REVIEW

of a DISSERTATION on Evaluation of the Potential of New Derivatives of Ethambutol and Isoniazid for the Study of Their Antitubercular Activity for the award of an educational and scientific degree "PhD" in the scientific specialty of: in the field of higher education 4. Natural Sciences, Mathematics, and Informatics, specialty 4.3 Biological Sciences, in the scientific specialty Microbiology, code 01.06.12, by Simeon Emilov Dimitrov, full-time doctoral student.

From Assoc. Prof. Denitsa Stefanova Stefanova, PhD, Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University–Sofia

Elected as a member of the scientific jury in compliance with Order № I-116/04.08.2025 of the Director of the Institute of Microbiology "Stefan Angelov" at the Bulgarian Academy of Sciences Bulgaria

I. Relevance and Scientific Significance of the Topic

The doctoral dissertation of Simeon Emilov Dimitrov addresses a topic of extremely high relevance and significant public health priority—the development of new therapeutic compounds for the treatment of tuberculosis. The disease, caused by *Mycobacterium tuberculosis*, remains one of the most serious challenges to global health. Despite the availability of established drug regimens, tuberculosis continues to cause substantial morbidity and mortality. The prevalence of multidrug-resistant and extensively drug-resistant strains calls into question the effectiveness of standard therapeutic approaches and necessitates the urgent search for new pharmacological solutions.

The targeted design of molecules with improved efficacy, minimal toxicity, and the ability to overcome drug resistance is of particular importance to modern medical science and clinical practice. In this context, the research of Simeon Dimitrov aligns with current international trends and possesses indisputable scientific and social value.

II. Aims and Objectives of the Dissertation

The primary aim of the dissertation is the systematic pharmacological screening of pre-synthesized chemical compounds—derivatives of ethambutol and isoniazid—with a view to a comprehensive assessment of their antimycobacterial activity, toxicological profile, pharmacokinetic characteristics, and proposed molecular mechanisms of action.

The author's tasks are multifaceted and include determining in vitro antimycobacterial activity against reference strains *M. tuberculosis* H37Rv and *M. smegmatis* MC2155; analyzing interactions of the compounds with key enzymatic and structural targets via molecular docking; evaluating transmembrane permeability and intracellular transport; studying in vitro cytotoxicity; and conducting in vivo acute and subacute toxicity tests accompanied by pathomorphological analysis of the liver, kidneys, and small intestine.

Additional objectives include evaluating the antioxidant activity and redox-modulating potential of selected molecules through the determination of key biochemical parameters, induced in vitro mutagenesis of resistant mutant clones, and whole-genome sequencing to clarify the molecular-genetic mechanisms of drug resistance. All these directions build a comprehensive and integrated characterization of the tested compounds as potential therapeutic candidates.

III. Structure, Methodology, and Scientific Supervision

The dissertation is structured in a rigorous academic format, including an introduction, an in-depth literature review, detailed materials and methods, experimental results, analytical discussion, and formulated conclusions. The work contains 40 figures and 20 tables, with a bibliography based on 346 references. The methodological section is impressive in its thoroughness and precision, ensuring the reliability and reproducibility of the data.

The dissertation represents an in-depth and interdisciplinary study dedicated to the comprehensive pharmacological and microbiological characterization of newly synthesized aroylhydrazones and nitrofuranilamides as antimycobacterial agents. The research was conducted under the supervision of prominent experts in microbiology and pharmacology—Assoc. Prof. Dr. Violeta Vulcheva Ruseva and Prof. Dr. Milka Milcheva Mileva—who provided continuous scientific support and expert consultation in interpreting the results and selecting the methodological approach. Thanks to their professionalism and methodological guidance, the author successfully integrated various experimental techniques and produced a logically coherent and scientifically grounded dissertation.

The applied multidisciplinary approach—combining microbiological, pharmacological, biochemical, and molecular-genetic techniques—gives the work solid scientific value and confirms its validity and applicability.

IV. Main Results and Scientific Contributions

Among the most significant achievements of the study is the identification of four compounds with leading antimycobacterial potential—two aroylhydrazones (3a and 3d) and two nitrofuranilamides (DO190 and DO209). These structures are distinguished by high activity, favorable toxicological profiles, and optimal intracellular transport, defining them as promising

candidates. Molecular docking shows specific binding of aroylhydrazone 3d to NAD^+ and Tyr158, explaining its efficacy against multidrug-resistant *M. tuberculosis* strains.

Safety assessment was performed through in vivo acute and subacute toxicity tests, as well as pathomorphological analyses of the liver, kidneys, and small intestine, with no significant pathological changes observed. Data on antioxidant capacity and redox-modulating properties of the molecules indicate potential protective effects against oxidative stress—a factor of importance in chronic infections such as tuberculosis.

The scientific novelty of the work also lies in the first application in Bulgaria of induced in vitro mutagenesis of reference strains, followed by chromosomal DNA isolation and whole-genome sequencing. This approach allowed the identification of a mutation in the *Rv3755c* gene associated with drug resistance, revealing new perspectives for understanding the molecular mechanisms of pathogen adaptation and for identifying potential therapeutic targets.

V. Literature Review and Methodological Competence

The literature review demonstrates a systematic and in-depth approach, covering global and national distribution of resistant forms of tuberculosis, mechanisms of drug resistance, and current directions in the development of new antimycobacterial molecules. Modern preclinical technologies such as molecular docking, transmembrane permeability analysis, and genome sequencing are included, highlighting the author's scientific competence and the alignment of the dissertation with international standards.

The methodology section is exemplary, with protocols described in detail, allowing full reproducibility of experiments. This testifies to the high level of methodological maturity and competence of the doctoral candidate.

VI. Critical Remarks on the Dissertation

I would like to recommend improving the quality of some of the presented figures. Specifically, the figure illustrating the synthesis and chemical structures of the nitrofuranilamide series, as well as the figure demonstrating cell viability assessment after treatment with nitrofuranilamides DO190 and DO209, could be visually refined and further detailed to improve clarity and facilitate reader comprehension. This would contribute to an even higher quality and reproducibility of the experimental data.

The choice of tumor cell lines for cytotoxicity evaluation is of interest. It would be helpful to clarify the criteria for selecting these specific lines and the logic behind determining the concentration range used for treatment with nitrofuranilamides. Such information would provide valuable context for interpreting the toxicological profile and potential safety of the newly synthesized aroylhydrazones and nitrofuranilamides.

VII. Publications and Citations

The scientific output resulting from the dissertation has been published in international peer-reviewed journals, highlighting the high scientific value and originality of the research. The fact that these publications have been cited by other researchers demonstrates the recognition and impact of the results in the international and national scientific community, as well as their applied and conceptual significance. Presenting the results at multiple scientific forums further confirms their scientific relevance and broad interest in their interpretation and subsequent research.

Conclusion:

Overall, the dissertation represents an in-depth, original, and methodologically precise study, contributing significant new knowledge in the field of antimycobacterial pharmacology and medicinal chemistry. The author demonstrates high scientific maturity, skills in planning and conducting complex experimental studies, and impressive competence in interpreting results.

The scientific output reflected in the dissertation and its associated scientometric indicators meet the requirements for awarding the educational and scientific degree of "Doctor." Particularly impressive is that the published articles are already citable and that the results have been presented at prestigious international and national scientific forums, demonstrating the high scientific value and significance of the research.

Based on these findings, I recommend to the esteemed scientific jury to award the educational and scientific degree of "PhD" to Simeon Emilov Dimitrov.

29/09/2025

Sofia

Reviewer:

(Assoc. Prof. Denitsa Stefanova, PhD)