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In Vitro Evaluation of the Antituberculosis Potential of Basil Leaf Ethanol Extract Using the Resazurin Microtiter Assay (REMA) Method

Nurlina Setiadi[1], Humairah Medina Liza Lubis[2], Ikhfana Syafina[3]

AFFILIATIONS

1. Department of Biomedical, Faculty of Medicine and Health Science Universitas Muhammadiyah Sumatera Utara
2. Department of pathological anatomy, Faculty of Medicine and Health Science Universitas Muhammadiyah Sumatera Utara
3. Department of Pulmonology, Faculty of Medicine and Health Science Universitas Muhammadiyah Sumatera Utara

ABSTRACT

***Background**: Tuberculosis (TB) remains a global health challenge and is the second leading infectious cause of death. Indonesia ranks among the top three countries with the highest TB burden. Efforts to discover safer and more effective antituberculosis agents include exploring natural products such as basil (Ocimum basilicum), which contains antimicrobial bioactive compounds. **Objective**: To determine the minimum inhibitory concentration (MIC) of basil leaf ethanol extract against Mycobacterium tuberculosis H37Rv using the Resazurin Microtiter Assay (REMA). **Methods**: Basil leaves were extracted by maceration using 96% ethanol and tested at concentrations of 2.5%, 1.25%, and 0.625%. Antimycobacterial activity was evaluated using REMA, with rifampicin as the positive control and untreated media as the negative control. Color change from blue to pink indicated bacterial growth. **Results**: All concentrations of basil extract produced a pink color, indicating continued bacterial growth, whereas rifampicin remained blue, confirming assay validity. The MIC of the extract was >2.5 µg/mL, indicating no inhibitory effect at the tested concentrations. **Conclusion**: Basil leaf ethanol extract did not inhibit M. tuberculosis H37Rv at the evaluated doses. Further research using higher concentrations, phytochemical profiling (LC–MS/GC–MS), and fractionation of active compounds is recommended to explore its potential antimycobacterial activity.*

KEYWORDS:

Basil Leaves, Minimum Inhibitory Concentration, Mycobacterium tuberculosis, REMA

CORRESPONDING AUTHOR:
Nurlina Setiadi
nurlinasetiadi@gmail.com

INTRODUCTION

Tuberculosis (TB) remains a major global health problem and, in 2022, was the second leading cause of death from infectious diseases after COVID-19, causing twice as many deaths as HIV/AIDS. (1) More than 10 million people develop TB annually, with 30 high-burden countries accounting for 87% of global cases; two-thirds of these occur in eight countries, including India (27%), Indonesia (10%), China (7.1%), the Philippines (7%), Pakistan (4.5%), Bangladesh (3.6%), and the Democratic Republic of Congo (3%). Indonesia ranks second after India, reporting an estimated 969,000 TB cases in 2021 (354 per 100,000 population). Within Indonesia, North Sumatra Province holds the third-highest TB burden after West Java and East Java, with an estimated 83,949 cases. (2,3)

Drug-resistant tuberculosis (DR-TB) remains one of the most significant challenges to global TB control efforts. Globally, the annual number of MDR/RR-TB cases remained stable from 2020 to 2023, with an estimated 400,000 cases in 2023. (3) Drug-resistant TB cases occur annually, placing a heavy burden on health systems, prolonging treatment duration, increasing treatment failures,

and contributing substantially to global antimicrobial resistance. In many high-burden and resource-limited countries, managing drug-resistant TB consumes a large portion of healthcare budgets and often exceeds available diagnostic and treatment capacities. (4)

These challenges highlight the urgent need to identify new therapeutic candidates capable of combating *Mycobacterium tuberculosis* strains that no longer respond to conventional drugs. One promising approach is the exploration of natural products with potential antimycobacterial properties. Medicinal plants are widely used in traditional medicine across various regions and contain diverse bioactive compounds that may serve as alternative or complementary therapies for TB. (5)

To systematically evaluate the antimycobacterial activity of plant extracts, the Resazurin Microtiter Assay (REMA) has become an established and reliable phenotypic method. REMA is simple, rapid, cost-effective, and highly sensitive, making it especially suitable for screening natural product libraries, including herbal extracts. This method uses resazurin as an indicator with the principle of oxidation-reduction. (6,7) Previous studies have shown that aqueous extracts of *Ocimum sanctum, Adhatoda vasica, Leptedinia reticulata*, and *Cocculus hirsutus* exhibit strong antimycobacterial activity when evaluated using the REMA assay compared with the MTT assay. (8)

One of the plants that has many benefits is basil leaves (*Ocimum basilicum*) which has an abundant source of polyphenols and has long been known to be used to treat various types of diseases through its function as an anti-inflammatory, anti-oxidant, immunomodulator, anti-microbial, analgesic and diuretic. (9,10)

In Indonesia, basil leaf plants are widely used on large islands, such as Sulawesi, Sumatra, and Kalimantan. The tribe in Kolaka, East Kolaka, Southeast Sulawesi, uses basil leaves to treat tuberculosis. (11) In addition, basil leaves are also used to treat acute lung diseases, including bronchitis, cough, and sore throat, in Brazil, as well as tuberculosis and acute lung diseases such as bronchitis in Ethiopia. (12)

Previous studies reported that antimycobacterial activity of the essential oil from African basil leaves can damage microbial cell membranes, increase the formation of ROS which causes oxidative damage to microbes, damage microbial DNA and its replication wheels, denatured proteins essential for microbial survival. Through hydrodistillation methods and characterized using GC-MS, it showed antibacterial potential against *S. aureus, S. enteritidis, E. coli, and P. aeruginosa*. The minimum inhibitory concentration (MIC) ranges from 2 to 4 µg/mL, and the inhibitory zone is between 5 to 10. (13)

By applying REMA, a sensitive, rapid, and cost-efficient phenotypic assay, this study provides new

and standardized evidence on the antimycobacterial potential of basil extract, offering a more reliable assessment compared with earlier approaches.

METHODS

This study uses an experimental research design in *vitro* with *a non-randomized pos test only controlled group design* method. It was held from September 2024 to April 2025 at the Microbiology Laboratory of Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada and the Biochemistry Laboratory of Faculty of Medicine and Health Science, Universitas Muhammadiyah Sumatera Utara.

The stages of the procedure carried out are: first, the extraction of basil leaf simplicia is macerated with 96% ethanol for 72 hours, filtered, and evaporated until a thick extract is obtained, then diluted with 10% DMSO to obtain a concentration of 2.5%, 1.25%, 0.625%.(14) Phytochemical tests were carried out on basil leaf extract using qualitative methods to determine the content of compounds such as flavonoids, alkaloids, tannins, saponins, triterpenoids and steroids. (15)

The quantitative in vitro antimycobacterial activity of basil leaf ethanol extract at concentrations of 2.5%, 1.25%, and 0.625% was evaluated using 96-well microtiter plates with resazurin as an indicator of bacterial viability. A total of 100 µL of Middlebrook 7H9-S medium was added to each well, followed by 100 µL of plant extract at the designated concentrations and mixed thoroughly. Rifampicin as the positive control, was thawed and subsequently diluted in 7H9-S medium. Twofold serial dilutions were prepared directly in 96-well plates by adding the drug to 100 µL of 7H9-S medium, yielding final concentrations ranging from 2.0 to 0.06 µg/mL. Wells containing medium without antibiotics served as growth controls, while uninoculated wells functioned as sterility controls. The plates were then sealed and incubated at 37°C. After 7 days of incubation, 30 µL of 0.01% resazurin solution was added to each well, followed by overnight incubation. On day 8, color changes from blue (oxidized form) to pink (reduced form) were observed to determine bacterial growth. The results were subsequently analyzed using a microplate reader at 600 nm.

The experiment was performed in triplicate for each treatment. Minimum inhibitory concentration values were reported as mean values. MIC was defined as the lowest concentration of extract or drug that prevented the change in resazurin color from blue to pink (visual determination). A blue color indicated "no mycobacterial growth," whereas a pink color indicated "mycobacterial growth." (16)

RESULT AND DISCUSSION

Tuberculosis remains one of the most challenging infectious diseases globally, particularly

in low- and middle-income countries such as Indonesia. (4)

The increasing incidence of resistance to both first-line and second-line anti-tuberculosis drugs underscores the urgent need for new, effective, non-toxic, and affordable antimycobacterial agents. Medicinal plants are recognized as an important reservoir of structurally diverse bioactive compounds, making them a promising source for the discovery of antimycobacterial candidates. (17)

Based on the results of phytochemical screening of basil leaf ethanol extract, the following results were obtained:

Table 1. Phytochemical Screening Results

Compound	Result	Information
Flavonoid	Black	+
Alkaloid	Brownish-yellow and there are brown deposits	+
Saponin	Stable foam formation	+
Tanin	Blackish green	+
Phenol	Blackish blue	+
Steroids	Purple, blue or greenish	+

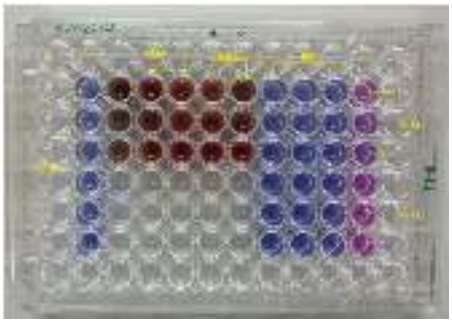
Based on the results of phytochemical screening presented in table 1, it was found that basil leaf extract contains flavonoid compounds, alkaloids, saponins, tannins, phenols and steroids. The consistent presence of these bioactive compounds aligns with previous phytochemical studies on basil leaves. (18)

Previous research has shown that basil leaves have promising antimicrobial activity against *the strains of M. tuberculosis* and *M. bovis*. This activity is associated with the bioactive ingredients contained in it, such as saponins, tannins, alkaloids, flavonoids and polyphenols with *a minimum inhibitory concentration* (MIC) ranging from 25-100 µg/mL and 25-50 µg/mL, respectively. (19)

A study reported that the administration of flavonoids significantly decreased the survival of intracellular *M. tuberculosis*, increasing cell density, aggregation, and granuloma formation. (20) While saponins work by lowering the surface tension of the bacterial cell wall, thereby impairing the permeability of the cell membrane, which leads to lysis, making enzymes and proteins out of the cell. The release of these cell contents causes the death of bacterial cells. (21) Tannins have a role in forming complex compounds with proteins through hydrogen bonds, if a hydrogen bond is formed between tannins and proteins then the protein will be denatured so that bacterial metabolism is disrupted. Meanwhile, alkaloids can inhibit DNA synthesis by inhibiting the topoisomerase enzyme in bacterial cells. Alkaloids exhibit antibacterial activity while inhibiting the transport of cell membrane-dependent ATP compounds. (22,23)

(a)

(b)



(c)

Figure 1. Minimum inhibitory Concentration (a) Extraction of compound extract solution with *M. tuberculosis* H37Rv bacteria; (b) Addition of Resazurin to each group; (c) Color observation that occurs in each group

Based on the image above, it was found that in the ethanol extract of basil leaves the concentrations of 2.5%, 1.25% and 0.625% changed to red, while in the group given rifampicin it remained blue.

Table 2. Results of the test of the potency of basil leaf extract against *M. tuberculosis*

Test	C (B)	C (M)	C (S)	Concentration of ethanol extract of basil leaves (%)		
				2,5	1,25	0,625
I	Grow	Not growing	Grow	Grow	Grow	Grow
II	Grow	Not growing	Grow	Grow	Grow	Grow
III	Grow	Not growing	Grow	Grow	Grow	Grow

Information:
C (B) : Control Bacteria
C (M) : Control media (Rifampicin)
C (S) : Solvent Control

In these results, it was found that *M. tuberculosis* bacteria grew in a control that was not given anything [C(B)], did not grow in the medium given rifampicin compound [C(M)], still grew in the control given aquadest solvent [C(S)], while in the control media given basil leaf ethanol extract compounds, *M. tuberculosis* bacteria continued to grow at a concentration of 2.5%, 1.25% and 0.625% after three repeats of testing.

Table 3. Minimum Inhibitory Concentration

Test	Concentration (2.5%)	Concentration (1.25 %)	Concentration (0.625 %)	MIC (µg/mL)
I	Grow	Grow	Grow	>2.5
II	Grow	Grow	Grow	>2.5
III	Grow	Grow	Grow	>2.5

In the above results, the minimum inhibitory concentration of *M. tuberculosis* was obtained after the administration of basil leaf ethanol extract at concentrations of 2.5%, 1.25% and 0.625% which is >2.5 µg/mL, meaning that the concentration used is not enough to inhibit the growth of bacteria.

The REMA method was chosen because it has advantages in terms of sensitivity, relatively short time, and visually interpretable results. Based on previous research, the methanol extracts of *L. camara, C. sanguinolenta*, and *Z. leprieurii* inhibited the growth of *M. smegmatis* as demonstrated by the REMA assay. They exhibited greater activity against rifampicin-resistant strains. (24)

The results of the study in Pakistan, that methanol extract *of* basil leaves showed up to 49%

inhibition against *M. tuberculosis* at a concentration of 6.25 mg/ml. Based on the study, basil leaves that separate methanol extract into fractions and purify pure compounds, showed that only a few compounds exerted a maximum inhibition effect of 49% against *M. tuberculosis H37Rv* at a concentration of 6.25 µg/mL, rather than total inhibition.(25)

In a previous study conducted in Ethiopia, an extract of 80% methanol from basil leaves seeds showed promising antimycobacterial activity against strains of *M. tuberculosis* (H37Rv, SIT777, SIT73, SIT26, SIT37, SIT1688, SIT336, SIT149, SIT53 and SIT54) and *M. bovis* (SB1176, SB1953 and SB0133). In the study, using the REMA method, anti-mycobacterial activity against *M. tuberculosis* and *M. bovis* was shown with an average minimum inhibitory concentration of 6.25 to 100 µg/mL (26)

In previous studies, there was also a significant decrease in the growth rate of *M. tuberculosis using* Ocimum sanctum *alcohol extract* with doses of 0.25 µL and 0.5 µL. (27). Another study reported the anti-tuberculosis effects of the essential oil *Ocimum sanctum L*. (OsEO). OsEO inhibited the growth of H37Rv with a MIC of 3 ml (2,931 mg). OsEO also inhibited the growth of all nine clinical isolates of *M. tuberculosis* tested, with MICs ranging from 1.5 mL (1.4655 mg) to 6 mL (5.862 mg). (28)

Differences between our findings and earlier studies likely arise from variations in extraction methods, assay conditions, and phytochemical composition. Previous research commonly used methanol extracts, essential oils, or purified fractions that concentrate bioactive compounds (25–28), whereas our study evaluated crude ethanol extract at lower concentrations that may have contained insufficient active constituents. Although REMA is a highly sensitive and widely used assay (29), its colorimetric nature may underestimate slow-acting compounds or be affected by extract coloration. Phytochemical variability due to plant chemotype, origin, and extraction technique may further contribute to inconsistent results.(30)

CONCLUSION

Basil leaves ethanol extract concentrations of 2.5%, 1.25% and 0.625% showed no antibacterial activity against *M. tuberculosis*. Further research at higher doses is needed to obtain the expected results, it is necessary to standardize and analyze the phytochemical profile of the extract before the activity test, for example with LC-MS or GC-MS as well as *in vivo* studies on the benefits of basil leaf extract against *M. tuberculosis*.

LIMITATIONS

1. Only crude ethanol extract was tested, active components may have been too diluted to produce inhibitory effects.
2. No chemical profiling (LC-MS/GC-MS) was performed to quantify active compounds.

3. Concentration range may have been insufficient, as higher doses used in previous studies showed inhibition.
4. The REMA assay assesses metabolic activity, which may not fully reflect bactericidal effects.
5. Only one strain (*H37Rv*) was tested, clinical isolates may respond differently.

RECOMMENDATIONS

1. Use a broader concentration range (>2,5%).
2. Perform chemical profiling before activity testing.
3. Use fractions or purified compounds.
4. Combine REMA with confirmatory methods such as MGIT or CFU assay.
5. Ensure extract clarity to avoid interference with color reading.

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