antimicrobials influence the biocompatibility of the material. One of the biomaterials used in the dentistry field is hydroxyapatite. It is bioceramic with excellent biocompatibility, bioactivity, osteoinductive, and osteoconductive properties. This material is widely used to repair and reconstruct bone damage in the human skeleton. The process of filling the bone defect to repair was called augmentation or guided bone regeneration (Pandey et al. 2016). The hydroxyapatites from *Anadara granosa* and *Achatina fulica* are composed of calcium phosphate, both have antimicrobial activity against gram positive and negative bacteria, yet their combined mechanism of action has not been revealed (Berniyanti et al. 2007; Santana et al. 2012; Pandey et al. 2016). When two antimicrobial agents are combined, the effect of the combination may equal the sum of the antimicrobial effects of its components (addition), it may exceed this sum (synergism), or it may be smaller (antagonism) (Hafidh et al. 2011).

The study aims to determine whether the combination of hydroxyapatites from *A. granosa* and *A. fulica* has antimicrobial properties against *Prophyromonas gingivalis*.

MATERIALS AND METHODS

Materials

The sampling and collection of *Anadara granosa* and *Achatina fulica* was conducted in Samarinda, East Kalimantan, Indonesia; and identification was carried out in the Ecology and Animal Systematics Laboratory, Mulawarman University, Samarinda, East Kalimantan, Indonesia.

Calcination of *Anadara granosa* and *Achatina fulica*

The shells of *A. granosa* and *A. fulica* were cleaned and dried. The process was continued with calcination twice. The first calcination was done at the temperature of 1000°C for 12 hours to get calcium, and at the second calcination was done at the temperature of 1000°C for 24 hours (Anitasari et al. 2018).

Hydroxyapatite synthesis (HAp synthesis)

The HAp synthesis was carried as follows: 1 M Ca $(OH)_2$ solution was prepared by weighing 24.81 grams of CaO in a beaker glass and mixed with 600 mL of distilled water. The solution was stirred using a magnetizer at a speed of 150 rpm until homogeneous. After being homogenous, the solution was added with 300 mL of 1.8 M phosphoric acid, heated on a hot plate at 40°C, and stirred for 60 minutes at a speed of 300 rpm and then left for 24 hours. The product was filtered with Whatman membrane and dried at 100°C for 3 hours. Finally, the sintering process was carried out at 900°C for 5 hours (Anitasari et al. 2018).

Antimicrobial sensitivity test

The culture medium of Mueller-Hinton Agar was prepared as follows: 38 gram of Mueller Hinton Agar was suspended in one liter of distilled water and heated with frequent agitation. It was then boiled for one minute to completely dissolve the medium, followed by autoclaving at 121°C for 15 minutes. 20-25 mL of the freshly prepared and cooled MHA was poured into sterile Petri dishes. After solidification, 100 µL of *P. gingivalis* bacteria cultured in nutrient broth (with a concentration of 0.5 Mc Farland Standard) were swabbed evenly on each of the agar plates using a sterile cotton bud and clearly labeled. After that, holes were on the surface of agar were made with a punch hole. A different combination of HAp was put into the hole. Chlorhexidine used as a positive control and sterile saline as a negative control. Finally, Petri dishes were incubated at 37°C for 24 hours, and the clear zone of inhibition was observed after incubation (Sudarna et al. 2011).

Statistical analysis

The data was expressed as means ± SE, and the statistical analysis was performed according to SPSS software version 23.00.

RESULTS AND DISCUSSION

Table 1 shows that the diameter of the inhibitory zone of HAp from *A. granosa* was similar to positive control chlorhexidine. It indicated that the HAp of *A. granosa* had significant antimicrobial activity against *P. gingivalis*. *P. gingivalis* is gram-negative anaerobic bacterium as the main cause of Periodontitis. It utilizes cysteine protease, called gingipains. The gingipain family includes two related proteases, Arg-gingipain and Lys-gingipain. The Arg-gingipains include two members, namely Arg-gingipain A (*RgpA*) and Arg-gingipain B (*RgpB*), encoded by two closely related genes, *RgpA* and *RgpB*. The Lys-gingipain (*kgp*) is encoded by a single gene, *kgp*, which is the major virulence of the *P. gingivalis* (Guo et al. 2010; Tang et al. 2011; Jerala et al. 2012). The HAp of *A. granosa* may have neutralized the *kgp* gene due to its capability to inactivate the process of cationic antimicrobial peptides on their surface. Different conditions were observed in the HAp of *A. fulica*, as they could not kill or inhibit the growth of the bacteria (Viera et al. 2004; Dos Santos et al. 2006; Verma et al. 2016). The combination of HAp of *A. granosa* and *A. fulica* was antagonistic (Table 2). Increasing HAp from *A. fulica* cause in decreasing the inhibitory zone against *P. gingivalis*. The HAp of *A. fulica* hindered the effect of *A. granosa* and the circumstances that influenced their antagonism enabled us to understand their benefit better.

The shells of *A. granosa* and *A. fulica* contained glycosaminoglycan (GAGs). The common GaGs include glycosaminoglycans (heparin, keratan sulfate, heparan sulfate, and hyaluronic acid), galactosaminoglycan (chondroitin sulfate and dermatan sulfate) and acharan sulfate which can only be found in snails (*A. fulica*) (Verma et al. 2016).

The glycosaminoglycans can inhibit protease enzymes, such as gingipain, but their activity depends on the pH and temperature of hydroxyapatites because there are several sensors in the proteases that can be activated by pH and temperature. Hence, it is still unexplainable that the HAp of *A. granosa* inhibits the growth of *P. gingivalis*, but the other one from *A. fulica* did not inhibit the growth of *P. gingivalis* (Hocevar et al. 2018).

Table 1. Antimicrobial activity of HAp of *Anadara granosa* and HAp of *Achatina fulica* by Kirby-Bauer Method (n=3).

	HAp *A. granosa*	**HAp *A. fulica***	**Control +**	**Control-**
Inhibition zone in diameter (mm)	8.3±0	0	8.5±0.1	0

Table 2. Antimicrobial activity of various combination of hydroxyapatites (*Anadara granosa: Achatina fulica*) by Kirby-Bauer Method (n=6).

	The ratio of Ag: Af										
	9: 1	**8: 2**	**7: 3**	**6: 4**	**5: 5**	**4: 6**	**3: 7**	**2: 8**	**1: 9**	**C+**	**C-**
Inhibition zone in diameter (mm)	7.8±0.2	5.1±0.	5±0	3.5±0.5	0	0	0	0	0	8.5±0.1	0

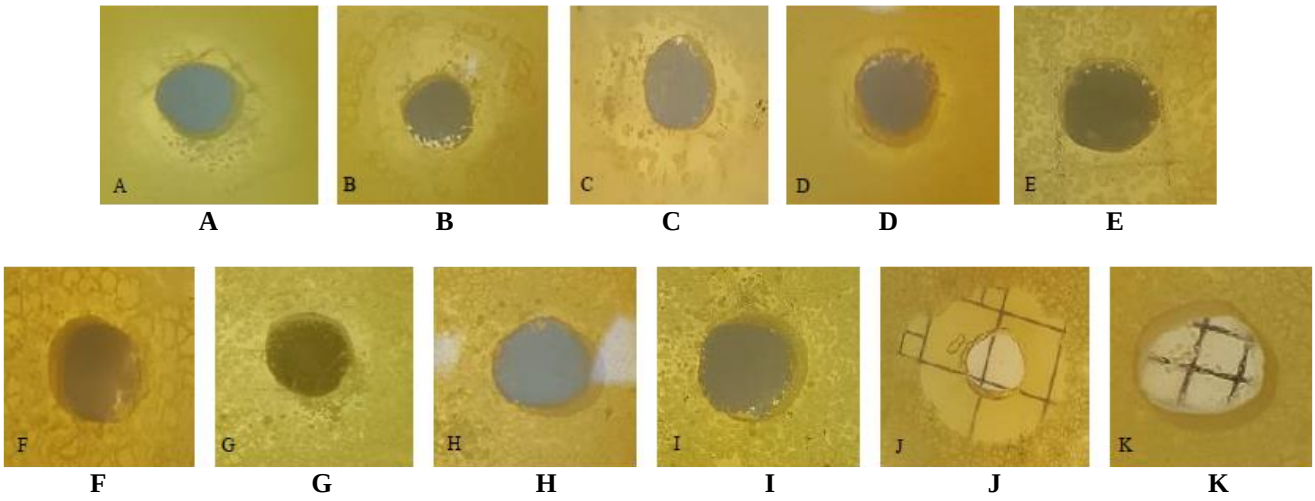

Figure 1. Photographs of inhibitory zone of various hydroxyapatite combination of *Anadara granosa* (Ag) and *Achatina fulica* (Af). A. Ag: Af (9: 1), B. Ag: Af (8: 2), C. Ag: Af (7: 3), D. Ag: Af (6: 4), E. Ag: Af (5: 5), F. Ag: Af (4: 6), G. Ag: Af (3: 7), H. Ag: Af (2: 8), I. Ag: Af (1: 9), J. C+, K. C-.

A combination of HAp of *A. granosa* with *A. fulica* showed antagonism due to the presence of acharan sulfate that could inhibit the GAGs on the *A. granosa* (Adhya et al. 2016; Chernikov et al. 2013; Shyamasree 2017; Toda et al. 1980; Tuane et al. 2004). Acharan sulfate, the new GAGs, was first isolated and characterized by Kim et al (1996). The inhibitory zones of various combinations of the HAp from *A. granosa* and *A. fulica* were presented in Figure 1. Antibacterial ability is very important in the field of dentistry when using them on the human body (Dos Santos et al. 2006; Kim et al. 2007).

The use of biomaterials in the field of dentistry is influenced by many factors, including antimicrobial characteristics and its toxicity to tissues. Hence, it cannot be proposed as a dental material if it is toxic to tissues. Therefore, employing the right ratio between HAp *A. granosa* and HAp *A. fulica* was important to prevent tissue toxicity due to the long-term use of the material. The best ratio between two hydroxyapatites was 6: 4, because it was the smallest concentration for inhibiting or killing bacteria (Leekha et al. 2011; Li et al. 2018; Yousef et al. 2018).

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