








The Effectiveness of Chitosan and Snail Seromucous as Anti Tuberculosis Drugs

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Abstract

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BACKGROUND: Tuberculosis (TB) disease is an infection caused by *Mycobacterium tuberculosis* (MTB) and is transmitted through sputum droplets of sufferers or suspect TB in the air. Chitosan has been widely used in the biomedical and pharmaceutical fields because it is a biocompatible, biodegradable, non-toxic, antimicrobial, and hydrating agent with positive effects on wound healing. Seromucous of snail has anti-tumor bioactivity and is non-toxic to lymphocyte cells, and can even stimulate lymphocyte proliferation. Seromucous of snail as glycoprotein containing carbohydrates; α -1 globulin-OROMUCOID fraction; glycans, peptides, glycopeptides, and chondroitin sulfate.

AIM: This study was to determine the effectiveness of snail seromucous and chitosan as anti TB drugs (ATD) *in vitro*.

METHODS: The research method is based on an experimental laboratory. MTB isolates in this research from sputum samples of patients suspected of TB in Surakarta Regional General Hospital. The stages of the study were performed MTB culture and identification, management sampling, and drug susceptibility testing.

RESULTS: The research results showed chitosan 5%; a combination of chitosan 9% and snail seromucous 50% (ratio 1:1) is a microbicide against MTB TB patient isolates. Snail seromucous was ineffective as a microbicide against MTB TB patients.

CONCLUSION: The effectiveness as a bactericide against MTB, chitosan, and its combination with snail seromucous has the potential to be an ATD alternative.

Introduction

Tuberculosis (TB) disease is an infection caused by *Mycobacterium tuberculosis* (MTB) and is transmitted through sputum droplets of sufferers or suspect TB in the air. TB treatment lasts quite a long time, namely, at least 6 months of treatment which results in the emergence of germ resistance so that TB treatment is not successful because patients drop out of treatment or undergo treatment irregularly resulting in Multi Drugs Resistance TB (MDR-TB).

Chitosan or β - (1.4) -2 amino-2deoxy D-glucopyranose is a polysaccharide compound that can be obtained through the process of deacetylation of chitin compounds that are found in shrimp shells, crab shells. Chitosan synthesis uses samples of shrimp shells or crab shells, through the process of deacetylation with 60% NaOH at 60–100°C; deproteinization with 3.5% NaOH, decalcification with HCl 2N, and color removal with acetone and 2% NaOCl [1]. Chitosan has been widely used in the biomedical and pharmaceutical fields because it is a biocompatible, biodegradable, non-toxic,

antimicrobial, and hydrating agent with positive effects on wound healing.

Seromucous of snail has anti-tumor bioactivity and is non-toxic to lymphocyte cells, and can even stimulate lymphocyte proliferation. Seromucous of snail as glycoprotein containing carbohydrates; α -1 globulin-OROMUCOID fraction; glycans, peptides, glycopeptides, and chondroitin sulfate. Chondroitin sulfate can function as an immunomodulator and immunosuppressant [2]. The content of Glycosaminoglycans, heparin, heparin sulfate, chondroitin sulfate, dermatan sulfate, and hyaluronic acid in snail seromucous can function as stabilizer cofactors and/or coreceptors for growth factors, cytokines, chemokines; enzyme activity regulator; molecular labeling in response to cellular damage in the process of wound healing, infection, tumorigenesis; targets for virulence factors of bacteria, viruses, parasites; as well as the immune system [3]. The antimicrobial activities of peptides isolated from the hemolymph of the molluscan garden snail *Helix lucorum*, which exhibited inhibition effects against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli*. The achasin protein in the

Achatina fulica Ferussac snail has important biological functions, including as a bacterial enzyme protein binding receptor [4]. Seromucous of snail 100% and 5% snail mucus cream preparation have an effective effect on accelerating the healing duration of second degree (A) burns. The combination of 100% snail mucus and 1.5% chitosan = 1:2 gave the optimum wound healing rate in the *in vivo* test. There is a synergistic effect of chitosan and seromucous of snail against *S. aureus in vitro* [5].

The diagnosis of TB can be performed based on clinical symptoms, chest X-ray, microscopic examination of smear sputum, smear culture on culture media as well as the sensitivity test of MTB isolates to anti TB drugs (ATD) and Drug Susceptibility Testing (DST). Until now there has been no research related to the effectiveness of snail chitosan and seromucous of snail as an alternative to ATD so that research related to this needs to be done.

The purpose of this research was to assess the effectiveness of chitosan and seromucous of snail as ATD *in vitro*. The essence of the research results is expected to be the potential of chitosan and seromucous of snail as an alternative to ATD.

Materials and Methods

This study and laboratory examinations were conducted and performed at Surakarta General Center Hospital from January to March 2020. All strains were isolated from culture-positive MTB cases. The TB diagnostic criteria were based on the Ministry of Health of the Republic of Indonesia (2014) and the corresponding WHO guidelines [6].

Seromucous of snail were collected from local snails (*A. fulica* Ferussac) and chitosan powder was performed from Biotechsurindo factory, Cirebon, Indonesia. The dosage of ATD including were performed rifampicin (RIF) (8000 mg/L), isoniazid (INH) (20 mg/L), ethambutol (EB) (200 mg/L), and streptomycin (SM) (800 mg/L).

Clinical specimens including sputum were collected from patients with suspected TB of Surakarta General Center Hospital. The screening test was performed by microscopic examination of Ziehl Nelson staining and Molecular Quick Test - Genexpert instrument by following under relevance guidelines. The positive MTB isolates were subjected to cultivation with Lowenstein-Jensen medium (HiMedia, M162 product). All the MTB isolates were validated by both the growth test on p-nitrobenzoic acid (PNB) and MPT-64 antigen detection kit. Non-TB mycobacteria were excluded.

DST was performed using the MTB system. The colonies of MTB were swept from the agar plates

and suspended in sterile saline containing 0.2% Tween and glass beads. After vortexing for at least 30 s to break up organisms clumps, the bacterial suspension was stayed for 15 min at room temperature to allow any remaining clumps to settle to the precipitations, and the supernatant was adjusted to then a suspension with a turbidity of 0.5–1.0 Mc. Farland standard using a nephelometer. The dilution suspension was performed to 10^{-3} and 10^{-5} . Stock solutions and working solutions were prepared. 100 μ l suspension of the subsequent suspension was inoculated to each tube of Lowenstein Jensen's media that contained freeze-dried seromucous, chitosan, and ATD including SIRE. The tube culture was incubated at 37°C for 28–42 days. All steps were performed by trained and specialized persons in a biosafety cabinet by following under relevant guidelines [7].

The study results were analyzed using the statistical program of Statistical Package for the Social Sciences version 20.0.

Results

Seromucous of snail collected from the amount of 10–50 snails, opened the end of the shell, and given the electric shock of 5–10 volts, for 30–60 s, and the liquid that comes out is collected in the flask container. Next, the liquid is centrifuged at 3500 rpm for 10 min as hemolymph fluid or seromucous. Chitosan used in this research was dissolved in 5% acetic acid solution [8].

Isolation and identification of MTB based on positive screening test results from sputum samples of suspect TB patients with the microscopic examination of the Ziehl Nelson method based on the scale International Union Against TB and Lung Disease scale, Genexpert test, culture on PNB media and MPT64 rapid test.

MTB resistance to Chitosan, Seromucous, Combination of Chitosan and Seromucous of Snail; ATD against MTB isolates as shown Table 1.

Discussion

Based on Table 1 shows that chitosan is 5%; a mixture of chitosan 9% and seromucous of snail 50% (ratio 1:1) is a microbicide against MTB TB patient isolates. All MTB isolates of suspected TB patients were resistant to a single preparation of 100% seromucous of snail and 2% chitosan compared to ATD. Meanwhile, the most effective type of ATD as RIF is compared to other types of ATD, namely, SM, INH, and EB.

Table 1: Percentage of MTB resistance to Chitosan, Seromucous, Combination of Chitosan and Seromucous of Snail; ATD against MTB isolates

Group	Dosage (%)	% Resistance of MTB	Mean	p-value	CI		
					Upper	Lower	
Chitosan	2	100	5.5	1.000	1.0	1.0	
	5	0	8.0	0.000	0.011	0.006	
	6	0	8.0	0.000	0.011	0.006	
	7	0	8.0	0.000	0.011	0.006	
	8	0	8.0	0.000	0.011	0.006	
	9	0	8.0	0.000	0.011	0.006	
Seromucous of Snail	10	0	8.0	0.000	0.011	0.006	
	25	100	5.5	1.000	1.000	1.000	
	30	100	5.5	1.000	1.000	1.000	
	35	100	5.5	1.000	1.000	1.000	
	40	100	5.5	1.000	1.000	1.000	
	45	100	5.5	1.000	1.000	1.000	
	50	100	5.5	1.000	1.000	1.000	
	100	100	5.5	1.000	1.000	1.000	
	Ratio Chitosan:Seromucous of snail = 1:1	C 2:SS 25	100	5.5	1.000	1.000	1.000
		C 5:SS 30	100	5.5	1.000	1.000	1.000
C 6:SS 35		100	5.5	1.000	1.000	1.000	
C 7:SS 40		100	5.5	1.000	1.000	1.000	
C 8:SS 45		100	5.5	1.000	1.000	1.000	
C 9:SS 50		0	8.0	0.000	0.011	0.006	
C 10:SS 100		0	8.0	0.000	0.011	0.006	
ATD		S (SM)	60	6.5	0.134	0.462	0.436
		I (INH)	20	7.5	0.014	0.510	0.400
		R (RIF)	0	8.0	0.000	0.011	0.006
	E (EB)	100	5.5	1.000	1.000	1.000	
Negative control		100	5.5	1.000	1.000	1.000	

ATD: Antituberculosis drugs, MTB: *Mycobacterium tuberculosis*, SM: Streptomycin, INH: Isoniazid, RIF: Rifampicin, EB: Ethambutol.

The ineffectiveness of the snail seromucous as ATD *in vitro* is due to the physicochemical properties of the preparation, namely, the solubility or polarity of the bioactive compound which is not able to penetrate the permeability of MTB cells in order that the dosage of the bioactive compound of snail seromucous is used, it not optimal as a bactericide. Furthermore, the difference within the variation in antibacterial activity is influenced by differences in the resistance level of the inoculum strain associated with the resistance gene expression results.

Chitosan is a β - (1.4) -2 amino-2deoxy D-glucopyranose compound, as a product of chitin deacetylation. Chitosan has been widely utilized in the biomedical and pharmaceutical fields because its biodegradable, non-toxic, non-immunogenic, and biocompatible with animal body tissues. The effectiveness of chitosan as an antimicrobial is related to the role of the Chito-Oligosaccharide (COS) compound, a group of complex compounds glycan-binding protein that has a 1,4-b-glucosamine, which is a derivative of chitosan deacetylation of chitin. The effect of COS as the antimicrobial activity is highly dependent on the degree of deacetylation and polymerization of the types of bacteria and fungi. COS as a potential material as "alternative antibiotics" has a value of more effective without causing residue. The uniqueness of chitosan is that it's polycationic in order that it can suppress the growth rate of diarrheagenic *E. coli in vitro* [9].

The seromucous of snail contains various types of achasin proteins that have important biological functions, including receptors for binding bacterial enzymes. The effectiveness of the bactericide and or bacteriostatic snail mucus against the isolates of *Staphylococcus sp*, *Streptococcus sp*, and *Pseudomonas sp* showed varied results [10]. The

100% concentration of snail seromucous is capable of being bactericidal against *S. aureus*, *Candida albicans*, and *Pseudomonas aeruginosa* [11]. The results of the Minimal Inhibition Concentration test of meat protein extract from seven, different types of snails showed varied results because they were influenced by the ecological conditions of the snails [12]. Seromucous of snail is antibacterial against *Streptococcus mutans*; *E. coli* and inhibits the growth of Methicillin-Resistant *S. aureus* [13]. The 100% snail mucus concentration is capable of being bacteriostatic against the growth of *S. aureus* and *Salmonella typhosa* [14]. Some of the protein lectins are known to be contained in snails, namely, selectin, galectin, C-type lectin, and fibrinogen-related protein which are secreted by snails that plays a role within the pathogen agglutination process [15].

Based on these results, chitosan and its mixture with seromucous snail are potential candidates for ATD. The content of bioactive compounds in chitosan and seromucous snail can stimulate the function of cellular immunity, namely, lymphocyte proliferation and the production of reactive oxygen intermediated macrophages. The results of the characterization of the snail seromucous protein profile using the SDS-PAGE method showed that have been 3 protein subunits, namely the range of 55–72 kDa because the achasin sulfate group that acted as an antimicrobial and 1 specific protein subunit 43 kDa as adhesive protein. The 100% seromucous of snail and 5% snail slime cream showed optimum effectiveness against lymphocyte proliferation *in vitro* [16]. The research being carried out is freeze-drying seromucous of snail preparation.

The factor causing the ineffectiveness of a microbicidal agent is additionally influenced by the physiological characteristics of MTB isolates which have specific characteristics compared to the physiology of other bacterial cells, namely the presence of mycolic acid in the cell walls which acts as a virulence factor for MTB cells. The effectiveness of a bactericidal or bacteriostatic drug against MTB isolates is often influenced by the physiology of bacterial cells, namely genetic factors associated with the extent of resistance or virulence of cells to an agent or the mutation process caused by mutagenic agents, physically and chemically, from environmental factors. In MTB cells have mycolic acid (trehalose dimycolate) which plays an important role in pathogenic interactions with the host. Mycolic acid has a similar function with lipopolysaccharide in Gram-negative bacterial cells. Mycolic acid affects the function of macrophages, namely inhibiting the fusion of macrophages in host cells against pathogens. The presence of mycolic acid in MTB plays a crucial role within the level of germ resistance to host immune cells and drugs. Each type of ATD contains a different mechanism of action so that it affects the effectiveness of bactericide as ATD. The mechanism of action of SM interferes with the translation process by binding to 16 s rRNA in protein synthesis. INH inhibits the synthesis of

mycolic acid so INH is that the best ATD for the treatment and prevention of TB. INH resistant strains often appear with a frequency of around 90% and therefore the resistance is caused by mutations in one in every of the catalase-peroxidase (KatG), *inhA*, or *ahpC* genes. INH in cells will turn active in an oxidized form because of activation of the enzyme KatG which is encoded by the KatG gene. The KatG gene encodes the enzyme KatG which activates INH as a prodrug in order that mutations within the gene cause the enzyme to become inactive. Other mechanisms of resistance to INH and ethionamide (ETH) are changes in the expression of drug activators, redox changes, drug inactivation, and efflux pump activation [17]. Mode of action RIF a bactericide by inhibiting nucleic acid synthesis by binding to the β RNA polymerase subunit within the RNA transcription process. The resistance of MTB to RIF reaches 95% and occurs due to the mutation of the *rpoB* gene that encodes the β RNA polymerase subunit as a very important component within the transcription process. The transcription process is inhibited because RIF is specifically bound to the β RNA polymerase subunit. EB could be a bactericide by interfering with carbohydrate metabolism and cell wall biosynthesis. While pyrazinamide (PZA) as an analog of nicotinamide structure is bactericidal because pyrazinoic acid as a result of the activity of the PZA enzyme formed under acidic conditions. PZA resistance occurs because of mutations within the *pncA* gene that cause loss of PZA activity (PZAase) so that the mechanism of action of PZA becomes inactive when entering the cell of MTB resistance to EB because of mutations in the *embB* gene that encodes arabinosil transferase so that biosynthesis or wall polymerization is inhibited arabinan cells as arabinogalactans components and therefore the occurrence of the accumulation of decaprenol phosphoarabinose lipid carriers [18].

The level of resistance of MTB isolates to ATD can occur due to the physiological properties of cells or MTB resistant strains that are different in each region due to the occurrence of mutations that are not interdependent (independent mutation) in additional than one ATD coding gene and or gene coding enzyme for precursor activation enzymes ATD. Mutations occurred within the process of protein synthesis are transcription and or translational processes that have a control on the expression of those genes, resulting in changes within the structure of the target protein or changes in enzymatic activity needed to activate ATD compounds to function as bactericides.

The immune response plays an important role in MTB infection. Most of the people are infected with Mycobacteria, 90% don't develop TB. Macrophages in host cells play a vital role within the immune system, namely phagocytosis of cellular antigens. Bacteria within the lung are phagocytes by alveolar macrophages. MTB in macrophages can change the environment by inhibiting the acidification process

in phagosome maturation which ends within the phagosome maturation process being stopped. This ends up in phagosomes not having the ability to fusion with lysosomes in order that MTB cannot be destroyed and continues to replicate in macrophages [19]. Treatment of TB with ATD to date has been given the correct ATD; however, there are many strains of MTB resistant to two or more ATD called MDR-MTB strains. The prevalence of MDR-TB and extensively drug-resistant TB is higher in the case of recurrent TB treatment compared to the initial TB case and also the variation within the level of TB germ resistance to ATD is influenced by age, sex, and region [20]. There are side effects in MDR-TB therapy and also the correlation between cure rates and resistance to ATD so that a psychological social management approach is needed in MDR-TB patients and the presence of a bacterial profile related to resistance against antibiotics and TB treatment. This is often to the patient's ignorance of the disease, poor patient compliance, administration of monotherapy or effective drug regimens, inadequate doses, poor instructions, low medication regularity, poor patient motivation, irregular drug supply, poor bioavailability, and poor quality of the drug contributes to the occurrence of secondary drug resistance. Resistance encourages the employment of other more toxic alternative medicines, namely, ETH, aminosalicylic acid, cycloserine, capreomycin, ciprofloxacin, or ofloxacin. The emergence of MTB strains that are resistant to two or more ATD causes the failure rate of TB therapy to be high.

Conclusion

Chitosan 5%; a combination of chitosan 9% and snail seromucous 50% (ratio 1:1) could be a microbicide against MTB TB patient isolates. Seromucous of snail was ineffective as a microbicide against MTB TB patients. Given its effectiveness as a bactericide against MTB, chitosan and its mixture with snail seromucous have the potential to be an ATD alternative. Further research is required regarding the optimum dosage formulation and also the synergistic effect of seromucous and chitosan preparations with other ATD.

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