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Antibacterial Activity Test of the Combination of Bay Leaf (*Syzygium polyanthum*) Extract and Nanochitosan Against *Escherichia coli* and *Bacillus subtilis*

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Abstract

Indonesia is one of the world's most biodiverse countries, including in medicinal plants that contain active compounds with potential as sources of modern medicine, particularly for treating bacterial infections. The use of herbal plants as antibacterial agents offers an alternative to overcome antibiotic resistance and the side effects of synthetic drugs. Bay leaf (Syzygium polyanthum) and nanochitosan are known for their antibacterial properties due to their bioactive compounds. However, studies on their combined effects are still limited. This study aimed to evaluate the antibacterial effectiveness of combinations of bay leaf extract (15%, 25%, and 35%) and nanochitosan (0.5% and 1%) against Escherichia coli and Bacillus subtilis. A Completely Randomized Design (CRD) was applied, using sterile distilled water as a negative control and 0.1% chloramphenicol as a positive control. Antibacterial activity test was conducted using the Kirby Bauer disc diffusion method. The results showed that the positive control produced an inhibition zone of 25.45 ± 1.27 mm against E. coli and 25.05 ± 0.39 mm against B. subtilis. The best treatment was obtained in the combination of K3 (35% bay leaf extract and 0.5% nanochitosan) with an average inhibition zone diameter of 12.65 ± 0.52 mm against E. coli and 12.91 ± 0.55 mm against B. subtilis. Statistical analysis showed a significant difference based on the Tukey HSD further test. These findings indicate that the combination of bay leaf extract and nanochitosan has the potential to be developed as an alternative antibacterial agent.

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INTRODUCTION

Indonesia is a country with high biodiversity and has the potential in developing herbal medicines based on native tropical plants. In Indonesia, there are around 70,000 plant species, estimated around 7,000 have the potential as medicinal plants. There are around 1,000 to 1,200 species that have been utilized by the community. While the new traditional medicine industry uses about 300 species (Ramadhania et al., 2018). These plants contain active compounds that can be utilized as a source of modern medicine. The utilization of herbal plants as antibacterials is one of the alternative solutions in dealing with the problem of antibiotic resistance and side effects from the use of synthetic drugs. Active compounds contained in plants have the ability to inhibit bacterial growth naturally and are relatively safer for the body. Therefore, the development of local plant-based drugs is very important to support efforts to improve public health in a sustainable manner. As science develops, the utilization of medicinal plants continues to increase. Plants not only function as traditional ingredients, but can also be developed into modern products that are scientifically based. One of the potential plants is bay leaf

(*Syzygium polyanthum*). In Indonesia, bay leaves are known as a spice commonly used as a food flavoring (Agustina et al., 2022). Bay leaves are traditionally used to treat various health problems such as digestive disorders (diarrhea, abdominal pain), respiratory disorders (cough, asthma), and to help relieve fever (Shukla et al., 2023).

Various studies have proven that bay leaves have antibacterial activity. Chemical compounds contained in bay leaves that have functions as antibacterial agents are saponins, tannins, alkaloids, flavonoids, and steroids (Siagian & Sim, 2021). Research by Sapoetri et al., (2022) showed that bay leaf extract can inhibit the growth of *E. coli*, a Gram-negative bacteria that is often pathogenic. The results of Abobaker's research (2018) also reported that bay leaf extract can inhibit the growth of *B. subtilis*, a Gram-positive bacteria, with an inhibition zone between 10.00 and 16.00 mm and a minimum inhibitory concentration (MIC) between 0.02 and 0.08 mg/mL. *E. coli* is an example of Gram-negative bacteria, while *B. subtilis* is included in the group of Gram-positive bacteria which are distinguished based on cell wall structure and response to Gram staining.

Nanoparticle materials derived from animals are also widely developed as antibacterial agents. Chitosan, which is taken from the shells of marine animals, is a polysaccharide obtained from the final deacetylation process of chitin. Chitosan has a higher reactivity than chitin and can be easily provided in various forms, such as powder, gel, film, and fiber (Iber et al., 2022). The compounds contained in it can damage the bacterial cell membrane, making it antimicrobial. Chitosan is also known for its non-toxic, economical, biocompatible, naturally degradable, and soluble in solution properties. Chitosan nanoparticles also have many advantages such as high stability of use, non-toxic, large surface area, and can be used as a matrix for various types of drugs and plant extracts (Rizeq et al., 2019).

The combination of bay leaf extract with nanochitosan can significantly increase antibacterial activity, which will open up new potential in the development of more effective and sustainable antibacterial therapy. In addition, the combination of the two materials can also optimize the superior properties of each, such as the antibacterial properties of bay leaves and the biocompatible properties in chitosan solutions, resulting in a product that is more efficient and safe in its use.

METHODS

Materials used were bay leaf extracts, Nanochitosan (Merck), H_2O, Et-OH 70% (Merck), nutrient agar (NA), nutrient broth (NB), aluminum foil, bacterial strain of *B. subtitulis* (Bc), *E. coli* (Ec), paper disk (MN Germany), acetic acid (Merck), Chloramphenicol. The tools used in this study were blenders (Toshiba), oven (Memmert), incubator (Memmert), biological safety cabinet (Biobase), autoclave (Gea), varnier caliper, analytical scales, Ose Needles, Micropipette, Microtip, Dry glaski, Erlenmeyer, Petri dish, Bunsen burners, and general glass tools (Pyrex).

Preparation and Extraction

The preparation of unprocessed natural substance powder from bay leaves (*Syzygium polyanthum*) begins with wet sorting to remove dirt, followed by washing three times with running water. The cleaned leaves are then dried in an oven at 50°C to reduce moisture, prevent mold, and stop enzymatic

degradation (Putri et al., 2023). After drying, the leaves were sorted again and ground into a fine powder, which was sieved through an 80 mesh sieve to increase the extraction efficiency (Supriningrum et al., 2018). For the preparation of the extract, 600 grams of the fine powder was macerated using 96% ethanol for five days, with stirring every 24 hours. The mixture was then filtered, and the filtrate was dried in an oven at 50°C to obtain a concentrated bay leaf extract. The results were measured to evaluate the quality of the extract, as higher results indicate a greater concentration bioactive compounds (Tušek et al., 2018).

Preparation of Bay Leaf Extract Concentration Variations

The concentrated bay leaf extract formed (100% concentration) will be diluted using sterile aquadest with concentration levels of 15%, 25%, and 35%. In this research, aquadest was used as a diluent. This is intended to obtain the desired concentration of extract concentration as desired and distilled water can dissolve in ethanol and does not damage the active substances (Tammi et al., 2015). According to Kusuma et al., (2012), Making concentration using the following formula:

$$\% \text{ Concentration} = \frac{m}{v} \times 100\%$$

% Concentration : Desired extract concentration
M : Mass/weight of extract (grams)
v : Volume of solvent (ml)

Preparation of Nanochitosan Concentration Variations

Preparation of stock solution was done by dissolving 1 g of nanochitosan in 1% acetic acid to reach a volume of 100 mL. This solution was then stirred at room temperature for three hours at 400 rpm until a final concentration of 1% was obtained. Subsequently, dilution was carried out to get a concentration of 0.5% (Magani et al., 2020). Dilutions were made using the formula:

$$C1xV1 = C2xV2$$

C1 : Initial concentration
V1 : Volume to be taken from the stock solution
C2 : Desired (final) concentration
V2 : Final volume of the diluted solution

Preparation of Bay Leaf Extract – Nanochitosan Combination

The mixing was carried out at a 1:1 ratio for each group. Bay leaf extract (*Syzygium polyanthum*) at concentrations of 15%, 25%, and 35% was combined with nanochitosan at concentrations of 0.5% and 1%, with each solution contributing 10 mL to the mixture (Siagian & Sim, 2021).

Sterilization of Tools and Materials

The purpose of sterilizing equipment is to ensure that all laboratory tools are free from microorganisms that could potentially interfere with the research process. The sterilization was carried out using an autoclave. First, all tools were placed inside the autoclave, and both the door and the drain valve were opened to release water. Once the water began to boil, the valve was closed. The temperature was then raised to 121°C and maintained for 20 minutes for equipment and 15 minutes for materials.

Sterilization of solutions and media is carried out in suitable containers, such as Erlenmeyer flasks, with the mouth closed with a cotton plugs and wrapped with aluminum foil.

Preparation of Media

Nutrient Agar (NA) powder was weighed as much as 10 g and put into an Erlenmeyer flask. Then 500 mL of distilled water was added, and the mixture was heated until completely dissolved. The mouth of the Erlenmeyer flask was closed with cotton plugs, and the contents were sterilized in an autoclave at 121°C for 15 minutes. After that, the media was ready to be poured into a Petri dish (Munira et al., 2018).

Positive Control Preparation

Positive control was made from the capsule dosage form by opening one 500 mg chloramphenicol capsule and weighing the powder as much as 0.1% of the chloramphenicol capsule weight (±500 mg) and weighing the powder as much as 0.1% of the capsule weight, which is 5 mg, then dissolved in 100 ml of distilled water. (Isnawati et al., 2018).

Antibacterial Activity Test

The positive control used was chloramphenicol with a concentration of 0.1%. Using 0.1% of the capsule weight ensures the active ingredient is present at a level sufficient to demonstrate its expected biological or pharmacological effect without causing saturation or toxicity (Petersen et al., 2021). Chloramphenicol works by inhibiting protein synthesis in bacterial cells (Dinos et al., 2016). The negative control used was distilled water. The antibacterial activity test of the combination of bay leaf extract (*Syzygium polyanthum*) and nanochitosan used the paper disk diffusion method. A media inoculated by the bacteria was prepared, then the paper disk with a diameter of 6 mm is sterilized. The next step was to soak the paper disc with a solution of all treatments, positive controls, and negative controls. The paper disk were placed on the surface of the media using sterile tweezers, by pressing it gently to ensure good contact between the paper disk and the media. After that, the petri dish was incubated at 37°C for 24 hours. After the incubation period was completed, the diameter of the inhibition zone was measured on *E. coli* and *B. subtilis* bacteria. If the diameter of the extract inhibition zone was larger than the positive control inhibition zone, the extract showed high antibacterial effectiveness. Conversely, if the diameter of the extract inhibition zone was smaller than the positive control, the extract was still less effective as an antibacterial. However, increasing the concentration of the extract can increase its effectiveness, approaching or even exceeding the positive control (Oroh et al., 2015).

RESULT AND DISCUSSION

Antibacterial Activity

The results of the measurement of the inhibition zone diameter of the combination of bay leaf extract and nanochitosan are presented in Table 1.

Table 1. Antibacterial activity test of the combination of bay leaf extract and nanochitosan against *E. coli* and *B. subtillis.*

Treatment	Average diameter of zone of inhibition ± SD (mm)		Antibacterial Inhibition Category (Susanto et al., 2012)
	E. coli	*B. subtillis*	
Nanochitosan 0,5%+Bay leaf extract 15% (K1)	10.48 ± 0.48^{c}	10.85 ± 0.43^{c}	Strong
Nanochitosan 0,5%+Bay leaf extract 25% (K2)	11.46 ± 0.57^{bc}	11.53 ± 0.35^{bc}	Strong
Nanochitosan 0,5%+Bay leaf extract 35% (K3)	12.65 ± 0.52^{b}	12.91 ± 0.55^{b}	Strong
Nanochitosan 1%+Bay leaf extract 15% (K4)	8.80 ± 0.23^{d}	8.93 ± 0.37^{d}	Strong
Nanochitosan 1%+Bay leaf extract 25% (K5)	11.20 ± 0.41^{bc}	11.23 ± 0.30^{bc}	Strong
Nanochitosan 1%+Bay leaf extract 35% (K6)	12.25 ± 0.25^{b}	12.81 ± 0.72^{b}	Strong
Bay leaf extract 15% (K7)	7.67 ± 0.28^{e}	7.70 ± 0.18^{e}	Intermediate
Bay leaf extract 25% (K8)	8.67 ± 0.28^{de}	8.71 ± 0.15^{de}	Intermediate
Bay leaf extract 35% (K9)	9.61 ± 0.21^{d}	9.70 ± 0.15^{d}	Intermediate
Nanochitosan 0,5% (K10)	5.43 ± 0.35^{fg}	5.71 ± 0.47^{fg}	Intermediate
Nanochitosan 1% (K11)	6.43 ± 0.57^{f}	6.65 ± 1.68^{f}	Intermediate
Chloramphenicol (K12)	25.45 ± 1.27^{a}	25.05 ± 0.39^{a}	Very Strong
Aquadest (K13)	0.00 ± 0.00^{g}	0.00 ± 0.00^{g}	Weak

Note: Data are expressed as the mean ± standard deviation (SD) of inhibition zone diameters (mm) from three replicates. Superscript letters within the same column indicate significant differences among treatments based on Tukey's Honestly Significant Difference (HSD) post hoc test at a 95% confidence level ($p < 0.05$).

Table 1 shows that the combination of bay leaf extract and nanochitosan was able to inhibit the growth of *E. coli* and *B. subtilis* bacteria. This can be seen from the formation of an inhibition zone around the test disc paper. The average zone of inhibition of bay leaf extract and nanochitosan against bacteria *E. coli* and *B. subtilis* was highest in the K3 treatment, which is a combination of concentration of 0.5% nanochitosan with 35% concentration of bay leaf extract, with average inhibition zone ± SD of 12.65 ± 0.52; 12.91 ± 0.55, respectively. The combination of bay leaf extract and nanochitosan showed increased antibacterial activity compared to the single formulation. The combination of bay leaf extract and nanochitosan showed a synergistic effect, where the effect produced was greater than the effect of each component (Stan et al., 2021).

Based on the data presented in Table 1, it can be seen that bay leaf extract with a concentration of 35%, both in single and combination forms, shows the largest inhibition zone in its antibacterial activity. These results indicate that an increase in the concentration of bay leaf extract is directly proportional to an increase in the diameter of the inhibition zone against the growth of *E. coli* and *B. subtilis*. This study is in accordance with the study conducted by Siagian & Sim (2020), which states that the higher the concentration of bay leaf extract used, the larger the inhibition zone formed, thus indicating a stronger potential in inhibit bacterial growth.

The combination of bay leaf extract and nanochitosan produces an inhibition zone with a larger diameter compared to each formulation separately. The results of this study are in line with the findings of Khairunnisa et al., (2020), which reported that the mixture of nanochitosan with longan seed extract showed the greatest inhibition. Compared to longan seed extract, the combination of nanochitosan and longan seed extract is more effective in inhibiting the growth of *S. aureus*. This higher effectiveness is due to the role of nanochitosan as a carrier matrix that delivers active compounds from the extract, thereby increasing its antibacterial activity. The highest inhibition zone diameter is in the combination of 0.5%

nanochitosan and 35% bay leaf extract, 1% chitosan content has a higher viscosity compared to 0.5% concentration, so that the process of inhibiting the growth of *E. coli* and *B. subtilis* bacteria becomes slower. This is due to the diffusion process that taking a longer time in the NA medium containing the test bacteria. This is consistent with the findings of Magani et al., (2020), who reported that the greatest inhibition of *E. coli* and *S. aureus* growth occurred at a nanochitosan concentration of 0.5%, compared to concentrations of 1%, 1.5%, and 2%. Nanochitosan is adsorbed on the surface of bacterial cells and disrupts the membrane which causes leakage of intracellular components, thus killing bacteria (Kassem et al., 2019).

Statistical analysis of inhibition zones against *E. coli* and *B. subtilis* showed that the data followed a normal distribution according to the Shapiro-Wilk test with a significance value of > 0.05. The results of the Two-Way ANOVA test showed significant differences with a significance value of < 0.05, which prompted further analysis using the Tukey HSD post hoc test to identify specific differences between treatments. According to the Tukey HSD test, Group 3 (K3) and Group 6 (K6) were the most effective treatments, after K12 (positive control), in inhibiting the growth of *E. coli* and *B. subtilis*. Group 3 showed an inhibition zone of 12.65 mm for *E. coli* and 12.91 mm for *B. subtilis*, while Group 6 showed an inhibition zone of 12.25 mm for *E. coli* and 12.81 mm for *B. subtilis*. Groups K3 and K6 showed significant differences compared to groups with lower inhibition zones, such as K4, K7, K8, K9, K10, K11, and K13. However, no significant difference was observed between K3, K6, K2, and K5, indicating that the antibacterial effectiveness of these treatments is relatively comparable. Despite this, K3 and K6 demonstrated higher inhibition zones compared to K2 and K5, where K2 showed inhibition zones of 11.46 mm (*E. coli*) and 11.53 mm (*B. subtilis*), while K5 had 11.20 mm (*E. coli*) and 11.23 mm (*B. subtilis*). Thus, when compared to other treatments, K3 and K6 were the most effective, following K12, with K3 slightly outperforming K6 in inhibiting both bacterial strains.

The combination of bay leaf extract and nanochitosan is more effective in inhibiting the growth of *B. subtilis* than *E. coli*. This is due to differences in cell wall structure in Gram-positive and Gram-negative bacteria. Differences in bacterial cell wall structure determine the binding, penetration, and activity of antibacterial compounds (Egra et al., 2019).

Gram-positive bacteria have cell walls rich in peptidoglycan and polysaccharides (teichoic acids), and low lipid content compared to Gram-negative bacteria. The polysaccharides present in the Gram-positive cell wall function as polar polymers that facilitate the transport of positive ions, rendering the cell wall relatively more polar. Approximately 30% or more of the total cell mass consists of plasma membrane components (Malanovic & Lohner, 2016).

Gram-negative bacterial cells have a more complex structure than Gram-positive bacteria. The main difference lies in the presence of an outer membrane that encloses the peptidoglycan layer. This outer membrane makes the Gram-negative cell wall rich in lipids, offering protection from environmental influences and reducing the likelihood of peptidoglycan layer lysis. This structural feature makes Gram-negative bacteria more resistant to hypertonic environments compared to Gram-positive bacteria (Herlinawati, 2020).

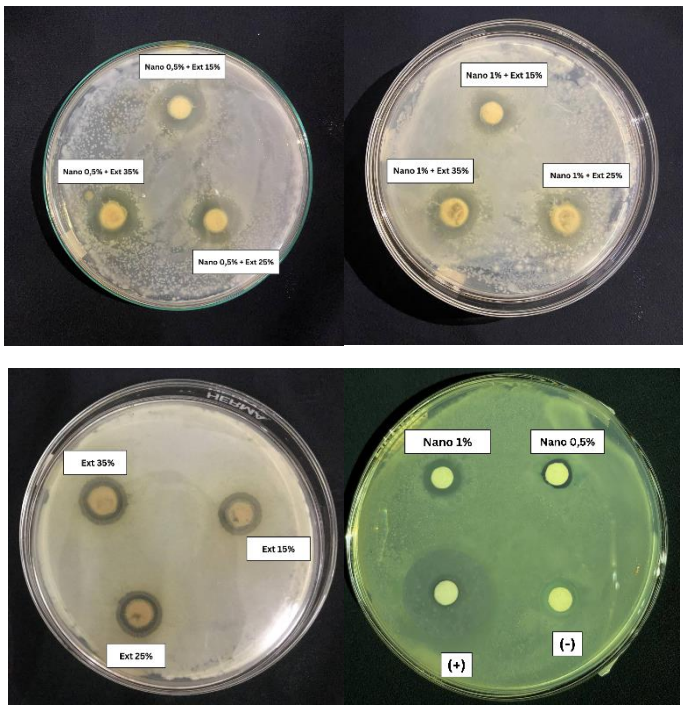

Figure 1. Inhibition zone of bay leaf extract and nanochitosan against *E. coli*.

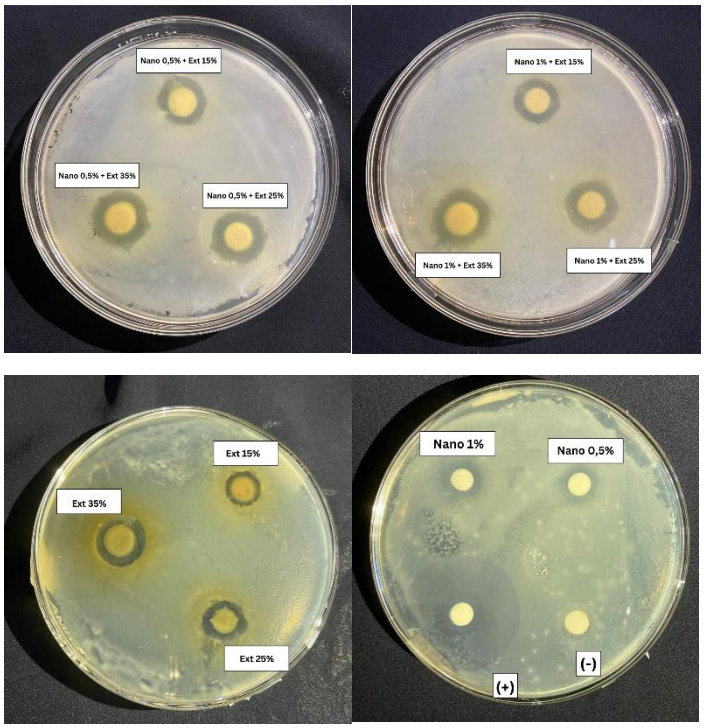

Figure 2. Inhibition zone of bay leaf extract and nanochitosan against *B. subtillis*.

In Figures 1 and 2, it can be observed that in the negative control treatment, distilled water (aquadest) did not produce any inhibition zone. This occurred because aquadest consists solely of hydrogen (H) and oxygen (O), the main components of pure water, which possess no antibacterial activity. Moreover, these elements serve as essential nutrients for microorganisms to survive. Instead of inhibiting bacterial growth, aquadest may actually support bacterial survival. Being a neutral compound,

aquadest does not affect bacterial growth because it lacks active substances capable of damaging or disrupting the bacterial cell wall.

Aquadest was used as the negative control because, according to Putri et al., (2023), a negative control in such studies refers to a solvent used to dilute the test compound—in this case, aquadest. The purpose of using aquadest as a negative control is to ensure that the solvent itself does not influence the antibacterial assay results of the tested substances.

Chloramphenicol was used as the positive control in this study to compare the effectiveness of the tested substances with a compound known to possess antibacterial activity. Chloramphenicol was selected because of its broad-spectrum antibacterial activity, making it effective against both Gram-positive and Gram-negative bacteria. Bacteria are considered resistant to chloramphenicol if the diameter of the inhibition zone is less than 20 mm, while bacteria are said to be sensitive if the diameter of the inhibition zone formed is greater than or equal to 20 mm (Giannopoulou et al., 2019). In this study, an inhibition zone of 25.45 ± 1.27 was produced in *E. coli* and 25.05 ± 0.39 in *B. subtilis*. From the inhibition zone produced, it can be seen that chloramphenicol is able to inhibit the growth of *E. coli* and *B. subtilis*, it can be seen that *E. coli* and *B. subtilis* have sensitivity to the chloramphenicol disc used in this study.

The mechanism of chloramphenicol in inhibiting bacterial growth is by interfering with protein synthesis through binding to the 50S subunit of the ribosome (Pathak et al., 2017). This binding disrupts the protein translation process by inhibiting the activity of the peptidyl transferase enzyme, which plays a role in the formation of peptide bonds during protein synthesis. As a result, bacteria are unable to synthesize essential proteins needed for their growth and survival, so that the metabolic process is disrupted, energy production decreases, and the bacterial cell structure becomes unstable, which ultimately causes inhibition or death of the bacteria (Admi et al., 2021). Further research is recommended through in vivo tests to evaluate the effectiveness of the combination of bay leaf extract and nanochitosan in more representative biological conditions.

CONCLUSION

The combination of bay leaf extract (*Syzygium polyanthum*) and nanochitosan showed antibacterial activity against *E. coli* and *B. subtilis*. This antibacterial activity indicates that the combination is able to inhibit the growth of Gram-positive and Gram-negative bacteria. The most effective treatment was the combination of 35% bay leaf extract and 0.5% nanochitosan (K3 treatment), which produced an average inhibition zone of 10.48 ± 0.48 mm against *E. coli* and 10.85 ± 0.43 mm against *B. subtilis*.

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