

REVIEW

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appointed as a member of the Scientific Jury with Order № I – 98/30.06.2026 issued by the
Director of the Institute of Microbiology, BAS and as a reviewer by the Scientific Jury with
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for evaluation of the thesis for academic and scientific degree "Doctor"(PhD)

PhD student: Eng. Saveta Georgieva Mladenova

PhD thesis title: Investigation of the anti-obesity 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. Dr. Milen I. Georgiev

Research field: 5.11. Biotechnology (Technology of biologically active molecules)

1. General description of the presented materials

The author of the PhD thesis, **Saveta Mladenova**, is a PhD student enrolled in a free form of the PhD program at the Metabolomics Laboratory of the Department of Biotechnology, Institute of Microbiology, BAS under the supervision of Prof. Milen Georgiev. She has submitted her dissertation work, in paper and in electronic version, and all relevant documents, according to the requirements of the Regulations for the Development of the Academic Staff of IMikB, BAS, namely: application for admission to thesis defense, copy of completed master's degree, certificate for PhD enrollment, records of exams and courses in the specialty, summary for credits, summary of scores according to the minimum requirements of the Bulgarian law and the additional requirements of IMikB, BAS, protocol and preliminary review of pre-defense, dissertation summary, list of publications, declaration of originality and curriculum vitae.

2. Significance of the research topic of the PhD thesis

According to the WHO definition, obesity is "a condition with an accumulation of fat," with a body mass index over 30 kg/m². Statistics show that 1 in 8 people suffers from obesity, and its prevalence has multiplied 4 times since 1990, reaching 38% in the USA. According to the National Centre for Public Health and Analyses: "Bulgaria ranks 6th in the EU for obesity, based on 2019 data. The reasons are many: unhealthy eating - high intake of saturated fats, high consumption of pastries and sweets; low intake of whole grains, fruits, and vegetables; low physical activity, and others." (Prof. Dr. Veselka Duleva¹)

The most striking statistics concern the young population with obesity, which includes 160 million registered children with this condition (Di Cesare et al., 2019²). Numerous studies show that the disease

¹ България е една от страните с най-високи нива на затлъстяване в Европейския съюз

² Di Cesare, M., Sorić, M., Bovet, P., Miranda, J. J., Bhutta, Z., Stevens, G. A., Laxmaiah, A., Kengne, A. P., & Bentham, J. (2019). The epidemiological burden of obesity in childhood: a worldwide epidemic requiring urgent action. *BMC medicine*, 17(1), 212. <https://doi.org/10.1186/s12916-019-1449-8>.

is accompanied by a higher incidence of illnesses such as diabetes and cardiovascular diseases (including heart attacks), steatosis, and liver dysfunction. There is also evidence that anti-inflammatory responses in obesity patients are often compromised or correlate with the severity of accompanying infectious diseases (Nyberg S. et al., 2026³) and cancer (Calle et al., 2003⁴) leading to increased mortality. This shows that the health problem of obesity for society and the healthcare system is greater than described. In this regard, any new study on obesity is essential for finding better early diagnostic tools as well as for novel therapeutic approaches and disease monitoring.

The main pathological mechanism in the disease involves the fat storage in adipocytes, followed by adipocyte's hypertrophy and hyperplasia but additional dysfunction such as abnormal insulin signalling and insulin resistance, neuro-hormonal dysregulation with leptin resistance, changes in the hunger hormone ghrelin, and intense fat tissue accumulation can co-occur in obesity. The main pathways described in obesity are through the mitogen-activated protein kinases (MAPKs, incl. ERK1/2, JNK, p38 MAPK) and signaling via PI3K/AKT, JAK/STAT, TGF- β , AMPK, and Wnt/ β -catenin, regulating appetite, fat tissue metabolism, glucose homeostasis and energy expenditure. As the disease is heterogenous, the mechanisms of obesity can be divided into that with the anti-obesity effects (thermogenesis, lipolysis, and hypophagia), and anti-obesity effects (inflammation in fat tissue, insulin resistance and adipogenesis).

Due to the involvement of multiple signalling pathways and mechanisms, the disease therapy involves more than 20 different agonists or inhibitors targeting the above mentioned signalling pathways. Most of them have side effects, outlining the remain necessity of new approaches and increasing a focus on studying active substances among plant-derived biologically active molecules. In this regard, the dissertation is significant, as it provides hypothesis and evidence for a novel therapeutic approach targeting key signalling activated in adipocytes and in obesity

3. Relevance of the PhD thesis goal and tasks

A clear and well-formulated goal has been defined in the PhD study of Saveta Mladenova, namely to investigate the pharmacological potential of the extracts from *Alchemilla monticola* Opiz and *Ononis spinosa* L. and their secondary metabolites on adipogenesis and lipid accumulation in human adipocytes, differentiated *in vitro*, followed by validation of the most significant results showing anti-obesity potential in *Caenorhabditis elegans in vivo*.

Six tasks have been formulated to achieve the goal following the consecutive experimental steps - an in-depth search of the available databases, based on the traditional application of plants and their extracts in obesity and other metabolic conditions with established dysfunction and pathology; selection of plants and metabolites with biological activity for inclusion in the study; obtaining extracts from the selected plants; metabolomic analysis of the plant-derived extract, *in silico* analysis for potential interactions of metabolites with molecular targets from signalling pathway; investigation of the anti-adipogenic potential *in vitro* in Simpson-Golabi-Behmel Syndrome (SGBS) differentiated adipocytes; proof

³ Nyberg, S. T., Frank, P., Ahmadi-Abhari, S., Pentti, J., Vahtera, J., Ervasti, J., Suominen, S. B., Strandberg, T. E., Sipilä, P. N., Meri, S., Sattar, N., & Kivimäki, M. (2026). Adult obesity and risk of severe infections: a multicohort study with global burden estimates. *Lancet* (London, England), 407(10532), 951–962. [https://doi.org/10.1016/S0140-6736\(25\)02474-2](https://doi.org/10.1016/S0140-6736(25)02474-2).

⁴ Calle, E. E., Rodriguez, C., Walker-Thurmond, K., & Thun, M. J. (2003). Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *The New England Journal of Medicine*, 348(17), 1625–1638. <https://doi.org/10.1056/NEJMoa021423>

of their action on the targeted kinases and pathways and validation of the observed beneficial effects *in vivo* in a model of glucose-induced lipid accumulation and chemotaxis in *Caenorhabditis elegans*.

Notably, it makes an excellent impression the careful and adequate selection of the *in vitro* and *in vivo* model systems – for example, the use of adipocytes differentiated from SGBS cells is considered a versatile modern approach for studying adipogenesis, insulin sensitivity, inflammatory response and adipokine production (Tews et al., 2022⁵). Genetically amenable organisms such as *Caenorhabditis elegans* are powerful tools for studying fundamental molecular mechanisms that govern adipose tissue functions (Mora et al., 2024⁶).

4. Level of the author's competence

Saveta Mladenova has prepared a detailed literature review of current state on the research problem, presenting a description of the biological activity, use and phytochemical characteristics of *Alchemilla monticola* Opiz and *Ononis spinosa* L. The literature review focuses on the pathological growth of adipose tissue and its role in obesity, experimental models of obesity and approaches to control and therapy of obesity. The review is illustrated with 5 figures. Figure 5 makes a good impression, as the key signalling pathways affecting adipogenesis are described in friendly manner - WNT/ β -Catenin, Insulin/AKT, Hedgehog (Hh) signaling and BMP/SMAD4 pathway, as the key connection with the PPAR γ /C/EBP α complex is indicated. The literature review cited articles from both, older sources and those published in the period 2020-2026 - more than 149 citations, showing the comprehensive coverage of the problem by the PhD student. The use of multiple experimental approaches in the PhD thesis provides basis of high-quality results aligned objectively with the discussion.

5. Research methodology

Saveta Mladenova has applied modern approaches and diverse methodology in the dissertation, namely preparation of plant extracts, phytochemical analysis of extracts, use of metabolic profiling based on NMR spectroscopy, *in silico* docking analysis, cell cultivation of SGBS pre-adipocytes and their differentiation, analysis of their quantitative accumulation, RT-PCR for quantitative determination of the expression of a panel of genes related to low-grade inflammation and adipocyte signalling pathways, immunoblot for identification of signalling molecules and quantitative determination of a model of glucose-induced lipid accumulation in nematodes by accumulated lipids staining and functional changes in nematodes - such as viability, motor activity and chemotaxis.

The methods used are correctly described so that they can be repeated, appropriate controls are included. This shows a good methodological level of competency achieved by the PhD student and skills for careful planning of the experimental work. The statistical methods are adequate and guarantee repeatability of the obtained data.

6. Characterization and evaluation of the dissertation work

The dissertation has a structure according to the requirements of the Regulations for the Development of the Academic Staff of the Institute of Microbiology and Biochemistry, Bulgarian Academy

⁵ Tews, D., Brenner, R. E., Siebert, R., Debatin, K. M., Fischer-Posovszky, P., & Wabitsch, M. (2022). 20 Years with SGBS cells - a versatile *in vitro* model of human adipocyte biology. *International journal of obesity* (2005), 46(11), 1939–1947. <https://doi.org/10.1038/s41366-022-01199-9>

⁶ Mora, I., Puiggròs, F., Serras, F., Gil-Cardoso, K., & Escoté, X. (2024). Emerging models for studying adipose tissue metabolism. *Biochemical pharmacology*, 223, 116123. <https://doi.org/10.1016/j.bcp.2024.116123>

of Sciences. It contains the following chapters: Title; Abbreviations used; Introduction; Literature review; Goals and objectives; Materials and methods; Results and discussion with 15 figures; Discussion with 4 summarized figures; Conclusion; Conclusion Summary and Contributions; Literature sources – with over 140 sources, over 51% of which in the period 2020-2026.

The results obtained demonstrate the effect of plant extracts from *Alchemilla monticola* Opiz and *Ononia spinosa* L. on adipocyte function including the expression of a panel of factors associated with the maintenance of low-grade chronic inflammation. It was found that the extract of *Alchemilla monticola* Opiz suppresses the PI3K/AKT signaling pathway and negatively regulates PPAR γ and C/EBP α , suppresses the expression of the adiponectin genes *ADIPOQ* and the transcription factor *SREBP* (sterol-regulatory element-binding protein) and the lipogenic genes *ACC* (encoding encodes acetyl-CoA carboxylase alpha) and *FASN* (encoding fatty acid synthase) in human adipocytes. The effects of the metabolites identified in the plant extract, astragalín (AST) and quercitrín (QUE), were studied. The anti-adipogenic effect of AST is more likely related to a direct interaction with PPAR γ or C/EBP α than to the modulation of PI3K and its signaling. Furthermore, AST shows a bidirectional action on PI3K at low and high concentrations. QUE positively affects C/EBP α and adiponectin levels, while weakly inhibiting PPAR γ , indicating a different effect on the intracellular pathway in comparison to AST, but demonstrating anti-adipogenic potential, related and probably determining that of the extract.

The thesis also investigates the anti-adipogenic effect of an extract of *O. spinosa* and its secondary metabolites – Ononin (ONON) and Maackianin (MACK). The extract, unlike the two metabolites, does not significantly suppress the adipogenic differentiation and does not drastically affect the later phase of differentiation affecting lipogenesis. ONON and MACK inhibit PI3K, but their action is probably related to an effect on the stability of mRNA and proteins. ONON suppresses adipogenesis by stimulating SIRT1 and inhibiting PPAR γ , while MACK acts on PPAR γ signaling and suppresses SREBP1-dependent lipogenesis. The data showed that MACK increased the transcription of *aak-2*, *sir-2.1*, *mdt-15*, *nhr-49*, *cebp-2* and *sbp-1*, which are associated with calorie restriction and mitochondrial functions in *C. elegans*. This effect was not mediated through AMPK, but was likely due to a decrease in the protein level of AAK-2 and suppression of microRNAs miR-60 and lin-4, which are associated with lipid metabolism in nematodes. These effects of MACK were antagonized by the simultaneous administration of the lipase inhibitor orlistat with MACK.

7. Contributions and research significance

I accept the conclusions drawn in the dissertation, as well as support the formulated contributions. I would summarize that the significance of the work is due to:

- For the first time, the anti-adipogenic activity of metabolites from *Alchemilla monticola* Opiz extract and the anti-obesogenic activity of metabolites from *Ononis spinosa* L. extract have been characterized.
- For the first time are proven the effects of *Alchemilla monticola* Opiz extract and its metabolites AST and QUE on PI3K signaling and the transcription factors C/EBP α and PPAR γ , regulating genes such as adiponectin *ADIPOQ*, transcription factor *SREBP* and *SREBP*-dependent lipogenic genes.
- A mechanism of the anti-adipogenic action of ononin (ONON), found in an extract of *Ononis spinosa* L., has been shown to involve the upregulation of SIRT1 and the inhibition of PPAR γ .
- The potential of maackianin (MACK) on signaling pathways related to caloric restriction, *aak-2/sir2.1*, and energy expenditure and lipid metabolism, *nhr-49/mdt-15*, and a mechanism involving MACK in the regulation of lipid metabolism in nematodes via microRNAs have been proven.

8. Evaluation of publications on the dissertation work and research score of the author

The dissertation ends with 3 publications. All publications are in Q1, and in all of them, the impact factor is above 4. In this aspect, the criteria of the Institute of Microbiology for publication activity are excellently met by the doctoral student and exceed the quantitative criteria for acquiring the educational and scientific degree "DOCTOR".

9. Critical remarks and recommendations

Question: Is it possible to hypothesize that the studied metabolites from *Ononis spinosa* ONON and MACK can be used in combination therapy in patients with metabolic problems, not only in those with obesity?

CONCLUSION

The dissertation of Saveta Mladenova contains fundamental and scientific-applied results that represent **an original contribution to the research field and meet all the requirements** of the Law on the Development of the Academic Staff in the Republic of Bulgaria (ZRASRB), the Regulations for the Implementation of the ZRASRB and the Regulations for the Implementation of the ZRASRB of the Bulgarian Academy of Sciences. The presented dissertation results fully comply with the specific requirements of the Rules of the IMikB for the application of ZRASRB.

Saveta Mladenova's dissertation work demonstrates skills and characteristics **required for the independent conduct of scientific research**, proving that she possesses in-depth theoretical knowledge and professional skills.

Due to the aforementioned, **I can confidently state my positive assessment** of the research that was done and **I strongly recommend to award** Saveta Georgieva Mladenova the educational and scientific degree of "Doctor" in the field of 5.11 Biotechnologies (Technology of biologically active substances).

31/08/2026

Reviewer:

Assoc. Prof. Petya Dimitrova