THE SENSITIVITY TEST OF MYCOBACTERIUM TUBERCULOSIS ISOLATES FROM SUSPECT TUBERCULOSIS PATIENTS TO THE SEROMUCOUS OF SNAIL AND CHITOSAN AS AN ALTERNATIVE ANTI-TUBERCULOSIS DRUGS

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ABSTRACT

Objective: The purpose of this research is to study the sensitivity of Mycobacterium tuberculosis (MTB) isolates from suspect TB patients to seromucous of snail and chitosan as an alternative to anti-TB drugs.

Methods: The research methods include management specimen, freeze-drying of snail seromucous; formulation of dosage preparation; identification of MTB isolates; and sensitivity testing of MTB isolates to snail seromucous, chitosan, and streptomycin, isoniazid, rifampicin, and ethambutol (SIRE).

Results: The characteristics of respondents by sex and age are the majority of male respondents and productive adult age that is 26 years–52 years. MTB isolates used in the study were obtained from the results of the screening of sputum samples of suspect TB patients through microscopic smear examination and molecular rapid test using GeneXpert tools. MTB isolates in patients suspect TB are resistant against seromucous of snails and chitosan that it is compared with SIRE. The dosage of snail seromucous is 8000 mg/l, chitosan 2% is 800 mg/l and SIRE, respectively (rifampicin 8000 mg/l, isoniazid 20 mg/l, ethambutol 120 mg/l, streptomycin 800 mg/l).

Conclusion: MTB isolates from patients suspect who is resistant to seromucous of snail (8000 mg/l) and chitosan (800 mg/l).

Keywords: Sensitivity, Isolates, Mycobacterium tuberculosis, Snail seromucous, Chitosan, Streptomycin, Isoniazid, Rifampicin, Ethambutol.

INTRODUCTION

Tuberculosis (TB) is an infection caused by Mycobacterium tuberculosis (MTB) and transmission through droplets of sputum sufferers or suspect TB in the air. Organs that are infected are generally the lungs so that it is called pulmonary TB, but it can also attack other organs, namely, lymph, brain membranes, skin, bones, joints, intestines, and kidneys, so it is called extrapulmonary disease. TB can be identified through suspected TB, including coughing continuously for 2–3 weeks or more accompanied by blood, shortness of breath, weak body, decreased appetite, weight loss, night sweats even without activity, and fever for more than a month.

TB treatment lasts long enough that is at least 6 months of treatment which has an impact on the emergence of germ resistance and TB treatment does not work because patients drop out of treatment or take medication irregularly so that multi drugs resistance tuberculosis (MDR-TB) occurs. Most MDR-TB occurs due to lack of adherence in the treatment of T. The resistance that occurs can be in the form of primary resistance and secondary resistance. Early detection of MDR-TB and starting therapy as early as possible is an important factor for achieving therapeutic success.

TB can be cured by administering appropriate anti-tuberculosis drugs (ATD). First-line treatment for TB usually uses isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EB) or streptomycin (SM) as the main choice. There are side effects in MDR-TB therapy and the correlation between cure rates and resistance to anti-tuberculosis medication 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 against TB treatment.

Snails (Achatina fulica Ferussac) contain bioactive compounds found in seromucous or hemolymph. Seromucous of snails have bioactivity as HeLa antitumor and are non-toxic to lymphocyte cells, it can even stimulate lymphocyte proliferation. Snails seromucous as glycoprotein containing carbohydrate; fraction α-1 globulin oronucoid, glycans, peptides, glycopeptides, and chondroitin sulfate. The snail chondroitin sulfate can function as immunomodulation and immunosuppressant [1]. Another substance of snails seromucous is content of glycosaminoglycans, heparin, heparin sulfate, sulfate dermal, and hyaluronic acid; can function as stabilizer cofactors and/or coreceptor for growth factors, cytokines, and chemokines; enzyme activity regulator; molecular labeling in response to cellular damage in the process of wound healing, infection, tumorigenesis; target for bacterial, viral, and parasitic virulence factors; as well as the immune system [2]. Achainis protein in the snail has important biological functions, among others, as a bacterial binding protein (enzyme) receptor [3]. The concentration snail mucus 100% and 5% snail slime cream have an effective effect on accelerating the duration of healing of second-degree (A) burns [4].

Chitosan is a polysaccharide that is found in abundant quantities in nature, especially in waste shrimp shells, crab skin. Chitosan is β-(1-4)-2 amino-2 deoxy D-glucopyranose as a product of chitin deacetylation. The uniqueness of chitosan is polycationic so that it can suppress the growth rate of diarrheagenic Escherichia coli in vitro [5]. Symbiotic (Chirodoiosaccharide and Lactobacillus acidophilus) bio preparation in yogurt can reduce cholesterol levels in vitro and in vivo [6,7]. Chitosan has been widely used in biomedical and pharmaceutical fields because it is biodegradable, non-toxic, non-immunogenic, and biocompatible with animal body tissues [8]. The combination of 100% snail mucous...
and chitosan. 1.5% with ratio 1:2 gives the optimum wound healing rate in the in vitro test. The synergistic effect of snail mucous and chitosan on Staphylococcus aureus in vitro [9].

The presence of bioactive compounds in snail serum mucous and chitosan can stimulate cellular immune function in lymphocyte proliferation and the production of reactive oxygen intermediated macrophages which can increase the body’s immunity against intracellular facultative bacterial infection including TB infection. The high incidence of TB infection is related to the level of resistance of MTB to ATD and there is no research conducted on the potential of snail and chitosan seromucous as an alternative to ATD. Research on the study of the sensitivity of MTB isolates from patients suspected of TB to snails seromucous and chitosan as an alternative to ATD needs to support the treatment of TB infection and the development of new ATD other than SIRE that have been used.

The purpose of this research is to study the sensitivity of MTB isolates from suspect TB patients against seromucous of snails and chitosan as an alternative to ATD.

METHODS

Management sampling

Seromucous freeze-dried of snail was collected from local snails (A. fulica Ferruscasea) as many as 10-50 snails then seromucous or hemolymph of snail taken by opening the tip of the shell and the liquid that comes out is collected in a flask then centrifuged 3000 rpm 30 min. Seromucous fluid obtained was then carried out the freeze-drying process at 48°C for 24 h. The freeze-drying process of snail seromucous is carried out at the Laboratory of Pharmaceutics at Muhammadiyah University, Surakarta.

Chitosan powder was performed from Biotecherusindo, Indonesia. Chitosan used 2% was dissolved in 5% acetic acid solution.

Clinical specimens were collected from patients with suspected TB at Surakarta City Central General Hospital.

MTB culture and identification

The screening test was performed by microscopic examination of Ziehl–Neelsen (ZN) staining and Molecular Quick Test – GeneXpert instrument in accordance with relevance guidelines. The positive MTB isolates were subjected to cultivation with Lowenstein–Jensen (LJ) medium (HiMedia, M162 product). Suspension of bacteria obtained a concentration of 1 mg/mL or McFarland 0.5–1.0. Suspension of germs medium (HiMedia, M162 product). Suspension of bacteria obtained a concentration of 1 mg/mL or McFarland 0.5–1.0. Suspension of germs was performed by trained and specialized persons in a biosafety cabinet in accordance with relevant guidelines. 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 30s 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 a concentration with a turbidity of 0.5–1.0 McFarland standard using a nephelometer. The dilution suspension was performed to $10^{-2}$ and $10^{-3}$. Stock solutions and working solutions were prepared. One hundred microliter suspension of the subsequent suspension was inoculated to each tube of LJ media that contained freeze-dried seromucous, chitosan, and ATD, including SIRE. The tube culture was incubated at 37°C for 28–42 days and results of the percentage of resistance can be known.

RESULTS AND DISCUSSION

Isolation and identification of MTB isolates

The screening tests conducted in this study included microscopic examination according to IUALTD; culture on LJ media added PNB and MPT-64 rapid test on five TB isolates. Isolates of MTB were obtained from culture-positive MTB cases and non-tuberculosis mycobacteria (NTM) were excluded from the study. All the MTB isolates were validated by both the growth test on p-nitrobenzoic acid (PNB) and MPT-64 antigen detection kit, as shown in Table 1.

The most TB diagnosis measures practiced in the area are direct microscopy for acid-fast bacilli (AFB). AFB microscopic examination is a simple examination by using the ZN staining method, which is from a sputum sample made smear preparations. The results of smear microscopic carried out using the International Union against Tuberculosis and Lung Disease scale. MTB is known AFB in the form of a straight rod with a width of 0.3–0.6 µ and a length of 1–4 µ, non-motile, non-sporicidal, and not encapsulated. The cell wall is complex, consisting of 60% of the fat layer and is composed of mycolic acid, complex waxes (complex-waxes), trehalose-6,6-dimikolat, arabinomannan, and mycobacterial sulfolipid that play an important role in virulence. Mycolic acid is a chain of fatty acids. Mycolic acid is a long-chain fatty acid (C60-C90) that binds to arabinogalactan. The basic structure of a typical and complex cell wall such as Gram-positive bacteria, the inner layer of the membrane is covered with a thick layer of peptidoglycan but has a more complex structure compared to other Gram-positive so that MTB is known to be acid-resistant. The microscopic method as a non-expensive and suitable method for the diagnosis of TB. The overall sensitivity and false-negative results of the microscopic examination were 66.0% and 34.0%, respectively. In addition, the overall specificity and false-positive results would be 94.6% and 5.4%, respectively. There was a report for comparing the positive samples in microscopy and culture methods regarding the culture as a gold standard method and AFB were found in only 46% of culture-positive smears and in the range of 53–80% of the first smear of positive sputum samples. The sensitivity and specificity of the sputum smears compared to the culture method were 80% and 94.1% [11]. Rapid MPT64 ICT test is an immunological test that can be used to identify new and old MTB isolates with the record that the isolate is still alive; it has high sensitivity and specificity, so it becomes an alternative for the identification of MTB and MDR-TB. The antigen component in the cell wall and cytoplasm consists of lipid, polysaccharide, and protein components. There are also groups of

| No | Code sample | Sex | Age (year) | Culture results | PNB | MPT-64 | Result |
|----|-------------|-----|------------|----------------|-----|--------|--------|
| 1  | 122         | Male| 40         | 7 colonies     | Negatif| Positif | MTb    |
| 2  | 172         | Female| 52       | 12 colonies    | Negatif| Positif | MTb    |
| 3  | 197         | Male| 26         | 2+             | Negatif| Positif | MTb    |
| 4  | 200         | Male| 44         | 2+             | Negatif| Positif | MTb    |
| 5  | 218         | Male| 38         | 4+             | Negatif| Positif | MTb    |
Based on Table 2, it showed that all MTB isolates in patients suspected TB are resistant against seromucous of the snail and chitosan that it is compared with SIRE. The drug-resistance pattern of all MTB isolates that 100% were resistant against freeze-dried seromucous of the snail, chitosan, and vary with ATD as follows: SM 80%, INH 0%, RIF 0%, and EB 80%. This is thought to be related to the mechanism of action of seromucous seromucous of the snail and chitosan which has not been effective. The resistance level of each organism is influenced by internal factors and external factors of the cell. Internal factors include physiological factors, namely the structure and composition of cell walls, resistance factors associated with resistance coding genes. While external factors include environmental factors, namely chemical and biological-physical agents that can affect the physiology of microbial cells. In addition, the factor causing the ineffectiveness of the test preparation as an alternative to ATD is the quantitative factor of the test preparation which is not optimal dosage. The ineffectiveness as an alternative to ATD is due to the biochemical, physical properties of the preparations as the solubility and dispersion compound active of seromucous freeze-dried, and chitosan which is unable to penetrate the permeability of MTB cells so that the dosage of the test preparation used is not optimal as bactericidal agents. Besides that, the factor of physiological characteristics of MTB cells as AFB cells that have specific characteristics compared to other physiological bacterial cells with the presence of mycopic acid in cell walls acts as a factor for the virulence of MTB bacterial cells. The sensitivity test results of MTB isolates against SIRE showed that RIF and INH were the most effective ATD because all MTB isolates were sensitive so that there was no growth in LJ medium with the drug. Each type of ATD has a different mechanism of action so that it has an effect on the effectiveness of bactericidal as ATD. RIF and SM are drugs as first-line ATD which are commonly used today. The mechanism of action of SM with the translation process by binding to 16 s RNA in protein synthesis. INH inhibits the synthesis of mycopic acid so that INH as the most effective ATD for the treatment and prevention of TB. INH resistant strains often appear with a frequency of around 90% and the resistance is caused by mutations in one of the katG, inhA, or ahpC genes. INH in cells will turn into active in an oxidized form due to activation of the enzyme catalase-peroxidase (KatG) which is encoded by the katG gene. The katG gene encodes the enzyme catalase-peroxidase which activates INH as a prodrug so that mutations in 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 [16]. RIF as bactericidal by inhibiting nucleic acid synthesis by binding to the β RNA polymerase subunit in 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 an important component in the transcription process. The transcription process is inhibited because RIF is specifically bound to the β RNA polymerase subunit. EB is a bactericidal by interfering with carbohydrate metabolism and cell wall biosynthesis. While PZA as an analog of nicotinamide structure is bactericidal because pyrazinoic acid as a result of the activity of the pyrazinamidase enzyme formed under acidic conditions. PZA resistance occurs due to mutations in the pncA gene that causes loss of pyrazinamidase activity (PZAAse) so that the mechanism of action of PZA becomes inactive when entering the cell MTB resistance to EB occurs due to mutations in the embL gene that encodes arabinosyltransferase so that the biosynthesis of PZA is not performed with the translation of the compound active as arabinofuranosyl components and the occurrence of the accumulation of decaprenyl-phospho-arabinose lipid carriers [17]. The potential of snail mucus in the wound healing process was due to its active substance content. Snail mucus contained chemicals such as Achaen isolates, heparan sulfate, and calcium. The Achaen isolates were beneficial as antibacterial and analgesic, while calcium played a role in hemostasis. The effects of snail mucus as antibacterial and anti-inflammatory accelerated the inflammatory phase, either
the proliferation phase of wound healing. The seromucous of snail preparations that have been used in this study as freeze-dried preparation because based on the results of research that is being done shows that snail seromucous freeze-dried has been significant activity on lymphocyte proliferation in vitro compared to snail seromucous without freeze-dried. The results of previous studies showed that snail seromucous concentration of 100% is antibacterial against S. aureus, Candida albicans, and Pseudomonas aeruginosa [18]. The difference in the variation of antibacterial agents because it is influenced by inoculum strains related to the level of resistance of microorganisms as well as the type of antibacterial Achaemin protein of genetic expression each strain varies. Antibacterial and antifungal test results of meat protein extract from seven different types of snail with diffusion and dilution methods showed that varied minimal inhibition concentration and influenced by snail's ecological conditions [19]. Various types of proteins or known as Achaemin proteins in snails have important biological functions, including as a protein-binding receptor (enzyme) for bacteria. Snail mucous is able to be as antibacterial against Streptococcus mutans and E. coli and inhibits the growth of mithiclin-resistant S. aureus [20]. The concentration of 100% snail mucus is bacteriostatic against the growth of S. aureus and Salmonella typhosa [21]. The effectiveness of bactericidal and bacteriostatic snail mucus varies with isolates Staphylococcus Sp., Streptococcus Sp., and Pseudomonas Sp. [22]. A number of protein lectins are known to be contained in snails, namely selectin, galectin, C-type lectins, and fibrinogen-related proteins secreted by snails after an infection which has a role in the process of pathogen agglutination [23]. The results of the characterization of small protein seromucous profile SDS-PAGE method showed that there were three subunits of protein, namely the range of 55-72 kDa as the Achaemin sulfate group that played an antimicrobial role and 1 specific protein subunit 43 kDa as an adhesive protein which was still further investigated [24].

Chitosan synthesis uses samples of shrimp shell 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% NaClO [25]. Chitosan has biocompatible, biodegradable, non-toxic, antimicrobial, and hydrating agent properties. Chitosan also influences the process of blood clotting so that it can be used as a hemostatic and positive effect on wound healing.

The content of bioactive compounds in snail seromucous and chitosan can stimulate the function of cellular immunity, namely lymphocyte proliferation and the production of reactive oxygen intermediates macrophages. Seromucoid freeze-drying and chitosan preparations showed significant activity on lymphocyte proliferation in vitro compared to snail slime without freeze-drying. Chitosan 5% gave the most effective result toward lymphocyte proliferation activity compared to 100% snail slime and 5% snail slime cream. The snail mucus cream 5% provides a higher proliferative activity than the 100% snail mucus. The mixture of chitosan 5% and snail slime 100%; snail slime 5% cream showed optimum effectiveness against lymphocyte proliferation in vitro [26].

The effectiveness of a bactericidal or bacteriostatic drug against MTB isolates can be influenced by the physiological bacterial cells as genetic factors related to the level of resistance or cell virulence and mutation process caused by mutagenic agents in physical chemistry from environmental factors. MTB cells have mycolic acid (trehalose dimycolate) which plays an important role in the interaction of pathogens with the host. Mycolic acid functions were equivalent to lipopolysaccharides in Gram-negative bacterial cells. Mycolic acid influences the function of macrophages which inhibits the fusion of macrophages in host cells against pathogens. The presence of mycolic acid content in MTB plays an important role in the level of resistance of germs to host immune cells and drugs.

The immune response also plays an important role in MTB infection. The immune system plays an effective role in most infections. The risk of developing TB increases if there are conditions that interfere with the immune system such as coinfection with HIV. Macrophages cells play an important role in the immune system host by phagocytosis of cellular antigens. Bacteria in the lungs will be phagocytosed by alveolar macrophages, but MTB found in macrophages can change the acidity of the environment so that the phagosome maturation process stops. This results in a phagosome not being able to fuse with lysosomes and MTB cannot be destroyed and subsequently replicates in macrophages. MTB cells secrete virulence factors such as ESAT-6, CFP-10, and MPB-64. MTB cells have many protein antigens, some of which are in the cytoplasm, cell walls, and others are secreted. Proteins secreted into the extracellular environment by MTB, namely ESAT-6, CFP-10, MPB-70, MPB-64, MPT-63, and MPT-80 can cause an immune response and have diagnostic values [27].

Treatment of TB with ATD so far has been given the right ATD; however, lately, there are many strains of MTB resistant to two or more ATD known as 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 the variation in the level of TB germ resistance to ATD is influenced by age, sex, and region [28]. There are side effects in MDR-TB therapy and 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 against TB treatment. This is due to the patient’s ignorance of the disease, poor patient compliance, administration of monotherapy or ineffective drug regimens, inadequate doses, poor instructions, low medication regularity, poor patient motivation, irregular drug supply, poor bioavailability, and poor quality the drug contributes to the occurrence of secondary drug resistance.

The characteristics of respondents by sex and age are the majority of male respondents and productive adult age that is 26 years–52 years. Respondents generally suspect TB is more common in men because the occurrence of cases of TB infection is closely related to the respondent's internal factors or physiological factors, namely genetic, nutritional, stressor, respondent behavior, and external factors, namely

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**Table 2: Resistance of Mycobacterium tuberculosis isolates against seromucous of snail, chitosan, and SIRE**

| No. | Sample | Dilution | Negative control Chitosan | Seromucoid of snail | Streptomycin (S) | Isoniazid (I) | Rifampicin (R) | Ethambutol (E) |
|-----|--------|----------|---------------------------|---------------------|------------------|---------------|---------------|---------------|
| 1   | 122    | 10^-3    | 2+                        | 2+                  | 2+               | Negative      | Negative      | 2+            |
| 2   | 172    | 10^-3    | 1+                        | 1+                  | 1+               | Negative      | Negative      | 1+            |
| 3   | 197    | 10^-3    | 15 colonies 3+            | 3+                  | 5 colonies       | Negative      | Negative      | 1+            |
| 4   | 200    | 10^-3    | 1+                        | 1+                  | 1+               | Negative      | Negative      | 1+            |
| 5   | 218    | 10^-3    | 3+                        | 3+                  | 3+               | Negative      | Negative      | 1+            |

% Resistance 100 100 100 80 0 0 80
CONFLICTS OF INTEREST
The author declared that there are no conflicts of interest related to this study.

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