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**Re: Evaluation of the doctoral thesis by Ms. Aleksandra Maria Juszcak**

This evaluation reports has been prepared as a reply to the letter of Prof. Wojciech Miltyk, the Dean of the College of Pharmaceutical Sciences, which followed the decision of the Senate of the Medical University of Białystok (204/2022 of 26.05.2022).

**General information**

The basis for the application of Ms. Aleksandra Maria Juszcak for the doctoral degree is the dissertation entitled "Phytochemical analysis and biological activities of *Jasione montana* L. (Campanulaceae)". The supervisors of the thesis are: Prof. Dr. Michał Tomczyk from the Department of Pharmacognosy, Medical University of Białystok, Poland, and Prof. Dr. Marijana Zovko Končić from the Department of Pharmacognosy, University of Zagreb, Croatia. The thesis is based on a series of four published manuscripts in well respected journals, between 2019 – 2022.

The dissertation is structured in 12 chapters, starting with the publication list comprising the doctoral dissertation. All four papers are published in international journals from JCR list (indexed by Clarivate Analytics), two are original research articles and two are review papers. One review paper is published in the renowned journal Medicinal Research Reviews having an impact factor of 12.944. The total Impact Factor of the presented articles is 29.556, and the total number of MES scores is 480 points. In all papers the candidate is the first author, and her leading contribution is stated in separate documents (chapter 10), and confirmed by the Supervisor(s). Also, the statements of contribution from all co-authors are provided (chapter 11) and are in line with the first author's statements.

Following the publications, the thesis includes chapters like introduction (a review of the literature on the subject of the dissertation), the objective of the work, the achievement of scientific objectives, conclusions, references and a summary in English as well as in Polish. Finally, the publications comprising the doctoral dissertation are presented, and a list of scientific achievements of the candidate.

The dissertation is well organized, written in a clear and logical style, with a good level of English, and easy to be read.



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### Assessment of the doctoral thesis

The scientific aim of the doctoral dissertation was the detailed characterization of the chemical composition and of the aerial parts of *Jasione montana* L. (Campanulaceae), including the preparation of selected extracts and fractions, attempting to isolate the biologically active compounds and assess their biological activity. From an ethnopharmacological point of view, *J. montana* is given by some reports from the Bellorussian folk medicine to be used as a plant with sedative effects in the case of restless sleep in children. The very limited reports regarding the chemical composition of the aerial parts of *J. montana* led the author of this dissertation to identify possible scientific gaps and to try to solve them using modern scientific approaches, in order to elucidate the presence of the biologically active compounds from the aerial parts of *J. montana* and to evaluate their biological activity.

In the introduction section the author of the thesis presents a short review of the literature concerning *J. montana*, starting with its botanical description, distribution and ecological parameters. Moreover, the uses of this plant, as well as its ethnopharmacological significance in the Bellorussian folk medicine are presented. The author clearly identified the limited literature reports on the chemical composition of the plant, as well as of the lack of pharmacological characterization of its eventual phytochemical constituents.

The objective of the work, the structure of the research conducted in the thesis are clearly presented in chapter 3, in a logical way, making reference to the 4 publications realized by the candidate.

In the 4th chapter, the candidate presents the achievement of the scientific objectives, alongside with a concise summary of research materials and methods, results, everything being well described (geographical coordinates) and presented in a logical manner. The origin of the plant material is well presented, and the candidate clearly presents the place of collection, period of collection, processing of the herbal material. I must positively appreciate the use of scientific botanical literature and morphological depiction for the confirmation of the species' identity. Different extracts and fractions were prepared, which were further analyzed by LC-ESI-MS. Qualitative analysis of extracts/fractions revealed the presence of 25 compounds (Table 1), which were identified or tentatively identified using UV-Vis data and MS fragmentation patterns; moreover quantitative analysis was performed by HPLC-DAD. Furthermore, the separation, isolation and identification of the main flavonoids (compounds 9, 12, 22 – luteolin derivatives – Table 1, and Figure 2) was made by LPLC, TLC and NMR. The use of modern spectral techniques represents a very important step in the characterization of botanical extracts, and the identification of the main bioactive compounds



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by MS and NMR shows that the candidate possesses an excellent knowledge of phytochemistry, which is an essential feature of a researcher in pharmacognosy.

In the first article, published in 2021 in International Journal of Molecular Sciences (IF 5.923, MES score: 140) the activities of *J. montana* extracts, their fractions and main compounds were evaluated in amelanotic melanoma C32 (CRL-1585) cells and normal fibroblasts (PCS-201-012). The extracts and fractions were analyzed using liquid chromatography–photodiode array detection–electrospray ionization–mass spectrometry (LC–PDA–ESI–MS/TOF) to characterize 25 compounds. Further, three major and known constituents, luteolin (**22**) and its derivatives such as 7-*O*-glucoside (**12**) and 7-*O*-sambubioside (**9**) were isolated and identified. The cytotoxic activities against fibroblasts and the amelanotic melanoma cell line were determined using the fixable viability stain (FVS) assay. The influence of diethyl ether (Et<sub>2</sub>O) fraction (**JM4**) and **22** on apoptosis induction was investigated using an annexin V binding assay. The obtained results showed significant cytotoxicity of **JM4** and **22** with IC<sub>50</sub> values of 119.7 ± 3.2 and 95.1 ± 7.2 μg/mL, respectively. The proapoptotic potential after **22** treatment in the C32 human amelanotic melanoma cell line was comparable to that of vinblastine sulfate (VLB), detecting 29.2 ± 3.0% apoptotic cells. Moreover, **22** displayed less necrotic potential against melanoma cells than VLB. In addition, the influences of **JM4** and **22** on the dysfunction of the mitochondrial membrane potential (MMP), cell cycle and activity of caspases 3, 8, 9, and 10 were established. The effects of **JM4** on MMP change (74.5 ± 3.0% of the cells showed a reduced MMP) corresponded to the results obtained from the annexin V binding assay and activation of caspase-9. **JM4** and **22** displayed a significant impact on caspase-9 (40.9 ± 2.4% of the cells contained active caspase-9 after **JM4** treatment and 16.6 ± 0.8% after incubation with **22**) and the intrinsic (mitochondrial) apoptotic pathway. Moreover, studies have shown that **JM4** and **22** affect the activation of external apoptosis pathways by inducing the caspase-8 and caspase-10 cascades. Thus, activation of caspase-3 and DNA damage via external and internal apoptotic pathways were observed after treatment with **JM4** and **22**. The results obtained by the candidate suggest that *J. montana* extracts could be developed as new topical preparations with potential anticancer properties due to their promising cytotoxic and proapoptotic potential. The candidate has shown through this study that she possesses good knowledge both in terms of phytochemical analysis as well as in biological prospecting of natural extracts, fractions and isolated compounds, trying to elucidate some potential pharmacological activity/molecular mechanisms of the isolated compounds/fractions.

In the second publication, issued in 2022 in Frontiers in Pharmacology (section Ethnopharmacology – IF 5.810, MES score: 100) the effects of different extracts obtained from *J. montana* (JM1–JM6) and their main metabolites on biological processes during wound healing were evaluated. The effect on wound closure in the scratch test was



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established, and collagen type I synthesis and anti-inflammatory effects were assessed by flow cytometry in a human dermal fibroblast model (PCS-201-012). Additionally, the antioxidant activity (DPPH and FRAP) and degree of inhibition of elastase participating in the proliferation processes of skin fibroblasts were determined in an *in vitro* model. The extracts and fractions were analyzed using high-performance liquid chromatography–photodiode array detection (HPLC–PDA) to quantitatively characterize their main polyphenolic compounds. The high antioxidant activity of the JM4–JM5 fractions correlated with the content of luteolin and its derivative 7-*O*-glucoside. Luteolin also showed the highest anti-elastase activity with an IC<sub>50</sub> value of  $39.93 \pm 1.06 \mu\text{g/mL}$ , and its substantial content in the JM4 fraction presumably determines its activity ( $359.03 \pm 1.65 \mu\text{g/mL}$ ). At lower concentrations ( $<50 \mu\text{g/mL}$ ) of all extracts, cell proliferation and migration were significantly stimulated after 24 h of treatment. The stimulation of cell migration was comparable with that of allantoin, which was used as a positive control. However, most of the tested extracts showed limited capacity to affect collagen type I biosynthesis. Moreover, the tested samples exhibited a complex effect on cytokine secretion, and the strongest anti-inflammatory activity through the moderation of IL-1 $\beta$ , IL-6 and IL-8 was observed for JM4 and luteolin. Based on the obtained results of the quantitative analysis, the anti-inflammatory activity of JM4 may be due to the high content of luteolin. In summary, extracts from *J. montana*, which is flavonoid-rich, promote the viability and accelerate the migration of fibroblasts as well as moderate oxidant and inflammatory processes and elastase activity. Therefore, they may be potentially useful for topical therapeutic applications to stimulate the wound healing process. Through this publication, which is issued in a very well established journal in the area of pharmacology/ethnopharmacology (Q1) the author has demonstrated a good understanding of the mechanisms involved in wound healing process as well as the importance of the link between the ethnopharmacological relevance of a herbal drug, its chemical composition and possible novel biological activities which can be prospected, taking into account the detailed chemical identity of the complex herbal matrix.

In the third publication, which is a review paper (Biomolecules, IF – 4.879, MES score: 100, WoS/Scopus citations: 18/21) the candidate and her mentors present the recent trends in the application of chromatographic techniques in the analysis of luteolin and its derivatives. I appreciate this approach, and the fact that chronologically this is the first published paper, which comprises the dissertation, as this shows clearly the capacity of the candidate to analyze the current state of the art of the scientific literature concerning the topic of her research, as well as to improve her knowledge in terms of chromatographic analysis of flavonoids, in particular luteolin and its derivatives, which are also the major compounds of *J. montana* aerial parts. The paper provides the first comprehensive review, according to the authors, of the current analytical methods that were developed and validated for the quantitative determination of luteolin and its C- and O-derivatives including orientin,



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isoorientin, luteolin 7-O-glucoside and others. It provides a systematic overview of chromatographic analytical techniques including thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC), liquid chromatography (LC), high performance liquid chromatography (HPLC), gas chromatography (GC) and counter-current chromatography (CCC), as well as the conditions used in the determination of luteolin and its derivatives in plant material.

The forth paper comprising the dissertation is a review paper published in the prestigious journal *Medicinal Research Reviews* (IF – 12.944, MES score: 140) where the candidate and her coauthors discuss the relevance of luteolin derivatives in the treatment of skin tumors and the involved molecular mechanisms. The review focuses on an exhaustive overview of the therapeutic effects of luteolin and its derivatives in the treatment and prevention of skin cancers. The bioavailability and structure–activity relationships of luteolin derivatives are also discussed. The candidate has shown that she possesses a critical and mature overview about the possible therapeutic uses of natural products i.e. polyphenols, and particularly luteolin derivatives (the major compounds of *J. montana*), taking into account not only the natural sources of these compounds, or eventual pharmaceutical formulations but also crucial aspects such as bioavailability or structure – activity relationships, demonstrating the approach of a real health professional specialist.

### **Concluding remarks**

According to my expertise, the dissertation presented by Ms. Aleksandra Maria Juszczak as her doctoral thesis constitutes a well-designed experimental approach, coherent, and of great scientific value. I also appreciate that the results obtained through her research are published in well respected journals, and some of the papers got an appreciable number of citations so far, showing that the research conducted by the candidate is also relevant for the scientific community overall. The thesis is well written, with a good level of English and very easy to follow, being concise and well-structured.

As a reviewer, I consider after seeing the list of scientific accomplishments of the candidate (chapter 12), that undoubtedly her overall scientific and academic activity had a strong positive influence on the quality of research she conducted for this PhD thesis. Besides the publications from her PhD thesis, she co-authored other articles demonstrating that she is well integrated in her group, as well as she presented her results in several conference communications. The scientific internships she made demonstrate that she is an open person, always with the desire of improving her scientific knowledge and sharing valuable ideas with colleagues from the same academic area of research and teaching. Her involvement in scientific projects is also appreciable as well as her teaching activity in the field of Pharmacognosy.



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To sum up, having in mind the results and facts presented in the doctoral thesis, I conclude that it represents a significant contribution to the development of the field of Medical Sciences and Health Sciences, particularly the discipline of Pharmaceutical Sciences (pharmacognosy), and fully meets all the academic, scientific and formal requirements for a doctoral thesis. I would also have two questions for the candidate, because an interesting and well-conducted research always generates curiosities:

1. What was the reason behind choosing to do research on *J. montana*?
2. Was it a difficult job to transfer the analytical HPLC method into the preparative HPLC? Did you had problems in separating the three compounds?

Therefore, I recommend and propose to the Senate of the Medical University of Białystok that Ms. Aleksandra Maria Juszcak should be conferred with the doctoral degree in the field of Medical Sciences and Health Sciences, and *in the discipline of Pharmaceutical Sciences*.

Cluj-Napoca,

31.07.2022

Assoc. Prof. Dr. Pharm. Andrei Mocan

*Andrei Mocan*  
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