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Evaluation of the doctoral thesis entitled: "*Effects and underlying mechanism of plant secondary metabolites on insulin secretion regulation*" submitted for the degree of doctor of medical and health sciences in the discipline of pharmaceutical sciences. The thesis was carried out within the Department of Pharmaceutical Biology, Faculty of Pharmacy of the Medical University of Warsaw under the supervision of Prof. Anna Karolina Kiss and the Cellular Pharmacology team (F13) of the Institut des Biomolecules Max Mousseron, Université de Montpellier under the supervision of Prof. Catherine Oiry-Cuq.

In recent decades, Type 2 Diabetes Mellitus (T2DM) has become a global health crisis, marked by chronic high blood sugar and insulin sensitivity issues. The current pharmaceutical treatments have limitations and side effects, prompting the exploration of plant secondary metabolites as potential therapies. These plant compounds offer a unique approach that combines natural product chemistry and pharmacology to manage T2DM and an example which should be mentioned here is the plant with the most notable success in antidiabetic therapy - *Galega officinalis* L., and its metabolites. It was historically used in traditional medicine for various ailments, including what we now associate with T2DM symptoms. Metformin, a synthetic derivative of one of major compound, is now a widely prescribed antidiabetic drug due to its glucose-lowering efficacy with minimal side effects.

The hypoglycemic effects of plant extracts and their metabolites can result from various mechanisms, including, among others, enhancement of β-cell function, a crucial element in regulating glucose levels. In this thesis, the PhD student focused on searching secondary plant metabolites that can potentially protect β-cell function and induce glucose-dependent insulin secretion. To obtain his aim, Mr. Patyra first tested series of plant extracts containing lignans, flavonoids and coumarins to select extracts for further isolation of bioactive molecules. Then pure plant secondary metabolites were characterized and screened on a β-cell model to assess their effects on insulin secretion. The mechanism of action of these active compounds was further elucidated by studying their effects on key stages in insulin secretion, such as cell electrical activity, intracellular calcium levels, and ion channel currents. Additionally, some preliminary structure-activity relationship studies were carried out.

The experiments presented in the thesis were performed under financial support from:

- National Science Centre, Poland, PRELUDIUM 20 project 2021/41/N/NZ7/00313, entitled: "Wood from pine family (Pinaceae) native to Poland as a potent source of pharmacologically active lignans"; Principal Investigator: Andrzej Patyra.
- The Ministry of Education and Science, Poland, Studenckie Koła Naukowe tworzą innowacje project SKN/SP/533710/2022, entitled: "Searching for new innovative drugs among compounds of natural origin"; Project Manager: Sebastian Granica; Project Executive: Andrzej Patyra.

- French Government scholarship for doctorate degree training in Institute des Biomolecules Max Mousseron, Université de Montpellier, France, granted to Andrzej Patyra by Campus France, No 132828P.
- Two short research visits were funded by the Polish National Agency For Academic Exchange, Poland, through the PROM program "International scholarship exchange of PhD candidates and academic staff" and the STER program "Internationalization of Doctoral Schools

The thesis is presented on 209 pages. The work is supplemented by tables (11), figures (57) and the list of abbreviations. The thesis starts with a short introduction, followed by state of the art, summarizing existing data on the topic. The pancreatic β-cell, anatomy, histology, physiology, and mechanisms on glucose-induced insulin secretion are discussed. There is a detailed description of T2DM including available treatments and the role of natural products with known for their modulation of insulin secretion, including flavonoids, lignans, coumarins. Overall, this theoretical part is consistent and informative, what makes it an excellent introduction to the subject of the work.

In his research Mr. Patyra used various research techniques, both analytical and a wide range of biological tests, which are briefly described in section *Material and Methods*. The results are carefully presented and illustrated on the next 65 pages. The phytochemical characterisation of lignans- and coumarin-rich extracts, as well as step-by-step isolation of single molecules were described in detail. The results on the screening of molecules able to affect insulin secretion and their influence on β-cells (toxicity and protection) are shown, together with an attempt to unravel possible of mechanism of action for those indicated as most active, are presented. The discussion of the obtained results is conducted in a mature and informative manner, with the necessary reference to the latest literature.

The thesis is structured around three already published manuscripts

1. Patyra A, Dudek MK, Kiss AK. LC-DAD–ESI-MS/MS and NMR Analysis of Conifer Wood Specialized Metabolites. Cells 2022, 11(20), 3332. IF=6.0; MEiN=140.

2. Patyra A, Kołtun-Jasion M, Jakubiak O, Kiss AK. Extraction Techniques and Analytical Methods for Isolation and Characterization of Lignans. Plants 2022, 11(17), 2323. IF=4.5; MEiN=70.

3. Patyra A, Kołakowski M, Dudek MK, Kiss AK. Isolation of trachelogenin 4-O-b-D-glucoside from the fruits of *Carthamus tinctorius* L. Prospects in Pharmaceutical Sciences 2022, 20(2), 24-30. IF=0.1; MEiN=20.

One is in review and 4 other are ready to be submitted. PhD candidate presented results on international conferences both as oral presentations as well as posters. Additionally, Mr. Patyra was awarded for the presented topic several times.

After conducting a large amount of experiments, the Author isolated 17 lignans and 23 coumarins which, together with supplied flavonoids, he tested, which allowed him to formulate the most important conclusions:

- Flavonoids' impact on pancreatic β-cell function depends on the presence of a double bond between C2 and C3,
- Studied lignans do not influence insulin secretion or protect pancreatic β-cells from dysfunction or cell death,

- Coumarins that have an isoprenyl sidechain have been observed to influence the function of pancreatic β-cells.

The findings reported in the papers provide valuable insights into the effects of flavonoids, lignans, and coumarins on pancreatic β-cell function and may have implications for potential therapeutic applications in the context of diabetes and insulin regulation. The PhD candidate has made a valuable contribution by elucidating a structure-activity relationship associated with the effects of coumarins on this process. Notably, it has been observed that imperatorin and isoimperatorin, along with structurally related coumarins, exhibit the potential to amplify glucose-mediated insulin secretion. This enhancement involves a multitude of still inadequately understood interactions with various ion channels. If this effect can be replicated in vivo, it holds the promise of clinical translation. One intriguing avenue for investigation arises from the existence of furanocoumarin-containing phytomedicines, which could be directly tested in human subjects using a blood glucose tolerance test. Given the PhD candidate's suggestion that certain natural product scaffolds, especially isoimperatorin, might serve as lead structures for the development of drugs targeting insulin resistance, it is imperative to delve into the pharmacokinetics of these molecules. This aspect was somewhat neglected in the discussion.

One notable concern is the substantial disparity between the very high concentrations (20 microM) of natural products used *in vitro* and the concentrations typically encountered *in vivo* following the ingestion of these compounds. This incongruity warrants a thorough and critical discussion. It is essential to consider whether the PhD candidate has considered this aspect comprehensively.

Regarding the primary assay and its characterization:

- The utilization of rat insulinoma INS-1 cells for investigating insulin secretory mechanisms is a well-established approach. Notably, a subset of INS-1 cells exhibits a bi-hormonal profile, co-expressing insulin and potentially proglucagon proteins. To ensure robust glucose responsiveness and reliable induction of insulin secretion, it is crucial to compare insulin secretion at different seeding densities and time points. One notable limitation in the PhD candidate's approach is the absence of known activators and inhibitors of glucose-stimulated insulin secretion in the assay. These modulators, such as glibenclamide or other KATP channel blockers, are crucial for assessing the kinetics of the assay and provide a vital benchmark for validating the results.

Some specific questions:

- Flavonoids and lignans were tested at a fixed concentration of 20 µmol/L on insulin secretion in response to glucose stimulation (8.3 mmol/L glucose) in INS-1 cells. A notable question arises regarding the range of concentrations tested; why were lower concentrations not included in the study? It would be valuable to explore the dose-dependency of the observed inhibition and its potential correlation with cytotoxic effects. In some cases, the effects of compounds can exhibit a biphasic nature, where lower concentrations have specific effects, while higher concentrations yield non-specific outcomes. This aspect merits further investigation to gain a more comprehensive understanding of the compounds' impact on insulin secretion.
- The PhD candidate assumes that insulin releasing activity of coumarins is associated with or even determined by an isoprenyl sidechain attached to the coumarin – no matter whether it is a simple or furano ring. *"Neither the position of substitution (both C5 and C8) nor its character (C-prenylation and O-prenylation) seem to be important for this activity. However, any substitution or prolongation of the isoprenyl sidechain leads to loss of function"*. Thus, the isoprenyl chain was identified as crucial also for other coumarins'activity. However, the

importance of the prenylation of natural products in general, and coumarins in particular for this effect was not discussed. I would appreciate the discussion during defense.

- Why different extracts were used for coumarins isolation (n-hexane) and biological studies (60% ethanolic extract)?
- The PhD student reports that all isolated compounds were of purity higher than 95%, which was deemed sufficient for pharmacological studies, however there is no information how the purity was checked.

As a summary I want to highlight that the above comments and questions are just feedbacks for potential further improvement, but they do not influence the final assessment of the thesis. What was presented is obviously a significant contribution to knowledge and understanding of the field. Mr. Patyra performed a significant amount of work, wrote a comprehensive thesis and has a very good understanding of the context of the work.

To sum up, I state that the doctoral dissertation of Mr. Andrzej Patyra meets the formal and substantive requirements specified in the Act on Higher Education and Science. In connection with the above, I recommend to the Board of the Discipline of Pharmaceutical Sciences of the Warsaw Medical University to accept this dissertation and admit the PhD student to further stages of the procedure for awarding a doctoral degree in the field of medical and health sciences in the discipline of pharmaceutical sciences.

At the same time, given the high value of the results obtained, I am requesting that the doctoral dissertation be considered for an appropriate award.

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