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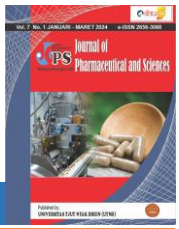

Antimycobacterial Activity and MIC–MBC Determination of *Clerodendrum minahassae* Ethanolic Leaf Extract Against *Mycobacterium smegmatis*

Aktivitas Antimikobakteri serta Penentuan MIC–MBC Ekstrak Etanol Daun *Clerodendrum minahassae* terhadap *Mycobacterium smegmatis*

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Abstract

Tuberculosis remains a major global health challenge, particularly due to the increasing emergence of drug-resistant strains, highlighting the need for new antimycobacterial agents from natural sources. Clerodendrum minahassae, a medicinal plant traditionally used in North Sulawesi, has demonstrated antibacterial activity; however, its antimycobacterial potential has not been previously explored. This study aimed to evaluate the antimycobacterial activity of the ethanolic leaf extract of C. minahassae against Mycobacterium smegmatis as a surrogate model and to estimate its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The extract was tested at concentrations of 5, 7.5, and 10 µg/disc using the disc diffusion method. Inhibition zone diameters increased in a concentration-dependent manner, ranging from 9.40 ± 0.52 mm to 10.60 ± 0.92 mm. Quantitative analysis using Bloomfield-based linear regression revealed a strong dose–response relationship (R^2 = 0.9926), from which MIC and MBC values of 0.63 µg/disc and 2.52 µg/disc were estimated, respectively. These findings indicate that the ethanolic extract of C. minahassae exhibits measurable inhibitory and bactericidal activity against M. smegmatis at relatively low concentrations. While this study represents an exploratory screening, the results support the potential of C. minahassae as a source of bioactive compounds with antimycobacterial relevance. Further investigations involving fractionation, compound identification, toxicity assessment, and validation against pathogenic Mycobacterium tuberculosis strains are warranted.

Keywords: Clerodendrum minahassae; antimycobacterial activity; Mycobacterium smegmatis; MIC; MBC.

Abstrak

Tuberkulosis masih menjadi tantangan kesehatan global, terutama akibat meningkatnya kasus resistensi obat, sehingga diperlukan pencarian agen antimikobakteri baru berbasis bahan alam. Clerodendrum minahassae, tanaman obat yang digunakan secara tradisional di Sulawesi Utara, telah dilaporkan memiliki aktivitas antibakteri, namun potensi antimikobakterinya belum banyak diteliti. Penelitian ini bertujuan untuk mengevaluasi aktivitas antimikobakteri ekstrak etanol daun C. minahassae terhadap Mycobacterium smegmatis sebagai organisme model, serta mengestimasi nilai MIC dan MBC. Ekstrak diuji pada konsentrasi 5, 7,5 dan 10 µg/disc menggunakan metode difusi cakram. Diameter zona hambat meningkat seiring kenaikan konsentrasi ekstrak, dengan rentang 9,40 ± 0,52 mm hingga 10,60 ± 0,92 mm. Analisis kuantitatif menggunakan regresi linear berbasis metode Bloomfield menunjukkan hubungan dosis–respon yang sangat kuat (R^2 = 0,9926), yang digunakan untuk mengestimasi nilai MIC sebesar 0,63 µg/disc dan MBC sebesar 2,52 µg/disc. Hasil penelitian ini menunjukkan bahwa ekstrak etanol C. minahassae memiliki aktivitas penghambatan dan bakterisidal yang terukur terhadap M. smegmatis pada konsentrasi relatif rendah. Meskipun bersifat eksploratif, temuan ini mendukung potensi C. minahassae sebagai sumber senyawa bioaktif dengan relevansi

antimikobakteri. Penelitian lanjutan diperlukan untuk fraksinasi terpandu bioaktivitas, identifikasi senyawa aktif, evaluasi toksisitas, serta validasi terhadap strain patogen Mycobacterium tuberculosis.

Kata kunci: Clerodendrum minahassae; aktivitas antimikobakteri; Mycobacterium smegmatis; MIC; MBC.

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Introduction

Tuberculosis (TB) continues to be one of the most enduring infectious diseases globally, leading to significant illness and death, even with the availability of established chemotherapy[1]. According to the latest Global TB Report from the World Health Organization (WHO), approximately 10 million people developed TB in 2020, resulting in 1.4 million deaths. This makes TB the second leading infectious killer after COVID-19[2]. The rising prevalence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains further complicates TB management. Resistance to first-line medications, such as isoniazid and rifampicin, greatly decreases treatment success rates and necessitates the search for new therapeutic alternatives[3].

Indonesia ranks among the top three countries globally with the highest burden of tuberculosis (TB), with Southeast Asia representing nearly 44% of the world's TB cases[4]. Despite progress in national TB control programs, challenges such as drug resistance, prolonged treatment regimens, medication-induced hepatotoxicity, and patient non-compliance impede effective TB eradication efforts[5]. These challenges highlight the pressing need for new anti-tuberculosis agents that are safer, more accessible, and sustainable. Natural products continue to play a pivotal role in drug discovery initiatives targeting *Mycobacterium* species.

Plant-derived metabolites, including flavonoids, terpenoids, alkaloids, phenolics, and tannins, are widely recognized for their antimycobacterial properties. These compounds target various aspects of *Mycobacterium tuberculosis*, such as cell wall integrity, energy metabolism, redox homeostasis, and protein synthesis[6]. While *several* ethnomedicinal plants from Indonesia have shown effectiveness against rapidly growing mycobacteria, scientific validation for many local species is still limited[7]. One such understudied endemic plant is *Clerodendrum minahassae* L., commonly known as leilem, which is traditionally used in North Sulawesi for its anti-inflammatory, hematinic, metabolic, and restorative properties. Phytochemical analyses of its leaves have revealed the presence of flavonoids, alkaloids, tannins, steroids, and phenolic compounds, many of which are linked to antibacterial and antimycobacterial activities[8–10].

Previous studies on *C. minahassae* have demonstrated its antioxidant, anti-inflammatory, antidiabetic, and general antibacterial activities[11–17]. However, no scientific report to date has examined its activity against *Mycobacterium smegmatis*, a non-pathogenic model organism commonly used as a surrogate for *M. tuberculosis* due to similarities in cell wall composition and antimicrobial response patterns. This gap in research is particularly noteworthy given the strong ethnopharmacological relevance and the presence of bioactive compounds that may interact with mycobacterial targets.

Therefore, assessing the antimycobacterial potential of *C. minahassae* leaves is both scientifically and regionally significant. This study aims to evaluate the anti-tuberculosis activity of the ethanol extract of *C. minahassae* against *M. smegmatis* using disc diffusion and linear regression analysis to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The results are expected to provide foundational evidence for the development of natural-product-based antimycobacterial candidates derived from Indonesian endemic flora.

Experimental Section

Materials and Apparatus

Dried leaves of Clerodendrum minahassae L. were collected from Kiawa Dua Village in North Sulawesi, Indonesia, and identified based on their morphological characteristics. The chemicals utilized in this study included 96% analytical-grade ethanol, Middlebrook 7H9 broth, Middlebrook 7H10 agar, Middlebrook ADC supplement, and streptomycin sulfate as a reference standard. The bacterial strain *Mycobacterium smegmatis* ATCC 19420 was used as the test organism.

Instrumentation consisted of a rotary evaporator (IKA® RV 10), autoclave, incubator, micropipettes (Eppendorf®), analytical balance (Sartorius®), desiccator, vortex mixer, sterile Petri dishes, digital caliper, and 8 mm paper discs (Advantec®). All glassware used was sterilized prior to experimentation.

Sample Preparation

A total of 750 g of C. minahassae leaves were washed, air-dried, and pulverized. Maceration was conducted with 1.5 L of 96% ethanol over three 24-hour periods, with periodic agitation. The filtrate was collected and concentrated using a rotary evaporator at 40 °C to yield a thick ethanolic extract. The extract was then stored at 4 °C until further use[18].

Selection of Test Organism

Mycobacterium smegmatis was selected as the test organism due to its non-pathogenic nature, rapid growth characteristics, and structural similarity to *Mycobacterium tuberculosis*, particularly with respect to its lipid-rich cell wall. These features make *M. smegmatis* a widely accepted surrogate model for preliminary antimycobacterial screening prior to confirmatory testing on pathogenic strains [19].

Antibacterial Assay Procedure

A crude leilem extract weighing 61 mg was dissolved in 610 µL of 96% ethanol. Serial dilutions were performed to achieve final concentrations of 5, 7.5, and 10 µg/disc. Streptomycin at 5 µg/disc served as the positive control, while 5 µL of 96% ethanol acted as the negative control. Each solution was applied to sterile paper discs using a micropipette, and the discs were then placed on a clean spot plate. They were dried in a desiccator for 24 hours. Since ethanol was the solvent for both the extract and the negative control, it was crucial to ensure that all discs dried thoroughly in the desiccator. This step guaranteed that any observed inhibition zones were due to the extract itself and not residual solvent.

A 1 mL aliquot of *Mycobacterium smegmatis* suspension was mixed with 100 mL of sterile medium. The inoculated medium was poured into sterile Petri dishes and allowed to solidify. Extract-impregnated discs were placed on the surface of the solidified agar in labeled Petri dishes and incubated at 37 °C for 48 hours. After incubation, the diameters of the inhibition zones were measured using a digital caliper[20–22].

Rationale for Antimycobacterial Assay and MIC–MBC Determination

The disc diffusion method was employed as an initial screening approach to assess antimycobacterial activity. To obtain quantitative estimates of inhibitory and bactericidal thresholds, inhibition zone data were further analyzed using linear regression based on the Bloomfield method, correlating the natural logarithm of extract concentration with the squared inhibition zone diameter. This approach is appropriate for exploratory studies of plant extracts, while further validation using broth microdilution assays is recommended for future investigations.

Inhibition Zone Measurement Method

The diameter of the inhibition zone was calculated according to the method of Davis and Stout,. The formula used is as follows:

$$d = \frac{A + B + C}{3} \qquad (1)$$

Description:

- d = inhibition zone diameter
- A = vertical diameter
- B = horizontal diameter
- C = diagonal diameter

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To classify the antibacterial activity of the extract, the inhibition zone values were interpreted based on the criteria described as shown in Table 1.

Tabel 1. Classification of Antibacterial Activity Based on Inhibition Zone Diameter

Inhibition Zone Diameter (mm)	Antibacterial Activity
2–5	Very Weak
5–10	Moderate
10–20	Strong
≥ 20	Very Strong

Determination of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration)

The determination of MIC and MBC followed the method of Bloomfield (1991) as cited by Kanter [22]. MIC represents the lowest concentration of extract capable of inhibiting bacterial growth, whereas MBC represents the minimum concentration required to kill the bacteria.

A linear regression curve was constructed using the natural logarithm of the extract concentration (Ln M_0) plotted on the X-axis and the squared inhibition zone diameter (Z^2) on the Y-axis. The X-intercept of the regression line corresponded to the value of Ln Mt. The MIC value was obtained by multiplying Mt by 0.25, while MBC was calculated as four times the MIC value.

Data Analysis

Data collection was carried out by observing and measuring the inhibition zones formed on each Petri dish, followed by tabulation. Observations were conducted after 48 hours of incubation. The diameter of the inhibition zones was measured using a digital caliper. The collected data were analyzed descriptively and presented in tables and figures.

Results and Discussion

Extraction Rendement

The ethanolic maceration of *Clerodendrum minahassae* leaves yielded 1.12 g of concentrated extract from 750 g of dried simplicia, demonstrating the efficiency of 96% ethanol in solubilizing polar to semi-polar metabolites. Maceration was selected because it operates at ambient temperature, minimizing thermal degradation of thermolabile constituents such as flavonoids, alkaloids, phenolics, and tannins[23]. Ethanol 96% provides an optimal polarity range for extracting bioactive compounds associated with antimycobacterial properties, while offering advantages in safety, volatility, and compatibility with subsequent biological assays[24][25].

Inhibition Zone Profile of *C. minahassae* Extract Against *M. smegmatis*

The antimycobacterial activity of the ethanolic extract of *Clerodendrum minahassae* against *Mycobacterium smegmatis* was demonstrated by the formation of inhibition zones at all tested concentrations.

Table 2. Inhibition Zone Diameter of *C. minahassae* Extract Against *M. smegmatis* (Mean ± SD)

Concentration (µg/disc)	Treatment	Mean (mm)	SD (mm)
5 µg/disc	*C. minahassae* extract	9.40	0.52
7.5 µg/disc	*C. minahassae* extract	10.03	0.89
10 µg/disc	*C. minahassae* extract	10.60	0.92
5 µg/disc	Streptomycin (positive control)	17.16	0.15
5 µg/disc	Ethanol (negative control)	0.00	0.00

The ethanolic leaf extract of *Clerodendrum minahassae* exhibited measurable antimycobacterial activity against *Mycobacterium smegmatis* at all tested concentrations. As summarized in Table 1, inhibition zone diameters increased from 9.40 ± 0.52 mm at 5 µg/disc to 10.03 ± 0.89 mm at 7.5 µg/disc and 10.60 ± 0.92 mm at

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10 µg/disc, indicating a clear concentration-dependent response. These quantitative data are consistent with the visual observations presented in Figure 1.

The positive control, streptomycin (5 µg/disc), produced a markedly larger inhibition zone (17.16 ± 0.15 mm), confirming the responsiveness of the test organism, whereas the negative control (96% ethanol) showed no inhibitory effect. Collectively, the integration of quantitative measurements and visual documentation confirms that the observed antimycobacterial activity is attributable to the bioactive constituents of C. minahassae extract rather than solvent effects or experimental artifacts.

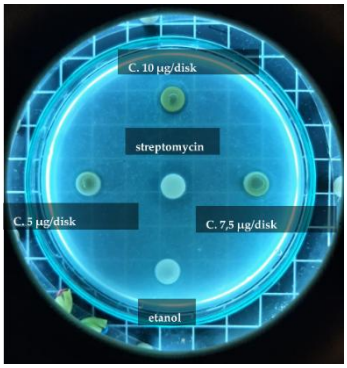

Figure 1. Inhibition zones produced by *Clerodendrum minahassae* leaf extract at concentrations of 5, 7.5, and 10 µg/disc against *Mycobacterium smegmatis*, compared with streptomycin (positive control) and 96% ethanol (negative control), as observed using the disc diffusion method.

The linear regression plot shows a strong, statistically significant relationship between the natural logarithm of extract concentration and the squared inhibition zone diameter. This relationship is represented by the regression equation y = 34.334x + 32.609, with a coefficient of determination R^2 = 0.9926. Such a high R^2 value indicates that nearly all variability in the inhibition response is explained by changes in extract concentration, confirming a stable and reliable dose–response pattern. This linear relationship provides evidence that increasing concentrations of *Clerodendrum minahassae* extract lead to greater inhibitory effects against *Mycobacterium smegmatis*.

The robustness of this regression model offers a solid mathematical basis for determining the minimum inhibitory threshold (Mt) through the X-intercept, which is then used to calculate the MIC and MBC values according to the Bloomfield method. The determined MIC of 0.63 µg/disc and MBC of 2.52 µg/disc indicate substantial antimycobacterial activity of the extract. The agreement between the experimental inhibition pattern and the mathematical estimations reinforces that these MIC and MBC values accurately reflect the extract's capacity to inhibit and kill *M. smegmatis*.

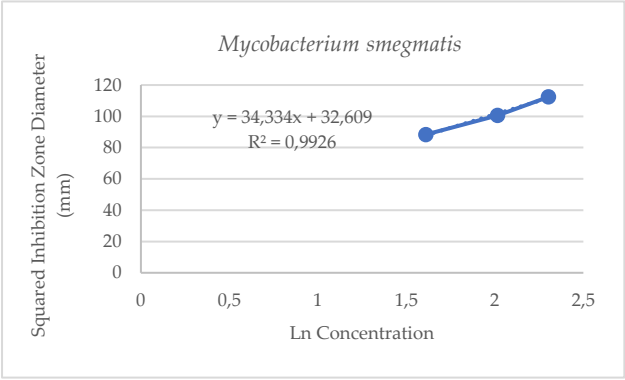

Figure 2. Linear regression plot showing the relationship between Ln extract concentration and the squared inhibition zone diameter for MIC determination of *Clerodendrum minahassae* extract against *Mycobacterium smegmatis*.

The findings of this study demonstrate that the ethanolic extract of *Clerodendrum minahassae* exhibits significant antimycobacterial activity against *Mycobacterium smegmatis*, as evidenced by a consistent increase in inhibition zone diameter with rising extract concentrations. The dose–response relationship is strongly supported by a linear regression model, which yielded an exceptionally high coefficient of determination (R^2 = 0.9926), indicating that nearly all observed variation in inhibitory activity is attributable to differences in

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extract concentration. The regression-based determination of inhibitory thresholds resulted in remarkably low values for MIC (0.63 µg/disc) and MBC (2.52 µg/disc), suggesting that the extract contains potent bioactive constituents capable of inhibiting and killing mycobacterial cells at minimal concentrations.

The use of *Mycobacterium smegmatis* as a surrogate model in this exploratory study is justified by its non-pathogenic nature, rapid growth rate, and well-documented structural similarities to Mycobacterium tuberculosis, particularly with respect to the lipid-rich mycolic acid–containing cell wall, which plays a critical role in mycobacterial drug susceptibility[26]. The biological relevance of *Mycobacterium smegmatis* as a surrogate model is further supported by evidence showing that heterologous expression of Mycobacterium tuberculosis PPE family proteins in M. smegmatis induces significant alterations in cell wall lipid composition, membrane integrity, and stress tolerance, while also modulating host immune responses. These findings highlight that M. smegmatis can recapitulate key cell wall– and host interaction–related features of pathogenic mycobacteria, reinforcing its suitability for preliminary antimycobacterial investigations[27].

The antimycobacterial activity demonstrated by the ethanolic extract of *Clerodendrum minahassae* in this study is strongly supported by previous investigations on the antimicrobial potential of this species and the broader *Clerodendrum* genus. Several studies have consistently shown that *C. minahassae* contains bioactive metabolites particularly flavonoids, phenolics, alkaloids, steroids, and terpenoids which contribute to its antimicrobial effects. Evidence from Egam *et al*. (2023) revealed that the ethyl acetate fraction of *C. minahassae* incorporated into toothpaste formulations produced inhibition zones of 9.75–13.08 mm against *Streptococcus mutans*, confirming the plant's antibacterial properties and its richness in antimicrobial phytochemicals[28] . Similarly, Bontjura *et al*. (2015) demonstrated that crude extracts of *C. minahassae* effectively inhibited *S. mutans*, reinforcing the spectrum of antibacterial activity previously attributed to this species . These findings align with the present study, in which inhibition zones of 9.40–10.60 mm were observed, although against a more resilient organism, *Mycobacterium smegmatis*[29]. The ability of the extract to inhibit a mycobacterial species at low concentrations underscores a potentially higher biological potency compared to its activity against Gram-positive cocci.

Beyond *C. minahassae*, investigations into related species within the *Clerodendrum* genus further contextualize the present findings. For example, Sapiun et al. (2020) reported high total flavonoid content (13.47%) in *Clerodendrum fragrans*, supporting the assertion that members of this genus are chemically rich in flavonoids and phenolic compounds known for antimicrobial activity through membrane disruption or enzyme inhibition pathways[30] .

Likewise, Rabha et al. (2024) demonstrated significant antibacterial activity of *Clerodendrum japonicum* leaf extract-supported silver nanoparticles, producing zones of inhibition up to 36 mm against *Escherichia coli* and 25 mm against *Staphylococcus aureus* further confirming that *Clerodendrum* species possess broad-spectrum antimicrobial bioactivity, and that their metabolites can significantly enhance antibacterial performance even when integrated into advanced delivery systems such as nanoparticles [31].

This body of evidence strengthens the biological plausibility of the strong antimycobacterial activity observed in the present study. In comparison with these prior studies, the remarkably low MIC (0.63 µg/disc) and MBC (2.52 µg/disc) values obtained in this work suggest that *C. minahassae* may exert relatively higher potency against mycobacteria than against non-mycobacterial bacterial species reported earlier. This enhanced effect may be attributed to the affinity of its phytochemical constituents-particularly flavonoids and tannins for targeting the unique lipid-rich mycobacterial cell wall or inhibiting proteins essential to cell division, such as FtsZ. The stronger potency observed here aligns with the genus-level trend of strong pharmacological potential but positions *C. minahassae* as a promising candidate for antimycobacterial drug exploration.

These findings extend previous reports describing the antibacterial activity of Clerodendrum minahassae against non-mycobacterial pathogens and are consistent with antimicrobial properties reported for other species within the Clerodendrum genus, thereby reinforcing the broader pharmacological relevance of this plant group [32]. Collectively, the consistency between the present findings and previous antimicrobial reports across the genus *Clerodendrum* provides a coherent scientific narrative supporting the relevance and novelty of this study's contribution.

The combination of disc diffusion assays with Bloomfield-based linear regression analysis provides a practical and quantitative approach for estimating MIC and MBC values in early-stage screening of plant extracts, particularly when used as a preliminary method prior to validation by standard broth microdilution techniques [33].

Despite the promising antimycobacterial activity observed in this study, several limitations should be acknowledged. First, Mycobacterium smegmatis was used as a surrogate model rather than Mycobacterium

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tuberculosis. Although M. smegmatis is widely accepted for preliminary antimycobacterial screening due to its non-pathogenic nature and structural similarities to pathogenic mycobacteria, the results cannot be directly extrapolated to M. tuberculosis without further validation using virulent strains. Second, the study employed a crude ethanolic extract, and therefore the specific bioactive compounds responsible for the observed activity could not be identified.

The antimycobacterial effect may result from synergistic interactions among multiple phytochemicals rather than a single active constituent. Bioassay-guided fractionation and compound isolation are necessary to elucidate the active principles and their mechanisms of action. Third, MIC and MBC values were estimated using a disc diffusion assay combined with Bloomfield-based linear regression, which is suitable for exploratory screening but does not replace standardized broth microdilution methods. Consequently, future studies should validate these findings using reference susceptibility assays. In addition, toxicity and selectivity toward host cells were not evaluated, which limits direct translational interpretation. Addressing these limitations through further in vitro and in vivo studies will be essential to fully assess the therapeutic potential of *Clerodendrum minahassae*.

Conclusions

This study demonstrates that the ethanolic extract of *Clerodendrum minahassae* exhibits measurable antimycobacterial activity against *Mycobacterium smegmatis*, as indicated by a clear concentration-dependent inhibition pattern and supported by linear regression analysis ($R^2 = 0.9926$). The low MIC (0.63 μg/disc) and MBC (2.52 μg/disc) values observed suggest that the extract possesses both inhibitory and bactericidal effects at relatively low concentrations, supporting its potential as a source of bioactive compounds with antimycobacterial relevance. Considering the urgent need for new antimycobacterial agents, particularly in the context of increasing drug resistance, *C. minahassae* warrants further investigation. Future studies focusing on bioassay-guided fractionation, identification of active constituents, mechanistic evaluation, toxicity profiling, and validation against pathogenic *Mycobacterium tuberculosis* strains are required to substantiate and extend the findings of this preliminary study.

Conflict of Interest

The authors declare that there are no financial, personal, or organizational conflicts of interest that could inappropriately influence the work reported in this manuscript. All experimental procedures, data analyses, and interpretations were conducted independently without external bias.

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Supplementary Materials

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