REVIEW

by assoc. prof. **Maria Zhelyazkova-Savova**, MD, PhD

Department of pharmacology and clinical pharmacology and therapy at the MU of Varna

Regarding:

The dissertation work of

Elis Rafailova Gasanzadeeva, MD,

a self-study PhD student

at the Department of pharmacology and clinical pharmacology and therapeutics,

Medical University of Varna,

for awarding an educational and scientific degree "Doctor",
in the area of Higher Education 7. Health care and sports,
Professional direction 7.1. Medicine,
Doctoral program "Pharmacology (including Pharmacokinetics and Chemotherapy)"

Topic of the PhD thesis:

PHARMACOLOGICAL EXAMINATION WITH ANETHOLE IN AN EXPERIMENTAL MODEL OF OBESITY

By order No P-109-168/May 22 2024 of the Rector of MU of Varna, and with the decision of the Faculty Council of Medical Faculty at the MU of Varna (protocol No 21/May 16 2024), I was designated as a Member of the Scientific Jury for the above procedure. Herewith, I submit my review on the dissertation work of **Elis Rafailova Gasanzadeeva** entitled **"Pharmacological examination with Anethole in an experimental model of obesity"**.

The documents presented by the applicant are in accordance with the requirements of the regulation for the acquisition educational and scientific degree "Doctor" and the rules of the Medical University of Varna. All presented materials are duly arranged and described.

Education and professional development of the applicant

Elis Rafailova graduated from the High school with foreign language teaching "Nikola Yonkov Vaptsarov" in the town of Shumen in 2012 and then in 2018 from the Medical University of Varna.

After a brief period as a physician in the medical oncology department at the "Virgin Mary" Hospital in Burgas, in 2019 she joined the Department of Pharmacology and Clinical

Pharmacology and Therapeutics at the Medical University of Varna – initially as a part-time, and then as a full-time assistant. She is conducting seminars in pharmacology for medical and dental students and clinical pharmacology for medical students, including those in the English language program. She speaks English and Russian. She works with basic office suite functions and GraphPad Prizm statistical and graphics software.

Relevance of the topic

Obesity is among the most serious modern health problems worldwide. Particularly alarming are the trends of its continuous increase among children and young people. Its role as a risk factor for metabolic-endocrine, cardiovascular, hepatic, neurodegenerative and malignant diseases is well recognized and are considered to be secondary to the development of chronic low-grade inflammation in visceral adipose tissue and through other mechanisms. This determines the importance of measures that can effectively limit or prevent it. Before considering a medication treatment of the problem, which may be associated with serious adverse effects, non-pharmacological approaches have an important role, especially for prevention purposes. In this regard, along with increased physical activity and caloric restriction, a healthy diet encouraging the consumption of plant products has a proven beneficial effect. Phytopreparations, with their valuable qualities such as anti-inflammatory, antioxidant and other beneficial properties based on the biologically active substances contained in them, are increasingly popular. More and more natural plant-based products are undergoing extensive scientific research to prove their health benefits.

Anethole, as a component of essential oils contained in widely used spices and drinks in our national cuisine, is also of medical interest. The present work aims at elucidation the potential effects of this monoterpene in the conditions of an experimental model of obesity in rats.

Review of the dissertation and analysis of results

Dissertation structure

The dissertation is written on 157 pages. It is built according to the standards adopted in our country and contains the following sections: Introduction – 2 pages, Literature review – 33 pages, Aim and objectives – 2 pages, Material and methods – 9 pages, Own results with discussion – 72 pages, Conclusions – 4 pages, Contributions – 1 page, List of publications and participations – 4 pages, Bibliography (309 sources in English, 2 in Bulgarian) – 22 pages. The work is well balanced structurally. Summary discussion as an essential part of the exposition is not presented.

Knowing the literature

The literature review is extensive and well structured. It reveals a good awareness of the author on the problems of interest. Up-to-date contemporary data is included: over 30% of the cited literature is from the last 5 years, and more than 50% is from the last 10 years.

The first part examines obesity as a medical problem: the definition and epidemiology, risk factors, and associated socially significant diseases are discussed. The current drug treatment possibilities, as well as non-pharmacological measures, are discussed. Surgical methods are also described, in unnecessary detail.

The following part is dedicated to a list of natural products containing monoterpenes and essential oils or the active principles themselves, accompanied by brief quotations of their beneficial metabolic effects in various aspects. This part could be presented in a more comprehensive way by providing an overview of these compounds, summarizing the possible mechanisms of action and illustrating them with specific examples to achieve an overall systematic view.

The next section of the literature review is devoted to the characterization of anethole from a physicochemical, pharmacokinetic and pharmacodynamic point of view. Effects of anethole known thus far on various organs and systems established in experimental settings *in vivo* or *in vitro*, and revealing its organoprotective potential, are presented. Again, a more unified approach would be appropriate in summarizing this information.

Finally, the literature review focuses on the different experimental models of obesity and the methods for their induction.

Then the aim and tasks of the dissertation are formulated. The objective is set to investigate and generalize the pharmacological effects of anethole administered orally at increasing doses in rats in a high-calorie diet-induced obesity model. The tasks are divided into two groups: (1) Verifying the obesity in the experimental model, and (2) Investigating the effects of anethole in the experimental model induced. The interest is focused on multiple parameters: physical – such as body weight and food, fluid, and calorie intake; behavioral changes; biochemical measures; indicators of oxidative stress and antioxidant protection; tissue indices; histological analysis of organs; immunohistochemical markers of apoptosis and inflammation; carrageenan-induced acute inflammation.

Methods used and design of the experiments

The diversity of the assigned tasks also implies methodological diversity. Indeed, the methods used are adequately selected. While in the case of the routine pharmacological methods, such as the study of physiological, biochemical, clinical-laboratory and behavioral indicators, their informative value is *a priori* clear, but also specifically indicated, in tests such as immunohistochemical determination of indicators of inflammation and, especially, apoptosis, which give additional weight to the investigation, it is necessary to point out purposefully what the working hypothesis is and why they are used. This would help when discussing the results.

For the study, 50 male Wistar white rats were used, divided into 5 groups – control, obesity (through a high-calorie diet, HCD), three groups with obesity and treatment. Anethole has been administered daily intra-gastrically, in three different doses (62.5, 125 and 250 mg/kg), dissolved in sunflower oil.

All the procedures have been performed in accordance with the European directive 2010/63/EU and with the allowance issued by the Bulgarian agency of food safety (No 177/07.07.2017).

The experiment is chronic and labor-intensive, lasting 10 weeks. Food and fluid intake has been measured every 3 days, which is a bit disconcerting given the limited shelf life of lard added to food. Body weight has been monitored weekly.

Statistical processing of the results was performed with one-factor and two-factor analysis of variance with Dunnett's post-test and linear post-test, as well as Student's t-test. For the latter, however, it is not clear when it has been used, as this has not been marked.

Analysis and evaluation of results

Own results are presented sequentially and according to the set tasks. They are visualized by 38 figures and 27 tables. Each part includes the results of the experimental setup, presented in text and illustrated in tables and figures, along with a discussion.

In my opinion, the results of the dissertation work are interesting, worthy of attention and thought-provoking. The following beneficial effects of anethole, demonstrated in the study, stand out:

- Weight loss at the highest dose of anethole (paradoxically, given the higher food and caloric intake compared to the HCD group)
- Improved spatial memory; tendency to antidepressant and anti-anxiety action
- Tendency to improve glucose tolerance at the highest dose
- Tendency to improve antioxidant defense (increased SOD in serum and decreased TBARS in brain homogenate)
- Adipose tissue: decrease in adipose tissue index from the highest dose (indicator of obesity reversal); histologically verified dose-dependent reduction in adipocyte size; marked apoptosis of adipose tissue (BAX/Bcl-2 strongly increased at the highest dose, but also in HCD?)
- Liver: increased pro-inflammatory marker MAS387 with HCD and a decrease from the small dose of anethole; BAX/Bcl-2 strongly increased at the highest dose (both vs. control and HCD – interpretation?)

At the same time, some of the parameters studied did not change or demonstrated the potential for deterioration compared to the HCD (increased food consumption and caloric intake; acute increase in blood sugar at the 30th minute of glucose loading from the medium dose of anethole; increase in TBARS in serum from the small dose (relative to control); increase in liver index at the middle dose; increase in ALT at the middle dose). The author usually correctly notes these results, but the evaluative analysis could be more prevalent.

Dr. Elis Rafailova has done a good job in discussing the results section by section, as far as including sufficient literature relevant to each issue. Still, there remain questions that really

don't have an easy answer. This is, for example, the case with the dose-dependently increased food and calorie consumption relative to the HCD group of treated animals and the paradoxically reduced body weight at the highest dose of anethole. Against the background of the many cited studies with divergent data, an attempt could be made to comment on these really difficult-to-analyze results.

The results from the study of the pro/anti-apoptotic factors BAX and BCL-2 and their ratio in adipose and liver tissue are also interesting. I welcome the idea of including these relatively innovative markers in the dissertation. It is here that it would be good to know what the author's expectations are and what his working hypothesis is when choosing these parameters. For example, in the adipose tissue, the significantly increased BAX/BCL-2 ratio is interpreted as a potentially useful opportunity to reduce body weight in obesity, but the fact that in the HCD group itself the deviation of this indicator is in the same direction is not commented on. On the other hand, the analogous increase of BAX/BCL-2 in the liver lacks comment or evaluation.

Discussion of the results by sections allows the author to focus on the corresponding part of the work. Thus, the ability of the PhD student to consider the specific results in the context of the already existing information and to search and find in the literature data for comparison, as well as for a possible explanation of potential mechanisms that may underlie the results in his work is manifested. At the same time, many of the investigated parameters are interrelated and inevitably, the discussion of the results of one of them rests on another, which, however, is commented on in a separate section. For example, the neuroprotection, which in this case is manifested by a promnestic effect (one of the indicators studied), can be due to the antioxidant action (another indicator studied). Here is the role of the general discussion, which will allow to integrate the entire work and summarize the results (the intended purpose of the work has been not only to conduct the research, but also to ***generalize*** the pharmacological effects of anethole). Such a summary analysis, unfortunately, is not presented.

I believe that an important aspect of the quality of a scientific study is the correct interpretation of statistical data. In my opinion, the work will not lose by commenting on the results as they are, regardless of whether they are in harmony with or contradict the main idea and the author's expectations. Trends that are established in one direction or another by statistically non-significant results can also contribute to the characterization of a product, especially when considered in an appropriate context.

Analysis and assessment of conclusions and contributions

Two main conclusions are drawn, following the formulated tasks. The first one documents the verification of the experimental model of obesity, and the second duly summarizes the effects of anethole in relation to the investigated indicators. I accept the conclusions with a caveat regarding the categorical statements concerning the statistically non-significant results. It is also my belief that the conclusions should not be presented in a so fragmented

and factual manner, but more compact and summarized, giving them a more evaluative character, which could compensate for the absence of a final discussion.

The contributions are well organized and presented in six points. I agree with the original nature of the results.

Evaluation of publication activity

In connection with her dissertation, Dr. Elise Rafialova has published five full-text articles. She is the lead author on all publications. 14 participations in scientific events at home and abroad are also presented. Dr. Elise Rafailova also has three awards from scientific conferences, two of which are from ECNP congresses (the 35th in Vienna 2022 and the 36th in Barcelona 2023).

Notes, omissions, recommendations

I would like to make it clear that the criticisms and recommendations I address to the author are principled and aim to improve her work in the future. The critical remarks in no way detract from my overall positive impression of the work. The most important of these were mentioned above. In addition, I would also note:

- In the section "Own results and discussion" I think it would be better if at the very beginning, after presenting the results for body weight, food and fluid consumption and caloric intake, the results of the adipose tissue examination should be discussed next, because it is through the accumulation of visceral adipose tissue that the model of obesity is verified, and accordingly the effect of anethole on this indicator is of the greatest importance and makes sense of the rest of the experimental setups.
- Duplicates are found among the literature sources (at least eight references are repeated), while some mentioned in the text are missing from the bibliography list (e.g., Tappy et al, 2010, p. 118)
- The list of abbreviations presented at the beginning is not exhaustive; many abbreviations are introduced repeatedly in the text, while others are not explained.
- Editing of the text is needed in places to improve the style and linguistics.

Evaluation of the abstract

The abstract, written on 78 pages, meets the requirements of the regulatory framework and correctly reflects the content of the dissertation work.

Personal impressions of the candidate

I have known Dr. Elise Rafailova since she joined the Department of Pharmacology at MU-Varna. She shows zeal and diligence both in teaching and in experimental work. Warm-hearted and smiling, she enjoys a good attitude from the Department team and students.

In conclusion, I believe that Dr. Elis Rafailova's dissertation represents an interesting experimental work with original scientific and applied contributions. The PhD student shows

a good knowledge of the researched issues, as well as the ability to independently conduct an experimental study. The scientific indicators satisfy the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria and the Regulations for the Development of the Academic Staff at the MU-Varna for awarding the scientific and educational degree "DOCTOR". With these arguments in mind, I am giving my positive vote for awarding the scientific and educational degree "DOCTOR" to Dr. Elis Rafailova Gasanzadeeva.

Varna
22. 06. 2024

Заличено на основание чл. 5, §1, б. „В“ от Регламент (ЕС) 2016/679

Reviewer:

/ Assoc. prof. Dr. Maria D. Zhelyazkova-Savova, MD/