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Antibacterial Activity Test of Combination of Chitosan and Ethanol Extract of Cocoa Leaves (Theobroma Cacao L.) Against Salmonella Typhi Bacteria

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ABSTRACT

Introduction: Typhoid fever is a systemic infectious disease caused by the bacterium Salmonella typhi. The increasing antibiotic resistance of Salmonella typhi has spurred interest in natural antibacterial agents. This study examines the antibacterial activity of a combination of chitosan and ethanol extract of cocoa leaves (Theobroma cacao L.) against Salmonella typhi. Methods: The research involved extracting cocoa leaves using ethanol and combining the extract with chitosan. The antibacterial activity was evaluated using the well diffusion method on Salmonella typhi. The combination of chitosan and cocoa leaf extract was tested at various concentrations, and chloramphenicol was used as a positive control. Results: The combination of chitosan and cocoa leaf extract showed significant antibacterial activity, with the best results obtained at higher concentrations. The zone of inhibition for the combination at 600 ppm was comparable to that of the positive control of chloramphenicol. Conclusion: The study suggests that the combination of chitosan and cocoa leaf extract has the potential to be developed as an alternative treatment for typhoid fever, particularly against antibiotic-resistant strains of Salmonella typhi.

Keywords: Chitosan, Cocoa leaves, Salmonella typhi, Antibacterial activity, Well diffusion method.

INTRODUCTION

Typhoid fever is a disease caused by the bacteria Salmonella enterica serovar typhi (Salmonella typhi). According to the World Health Organization (WHO), there are 17 million cases of typhoid fever worldwide with an incidence of 600,000 deaths each year. Based on epidemiological data, typhoid fever occurs in around 9 million people worldwide, with mortality reaching 110,000 cases per year (Bhandari J, 2023).

*Salmonella typhi*is a foodborne bacterium that can be transmitted to humans through contaminated water or food, causing disease in both sexes and infecting healthy and immunocompromised patients. It causes enteric fever, which remains a major public health problem (Jaafar, 2022).

*Chitosan*is a natural biopolymer that can be obtained from chitin, one of the most abundant polysaccharides in nature. Chitosan has several advantages such as accessibility, biocompatibility, biodegradability, and no toxicity, thus showing significant antibacterial potential (Cristina., et al, 2021). Chitosan polysaccharide has two types of active groups, namely amino groups and hydroxyl groups, which can form various derivatives, and the properties of these derivatives can be further improved. Chitosan polysaccharide and its derivatives have antimicrobial and antitumor applications (Dawei., et al, 2022). Antibacterial activity can occur through several mechanisms in chitosan. One of them is through binding to the bacterial cell wall which has a negative charge, which causes disruption to the cell. In addition, chitosan can also change the permeability of the bacterial membrane, as well as attach to DNA, inhibit DNA replication, and ultimately cause cell death (Yilmaz, 2020). Another possible mechanism involved is the ability of chitosan as a metal-binding agent, which selectively binds trace metal elements. This can inhibit toxin production and microbial growth. The importance of the polycationic structure of chitosan is also a prerequisite for antibacterial activity. Electrostatic interactions between the polycationic structure of chitosan and the predominantly anionic surface

components of microorganisms play a major role in supporting antibacterial activity (Yilmaz, 2020).

Cocoa (Theobroma cacao L.) is one of the plantation commodities in Indonesia. Cocoa plantation land in Indonesia is increasing every year reaching 1,722,315 hectares in 2014 (Directorate General of Plantations, 2014). Cocoa leaves (Theobroma cacao L.) are taken from the third to eighth stalk leaves because at that stage, the leaves still contain a higher amount of secondary metabolite compounds compared to older stalks (Mandhaki, N., et al., 2021). The results of pruning cocoa leaves are used by a small number of farmers for compost, but many farmers still consider the results of pruning leaves as waste (Directorate General of Plantations, 2014).

Secondary metabolite compounds found in cocoa leaves such as flavonoids, saponins and tannins have various potential benefits as antimicrobials against Staphylococcus aureus bacteria (Rahman, MS, et al., 2018). Flavonoid compounds act as antibacterials by disrupting the cytoplasmic membrane, leading to the leakage of essential metabolites and the activation of bacterial enzyme systems. This disruption causes the release of nucleotides and amino acids, preventing active substances from entering the cell, ultimately resulting in bacterial death. Saponins function as antibacterials by lowering the surface tension of bacterial cell walls. The mechanism of action of tannins as antibacterials is that tannins cause the bacterial cell wall to become lysed, so that the formation of the bacterial cell wall is inhibited and causes the bacterial cell to die. (Noviana., et al., 2021).

This study aims to test the antibacterial activity of the combination of chitosan and ethanol extract of cocoa leaves against Salmonella typhi in the process of testing the combination of chitosan and ethanol extract of cocoa leaves.

METHODS

This study is a laboratory experiment conducted at the Pharmaceutical Microbiology Laboratory of Strada Indonesia University. The cocoa leaves used in this study were obtained from plantations in Nangkaan, Bondowoso, East Java. After being dried and ground, the cocoa leaves were extracted using 70% ethanol through the maceration method. The extract obtained was then combined with chitosan powder, which was obtained from the chitin of Litopenaeus vannamei shrimp.

Antibacterial activity was tested using the well diffusion method on Mueller Hinton Agar (MHA) media inoculated with Salmonella typhi. The concentrations of chitosan and cocoa leaf extract combinations tested were 200 ppm, 400 ppm, and 600 ppm. The inhibition zones formed around the wells were measured to assess antibacterial activity.

The materials used in this study include cocoa leaves (Theobroma cacao L.), which are extracted using 70% ethanol, chitosan powder. In addition, other chemicals used include distilled water, $FeCl_3$, NaCl, $HgCl_2$, Crystal Violet, acetic acid, ammonia, HCl, 70% ethanol, Mg powder, ethyl acetate, and n-hexane. Salmonella typhi bacteria used as test subjects were obtained from cultures that had been conditioned in McFarland solution, with growth media such as Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), and Salmonella Shigella Agar (SSA). 10% DMSO was used as a solvent in several stages of testing.

The equipment used in this study included various standard laboratory equipment, such as Erlenmeyer flasks, test tubes, funnels, measuring flasks, droppers, glassfirn, volume pipettes, petri dishes, stirring rods, measuring cups, and spoons. In addition, special tools such as well punches, autoclaves, rotary evaporators, incubators, microbiology safety cabinets, busen, ose needles, filter paper, analytical scales, rulers, sieves no. 40, and silica gel plates F254 were also used. The use of these tools aims to ensure that extraction, fractionation, and antibacterial testing can be carried out accurately and efficiently.

The research was conducted at the Pharmacy Study Program Laboratory, Strada Indonesia University for 1-3 months.

Data obtained from the measurement of the inhibition zone diameter were analyzed using SPSS for Windows software. The analysis was carried out using the One Way Analysis of Variance (ANOVA) method to compare the average data between different treatment groups. The ANOVA test was chosen because the data analyzed were numerical with one independent variable and one treatment group.

Before the ANOVA test was conducted, a normality test was conducted to ensure that the data distribution of the inhibition zone diameter followed a normal distribution. The results of the normality test showed that the data were normally distributed, so that the analysis could be continued with the

ANOVA test. In addition, a homogeneity test was also conducted to ensure uniformity of data across all treatment groups.

If the ANOVA test results show a significant difference ($P<0.05$), the Duncan Post Hoc test is performed using the LSD (Least Significant Difference) method. This test aims to identify significant differences between the test and control groups, and to determine which group shows the most effective antibacterial activity.

RESULTS AND DISCUSSION

The results showed that the combination of chitosan and ethanol extract of cocoa leaves had significant antibacterial activity against Salmonella typhi. At the highest concentration (600 ppm), this combination produced an inhibition zone with a diameter of 27.30 mm, which was almost the same as the inhibition zone produced by chloramphenicol at a concentration of 400 ppm (27.70 mm). These results indicate that the combination has strong potential as an antibacterial agent.

The mechanism of action of this combination is thought to involve several aspects that work synergistically. First, positively charged chitosan will interact with the negatively charged cell wall of Salmonella typhi, causing disruption to the structure of the cell wall. This disruption makes the bacterial cell wall more permeable, so that the active compounds from cocoa leaf extract can more easily enter and penetrate the bacterial cells.

Flavonoids in cocoa leaf extract can then interact with bacterial cell membranes, disrupting membrane integrity and causing leakage of important cellular components such as ions, nucleic acids, and proteins. In addition, flavonoids can also inhibit enzymes involved in nucleic acid synthesis, which are essential for bacterial DNA replication and transcription. This will result in disruption of the bacterial cell reproduction process, effectively inhibiting the growth of bacterial colonies.

Tannins, which are also found in cocoa leaf extract, will bind metal ions needed by Salmonella typhi for enzymatic and metabolic activities. Chelation of these ions inhibits the function of enzymes essential for bacterial growth, and ultimately leads to bacterial cell death. Saponins in the extract also play a role in disrupting bacterial cell membranes, causing lysis and cell leakage, which accelerates bacterial death.

This study is in line with previous studies showing that chitosan and plant extracts have significant antibacterial potential. However, what distinguishes this study is the use of a combination of chitosan with ethanol extract of cocoa leaves, which has not been widely studied before. The results obtained indicate that this combination has the potential to be further developed as a natural antibacterial agent, especially in the treatment of typhoid fever caused by Salmonella typhi.

The clinical implications of this study are highly relevant, especially in the context of increasing antibiotic resistance. The development of natural antibacterial agents such as a combination of chitosan and cocoa leaf extract can be an effective and safe alternative treatment, and reduce dependence on synthetic antibiotics that are increasingly losing their effectiveness due to bacterial resistance. In addition, the use of natural ingredients such as chitosan and cocoa leaves is also more environmentally friendly and potentially cheaper, which is very beneficial for developing countries such as Indonesia.

Although the results of this study are very promising, there are some limitations that need to be considered. First, this study only used one type of pathogenic bacteria (Salmonella typhi). Further research is needed to explore the effects of this combination on a variety of other pathogenic bacteria, including Gram-positive bacteria. Second, this study was conducted in laboratory conditions (in vitro), so further clinical trials are needed to confirm the effectiveness and safety of this combination in human treatment.

In addition, further research can also be done to optimize the dosage and formulation of the combination of chitosan and cocoa leaf extract, as well as to study the mechanism of action in more depth through molecular studies. Testing for bacterial resistance to this combination is also important to ensure that long-term use will not cause the emergence of resistant bacterial strains.

CONCLUSION

This study concluded that the combination of chitosan and ethanol extract of cocoa leaves (Theobroma cacao L.) showed significant antibacterial activity against Salmonella typhi, with the inhibition zone formed increasing with concentration, where a concentration of 600 ppm gave results close to the effectiveness of chloramphenicol. The results of statistical analysis showed significant differences between treatment groups, supporting the potential of this combination as a natural

antibacterial agent that can be further developed. Suggestions for further research include testing on other bacteria, optimization of formulation and dosage, and in vivo and clinical trials to ensure safety and effectiveness in humans. In addition, the development of pharmaceutical products based on natural ingredients such as this combination can be an environmentally friendly and safer alternative compared to synthetic antibiotics.

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