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Antibacterial Activity of Ketapang Leaf Extract (*Terminalia catappa*) Against *Streptococcus pneumoniae* In Vitro

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Abstract

Background: *Streptococcus pneumoniae* is a Gram-positive bacterium typically residing as normal flora in the respiratory tract, which can transform into a pathogen causing severe infections such as pneumonia and meningitis. Although amoxicillin is widely utilized as the primary therapy, its effectiveness has progressively declined due to the rising incidence of antibiotic resistance. Consequently, exploring the antibacterial potential of natural resources, such as ketapang leaves (*Terminalia catappa*), has become crucial as a viable alternative solution. **Objectives:** . This study aimed to determine the antibacterial activity of ketapang leaf extract (*T. catappa*) against *S. pneumoniae* isolates in vitro. This experimental study used a post-test group only method with ketapang leaf extract obtained through maceration. **Methods:** The antibacterial activity test was conducted using the disc diffusion method (Kirby-Bauer) on Mueller-Hinton agar media against S. pneumoniae bacteria collected from the Microbiology Laboratory of the Faculty of Medicine, Unsoed. This experimental consisted of seven groups, namely the treatment group (*T. catappa* leaf extract) at concentrations of 10%, 20%, 40%, and 80%; the positive control group (amoxicillin); the negative control group (distilled water); and the solvent group (10% DMSO) with four replicates. The inhibition zone diameter was measured and analyzed using Shapiro-Wilk, Kruskal-Wallis, and post hoc Bonferroni test to determine its antibacterial activity. **Key findings:** The results showed that ketapang leaf extract at concentrations of 10%, 20%, 40%, and 80% was unable to form an inhibition zone against the growth of *S. pneumoniae*. This finding contrasted with the positive control, amoxicillin (25 µg/disk), which produced an inhibition zone with an average diameter of 12.5 mm. The absence of inhibitory activity by the extract is suspected to be related to physical factors, such as molecular size and extract viscosity that hinder diffusion, or suboptimal bacterial growth. **Conclusions:** Ketapang leaf extract shows no antibacterial activity against *S. pneumoniae* in vitro.

Keywords: Antibacterial, Ketapang Leaf (*Terminalia catappa*), Disk Diffusion, Maceration, *Streptococcus pneumoniae*

Introduction

Streptococcus pneumoniae is a Gram-positive bacterium that is an opportunistic pathogen that causes severe infections such as pneumonia, otitis media, and meningitis. Based on Global *Burden of Disease data* in 2021, pneumonia causes 2.1 million global deaths, with the number of cases in Indonesia according to the Indonesian Health Survey reaching more than 800 thousand people in 2023. The pathogenicity of these bacteria is supported by virulence factors such as polysaccharide capsules that prevent phagocytosis, adhesion proteins (PspA and PspC) for colonization, as well as the release of pneumolysin that damages host tissue. The ability *of S. pneumoniae* to form biofilms also contributes to exacerbating systemic invasion and increasing bacterial resistance to medical therapy (1,2). Although penicillin antibiotics are the first line of treatment, the increasing cases of resistance in *S. pneumoniae* is now a serious global threat (3). This condition decreases the effectiveness of therapy and increases the risk of drug side effects, thus driving the need to search for antibacterial alternatives from natural ingredients. One of the plants that has great potential to be developed is ketapang (*Terminalia catappa*). Ketapang leaves contain bioactive compounds such as flavonoids, tannins, saponins, alkaloids, and steroids that are theoretically capable of damaging the integrity of the cell wall, inhibiting the synthesis of peptidoglycan, and interfering with bacterial homeostasis (4).

Several previous studies have proven the effectiveness of ketapang leaf extract against other Gram-positive bacteria. For example, testing on *Propionibacterium acnes* and *Staphylococcus aureus* showed significant inhibition zones (5,6). However, to date, studies that specifically evaluate the antibacterial activity of taceo leaves against the respiratory pathogen *S. pneumoniae* have not been found. This shows that there are research gaps that are important to be followed up immediately.

Based on the urgency of the high morbidity rate due to pneumonia and the challenge of antibiotic resistance, this study was conducted as the first step in the exploration of *T. catappa* against *S. pneumoniae*. The novelty of this study lies in testing the inhibition of ketapang leaf extract in *vitro* which has never been done before against these bacteria. The results of this study are expected to provide strong scientific evidence for the development of

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alternative antibacterial therapies that are more effective and have minimal side effects for the community.

Materials and Methods

This study is an *experimental design* study with *a posttest only control group design*. This study will use two groups to be tested, namely the control group and the treatment group. The control group consisted of solvent control (DMSO 10%), negative control (*aquadest*), and positive control (amoxicillin). The treatment group consisted of 10%, 20%, 40%, and 80% concentrations of tatapang leaf extract. The research was carried out by repeating 4 times per group of *S. pneumonia bacteria* in the collection of the Microbiology Laboratory of FK Unsoed. The diameter of the inhibition zone is measured to determine its antibacterial activity. Data were analyzed using SPSS software with *the Saphiro-wilk normality test*, the Kruskal-wallis *nonparametric test*, and the Post hoc Bonferroni *follow-up test*.

Tools and Materials

The tools used in this study include maceration devices, *rotary evaporators*, autoclaves (*All American Model No 25 x*), bunsen, petri dishes (*Pyrex*), ose needles (*Germany* handles), micropipettes, incubators, *vortex mixers*, digital scales (*OHAUS)*, microscopes (*Olympus*), and laboratory standard glassware. The main ingredients of the study were deciduous ketapang leaves (*Terminalia catappa*), 96% ethanol solvent, 10% DMSO%, *sterile* aquadez, *Mueller-Hinton Agar* (*MHA*) and *Blood Agar Plate* (*BAP)* media, empty disc paper, 25 µg amoxicillin antibiotic disc as a positive control, 5 µg optochin antibiotic disc paper, and bacterial pure *isolate Streptococcus pneumoniae*.

The Course of Research

The extraction process is carried out by the maceration method using 96% ethanol solvent for 3x24 hours with periodic stirring. The obtained filtrate is then filtered and concentrated using *rotary evaporator* at 50°C and followed by the above *water bath* until a thick extract is obtained. The extract was then diluted using a 10% DMSO solvent to obtain a series of concentrations of 10%, 20%, 40%, and 80% as the treatment group.

Antibacterial activity testing was performed using the disc diffusion method (Kirby-Bauer) on *Mueller-Hinton Agar* (MHA) media. Purified *Streptococcus pneumoniae* bacteria are suspended into a physiological NaCl solution until they reach *the McFarland turbidity standard of 0.5*. Inoculation of bacteria in the media is carried out evenly using sterile cotton skewers (*swab method*). The sterile disc paper is saturated into each extract concentration, negative control (*aquadest*), and solvent control (DMSO 10%). Then the disc paper is placed on the surface of the agar medium that has been inoculated with bacteria. The entire petri dish is then incubated at 37°C for 24 hours. The observed parameter is the diameter of the barrier zone formed around the perimeter of the disc, which is measured precisely using a digital caliper.

Data Analysis

The data from the measurement of the diameter of the inhibition zone obtained from the antibacterial test were processed and statistically analyzed using the SPSS device. The initial step of the analysis begins with a normality test using the *Shapiro-Wilk* given the small sample count. If the data showed abnormal distribution or inhomogeneous variance, the analysis was continued with a non-parametric test *Kruskal-Valais* to evaluate the presence of significant differences in antibacterial activity between different concentrations of ketapang leaf extract, positive control of amoxicillin, and negative control. The test continued using *Post Hoc* for double comparison with Bonferroni correction to minimize the risk of error. All statistical test results are then presented in the form of tables and narratives to provide a comprehensive picture of the effectiveness of each treatment group against bacteria *Streptococcus pneumoniae*.

Results and Discussion

This research began with the extraction process of deciduous ketapang leaves (*Terminalia catappa*) using the maceration method with 96% ethanol solvent. From the results of the extraction, a thick blackish-brown extract with a distinctive aroma was obtained. This extract is then used for the manufacture of 10%, 20%, 40%, and 80% concentration series by using 10% DMSO as a solvent so that the consistency of the extract can be completely dissolved before being applied to disc paper.

Identification of *Streptococcus pneumoniae bacteria* is carried out to ensure the purity and characteristics of the isolate before antibacterial activity tests are carried out. The results of macroscopic observations on the media show the growth of small, round, grayish-white colonies, mucoid appearance, and there is a green zone around the colony (alpha hemolytic). Based on Gram staining, the isolates exhibit characteristics of Gram-positive bacteria characterized by diplococci-shaped cells that resemble *purple lancet-shaped* cells . In addition, the sensitivity test to optochin showed the formation of an inhibitory zone around the disc, which specifically distinguishes *S. pneumoniae* from other alpha-hemolytic *Streptococcus strains*. All of these identification results confirm that the isolates used in the study are pure *S. pneumoniae* and are suitable for use as test organisms.

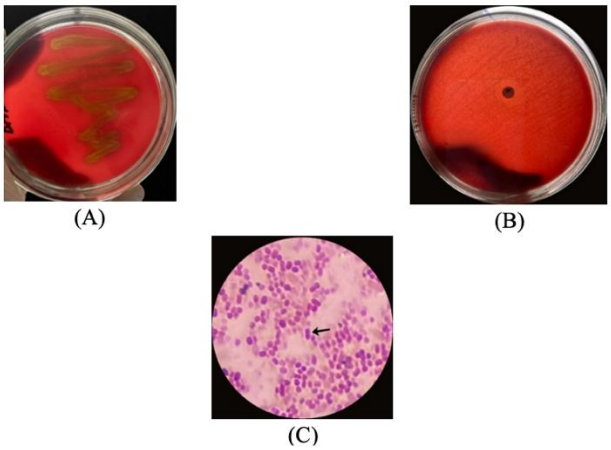

Figure 1. Morphology of *S. pneumoniae* colony (A) Inhibition zone of optochin sensitivity assays (B) Cell morphology *of S. pneumoniae* (C)

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The test results showed that the almond leaf extract at the entire concentration of 10%, 20%, 40%, and 80% had no antibacterial activity against *S. pneumoniae*. This can be seen from the absence of an inhibition zone around the disc paper, with a fixed average diameter of 6 mm which is identical to the negative control group (*aquadest*) and solvent control (DMSO 10%). The value of 6 mm formed is equivalent to the diameter of the disc paper used so it has the meaning that an obstacle zone is not formed. In contrast, the positive control of amoxicillin 25 µg showed clear activity with an average inhibition zone of 12.5 mm. This confirms that the bacteria are in a vicious state.

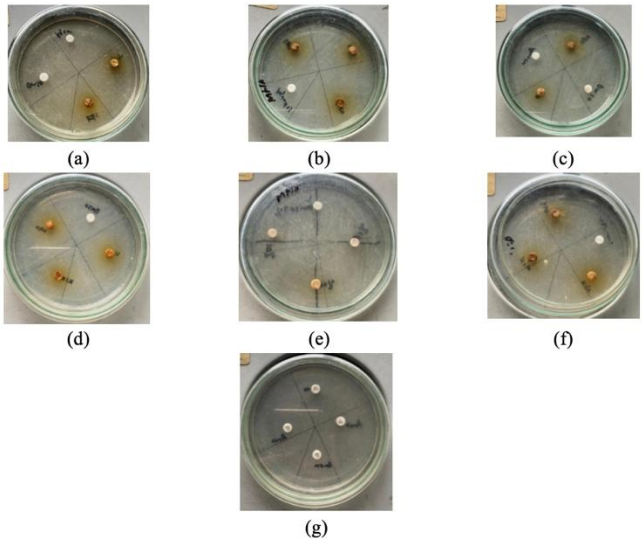

Figure 2. Antibacterial Activity Test Results (a) DMSO, *Aquadest*, K40, K80 (b) *Aquadest*, K10, K20, K80 (c) *Aquadest*, DMSO, K40, K80 (d) DMSO, K10, K20, K40 (e) DMSO, K10, K20, K40 (f) *Aquadest*, K10, K20, K80 (g) positive control (amoxisilin). K = Palm leaf extract**;** *Aquadest* = negative control**;** DMSO = solvent control (DMSO 10%)**;** The number is the concentration of the extract (%)

Statistical analysis began with the *Shapiro-Wilk* normality test which showed that the data were not distributed normally ($p < 0.05$). Based on these results, the test was continued using the non-parametric *Kruskal-Wallis test* which resulted in a significance value *of* $p < 0.001$, indicating a significant difference in the mean diameter of the inhibition zone between the treatment groups. As a final stage, *a Post Hoc* test with *Bonferroni correction* was carried out to see specific comparisons between groups. The results showed that significant differences were only found between positive controls (amoxicillin) against the other groups ($p = 0.002$), while comparisons between extract concentrations (10%, 20%, 40%, and 80%) showed no significant differences as they all gave identical results without inhibitory zones.

The identification of the isolates showed that the bacteria used had the typical characteristics of *S. pneumoniae*, namely small, round, mucoid colonies with alpha hemolytic zones and the *lancet-shaped cell shape of purple diplococcus* on Gram staining. These results are in accordance with the characteristics of colonies and *cells of S. pneumoniae* (7,8). The validity of the isolate was strengthened through the Optochin sensitivity test with an inhibition zone of 16 mm, which exceeded the standard threshold of CLSI M100 (≥14 mm) (15). This confirms that the bacteria are in a viable state and sensitive to standard controls before the extract test is performed.

Ketapang leaf extract obtained through 96% ethanol maceration has a yield of 10% and contains secondary metabolite compounds such as flavonoids, alkaloids, tannins, and saponins. This figure meets the yield standards of thick extracts (4,9). Although phytochemical screening showed positive results for antibacterial compounds, the entire concentration of the extract (10%–80%) did not result in an inhibitory zone against *S. pneumoniae*. These results were inversely proportional to the positive control of amoxicillin which showed an average inhibition zone of 12.5 mm.

The inability of the extract to inhibit *S. pneumoniae* is different from the research of Putriani *et al.* (6) which found antibacterial activity in *the Staphylococcus aureus group*. This difference is influenced by the specific characteristics *of S. pneumoniae* which have a thick, negatively charged polysaccharide capsule. This difference is in line with research by Sanchez-Rosario & Johnson (2021) which states that *S. pneumoniae* has a complex metabolism and is very easily influenced by its growing environment. The capsule serves as a physical and electrostatic barrier that prevents large molecules such as flavonoids from reaching cellular targets, especially when bacteria are subjected to nutritional stress on the media without blood supplementation (10).

In addition to the bacterial factor, technical constraints in the disc diffusion method also affected the results. The high viscosity of the viscous extract due to the accumulation of tannins and glucose inhibits the rate of horizontal diffusion of the active compound in agar media. Polyphenol molecules tend to accumulate vertically under the disc paper, so no representative barrier zones are formed. This is exacerbated by a 96% ethanol solvent that attracts hydrophobic components such as chlorophyll, which can impede the diffusion of the active compound to the hydrophilic MHA media (11,12).

Biochemically, the production of hydrogen peroxide (H2O2) by *S. pneumoniae* is thought to inactivate flavonoid compounds in the extract. Flavonoids that are supposed to damage the cell wall actually act as antioxidants to neutralize H2O2, so that their antibacterial power is weakened (13). This phenomenon does not occur with amoxicillin because the antibiotic acts specifically on *Penicillin-Binding Proteins* (PBP) in the *process of cross-linking* peptidoglycan without being disturbed by the oxidative mechanisms of the bacteria (14).

The study concluded that although ketapang leaves contain active compounds, their effectiveness in coarse diffusion methods is severely limited by the bacteria's specific diffusion and defense factors. Researchers are aware of limitations in the use of coarse extracts and qualitative phytochemical methods that have not been able to ensure effective dosage. For future studies, it is recommended to use pure fractions and dilution methods to obtain more accurate minimum inhibitory concentration (KHM) data.

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Table 1. Results of Jamming Zone Diameter Calculation

Treatment	Repetition				Average diameter (mm)
	I	II	III	IV	
Positive control (amoxicillin)	15	12	11	12	12,5
Negative control (*aquadest*)	6	6	6	6	6
Solvent control (DMSO 10%)	6	6	6	6	6
Palm oil leaf extract 10%	6	6	6	6	6
Ephemeral leaf extract 20%	6	6	6	6	6
Pumpkin leaf extract 40%	6	6	6	6	6
Ephesus leaf extract 80%	6	6	6	6	6

Conclusion

This study concluded that ethanol extract of ketapang leaves (*Terminalia catappa*) has no antibacterial activity against *Streptococcus pneumoniae* at concentrations of 10%, 20%, 40%, and 80% by disc diffusion method. The absence of an inhibition zone is suspected to be caused by the inhibition of diffusion of the active compound due to the high viscosity of the extract and the characteristics of the bacteria that are *fastidious*. Although the results of this test are negative, this study provides important basic data regarding the limitations of the use of diffusion methods in coarse extract of ketapang leaves. It is recommended for the next researcher to use the liquid dilution method and perform compound purification (fractionation) to evaluate the antibacterial potential of this plant in more depth.

Supplementary Material

None

Author Contributions

DAF : Conceptualization, Methodology, Writing-Original Draft. **RANH** : Data Curation, Formal Analysis, Visualization. **APKNW** : Supervision, Funding Acquisition, Writing- Review & Editing.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

Acknowledgement

None

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