

To the Chairman of the Scientific Jury
appointed with Order No. I-64/29.05.2023
of the Director of Institute of Microbiology
"Stefan Angelov" - BAS

REVIEW

from Prof. Tsvetalina Tankova, MD, PhD, DMedSc,
Scientific specialty - endocrinology
Head of Department of Endocrinology, Faculty of Medicine,
Medical University – Sofia

regarding the dissertation work for acquiring educational and scientific degree "doctor"
in the field of higher education 5. Technical sciences, professional direction 5.11.
Biotechnologies, doctoral program Technology of biologically active substances

for Martina Stoyanova Savova, Master of pharmacy, full-time doctoral student at
Department of Applied Microbiology, Institute of Microbiology "Stefan Angelov", BAS
Topic of the dissertation: "Mechanisms of modulating obesity processes in an in vitro
model of human adipocytes by adding biologically active molecules"

Supervisor: Prof. Milen Ivanov Georgiev,
Institute of Microbiology "Stefan Angelov" - BAS

1. General presentation of the procedure

By decision of the Research Council of the Institute of Microbiology "Stefan Angelov" - BAS, Protocol No.46/25.05.2023 and Order No. I-64/29.05.2023 of the Director of the Institute of Microbiology – BAS, I have been appointed as a member of the scientific jury in the procedure for the defense of a dissertation work on the topic "Mechanisms of modulating the processes of obesity in an in vitro model of human adipocytes by applying biologically active molecules" for the acquisition of the educational and scientific degree "doctor" in the field of higher education 5. Technical sciences, professional direction 5.11. Biotechnologies, doctoral program Technology of biologically active substances.

The author of the dissertation is Martina Stoyanova Savova, Master of pharmacy, full-time doctoral student at the Department of Applied Microbiology at the Institute of Microbiology "Stefan Angelov" - BAS, supervised by Prof. Milen Ivanov Georgiev,

Institute of Microbiology "Stefan Angelov" - BAS. Martina Savova has been dismissed with the right of defense of the doctoral thesis by Order No.I-136/27.09.2022 of the Director of the Institute of Microbiology "Stefan Angelov" - BAS.

The set of materials, presented by Martina Stoyanova Savova, in paper form and in electronic version, is in accordance with the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LAD), the Regulations for the Implementation of the LAD and the Regulations for the Implementation of the LAD of the Bulgarian Academy of Sciences, and meets the criteria in the Regulations for the Terms and Procedures for the Acquisition of Scientific Degrees and occupying academic positions at the Institute of Microbiology "Stefan Angelov" - BAS for the acquisition of an educational and scientific degree of "doctor".

The dissertation work on the topic: "Mechanisms of modulating obesity processes in an in vitro model of human adipocytes by adding biologically active molecules" passed a preliminary defense at a meeting of the seminar "Applied Microbiology and Microbial Biotechnologies" at the Institute of Microbiology "Stefan Angelov" – BAS on 18.05.2023, which was judged to meet the minimum requirements of BAS and the requirements of the Institute of Microbiology "Stefan Angelov" – BAS. The dissertation work has been directed for public defense by decision of the Research Council of the Institute of Microbiology "Stefan Angelov" – BAS, Protocol No. 46/25.05.2023.

The review is compiled in accordance with the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LAD), and Chapter II, Section II of the Regulations for the Implementation of LAD.

2. Presentation of the doctoral student

Martina Stoyanova Savova was born on August 10, 1994 in the city of Plovdiv. She is Master of Pharmacy from the Faculty of Pharmacy, MU-Plovdiv since 2019. Since 2019, she has been working as a biologist at the Center for Plant System Biology and Biotechnology, Plovdiv. She has been a full-time doctoral student in direction 5.11. Biotechnologies at the Metabolomics Laboratory at the Department of Applied Microbiology, Institute of Microbiology - BAS since 2019. She is co-author in 14 publications referenced in Scopus, cited 176 times in the same database. H-index 7. She has participated in 14 scientific conferences and congresses so far. Martina Savova received the "Ivan Evstratiev Geshov" award for the youngest scientists in the field of "Biomedicine and quality of life" in 2021.

3. **Relevance of the dissertation topic and expediency to the set goals and objectives**

Obesity is a progressive chronic disease that underlies over 230 comorbidities. 1.9 billion people will be living with obesity in 2035, with one in two adults expected to be overweight and one in four adults to be obese. Between 2020 and 2035, childhood obesity is expected to increase by 100%. This prediction calls for global efforts to reduce, prevent and treat obesity.

The modern approach to obesity includes lifestyle changes with diet and physical activity, drug therapy, and metabolic surgery. In Europe, there are 4 medications approved for the treatment of obesity - orlistat, naltrexone/bupropion, liraglutide at a dose of 3.0 mg daily and semaglutide at a dose of 2.4 mg weekly.

It should be considered that less than 40% of people with obesity have diagnosis, less than 20% undergo treatment, and only about 1% of them are on drug therapy, despite the available options for obesity treatment.

A significant percentage of obese people do not achieve a significant effect on their body weight with modern therapies. Maintenance of the achieved weight loss over time also turns out to be a serious problem. It is necessary to be aware of the current options for the treatment of obesity in order to improve the prognosis of people with this disease. The complex nature of obesity as a disease necessitates the search for therapeutic options targeting various mechanisms involved in its pathogenesis. That is why extremely intensive work is being performed in the direction of finding new options for body weight control. Plant extracts and their secondary metabolites have the potential to affect the signaling pathways and mechanisms that underlie the function and physiology of fat cells, as the main structure units of adipose tissue, and thus influence the development of obesity.

The still unresolved questions surrounding the effectiveness of the approved therapeutic agents in obesity determine the relevance of the presented dissertation work of Martina Savova, which aims to investigate the effects of extracts from *Z. jujuba* (jujube), *P. aviculare* (pacha grass), *P. hydropiper* (water pepper) and their secondary metabolites on the processes of adipogenesis and lipid accumulation in vitro in a human adipocyte model and validating the effect of the most promising metabolites obtained in an in vivo model of obesity in the nematode species *C. elegans*. In order to fulfill the main goal of the current dissertation, 7 main tasks have been defined precisely and specifically, targeting the selection of plants, according to available ethnopharmacological data on the effect on obesity; determination of the phytochemical

composition of selected plant extracts by metabolic profiling using nuclear magnetic resonance (NMR); assessment of the effect of *Z. jujuba*, *P. aviculare* and *P. hydropiper* extracts on the processes of adipogenesis and lipolysis in an in vitro model of human adipocytes; study of the mechanism of action of *Z. jujuba*, *P. aviculare* and *P. hydropiper* extracts on the processes of adipogenesis and lipolysis in an in vitro model of human adipocytes; conducting an in silico docking simulation with natural molecules selected according to the data on the phytochemical composition of the studied extracts; establishing the mechanism of action of selected secondary metabolites – apigenin, betulinic and olive acid in an in vitro model of human adipocytes; confirming the anti-adipogenic effect of betulinic acid and elucidating signaling mechanisms in a *C. elegans* obesity model.

4. Characteristics and evaluation of the dissertation work

The dissertation is written on 131 pages, well organized in the following main sections: Title page (1 page), Table of Contents (4 pages), Abbreviations used (2 pages), Introduction (3 pages), Literature review (25 pages), Aim and objectives (2 pages), Material and methods (12 pages), Results with 3 tables and 29 figures (38 pages), Discussion of the results, illustrated with 3 figures (19 pages), Conclusions (1 page), Contributions (1 page), Acknowledgments (1 page), Bibliography with 218 literature sources (20 pages).

5. Knowledge of the problem

The literature review is very well structured and presents up-to-date information on the epidemiology, etiology, pathogenesis, complications, prevention and pharmacological treatment of obesity. The structure and function of adipose tissue, as well as the characteristics of white, brown and beige adipose tissue, are discussed in detail. The role of visceral adipose tissue as an endocrine organ is thoroughly analyzed. The molecular mechanisms of adipocyte differentiation, the role of key transcription factors, signaling pathways and microRNAs are presented, and these are very well illustrated. Experimental models of obesity are analyzed - an in vitro model in human adipocytes (cell line 3T3-L1), as well as an in vivo model system in the nematode *C. elegans*. Special emphasis is placed on ethnopharmacological solutions in obesity, evaluation of the quality and composition of herbal products as an alternative in the therapy of obesity, in order to ensure their safe use. The application of medicinal plants in obesity and related metabolic disorders is discussed, and three plants are presented -

Polygonum aviculare L (pacha grass), *Polygonum hydropiper* L (water pepper) and *Z. Jujuba* Mill (jujube). General information, phytochemical composition, biological activity and secondary metabolites in extracts of the presented plants, grouped according to their chemical structure, are presented in detail.

218 references are cited, 61% of them are from the last 5 years, 89% from the last 10 years; they are from foreign journals, and authors from the country with publications in international journals are also cited.

The doctoral student is quite well acquainted with the state of the problem and has presented a deep thorough assessment of the analyzed literature sources.

6. Research methodology

A strategy in several stages has been used in the dissertation work: an ethnopharmacological approach in the selection of plants and metabolic profiling of the obtained extracts; screening for anti-adipogenic potential of plant extracts as well as of selected secondary metabolites in human adipocytes and confirming the effect of selected secondary metabolites in vivo in *C. elegans*.

Plant collection and extraction processes are described in detail. An in vitro evaluation of the anti-adipogenic potential in human adipocytes has been performed, with cell culture and treatment, cell viability assessment, and intracellular lipid accumulation assay being presented in detail. An in vivo model of glucose-induced obesity in *C. elegans* has been used.

Modern investigational methods have been used - high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) spectroscopy, in silico docking analysis, polymerase chain reaction in real time (RT-qPCR) of mRNA and microRNAi, Western blot technique. Statistical analysis of the data is performed using SigmaPlot v11.0 from Systat Software GmbH (Erkrath, Germany).

The selected research methodology allows for achieving the main goal and obtaining an adequate answer to the objectives to be solved in the dissertation work.

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

In the present dissertation, data on the phytochemical composition of extracts from leaf mass of *Z. jujuba* (jujube), and aerial parts of *P. hydropiper* (water pepper) and *P. aviculare* (pacha grass) have been obtained by performing metabolic profiling with NMR.

The implementation for the first time in Bulgaria of an in vitro model of obesity in human SGBS adipocytes as a screening platform for evaluating the anti-adipogenic potential by influencing the lipid accumulation (lipogenesis) and degradation (lipolysis) of plant extracts and natural molecules is of a significant contributing nature. All three plant extracts (jujube, water pepper and pacha grass) have been found to suppress the differentiation and accumulation of lipids in fat cells, which supports their potential therapeutic effect in obesity. The most pronounced anti-adipogenic activity and the strongest inhibitory effect on adipogenesis and lipolysis have been observed in the extract of *Z. jujuba*. The experimental platform structured in this way allows for the evaluation of the molecular mechanisms of action of natural molecules. The results of the experiments conducted in the present work provide for the first time data on the mechanism of action of jujube leaf extract in a model of obesity in human adipocytes. Its anti-adipogenic effect has been found to be associated with inhibition of PPAR γ , C/EBP α , adiponectin and PI3K/AKT signaling pathway, while extracts of pacha grass and water pepper only affect PI3K and PPAR γ . Inhibition of the PI3K/AKT signaling pathway has been identified as the underlying molecular mechanism of the effect of jujube extract on adipogenesis. The observed effect on the expression of PPAR γ and C/EBP α indicates the involvement of these transcription factors in the established anti-adipogenic effect.

Screening for the assessment of the anti-adipogenic potential of plant secondary metabolites - apigenin, betulinic, olive and rosmarinic acid has been carried out in the thesis. Among the natural molecules of jujube extract, betulinic acid stands out with the most pronounced anti-adipogenic effect. The obtained results show that betulinic acid has an effect on key signaling pathways, interacting with the PI3K/AKT signaling cascade and modulating PPAR γ in an in vitro model of obesity in human adipocytes.

The molecular pathways involved in the mechanism of anti-adipogenic action of apigenin, betulinic and oleic acid in human adipocytes represent fundamental scientific contribution.

In the current dissertation, for the first time in Bulgaria, an in vivo model for studying the phenotype and lipid accumulation in obesity in nematodes of the species *C. elegans* has been introduced, which represents an in vivo platform for studying metabolic diseases and evaluating the anti-obesogenic potential of molecules of different origins.

Among the investigated pure molecules from jujube extract, betulinic acid has demonstrated an exceptional potency to lower lipid accumulation at the cellular level,

which necessitated conducting an experiment at the organismal level - in a glucose-induced obesity model *C. elegans*, to validate the obtained in vitro results. Betulinic acid has been proven to affect various aspects of lipid metabolism both in the cell model used and at the organismal level, thus representing a natural compound with potential for use in the pharmacotherapeutic approach for the treatment and prevention of obesity. At the highest concentration used, betulinic acid suppressed lipid accumulation to a degree comparable to the results achieved with the reference obesity drug orlistat.

In the final experiment of the present work, the expression of a panel of genes involved in both lipogenesis and lipid hydrolysis and oxidative degradation has been investigated. Betulinic acid has been found to suppress lipid metabolism in vivo in the model organism *C. elegans* by modulating transcription factor *nhr-49*, regulating the processes of lipid synthesis and hydrolysis.

Of serious scientific-applied contribution nature is the proven inhibitory effect of betulinic acid (10 μ M) on lipid depots, associated with stimulation of the expression of *nhr-49* and *acs-2*, while at a concentration of 50 μ M betulinic acid affects genes related to lipid hydrolysis and the action of desaturases in *C. elegans*. Of serious importance is the established effect of betulinic acid on the expression of microRNAs whose target genes are involved in the processes of lipolysis and lipogenesis in *C. elegans*. In summary, betulinic acid appears to regulate insulin-mediated adipocyte differentiation via the PI3K/AKT signaling pathway, the expression of the transcription factor *nhr-49*/PPARs, and microRNAs related to lipid metabolism. The data obtained on the molecular mechanism of action of betulinic acid could serve as a basis for the development of preparations for body weight control and for the prevention of obesity. Betulinic acid emerges as the most promising natural molecule among those investigated in the present dissertation, whose effect is also validated at the organismal level.

The characterization of the effect of rosmarinic acid on adipogenesis and lipolysis in SGBS adipocytes by inhibiting factors key to adipogenesis such as *C/EBP α* , *PPAR γ* and adiponectin, as well as on the expression of inflammatory factors during adipocyte differentiation, is of scientific fundamental contribution, which outlines future possibilities for the inclusion of this plant secondary metabolite in therapeutic approaches in obesity.

8. Evaluation of the publications in relation to the dissertation work

In relation to the topic of the dissertation work, Martina Savova has presented 6 publications in international scientific journals, 4 of which in journals with impact factor

- overall IF 28.068, individual IF 5.06, all in Q1. Martina Savova is the first author in three of the publications, two of which have an impact factor in Q1, which confirms her leading role in the research conducted. 4 of the scientific publications on the topic of Martina Savova's dissertation have 79 citations (Scopus). Different parts of Martina Savova's dissertation have been presented at 6 international scientific events, 2 as reports and 4 as posters, with Martina Savova being the lead author in 5 of these participations. Martina Savova has participated in one national research project funded by the National Research Fund (KP 06-H51/14) and in one international scientific project funded by the European Commission under the "Horizon 2020" program (739582 PlantaSYST).

9. Personal involvement of the doctoral student

The personal involvement of Martina Savova in the conducted dissertation research is evident from the provided documentation. The results obtained and the contributions formulated are her personal merit.

10. Abstract

The abstract of the dissertation of Martina Stoyanova Savova reflects the main results achieved in the dissertation and meets all the generally accepted requirements in the Regulations for the Implementation of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LAD)=

11. Critical remarks and recommendations

I have no critical remarks on the dissertation work.

12. Compliance with the requirements in the Regulations for the conditions and procedures for acquiring scientific degrees and holding academic positions at the Institute of Microbiology "Stefan Angelov" - BAS

Appendix 1. Number of points by indicators - 5. Technical sciences

Groups of metrics	Indicator	Required points	Martina Savova
A	1. Dissertation work for acquiring the educational and scientific degree "Doctor"	50	50
Γ	7. Publication in scientific journals, referenced and indexed in world-renowned databases of scientific information	30	42.3
	8. Scientific publication in non-referenced peer-reviewed journals or in edited collective volumes		5

Appendix 1. Number of points by indicators - 5. Technical sciences

Groups of metrics	Indicator	Required points	Martina Savova
Д	12. Citations or reviews in scientific journals, referenced and indexed in world-renowned databases of scientific information, or in monographs and collective volumes		790
E	18. Participation in a national research or educational project		10
	19. Participation in an international research or educational project		20
Total number		80	917.3

According to Appendix 1, a total of 80 points are required in Indicator Groups A and D, and Martina Savova has submitted a total of 917.3 points.

According to Appendix 2, two publications in impact factor journals, chapters of monographs, proceedings of international forums published in full text, patents are required, and Martina Savova has presented 4 publications in journals with impact factor, all in Q1.

Thus, with the data presented, Martina Savova repeatedly exceeds the minimum national requirements, as well as the requirements in the Regulations for the conditions and procedures for acquiring scientific degrees and holding academic positions at the Institute of Microbiology “Stefan Angelov” – BAS.

CONCLUSION

In conclusion, I do consider that the dissertation work of Martina Stoyanova Savova is complete, modern, in-depth, well-structured, with clear and precisely formulated and fulfilled tasks, with important conclusions and serious scientific-fundamental and scientific-applied results, which represent an original contribution to science in an interesting field – experimental obesity, on which relatively little work has been done in our country.

The dissertation meets all the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LAD), the Regulations for the Implementation of the LAD and the Regulations for the Implementation of the LAD of the Bulgarian Academy of Sciences. The presented materials and dissertation results fully comply with and significantly exceed the specific requirements of the Regulations for the terms and conditions for acquiring scientific degrees and holding academic positions at the Institute of Microbiology “Stefan Angelov” - BAS.

The dissertation shows that the doctoral student Martina Stoyanova Savova possesses in-depth theoretical knowledge and professional skills in the scientific specialty Technology of biologically active substances, demonstrating qualities and skills for independent conduct of scientific research.

In respect to the above, I confidently give my positive assessment of the conducted research, presented by the above-reviewed dissertation work, abstract, achieved results and contributions, and I propose to the members of the esteemed scientific jury to award the educational and scientific degree "doctor" to Martina Stoyanova Savova in field of higher education 5. Technical sciences, professional direction 5.11. Biotechnologies, doctoral program Technology of biologically active substances.

12.08.2023

Sofia

Reviewer:

Prof. Tsvetalina Tankova, MD, PhD, DMedSc