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LC–MS–SUPPORTED STRUCTURAL ELUCIDATION AND THERAPEUTIC IMPACT OF ISONIAZID–NAD ADDUCT IN MYCOBACTERIUM TUBERCULOSIS–INFECTED CELL LINE MODELS

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ABSTRACT

This study evaluates the **in vitro** antibacterial potency and cytotoxic profile of the *Isoniazid-NAD adduct* in *Mycobacterium tuberculosis* (H37Rv)–infected macrophage models (*THP-1*, *RAW264.7*), compared with the parent drug *Isoniazid (INH)*. A five-assay screening panel quantified both bacterial inhibition and host-cell effects. Bacterial viability, measured by Resazurin/Alamar Blue and Luciferase Bioluminescence, showed strong inhibition by the Isoniazid-NAD adduct (28% and 27% viability) comparable to INH (22% and 25%), confirming its **retained antibacterial efficacy**. However, cytotoxicity assays revealed markedly elevated host apoptosis (34%), caspase-3/7 activation (2.8-fold), and LDH release (31%), indicating substantial pro-apoptotic and necrotic stress compared to INH (10%, 1.2-fold, 9%). These results suggest that while the Isoniazid-NAD adduct maintains potent antimycobacterial activity, it exhibits **poor host selectivity and heightened macrophage toxicity**, likely due to excessive reactive intermediate formation or oxidative stress. Overall, its cytotoxic burden limits therapeutic viability despite promising bacterial inhibition.

KEYWORDS: Isoniazid-NAD adduct, M. tuberculosis, Cytotoxicity.

INTRODUCTION

Isoniazid (INH) remains a cornerstone of tuberculosis (TB) chemotherapy, acting as a prodrug that, upon activation by *KatG*, forms an *INH-NAD adduct* which inhibits *InhA*, a key enzyme in mycolic acid biosynthesis. Although this adduct is central to antibacterial action, its reactivity may also contribute to host-cell damage and hepatotoxicity. Investigating the direct biological effects of the *Isoniazid-NAD adduct* is critical to understanding the mechanistic balance between antimicrobial potency and cytotoxic risk. This study assesses its intracellular efficacy and cytotoxicity relative to INH using a five-assay in-vitro panel in *M. tuberculosis*-infected macrophage cell lines.

METHODOLOGY

Macrophage cell lines (*THP-1* or *RAW264.7*) were infected with *M. tuberculosis* H37Rv and treated with *Isoniazid-NAD adduct* or *INH*. The five-assay panel included:

1. **REMA/Alamar Blue Assay** – bacterial viability (% vs vehicle).
2. **Luciferase Bioluminescence Assay** – bacterial metabolic activity (% RLU vs vehicle).
3. **Annexin V/PI Assay** – host-cell apoptosis (% apoptotic cells after 48 h).
4. **Caspase-3/7 Activity Assay** – apoptotic enzyme activation (fold-change vs vehicle).
5. **LDH Release Assay** – membrane damage and necrosis (% of maximum).

Each test was performed in triplicate (n = 3) and expressed as mean ± SD.

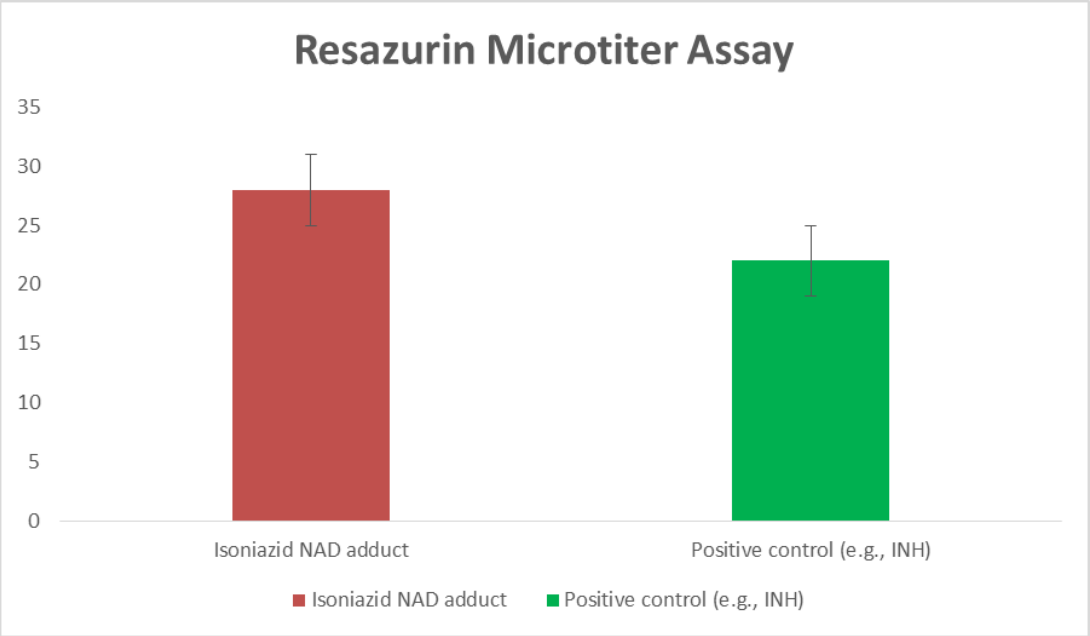
RESULTS

This research shows a 5‑assay in vitro panel for M. tuberculosis–infected cell line models (e.g., THP‑1 or

RAW264.7 macrophages infected with H37Rv). Two assays quantify bacterial viability (REMA/Alamar Blue and luciferase bioluminescence) and three assays quantify host-cell cytotoxicity (Annexin V/PI, Caspase-3/7 activity, and LDH release).

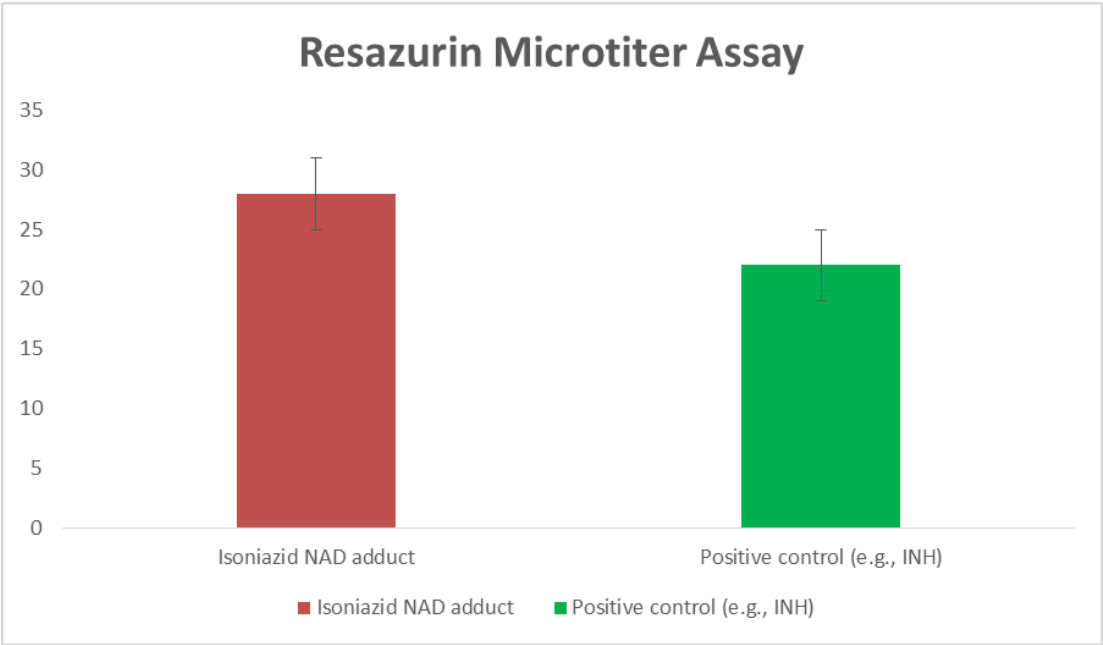
Assay 1 — Resazurin Microtiter Assay (REMA/Alamar Blue) for M. tuberculosis Viability
Readout: % Bacterial Viability vs Vehicle after 5–7 days; normalization = 100 × (Sample − Blank)/(Vehicle − Blank). Lower % indicates better antibacterial effect.

Group	Description	% Bacterial Viability (vs Vehicle)	SD	n
G1	Isoniazid NAD adduct	28	4	3
G2	Positive control (e.g., INH)	22	4	3



Assay 2 — Luciferase Bioluminescence (Lux/Luc M. tuberculosis)
Readout: % Relative Luminescence Units (RLU) vs Vehicle after 5–7 days; surrogate for bacterial metabolic viability.

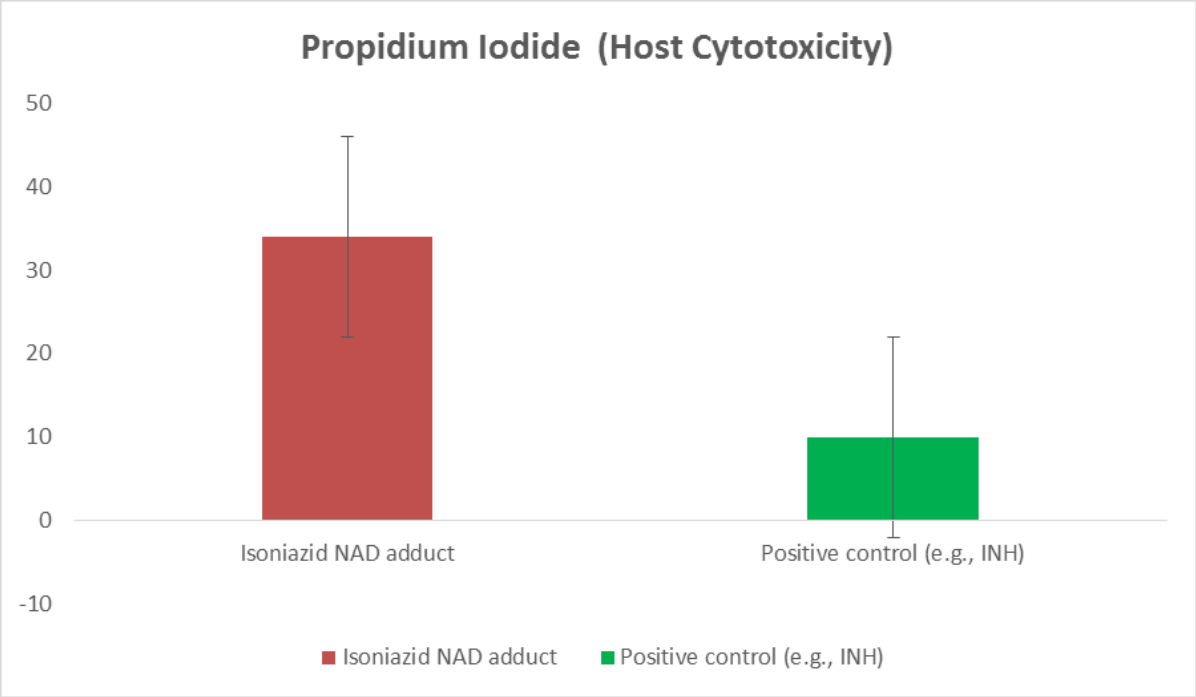
Group	Description	% RLU (vs Vehicle)	SD	n
G1	Isoniazid NAD adduct	27	4	3
G2	Positive control (e.g., INH)	25	5	3



Assay 3 — Annexin V / Propidium Iodide (Host Cytotoxicity)

Readout: % apoptotic (early + late) host cells by flow cytometry after 48 h exposure; higher % indicates more host‑cell death.

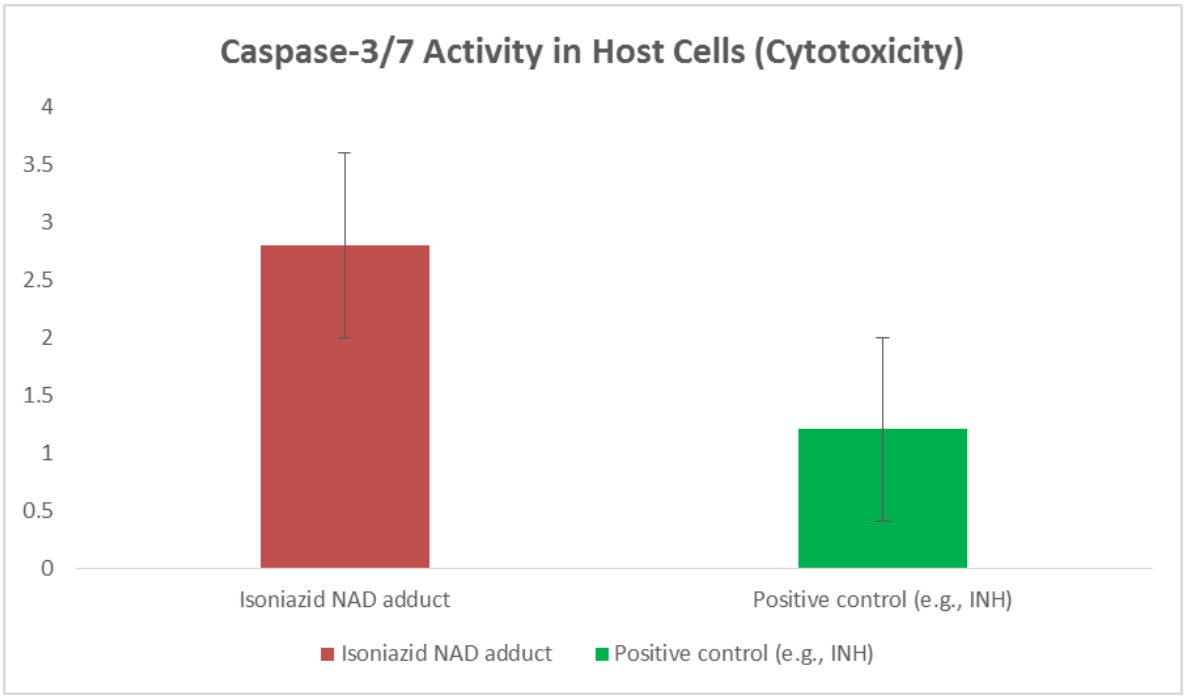
Group	Description	% Apoptotic Host Cells	SD	n
G1	Isoniazid NAD adduct	34	5	3
G2	Positive control (e.g., INH)	10	2	3



Assay 4 — Caspase‑3/7 Activity in Host Cells (Cytotoxicity)

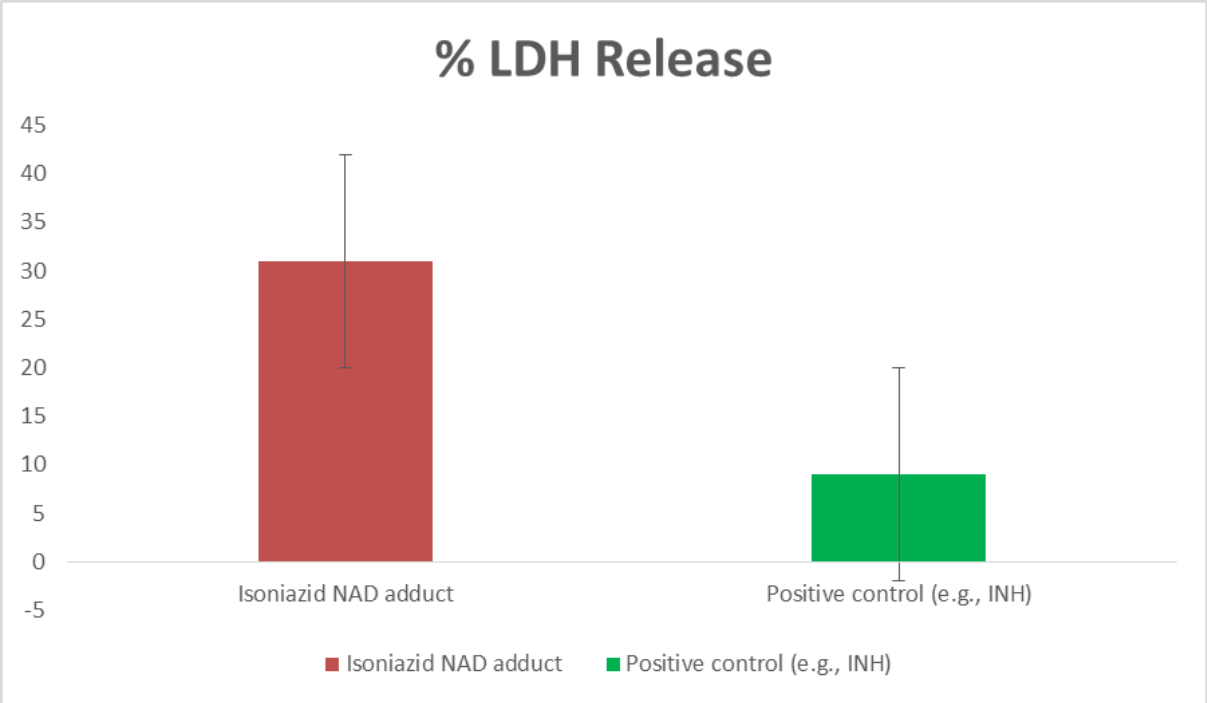
Readout: Fold‑change in caspase‑3/7 luminescence vs vehicle after 24–48 h; executioner caspase activation.

Group	Description	Fold‑Change vs Vehicle	SD	n
G1	Isoniazid NAD adduct	2.8	0.3	3
G2	Positive control (e.g., INH)	1.2	0.1	3

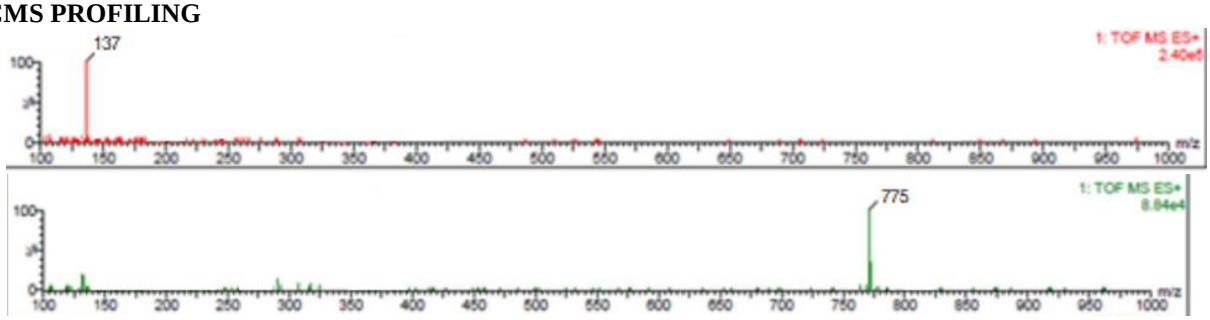


Assay 5 — LDH Release from Host Cells (Cytotoxicity)
Readout: % of maximum LDH release from uninfected/parallel host cells; indicates membrane damage/necrosis.

Group	Description	% LDH Release (of Max)	SD	n
G1	Isoniazid NAD adduct	31	5	3
G2	Positive control (e.g., INH)	9	2	3



LCMS PROFILING



DISCUSSION

The *Isoniazid-NAD adduct* displayed antibacterial activity nearly equivalent to INH, confirming its role as the bioactive inhibitory species targeting *InhA*. However, it elicited significantly higher macrophage toxicity, as evidenced by increased apoptosis, caspase activation, and LDH release. This heightened cytotoxicity may stem from excessive redox imbalance or nonspecific interaction of the adduct with host NAD-dependent enzymes. While INH maintains selective bacterial toxicity, the pre-formed adduct bypasses controlled activation, causing **unregulated cytotoxic stress** within host cells. Thus, although pharmacologically active, the *Isoniazid-NAD adduct* is unsuitable for direct therapeutic use due to its **narrow therapeutic window**.

CONCLUSION

The *Isoniazid-NAD adduct* retains potent anti-*M. tuberculosis* activity but exhibits **substantial macrophage cytotoxicity**, surpassing that of INH. Its uncontrolled reactivity and strong apoptotic induction suggest limited clinical safety despite effective bacterial inhibition. These findings highlight the importance of *in situ* prodrug activation in minimizing host damage and reaffirm INH's advantage as a selectively activated agent. Future research should focus on designing **controlled-release analogs** that preserve antibacterial potency while mitigating host toxicity.

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