REVIEW

by Assoc. Prof. Petya Asenova Dimitrova, Laboratory "Experimental Immunotherapy" at the Stephan Angeloff Institute of Microbiology, BAS

appointed as a member of the Scientific Jury with Order I-64/29.05.2023 issued by the Director of the Institute of Microbiology, BAS and as a reviewer by the Scientific Jury with Protocol №1/20.06.2023

for evaluation of the thesis for academic and scientific degree "Doctor"(PhD)

PhD student: MPharm Martina Stoyanova Savova
PhD thesis title: MECHANISM OF MODULATION OF OBESITY IN IN VITRO MODEL OF HUMAN ADIPOCYTES THROUGH APPLICATION OF BIOACTIVE COMPOUNDS
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, Martina Savova, is a full-time PhD student at the Metabolomics Laboratory of the Department of Biotechnology, Institute of Microbiology, BAS, supervised by Prof. Milen Georgiev. Martina Savova has submitted for her dissertation work, in paper and in electronic version, 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 ZRASRB and the additional requirements of IMikB, BAS, protocol and preliminary review of pre-defense, dissertation thesis and dissertation summary in Bulgarian and English, list of publications and citations, declaration of originality and curriculum vitae.

2. Significance of the research topic of the PhD thesis

According to the World Health Organization (WHO), obesity is a disease that affects all ages, with data showing that the incidence increases from 4 to 18% among adolescents aged 5-19 years and that about 39 million children under the age of 5 are overweight or obese at 2020. According to the Global Obesity Observatory initiative in Bulgaria, more than 20% of the adult population over the age of 18 is overweight with more men than women suffering from the disease. Approaches to pharmacological therapy in obesity are still limited, due to the complex pathology of the disease, which includes abnormalities in metabolism (redox and catabolite-metabolism), changes in energy stores and cellular respiratory activity, and underlying chronic inflammation. In addition to being overweight, some patients suffer from the condition named metabolic syndrome, described to include the 3 symptoms of increased risk of cardiovascular disease, stroke and type 2 diabetes. Based on this, I believe that the subject of the PhD thesis is important for contemporary research, particularly as it focuses on the need for new

pharmacological treatments for obesity. The choice of the strategy for pharmacological intervention by the use of secondary metabolites from plant extracts, with low molecular mass but potential for selective action on specific signalling pathways and/or on key mechanisms related to the pathophysiology of the disease, also contributes to the relevance of the dissertation. The present number of publications published really serves as a good indicator of the need for research to address the complicated illness. 12 139 publications are found in 2022 when the term "obesity" is entered in PUBMED, compared to 2 488 in 2002.

3. Relevance of the PhD thesis goal and tasks

The purpose of this dissertation is to examine the effects of extracts from *Z. jujuba* (jujube), *P. aviculare* (pacha grass), and *P. hydropiper* (water pepper) and their secondary metabolites on the processes of adipogenesis of human adipocytes and lipid accumulation *in vitro*, as well as to confirm the therapeutic effect of a chosen secondary metabolite with the strongest antiadipogenic action in a *C. elegans* nematode model of obesity.

Seven assignments have been made with the intention of consistently achieving the goal. A figure outlining the steps of the overall experimental strategy is provided with each task, which gives a very good indication of the level of problem knowledge and the sophistication of the scientific methodology.

4. Level of the author's competence

Martina Savova, a PhD student, shows excellent knowledge of the research conducted by other teams in the field and includes 11 literary sources published in 2023, 66 literary sources published in 2021, and 84 literary sources published in 2021 in the review and discussion. The literature review is written competently and succinctly, and it includes 4 figures that highlight significant molecular and cellular mechanisms and relationships important to the study on a *C. elegans* model. Additionally attached are the fourth tables listing the secondary metabolites found in the different plant extracts based on published data. Another indication of the authors' competence with the topic is the subsection Future directions that appears after the Discussion.

5. Research methodology

The PhD candidate employs a range of methodologies, including biotechnological approaches, cell culture and signaling, methods for adipocyte differentiation, activity, and function, methods for analyzing gene expression, and *C. elegans* growth and phenotypic monitoring. The data are repeatable and coherent according the statistical analyses. Martina will undoubtedly find use for the methods she used and benefited from in the future whenever she plans new experimental tasks for the implementation of scientific projects.

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 IMikB, BAS, namely it contains the following chapters:

- Title
- Abbreviations used
- Introduction – 3 pages: with clear outline of the problem

• Literature review – 24 pages, with subsections on adipose tissue, pathophysiology of obesity, modern approaches to prevention and therapy of obesity, experimental models of obesity, treatment and ethnopharmacological remedy for obesity. Martina has used 4 figures and 4 tables to facilitate the content.

• Goals and tasks – 2 pages, with 7 tasks and an illustrative figure

• Materials and methods – 11 pages, with methods used for nuclear magnetic resonance (NMR) spectroscopy, high performance liquid chromatography (HPLC), *in silico* docking analysis, *in vitro* evaluation of anti-adipogenic potential, cell culture, assay for intracellular lipid accumulation (adipogenesis) and glycerol release (adipolysis), real-time polymerase chain reaction (RT-qPCR) for quantification of mRNA and microRNAs, *in vivo* model of hyperglycemic shock-induced obesity in *C. elegans*, induction of lipid accumulation and treatment in *C. elegans*, assessment of viability and phenotypic indicators, staining of accumulated lipids. Tables are also used for a precise description of the primers in RT-PCR method used in the dissertation work.

• Results – 37 pages, 28 figures with several panels each (usually 4-6 panels), and when studying the effect of metabolites on the expression of genes related to metabolism – up to 14 panels. Attached in the results are 2 tables.

• Discussion – 15 pages with 3 figures summarizing the most important mechanisms discovered and described in the dissertation.

• Future directions – 3 pages with the outline of the research significance of the data in the PhD thesis

• Conclusions – 1 page with six conclusions from the experimental work

• Contributions – 1 page with 5 fundamental contributions and 3 scientific-applied contributions in the research field.

7. Contributions and research significance
General contributions to the research field

• The PhD student provides experimental data on the phytochemical profile of *Z. jujuba* leaf extracts, *P. aviculare* and *P. hydropiper* aerial parts. Triterpenes, including betulinic acid, apigenin, and rosmarinic acid are identified in the plant extracts by the author as having the potential to control adipogenesis. These findings are supported by other studies and the literature database.

• The data in the dissertation affirm that the extract of *Z. jujuba* have a strong inhibitory effect on the adipogenesis and lipolysis.

Fundamental contributions to the research field

• The inhibitory effect of rosmarinic acid on two parallel processes - adipogenesis and lipogenesis and on the expression of inflammatory factors during adipocyte differentiation - has been characterized for the first time.

• It has been shown for the first time that the inhibitory effect of a low dose of betulinic acid on lipid depots in *C. elegans* is associated with the stimulation of of *NHR-49* and *ACS-* expression.

• For the first time, it has been shown that the action of a high concentration of betulinic acid affects the expression of genes related to lipid hydrolysis processes in *C. elegans* and the action on desaturases.

• For the first time, the mechanism of action of betulinic acid on microRNAs, whose target genes are involved in the processes of lipolysis in *C. elegans*, has been proven.

Applied contributions to the research field

• An *in vitro* human adipocyte differentiation model with the potential for a screening platform for the anti-adipogenic potential of plant extracts and natural molecules was introduced and optimized.

• A model was introduced and exploited to study the phenotype, lipid accumulation in induction of obesity in *C. elegans* under hyperglycemic conditions. The model is an excellent *in vivo* platform for evaluating the anti-obesogenic potential of natural compound molecules in the model organism *C. elegans*.

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

The doctoral student has **6 publications**, of which she is **the first author in 3** and the three articles are based on the results of the doctoral student's experimental research on the dissertation and two of them have an impact factor **above 7 (Q1).** In the remaining articles, **Martina again contributes to the experimental work and to the discussion** and writing of the articles (as described in the Contribution of the authors section of each article). Martina has over **80 citations** to published articles.

9. Critical remarks and recommendations

My questions to Martina Savova are:

1. Why recent therapies of obesity are not effective and failed?

CONCLUSION

The dissertation 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.

Martina Savova'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** Martina Savova the educational and scientific degree of "Doctor" in the field of 5.11 Biotechnologies (Technology of biologically active substances).

11/08/2023   Reviewer: ....

                                            Assoc. Prof. Petya Dimitrova