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The Potential of Snail Seromucous and Chitosan as Bioimunomodulator for Tuberculosis Therapy

(Potensi Seromukus Siput dan Kitosan sebagai Bioimunopemodulat untuk Terapi Tuberkulosis)

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ABSTRACT

Tuberculosis (TB) as a global emergency is a chronic disease caused by Mycobacterium tuberculosis *(Mtb). Mtb plays an important role in inducing or suppressing the production of Interferon Gamma (IFNG) and IL-4 in the regulation of TB homeostasis and pathogenesis. The bioactive compounds of the snail seromucous* (Achatina fulica *Ferussac) and chitosan function as biological response modifiers. The study aimed to determine the potential effectiveness of snail seromucous and chitosan as bio-immunomodulator for TB therapy. The research method was based on the results of laboratory experiments with the physic-chemical, biochemical, microbiological examination, snail seromucous protein profile, lymphocyte proliferation, measurement of IFNG, and IL-4 levels. The results of the physic-chemical examination of the snail seromucous showed a specific gravity of 1.010; pH 8, glucose 16 mg/dL; cholesterol 9 mg/dL; protein 2.8 mg/dL and heavy metals (Pb, Cu, Hg, Al) negative. The results of microbiological tests showed that a 100% concentration of snail seromucous was antimicrobial against* Staphylococcus aureus, Candida albicans, *and* Pseudomonas aeruginosa*. The protein profile of snail seromucous shows that there are 3 protein subunits, namely the range 55 - 72 kDa and 1 specific protein sub-unit 43 kDa as a bioactive compound achasin sulfate. Addition of chitosan dose of 65 µg/mL; snail seromucous dose of 65 µg/mL and a mixture of chitosan (65 µg/mL): snail seromucous (65 µg/mL) ratio 1: 1, can increase lymphocyte proliferation; optimum levels of IFN-γ and IL-4. Snail seromucous and chitosan are effective immunomodulators and potential candidates for TB therapy.*

Keywords: Chitosan; IFNG; IL-4; immunomodulator; Mtb; snail seromucous

ABSTRAK

Tuberkulosis (TB) ialah penyakit kronik kecemasan global yang disebabkan oleh Mycobacterium tuberculosis *(Mtb). Mtb memainkan peranan penting dalam menekan pengeluaran Interferon Gamma (IFNG) dan IL-4 untuk pengaturan homeostasis TB dan patogenesis. Sebatian bioaktif seromukus siput* (Achatina fulica *Ferussac) dan kitosan berfungsi sebagai pengubah tindak balas biologi. Objektif kajian ini adalah untuk menentukan potensi keberkesanan seromukus siput dan kitosan sebagai bioimunopemodulat untuk terapi TB. Kaedah penyelidikan berdasarkan hasil makmal uji kaji dengan tahap penyelidikan fizikokimia, biokimia, pemeriksaan mikrobiologi, profil protein seromukus siput, aktiviti imunopemodulat seromukus siput dan kitosan, percambahan limfosit, pengukuran tahap IFNG dan IL-4. Hasil pemeriksaan fizik-kimia seromukus siput menunjukkan graviti khusus 1.010; pH 8, glukosa 16 mg/dL; kolesterol 9 mg/dL; protein 2.8 mg/dL dan logam berat (Pb, Cu, Hg, Al) negatif. Hasil ujian mikrobiologi menunjukkan bahawa kepekatan seromukus siput 100% adalah antimikrob terhadap* Staphylococcus aureus, Candida albicans *dan* Pseudomonas aeruginosa*. Profil protein kaedah SDS-PAGE menunjukkan bahawa terdapat 3 sub-unit protein berkisar 55 - 72 kDa dan 1 sub-unit protein khusus 43 kDa sebagai sebatian bioaktif achasin sulfat. Kitosan (65 ug/mL); lendir siput (65 µg/mL) dan campuran kitosan (65 µg/mL) dengan lendir siput (65 µg/mL) nisbah 1: 1, dapat meningkatkan percambahan limfosit juga tahap optimum IFN-γ dan IL- 4. Seromukus siput dan kitosan adalah imunopemodulat yang mengagumkan dan calon yang berpotensi untuk terapi TB.*

Kata kunci: IFNG; IL-4; imunopemodulat; kitosan; Mtb; seromukus siput

INTRODUCTION

Tuberculosis (TB) as a global emergency is a chronic disease caused by *Mycobacterium tuberculosis* (Mtb). TB transmission occurs through the air and TB has infected one-third of the world's population. In individuals infected with active TB, bacterial replication occurs intracellularly

or extracellularly (WHO 2014). Mtb could initiate the formation of an adaptive response through the process of presenting antigens to T lymphocytes. The spread of Mtb to other peripheral organs can occur through the bloodstream and is in a latent state several decades before experiencing reactivation and the occurrence of extrapulmonary TB.

The cellular immune response plays an important role in the elimination process of Mtb. The cellular immune response is largely determined by the function and activity of lymphocytes. There are 2 ways to assess lymphocytes, namely by examining the quantity, and function of cells. The response to several types of extracellular bacteria requires specific antibodies that are produced by B lymphocytes with or without the help of T cells (helper T cells). Differentiation occurs due to the response of T cells to CD4 and CD8 cells based on the aspect of recognition of antigens presented by different MHC molecules. The class I MHC molecules will be recognized by CD8 cells and MHC class II molecules will be recognized by CD4 cells. CD4 and CD8 cells serve very different effector mechanisms. CD4 cells are known as helper T cells, which function to stimulate the growth of many hemopoietic cells with the production of cytokines, including IL-2 as a T cell growth factor, which plays an indispensable role in intracellular bacterial infections. Meanwhile, CD8 cells function as cytotoxic cells that do not require cytokines but through cell contact with one another. CD4 is cytokine-mediated while CD8 is cell-mediated (Abbas et al. 2014).

The inflammatory response in the body is characterized by the presence of various mediators, such as pro-inflammatory cytokines in the form of IL-1, Tumor Necrosis Factor (TNF), Interferon (IFN), IL-6, IL-12, and IL-18. Besides, Nitric Oxidase and COX-2 can stimulate the production of pro-inflammatory mediators. Anti-inflammatory cytokines such as IL-4, IL-10, IL13, and IFN-α act antagonistically against pro-inflammatory cytokines. Th2 cytokines such as IL-4 and IL-13 can inhibit autophagy due to IFN-γ induction. Diagnostic tools for measuring various types of cytokines produced by lymphoid cells and preferably in assessing cell function and cell response to various stimuli including the ELISA method.

Interferon (IFN) is a type of protein in the cytokine group. IFN-gamma (IFNG) plays a very important role in protective immunity against Mtb infection (Deretic et al. 2009). IFNG or IFN type II is secreted by cells in response to various inflammatory stimuli or other immune reactions. At 3 weeks after the initial infection, cellular adaptive immunity, which is dominated by CD4 + T lymphocytes, will then differentiate into Th1 so that Mtb growth in the lungs will be inhibited, and the disease progression process will stop temporarily. Activated Th1 cells will secrete IFNG which will activate macrophages for Mtb cytolytic. IFNG is an immunomodulator in the immune response that can increase autophagy against Mtb antigen in active TB patients. IFNG can increase the polarization of Th2 and cells that produce IL-4 during the initial priming of T cells and can induce an autophagy mechanism in cells infected with Mtb (Rovetta et al. 2014).

Chitosan is a complex compound of chitin derivatives in the glycoprotein group as a result of the deacetylation process of chitin which has 1,4 glucosamine bonds. The potential of chitosan as an antimicrobial agent can be used in the biomedical sector because chitosan has several hydroxyl groups (OH) and amine groups (NH2) (Harti et al. 2018).

Snails seromucous contain bioactive compounds such as glycans, peptides, glycopeptides, and chondroitin sulfate. Chondroitin sulfate can function as an immunomodulator and immunosuppressant. Gastropod hemocytes play an important role in cell defensive reactions, namely phagocytosis, encapsulation, nodulation and neutralization of parasites, blood coagulation processes, and wound healing. The bioactive compound of snail hemolymph has the potential as a medicinal derivative that can be used in the medical field, including skin smoothing, treatment of respiratory infections, and wound healing (Benkendorff et al. 2015).

The use of bioactive compounds based on natural ingredients as immunomodulators aims to change the activity of the body's immune system by dynamizing the regulation of immune system cells such as cytokines. The bioactive compounds in 100% snail seromucous and 1.5% chitosan can be used as immunostimulants for wound healing (Harti et al. 2016). Mtb plays an important role in inducing or suppressing the production of IFNG and IL-4 *in vivo*, especially related to the inhibition of cell-mediated immunity that produces cytokines in the regulation of TB homeostasis and pathogenesis (Nisha et al. 2018). Based on this, the study aimed to determine the potential and effectiveness of snail seromucous and chitosan as bio-immunomodulator for TB therapy.

Materials and Methods

The sample was a local snail (*Achatina Fulica* ferussac) by 10 - 50 snails. The snail seromucous isolated from the end of the shell was opened and the liquid out was collected in a container then centrifuged at 3000 rpm for

30 min. The seromucous liquid of the snail was carried out by freeze-drying process at -48 °C for 24 h. The results of freeze-drying were weighed. The process of freeze-drying the snail seromucous was carried out at the Pharmacy Laboratory of the Muhammadiyah University of Surakarta.

Chitosan was obtained from the Biotechsurindo factory, Cirebon Indonesia. The synthesis of chitosan comes from crab or shrimp shell samples through the stages of deacetylation, demineralization, and chitin deproteination. Chitosan can be obtained through the deacetylation process, namely the conversion of chitin with the addition of an alkaline NaOH solution and heating at 60-100 °C. Chitin isolation was carried out in three stages, namely, deproteinization with 3.5% NaOH, decalcification with 2N HCl, and decolouration with acetone and NaOCl. 2%. The transformation of chitin into chitosan used 60% NaOH at 90 °C. 2% w/v chitosan was dissolved in 2% acetic acid solution.

RESEARCH STAGES

This type of research used a laboratory experimental design. The research variables included independent variables of the snail seromucous formulation and chitosan. While the dependent variable was lymphocyte proliferation, measurement of IL-4, and IFNG levels. Measurement of lymphocyte proliferation using the MTT assay method, measurement of IL-4 and IFN-γ levels using the ELISA method in each treatment group. All data collected in the study were arranged in tables, diagrams, and graphs. The data analysis used one-way ANOVA with a significance level of $p < 0.05$.

CHARACTERIZATION OF SEROMUCOUS SNAILS

Seromucous characterization of snails includes examination physical, namely: colour, smell, consistency, viscosity, and specific gravity. Biochemistry test includes pH, heavy metals, proteins, carbohydrates, and lipids. Microbiology test includes antimicrobial activity test by Kirby Bauer method and SDS-PAGE method for biomolecular protein profile.

LYMPHOCYTE PROLIFERATION TEST

Lymphocyte cells were isolated using RPMI 1640 medium containing 10% Fetal Bovine Serum (FBS), 0.5% fungizone, and 2% Penicillin-Streptomycin and homogenized, then counting the number of cells using a hemocytometer. To determine the correct number of lymphocytes and the concentration of Con A used in the lymphocyte cell proliferation activity test, a preliminary test was performed. This test used lymphocyte cell cultures of 1×10^4 cells/mL, 2×10^4 cells/mL, 4×10^4 cells/mL, and 10×10^4 cells/mL. Meanwhile, the concentration of ConA used 10 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL, and 100 µg/mL. The test used a complete RPMI medium and incubated on cultured cell plates for 48 h. The results that provide the highest average number of lymphocytes will be used in future studies. The effectiveness of snail seromucous and chitosan on lymphocyte proliferation was carried out by the MTT assay method. Cell proliferation was detected by colorimetric method using Methyltiazoltetrazolium solution (Sigma) in PBS solution. The principle of cells experiencing proliferation is that their mitochondria will absorb MTT so that these cells will be dark purple due to the formation of tetrazolium crystals. Based on this principle, 4 h before the 72-h incubation period, the micro-culture plates were removed from the CO_2 incubator. Each well was given 20 µL (100 µg) of MTT solution, then the cell cultures were incubated again. A total of 200 µL of the medium from each well at the end of the 72-h incubation period, was carefully aspirated using a micropipette. Furthermore, each well was added 100 µL of isopropanol containing 0.04 N HCl to resuspend the tetrazolium crystals formed. After the crystals dissolve, a purple-coloured solution is formed with an intensity proportional to the rate of cell proliferation. Colour intensity was measured using an ELISA photo reader at 570 nm. The number of lymphocyte cells used was 2×105 cells per well. Determination of the percentage of lymphocyte proliferation using the formula:

$$\text{Lymphocyte proliferation} = \frac{\text{normal absorbance}}{\text{control absorbance}} \times 100\%$$

GAMMA AND INTERLEUKIN-4 INTERFERON LEVEL MEASUREMENT TEST

The IL-4 and IFNG examination methods used the ELISA solid-phase immunoassay method. Measurement of plasma IL-4 and IFNG levels of mice was carried out individually and in the population for each treatment group. The principle of measurement was based on the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific to IL-4 or IFNG in each microplate well binds to the IL-4 or IFNG present in the sample or standard solution. Then, in each well was

added an enzyme-linked polyclonal antibody against IL-4 or IFNG and a dye substrate so that the colour change will occur. The colour intensity was measured in optical density (OD) using an ELISA microplate reader.

RESULTS AND DISCUSSION

The physic-chemical test results of the snail seromucous as Table 1 showed a specific gravity of 1.010; pH 8, glucose 16 mg/dL; cholesterol 9 mg/dL; protein 2.8 mg/dL and heavy metals (Pb, Cu, Hg, Al) negative. Snail seromucous contains chemicals including *Achatina fulica* isolate, heparan sulfate, and calcium. The *Achatina fulica* content of the isolate is useful as an antibacterial and painkiller, while calcium plays a role in hemostasis. The effect of snail seromucous as an anti-inflammatory agent will further accelerate the inflammatory phase so that the lymphocyte proliferation phase will also accelerate in wound healing. Snail seromucous contains bioactive compounds such as glycans, peptides, glycopeptides, and chondroitin sulfate (Dolaskha et al. 2015, 2014). Snail chondroitin sulfate can function as an immunomodulator and immunosuppressant. The content of glycosaminoglycans (GAGs), heparin, heparin sulfate, chondroitin sulfate, dermatan sulfate, and hyaluronic acid in snail hemolymph can function as a major biological response modifier, namely acting as a stabilizer co-factor, and co-receptor for growth factors, cytokines, and chemokines; enzyme activity regulator; molecular labeling in response to cellular damage, especially in the process of wound healing, infection, and tumorigenesis; targets for virulence factors of bacteria, viruses, parasites; as well as the immune system.

TABLE 1. The result of physicochemical and biochemistry of seromucous snail test

Test types	Results
Consistency	Liquid
Colour	transparent white to brownish-yellow
Smell	odorless - a little fishy
Density	1.010
pH	8
Heavy metals (Pb, Hg, Cu, Al)	negative
Glucose	16 mg/dL
Cholesterol	9 mg/dL
Protein	2,8 mg/dL

The results of microbiological tests as shown in Tables 2 and 3 show that the 100% concentration of snail seromucous is MIC (Minimal Inhibition Concentration) against *Staphylococcus aureus, Candida albicans*, and *Pseudomonas aeruginosa*.

TABLE 2. Results of seromucous antimicrobial activity with diffusion method

No	Material	The diameter of resistance (mm)								
		Staphylococcus aureus			*Pseudomonas aeruginosa*			*Candida albicans*		
		1	2	x	1	2	x	1	2	x
1	Snail slime	18	16	17	18	18	18	19	19	19
2	Seromucous	16	18	17	15	14	14,5	18	17	17,5
3	Snail slime cream	32	20	26	22	21	21,5	16	18	17
4	Positive control	20	20	20	21	21	21	19	20	19,5
5	Negative control	0	0	0	0	0	0	0	0	0

TABLE 3. The results of snail seromucous antimicrobial activity with dilution test

Concentration (%)	Observation results
100	- (Clear, No Growth)
90	+ (Cloudy, Growth)
80	+ (Cloudy, Growth)
70	+ (Cloudy, Growth)
60	+ (Cloudy, Growth)
50	+ (Cloudy, Growth)
40	+ (Cloudy, Growth)
30	+ (Cloudy, Growth)
20	+ (Cloudy, Growth)
10	+ (Cloudy, Growth)

Chitosan is a β-(1.4)-2 amino-2deoxy D-glucopyranose compound, as a product of chitin deacetylation. Chitosan has been widely used in the biomedical and pharmaceutical fields because it is biodegradable, non-toxic, non-immunogenic, and biocompatible with animal tissues. The effectiveness of chitosan as an antimicrobial is related to the role of the Chito-Oligosaccharide (COS) compound, which is a group of glycan-binding protein complexes that have 1,4-b-glucosamine which is a deacetylated chitosan derivative of chitin (Ibrahim et al. 2016). The effect of chitosan as an antimicrobial activity is highly dependent on the degree of deacetylation and polymerization of bacteria and fungi. COS as a potential ingredient as an 'alternative antibiotic' has a more effective value without causing residue. The uniqueness of chitosan is polycationic. Therefore, it can reduce the growth rate of diarrheagenic *Escherichia coli in vitro*.

Snail seromucous contains bioactive compounds such as glycans, peptides, glycopeptides, and chondroitin sulfate. Snail slime protein with a molecular weight of 50.81 kDa, 15 kDa, 11.45 kDa as achasin protein has antimicrobial activity on *Streptococcus mutans*, and *Actinobacillus actin* (Bonnemain 2005; Vieira et al. 2004). The effect of snail seromucous as an anti-inflammatory agent will further accelerate the inflammatory phase so that the lymphocyte proliferation phase will also be faster in healing wounds (El Mubarak et al. 2013). The inhibition and antibacterial potential of snail mucus against the wound isolates of *Staphylococcus* sp., *Streptococcus* sp., and *Pseudomonas* sp. were varied. Snail seromucous is antibacterial against *Streptococcus mutans, Escherichia coli*, and inhibits the growth of Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Etim et al. 2015).

The difference in the variation of antibacterial power as the type of antibacterial achasin protein produced is related to the level of resistance of microorganisms and the type of antibacterial achasin protein resulting from genetic expression of different snail strains and is influenced by the ecological conditions of snail (Ulagesan & Kim 2018). Achacin glycoprotein as an antibacterial factor in *Lissachatina fulica* is known as the African giant snail and *Pomacea canaliculata* as an anti-bacterial golden snail on cell membranes against Gram-positive and Gram-negative bacteria, namely against *Staphylococcus aureus, S. epidermidis*, Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis,* and *Corynebacterium* sp. (Nantarat et al. 2019).

Based on the results of the characterization of the snail seromucous protein profile with the SDS-PAGE method as shown in Figure 1, it showed that there are 3 protein subunits, namely the range of 55 - 72 kDa and 1 specific protein subunit 34 kDa which is suspected as protein adhesion and functions as immunostimulatory biological response modifiers. The snail seromucous protein was purified and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to obtain a dominant band with a certain

molecular weight. The Vivantis brand protein molecular weight ranges from 10 to 180 kDa were used as markers. The results of the Bradford method of total protein analysis at a wavelength of 595 nm showed a protein content of 6.99 ug/uL.

Several protein lectins in snail mucus, namely selectin, galectin, C-type lectin, and fibrinogen-related protein (FREPs) function in the agglutination process of pathogens (Dang et al. 2015). The presence of aldolase and myosin were identified as proteins that play a role in the regulation of hemocyte migration and impact the process of killing pathogens through cytotoxic reactions and phagocytosis. Bioactivity of the snail seromucous against lymphocyte proliferation can be carried out against lymphocyte cells which are treated as normal human cells. If an agent is not toxic to lymphocytes, it can be concluded that the agent is also not toxic to normal cells. Three proliferation mechanisms, namely mitosis, amitosis, and cytoplasmic fragmentation (Sallam et al. 2009).

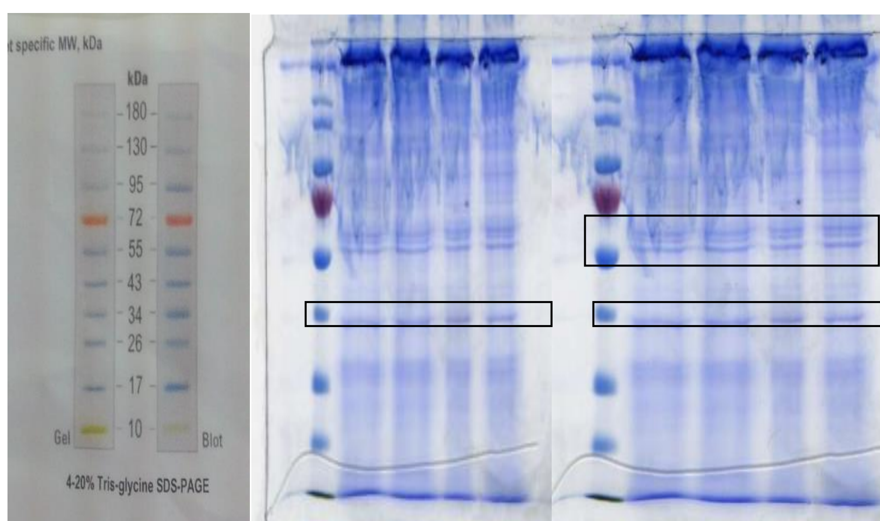

FIGURE 1. Profile of seromucous snail protein with SDS-PAGE method

The results of statistical tests in Table 4 showed that there were significant differences between the treatment groups. It referred that the addition of a single dose of (65 µg/mL) of chitosan and single-dose snail seromucous (65 µg/mL) and a mixture of chitosan (65 µg/mL): seromucous snail (65 µg/mL) = 1: 1, can increase lymphocyte proliferation optimally. While the most optimum lymphocyte proliferation activity was a mixture of chitosan (65 µg/mL): snail seromucous (65 µg/mL) with a ratio of 1: 1. It indicated that the seromucous mixture of snail and chitosan was effective as a biological response modifier and a potential candidate for anti-inflammatory cell therapy drugs.

TABLE 4. Effectiveness of snail seromucous and chitosan on lymphocyte proliferation

Group	Dosage	O.D	Mean difference	Std. error	Sig	95% Confidence interval	
						Lower bound	Upper bound
Chitosan	30 µg/mL	0,097	.564000*	.022212	.000	.51767	.61033
	35 µg/mL	0,142	.519333*	.022212	.000	.47300	.56567
	40 µg/mL	0,166	.495333*	.022212	.000	.44900	.54167
	45 µg/mL	0,206	.455333*	.022212	.000	.40900	.50167
	50 µg/mL	0,243	.417667*	.022212	.000	.37133	.46400
	55 µg/mL	0,261	.400000*	.022212	.000	.35367	.44633
	60 µg/mL	0,466	.195000*	.022212	.000	.14867	.24133
	65 µg/mL	0,491	.170000*	.022212	.000	.12367	.21633

Seromucous of snail	30 µg/mL	0,180	.481333*	.031185	.000	.41582	.54685
	35 µg/mL	0,191	.470333*	.031185	.000	.40482	.53585
	40 µg/mL	0,228	.432667*	.031185	.000	.36715	.49818
	45 µg/mL	0,210	.451333*	.031185	.000	.38582	.51685
	50 µg/mL	0,329	.332000*	.031185	.000	.26648	.39752
	55 µg/mL	0,358	.303000*	.031185	.000	.23748	.36852
	60 µg/mL	0,396	.265000*	.031185	.000	.19948	.33052
	65 µg/mL	0,462	.198667*	.031185	.000	.13315	.26418
Ratio Chitosan : Seromucous of snail = 1 : 1	(C 30 µg/mL : SS 30 µg/mL)	0,271	.390000*	.038922	.000	.30823	.47177
	(C 35 µg/mL : SS 35 µg/mL)	0,471	.189667*	.038922	.000	.10789	.27144
	(C 40 µg/mL : SS 40 µg/mL)	0,524	.136667*	.038922	.002	.05489	.21844
	(C 45 µg/mL : SS 45 µg/mL)	0,656	.005000	.038922	.899	-.07677	.08677
	(C 50 µg/mL : SS 50 µg/mL)	0,630	.031667	.038922	.427	-.05011	.11344
	(C 55 µg/mL : SS 55 µg/mL)	0,644	.017000	.038922	.667	-.06477	.09877
	(C 60 µg/mL : SS 60 µg/mL)	0,758	-.096667*	.038922	.023	-.17844	-.01489
	(C 65 µg/mL : SS 65 µg/mL)	1,103	-.441667*	.038922	.000	-.52344	-.35989
Positive control		0,661	.35050	.138945	.050	-.00667	.70767
Negative control		0,330	.18517	.065041	.050	.01797	.35236

*. The mean difference is significant at the 0.05 level

The mechanism of an anti-inflammatory agent could induce apoptosis through the inhibition of several cell-signaling pathways, including transcription factors, oncogenes, and protein signaling. The activity of lymphocyte prophylaxis against exposure to the agent was influenced by the quality and quantity of cells. The inducing agent included the type and number of lymphocytes, the active compound of an agent. The lectin or *Helix pomatia* agglutinin (HPA) compound in the *Helix pomatia* snail type can be used as a prognostic indicator in several cases of cancer, namely breast, stomach, and intestinal cancer, namely the presence of HPA in the fixation of tissue preparations as glycoproteins associated with cancer metastasis (Bismili et al. 2013; Greistorfer et al. 2017). African snails, giant snail mucus, contain substances that are antimicrobial peptides, namely mytimacin-like antimicrobial and glycolic acid (Suwannatri et al. 2016). Acharan sulfate as a glycoaminoglycans in giant African snails, structurally similar to heparin and heparan sulfate; widely used in medical preparations (Zhong et al. 2013). Besides, snail mucus can induce the accumulation of Calcium needed to repair the snail shell. Crystalline Calcium in snails has a similar structure contained in bones and teeth which were developed as tooth and bone material (Zhuang et al. 2015). 100% snail slime and 5% cream snail mucus against lymphocyte proliferation *in vitro* (Harti et al. 2019).

The results of the one-way ANOVA statistical test in the measurement of Interferon Gamma (IFNG) and Interleukin-4 (IL-4) level as Tables 5 and 6 showed that there were significant differences between the treatment groups. It indicated a single dose of 65 µg/mL of chitosan and a single dose of 65 µg/mL of snail seromucous and a mixture of chitosan (65 µg /mL): seromucous snail (65 µg/mL) = 1: 1, can increase levels of IFNG and IL-4 simultaneously optimum. Meanwhile, the optimum levels

of IFNG and IL-4 were a mixture of chitosan (65 µg/mL): snail seromucous (65 µg/mL) with a ratio of 1: 1. It showed that the mixture of snail seromucous and chitosan was effective as an immunomodulator.

TABLE 5. Effectiveness of snail seromucous and chitosan on interferon gamma levels

Group	Dosage	Mean OD	Concentration pg/mL	Mean difference	Std. Error	Sig.	95% Confidence interval	
							Lower bound	Upper bound
Chitosan	30 µg/mL	0,2220	8,667	-.031500	.015873	.075	-.06687	.00387
	35 µg/mL	0,2880	228,667	-.097500*	.015873	.000	-.13287	-.06213
	40 µg/mL	0,3195	333,667	-.129000*	.015873	.000	-.16437	-.09363
	45 µg/mL	0,3455	420,333	-.155000*	.015873	.000	-.19037	-.11963
	50 µg/mL	0,4175	660,333	-.227000*	.015873	.000	-.26237	-.19163
	55 µg/mL	0,4330	712,000	-.242500*	.015873	.000	-.27787	-.20713
	60 µg/mL	0,4575	793,667	-.267000*	.015873	.000	-.30237	-.23163
	65 µg/mL	0,5365	1057,000	-.346000*	.015873	.000	-.38137	-.31063
Positive control		0,1905	-96,333	-.131000*	.015873	.000	-.16637	-.09563
Seromucous of snail	30 µg/mL	0,2435	80,333	-.053000*	.012859	.002	-.08165	-.02435
	35 µg/mL	0,2910	238,667	-.100500*	.012859	.000	-.12915	-.07185
	40 µg/mL	0,3275	360,333	-.137000*	.012859	.000	-.16565	-.10835
	45 µg/mL	0,3665	490,333	-.176000*	.012859	.000	-.20465	-.14735
	50 µg/mL	0,4015	607,000	-.211000*	.012859	.000	-.23965	-.18235
	55 µg/mL	0,4505	770,333	-.260000*	.012859	.000	-.28865	-.23135
	60 µg/mL	0,4950	918,667	-.304500*	.012859	.000	-.33315	-.27585
	65 µg/mL	0,6280	1362,000	-.437500*	.012859	.000	-.46615	-.40885
Positive control		0,1905	-96,333	-.131000*	.012859	.000	-.15965	-.10235
Ratio Chitosan : Seromucous of snail = 1 : 1	(C 30 µg/mL : SS 30 µg/mL)	0,3605	470,333	-.170000*	.018699	.000	-.21166	-.12834
	(C 35 µg/mL : SS 35 µg/mL)	0,3820	542,000	-.191500*	.018699	.000	-.23316	-.14984
	(C 40 µg/mL : SS 40 µg/mL)	0,4550	785,333	-.264500*	.018699	.000	-.30616	-.22284
	(C 45 µg/mL : SS 45 µg/mL)	0,5310	1038,667	-.340500*	.018699	.000	-.38216	-.29884
	(C 50 µg/mL : SS 50 µg/mL)	0,5945	1250,333	-.404000*	.018699	.000	-.44566	-.36234
	(C 55 µg/mL : SS 55 µg/mL)	0,6755	1520,333	-.485000*	.018699	.000	-.52666	-.44334
	(C 60 µg/mL : SS 60 µg/mL)	0,7530	1778,667	-.562500*	.018699	.000	-.60416	-.52084
	(C 65 µg/mL : SS 65 µg/mL)	0,8530	2112,000	-.662500*	.018699	.000	-.70416	-.62084
Positive control		0,1905	-96,333	-.131000*	.018699	.000	-.17266	-.08934

*The mean difference is significant at the 0.05 level

TABLE 6. Effectiveness of snail seromucous and chitosan on il-4 levels

Group	Dosage	Mean OD	Concentration pg/mL	Mean difference	Std. Error	Sig.	95% Confidence interval	
							Lower bound	Upper bound
Chitosan	30 µg/mL	0,5605	1137,000	-.138000*	.014940	.000	-.17129	-.10471
	35 µg/mL	0,6310	1372,000	-.208500*	.014940	.000	-.24179	-.17521
	40 µg/mL	0,6690	1498,667	-.246500*	.014940	.000	-.27979	-.21321
	45 µg/mL	0,7430	1745,333	-.320500*	.014940	.000	-.35379	-.28721
	50 µg/mL	0,7860	1888,667	-.363500*	.014940	.000	-.39679	-.33021
	55 µg/mL	0,7845	1883,667	-.362000*	.014940	.000	-.39529	-.32871
	60 µg/mL	0,7930	1912,000	-.370500*	.014940	.000	-.40379	-.33721
	65 µg/mL	0,8675	2160,333	-.445000*	.014940	.000	-.47829	-.41171
Positive control		0,4225	677,000	.208500*	.014940	.000	.17521	.24179
Seromucous of snail	30 µg/mL	0,3965	590,333	.026000	.013925	.091	-.00503	.05703
	35 µg/mL	0,4835	880,333	-.061000*	.013925	.001	-.09203	-.02997
	40 µg/mL	0,5105	970,333	-.088000*	.013925	.000	-.11903	-.05697
	45 µg/mL	0,5500	1102,000	-.127500*	.013925	.000	-.15853	-.09647
	50 µg/mL	0,6260	1355,333	-.203500*	.013925	.000	-.23453	-.17247
	55 µg/mL	0,6375	1393,667	-.215000*	.013925	.000	-.24603	-.18397
	60 µg/mL	0,6560	1455,333	-.233500*	.013925	.000	-.26453	-.20247
	65 µg/mL	0,7445	1750,333	-.322000*	.013925	.000	-.35303	-.29097
Positive control		0,4225	677,000	.208500*	.013925	.000	.17747	.23953
Ratio Chitosan : Seromucous of snail = 1 : 1	(C 30 µg/mL : SS 30 µg/mL)	0,6000	1268,667	-.177500*	.013901	.000	-.20847	-.14653
	(C 35 µg/mL : SS 35 µg/mL)	0,6895	1567,000	-.267000*	.013901	.000	-.29797	-.23603
	(C 40 µg/mL : SS 40 µg/mL)	0,7615	1807,000	-.339000*	.013901	.000	-.36997	-.30803
	(C 45 µg/mL : SS 45 µg/mL)	0,7995	1933,667	-.377000*	.013901	.000	-.40797	-.34603
	(C 50 µg/mL : SS 50 µg/mL)	0,9125	2310,333	-.490000*	.013901	.000	-.52097	-.45903
	(C 55 µg/mL : SS 55 µg/mL)	0,9300	2368,667	-.507500*	.013901	.000	-.53847	-.47653
	(C 60 µg/mL : SS 60 µg/mL)	0,9900	2568,667	-.567500*	.013901	.000	-.59847	-.53653
	(C 65 µg/mL : SS 65 µg/mL)	1,1370	3058,667	-.714500*	.013901	.000	-.74547	-.68353
Positive control		0,4225	677,000	.208500*	.013901	.000	.17753	.23947

*The mean difference is significant at the 0.05 level

The immune response presented an important role in Mtb infection. The risk of developing TB disease increased when conditions interfere with the immune system, such as co-infection with HIV. It was recognized that BCG vaccination cannot provide effective prevention against pulmonary TB. Macrophages in host cells play an important role in the immune system, namely phagocytosis of cellular antigens. In the lungs, bacteria are phagocytes by alveolar macrophages; however, Mtb in macrophages can change the environment by inhibiting the acidification process in phagosome maturation which results in a halted phagosome maturation process. It resulted in phagosomes unable to fuse with lysosomes so that Mtb cannot be destroyed and continues to replicate in the macrophages. It was not clear the causes of the termination of phagosome maturation. It was suspected that Mtb cells secrete virulence factors such as ESAT-6, CFP-10, MPT-64. Mtb cells had many protein antigens, some of them presented in the cytoplasm and cell walls, and others were secreted. Proteins secreted into the extracellular environment by Mtb, namely ESAT-6, CFP-10, MPB-70, MPT-64, MPT-63, MPT-80 created an immune response and have a diagnostic value (Gustiani et al. 2014).

Cytokines are specific molecules that can function as mediators, regulators of immunity, inflammation, and hematopoiesis in specific immunity. Specific immunity to pathogenic microbes is generated when CD4 + cells recognize the antigen presented by MHC class II molecules. CD4 + cells secrete the most important cytokines, namely IFNG, IL-4, and TNF-α which activate macrophages to destroy pathogens (Sutanto et al. 2021). Furthermore, IL-4, IL-5, and IL-6 induce differentiation of B lymphocytes into memory cells and plasma cells for antibody production. Cytokines can react synergistically with two or more other cytokines, together or antagonistically. Cytokines trigger the release of other cytokines, and they can also play a role in preventing inflammatory overreaction. Cytokines are important signals to activate the work of other cells so that the type of cytokines produced affects the target cell. Immunological type 1 or Th1 cell cytokines that enhance cellular immune response (IFN-γ, TNF-a, TGF-ß, IL-1, IL-2, IL-11, IL-12, IL-18). Th-1 cytokines activate macrophages, form pro-inflammatory cytokines,and induce cytotoxic effector immune mechanisms of macrophages. Th2-type cells that support antibody response (IL-4, IL-5, IL-6, IL-10, IL-13). Th-2 cytokines induce antibody formation, as well as inhibiting macrophage function, referred to as anti-inflammatory cytokines. IFNG is produced by helper T cell lymphocytes and acts on macrophage cells, endothelial cells, fibroblasts, cytotoxic T cells, and B lymphocytes which are capable of being anti-viral (Sudiana 2014). IFNG is generated during the immune response by the presence of T cell-specific antigens and natural killer cells (NK cells) which are stimulated by IL-2. IFNG will activate macrophages to increase phagocytosis and the ability to kill tumor cells, increase the growth of cytolytic T cells and NK cells. Other IFNG activities are increasing antigen presentation by macrophages, activating lysosomal activity in macrophages, increasing Th2 activity, influencing normal cells to increase the expression of MHC class I molecules, promoting adhesion and binding of migrating leukocytes, promoting NK cell activity and activating APCs, stimulating differentiation. Th1 with transcription factor T. IFNG regulates the expression of MHC-1 antigen and induces MHC class II. By activating MHC class II in endothelial cells, these cells become sensitive to the action of specific class II cytolytic T cells (Levinson & Jawetz 2003). IFNG and IL-4 are cytokines that play a role in increasing the activity of macrophage cells in the phagocytosis process against inflammation. Measurement of IFNG and IL-4 cytokines can be used as an indicator of a protective immune response in the phagocytosis process against bacterial infections. This indicates that there is a significant increase in IFNG and IL-4 levels so that the chitosan and snail seromucous are potential candidates and potential as immunomodulators and candidates for anti-inflammatory cell therapy drugs including TB therapy.

Conclusion

The results of the physic-chemical examination of the snail seromucous showed a specific gravity of 1.010; pH 8, glucose 16 mg/dL; cholesterol 9 mg/dL; protein 2.8 mg/dL and heavy metals (Pb, Cu, Hg, Al) negative. The results of microbiological tests showed that a 100% concentration of seromucous was antimicrobial against *Staphylococcus aureus, Candida albicans*, and *Pseudomonas aeruginosa*. The protein profile of the SDS-PAGE method confirmed that there were 3 protein subunits, namely the range of 55 - 72 kDa as a bioactive compound of Achasin sulfate, and 1 specific protein subunit of 34 kDa. A single dose of 65 µg/mL of chitosan and a single dose of 65 µg/mL of snail seromucous and a mixture of chitosan (65 µg/mL): snail seromucous (65 µg/mL) = 1: 1, can increase lymphocyte proliferation, optimum levels of IFNG and IL-4. Meanwhile, the optimum levels of IFNG and IL-4 were a mixture of chitosan (65 µg/mL): snail seromucous (65

μg/mL) with a ratio of 1: 1. The result indicated that the mixture of snail seromucous and chitosan is effective as an immunomodulator and has the potential as a candidate for drug therapy for TB, so further research is needed.

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