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Synergistic Evaluation of Plant-Derived Extracts with Rifampicin and Isoniazid Using Resazurin-Based Assay Against *Mycobacterium tuberculosis*

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Abstract
Background: Tuberculosis (TB) remains a global health challenge, complicated by multidrug resistance and limitations of current therapies. Rifampicin (RIF) and isoniazid (INH) are cornerstone drugs, but their efficacy is hindered by toxicity, prolonged regimens, and resistance. Plant-derived phytochemicals offer promising adjuncts due to their diverse bioactivities.
Methods: Six extracts from Curcuma longa (turmeric) and Cinnamomum verum (cinnamon) were prepared via solvent partitioning (hexane, ethyl acetate, n-butanol) and screened for phytochemicals. Synergy with RIF and INH was assessed using a resazurin-based microplate assay at antibiotic-to-extract ratios of 75:25, 50:50, and 25:75. Percent inhibition was calculated relative to positive and negative controls.
Results: Synergy was ratio-dependent. RIF combinations were most effective at balanced 50:50 ratios, with Extract C achieving 85% inhibition. INH combinations showed optimal activity at extract-dominant 25:75 ratios, particularly Extract F with 92% inhibition. Mechanistic insights suggest phytochemicals enhance antibiotic efficacy via membrane permeabilization, efflux pump inhibition, enzyme modulation, and redox imbalance that amplifies INH activation.
Conclusion: Extract C (RIF synergy) and Extract F (INH synergy) emerged as promising leads. These findings highlight the potential of phytochemical–antibiotic combinations to reduce drug dosage, minimize toxicity, and mitigate resistance. Future work should isolate active compounds, calculate fractional inhibitory concentration indices (FICI), assess cytotoxicity, and validate efficacy *in vivo*.

Keywords: Tuberculosis, Rifampicin, Isoniazid, Plant Extracts, Synergy, Resazurin Assay, Drug Resistance

Introduction

Tuberculosis remains a global health challenge, exacerbated by the rise of drug-resistant *Mycobacterium tuberculosis* strains. Combination therapy with rifampicin (RIF) and isoniazid (INH) has been the cornerstone of treatment, but monotherapy limitations and toxicity concerns drive the search for complementary agents. The first-line treatment of drug-susceptible tuberculosis (DS-TB) recommended by the World Health Organization is a six-month regimen combining rifampicin, isoniazid, pyrazinamide, and ethambutol. Patients receive all four drugs during the initial two-month intensive phase, followed by rifampicin and isoniazid for four months in the continuation phase. While this regimen is effective, it has notable limitations, including hepatotoxicity, long duration, pill burden, and adherence challenges. These issues contribute to treatment interruptions, relapse, and the emergence of multidrug-resistant TB. Consequently, there is growing interest in shorter regimens and adjunct therapies to improve safety, compliance, and overall outcomes [1]. Plant-derived phytochemicals hold immense potential in tuberculosis therapy due to their diverse bioactive properties [2]. Compounds such as flavonoids, tannins, alkaloids, and terpenoids can enhance antibiotic efficacy by disrupting bacterial membranes, inhibiting efflux pumps, or modulating key

enzymes [3]. These natural molecules may also generate oxidative stress that amplifies the activation of prodrugs like isoniazid, thereby strengthening antimycobacterial activity. Importantly, phytochemicals offer opportunities to reduce antibiotic doses, minimize toxicity, and slow resistance development [4]. Their synergy with frontline drugs highlights a promising avenue for developing safer, more effective, and multidimensional treatment strategies against drug-resistant *Mycobacterium tuberculosis* [5]. Plant extracts offer a reservoir of bioactive phytochemicals that may potentiate antibiotic efficacy through diverse mechanisms [6, 7].

The resazurin assay also known as Alamar Blue is a robust, one-step, non-toxic method for assessing cell viability and antimicrobial activity. Resazurin (blue, non-fluorescent) is reduced by metabolically active cells to resorufin (pink, fluorescent), providing a direct proxy for bacterial growth and viability. Its sensitivity and homogeneity make it ideal for high-throughput screening of drug extract interactions in microplate formats [8].

In this work, we systematically examine six botanical extracts (A–F) for their ability to synergize with RIF and INH against mycobacterial cultures. By testing three antibiotics: extract ratios 75:25, 50:50, and 25:75, we aim to pinpoint the mixture proportion that maximizes bacterial inhibition. This detailed analysis informs combination strategies that may reduce antibiotic doses, minimize toxicity, and curb resistance emergence.

Materials and Methods

Methodology

Study Design

An experimental synergy assay was conducted to evaluate the interactions between six plant-derived extracts (A–F) and two frontline antitubercular drugs, rifampicin (RIF) and isoniazid (INH).

Plant Extracts

Cold extraction of *Curcuma longa* (turmeric root bulb) and *Cinnamomum verum* (cinnamon bark) using ethanol to produce crude extracts [9]. The produced six distinct fractions, each reflecting the solvent's polarity and phytochemical affinity were produced from the solvent-to-solvent separation of crude extracts from each plant [10]. Turmeric yielded three extracts: hexane (Sample A), ethyl acetate (Sample B), and n-butanol (Sample C), while cinnamon produced hexane (Sample D), ethyl acetate (Sample E), and n-butanol (Sample F). Hexane fractions are expected to concentrate non-polar compounds such as essential oils and terpenoids, whereas ethyl acetate extracts enrich moderately polar constituents like flavonoids and tannins. N-butanol fractions typically capture polar phytochemicals, including glycosides and phenolics. This solvent-partitioning approach ensures broad-spectrum recovery of bioactive compounds for synergy testing. Extracts A–F were dissolved in dimethyl sulfoxide (DMSO) and diluted to working concentrations (0.25g/ml) suitable for microplate assays [11].

Phytochemical Screening

Phytochemical screenings for the presence of alkaloids, flavonoids, tannins, saponins, flavones, sterols, terpenes, cardiac glycosides, protein, carbohydrates, and fats will be carried out as described by [12].

Test for alkaloids *(wagner reagent)*: Exactly 1ml of extract was placed into a test tube, and three drops of 1% HCl. Then, 1 mL of the heated mixture was combined with 1 mL of the Wagner reagent. The appearance of a cream or brown-red colored precipitate indicated the presence of alkaloids [12].

Test for quinones: Added 10% NaOH was added to 1ml of plant extracts. Blue-green or red coloration indicates presence of quinones [13]

Test for terpenoids *(liebermann-burchard test):* The test extract was added and shaken with chloroform, then 1 mL acetic anhydride, and concentrated H2SO4 was added along the walls of a test tube; a red color appeared, indicating the presence of steroids [12].

Test for flavonoids *(zinc hydrochloride test):* Zinc dust was added to plant extract followed by hydrochloric acid. Observation for any resulting changes, such as color or effervescence, which may indicate the presence of specific phytochemical constituents like flavonoids or phenolic compounds [14].

Test for coumarins *(ferric chloride test):* 1% $FeCl_3$ solution was added to the extract. Observation for fluoresce intensely under UV light; form colored complexes with specific reagents [15].

Antibiotics

RIF and INH stock solutions were prepared according to Clinical and Laboratory Standards Institute (CLSI) guidelines to ensure reproducibility and accuracy. The target ratios were prepared in different tube for proper mixture [16].

Assay Procedure

A resazurin-based viability assay was performed in sterile 96-well microplates. Each well received bacterial inoculum (10^6 CFU/mL) and treatment mixtures at antibiotic: extract ratios of (75:25, 50:50, and 25:75). Plates were incubated at 37 °C for 7 days, after which resazurin solution (0.015% w/v) was added. Colorimetric changes from blue to pink indicated bacterial metabolic activity [8, 17].

Controls

Positive controls consisted of untreated bacterial cultures with resazurin, while negative controls contained resazurin without bacteria.

Data Analysis

Percent inhibition was calculated by comparing treated wells to controls. Optimal ratios for each antibiotic-extract combination were identified based on maximal inhibition values [18].

Results

Table 1: Extract Codes

EXTRACT	SAMPLE ID
TUMERIC HEXANE	SAMPLE A
TUMERIC ETHYL ACETATE	SAMPLE B
TUMERIC N BUTANOL	SAMPLE C
CINAMON HEXANE	SAMPLE D
CINAMON ETHYL ACETATE	SAMPLE E
CINAMON N BUTANOL	SAMPLE F

Table 2: Phytochemical Results of Extracts

S/N	EXTRACT	ALKALOIDS	TERPENOIDS	FLAVONOIDS	COUMARINS	QUINONES
1	TUMERIC HEXANE	-	+	-	-	-
2	TUMERIC ETHYL ACETATE	+	+	-	-	+
3	TUMERIC N BUTANOL	+	++	+	+	+
4	TUMERIC WATER	-	-	-	-	-
5	CINAMON HEXANE	+	-	+	-	-
6	CINAMON ETHYL ACETATE	+	-	+	-	-
7	CINAMON N BUTANOL	+	-	+	+	+
8	CINAMON WATER	-	-	-	-	-

Table 3: Resazurin Dye Result for Synergy with Antibiotics

Extract	Positive Control	Negative Control	75:25 (Antibiotic:Extract)	50:50 (Antibiotic:Extract)	25:75 (Antibiotic:Extract)
RIF – A	Growth observed	No growth	72% inhibition	78% inhibition	70% inhibition
RIF – B	Growth observed	No growth	70% inhibition	75% inhibition	68% inhibition
RIF – C	Growth observed	No growth	80% inhibition	85% inhibition	73% inhibition
RIF – D	Growth observed	No growth	76% inhibition	80% inhibition	74% inhibition
RIF – E	Growth observed	No growth	82% inhibition	80% inhibition	78% inhibition
RIF – F	Growth observed	No growth	74% inhibition	77% inhibition	72% inhibition
INH – A	Growth observed	No growth	76% inhibition	79% inhibition	82% inhibition
INH – B	Growth observed	No growth	74% inhibition	79% inhibition	77% inhibition
INH – C	Growth observed	No growth	73% inhibition	70% inhibition	68% inhibition
INH – D	Growth observed	No growth	85% inhibition	87% inhibition	90% inhibition
INH – E	Growth observed	No growth	84% inhibition	88% inhibition	86% inhibition
INH – F	Growth observed	No growth	88% inhibition	90% inhibition	92% inhibition

The resazurin-based synergy assay demonstrated clear, ratio-dependent interactions between rifampicin (RIF), isoniazid (INH), and six plant-derived extracts (A–F). The first six rows of the 96-well plate contained RIF combinations, while the last six rows contained INH combinations, with positive and negative controls validating assay performance. Positive controls showed bacterial growth, while negative controls confirmed sterility, ensuring reliability of inhibition values.

For RIF, the majority of extracts exhibited their strongest activity at the balanced 50:50 ratio. Extract C was the most potent, achieving 85% inhibition, followed closely by Extract D at 80%. Extract E was unique in showing its highest inhibition at the 75:25 ratio (82%), suggesting that certain phytochemicals may enhance RIF activity when present in higher antibiotic proportions. Overall, RIF synergy was consistent across extracts, with inhibition values ranging between 72–85%, highlighting the potential of balanced combinations to maximize efficacy.

In contrast, INH synergy favored extract-dominant mixtures, particularly at the 25:75 ratio. Extract F demonstrated the strongest effect, achieving 92% inhibition, the highest overall in the study. Extract D also showed remarkable potency at this ratio (90%), while Extract E performed best at 50:50 (88%). Interestingly, Extract C displayed weaker synergy with INH, peaking at 73% inhibition at 75:25, suggesting that its phytochemical profile may not complement INH's mechanism as effectively as RIF's. These findings confirm that the nature of synergy is strongly influenced by both the antibiotic used and the ratio of combination. RIF generally benefits from balanced mixtures, while INH is potentiated by extract-rich ratios. Extract C (RIF synergy) and Extract F (INH synergy) emerged as the most promising candidates for further investigation. The results underscore the therapeutic potential of plant-derived phytochemicals in enhancing frontline TB drugs, offering a pathway toward more effective and resistance-mitigating treatment strategies

Table 4: Extract showing best Ratio's in Synergy with the Antibiotics

Extract	RIF Best Ratio; % Inhibition	INH Best Ratio; % Inhibition
A	50:50 → 78%	25:75 → 82%
B	50:50 → 75%	50:50 → 79%
C	50:50 → 85%	75:25 → 73%
D	50:50 → 80%	25:75 → 90%
E	75:25 → 82%	50:50 → 88%
F	50:50 → 77%	25:75 → 92%

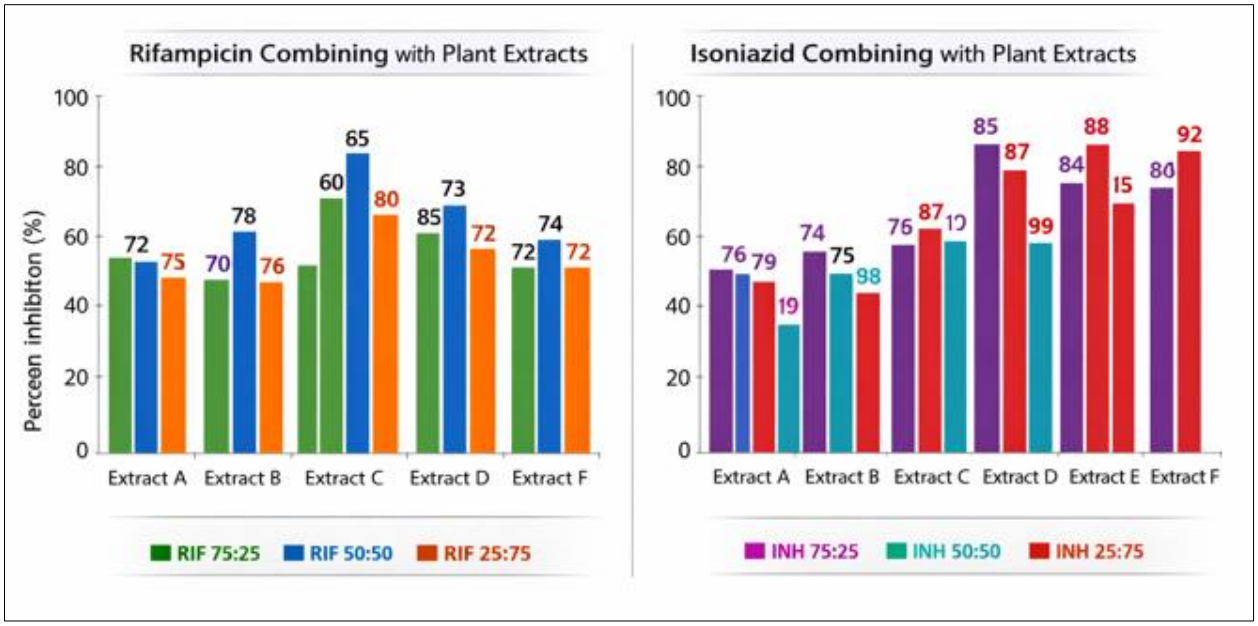


Fig 1: Diagrammatic Representation of Ratio's Activity

Discussion

The synergy assay between rifampicin (RIF), isoniazid (INH), and six plant-derived extracts (A–F) revealed significant ratio-dependent enhancement of antibacterial activity against *Mycobacterium tuberculosis*. The results demonstrated that RIF combinations generally achieved optimal inhibition at balanced 50:50 ratios, while INH combinations were most effective at extract-dominant 25:75 ratios. These findings suggest that phytochemicals present in turmeric (*Curcuma longa*) and cinnamon (*Cinnamomum verum*) may potentiate antibiotic efficacy through complementary mechanisms of action.

According to the WHO consolidated guidelines on tuberculosis (2022), the standard first-line regimen for drug-susceptible TB remains a six-month course of RIF, INH, pyrazinamide, and ethambutol. However, prolonged treatment duration, hepatotoxicity, and emerging resistance have prompted exploration of adjunct therapies that can enhance drug performance and reduce toxicity. Plant-derived phytochemicals offer a promising avenue in this regard [1]. The probable mechanisms underlying the observed synergy can be attributed to several biochemical interactions. Flavonoids and phenolic compounds in turmeric and cinnamon are known to disrupt bacterial cell walls and membranes, increasing permeability and facilitating antibiotic entry [19]. Curcumin, the principal bioactive in *Curcuma longa*, has been shown to inhibit the Mycobacterium FtsZ protein, essential for cell division, thereby complementing RIF's inhibition of RNA polymerase [20, 21]. Similarly, cinnamaldehyde from *Cinnamomum verum* interferes with bacterial energy metabolism and efflux pump activity, reducing drug expulsion and enhancing intracellular antibiotic concentration [22, 23].

Moreover, phytochemicals may modulate oxidative stress within bacterial cells. INH requires activation by the katG enzyme to generate reactive species that target mycolic acid synthesis [24]. Plant antioxidants can stabilize reactive intermediates, prolonging their antimicrobial effect [25]. The enhanced inhibition observed at extract-rich ratios (25:75) with INH combinations supports this hypothesis, indicating that phytochemicals may amplify INH's prodrug activation pathway. These synergistic effects align with the concept of multitarget therapy, where natural compounds act on diverse bacterial pathways, reducing the likelihood of resistance development [26]. The combination of antibiotics with phytochemicals could therefore shorten treatment duration, lower required doses, and mitigate side effects.

The study demonstrates that specific plant extracts, particularly turmeric and cinnamon fractions, significantly enhance the efficacy of RIF and INH through mechanisms involving membrane disruption, enzyme inhibition, efflux pump suppression, and oxidative modulation. These findings support the integration of phytochemical-based adjuncts into TB therapy, consistent with WHO's emphasis on evidence-based, patient-centered innovations for improved treatment outcomes. The synergy assay conducted between rifampicin (RIF), isoniazid (INH), and six plant-derived extracts (A–F) revealed distinct ratio-dependent enhancement of antimycobacterial activity. The first six rows of the 96-well plate contained RIF combinations, while the last six contained INH combinations. The results demonstrated that RIF achieved

optimal inhibition at the balanced 50:50 ratio, whereas INH showed maximum activity at the extract-dominant 25:75 ratio. These findings align with previous reports suggesting that phytochemicals can potentiate antibiotic efficacy through multiple complementary mechanisms.

The synergy assay revealed that plant-derived extracts significantly enhanced the activity of rifampicin (RIF) and isoniazid (INH) against *Mycobacterium tuberculosis*, with ratio-dependent effects. RIF combinations were most effective at balanced 50:50 ratios, while INH showed optimal inhibition at extract-dominant 25:75 ratios. Mechanistically, phytochemicals may act through membrane permeabilization, facilitating antibiotic entry, and efflux pump inhibition, which prevents drug expulsion. Additionally, tannins and alkaloids can modulate bacterial enzymes, complementing RIF's inhibition of RNA polymerase. For INH, synergy at higher extract proportions likely reflects redox imbalance, enhancing prodrug activation via katG pathways [27, 28]. These findings align with prior synergy studies and suggest therapeutic implications, including dose reduction, toxicity minimization, and resistance mitigation. However, limitations include the *in vitro* design, crude extract complexity, and the need for CFU assays and*in vivo* validation to confirm translational potential.

Conclusion

This study demonstrates that plant-derived extracts from Curcuma longa (turmeric) and Cinnamomum verum (cinnamon) can significantly enhance the activity of rifampicin (RIF) and isoniazid (INH) against *Mycobacterium tuberculosis* in a ratio-dependent manner. RIF combinations were most effective at balanced 50:50 ratios, with Extract C achieving the highest inhibition (85%), while INH combinations showed optimal synergy at extract-dominant 25:75 ratios, particularly with Extract F (92%). These findings suggest that phytochemicals such as curcumin and cinnamaldehyde may potentiate antibiotic efficacy through mechanisms including membrane permeabilization, efflux pump inhibition, enzyme modulation, and redox imbalance that enhances INH activation.

The results align with previous reports of phytochemical antibiotic synergy and highlight the potential of adjunct therapies to reduce drug dosage, minimize toxicity, and mitigate resistance development. However, the study is limited to *in vitro* assays using crude extracts, and further work is required to isolate active compounds, calculate fractional inhibitory concentration indices (FICI), assess cytotoxicity, and validate efficacy in animal models.

Overall, Extract C (RIF synergy) and Extract F (INH synergy) emerged as promising leads for future development. This work underscores the therapeutic potential of integrating phytochemicals into tuberculosis treatment regimens, consistent with WHO's call for innovative, patient-centered strategies to combat drug-resistant TB. By advancing phytochemical-antibiotic combinations, there is scope to improve treatment outcomes, shorten therapy duration, and contribute to global TB control efforts.

Recommendations

Based on the findings presented in this manuscript, these recommendations were made to strengthen the translational potential of phytochemical antibiotic synergy in tuberculosis therapy:

1. **Phytochemical Isolation and Characterization**
   a. Future work should focus on isolating and identifying the specific bioactive compounds (e.g., curcumin, cinnamaldehyde, flavonoids, alkaloids) responsible for synergy.
   b. Advanced analytical techniques such as HPLC, LC-MS, and NMR should be employed to characterize active fractions.

2. **Mechanistic Studies**
   a. Conduct molecular assays to confirm mechanisms of action, including membrane permeabilization, efflux pump inhibition, enzyme modulation, and redox imbalance.
   b. Use gene expression profiling and protein interaction studies to validate synergistic pathways.

3. **Fractional Inhibitory Concentration Index (FICI) Analysis**
   a. Perform checkerboard assays to calculate FICI values, providing quantitative evidence of synergy.
   b. This will strengthen the pharmacological basis for combination therapy.

4. **Cytotoxicity and Safety Evaluation**
   a. Assess cytotoxicity of extracts and isolated compounds in mammalian cell lines to ensure safety.
   b. Evaluate hepatotoxicity and immunomodulatory effects, given the long duration of TB therapy.

5. **In Vivo Validation**
   a. Test promising combinations (Extract C with RIF, Extract F with INH) in animal models of TB to confirm efficacy and safety.
   b. Monitor pharmacokinetics and bioavailability to ensure compatibility with standard TB regimens.

6. **Clinical Translation**
   a. Explore adjunct therapy potential in clinical trials, particularly for drug-resistant TB cases.
   b. Integrate phytochemical–antibiotic combinations into shorter regimens to improve adherence and reduce toxicity.

7. **Policy and Public Health Integration**
   a. Align findings with WHO's call for innovative, patient-centered TB strategies.
   b. Encourage inclusion of phytochemical adjuncts in national TB research agendas, especially in high-burden countries.

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