UNIWERSYTET MEDYCZNY
IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

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by Mr. ANDRZEJ PATYRA

Dissertation title

WPŁYW WTÓRNYCH METABOLITÓW ROŚLINNYCH NA REGULACJĘ WYDZIELANIA INSULINY I MECHANIZM LEŻĄCY U JEJ PODSTAW (Polish)

EFFETS ET MÉCANISME D'ACTION DE MÉTABOLITES SECONDAIRES DES PLANTES SUR LA RÉGULATION DE LA SÉCRÉTION D'INSULINE. (French)

prepared under direction of Prof. Dr. Anna K. Kiss and Prof. Dr. Catherine Oiry-Cuq

This evaluation has been completed on request of le Président de l'Université de Montpellier and the President of the Board of Pharmaceutical Sciences at the Warsaw Medical University

1. Formal evaluation

The submitted thesis in form of the printout from the PDF file has been written in English with French and Polish title pages as well as English, French and Polish summary.
The experimental part of the study has been performed at the Department of Pharmaceutical Biology, Warsaw and Institut des Biomolécules Max Mousseron, Montpellier. The dissertation consists of following parts: Acknowledgements, Funding sources, Table of Contents, List of figures, tables and abbreviations, Introduction, State-of-the art, Experimental, Conclusions, Scientific dissemination, References and Abstracts. The volume of the printout is 207 standard pages. The subject matter of the dissertation fulfills the requirements of a doctor degree in both: "Nauki Medyczne in Nauki o Zdrowiu w dyscyplinie Nauki Farmaceutyczne – Scientific field – Health and Medical Sciences in the discipline of Pharmaceutical Sciences" and "Biologie Santé – Health Biology."

2. Relevance of the subject matter to the state-of-the art

Plant-derived natural products have a strong position in pharmacy both in form of isolated native structures and as lead compounds modified for improved pharmacokinetic properties. Various estimates exist, but no less than 1/3 of clinically used drugs trace their origin to natural sources and plants are the most versatile. Mostly, the pharmacologically active compounds are products of so called 'specialized metabolism' which provides plants with adaptive tools to combat various environmental challenges. It has been and will be in future a major rationale behind bioprospecting. However, the enormous diversity of structures with concurrent similarities of physicochemical properties makes the full characterization of this diversity very challenging. Even the powerful recent techniques are not good enough to identify and quantify all constituents without prior isolation and purification. And the latter task, although made easier by recent technology advance, still requires tedious work and a lot of skill to obtain sound and reliable results. Therefore, every project aimed at enriching our knowledge of phytochemical diversity and pharmacological properties of the natural products is valid. Especially, if there is a knowledge gap in understanding the mechanisms of action within a certain important direction. In Mr. Patyra's thesis, the focus was on diabetes management in form of studying influence on insulin secretion and pancreatic β-cells viability by preselected isolated derivatives of phenylpropanoid metabolism, viz. flavonoids, lignans, and coumarins.
These compounds are highly relevant for pharmaceutical sciences as many of them are the active principle in officinal and folk herbal medicines. Therefore, the continuing search for novel activities even in already known structures is timely and of interest for natural drug discovery.

The topic of this thesis is also a logical and well-fit part of the years long expertise of both scientific mentors of this dissertation involving both natural product chemistry and analysis, anti-inflammatory and lifestyle disease-preventive and therapeutic properties.

In pursue of adding to the role of natural products from traditional and alternative medicinal plants, Mr. Patyra chose three classes of compounds from carefully selected sources. firstly, the flavonoids previously known to modulate insulin secretion were collected mainly from commercial vendors to provide an assortment of structural diversity. In this way, a contribution to the structure-activity relationships was envisaged. On the other hand, the hypothesis that two other classes, i.e. lignans and coumarins may prove active in this respect, required a good reconnaissance among various plant sources. They were for lignans: the branch wood of several conifers, and Traditional Chinese Medicine herbal drugs *Arctii fructus*, *Carthami fructus* and *Eleuterococci radix*. These, apparently remotely related taxons are, however, recognized for high content and diversity of lignans. The other group- coumarins were screened and obtained in a couple of Angelica species – typically known for high content of these compounds with some traditional indications in diabetes management.

The Author has decided to follow a rather uncanonical, yet efficient way to achieve his goals. First, he performed a comprehensive phytochemical characterization of extracts from the plant substances – wood, fruits and roots – using LC-MS/MS (ion trap) technique. Only then, a target compounds for isolation were chosen based not only on their potential activity or potential high yield but also to maximize the structural diversity. This way, a library of isolated compounds was to be created for using in pharmacological studies. The strength of this research is not in discovery of new natural products or finding an extraordinarily powerful molecule but in the comprehensive screening towards a well defined and somehow understudied activity of modulating insulin secretion by the β-cells *in vitro*. This is a convincing rationale, because there are so many studies, including *in vivo* where a final effect of lowering blood glucose in animals complemented with many molecular mechanisms performed on standardized (or not) extracts or single compounds, while there is not enough data on well-defined specific pharmacological target. The physiological background has been extensively reviewed by the Author in the "State of the Art" chapter, including a nicely and concisely explained details of pancreatic secretion, glucose homeostasis and role of ion channels. Further, the pathophysiology and management of type 2 DM has been depicted, using also a set of color diagrams and drawings.

In the second part of this chapter, Mr. Patyra attempts to convince the reader about the right choice he'd made on the plant sources of tested compounds. This has been also successful, despite a risk of mixing a rather diverse species in one thesis. The use of conifer wood, especially the sawmills or wood industry by-products has been postulated since the basal lignans have been isolated thereof – such as pinoresinol, lariciresinol and many derivatives. However, the antidiabetic direction has not been explored enough. Also, the use of Arctium and Carthamus fruits is based on their history of traditional use and high content of other kinds of lignans. Only choice of *E. senticosus* may rise some doubts as it was based on scarce previous data, such as a single study on an *in vivo* activity of a rare syringaresinol derivative. Nonetheless, it was worth trying and the comprehensive phytochemical profiling provided some new data on this quite well-studied adaptogenic plant.

Last but not least, the genus Angelica has been selected as an abundant source of coumarins and not as traditional antidiabetic agents. On the contrary, only a few examples of plants from this genus have been shown to have some antidiabetic properties, including insulin secretion stimulation by extract of *A. japonica* (not studied here) and *A. dahurica*. Notwithstanding, the introduction of less common activities may bring about extended scope of indications, even for such an established pharmacopoeial herb as *Archangelicae radix*. The other species were typically used in the TCM but with varied indications and with varied composition of bioactive phytochemicals. The aim of the thesis has been placed in the Experimental part and is quite clear. However, its introductory paragraphs largely repeat what has been previously written in the "State of the art" trying to justify the subject matter again – in my opinion it is unnecessary and could be skipped from the section 4 and written more explicitly in the previous chapter – which is actually lacking some sort of summing up the state of the art and is somehow abruptly finished.

3. Scientific content and contribution to the advancement of research area

Firstly, I would like to assess the methodology used in this thesis. Generally, iit hardly could have been better. In the phytochemical part, a classical approach to extraction, separation, analysis and isolation of target compounds was used. The solvent was basically methanol which is a reasonable choice. Further, a set of classical liquid-liquid extraction was performed to separate compounds of different polarities. The gravitationally eluted columns filled with three different beds were used to obtain selectively enriched fractions to be finally purified using preparative HPLC. The phytochemical analysis was performed using LC-MS/MS based on ion trap, so there was no high resolution mass spectra – this is a drawback in this study as some important information was missed that could facilitate identification. On the other hand, the material was mostly well-known and there were reference data in the literature and many commercial standards were available (not all were used, though).

Here, one important comment regarding taxonomy: the binomial *Larix polonica* Rac. is now recognized only at the level of variety of European larch and there is no substantiated reason to consider it a species, even if it is so in some local sources, perhaps for patriotic motivation. The current systematic should have been consulted with relevant acknowledged sources, such as POWO database of the Kew gardens. Therefore, it should have been only regarded as two accessions of one species – which infraspecific taxon was the other *Larix decidua* has not been disclosed in the thesis. If it was of Polish origin, one can assume either a typical or Carpathian variety. However, this small issue does not negatively impact the value of the results and even suggests a topic for further research into the chemophenetics of European larch populations. Also, for sake of accuracy, infraspecific taxa of other plants should be specified when applicable, before publication of the phytochemical results.

Another small issue was noticed in the HPLC gradient where the isocratic 30% B elution between 30-35 minutes is missing. I would also argue against calling the developed TLC plate "a chromatogram", like in the section 5.4. "the chromatogram was treated with ...". I'd rather say that chromatogram is what we see upon treatment. It is of course a matter of custom and not a crucial problem.

A more important comment is about one peak in the *C. tinctorius* extract, namely m/z 595 (tR 15.2) which is defined as $[M-H]^+$, which is definitely not. I suppose it was a formate adduct based on the neutral loss, since the m/z of the above would be 549!

The preparative part proved the Authors capability to successfully use both classical and instrumental methods and confirms his competence in phytochemical research. However, there are some comments to this part. First, some more details would be useful, such as volumes of the solvents used in fractioning (including liquid-liquid) or information on water fraction drying method. Also, the *Arctii fructus* fractioning seems to be somehow imperfect. The main compound (>20% of extract mass) arctiin was both in water and ethyl acetate subextracts in ca. equal mass. Maybe the EA extraction was incomplete? Alternatively, this step could be skipped and direct CC performed if arctiin was the major target.

The pharmacological approach and cell model was chosen very reasonably, starting with the INS-1 cells stimulated with glucose in various concentrations and detection of insulin using immunofluorescence (FRET). Further pharmacological approach included a use for channel blockers to reveal specific targets. The cell viability was assayed with a common MTT test. A logical development of this general screening was to seek potential mechanisms of action and the most adequate choice was made , i.e calcium channels activity studied with intracellular calcium indicator and whole-cell patch clamp, both being insightful and state-of-the-art techniques.
One question arises when reading the method of cytoplasmic calcium fluorescence microscopy – how exactly were the compounds added (and dissolved) – one could assume that they were in the extracellular medium but it is not fully clear. also, the time course of the experiment can be deduced from the results but has not been explained in the section 5.17. It seems that the treatments were on the same coverslip (same cells) one after another , i.e KCl, and compounds concentrations, including blockers. Here, in figure 45 A it looks like it was from the highest concentration of imperatorin in decreasing order while isoimperatorin was otherwise. Was it indeed so? Also in this section, I would like to ask why psoralen was used as an apparent inactive compound most closely resembling their (imperatorins) structure, while xanthotoxin as a methoxy-derivative would be even closer? It is, however, only a minor comment as both psoralen and xanthotoxin were only inactive counterparts.
Regardless the above comments, the pharmacological part is novel and provides interesting explanations about the potential mechanisms of action of those two coumarins. It is even more sound if we recall that tens of other compounds, including almost all lignans, were rather weakly or completely inactive.

The Discussion part is insightful and considers various pharmacological aspects of insulin secretion regulation within the complex cell signaling context. However, the opening paragraph also repeats the ideas already put out in the Introduction and "State of the Art" chapters. Then, the specific parts of the experiments were discussed, such as structural requirements of flavonoids (double 2-3 bond and inconclusive other substitution patterns. Next, the phytochemical considerations about the lignans and coumarins follow, emphasizing the differences to the previous results on the same species. Here, it is worth mentioning that some of the conifers, including Polish larch variety and some pines have not been studied in such a way before. Also, the annotation of compounds from *C. tinctorius* are novel. However, the presence of not so common serotonin-hydroxycinnamic conjugates could have been given more attention, even if not a main topic of this thesis. The preparative protocols developed during this part of the study shall be also useful for obtaining compounds for other pharmacological experiments where these lignans would be tested.
During discussion on the ion channels role in insulin secretion modulation by coumarins, the Author has proven his competence by coming to several conclusions/suggestions for further directions of research. In particular, the importance of deepening the insight into the cellular mechanisms using a variety of complementary techniques, usually not available in one place is worth noting.

4. Technical quality

The entire thesis is technically also well-prepared with high-quality and easy to read illustrations and quite correct English writing. In the printed dissertation, a moderate number of typographic and technical errors occur. For example, the References format is not completely uniform (and it should be) - some journals titles are abbreviated, some aren't, some are lacking sufficient bibliographic details or URLs (for web resources). The references requiring the Author's attention for formatting are: 15, 43, 53, 59, 75, 84, 90, 97, 99, 139-141, 148, 160, 162, 164-166, 183, 208, 214, 225, 226, 228, 244, 249, 250, 261, 262, 264-266, 288-290, 301, 306, 323, 329, 356, 366, 370, 372, 376, 390, 397, 398, 399, 407, 415.
Beside this, the thesis is well written, with the number of typographic and technical shortcomings kept to the minimum.

5. Final conclusion

The submitted thesis presents a substantial contribution to the area of pharmacognosy and natural product pharmacology. The results significantly extend the knowledge of the composition of specialized metabolites in the studied plant species. The thorough literature review provided in this dissertation is also of high value.

As such, the dissertation meets all requirements for the academic title and proves Mr. Andrzej Patyra's ability to conduct independent experimental scientific research, carefully interpret and discuss the complex results and provide comprehensive review on a state-in-the art in pharmaceutical sciences and health biology.

Hereby, I recommend this dissertation to be accepted by the Board of Pharmaceutical Sciences (Rada Dyscypliny Nauk Farmaceutycznych) at Warsaw Medical University and École doctorale Sciences Chimiques et Biologiques pour la Santé de l'Université de Montpellier and admit Mr. Andrzej Patyra to the defense of the degree of Doctor of Medical and Health Sciences in the discipline of Pharmaceutical Sciences according to the respective regulations, including a Polish Act on Higher Education and Science.

Finally, I also call for distinguishing the dissertation with *cum laude* distinctions (called "wyróżnienie rozprawy doktorskiej") in accordance with Polish law and Warsaw Medical University bylaws.

at Wroclaw, 11/11/2023

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