IOSUD - Universitatea Politehnica Timişoa
Şcoala Doctorală de Studii Inginereşti

NANOENGINEERING APPROACHES TO ENHANCE THE BIOAVAILABILITY OF PUERARIN FOR IMPROVED PHARMACOLOGICAL POTENTIAL

PhD Thesis
for obtaining the scientific title of doctor at
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in the field of CHEMICAL ENGINEERING

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Natural compounds continue to represent an essential component of everyday life and health-related fields, since a wide range of materials and products (e.g., pharmaceuticals, foods, perfumes, flavors, cosmetics) originate, directly or indirectly, from these natural resources [1]. This structural reliance on materials and products of natural origin supports the scientific relevance of natural-compounds research as a modern strategic domain with long-term importance [2].

Thus, the PhD thesis is structured into two main components:

1. Literature study, which provides a modern perspective on natural compounds, with an emphasis on the class of flavonoids, together with modern strategies for developing new products and materials through nanotechnology and biocatalysis. A marked evolution of the literature data regarding the growing importance of the isoflavone Puerarin in biomedical applications for cardiovascular health, neuroprotection, diabetes, or hepatoprotection was highlighted, which justifies the selection of this compound as the central pillar of the thesis, given that the main problem is its limited oral bioavailability.

This component of the thesis was valorized through the publication of 4 review articles [3,4,5,6], two of which [3,4] are among the most cited scientific works in the field (Highly Cited Papers, according to the Web of Science database, 2024).

2. Original contributions, in which the results obtained during the scientific research are presented, starting with the formulation of the objectives of the doctoral thesis. The central direction of the thesis is the increase of the solubility and bioavailability of the flavonoid puerarin, by applying the principles of green chemistry and by exploiting two modern research directions that are intensively explored today, namely nanotechnology and biocatalysis. The doctoral thesis pursues, as main objectives, the synthesis and physico-chemical and biological characterization of nanoparticles or nanobiocomposites based on puerarin, as well as the obtaining of compounds derived from it through enzyme-catalyzed transformations. Through the integrated approach of these two directions, the work proposes complementary strategies for improving the biopharmaceutical properties of puerarin and for increasing its pharmacological potential.

The PhD thesis is structured into the following chapters:

2.1. *In silico* computational studies on the flavonoid Puerarin

Within this subchapter, several in silico methods were investigated for the evaluation of reactivity and the optimization of the structure of the isoflavone puerarin, as well as for the prediction of its associated biological activities. The computational studies aimed to estimate the impact of structural modifications on the biological profile of puerarin, highlighting how these can influence and modulate different biological effects, with emphasis on anticancer activity against human melanoma and anti-inflammatory activity.

2.2. Synthesis, characterization, and biological activity of Puerarin-zinc oxide nanobiocomposites

Within this subchapter, the synthesis of new zinc oxide nanobiocomposites based on puerarin was optimized. Their advanced characterization was performed using instrumental methods such as X-ray diffraction analysis, UV–Vis, and FT-IR. Scanning electron microscopy (SEM) indicated that the size of these nanobiocomposites falls within the 200-400 nm range, while atomic force microscopy (AFM) highlighted the specific surface topography, which recommends them for use as delivery systems.

Following advanced physico-chemical characterization, the nanobiocomposites were investigated in terms of biological activity. The obtained results highlighted a promising biological profile, expressed by significant antimicrobial activity against pathogenic species (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Candida parapsilosis*), as well as in vitro anticancer activity against human melanoma cells (A375). In addition, the *in ovo* evaluation showed that the Puerarin-zinc oxide nanobiocomposites do not induce irritant effects and do not cause significant changes in angiogenesis processes. The results of these studies support preliminary biocompatibility and indicate their potential for use in the formulation of modern topical pharmaceutical products.

These results were published in the *Pharmaceutics* journal [7].

2.3. Synthesis, characterization, and complex biological evaluation of Puerarin-Ag_2O/Ag and Puerarin-Ag nanoparticles

Within this subchapter, the synthesis of new Puerarin-Ag_2O/Ag and puerarin-Ag nanoparticles, obtained using puerarin as a reducing agent, was optimized. Their characterization was performed by XRD, UV-Vis, and FT-IR. Morphological analyses (TEM, SEM, AFM) evidenced the formation of nanostructured systems and provided information on their size and surface topography. DLS analysis showed the tendency of these nanoparticles to form aggregates in suspension, resulting in large hydrodynamic sizes.

The biological evaluation of Puerarin-Ag_2O/Ag and Puerarin-Ag nanoparticles was performed through a multifactorial approach, including antibacterial activity against Gram-positive and Gram-negative nosocomial species (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*), antioxidant activity by the DPPH assay, as well as studies on the human melanoma cell line A375 and the human immortalized keratinocyte cell line HaCaT, using the MTT assay and the LDH release method, to investigate effects on mitochondrial activity and cytotoxicity. In addition, irritant potential and impact on angiogenesis were analyzed through in ovo tests. The ensemble of these investigations allowed outlining a complex biological

profile of the obtained nanosystems and demonstrated the possibilities for their use in biomedical and pharmacological applications.

These results were published in the *Journal of Functional Biomaterials* [8].

2.4. Preliminary studies on nanoparticle synthesis using alternative metal cations (Copper, Nickel, Iron)

The preliminary studies presented in this subchapter showed that, among the investigated alternative metal cations (Cu, Ni, Fe), copper is the most suitable for obtaining oxide nanoparticles in the presence of the isoflavone puerarin, with the formation of CuO nanoparticles, whereas Ni- and Fe-based systems led predominantly to intermediate hydroxide-type phases and require further optimization. Puerarin-CuO nanoparticles were characterized in terms of optical, structural, and morphological properties by XRD, UV-Vis, FT-IR, SEM, and AFM analyses, and their biological evaluation was performed by *in ovo* tests to assess the preliminary biocompatibility profile.

2.5. Niosome-based proniosomal gels as integrated delivery systems for Puerarin

Within this subchapter, for the first time, the formulation and physico-chemical and biological characterization of a puerarin-loaded proniosomal gel were achieved. The physicochemical properties and structural characteristics of the Puerarin-loaded proniosomal gel were systematically investigated to confirm the formation of the vesicular system and to evaluate its potential for topical administration. The optimized formulation was subjected to a complex physicochemical characterization through FT-IR analyses, differential scanning calorimetry (DSC), DLS, zeta potential determination, and rheological evaluation. Complementary to the experimental physicochemical investigations, biological studies were performed consisting of *in vitro* cytotoxicity tests, on the human melanoma cell line A375 and the immortalized keratinocyte cell line HaCaT, and *in ovo* evaluations on the chorioallantoic membrane (CAM), to investigate the initial biological performance and safety profile of the selected formulation.

These results were published in the *Gels* journal [9].

2.6. Use of enzymatic catalysis as a strategy for increasing the solubility and bioavailability of Puerarin

In this subchapter, the synthesis of Puerarin esters with different natural acyl donors was pursued in order to increase lipophilicity and, implicitly, bioavailability. In this regard, the synthesis of puerarin esters was investigated using several native and immobilized lipases, three acyl donors (hexanoic acid, octanoic acid, vinyl laurate), in esterification and transesterification reaction systems. The reactions were optimized using immobilized lipase B from *Candida antarctica*, and, as an additional original element, the lipase from *Pseudomonas stutzeri* was immobilized by sol-gel entrapment, subsequently used in the same transesterification reactions and evaluated comparatively with commercial biocatalysts.

3. The experimental part of the thesis includes a detailed description of the materials and experimental procedures used for the synthesis and characterization of nanoparticles, as well as the instrumental analysis methods employed.

4. Final conclusions and original contributions summarize the main results of the experimental and theoretical studies, in accordance with the proposed objectives and with emphasis on their innovative character.

Achieving the objectives of the PhD thesis, through the experimental research activity carried out, led to the formulation of the following conclusions and original contributions:

4.1. The literature study highlighted relevant aspects regarding the importance of natural compounds, with emphasis on flavonoids, as well as on modern strategies for increasing their solubility and bioavailability, such as nanotechnology and biocatalysis.

4.2. In silico studies enabled the structural optimization and reactivity analysis of Puerarin, as well as the prediction of relevant biological activities, in particular anticancer activity against human melanoma and anti-inflammatory activity.

4.3. For the first time, the synthesis and physico-chemical and biological characterization of Puerarin-zinc oxide (PUE-ZnO) nanobiocomposites were achieved and optimized.

- The possibility of using Puerarin as a reducing and stabilizing agent for the synthesis of zinc oxide nanoparticles was demonstrated.
- Morphological analyses evidenced particles in the 200-400 nm range, supporting their potential use as delivery systems.
- The anticancer potential of Puerarin against the A375 cell line (human melanoma) was demonstrated for the first time.
- PUE-ZnO nanobiocomposites presented a promising biological profile, expressed by antimicrobial activity against pathogenic species (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Candida parapsilosis*), as well as synergistic *in vitro* anticancer activity against human melanoma.
- *In ovo* study indicated the absence of irritant effects and the lack of a significant impact on angiogenesis under the investigated experimental conditions, supporting their potential for pharmaceutical applications.

4.4. For the first time, the synthesis of Puerarin-Ag_2O/Ag and Puerarin-Ag nanoparticles was optimized, followed by physicochemical and biological characterization.

- Physicochemical characterization by XRD, UV-Vis, and FT-IR confirmed nanoparticle formation and allowed highlighting structural and optical particularities.
- Morphological analyses by SEM and AFM provided complementary information regarding morphology, surface topography, and particle organization, confirming the obtainment of nanostructured systems suitable for biological evaluation.
- Biological evaluation was performed through a multifactorial approach, including antibacterial activity, antioxidant activity (DPPH), *in vitro* cytotoxicity/cell viability tests (MTT and LDH), and *in ovo* evaluations (irritant potential and impact on angiogenesis).
- At all tested concentrations, the nanoparticles showed pronounced cytotoxicity toward the HaCaT and A375 cell lines, resulting in a significant decrease in mitochondrial activity and increased cell death. The results obtained on the HaCaT line (human keratinocytes) indicate the need for careful evaluation of the therapeutic window and safety profile, especially in the context of potential topical or biomedical applications.
- *In ovo* evaluation complemented the biological profile of the nanoparticles, providing

preliminary information on irritant potential and impact on angiogenesis, useful for assessing initial biocompatibility.

4.5. Preliminary studies on the use of alternative metal cations (Cu, Ni, Fe) in the presence of Puerarin showed that copper is the most promising cation for obtaining oxide nanoparticles, with the formation of the CuO phase. Under the investigated experimental conditions, Ni- and Fe-based systems led predominantly to hydroxide-type species, indicating the need for further optimization to promote conversion toward the corresponding oxide phases.

4.6. For the first time, the formulation and physico-chemical and biological characterization of a Puerarin-loaded proniosomal gel were achieved, as an integrated niosome-based delivery system, with potential for topical pharmaceutical applications.

- Physicochemical characterization of the optimized formulation by FT-IR, DSC, DLS, zeta potential determination, and rheological evaluation confirmed the formation of the vesicular system and provided relevant information on its structural and colloidal properties.
- The performed analyses evidenced physicochemical characteristics compatible with the use of the formulation as a topical delivery system, supporting the stability and appropriate behavior of the proniosomal system under the investigated conditions.
- Preliminary in vitro biological studies on the human malignant melanoma cell line A375 and the immortalized keratinocyte cell line HaCaT enabled initial evaluation of the cytotoxicity profile of the Puerarin-loaded proniosomal formulation.
- *In ovo* tests were used for the preliminary evaluation of irritant potential and for assessing the safety profile of the formulation, in view of future topical applications.
- The obtained results support the potential of the Puerarin-loaded proniosomal gel as a promising platform for the development of modern topical delivery systems.

4.7. In the field of biocatalysis, the feasibility of synthesizing Puerarin esters using natural acyl donors (hexanoic acid, octanoic acid, vinyl laurate) in esterification and transesterification reaction systems was demonstrated, with the aim of improving its solubility and bioavailability.

- The results showed that the nature of the acyl donor and the type of reaction significantly influence conversion and ester yield, confirming the importance of optimizing reaction conditions.
- The reactions were optimized using immobilized lipase B from *Candida antarctica* (CalB GF-IM), confirming its efficiency in the synthesis of Puerarin ester derivatives.
- Sol-gel entrapment immobilization of *Pseudomonas stutzeri* lipase was performed, followed by evaluation of the obtained preparations in the transesterification reaction of Puerarin with vinyl laurate.
- Six enzymatic formulations were obtained and tested by sol-gel entrapment, using different silane precursors and ionic liquids at various molar ratios, enabling evaluation of the effect of matrix composition on biocatalytic activity.
- Among the investigated formulations, two formulations showed the best performance, with conversions > 50%, being identified as the most efficient enzyme formulations obtained.
- The biocatalysis study confirms that enzymatic modification of Puerarin is a promising direction for obtaining derivatives with improved biopharmaceutical potential, in accordance with green chemistry principles.

5. Selected references

[1] G. A. Cordell, "Sixty Challenges – A 2030 Perspective on Natural Products and Medicines Security", *Nat. Prod. Comm.*, vol. 12, no. 8, pp. 1371-1379, **2017**, doi:10.1177/1934578X1701200849.

[2] S. Daley, G. A. Cordell, "Natural Products, the Fourth Industrial Revolution, and the Quintuple Helix", *Nat. Prod. Comm.*, vol. 16, no. 3, pp. 1-31, **2021**, doi:10.1177/1934578X211003029.

[3] S. Liga, C. Paul, F. Péter, "Flavonoids: Overview of Biosynthesis, Biological Activity, and Current Extraction Techniques", *Plants*, vol. 12, no. 14, 2732, **2023**, https://doi.org/10.3390/plants12142732.

[4] S. Liga, C. Paul, E. A. Moacă, F. Péter, "Niosomes: Composition, Formulation Techniques, and Recent Progress as Delivery Systems in Cancer Therapy", *Pharmaceutics*, vol. 16, no. 2, 223, **2024**, https://doi.org/10.3390/pharmaceutics16020223.

[5] S. Liga, C. Paul, "Puerarin-A Promising Flavonoid: Biosynthesis, Extraction Methods, Analytical Techniques, and Biological Effects", *International Journal of Molecular Sciences*, vol. 25, no. 10, 5222, **2024**, https://doi.org/10.3390/ijms25105222.

[6] S. Liga, C. Paul, "Flavonoid-Based Nanogels: A Comprehensive Overview", *Gels*, vol. 11, no. 4, 267, **2025**, https://doi.org/10.3390/gels11040267.

[7] S. Liga, R. Vodă, L. Lupa, C. Paul, N. S. Nemeş, D. Muntean, Ș. Avram, M. Gherban, F. Péter, "Green Synthesis of Zinc Oxide Nanoparticles Using Puerarin: Characterization, Antimicrobial Potential, Angiogenesis, and *In Ovo* Safety Profile Assessment", *Pharmaceutics*, vol. 16, no. 11, 1464, **2024**, https://doi.org/10.3390/pharmaceutics16111464.

[8] S. Liga, R. Vodă, L. Lupa, E.-A. Moacă, D. Muntean, L. Barbu-Tudoran, M. Suciu, V. Socoliuc, F. Péter, "Synthesis of Ag2O/Ag Nanoparticles Using Puerarin: Characterization, Cytotoxicity, *In Ovo* Safety Profile, Antioxidant, and Antimicrobial Potential Against Nosocomial Pathogens", *J. Funct. Biomater.*, vol. 16, no. 7, 258, **2025**, https://doi.org/10.3390/jfb16070258.

[9] S. Liga, A. Tămaș, R. Vodă, G. Rusu, I. Bîtcan, V. Socoliuc, R. Pop, D. H. Ali, I.-A. Predescu, C. A. Dehelean, F. Péter, "Puerarin-Loaded Proniosomal Gel: Formulation, Characterization, *In Vitro* Antimelanoma Cytotoxic Potential, and *In Ovo* Irritation Assessment", *Gels*, vol. 12, no. 1, 72, **2026**, https://doi.org/10.3390/gels12010072.