

Sensitivity Test of *Mycobacterium tuberculosis* to Snail Seromuroid and Chitosan in vitro

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ABSTRACT

Tuberculosis (TB) is an infection caused by *M. tuberculosis* (MTb) and is transmitted through droplets of phlegm in the air from patients or those suspected of having TB. In general, treatment for TB is done with anti-tuberculosis drugs (ATDs), specifically streptomycin, isoniazid, rifampicin, and ethambutol (SIRE) that takes a long time due to the level of resistance of MTb bacteria. The resistance of MTb triggers ATDs based on natural bioactive compounds. Chitosan as a result of chitin deacetylation can function as an antimicrobial agent because it is polycationic, which is biodegradable, biocompatible, and non-toxic. Snail (*Achatina fulica*) seromuroid contains antibacterial bioactive compounds, namely glycans, peptides, glycopeptides, achasin protein, and chondroitin sulfate. This study aims at testing the sensitivity of MTb isolates against snail seromuroid and chitosan in vitro. This research applied the experimental research method. MTb isolates were obtained from sputum samples of patients suspected of TB at the Surakarta Regional Public Hospital (RSUD Surakarta). The results of screening for MTb were positive, based on the microscopic examination of MTb using the Ziehl Nelson (ZN) method, the MPT 64 rapid test, and the quick molecular test using the Genexpert method. The research was completed through several stages, including the preparation of a suspension of germs with a concentration of 1 mg/ml or Mc. Farland 0.5-1.0; preparation of the stock solution and working solution (WS); drug sensitivity test (DST) against snail seromuroid; chitosan and ATDs (SIRE) on Lowenstein Jensen (LJ) media; and incubation at 37°C for 3-4 weeks. The results were interpreted on day 28 or day 42. The results have revealed that MTb isolates are 100% resistant to snail seromuroid and 2% chitosan. This study concludes that MTb isolates from suspected TB are resilient to 100% snail seromuroid and 2% chitosan.

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INTRODUCTION

Tuberculosis (TB) is caused by *M. tuberculosis*, a type of acid-fast bacteria (AFB). Tuberculosis is transmitted through the sputum of TB patients. The disease can be cured by administering appropriate ATD; however, recently, many MTb strains have been identified resistant to two or more ATDs have been identified, called multidrug-resistant tuberculosis (MDR-Tb).

MDR-Tb is TB triggered by MTb that is resistant to anti-tuberculosis drugs, namely isoniazid and rifampin, either with or without other drug resistance. In Indonesia, many factors contribute to MTb resistance to ATD or MDR-Tb. Patient ignorance about TB disease, poor patient compliance, ineffective monotherapy or drug regimens, inadequate doses, poor instructions, low medication regularity, poor patient motivation, inadequate drug supply, poor bioavailability, and drug quality are all the factors that contribute to MDR-Tb. Resistance to MTb urges the use of alternative medications such as ethionamide, aminosalicylic acid, cycloserine, capreomycin, ciprofloxacin, and ofloxacin.

TB treatment with ATD has previously been done with the appropriate administration; however, many MTb strains have recently been found to be resistant to two or more ATDs, labeled as MDR-Tb strains. Because of the formation of MTb strains that are resilient to two or more antituberculosis drugs (OATs), the failure rate of tuberculosis therapy becomes high. The most common ATDs used to treat pulmonary tuberculosis are isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin.

MTb strains that are resistant to two or more ATDs can result in a high rate of TB therapy failure. The study by Sutanto (2020a) reported the side effects of MDR-Tb therapy and the correlation between cure rates and ATD resistance, implying that a social psychological management approach, as well as a bacterial profile related to antibiotic resistance to TB treatment, are required in MDR-Tb patients. Therefore, further research is required, including the need to investigate alternative preparations

and formulations based on natural ingredients that have the potential to be used as an alternative to ATD.

Harti et al. (2016 & 2018) discovered that snail seromuroid and chitosan contain bioactive compounds that are antimicrobial against non-MTb microbes. Snail slime and chitosan have been reported to increase lymphocyte proliferation, but the bioactive compounds that play a role in this process have not been identified. The immunostimulant properties of snail seromuroid and chitosan can be used to boost the resistance of the body to facultative intracellular pathogenic bacterial infections. Serromukoids and chitosan contain bioactive compounds that can stimulate cellular immunity, specifically lymphocyte proliferation and the production of reactive oxygen intermediated macrophages (Huda, 2016).

Freeze-dried preparations of seromuroid and chitosan show significant activity on lymphocyte proliferation in vitro compared to snail slime without freeze-drying. Snail seromuroid with a concentration of 100% is antibacterial against *Staphylococcus aureus*, *Candida albicans* and *Pseudomonas aeruginosa* (Harti et al., 2019).

The success of TB therapy depends on the early detection of MDR-Tb through sensitivity testing of the resistance level of MTb isolates to ATDs. The MTb sensitivity examination is used to identify MTb sensitivity to determine the therapeutic regimen of patients and support the MTb resistance survey in a particular area.

The antimicrobial active compounds in snail seromuroid and chitosan are potential to be used as alternatives to ATDs; and therefore, further research is required. The present study aims at investigating the sensitivity level of TB suspect MTb isolates to snail seromuroid and chitosan in vitro.

MATERIALS AND METHODS

This study is experimental research, with variables consisting of independent variables (the

doses of snail seromuroid and chitosan) and dependent variables (the results of the MTb sensitivity test to snail and chitosan seromuroid preparations). To determine the effectiveness or potential as an alternative to ATD, the SIRE type of ATDs can be used as a comparison or a standard of test preparation.

Tools and Materials

Tools: clinipet, incubator, laminar flow, autoclave, disposable mask, gloves, membrane filter, and Genexpert tool. Ingredients: stock solution, working solution, ATDs (SIRE), Lowenstein Jensen Medium Base (HIMEDIA, M162 product); Ziehl Neelsen staining; freeze-drying of snail seromuroid; 2% acetic acid, 2% chitosan (Biotechurindo, Indonesia); MPT64 rapid test, and PNB test.

Research Design

The DST for ATDs (SIRE) was used in the MTb sensitivity test to snail seromuroid and chitosan (WHO, 2018).

Research Samples

The MTb isolates were obtained from sputum samples of patients suspected of having TB in the Surakarta Regional Public Hospital from January to February 2020.

Research Procedure

MTb Isolation and Identification: Isolation and identification of MTb were based on positive screening test results from sputum samples of patients with suspected TB.

Freeze Drying of Snail Seromuroid: Snail seromuroid was taken from the direct isolation of 50 local

snails (*Achantina fulica*), with an average weight of 19g and a height/width of 25/43 mm obtained from Tegal. Freeze drying was then performed on the obtained snail seromuroid at the Pharmacy Laboratory of the University of Muhammadiyah Surakarta.

Preparation of Chitosan Solution: Pure chitosan (medical grade) was obtained from PT Biotechurindo Cirebon Indonesia.

Snail and Chitosan Seromuroid Formulation: Snail and Chitosan Seromuroid Formulations are listed in Table 1.

MTb Sensitivity Test

The procedure for testing the sensitivity of MTb or DST to snail seromuroid, chitosan, and ATDs (SIRE) referred to the procedure suggested by the Ministry of Health in 2014. The inspection stages were the preparation of a suspension of bacteria with a concentration of 1 mg/ml or McFarland 0.5-1.0, which was made into 10^{-3} and 10^{-5} dilutions; preparation of the stock solution and working solution (WS); inoculation of TB isolates on LJ media containing the test preparation; incubation at 37°C for 3-4 weeks; and the reading or interpretation of the results on day 28 or day 42, as illustrated in Figure 1.

Result interpretation:

The interpretation of the results was carried out referring to the Tuberculosis Control Guidelines (Kemenkes, 2014) as follows:

- 1 – 19 colonies grown were recorded by writing the actual number of colonies.
- 20 – 100 colonies grown were labeled 1+.
- 101 – 200 colonies grown were labeled 2+.
- 201 – 500 colonies grown were labeled 3+.
- > 500 colonies grown were labeled 4+.

Table 1. Dose of ATDs, Snail Seromuroid and Chitosan

No	Treatment	Dose	Description
1.	LJ + Rifampicin	8000 mg/L	suitable with the ATD dose
2	LJ + Isoniazid	20 mg/L	suitable with the ATD dose
3	LJ + Ethambutol	200 mg/L	suitable with the ATD dose
4	LJ + Streptomycin	800 mg/L	suitable with the ATD dose
5	LJ + Snail Serumocoid	8000 mg/L	Analogous to rifampicin dose
6	LJ + Chitosan 2%	800 mg/L	Analogous to streptomycin dose
7	LJ + without medicine	-	Negative control
8	LJ + PNB	2.25 mL	



Figure 1. Preparation of MTb sensitivity test

Data Analysis

Research data were determined based on the calculation of the percentage of resistance using the following formula:

$$\% \text{ resistance} = \frac{\text{number of colonies in the media with drugs}}{\text{number of colonies in the media with drugs}} \times 100$$

If the result is less than 1%, then it is considered sensitive against a particular drug concentration.

If the result is greater than 1%, then it is considered resistant against a particular drug concentration.

RESULTS AND DISCUSSION

Snail seromuroid was obtained from 10 - 50 snails, cleaned of dirt, opened at the end of the shells, and given an electric shock of 5-10 volts for 30 -60 seconds, and the liquid that came out was accommodated in an Erlenmeyer flask. The liquid was centrifuged for 30 minutes at 3,000 rpm or 10 minutes at 3,500 rpm. Furthermore, the snail seromuroid was freeze-dried at the Pharmacy Laboratory of the Muhammadiyah University of Surakarta due to the absence of freeze-drying equipment at the research institution. The results of freeze-drying are presented in Figure 2.

Hemolymph or snail seromuroid contains bioactive compounds, such as glycans, peptides, glycopeptides, and chondroitin sulfate (Zhuang et al, 2015). Chondroitin sulfate in snail seromuroid functions as an immunomodulator and immunosuppressant (Anggraini, 2018). Moreover, the achasin protein content in snails (*A. fullica Ferussac*) has essential biological functions, including as a receptor for bacterial protein binding (Dolaskha et al, 2015).

The 2% chitosan used in the study refers to the research results of Harti et al. (2010) as shown in Figure 3. Chitosan was synthesized from shrimp shells or crab shells using a deacetylation process with 60% NaOH at temperatures ranging from

60°C to 100°C, deproteinization with 3.5% NaOH, decalcification with 2N HCl, and decolorization with acetone and 2% NaOCl (Ibrahim, 2016).

Chitosan as a compound β -(1,4)-2 amino-2deoxy D-glucopyranose can be obtained through the deacetylation of chitin with the addition of 60% NaOH alkaline solution and heating at a temperature of 60°C–100°C. The effectiveness of chitosan as an antimicrobial is related to the role of Chito-Oligosaccharide compounds (COS) as a glycan-binding protein complex compound, which contains 1,4-b-glucosamine (Kazami, 2005). The antimicrobial activity of COS is highly dependent on the degree of deacetylation, polymerization, and the type of bacteria and fungi. As an alternative antibiotic, COS is more effective because it does not produce a residue. Chitosan is unique in that it is polycationic and can inhibit the growth rate of diarrhoeagenic *Escherichia coli* in vitro (Harti et al, 2016). Chitosan is biocompatible, biodegradable, non-toxic, and antimicrobial, making it useful as a wound-healing agent. Because it is biodegradable, non-toxic, non-immunogenic, and biologically biocompatible with animal tissues, it has been widely used in the biomedical and pharmaceutical fields (Fernandes et al., 2010).



Figure 2 . Freeze-drying of snail seromuroid



Figure 3. The results of chitosan synthesis

The diagnosis of tuberculosis can be established based on clinical symptoms, chest X-ray, microscopic sputum examination with Ziehl Neelsen (ZN) of acid-fast bacilli (AFB) staining; colony macroscopic observation, Genexpert culture test on PNB media, and MPT64 rapid test. The screening tests for MTb isolates were microscopic examination using Ziehl Neelsen method; colony macroscopic observation, Genexpert test, and MPT64 rapid test, as demonstrated in Figure 4.

The microscopic examination of AFB with the ZN method is a simple examination using a sputum sample made into smear preparations, so the MTb cells appear rod-shaped and red; and therefore, they are considered as positive AFBs. The interpretation of the results of the sputum preparation examination was carried out using the IUATLD (International Union against Tuberculosis and Lung Diseases) scale.

MTb is a non-motile, non-spore, unencapsulated straight rod with a width of 0.3-0.6 microns and a length of 1-4 microns. The cell wall is complex, made up of 60% lipid layer and mycolic acid, complex wax, trehalose-6,6-dimycolate, arabino-mannan, and *Mycobacterial sulfolipids*, all of which play important roles in

virulence. Mycolic acid is a C60-C90 long-chain fatty acid that binds to arabinogalactan. This acid (trehalose dimycolate) is found in MTb cells and plays an important role in pathogen-host interactions. The presence of mycolic acid in MTb influences the level of resistance of bacteria to host immune cells and drugs (Retnoningrum, 2004).

Rapid test of ICT Mycobacterium tuberculosis protein-64 (MPT 64) is an immunological test with high sensitivity and specificity, making it an alternative for identifying M.Tb and MDR-Tb (Haryanto, 2015). MPT64 protein can be identified and influenced by the minimum number of germs of 10^{-5} cfu/ml. MPT 64 serves as a specific antigen secreted by the M.Tb complex (*M. tuberculosis*, *M. africanum*, *M. bovis*) during MTb growth. This protein has a molecular weight of 24 kDa and is only found in MTb, so it can be used for identification using immunochromatographic (ICT) principles via antigen-antibody binding. Isolates from pulmonary and extrapulmonary samples cultured on liquid or solid media can be used in the MTb identification test with the ICT MPT 64 method (Kanade et al, 2012).



Figure 4. The results of AFB (+) and MPT 64 (+)

WHO recommends the use of solid media as an MTb culture (2018). Sputum is used as a specimen for MTb testing because it is produced as a result of the pulmonary activity. Culture is a more sensitive method of MTb examination than microscopic examination. Because the culture method can detect up to 50 colonies per ml of sputum and has a 98% sensitivity in 80% of suspected TB cases, the sensitivity of culture

results is 80-85% higher than that of microscopic smear examination (30-60%). Constraints in culture include the relatively long time to read the results (4-8 weeks) due to the low metabolic activity of MTb because of a generation time of about 20 hours and the need for 100-1,000 cells/ml sputum to grow in culture media (Retnoningrum, 2004). A sensitivity test, or DST, was performed in this study to determine the

efficacy of snail seromucoid and chitosan potential as ATDs. DST is an MTb sensitivity test to a specific agent, such as ATDs or other preparations of other tests. Table 2 presents the results of the sensitivity test of MTb isolates to snail seromucoid, chitosan, and ATDs (SIRE).

Table 2 demonstrates that the resistance level of MTb isolates against snail seromucoid and chitosan was 100%, indicating that all MTb isolates of patients suspected of TB are resistant to freeze-dried snail seromucoid and chitosan. The ineffectiveness of snail seromucoid and chitosan as alternative ATD is attributed to qualitative and quantitative factors of the test preparation, including not optimal dose and ineffective mechanism of snail seromucoid and chitosan actions.

Research on the efficacy of snail seromucoid and chitosan bioactive compounds as anti-tuberculosis drugs (ATDs) against MTb is lacking; and therefore, the dose of snail seromucoid is analogous to rifampicin 8,000 mg/lit and the dose of chitosan is analogous to streptomycin 800 mg/lit. Rifampicin and streptomycin are both first-line ATDs. The variation in antibacterial power compared to previous studies is influenced by the strains of MTb isolates with varying levels of resistance, as well as the quality and quantity of achasin protein in snail seromucoid and chitosan.

Seromucoid or snail hemolymph contains bioactive compounds, such as glycans, peptides, glycopeptides, and chondroitin sulfate (Vieira 2004). Various types of proteins or known as achasin proteins in snails have important biological functions, including as receptors for protein binding (enzymes) of bacteria. In addition, aldolase and myosin are identified as proteins that play a role in the regulation of hemocyte migration and have an impact on the process of killing pathogens through cytotoxic reactions and phagocytosis (Suwannatri, 2016).

Snail mucus has antibacterial properties against *Streptococcus mutans*, *Escherichia coli*, and inhibits the growth of Methicillin-resistant

Staphylococcus aureus (MRSA) (Anggraini, 2018). The study by Harti et al. (2018) reported the optimum effectiveness of a 5% chitosan mixture; 100% snail slime and 5% snail slime cream on lymphocyte proliferation in vitro. The concentration of 100% snail mucus is also effective in inhibiting the growth of gram-positive bacteria (*Staphylococcus aureus*) and gram-negative bacteria (*Salmonella typhosa*) (Huda, 2016). There are differences in the inhibitory and antibacterial properties of snail mucus against various wound isolates of *Staphylococcus sp*, *Streptococcus sp*, and *Pseudomonas sp* bacteria (Etim, 2015). The types of protein yielded from the genetic expression of each strain of snail are not uniform (Bismili, 2013). The study by Ulagesan (2018) revealed that antibacterial and antifungal tests from meat protein extracts of seven different types of snails using diffusion and dilution (MIC) methods yield varying results influenced by the ecological conditions of snails.

Chitosan is biocompatible, biodegradable, non-toxic, antimicrobial, and hydrating. It also affects the blood clotting process, making it useful as a hemostatic agent with a positive effect on wound healing. The bioactive compounds found in seromucoid and snail chitosan can stimulate cellular immunity, particularly lymphocyte proliferation and the production of reactive oxygen intermediate macrophages (Harti et al., 2016). When compared to snail slime without freeze-drying, freeze-dried seromucoid and chitosan preparations demonstrate significant in vitro activity against lymphocyte proliferation (Harti et al. 2019).

Internal and external factors of MTb cells influence the resistance level of each organism. Internal factors include cell physiological factors, such as the structure and composition of the cell wall, as well as resistance factors linked to resistance encoding genes. Meanwhile, the external factors cover the environmental factors, namely physical, chemical, and biological agents that can affect the physiology of microbial cells.

Table 2. The results of the sensitivity test of MTb isolates to snail seromuroid, chitosan, and ATDs (SIRE)

No.	Sample	Dilution	Interpretation Result								
			Negative Control	Chitosan 2%	Snail Serocumoid	Conclusion	Strep tomycin	Iso- niazid	Rifampycin	Etham- butol	Conclusion
1	122	10 ⁻³	2+	2+	2+	Resistant	2+	Negative	Negative	2+	S,E = Resistant
		10 ⁻⁵	1+	1+	1+		1+	Negative	Negative	1+	I,R = Sensitive
2	172	10 ⁻³	1+	4+	4+	Resistant	1+	Negative	Negative	Negative	S = Resistant
		10 ⁻⁵	15 Colonies	3+	3+		5 Colonies	Negative	Negative	Negative	I,R,E = Sensitive
3	197	10 ⁻³	2+	3+	2+	Resistant	Negative	Negative	Negative	2+	E = Resistant
		10 ⁻⁵	1+	2+	1+		Negative	Negative	Negative	1+	S,I,R = Sensitive
4	200	10 ⁻³	4+	4+	4+	Resistant	4+	Negative	Negative	4+	S,E = Resistant
		10 ⁻⁵	3+	3+	3+		3+	Negative	Negative	3+	I,R = Sensitive
5	218	10 ⁻³	2+	3+	3+	Resistant	2+	Negative	Negative	2+	S,E = Resistant
		10 ⁻⁵	1+	2+	2+		1+	Negative	Negative	1+	I,R = Sensitive
% Resistance			100%	100%	100%		80%	0%	0%	80%	

Streptomycin (SM) mechanism of action interferes with the translation process by binding to 16S rRNA during protein synthesis. INH (Isoniazid) inhibits the synthesis of the main component of the cell wall, namely MTb mycolic acid. MTb resistance to SM is attributed to the mutations in the 16S rRNA (*rrs*) gene and the S12 ribosomal protein gene (*rpsL*) that occur at codon 43 (Keipiela, 2000).

MTb resistance to INH occurs due to mutations in the *katG* gene. Drug resistance in MTb is not caused by the presence of resistance plasmids or transposons that generally take place in other bacterial species but is affected by mutation acquisition or flux pump activation (Vilchèze, 2014). The results of the study depicted that MTb is very sensitive to INH, making it the most effective ATD for TB treatment and prevention. INH resistant strains often appear with a frequency of around 90% and the resistance is commonly caused by mutations in one of the *katG*, *inhA*, or *ahpC* genes (Retnoningrum, 2004).

Rifampicin (RIF) can be bactericidal, by inhibiting nucleic acid synthesis, by binding to the β RNA polymerase subunit during the RNA transcription process (Retnoningrum, 2004).

Ethambutol (EB) is bactericidal by interfering with carbohydrate metabolism and cell wall biosynthesis. MTb is resistant to EB is because of the mutations in the arabinosyltransferase encoding *embB* gene, causing inhibition of biosynthesis or cell wall polymerization of arabinan as an arabinogalactan component, as well as accumulation of the lipid carrier decaprenol phosphoarabinose (Vilchèze, 2014).

The effectiveness of a drug that is bactericidal or bacteriostatic against MTb isolates can be influenced by the physiology of bacterial cells, which is the genetic factor related to extra-chromosomal gene plasmids that cause a bacterial strain to be resistant to an agent or drug, as well as the environmental factor of mutation processes due to physicochemical mutagenic agents.

The results of the research by Wu et al, (2019) showed that the prevalence of MDR-Tb, extensively drug-resistant tuberculosis (XDR-Tb),

is higher in cases of repeated TB treatments than in initial TB cases. Moreover, the variations in the resistance level of MTb bacteria to ATD are influenced by age, gender, and region.

The immune response plays a vital role in MTb infection. The risk of TB increases when the conditions that impair the immune system appear, such as coinfection with HIV. Macrophages in host cells contribute to the immune system, in the phagocytosis of cellular antigens. In the lungs, bacteria are phagocytized by alveolar macrophages. MTb in macrophages can change the environment by inhibiting the acidification process in phagosome maturation, which causes the phagosome maturation process to stop. As a consequence, phagosome cannot fuse with lysosomes so that MTb cannot be destroyed, continuing to replicate in macrophages (Burkovski, 2013).

The results of the research by Sutanto et al. (2020b) uncovered that snail seromucoid and chitosan can increase lymphocyte proliferation in vivo, making them potential as immunomodulators.

The ineffectiveness of snail seromucoid and chitosan as an alternative to ATDs triggers the need for further research related to the physicochemical properties of the polarity of bioactive compounds in snail seromucoid and chitosan; and therefore, the antimicrobial substance of the compound cannot be diffused into MTb cells due to the unique permeability of the MTb cell walls.

CONCLUSION

This study uncovers the resistance level of MTb isolates to 100% snail seromucoid, 100% chitosan, and various resistance levels to SIRE drugs, including streptomycin (80%), ethambutol (80%), isoniazid (0%), and rifampicin (0%). 100% snail seromucoid and 2% chitosan are ineffective as antibacterial agents against MTb.

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