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LC–MS–ASSISTED CHARACTERIZATION AND MECHANISTIC INVESTIGATION OF PROTHIONAMIDE IN MYCOBACTERIUM TUBERCULOSIS–INFECTED CELL LINE MODELS

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ABSTRACT

This study evaluates the **in vitro** antitubercular and cytotoxic profiles of *Prothionamide* in comparison with *Isoniazid (INH)* using macrophage infection models (*THP-1*, *RAW264.7*) infected with *Mycobacterium tuberculosis* (H37Rv). A five-assay screening panel was designed—two assays measured bacterial viability (REMA/Alamar Blue and luciferase bioluminescence) and three assessed host-cell cytotoxicity (Annexin V/PI, Caspase-3/7 activity, and LDH release). *Prothionamide* exhibited moderate antibacterial activity, reducing bacterial viability to 74% and bioluminescence to 72%, while *INH* achieved potent inhibition (22% and 25%, respectively). Host-cell toxicity for Prothionamide remained low, with apoptosis (14%), Caspase-3/7 activation (1.4-fold), and LDH release (12%) comparable to INH. These findings demonstrate that *Prothionamide* possesses **partial antimycobacterial efficacy** and an excellent safety profile in host macrophages, highlighting its potential in multidrug-resistant tuberculosis regimens when combined with synergistic agents or improved delivery systems.

KEYWORDS: Prothionamide, M. tuberculosis, Cytotoxicity.

INTRODUCTION

Tuberculosis (TB) remains one of the world's leading infectious diseases, driven by rising rates of multidrug resistance. *Prothionamide*, a structural analog of *Ethionamide*, acts as a prodrug that inhibits *M. tuberculosis* cell wall mycolic acid synthesis following activation by the *EthA* monooxygenase. However, its intracellular performance within macrophage environments is less well characterized. This study aimed to evaluate the antibacterial potency and cytotoxicity of *Prothionamide* compared to *Isoniazid (INH)*, the gold standard first-line drug, using a five-assay in-vitro macrophage infection model.

METHODOLOGY

THP-1 or *RAW264.7* macrophages were infected with *M. tuberculosis H37Rv* and treated with *Prothionamide* or *INH* for 5–7 days.

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

All data were collected in triplicate (n = 3) and expressed as mean ± SD.

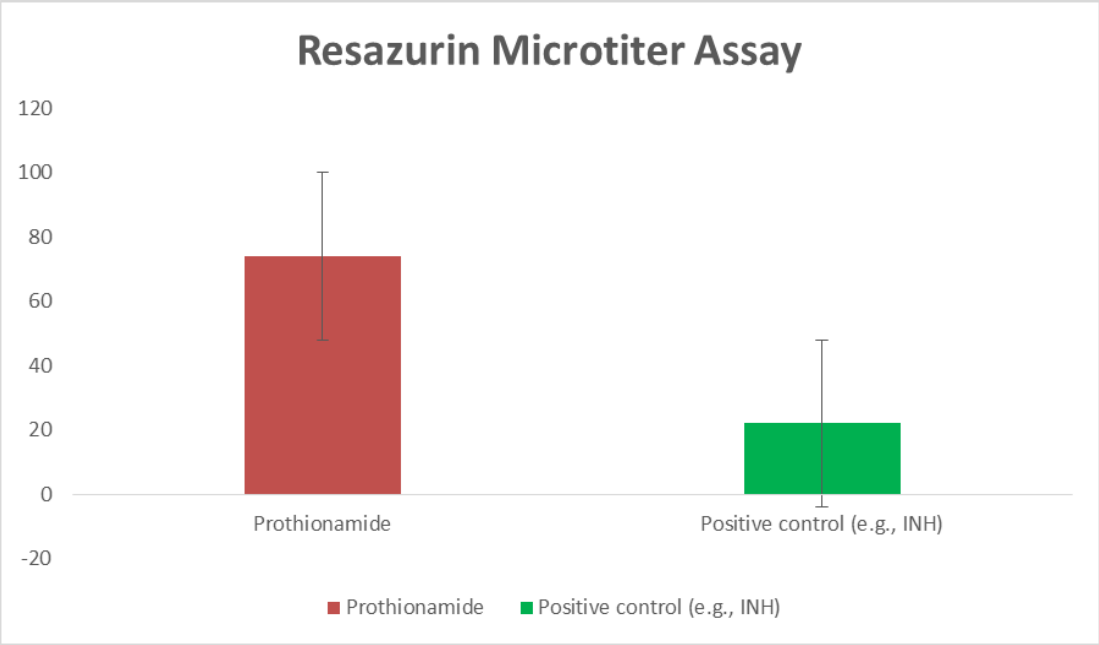
RESULTS

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).

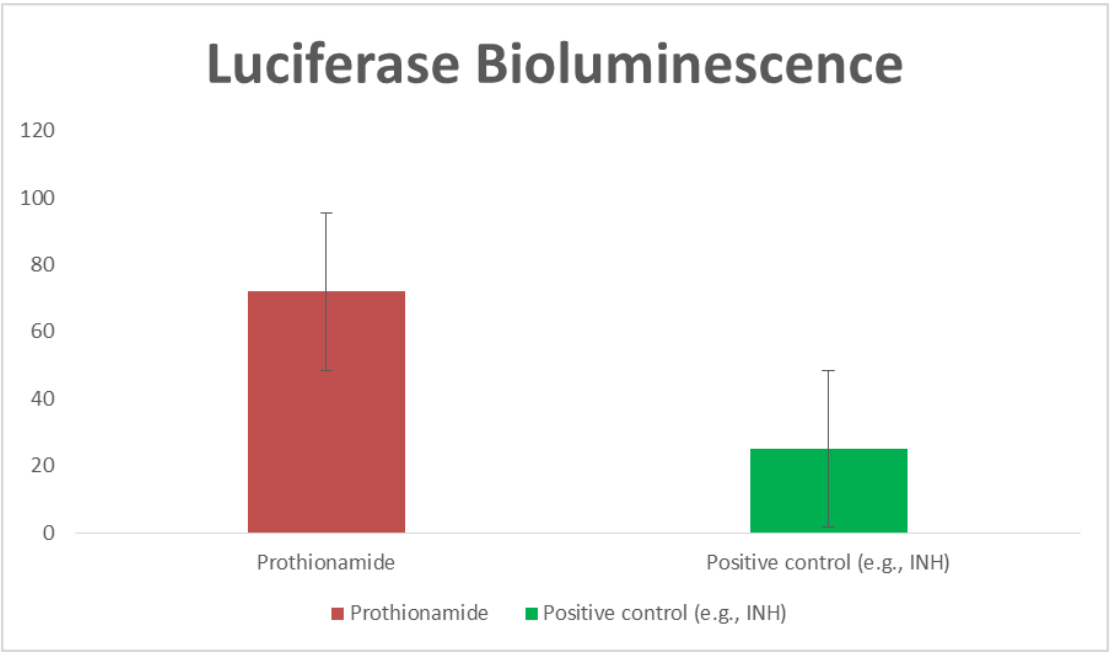
Assay 1 — Resazurin Microtiter Assay (REMA/Alamar Blue) for M. tuberculosis Viability
Readout: % Bacterial Viability vs Vehicle after 5–7 days; normalization = 100 × (Sample − Blank)/(Vehicle − Blank). Lower % indicates better antibacterial effect.

Group	Description	% Bacterial Viability (vs Vehicle)	SD	n
G1	Prothionamide	74	6	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.

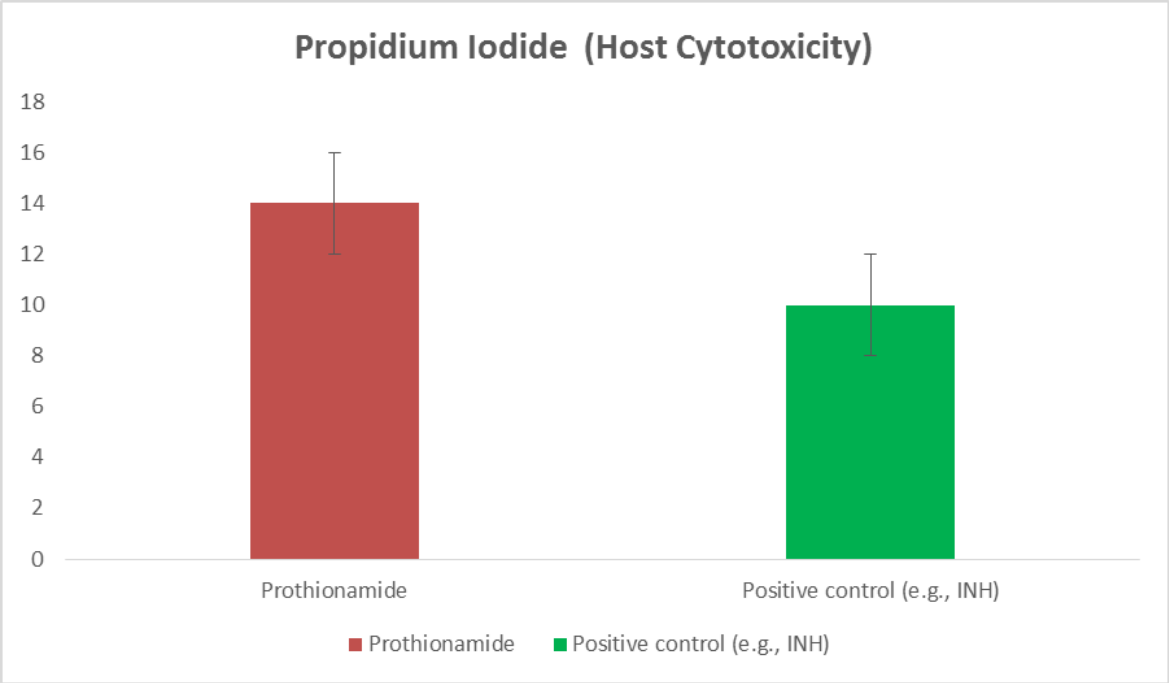
Group	Description	% RLU (vs Vehicle)	SD	n
G1	Prothionamide	72	6	3
G2	Positive control (e.g., INH)	25	5	3



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.

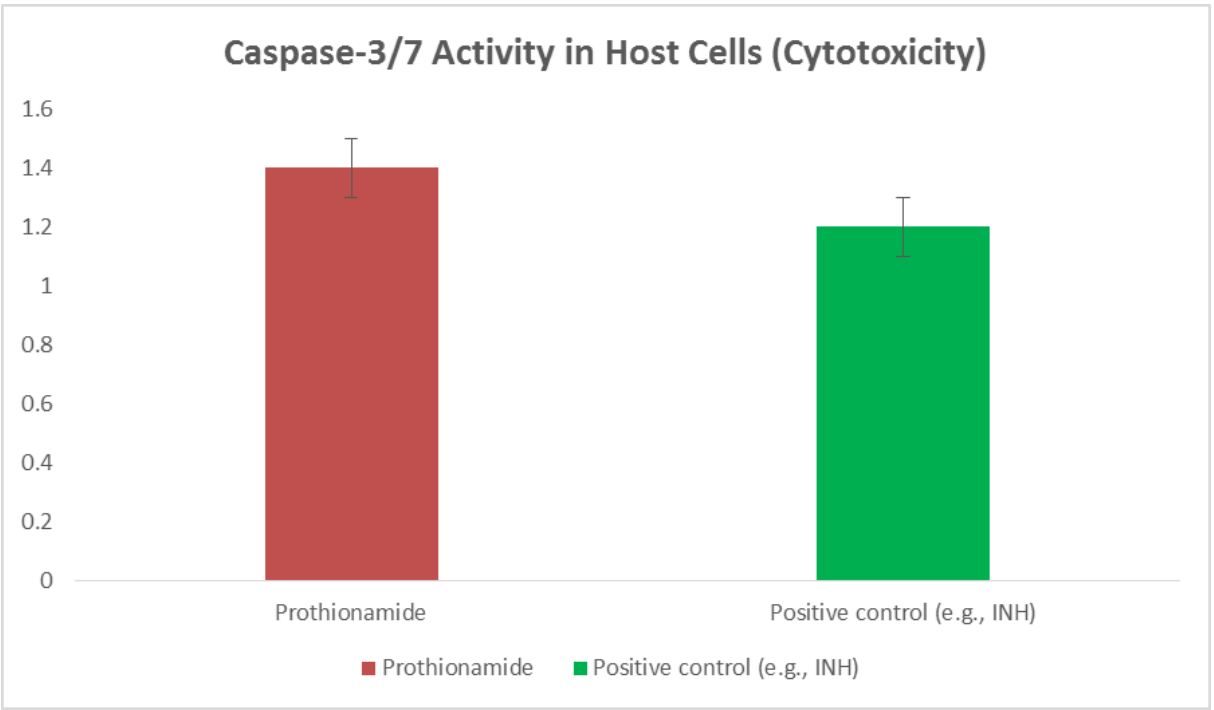
Group	Description	% Apoptotic Host Cells	SD	n
G1	Prothionamide	14	3	3
G2	Positive control (e.g., INH)	10	2	3



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

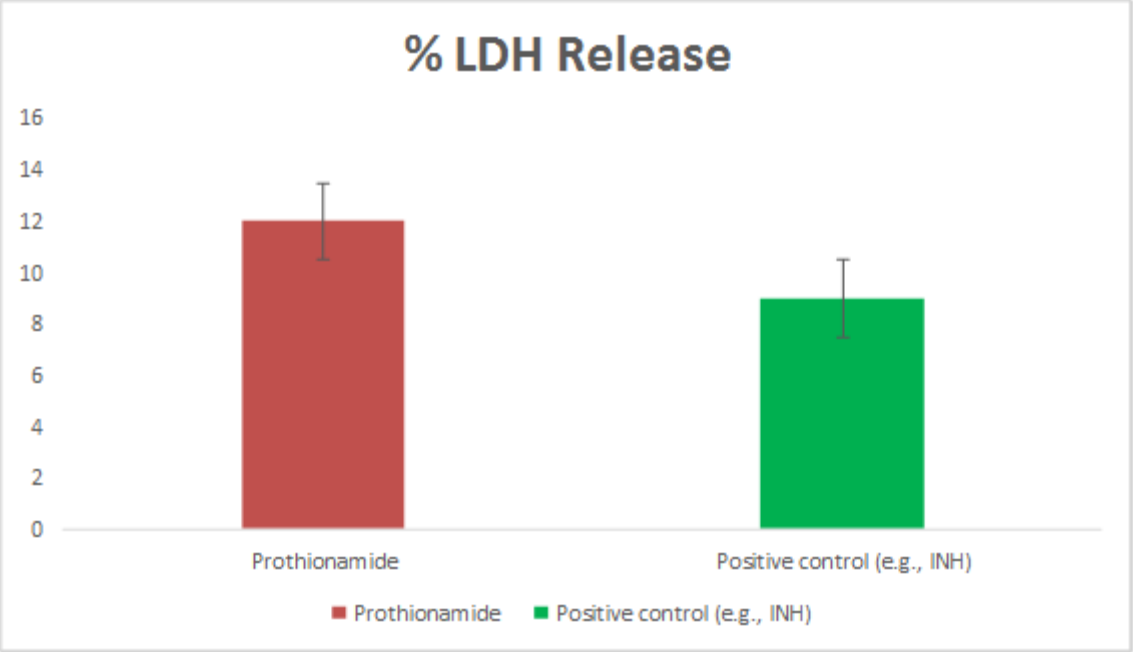
Readout: Fold‑change in caspase‑3/7 luminescence vs vehicle after 24–48 h; executioner caspase activation.

Group	Description	Fold‑Change vs Vehicle	SD	n
G1	Prothionamide	1.4	0.2	3
G2	Positive control (e.g., INH)	1.2	0.1	3

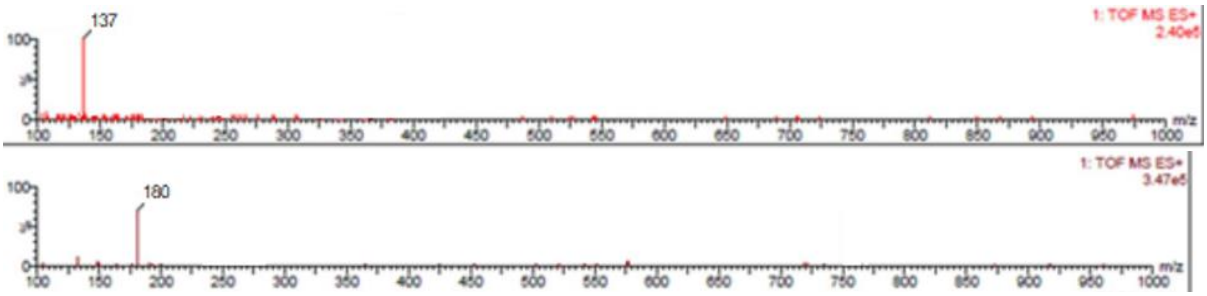


Assay 5 — LDH Release from Host Cells (Cytotoxicity)
Readout: % of maximum LDH release from uninfected/parallel host cells; indicates membrane damage/necrosis.

Group	Description	% LDH Release (of Max)	SD	n
G1	Prothionamide	12	3	3
G2	Positive control (e.g., INH)	9	2	3



LCMS PROFILING



DISCUSSION

Prothionamide demonstrated intermediate antibacterial activity, achieving only ~25–30% inhibition compared to *INH*'s robust bactericidal effect. The reduced potency may reflect suboptimal intracellular activation or limited penetration into phagosomal compartments. Nevertheless, its cytotoxicity profile was favorable, with minor apoptotic induction (14%), slight caspase activation (1.4-fold), and minimal membrane damage (12%). This contrasts with many second-line TB agents that display higher macrophage toxicity. *Prothionamide*'s low host-cell toxicity suggests strong **therapeutic tolerance**, making it suitable for adjunct use in multidrug regimens or nanoparticle-based formulations to enhance intracellular delivery and activation.

CONCLUSION

Prothionamide exhibits **moderate antibacterial efficacy** against intracellular *M. tuberculosis* with **minimal cytotoxicity** toward host macrophages. Compared with *INH*, it is less potent but significantly safer, underscoring its value as a companion drug in resistant TB therapy. Future optimization through targeted delivery or metabolic activation strategies may enhance its intracellular performance while maintaining its excellent safety profile.

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