

REVIEW
of the official reviewer
for the dissertation of Izaz Ahmad on the topic “Using Advanced Spectroscopic, and Chromatographic Techniques to Study the Biotransformation of Steroidal Drugs, and Phytochemical Investigation of Tussilago farfara L.” presented for the degree of Doctor of Philosophy (PhD) in the specialty “8D05301 – Chemistry”

№	Criteria	Eligibility (one of the options must be checked)	Justification of the position of the official reviewer
1.	The topic of the thesis (as of the date of its approval) corresponds to the directions of development of science and/or state programs	1.1 Compliance with priority areas of science development or government programs:	No state-budget project number or target-program code is indicated in the dissertation materials made available to the reviewer. On the basis of the topic and content, the work is best classified as corresponding to the priority directions.
		1) The thesis was completed within the framework of a project or target program financed from the state budget (indicate the name and number of the project or program) 2) The thesis was completed within the framework of another state program (indicate the name of the program) <u>3) The dissertation corresponds to the priority direction of the development of science, approved by the Higher Scientific and Technical Commission under the Government of the Republic of Kazakhstan (indicate the direction)</u>	The dissertation is aligned with priority direction 5: Science of life and health, in specialized and scientific field 4: Pharmacy and biologically active substances, functional products, medical and biological preparations. Particularly through the combined study of natural products, chromatographic/spectroscopic characterization, enzymatic assays and microbial biotransformation.
2.	Importance for science	<u>The work makes/does not make a significant contribution to science, and its importance is well disclosed/not disclosed</u>	The work makes a reasonable contribution at the PhD level. Its importance is adequately disclosed through the phytochemical

			investigation of <i>Tussilago farfara</i> L. and the microbial biotransformation study of methylprednisolone acetate. The thesis reports the isolation of twenty plant secondary metabolites, including three compounds reported for the first time in this species. In addition, the biotransformation study afforded eight steroidal metabolites, including one new derivative. Biological assays and computational analyses further support the experimental findings.
3.	The principle of independence	Self-reliance level: 1) High; 2) <u>Medium</u>; 3) Low; 4) No independence	The thesis indicates that the doctoral candidate independently performed the literature survey, chromatographic profiling and optimization, isolation and purification procedures, microbial transformation experiments, data analysis, and preparation of figures and tables. However, some experimental sections, particularly those related to the recombinant expression and biological evaluation of the <i>Leishmania</i> enzyme system, are described only briefly, making it difficult to fully assess the extent of the candidate's direct contribution to these activities. The level of independence is therefore considered medium, which is acceptable within the context of collaborative doctoral

			research, although a clearer specification of individual contributions in these sections would have strengthened the thesis.
4.	The principle of inner unity	4.1 Justification of the relevance of the thesis: 1) Justified; 2) Partially justified; 3) Not justified.	The relevance is justified by the need for sustainable sources of bioactive compounds, green chemistry approaches, and the continuing value of microbial biotransformation for producing modified steroidal derivatives.
		4.2 The content of the thesis reflects the topic of the thesis: 1) Reflects; 2) Partially reflects; 3) Does not reflect	The content reflects the declared topic: the first experimental part concerns <i>Tussilago farfara</i> phytochemistry and bioactivity, and the second part concerns fungal biotransformation of methylprednisolone acetate using <i>Fusarium oxysporum</i> and <i>Penicillium chrysogenum</i> .
		4.3. The purpose and objectives correspond to the topic of the thesis: 1) correspond; 2) partially correspond; 3) do not correspond	The aim and tasks correspond to the topic and cover plant/fungal selection, extraction and isolation, structural elucidation, in vitro bioassays and in silico validation. The objectives are realistic for a PhD dissertation.
		4.4 All sections and provisions of the thesis are logically interconnected: 1) completely interconnected; 2) the interconnection is partial; 3) there is no interconnection	The literature review, experimental sections, biological assays, computational studies, and SAR considerations are generally organized in a logical manner. However, the interconnection

			between the two main research blocks is only partial. The transition from the phytochemical investigation of <i>Tussilago farfara</i> L. to the biotransformation study of the commercial steroidal substrate is justified by the reported low efficiency in obtaining suitable compounds from the plant material, but this preliminary experimental approach is not at all described in the thesis. Moreover, the extraction and fractionation strategy actually employed for <i>T. farfara</i> appears mainly oriented toward polar constituents and is not clearly suitable for the isolation of lipophilic steroidal or sterol-like compounds. Therefore, although both parts share a common analytical and pharmacological framework, their experimental interconnection is only partially demonstrated.
		4.5 The new solutions (principles, methods) proposed by the author are reasoned and evaluated in comparison with the known solutions: 1) there is a critical analysis; 2) <u>partial analysis</u>; 3) the analysis does not represent one's own opinions, but quotes from other authors 4) the analysis is absent.	The author compares the work with known phytochemical and biotransformation knowledge and gives a reasonable rationale for the methods used. However, the critical comparison with previous solutions is not always deep; parts of the literature review and discussion are descriptive rather than fully analytical. This is a limitation, but

			not one that undermines the overall scientific sufficiency of the dissertation.
5.	Scientific novelty principle	5.1 Are the scientific results and provisions new? 1) completely new; <u>2) partially new (25-75% are new);</u> 3) not new (less than 25% are new)	The novelty is partial but real: some compounds are reported from <i>T. farfara</i> for the first time, and the fungal transformation of methylprednisolone acetate with the selected strains yielded one new metabolite. The thesis also integrates <i>in vitro</i> and <i>in silico</i> approaches. At the same time, several isolated metabolites are known and the methodological framework is established in the field.
		5.2 Are the dissertation findings new? 1) completely new; <u>2) partially new (25-75% are new);</u> 3) not new (less than 25% are new)	The findings are new in their specific combination of plant source, fungal strains, steroidal substrate, isolated compounds, bioactivity profile and computational interpretation. The novelty is not absolute, but it is adequate for a doctoral dissertation in chemistry.
		5.3 Technical, technological, economic or management decisions are new and reasonable: 1) completely new; <u>2) partially new (25-75% are new);</u> 3) not new (less than 25% are new)	The technical and technological decisions are absolutely reasonable and partly new in application, especially the chromatographic/spectroscopic workflow and the microbial transformation strategy. They are not fundamentally new methods, but their application to the studied materials and products is

			scientifically justified.
6.	The validity of the main findings	All main conclusions are/are not based on scientifically significant evidence or well-grounded (for qualitative research and areas of training in the arts and humanities)	The main conclusions are supported by chromatographic isolation, extensive spectroscopic/spectrometric characterization, enzymatic assays and computational studies. Some biological claims should be interpreted cautiously because the antileishmanial activity is moderate and further pharmacological validation would be required, but the conclusions are more than sufficiently supported for the degree.
7.	The main provisions for the defense	Provision 1 The plant material of <i>Tussilago farfara</i> L. contains structurally diverse compounds (1–20), including simple phenolic acids, flavonoids, and caffeoylquinic acid derivatives, such as vanillic acid (1), (1S,3R,4R,5R)-3,5-di-O-caffeoylquinic acid (14), and 3,4,5-tri-O-caffeoylquinic acid methyl ester (20). 7.1 Is the provision proven? <u>1) proven;</u> 2) rather proven; 3) rather not proven; 4) not proven 5) in its current wording, it is impossible to verify whether the statement has been proven. 7.2 Is it trivial? 1) yes; <u>2) no</u> 3) in its current wording, it is impossible to verify whether the statement is trivial. 7.3 Is it new?	Provision 1 is supported by the isolation and characterization of twenty compounds from <i>Tussilago farfara</i> L., including compounds reported for the first time from this species. The statement is not trivial and has a medium application level because it expands the phytochemical profile of the plant and provides a basis for further bioactivity and pharmacological studies. The work is strengthened by the extensive use of advanced chromatographic and spectroscopic methodologies, including HPLC, UPLC, and multidimensional NMR and MS techniques, which were appropriately applied for compound isolation and structural elucidation.

		<u>1) yes;</u> 2) no 3) in its current wording, it is impossible to verify the novelty of the statement. 7.4 Application level: 1) narrow; <u>2) medium;</u> 3) wide 4) in its current wording, it is impossible to determine the scope of application of the statement. 7.5 Is it proven in the article? <u>1) yes;</u> 2) no 3) in its current wording, it is impossible to verify whether the statement has been proven in the article.	Overall, the analytical approach is technically sound and represents a valuable aspect of the thesis. Provision 1 is further supported by the peer-reviewed publications: Ahmad I. et al., “<i>Caffeoylquinic acid derivatives isolated from Tussilago farfara L. as potential anti-leishmanial agents; in vitro and in silico studies</i>”, Natural Product Research (2026, DOI: 10.1080/14786419.2026.2613766); Ahmad I., Atif M., Aldibekova G., Balpanova D., Zafar H., Sadia M., Ahmad M., Atia-tul-Wahab, Kudaibergenova B., Choudhary M.I. <i>Aromatase (CYP19A1) Inhibitory Activity of Coltsfoot (Tussilago farfara L.) Phytochemicals: In Vitro and In Silico Evaluation</i>. Eurasian Chemico-Technological Journal. 27 (2025). DOI: 10.18321/ectj1678. The articles confirm the isolation, structural characterization, and biological evaluation of the investigated compounds, thereby providing independent scientific validation of the main findings presented in the dissertation.
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	Provision 2 Microbial biotransformation of the selected steroidal substrate using the fungal strains <i>Fusarium oxysporum</i> and <i>Penicillium chrysogenum</i> affords regio- and stereoselective structural transformations, yielding one new compound, 20-(4',5'-dimethyl-1',3'-dioxolan-2-yl)-11β-hydroxypregn-4-ene-3,20-dione, and seven known metabolites. 7.1 Is the provision proven? <u>1) proven;</u> 2) rather proven; 3) rather not proven; 4) not proven 5) in its current wording, it is impossible to verify whether the statement has been proven. 7.2 Is it trivial? 1) yes; <u>2) no</u> 3) in its current wording, it is impossible to verify whether the statement is trivial. 7.3 Is it new? <u>1) yes;</u> 2) no 3) in its current wording, it is impossible to verify the novelty of the statement. 7.4 Application level: 1) narrow; <u>2) medium;</u> 3) wide 4) in its current wording, it is impossible to determine the scope of application of the statement. 7.5 Is it proven in the article? <u>1) yes;</u> 2) no	Provision 2 is supported by the microbial transformation experiments performed with <i>Fusarium oxysporum</i> and <i>Penicillium chrysogenum</i>, followed by chromatographic purification and detailed spectroscopic characterization of the resulting metabolites, as well as by the related peer-reviewed publication dedicated to the fungal-mediated transformation of methylprednisolone acetate. The study makes appropriate use of advanced analytical methodologies for structural elucidation and includes an appreciated effort to rationalize the possible biochemical mechanisms underlying the observed biotransformation pathways. The statement is not trivial and presents a medium application level in the context of steroid biotransformation and drug-derivative development. Overall, the provision can be considered proven on the basis of the analytical and experimental evidence presented, although broader biological validation and further independent confirmation would strengthen the potential applicability
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		3) in its current wording, it is impossible to verify whether the statement has been proven in the article.	of the findings. Provision 2 is further supported by the peer-reviewed publication: Ahmad I. et al., “<i>Fungal-mediated transformation of methylprednisolone acetate into potent aromatase inhibitors: In vitro and in silico studies</i>”, Journal of Molecular Structure (2026, DOI: 10.1016/j.molstruc.2026.145890). The article documents the microbial biotransformation of methylprednisolone acetate by <i>Fusarium oxysporum</i> and <i>Penicillium chrysogenum</i>, leading to the identification of one new metabolite together with several known derivatives, supported by extensive spectroscopic characterization, biological evaluation, docking analyses, and a proposed mechanistic interpretation of the fungal-mediated transformations. These results provide substantial support for the claims presented in the dissertation and independently validate the relevance of the biotransformation approach.
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8.	The principle of reliability Reliability of sources and information provided	8.1 Choice of methodology - is justified or the methodology is described in sufficient detail 1) yes; <u>2) no</u>	The phytochemical, chromatographic, spectroscopic, and computational methodologies are generally well justified and described in adequate detail, particularly regarding extraction procedures, isolation strategies, UPLC/HPLC conditions, structural elucidation, and docking/MD analyses. However, the description of the in vitro biological assays, especially those related to enzyme expression and inhibition studies, is comparatively limited and does not always provide sufficient experimental detail to fully assess reproducibility and direct methodological contribution. For this reason, the methodology cannot be considered completely described in all sections of the thesis.
		8.2 The results of the thesis were obtained using modern methods of scientific research and methods of processing and interpreting data using computer technologies: <u>1) yes;</u> 2) no	The thesis uses modern scientific methods, including TLC, column chromatography, HPLC, UPLC, 1D/2D NMR, MS/HRMS, FT-IR, UV/Vis, in vitro enzymatic assays, molecular docking, molecular dynamics and MM-GBSA calculations.

		8.3 Theoretical conclusions, models, identified relationships and patterns have been proven and confirmed by experimental research (for areas of training in pedagogical sciences, the results have been proven on the basis of a pedagogical experiment): <u>1) yes;</u> 2) no	The theoretical conclusions, binding models and proposed structure-activity interpretations are supported by experimental isolation/identification and biological assays. Computational results are used appropriately as supportive evidence, not as a substitute for chemical characterization.
		8.4 Important statements are confirmed / partially confirmed / not confirmed by references to current and reliable scientific literature	The dissertation contains a large and generally appropriate reference base and cites relevant literature for natural products, <i>T. farfara</i> , biotransformation, enzymatic assays and computational approaches. Nevertheless, some general claims could be supported by more recent or more precise references, and the discussion could more clearly distinguish previous knowledge from the author's own contribution.
		8.5 Used literature sources are sufficient/not sufficient for a literature review	The literature review is broad and sufficient, covering natural products, <i>T. farfara</i> phytochemistry/pharmacology, biotransformation and steroidal microbial transformations. The review could be more selective and critical, but the number and scope of references are adequate.
9	Practical value principle	9.1 The thesis has theoretical value: <u>1) yes;</u> 2) no	The thesis has theoretical value because it expands the chemical knowledge of <i>T. farfara</i> and

			contributes to the understanding of fungal transformations of a steroidal substrate.
		9.2 The thesis is of practical importance and there is a high probability of applying the results obtained in practice: 1) yes; 2) no	The work has practical importance as a source of characterized phytochemicals and steroidal derivatives with enzyme-inhibitory activity. Practical application is plausible in medicinal chemistry and lead-development research, although direct pharmaceutical application would require additional pharmacological, toxicological and preclinical evaluation.
		9.3 Are the practice suggestions new? 1) completely new; 2) partially new (25-75% are new); 3) not new (less than 25% are new)	The practice-oriented suggestions are partly new. The thesis does not deliver a final product or clinical candidate, but it provides experimentally supported chemical leads, bioactivity data and a sustainable transformation approach that can guide further research.
10.	The quality of writing and design	Academic writing quality: 1) high; 2) average; 3) below average; 4) low.	The academic writing is generally understandable and the dissertation is sufficiently structured.
11.	Comments on the thesis	The dissertation is scientifically sound for the award of the PhD degree, while not being free from weaknesses, and minor revisions not requiring additional experimental work would undoubtedly improve the overall quality, clarity, and critical discussion of the thesis. 1. Introduction and scientific rationale The Introduction provides a broad overview of natural products, <i>Tussilago farfara</i> L., and microbial	

		biotransformation. However, the biological context and therapeutic rationale could be strengthened. In particular, a more focused state-of-the-art discussion on the biological assays employed, the medical relevance of leishmaniasis and aromatase-related pathologies, the cultural/traditional context supporting the selection of <i>T. farfara</i>, and the justification for the chosen therapeutic targets (PTR-1 and aromatase) would improve the scientific framing of the study. 2. Link between the two research blocks and extraction strategy The transition between the phytochemical investigation and the steroid biotransformation section would benefit from additional clarification. The thesis mentions difficulties in obtaining suitable compounds from <i>Tussilago farfara</i> for further transformation studies, which motivated the selection of methylprednisolone acetate as an alternative substrate, but the preliminary experimental attempts leading to this decision are not described. This aspect is particularly important because the extraction and fractionation strategy actually presented in the thesis appears mainly optimized for relatively polar constituents, such as caffeoylquinic acid derivatives and other phenolic compounds identified among the isolated metabolites. Consequently, any attempt aimed at isolating steroidal or phytosterol-like constituents would likely have required substantially different extraction and purification conditions due to their distinct physicochemical properties. Including at least a concise description of these preliminary investigations would improve the internal coherence and methodological transparency of the dissertation. 3. Production and use of recombinant PTR-1 enzyme The sections describing the recombinant expression and preparation of Leishmania pteridine reductase-1 (PTR-1) require additional detail. It should be clarified whether the recombinant enzyme production was performed directly by the doctoral candidate or in collaboration with another laboratory or research group. If collaborative work was involved, this should be explicitly acknowledged. Otherwise, the experimental procedure should be described in greater detail both in the Experimental section and in the Results and Discussion section to allow proper evaluation of reproducibility and contribution. 4. Description of biological assays and controls The methodology of the enzymatic inhibition assays would benefit from a more detailed description of the experimental controls. In particular, the composition and role of the negative controls should be explicitly reported (e.g., assay mixture lacking enzyme or substrate, solvent controls, etc.) to improve methodological transparency. 5. IC₅₀ determination and graphical representation The procedure used to generate the dose–response curves and IC₅₀ plots presented in Figure 16 should be described more clearly. The thesis should specify the software or mathematical model used for curve fitting and explain the concentration scale reported on the x-axis, which is not immediately intuitive for
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	the reader. 6. Structure–activity relationship (SAR) discussion The SAR analysis mainly focuses on active compounds. The discussion would be more convincing if at least some inactive or weakly active compounds were also included as comparative controls to better support the proposed structure–activity interpretations. 7. Biotransformation section: selection of microbial strains In the Experimental section, ten microbial strains are listed together with their cultivation procedures, whereas only two strains (<i>Fusarium oxysporum</i> and <i>Penicillium chrysogenum</i>) are subsequently discussed in the Results section. A short explanation regarding the preliminary screening process and the criteria used to select these two strains for detailed study would improve the clarity and completeness of the experimental design. 8. Source of aromatase enzyme The source of the aromatase enzyme employed in the inhibition assays is not clearly indicated. The thesis should specify whether the enzyme was commercially obtained or produced in-house and, if commercial, provide the supplier and relevant specifications. 9. Results and Discussion: critical interpretation In several parts of the Results and Discussion sections, the interpretation remains predominantly descriptive. The thesis would benefit from a more critical comparison with previous literature data, especially regarding biological activity values, transformation selectivity, and the significance of the newly identified metabolites. 10. Computational studies The molecular docking and molecular dynamics sections are generally well presented and technically adequate. Nevertheless, the relationship between docking scores, MM-GBSA values, and experimental biological activity could be discussed more critically, particularly where the computational predictions only partially correlate with the experimental inhibition data. 11. Language and academic style The dissertation is overall understandable and scientifically structured, but the language would benefit from careful linguistic. Some grammatical inaccuracies, repetitive phrasing, inconsistent terminology, and typographical issues are present. Improving sentence structure and conciseness would significantly enhance readability and academic presentation. 12. Formatting and consistency Minor improvements in formatting consistency are recommended, particularly regarding compound numbering style, abbreviation usage, punctuation, italicization of species names, and uniform presentation
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		of spectroscopic data and units throughout the dissertation.	
12.	Scientific level of the doctoral student's articles on the topic of research (in case of defense of the dissertation in the form of a series of articles, the official reviewers comment on the scientific level of each article of the doctoral student on the topic of research)	The scientific level of the doctoral student's articles is good and adequately supports the dissertation. The dissertation lists four publications related to the topic: three original research papers and one review article in international peer-reviewed journals indexed by Scopus. The articles address caffeoylquinic acid derivatives from <i>T. farfara</i>, a review on coltsfoot, aromatase inhibitory activity of coltsfoot phytochemicals, and fungal-mediated transformation of methylprednisolone acetate. Taken together, they demonstrate that the main research results have undergone external peer review. The publication record is adequate for the defense.	
13.	Decision of the official reviewer (pursuant to paragraph 28 of the present Model Regulations)	Choose one of the following decisions: 1) to award the degree of the Doctor of Philosophy (PhD); <u>2) to send the dissertation for revision;</u> 3) refuse to award the degree of the Doctor of Philosophy (PhD).	Minor revisions addressing at least some of the points raised under Criterion 11 ("Comments on the thesis"), without the need for additional experimental work, would further strengthen the overall quality, clarity, and critical discussion of the dissertation.

Copies of the reviews of the official reviewers are handed over to the doctoral student no later than 5 (five) working days before the defense of the thesis.

Official Reviewer:

University of Rome La Sapienza
Full Professor of Pharmaceutical Biology


(signature)

LUCA SANTI
(FULL NAME)