OPINION

by Prof. Velizar Kostadinov Gochev, PhD,

Faculty of Biology, Plovdiv University "Paisii Hilendarski"

on a dissertation submitted for the award of the educational and scientific degree "Doctor"
Field of higher education: 5. Technical Sciences; professional field: 5.11 Biotechnology; doctoral programme: "Technology of Biologically Active Substances"

Author: Eng. Saveta Georgieva Mladenova

Title: "*Investigation of the Anti-Obesogenic Potential of Extracts from Alchemilla monticola Opiz and Ononis spinosa L. for the Control of Obesity in In Vitro and In Vivo Models*"

Scientific supervisor: Prof. Milen I. Georgiev, PhD, Stefan Angelov Institute of Microbiology – Bulgarian Academy of Sciences

1. General description of the submitted materials

By Order No. I-98/30.06.2026 of the Director of the Stefan Angelov Institute of Microbiology (IMicB), I was appointed as a member of the scientific jury in the procedure for the defence of the dissertation of Eng. Saveta Georgieva Mladenova. The set of materials submitted by the doctoral candidate in electronic and hard-copy form complies with the regulatory requirements and includes the following documents: an application for opening a procedure for the defence of a dissertation; a curriculum vitae in European format; minutes related to reporting readiness to open the procedure and to the preliminary discussion of the dissertation; the dissertation; an abstract; a list of scientific publications on the topic of the dissertation; copies of the scientific publications; and a declaration of originality and authenticity of the submitted documents. The submitted documents give me full grounds to consider the procedure compliant with all national and institutional regulatory requirements.

2. Brief biographical information about the doctoral candidate

The author of the work is Eng. Saveta Georgieva Mladenova, who completed her higher education at the University of Chemical Technology and Metallurgy, where she obtained a Master's degree with the professional qualification "Engineer-Biotechnologist". She is carrying out her dissertation research at IMicB under the scientific supervision of Prof. Milen I. Georgiev, PhD. Her professional development has included positions at "Bul-Bio NCIPD" EOOD, "Comac Medical" EOOD and "C 7 Commercial" EOOD. The doctoral candidate's education and professional competence are entirely within the field of the doctoral programme.

3. Relevance of the topic and appropriateness of the stated aim and objectives

The topic is unquestionably relevant. Obesity is a complex metabolic disease with increasing prevalence and a direct association with type 2 diabetes, cardiovascular, inflammatory and other chronic diseases. Alongside the development of contemporary pharmacotherapy, there remains a scientific need to identify new molecules and mechanisms capable of modulating adipogenesis, lipogenesis and energy metabolism. The selection of *Alchemilla monticola* and *Ononis spinosa* combines an ethnopharmacological rationale with the potential to discover and mechanistically characterize specific secondary metabolites.

The aim is clearly formulated: to investigate the effects of the two plant extracts and their secondary metabolites on adipogenesis and lipid accumulation in human adipocytes and to validate the most significant results in a *C. elegans* model. The six objectives are arranged logically – literature and phytochemical characterization, *in silico* prediction, functional cellular screening, elucidation of molecular mechanisms and *in vivo* validation. They are appropriate to the stated aim and mutually complementary.

4. Knowledge of the research problem

The literature review demonstrates good knowledge of adipose tissue biology, the regulation of adipogenesis, the role of PI3K/AKT, PPARγ/C/EBP, AMPK/SIRT1 and SREBP-1 signalling, as well as the possibilities and limitations of different cellular and whole-organism models of obesity. I positively assess the connection made between the traditional use and phytochemistry of the studied plants and specific molecular targets. The review is not merely descriptive; it builds the rationale for the selection of the extracts, compounds and experimental models. The literature used includes both fundamental publications and a substantial number of recent sources.

5. Research methodology

The experimental design and methodological scope are clearly among the strengths of the dissertation. The phytochemical investigation includes ^{1}H and 2D NMR-based metabolic profiling, followed by molecular docking to C/EBPα, PPARγ, AKT, PI3K and AMPK. Biological activity was investigated in human SGBS preadipocytes/adipocytes, which provides an important advantage over the widely used murine 3T3-L1 cells when extrapolating to human adipocyte biology. The methods used include an MTT viability assay, Oil Red O staining for quantitative assessment of lipid accumulation, a free glycerol assay, RT-qPCR and immunoblotting for key adipogenic and lipogenic regulators.

Whole-organism validation in *C. elegans* includes glucose-induced lipid accumulation, Nile Red staining and confocal microscopy, viability, locomotor activity and chemotaxis assays, as well as

expression analysis of aak-2, sir-2.1, mdt-15, nhr-49, cebp-2, sbp-1 and selected microRNAs. The inclusion of orlistat as a reference compound allows functional comparison. The use of three independent biological experiments and statistical analysis by t-test/ANOVA with Bonferroni post hoc analysis is fully appropriate to the stated objectives. Overall, the methodological set is contemporary and multilayered and allows phenotypic observations to be linked to molecular changes.

6. Characterization and evaluation of the dissertation

The experimental section is coherent and comprises several clearly distinguishable lines of investigation. In *A. monticola* NMR profiling identified flavonoids and flavonoid glycosides, including astragalin and quercitrin. The extract reduces lipid accumulation in SGBS adipocytes and increases basal lipolysis, with the effect accompanied by reductions in PI3K and AKT and in the key adipogenic markers PPARγ, C/EBPα and adiponectin. Of particular interest is the bidirectional concentration-dependent effect of astragalin on PI3K/AKT – a result that warrants more detailed pharmacodynamic investigation.

A more complex pattern is observed with *O. spinosa*. The whole extract moderately increases lipid accumulation and suppresses basal lipolysis, whereas the isolated secondary metabolites ononin and, in particular, maackiain decrease lipid accumulation. Maackiain shows the most convincing concentration-dependent effect, accompanied by changes in SREBP1/ACC and PPARγ/C/EBPα, which logically supports its selection for whole-organism validation. In *C. elegans*, maackiain reduces glucose-induced lipid accumulation, increases locomotor activity and modulates genes associated with energy metabolism and caloric restriction – aak-2, sir-2.1, mdt-15 and nhr-49. At the same time, the absence of a change in AAK-2 phosphorylation at the examined time point is appropriately acknowledged and indicates that some of the proposed relationships should be regarded as well-supported hypotheses rather than definitively established causality.

7. Contributions and significance of the work for science and practice

I accept the main scientific contributions as formulated. In my view, those of greatest significance are:

- ✓ the comprehensive demonstration of the anti-adipogenic potential of *A. monticola* in human adipocytes and its association with modulation of PI3K/AKT and PPARγ/C/EBPα;
- ✓ the first systematic characterization of the anti-adipogenic activity of ononin and maackiain in human adipocytes;
- ✓ the demonstration of a concentration-dependent lipid-reducing effect of maackiain and its validation at the whole-organism level in *C. elegans*;
- ✓ the delineation of a relationship between maackiain action and the energy-metabolism regulators aak-2/sir-2.1 and nhr-49/mdt-15.

The scientific and applied significance lies primarily in identifying maackiain as a promising natural molecule for subsequent pharmacological and biotechnological development. At this stage, the data do not justify direct therapeutic application, but they provide a reliable basis for studies of pharmacokinetics, target engagement, safety and efficacy in higher animal models.

8. Assessment of the publications related to the dissertation

The results of the dissertation have been presented in three scientific publications in English in international peer-reviewed Q1 journals: International Journal of Molecular Sciences (2023, IF 4.9), Biomedicine and Pharmacotherapy (2022, IF 7.5) and Frontiers in Pharmacology (2021, IF 5.988). The total stated impact factor is 18.388. Eng. Saveta Mladenova is first author of all three articles, which is an important indicator of her personal contribution to the planning, execution, analysis and presentation of the research. Thematically, the three publications cover the main experimental lines of the dissertation. The publication record is fully commensurate with the scope and quality of the dissertation.

9. Personal contribution of the doctoral candidate

Based on the submitted materials, the integrated presentation of the experimental results and first authorship of all publications related to the dissertation, it can reasonably be concluded that the doctoral candidate's personal contribution is substantial. The work demonstrates an ability to integrate analytical chemistry, cell biology, molecular methods and work with a model organism within a unified experimental strategy, as well as the capacity for critical interpretation of discordant results at the levels of gene expression, protein and phenotype.

10. Abstract

The abstract meets the requirements and reflects all major results of the dissertation.

11. Critical remarks and recommendations

My remarks are primarily recommendatory in nature and do not alter the positive assessment of the work. In some places, the interpretation of the results shifts from probability to a categorical statement of direct molecular interaction, which requires additional studies. Docking and changes in the abundance of PI3K, PPARγ or other proteins are strongly suggestive, but by themselves do not demonstrate direct binding or enzymatic inhibition.

The terms "anti-obesogenic effect" and "safety" in *C. elegans* should be used with some caution: the model convincingly demonstrates a lipid-reducing effect and no decrease in viability within the tested range, but it does not reproduce the full pathophysiology of human obesity and does not constitute a comprehensive toxicological assessment.

Question to the doctoral candidate: How would you explain the fact that *O. spinosa* extract increases lipid accumulation in SGBS adipocytes, whereas two of its identified components – ononin and maackiain – decrease it, and what experimental design would you use to determine which

components of the extract determine the final phenotype and whether synergistic or antagonistic interactions occur among them?

12. Personal impressions

I have no personal impressions of the doctoral candidate beyond my assessment of the submitted scientific materials. The dissertation itself, however, gives the impression of consistent experimental work, good methodological preparation and an effort to interpret the results in the context of contemporary molecular biotechnology.

Conclusion

The dissertation of Eng. Saveta Georgieva Mladenova is a complete, timely and methodologically well-supported scientific study. Original scientific results of fundamental and prospective scientific and applied significance have been obtained, and the publications on the topic demonstrate their scientific visibility. The work demonstrates in-depth theoretical knowledge, mastery of contemporary biotechnological, cellular and molecular methods, and the ability to independently plan, conduct and interpret scientific research.

I consider that the dissertation meets the requirements for the award of the educational and scientific degree “Doctor”. I give a positive evaluation and propose that the esteemed scientific jury award Eng. Saveta Georgieva Mladenova the educational and scientific degree “Doctor” in professional field 5.11 Biotechnology, doctoral programme “Technology of Biologically Active Substances”.

Date: 31 August 2026 Scientific Jury Member: .....