

# In Vitro Synergistic Effects of Snail Slime and Chitosan against *Staphylococcus aureus*

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**Abstract**—Snail slime contains such active substances as isolates, heparan sulfate, and calcium. The isolate content is useful as antibacterials and analgesics, while calcium plays a role in hemostasis. Snail slime has antibacterial and antiinflammatory effects and therefore the proliferation phase will heal wounds immediately. Chitosan is a biopolymer with a wide range of biomedical and pharmaceutical applications. Chitosan fibers are used as threads in surgery and are easily absorbed by the human body so that they can be used as a bandage covering the wound and medication carrier. Chitosan can be biologically degradable, is non-toxic, nonimmunogenic and biocompatible with the body tissue of mammals. *Staphylococcus aureus* is a bacterium causing skin infection and pus formation in wound. This research aims at finding out the in vitro synergistic effects of snail slime and chitosan against *Staphylococcus aureus*. The research method involves isolation of snail slime, 2% chitosan synthesis, and in vitro effectiveness test using diffusion method. The research findings indicate that snail slime and 1.25% chitosan are proven to be effective bactericide against *Staphylococcus aureus*. The mixture of snail slime and 1.25% chitosan with ratio of 1:1 shows the synergistic effect as bactericide against *Staphylococcus aureus*. The research findings are expected to be applied in nursing, particularly wound treatment to prevent *Staphylococcus aureus* infection with natural and safe materials.

**Index Terms**—Chitosan, in vitro, snail slime, *Staphylococcus aureus*, synergistic

## I. INTRODUCTION

Every living creature biologically possesses immune system which protects against disease or wound infection; when a wound is found, one of the treatment methods can be done by covering or treating it using antimicrobial wound dressing. The best bandage is the patient's skin which is permeable to moisture and protects the inner body tissues against mechanical injury or infection. Biocellulose is a natural polymer which has the same characteristics as hydrogel, which cannot be found in natural cellulose. The characteristic of hydrogel from cellulose gives better absorption capacity, and provides the similar characteristics to human skin. Regarding its

medical applications, biocellulose is only used temporarily due to its low strength and bioactive character. Therefore, to support the reinforcement of the bioactive character of the biocellulose, a treatment combining such active polysaccharide as chitosan widely used in medical care needs to be applied. Chitosan fibers are used as threads in surgery and are easily absorbed by the human body so that they can be used as a bandage covering the wound and medication carrier. Chitosan also has influential role in the blood clotting and therefore it can be used as hemostatics; it can be biologically degradable, is non-toxic, nonimmunogenic and biocompatible with the body tissue of mammals [1].

Wound is a damage of skin anatomy structure which leads to skin disorder. When we have a cut on finger, the existing wound will cause damage on skin, so it cannot protect its inner layers. The wound infection can occur if the wound is contaminated by dust or bacteria; it is because the wound is not treated well [2]. One of the bacteria causing the wound infection either directly or indirectly is *Staphylococcus aureus*. This bacterium produces pus, and therefore it is called pyogenic bacterium. Reducing the risk of *Staphylococcus aureus* infection can be done by restoring the function of the injured part of body, while reducing the infection and minimizing the scars can be done by doing some basic actions, such as washing hands, cleaning the wound, cleaning the skin around the wound, covering the wound, frequently replacing the bandage, and applying gel containing antibiotics. However, the use of antibiotic often results in the bacterial resistance to antibiotic agent; it is the reason why a research on natural antibiotic obtained from natural ingredients such as snail slime needs to be conducted [3].

Wound healing is very important to immediately restore its integrity and it is both complex and dynamic processes with a predictable pattern. One of the crucial phases of wound healing is proliferation phase and this occurs after inflammatory phase. The proliferation or fibroblastic phase will immediately occur in case that there exists no infection and contamination in the inflammatory phase. The use of chemical compounds for wound healing or chemotherapy including povidone iodine sometimes gives a toxic effect in in vitro studies. Therefore, other alternative treatments using natural

materials which serve as antimicrobial factors, one of which is snail slime, are highly required. Wound healing with snail slime can be one of the alternatives because it is not only easy to use, but it also can spread well in the skin. In addition, it does not clog skin pores, and it has an antibacterial effect. Snail slime gives a positive reaction to test for protein contents, comprising amino acids and proteins which play role in cell regeneration and growth. Furthermore, it also acts to aid immune system and exerts a protective function to repair damaged cells. The animal protein content of snail slime is predicted to have a high biological value in wound healing and in the inhibition of inflammatory process [4]. Chitosan is mainly used as chelating agent in drinking water and wastewater treatment and is found in cosmetics, fungicides, and wound care products [5]. The present study aims at finding out synergistic effect of snail slime and chitosan on *Staphylococcus aureus*. It is expected that the research findings can be applied in fields of nursing, particularly in wound care to prevent staph infections using effective and safe natural materials.

## II. MATERIAL AND METHODS

### A. Material and Samples

The research was carried out at science laboratory of School of Health Sciences of Kusuma Husada Surakarta for period of three months.

Samples include snail slime, chitosan synthesized from crab and shrimp shell waste, *Staphylococcus aureus* isolate, Vogel Johnson Agar medium, Brown II standard solution, sterile physiological NaCl solution, chitosan manufactured by Biotechsurindo in Cirebon.

### B. Synthesis of Chitosan

Synthesis of chitosan as in Fig. 1 from samples of shrimp shells or crab shells was made through deacetylation, demineralization, deproteination of chitin [6].

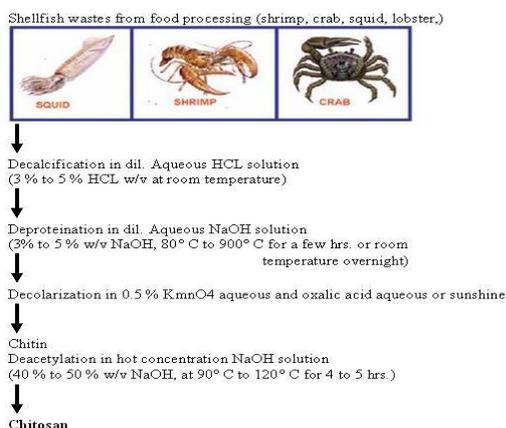


Figure 1. Synthesis of chitosan

Meanwhile, industrial chitosan as in Fig. 2 was obtained from PT. Biotech Surindo Cirebon Indonesia. Solution of 1.5% chitosan was then made in a solution of 10% acetic acid. Chitosan is insoluble in water but soluble in acidic solvents with a pH below 6.0.



Figure 2. Chitosan synthesized from crab and shrimps shell waste

### C. Isolation of Snail Slime

The snail slime isolation as in Fig. 3 was obtained from 10-50 local snails (*Achatina fulica*) using an electric shock from 5-10 volt power supply for 30-60 seconds. The slime was macerated in water for 24 hours in 40 °C. Fraction containing water-soluble slime was obtained from the procedure of mixing the water twice of the number of samples added to the slime. The supernatant was received as WSF (Water Soluble Fraction). The fraction of slime (mucin fraction) of the WSF was gained by using ethanol precipitation by mixing supernatant resulted from the water maceration with absolute ethanol ratio of 1: 3, and then it was centrifuged at 2900 r.p.m. for 30 minutes. The precipitation was re-dissolved with Tris -Cl and finally mucin fraction was obtained [7].



Figure 3. Isolation of snail slime

### D. The Making of *Staphylococcus aureus* Suspension

Pure culture of *Staphylococcus aureus* was obtained from isolated bacterial colonies undergoing incubation at 37 °C for 48 hours. The isolates were then inoculated and suspended in sterile physiological NaCl solution. This process resulted in turbidity level which fits to McFarland standards containing 10<sup>8</sup> CFU/ml of organism. Suspension used in the inoculation included disk diffusion method.

### E. Testing Stage of Diffusion Method

Snail slime and chitosan preparations that had been prepared were tested the activities using diffusion method, in which VJA (Vogel Johnson Agar) media were inoculated by spreading *Staphylococcus aureus* suspension using sterilized cotton buds. Sinks were later created using borer and each sink was filled with testing compounds, negative and positive controls. Sinks were filled with drops of preparations of snail slime galenic, snail slime cream, chitosan 1.25% and its 50 µl mixture,

and they later were incubated for 48 hours at 37 °C. Afterwards, the formation of clear areas around the sinks was observed and barrier areas were measured the diameters [8], [9].

### III. RESULTS AND DISCUSSION

Based on the results of research (as Table I) and statistical analysis (as Table II) showed a synergistic effect snail slime and chitosan is bactericidal against *Staphylococcus aureus*. The snail slime creams showed the most optimum bactericidal effect compared snail slime or chitosan. This is due to the preparation snail slime cream 5% in physicochemical be more effective in wound healing [10] compared to other preparation with snail slime 100% and or chitosan 1.25% and mixtures thereof as in Fig. 4.

TABLE I. SYNERGISTIC EFFECT TEST ON SNAIL SLIME AND CHITOSAN AGAINST *STAPHYLOCOCCUS AUREUS*

No	Treatment	Inhibition zone	
		I (cm)	II (cm)
1	Snail slime 100%	0.1	0.1
2	Snail slime cream 5%	0.3	0.4
3	Slime: Areca nut 5% = 1 : 1	0.1	0.1
4	Slime cream: Areca nut 5% = 1 : 1	0.3	0.2
5	Slime: Chitosan 1.25% = 1 : 1	0.2	0.2
6	Slime cream: Chitosan 1.25% = 1 : 1	0.2	0.2
7	Acetic acid 1.5%	0.2	0.1

TABLE II. MULTIPLE COMPARISONS

D) 1,2,3, 4,5,6,7	(J) 1,2, 3,4,5,6,7	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval		
					Lower Bound	Upper Bound	
LSD	Snail Slime cream 5%	Snail slime 100%	.25000*	.04629	.001	.1405	.3595
	Snail slime : Areca nut 5% = 1 : 1	.25000*	.04629	.001	.1405	.3595	
	Snail slime cream 5% : Areca nut 5% = 1 : 1	.10000	.04629	.068	-0.095	.2095	
	Snail slime 100% : chitosan 1.25% = 1:1	.15000*	.04629	.014	.0405	.2595	
	Snail slime cream 5% : chitosan 1.25% = 1:1	.15000*	.04629	.014	.0405	.2595	
	Acetic acid	.20000*	.04629	.003	.0905	.3095	

Dependent Variable: result



Figure 4. In vitro synergistic effect test on snail slime and chitosan against *Staphylococcus Aureus*

Chitosan is three-high-molecular weight natural polymer. It is nonpoisonous; it can accelerate wound healing, reduce blood cholesterol levels, stimulate the immune response and can be biologically decomposed. It has a stronger antimicrobial property compared to chitin in avoiding fungi because it has an active group that will bind to microbes, so it can inhibit microbial growth [11]. Chitosan has a good chemical reactivity because it has a number of hydroxyl (OH) and amine groups (NH<sub>2</sub>) attaching to its chain. One of its important characteristics

is that it has a positive charge in acidic solution. The substance is a stronger antifungal factor compared to chitin. In addition, chitosan is polycationic, so it can be used as a clotting agent.

Snail slime contains chemical substances including achatina isolates, heparan sulfate, and calcium. Achatina isolates can perform as antibacterials and analgesics, while calcium plays important roles in hemostasis. The effects of snail slime as antibacterial and anti-inflammatory agents will accelerate inflammatory phase, and hence this will speed up proliferation phase in wound healing [12], [13]. Heparan sulfate appears to be the most influential property in snail slime, especially in fibroblast proliferation. This property accelerates wound healing process by helping blood clotting and fibroblast cell proliferation processes. Heparan sulfate also plays important roles in angiogenesis, inhibiting vascular endothelial growth factor or reducing the mitogenic activities of FGF. Heparan sulfate as one of proteoglycans functions as binder and reservoir of *basic fibroblast growth factor* (bFGF) which is secreted into ECM (Extracellular Matrix). ECM can release bFGF which stimulates inflammatory cell recruitments, fibroblast activation and new blood vessel formation in every injury [14]-[17].

Chitosan's bactericide effects are attributable to its good chemical reactivity due to the chains of some hydroxyl groups (OH) and amino groups (NH<sub>2</sub>). Most of polysaccharides found in nature are neutral and alkaline such as cellulose, dextran, pectin, alginic acid and agar, while chitosan is a sample of alkaline polysaccharides which belongs to heteropolymer. Chitosan's significant nature is having a positive charge in acidic solution which gives it stronger antimicrobial effect than chitin does. In addition, chitosan is more polycathionic, and therefore it can perform as clotting agent. Important property of chitosan is having a positive charge in acidic solutions that are antifungal stronger than chitin. In addition, chitosan is polycationic so it can be used as a clotting agent. Activation of antimicrobial of chitosan is influenced by several intrinsic and extrinsic factors. A low molecular weight chitosan has better activity. Deacetylated chitosan is more perfect, and therefore it is more anti-microbial compared to chitosan which has a proportion of more acetylated amino group because of greater increased solubility. The activation of microorganisms in chitosan is determined by a number of intrinsic and extrinsic factors. Chitosans with lower molecule weight have better activities. More perfectly deacetylated chitosans will have better antimicrobial effects than those with more proportion of acetylated amino groups due to a greater increase in dissolution and density of the properties. Chitosan demonstrates in vitro antibacterial, antimetastatic, immunoadjuvant and biocompatible activities. Chitosan is capable of absorbing fats which reduce cholesterol [18], [19]. Chito-Olygosaccharide (COS), which can be obtained from chitosan waste from shrimps or crabs in Indonesia, is potential as a source of natural probiotics [20]-[24].

Staphylococci ('staph') are a common type of bacteria that live on the skin and mucous membranes of humans. *Staphylococcus aureus* is the most important of these bacteria in human diseases. Other staphylococci, including *Staphylococcus epidermidis*, are considered commensals, or normal inhabitants of the skin surface. About 15–40 per cent of healthy humans are carriers of *S. aureus*, that is, they have the bacteria on their skin without any active infection or colonisation. *Staphylococcus aureus* produces an enzyme called coagulase. Other species of staphylococci do not and thus are called coagulase-negative staphylococci. *S. aureus* is the most important type to be noticed because it infects human most frequently. Most of *Staphylococcus* strains are able to help the fermentation of mannitol and positive coagulase. However, coagulase-negative strains become more important since they often cause infections to human, particularly infections which lead to bacteremia on sufferers with catheterization. These happen to women with urinary tract and nosocomial infections. *Staphylococcus aureus* pathogenic factors relate to the production of coagulase enzyme. Negative coagulase performs as opportunistic pathogen [25], [26].

*Staphylococcus aureus* causes skin infections with highly variable clinical manifestations, starting from the appearance of pustules to sepsis which leads to death. At the beginning, lesions with pus occur which then develop into abscess. The virulence of strain *Staphylococcus* varies. These bacteria normally reside in the skin of all healthy people. These bacteria, although less dangerous than *Staphylococcus aureus*, can cause serious infections, usually when acquired in a hospital. The bacteria may infect catheters inserted through the skin into a blood vessel or implanted medical devices such as pacemakers or artificial heart valves and joints.

The Staphylococcal infections are caused by *Staphylococcus* bacteria, types of germs commonly found on the skin or in the nose of even healthy individuals. Most of the time, these bacteria cause no problems or result in relatively minor skin infections. Staphylococcal skin infections are usually diagnosed based on their appearance. Other infections require samples of blood or infected fluids, which are sent to a laboratory to culture the bacteria. Laboratory results confirm the diagnosis and determine which antibiotics can kill the staphylococci is called susceptibility testing. The diagnosis is based on the appearance of the skin or identification of the bacteria in a sample of the infected material. Thoroughly washing the hands can help prevent spread of infection. *Staphylococcus aureus* infections range from mild to life threatening. The bacteria tend to infect the skin causing abscesses. However, the bacteria can travel through the bloodstream or bacteremia and infect almost any site in the body, particularly endocarditis and osteomyelitis. The bacteria also tend to accumulate on medical devices in the body, such as artificial heart valves or joints, heart pacemakers, and catheters inserted through the skin into blood vessels. There are many strains of *Staphylococcus aureus*. Some strains produce toxins that can cause the symptoms of Staphylococcal food poisoning, toxic shock

syndrome, and scalded skin syndrome. Staphylococcal infection may be difficult to treat because many of the bacteria have developed resistance to antibiotics. These bacteria are often resistant to many antibiotics. Many strains have developed resistance to the effects of antibiotics. If carriers take antibiotics, the antibiotics kill the strains that are not resistant, leaving mainly the resistant strains. These bacteria may then multiply, and if they cause infection, the infection is more difficult to treat. Whether the bacteria are resistant and which antibiotics they resist often depend on where people got the infection: in a hospital or other health care facility or outside of such a facility in the community. Because antibiotics are widely used in hospitals, hospital staff members commonly carry resistant strains. When people are infected in a health care facility, the bacteria are usually resistant to several types of antibiotics, including all antibiotics that are related to penicillin is called beta-lactam antibiotics. Strains of bacteria that are resistant to beta-lactam antibiotics are called Methicillin-Resistant *Staphylococcus aureus* (MRSA). MRSA strains are common if infection is acquired in a health care facility, and more and more infections acquired in the community, including mild abscesses and skin infections, are caused by MRSA strains. Vancomycin, which is effective against many resistant bacteria, is used, sometimes with rifampin. Medical devices, if infected, often must be removed [27].

#### IV. CONCLUSION

There is a synergistic effect of snail slime and chitosan as a bactericide against *Staphylococcus aureus*. Snail slime cream 5% showed the most optimum bactericidal effect compared snail slime 100% or chitosan 1.25% and mixture thereof.

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