Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2022 Jan 06; 9(A):326-333.
https://doi.org/10.3889/oamjms.2022.8088
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Microbiology*

OPEN ACCESS

The Antimicrobial Effectiveness of Chitosan and Polysaccharide of Durian Peel Extract against *Mycobacterium tuberculosis* Isolates

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Edited by: Slavica Hristomanova-Mitkovska
Citation: Sutanto YS, Harti AS, Sutanto M, Puspawati N. The Antimicrobial Effectiveness of Chitosan and Polysaccharide of Durian Peel Extract against *Mycobacterium tuberculosis* Isolates. Open Access Maced J Med Sci. 2022 Jan 06; 10(A):326-333. https://doi.org/10.3889/oamjms.2022.8088
Keywords: Anti-microbial; Chitosan; Durian peel extract; *Mycobacterium tuberculosis*; Isolates
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Received: 26-Nov-2021
Revised: 12-Dec-2021
Accepted: 27-Dec-2021

Funding: This research did not receive any financial support
Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Tuberculosis (TB) disease is an infection caused by *Mycobacterium tuberculosis* and is transmitted through sputum droplets of sufferers or suspect TB in the air. Chitosan as an antimicrobial agent can be used in the biomedical field because it has a number of hydroxyl groups (OH) and amine groups (NH2). The chemical substance of durian peel extract (*Durio zibethinus* L.) contains pectin which is multifunctional and can be used in the pharmaceutical field. Chemically, pectin is a polysaccharide polymer of D-galacturonic acid linked by -1,4 glycosidic bonds which can dissolve in water to form colloidal solutions or gels.

AIM: This study was to determine the antimicrobial effectiveness of chitosan and polysaccharides from durian peel extract (*D. zibethinus* L.) against *M. tuberculosis* isolates *in vitro*.

METHODS: The research method is based on an experimental study *in vitro*. *M. tuberculosis* isolates in this research from sputum samples of patients suspected of TB in Surakarta Regional General Hospital. The stages of the research were performed that preparation chitosan gel (CG), bioformulation of CG, and polysaccharide gel (PG) durian peel extract are 5%, 10%, 15%, 20%, and 25%, and drug susceptibility testing against *M. tuberculosis* isolates.

RESULTS: CG 10% was effective as an antimicrobial against *M. tuberculosis* isolates but PG durian peel extract (5%; 10%; 15%; 20%; and 25%) was not effective as an antimicrobial against *M. tuberculosis* isolates. The types of anti-tuberculosis drug (ATD) that was effective against *M. tuberculosis* isolates were ethambutol 80% and streptomycin 40%, while isoniazid and rifampicin were not effective as ATD against *M. tuberculosis* isolates.

CONCLUSION: CG 10% was effective as an antimicrobial against *M. tuberculosis* isolates, while PG 25% durian peel extract was not effective as an antimicrobial against *M. tuberculosis* isolates. CG has the potential as an ATD based on natural bioactive ingredients for TB therapy.

Introduction

Tuberculosis (TB) disease is an infection caused by *Mycobacterium tuberculosis* 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 multidrugs resistance TB (MDR-TB). The diagnosis of TB can be performed based on clinical symptoms, chest X-ray, microscopic examination of smear sputum, and smear culture on culture media as well as the sensitivity test of *M. tuberculosis* isolates to ATD and drug susceptibility testing (DST). TB treatment has been using anti-tuberculosis drug (ATD) regimens, including streptomycin, isoniazid, rifampicin, and ethambutol (SIRE) with a long period, so there is a need for research on the potential of the plant and or animal galenic preparations that have the potential to contain safe and effective natural bioactive compounds as an alternative to ATD other than SIRE. Complete TB control can be achieved by implementing three strategies, namely, integrated patient-focused treatment and prevention; integrated support systems and policies and intensive TB research, and innovation in the search for TB drugs [1].

The results of the latest research conducted by the author's team on the antimicrobial effectiveness of polysaccharide gel (PG) and/or chitosan gel (CG) against non-*M. tuberculosis* microbes showed that PG concentration of durian peel extract is 100%, 50%, 25%, 12.5%, 1.5%, and CG 1.5% as well as a combination of durian skin extract PG (1.5%) and CG (1.5%) ratio 1:1; 1:2; and 2:1 was antimicrobial against test culture (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231). The PG durian peel extract concentration of 100% showed optimum effectiveness as an antimicrobial against *B. subtilis* ATCC 6633. Chitosan 1.5% showed optimum effectiveness as an antimicrobial against *P. aeruginosa*

ATCC 27853. The effectiveness of a mixture of PG 1.5% and CG 1.5% ratio 1:2 showed optimum effectiveness for all test cultures. Another research of study by Sutanto *et al*., 2021 [2] showed that 5% CG was effective as an antimicrobial against *M. tuberculosis* isolates of patients suspected of TB and was able to increase lymphocyte proliferation, levels of IFN and IL-4, so it is necessary to further investigate its potential as an alternative ATD.

Chitosan or -(1.4)-2 amino-2 deoxy D-glucopyranose is a polysaccharide compound as a natural-based general cationic polymer resulting from the deacetylation of chitin compounds from shrimp shells and crab shells and has been widely used in the pharmaceutical and biotechnology fields because of its biochemical properties, degradable, biocompatible, bio-renewable, and non-toxic [3]. Deacetylated chitin-derived chitosan is a bioactive polymer and has an active amino side group that can be modified into useful precursors and derivatives. The results showed that chitosan has multifunctional properties, namely, anti-hypercholesterolemia, anti-hyperlipidemia, anti-inflammatory, and antimicrobial [4], [5]. Chitosan has higher antimicrobial activity than chitin because chitosan has a high number of polycationic amines that can interact with negatively charged carbohydrate, lipid, and protein residues located on the surface of microbial cells [6]. Along with technological advances in the pharmaceutical and health fields, various kinds of galenic materials from plants and animals have been developed, including galenic anti-inflammatory gel preparations, wound dressings, hemostatic agents, preservatives, stabilizers, and others [7].

Durian (*Durio zibethinus* L.) is a tropical fruit native to Southeast Asia, especially Indonesia. Fruit peel is the highest composition of fruit (60–75%) which is considered as waste and has no economic value, in fact, it contains lignin (15.45%), holocellulose (73.45%), and cellulose (60.45%) which can be used materials for making bio briquettes with a carbon content of 77.87% and second-generation bioethanol as well as materials for making economical partition particle boards [8]. Another chemical content of durian peel that can be utilized is pectin as a polymer of D-galacturonic acid linked by -1,4 glycosidic bonds which are polar and non-toxic [9], [10]. Durian peel polysaccharides can be isolated and synthesized as PG which is useful in the food and pharmaceutical fields as jelly, tablet, suspension, and emulsion [11], [12].

Several diseases with pathogenic microbial etiology that are a problem in Indonesia include TB. The existence of bioactive compounds that are antimicrobial in chitosan and durian peel extract which are biodegradable, biocompatible, and non-toxic, it is necessary to develop the potential utilization of these natural ingredients as natural ingredients-based galenic preparations that have multifunctional and economical applications and can be applied in the medical field. Until now, there has been no research related to the effectiveness of snail chitosan and polysaccharide of durian peel extract (*D. zibethinus* L.) as an alternative to ATD, so it is necessary to do research related to this. Based on this, the purpose of this study was to determine the antimicrobial activity of chitosan and PG of durian peel extract (*D. zibethinus* L.) against *M. tuberculosis* isolates.

Materials and Methods

This type of research uses a laboratory experimental design. The research was carried out at the Microbiology Laboratory of the Surakarta Regional General Hospital and Microbiology Laboratory, Faculty of Health, Setia Budi University, Surakarta in August–December 2020.

Materials and media: Medical grade chitosan was obtained from PT Biotech Surindo, Indonesia. The raw material for durian skin is obtained from one of the durian fruit sales centers in the Surakarta area. The durian plant specimen used has been determined, verified, and approved by the person in charge of the Plant Determination Laboratory, Plant Systematics Laboratory, Setia Budi University, Surakarta Indonesia, based on letter number: 114E/DET/UPT-LAB/5.10.2020. *M. tuberculosis* isolates were obtained from the Microbiology Laboratory of the Surakarta Regional General Hospital. The screening test was performed by microscopic examination of Ziehl Nelson staining and Molecular Quick Test - Genexpert. 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. The standard ATD used is SIRE (streptomycin, isoniazid, rifampin, ethambutol).

The research stages were bio-preparation of CG; processing of durian peel extract, isolation of *M. tuberculosis* from patient suspect TB, and DST for TB microbes. Data analysis using one-way ANOVA with a significance level of $p < 0.05$. The analysis of different tests of two means using Mann Whitney test. Interpretation of results if p value < 0.05 means that there is a significant difference, while p value > 0.05 means that there is no significant difference. The study results were analyzed using the statistical program of Statistical Package for the Social Sciences version 20.0.

Results

Extraction of PG durian peel

The white part of the durian peel is taken and dried, blended, and then dissolved in a solution of

100 mM nitric acid in a ratio of 1:40 then heated at a temperature of 90–100°C for 10 min and then filtered to obtain the filtrate and then cooled. The filtrate was precipitated with 66% ethanol (v/v) then filtered to obtain a precipitate and then dried at 50°C. The precipitate was redissolved with water, filtered, concentrated, and re-precipitated to obtain PG powder. Furthermore, 0.5% concentration of PG durian peel extract was made as Figure 1 [13], [14].

Preparation of 1.5% CG

Medical grade chitosan was obtained from PT Biotech Surindo, Indonesia. Medical grade chitosan was weighed 1.5 g and dissolved in 2% acetic acid solution to form a CG.

Bioformulation of PG and CG

Bioformulation of CG and PG durian peel extract is 5%, 10%, 15%, 20%, and 25%.

DST method against M. tuberculosis

M. tuberculosis isolates were obtained from the Microbiology Laboratory of the Surakarta Regional General Hospital. Materials and media: Lowenstein Jensen media (HIMEDIA, M162 product), ATD SIRE, Genexpert, PNB, Ziehl Nelson staining, Gram staining, and NaCl physiological. The procedure for testing the sensitivity of *M. tuberculosis* to microbial agents refers to the DST method according to the applicable standards [15]. The inspection stages include the manufacture of suspensions of *M. tuberculosis* isolates with a concentration of 1 mg/ml or Mc. Farland 0.5–1.0 and made dilutions of 10–3 and 10–5; making stock solutions and working solutions; isolates were inoculated on LJ media containing the test preparation, incubation at 37°C for 3–4 weeks. Interpretation of results related to the percentage of resistance of MTb isolates based on the growth results of Mtb isolates in LJ culture media on day 28 and or day 42. All steps were performed by trained and specialized persons in a biosafety cabinet by following under relevant guidelines Ministry of Health Republic of Indonesia (2018).

The results of antimicrobial effectiveness of chitosan and polysaccharides from durian peel extract (*D. zibethinus* L.) against *M. tuberculosis* isolates *in vitro* are listed in Table 1.

Table 1: The level of resistance of *M. tuberculosis* isolates to PG durian peel extract, CG, and ATD

Group	Dosage	% Resistance of *M. tuberculosis*	Mean	p-value	CI	
					Upper	Lower
CG	5%	40	0.4	0.004	0.03	0.77
	10%	0	0.0	0.000	0.00	0.00
	15%	0	0.0	0.000	0.00	0.00
	20%	0	0.0	0.000	0.00	0.00
	25%	0	0.0	0.000	0.00	0.00
PG durian peel extract	5%	100	1.0	1.000	1.00	1.00
	10%	100	1.0	1.000	1.00	1.00
	15%	100	1.0	1.000	1.00	1.00
	20%	100	1.0	1.000	1.00	1.00
	25%	100	1.0	1.000	1.00	1.00
ATD	S (Streptomycin)	40	0.4	0.004	0.03	0.77
	I (Isoniazid)	0	0.0	0.000	0.00	0.00
	R (Rifampicin)	0	0.0	0.000	0.00	0.00
	E (Ethambutol)	80	0.8	0.146	0.50	1.10
Negative control		100	1.0	1.000	1.00	1.00

The analysis of different test of two means using Mann–Whitney test. CG: Chitosan gel, PG: Polysaccharide gel, ATD: Anti-tuberculosis drug, *M. tuberculosis*: *Mycobacterium tuberculosis*

Based on the data in Table 1 shows that 5% CG was effective as an antimicrobial against *M. tuberculosis* isolates, but the PG of durian peel extract in a single dose of 5%; 10%; 15%; 20%; and 25% was not effective as an antimicrobial against *M. tuberculosis* isolates. The types of ATD that was effective against *M. tuberculosis* isolates were ethambutol 80% and streptomycin 40%, while isoniazid and rifampicin were not effective as ATD against *M. tuberculosis* isolates.

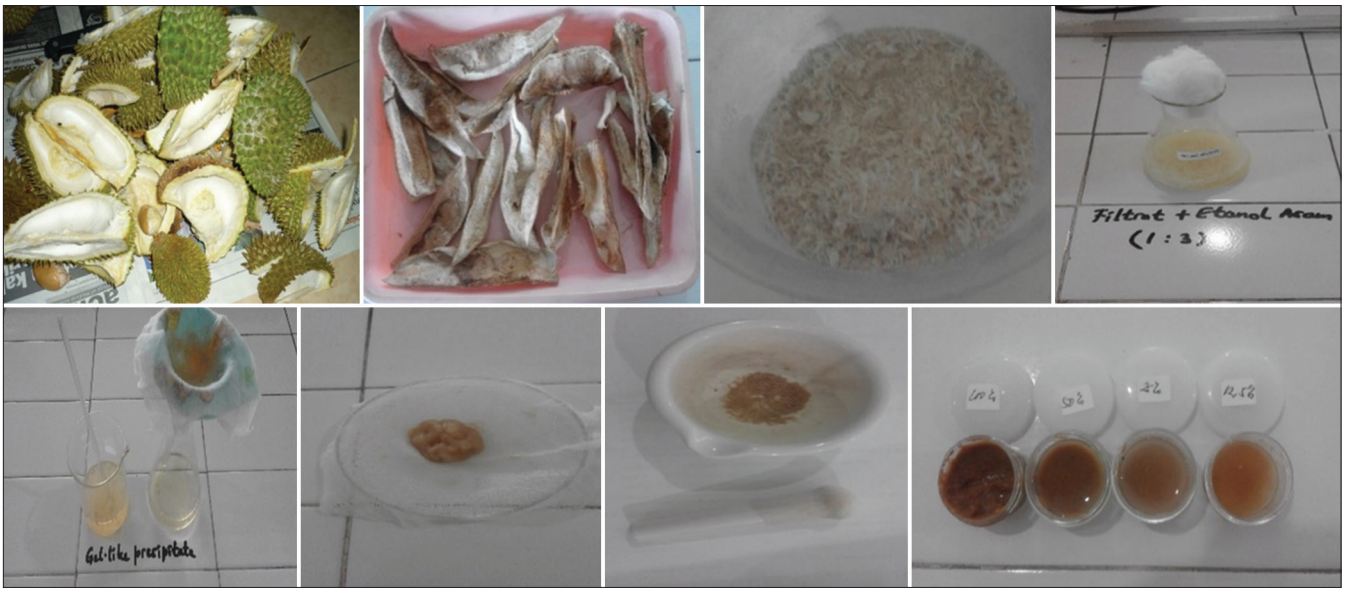

Figure 1: The process extraction of durian peel

Discussion

The diagnosis of TB can be established based on clinical symptoms, chest X-ray, microscopic sputum examination with BTA staining, culture on culture media, and the sensitivity test of *M. tuberculosis* isolates to OAT. In an effort to control TB nationally, the diagnosis of pulmonary TB must first be established by bacteriological examination is a direct microbiological examination, culture, and rapid tests. In addition, determining the level of resistance of *M. tuberculosis* to microbial agents can be done through DST. The DST method is a method recommended by Ministry of Health of the Republic of Indonesia and is used to determine the level of resistance of *M. tuberculosis* isolates to the ATD regimen.

Based on the results of the study in Table 1 showed, 5% CG was effective as an antimicrobial against *M. tuberculosis* isolates but PG of durian peel extract in a single dose of 5%; 10%; 15%; 20%; and 25% was not effective as an antimicrobial against *M. tuberculosis* isolates. Differences in the level of effectiveness of PG and CG are influenced by physicochemical factors of each preparation, including solubility, cationic charge, and molecular weight related to the process of diffusion of bioactive compounds into microbial cells. The composition of the type and amount of substances that make up the cell wall or plasma membrane plays an important role in the process of effective penetration of the diffused active compound into the cell so that the mode of action of the bioactive compound is microbiostatic and/or microbicidal. The process of diffusion of bioactive compounds into microbial cells is highly dependent on the permeability factor of the plasma membrane and/or cell wall as well as the physiology of each different type of microbe.

The effectiveness of a galenic material on CG or PG durian peel extract as an antimicrobial agent is also influenced by the type, and content of bioactive compounds, mode of action of bioactive compounds, and environmental factors such as incubation temperature, pH, humidity, and substrate or growth media. This research was conducted *in vitro* so that environmental factors are controlled variables, therefore, the difference in effectiveness as PG and CG is caused by physiological or biomolecular factors related to the expression of resistance genes of *M. tuberculosis* isolates.

Chitosan 10% was able to act as an antimicrobial against *M. tuberculosis* isolates due to the effectiveness of the mode of action of chitosan which was able to penetrate *M. tuberculosis* cell walls. There are three main mechanisms that have been recommended as the reason for the inhibition of microbial cells by chitosan [16]. The first mechanism refers to the interaction between positively charged chitosan molecules and the negatively charged surface of microbial cells. There is the mediation of electrostatic forces between the protonated NH3+ group and the negative residue, thereby inducing an internal osmotic imbalance that results in inhibition of the growth of microorganisms. The second mechanism is through the binding of chitosan to microbial DNA, which leads to the inhibition of mRNA and protein synthesis by penetration into the cell nucleus. Chitosan is thought to be able to penetrate the bacterial cell wall which is composed of multilayer murein that is cross-linked and reaches the plasma membrane and inhibits protein synthesis. The third mechanism is as a chelating agent of metal ions supplementing the basis of microbial cells; as a result, the rate of microbial growth is disrupted.

The effectiveness of chitosan as an antimicrobial is highly dependent on the degree of deacetylation and depolymerization of chitin derivatization as well as the different physiology of each microbe, including the type and amount of chemical substances that make up the cell walls of each microbe. The uniqueness of chitooligosaccharide (COS) which is polycationic is able to suppress the growth rate of diarrheagenic *E. coli in vitro* [17], [18]. Chitosan is a natural polycationic biopolymer consisting of glucosamine and N-acetyl glucosamine, as chitin derivatives. Chitosan is soluble in acetic acid, while chitin decomposes in lithium chloride and dimethylacetamide which are very toxic. Chitosan can be synthesized by partial deacetylation in alkaline conditions using NaOH or enzymatically using chitin deacetylase [19]. Chitosan as an antimicrobial is more effective than chitin because chitosan has a high number of polycationic amines that can interact with negatively charged carbohydrate, lipid, and protein residues located on the surface of microbial cells. The effectiveness of chitosan is antimicrobial related to the role of COS compounds which are glyco-binding protein complex compounds that have 1,4-b-glucosamine bonds. COS derived from chitosan is a potential substance as an “alternative antibiotic” that has a safer value without causing residue. Chitosan is able to interact in the protein flocculation process which is influenced by pH, namely, chitosan is able to absorb metal cations more effectively at high pH conditions [20]. Chitosan is able to function as a hemostatic agent in the form of chitosan salt. Chitosan salts can be synthesized by mixing chitosan with organic acids such as succinic or lactic acid. The mode of action of chitosan as a hemostatic agent is through the interaction between the erythrocyte cell layer (negative charge) and protonated chitosan (positive charge) which promotes platelet contribution and rapid thrombus formation. Chitosan salt can be mixed with different materials to make it chewier, for example, mixing with alginate or other materials that can change the solvency level and bioabsorbability of chitosan salt. Chitosan salt is biocompatible and biodegradable so it is valuable as an absorbable hemostat [21], [22]. The results of another study showed that chitosan was more effective against Gram-positive bacteria such as *Listeria* monocytogenes, *Bacillus megaterium*, *Bacillus cereus*,

S. aureus, *Lactobacillus brevis*, and *Lactobacillus bulgaris* than Gram-negative bacteria as *E. coli*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, and *Vibrio parahaemolyticus* [23].

The ineffectiveness of PG durian peel extract as an antimicrobial *M. tuberculosis* is thought to be due to the polarity of the active compound PG durian peel extract which is not able to diffuse into *M. tuberculosis* cells due to the unique physiology of *M. tuberculosis* related to cell wall composition so that it has high resistance to microbial agents. Another factor may be due to the dosage of the preparation that has not been optimal so that further research is needed regarding the determination of the optimal effective dose as an antimicrobial *M. tuberculosis* or bioformulation of other extract dosage forms, gel preparations, or liquid preparations. The ethanolic extract of durian skin (*D. zibethinus* Murr) contains secondary metabolites, namely, flavonoids, alkaloids, saponins, and tannins which have the potential to have antibacterial and antioxidant activity [24]. In general, secondary metabolites are a polar, so ethanol as a solvent is generally used for the extraction of active ingredients in a preparation containing these ingredients [25], [26]. The mode of action of flavonoids as an antibacterial is to damage the permeability of the cytoplasmic membrane. The mode of action of saponins is to interfere with the permeability of microbial cell membranes by changing the structure and function of the plasma membrane so that membrane protein denaturation occurs which results in cell damage and lysis. Saponins are hydrophilic and lipophilic so that they can reduce cell surface tension which in turn causes the destruction of germs. The mode of action of tannins as an antibacterial is interfering with cell permeability because tannins are astringent compounds causing shrinkage of cell walls or cell membranes and inducing the formation of complex compounds of microbial substrate enzymes, especially metal ions which will increase the toxicity of the tannins themselves so that cells cannot carry out living activities stunted or even dead. The mode of action of alkaloids as an antibacterial is to damage the peptidoglycan constituent components in the bacterial cell wall so that the cell wall layer is not intact and causes cell death. Another mechanism of antibacterial alkaloids is the alkaloid component known as DNA interchelator and inhibits the bacterial cell topoisomerase enzyme in the genetic process of microbial cells.

The effectiveness of durian peel extract as an antimicrobial is due to the active compound in durian peel, namely, polygalacturonase which can be synthesized as a PG that is polar and non-toxic [27]. PG (1.25%) of durian peel extract showed an inhibitory effect against *S. aureus* and PG (2.5%) against *E. coli.* The PG of durian peel extract did not seem to produce any inhibitory activity against *C. albicans* and *Saccharomyces cerevisiae* [28]. The active substance in durian peel extract, namely, polygalacturonan, is able to reduce the level of expression of the MMP-9 (matrix metalloproteinase 9) gene causing osteoarthritis which encodes gelatinase B as an enzyme capable of hydrolyzing skin protein components, namely, collagen, and elastin so that PG durian peel extract is used as fiber and biofilm in health and cosmetics [29], [30]. Another study showed that durian peel extract was able to inhibit the growth of *P. aeruginosa* at a concentration of at least 125 g/ml and the growth of *C. albicans* at a concentration of at least 25% [31]. The ethanol extract of 95% of durian fruit peel with 6% concentration was antibacterial against *S. aureus* and *Salmonella enteria* serovar typhi. The 96% ethanol extract of durian flesh *(D. zibethinus* Murr.) can act as an antibacterial against *Salmonella ATCC 14028* and *B. cereus ATCC 11778* which cause diarrhea [32], [33]. The antibacterial properties of PG durian peel extract against Gram-positive and Gram-negative bacteria have, therefore, begun to be used in the health and pharmaceutical industries [34], [35]. The results of the research showed that snail seromucous was not effective as antimicrobials against *M. tuberculosis* isolates using the DST method, but the results of *in vitro* lymphocyte proliferation tests showed that snail seromucous was able to function as biological response modifiers that play an important role as bio-immunostimulators [36].

In this study, to determine the potential of an agent against *M. tuberculosis* isolates, so ATD SIRE methods were used as positive controls. The types of ATD that was effective as antimicrobials against *M. tuberculosis* isolates were ethambutol 80% and streptomycin 40%, while isoniazid and rifampicin were not effective as ATD against *M. tuberculosis* isolates. The level of resistance of each organism to an antimicrobial agent is strongly influenced by internal factors and external factors of each cell. Internal factors include cell physiological factors, namely, the structure and composition of the cell wall; resistance factors are related to genes encoding resistance. Meanwhile, external factors that affect the physiology of microbial cells are environmental factors, namely abiotic factors including chemical physics such as temperature, humidity, environmental pH and biotic factors related to the association of species interactions. The resistance of microbial cells to antimicrobial compounds is influenced by the physiology of microbial cells related to the expression of resistance genes and mutations that occur in the DNA of microbial cells. The results of the study by Sutanto *et al*. (2020) [19] showed that chitosan (800 mg/L) and snail seromucoid (8000 mg/L) were not effective as an alternative ATD against *M. tuberculosis* isolates. This is due to the characteristics of *M. tuberculosis* known as acid resistant bacteria, rod-shaped, and Gram-positive and the cell wall of *M. tuberculosis* is composed of mycolic acid (trehalose dimycolate) which plays an important role in the pathogenesis of TB and the occurrence of the type of MDR-TB. Treatment of *M. tuberculosis* has been using drug regimens commonly used as anti-tuberculosis drugs or ATD, including SIRE. Selection of the right TB drug regimen can prevent the recurrence

of TB treatment [37]. *M. tuberculosis* is known as acid fast bacteria in the form of straight rods with a width of 0.3–0.6 microns and a length of 1–4 microns, non-motile, non-spore-forming, and not encapsulated. The cell wall is complex, consisting of 60% lipid layer and mycolic acid, complex wax, trehalose-6,6-dimycolate, arabinomannan, and mycobacterial sulfolipids which play an important role in virulence. Mycolic acid is a long-chain fatty acid (C60–C90) that binds to arabinogalactan. The level of resistance of *M. tuberculosis* isolates to OAT may occur due to the different physiological properties of *M. tuberculosis* resistant cells or strains in each region due to independent mutations in more than one OAT coding gene and or the gene encoding the enzyme activating OAT precursor. Mutations that occur in the protein synthesis process are transcription and/or translation processes that have an impact on the results of gene expression, resulting in changes in the target protein structure or changes in enzymatic activity needed to activate OAT compounds to function as bactericidal. The level of *M. tuberculosis* resistance is higher in cases of re-therapy TB than in cases of initial TB because of the presence of MDR-TB and extensively drug-resistant TB and variations in the level of resistance of *M. tuberculosis* bacteria to ATD are influenced by age, type gender, and region [38].

The results of this study showed the effectiveness of CG as an antimicrobial against *M. tuberculosis*, although PG durian peel extract was not effective against *M. tuberculosis in vitro*. The presence of bioactive compounds in CG and PG is biodegradable, biocompatible, and non-toxic, so further research is needed on the combination of CG and PG with a comparison of the optimal combination of the two doses so that it is expected to be applied as a companion or alternative to ATD in TB therapy.

Conclusion

CG 5% was effective as an antimicrobial against *M. tuberculosis* isolates but PG durian peel extract (5%; 10%; 15%; 20%; and 25%) was not effective as an antimicrobial against *M. tuberculosis* isolates. The types of ATD that was effective against *M. tuberculosis* isolates were ethambutol 80% and streptomycin 40%, while isoniazid and rifampicin were not effective as ATD against *M. tuberculosis* isolates.

Authors' Contributions

Y.S.S was responsible for constructed an idea for research, organized of the study, supervised the project, and critical review. M.S. and N.P. were responsible for biological materials, design of methodology, analysis, and interpretation of data. A.S.H. and N.P. performed the collection of clinical specimens, isolation of strains, identification and drug susceptibility testing, literature review, and cowriting of the article. Y.S., A.S.H., M.S., and N.P. were responsible for discussing the results and contribution to the final article.

Acknowledgments

The authors would like to thank the Dean of the Faculty of Medicine, Sebelas Maret State University of Surakarta, Unit Research and Community Service, Kusuma Husada University of Surakarta, Head of the Microbiology Laboratory of the Surakarta Regional General Hospital, Microbiology Laboratory, Setia Budi University of Surakarta; who have facilitated this research activity.

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