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Original Research Article

Vanillin Enhances the Efficacy of Isoniazid against *Mycobacterium smegmatis In Vitro*

Catrin Tania[1], Daniel Edbert[2], Agustina D. R. Nurcahyanti[1]*

[1]*Department of Pharmacy, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, Indonesia*
[2]*Department of Microbiology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, Indonesia*

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ABSTRACT

According to the World Health Organization's Global Tuberculosis Report 2023, Southeast Asia accounted for 46% of tuberculosis cases in 2022, with Indonesia ranking second after India. Although tuberculosis is treatable with antibiotics, such as isoniazid (INH), the development of antibiotic resistance by *Mycobacterium tuberculosis* (MTB), the causative agent of tuberculosis, can lead to treatment failure. Therefore, INH was combined with vanillin, an active natural compound, in an attempt to combat this resistance and tested against *Mycobacterium smegmatis* as a surrogate for MTB. Antibacterial activity was assessed by single-compound microdilution, followed by combination testing through the checkerboard microdilution and isobologram analysis. The minimum inhibitory concentrations for INH and vanillin were determined to be 9.14 µg/mL and 1610 µg/mL, respectively. Combination testing indicated an additive effect, with fractional inhibitory concentration indices and isobologram values showing a dose reduction index of 1.00 for INH and 255.56 for vanillin. These results suggest that vanillin may effectively inhibit *M. smegmatis*, but further studies should investigate vanillin's potential in combination with other anti-TB agents resistant to MTB.

Keywords: Tuberculosis, fractional inhibitory concentration index, isoniazid, vanillin, *Mycobacterium smegmatis*

Introduction

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis* (MTB), primarily affecting the lung parenchyma (pulmonary TB).[1] There is a high prevalence of TB in Southeast Asia, with an estimated 10.6 million cases globally in 2022, and Indonesia classified as one of the high-burden countries.[2] The mortality rate is further exacerbated by antibiotic resistance to first-line anti-TB drugs, including isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB).[3-8] TB patients resistant to INH who are treated solely with other first-line drugs experience poor therapeutic outcomes.[9] Consequently, combination therapy has been developed as a potential strategy to overcome the issue of antibiotic resistance. Treatment with two compounds that act synergistically enhances their antibacterial activity more than when used alone.[10] Combining anti-TB drugs with active compounds derived from natural sources holds promise for achieving such synergistic effects, owing to their unique chemical structures and broad-spectrum biological activities.[11] Previous studies have reported the potential of natural compounds, including isoimperatorin and eugenol, in combination with INH for treating TB.[12,13]Furthermore, the combination of a dichloromethane extract of *Piper corcovadensis* roots, combined with INH and RIF, exhibited synergistic effects.[14] Vanillin, a phenolic aldehyde derived from vanilla, has demonstrated antibacterial activity due to its phenol ring structure containing aldehyde, methoxy, and hydroxy groups.[15] Vanillin is active against *Mycobacterium smegmatis*, a surrogate model for MTB.[16]

*Corresponding author. Email: adr.nurcahyanti@atmajaya.ac.id
Tel: +62-21-6694366

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Subsequent lipidomics studies revealed that this activity may be due to alterations in the bacterial membrane lipids.[17,18]
M. smegmatis is commonly employed as a model organism for MTB in cell-based assays for tuberculosis drug research, as it is relatively easy to cultivate, grows rapidly, and is also sensitive to first-line anti-TB drugs, including INH.[19] *M. smegmatis* shares key similarities with MTB, including the presence of N-acetylmuramic acid (MurNac) and N-glycolymuramic acid (MurNGlyc), as well as a comparable cell structure and metabolic pathways.[20] Therefore, the synergistic effects of the combination of INH and vanillin against *M.smegmatis* were evaluated using the fractional inhibitory concentration index (FICI), and the INH dose was determined using the dose reduction index (DRI).

Materials and Methods

Bacterial strains and culture conditions
Mycobacterium smegmatis ATCC 19420 (Kwik-stik) was obtained from the American Type Culture Collection (ATCC), Saint Cloud, Minnesota, USA. The isolate was characterized using acid-fast staining techniques and cultured to a density of 0.5 McFarland, equivalent to 2 × 10^6 colony-forming units per milliliter (CFU/mL) in Middlebrook 7H9 Broth (M198-Himedia). A 1:1 dilution was prepared to achieve a final concentration of 1 × 10^6 CFU/mL for subsequent analyses.

Determination of minimum inhibitory concentration (MIC)
A microdilution antibacterial assay was conducted to determine the minimum inhibitory concentration (MIC) of vanillin and INH against *M. smegmatis* using ciprofloxacin (1 µg/mL) as the positive control and 8% DMSO (Sigma Aldrich, Germany) as the negative control.[21] Serial dilutions were prepared to achieve final concentrations of vanillin (80–0.625 mg/mL) ,[16] and of INH ranging from 409.6–0.2 µg/mL.

Checkerboard microdilution
A checkerboard dilution assay was conducted to evaluate the inhibitory activity of combinations based on the MIC values of the individual substances.[22] Vanillin and INH were serially diluted two-fold separately before being combined for the combination experiment (Table 1). The fractional inhibitory concentration index (FICI) was calculated as follows:

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$$FICI\ of\ two\ drug\ combination = \frac{MIC\ A_1}{MIC\ A_2} + \frac{MIC\ B_1}{MIC\ B_2} \quad (1)$$

Where A_1 is the MIC of vanillin in combination, A_2 is the MIC of vanillin alone, B_1 is the MIC of INH in combination, and B_2 is the MIC of INH alone. An FICI ≤ 0.5 was classified as synergistic, 0.5 < FICI ≤ 1.0 as additive, 1.0 < FICI ≤ 4.0 as indifferent, or FICI > 4.0 as antagonistic.

Isobologram analysis
The results of the checkerboard assay are illustrated in the isobologram, which displays the MICs for vanillin on the x-axis and INH on the y-axis. The line connecting these two points represents the indifference line, indicating no interaction between the substances. MIC values below the indifference line suggest additive (0.5 < FICI ≤ 1.0) or synergistic (FICI ≤ 0.5) interactions, whereas values positioned above the line indicate indifferent (1.0 < FICI ≤ 4.0) or antagonistic (FICI > 4.0) interactions.[23]

Dose reduction index (DRI)
The DRI refers to the permissible reduction in the drug dosage at a specified effect in the combination experiment.[24] The DRI also quantifies the fold reduction in the drug dosage to achieve a specific effect while maintaining the same efficacy as each drug administered individually.[25] The DRI was calculated according to the method established by Chou and Talalay:

$$DRI = \frac{(Dx)1}{(D)} \quad (2)$$

Where $(Dx)_1$ is the MIC of the substance alone, and (D) is the MIC of substances in the combination. A DRI > 1 indicates a reduction in concentration, DRI < 1 indicates an increase in concentration, and DRI = 1 indicates no reduction.

Data analysis
The data were analyzed in Microsoft Excel software.

Results and Discussion

The MICs of INH and vanillin are presented in Table 1 and are different from those reported in previous studies.[26–28] Variations in MIC values observed during drug susceptibility testing are influenced by the susceptibility profiles of the bacterial strains involved. The FICI of 1.00 indicates an additive effect, which signifies that the combined efficacy of the compounds is equivalent to the sum of their individual activities, with no significant enhancement observed. Isobologram analysis (Figure 1) confirmed the additive effect of the test combinations (Figure 2).

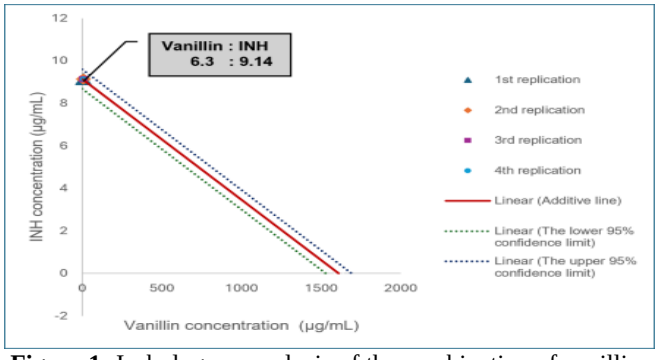
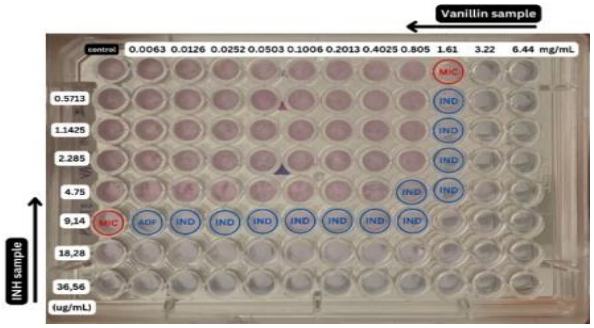
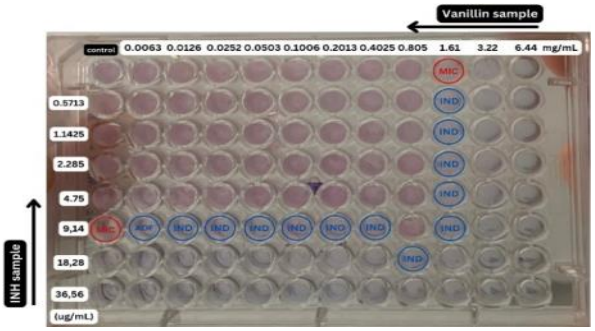


Figure 1: Isobologram analysis of the combination of vanillin and INH
Note: Data points from the 1st to 4th replications completely overlapped due to identical results.

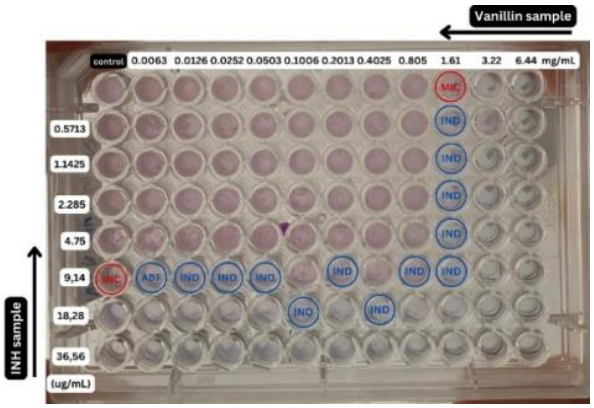


Replication 1



Replication 2

Replication 3



Replication 4

Figure 2: Checkerboard assay of the vanillin and INH combinations. The FICI values indicate an additive effect of vanillin and INH.

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Table 1: Summary of the antimicrobial effects (MIC, FICI, and DRI) of vanillin, INH, and the combination of vanillin and INH

Determination of MIC		**Checkerboard Dilution**								
MIC of drug alone (µg/mL)[a]		**Replication**	**Concentration of substance (µg/mL)**		**FICI[b]**	**Type of Interaction**	**DRI[c]**			
			Vanillin	**INH**			**Vanillin**		**INH**	
Vanillin	1610	1	6.3	9.14	1.00	additive	255.56	reduction in concentration	1.00	no reduction in concentration
		2	6.3	9.14	1.00	additive	255.56	reduction in concentration	1.00	no reduction in concentration
INH	9.14	3	6.3	9.14	1.00	additive	255.56	reduction in concentration	1.00	no reduction in concentration
		4	6.3	9.14	1.00	additive	255.56	reduction in concentration	1.00	no reduction in concentration

a MIC: Minimum inhibitory concentration; the lowest concentration of a compound that prevents visible microbial growth.
b FICI: Fractional inhibitory concentration index, used to determine drug interaction effects (synergy, additive, indifference, antagonism).
c DRI: Dose Reduction Index; a unitless parameter calculated as the ratio of a drug's MIC alone to its MIC in combination.

The DRI value for INH was 1.00, indicating that there was no reduction in concentration following the combination (Table 1). Conversely, the DRI value for vanillin of 255.56 was > 1, indicating a substantial decrease in concentration post-combination. The percentage change in the MIC value for INH in the combination was 0%, indicating that the MIC concentration remained unchanged whether administered alone or in combination. In contrast, the percentage change in the MIC value for vanillin was -99.61%, reflecting a highly significant reduction in concentration relative to the single-compound MIC.

Taken together, these results indicate an additive effect of vanillin and INH on *M. smegmatis*. Mechanistically, INH functions as a pro-drug that passively diffuses into *Mycobacterium* cells. It is activated by the KatG, forming the active INH-NAD adduct, which inhibits enoyl reductase InhA within the mycolic acid synthesis pathway. This disruption of mycolic acid synthesis compromises the integrity of the bacterial cell wall, ultimately leading to cell death.[29,30] In contrast, vanillin exerts its effects on *M. smegmatis* by targeting the cell membrane, causing lipid alterations that destabilize the bacterial membrane. This destabilization results in increased passive diffusion, disruption of the surface phenotype, heightened cell sedimentation rates, and changes in colony morphology.[16,17] Vanillin demonstrates bacteriostatic activity, allowing bacterial cells to remain viable despite exposure to the compound.[17] The combination of a bactericidal agent (INH) with a bacteriostatic agent (vanillin) may yield either synergistic or non-synergistic effects. Previous studies investigating the combination of these compounds against *Acinetobacter baumannii*, *Mycobacterium tuberculosis* H37Rv, and *methicillin-resistant Staphylococcus aureus* (MRSA) have reported synergistic, additive, and antagonistic effects, respectively.[31–33] As a phenolic compound, vanillin is frequently associated with additive or synergistic effects when combined with other antimicrobial agents due to its unique cellular targets. This enhances antimicrobial efficacy in combination therapies by disrupting bacterial functions through multiple pathways.[34]

Conclusion

The combination of vanillin and INH exerts an additive antimicrobial effect, but further research is necessary to gain a precise understanding of their mechanisms of action when used in combination. Vanillin exhibits inhibitory activity against *M. smegmatis*; thus, it should be further investigated in combination with other anti-TB drugs, particularly those facing resistance challenges.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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