REVIEW

By Prof. Margarita Ivanova Traykova, PhD

Medical College, Medical University of Plovdiv

Designated as a member of the scientific jury based on Order No. R-109-476/18.12.2024
Of the Rector of the Medical University of Varna

Regarding: Dissertation work on the topic ***"Nanostructured Lipid Carriers Loaded with Hypericum perforatum L. Extract for Dermal Application and Accelerated Wound Healing"*** written by ***Yoana Nikolova Sotirova*** for the acquisition of educational and scientific degree "Doctor of Philosophy" in Higher Education Area *7. Healthcare and Sports*, Professional Field *7.3. Pharmacy*, Doctoral Programme *Pharmacology (including Pharmacokinetics and Chemotherapy)*

General presentation of the procedure

The submitted set of documents by the PhD student Yoana Nikolova Sotirova is in accordance with the requirements of the Regulations on Academic Staff Development at the Medical University of Varna, Chapter II, Section IV—Conditions and procedure for award of a PhD scientific degree. She is a full-time PhD student (enrolled with Order No. R-109-546/03.12.2021) in the Department of Pharmacology, Toxicology, and Pharmacotherapy of the Faculty of Pharmacy, with scientific supervisors Prof. Kaloyan Georgiev, PhD, DSc, and Assoc. Prof. Velichka Andonova, PhD. Yoana Sotirova has fulfilled all the requirements regarding full-time doctoral studies, which is evident from the submitted documents. She was deregistered with the right to defend the PhD thesis with Order No. R-109-476/18.12.2024.

Short biographical data of the candidate

Yoana Nikolova Sotirova was born on November 30, 1996 in Yambol. She graduated from the Atanas Radev Mathematics High School in Yambol. In 2020, she graduated from the Medical University of Varna and obtained a Master's Degree in Pharmacy. As an excellent student, she received the annual scholarship of the Bulgarian Pharmaceutical Union for Pharmacy students in 2019. She graduated *summa cum laude* and was awarded the Golden Galen of the 2020 class of the Medical University of Varna.

After graduating, Yoana Sotirova worked as a pharmacist in a community pharmacy. After winning a competition for an assistant professor position in February 2021, she was appointed to the Department of Pharmaceutical Technologies of MU-Varna. In 2021, she was enrolled as a full-time PhD student in the doctoral programme "Pharmacology (including pharmacokinetics and chemotherapy)", Department of Pharmacology, Toxicology, and Pharmacotherapy of the Faculty of Pharmacy at MU-Varna. In May 2024, Yoana Sotirova acquired a specialty in "Pharmaceutical Technology with Biopharmacy".

Relevance of the topic and appropriateness of the set aim and objectives

The thesis is dedicated to a current problem related to introducing effective dosage forms comprising new therapeutic agents to solve unresolved therapeutic practice issues. Wound healing is a complex, dynamic physiological process. Its course is influenced by several exogenous and endogenous factors. The currently used therapeutic agents and approaches do not always manage to balance the necessary microenvironment in the damaged skin tissue in time. The latter necessitates the search and introduction of new medicinal substances with improved activity and broad-spectrum action, such as hyperforin—a biologically active substance (BAS) that is responsible for the wound-healing properties of St. John's wort (*Hypericum perforatum* L.). The medicinal plant is used both in cases of mental disorders (depression, anxiety, and insomnia) and in the therapy of skin pathological conditions, including wounds of various natures. Hyperforin is characterized by high photosensitivity and low water solubility, which is the basis for searching for an appropriate technological approach to improve these physicochemical characteristics. Nanostructured lipid carriers have been used to solve these issues. They can potentially improve chemical stability and provide an extended release of BAS. Bigels, as a semi-solid dosage form for application to the skin, were chosen as a suitable carrier for hyperforin-rich extract-loaded lipid nanoparticles. This choice is logical, considering that bigels combine the advantages while limiting the disadvantages of hydro- and oleogels. In this sense, the topic of the PhD thesis of Yoana Sotirova, namely the possibility of including a hyperforin-rich St. John's wort extract in nanostructured lipid carriers as a general strategy for accelerated wound healing, is particularly relevant.

Knowledge of the problem

The thesis contains 123 pages and is illustrated with 38 figures and 17 tables. The reference list comprises 387 papers, mainly from the last 5 years, one of which is in Cyrillic. The scientific work includes the following sections: Introduction, Literature Review, Aim and Objectives, Materials and Methods, Results and Discussion, Conclusions, Contributions, Reference List, and List of Publications and Scientific Participations. The structure of the dissertation is in accordance with the requirements of the Conditions and procedure for award of a PhD scientific degree at MU-Varna.

The literature review is written in 36 pages and is based on rich literature material—298 sources. The review shows a broad knowledge of the subject. The thesis has logically and consistently included in the literature review anatomical and physiological features of the skin as a chosen route of administration. This is followed by a brief characterization of wounds, stages of the wound-healing process, and factors influencing them. Next, a botanical characterization of *Hypericum perforatum* L., historical information about its application in medical practice, and its phytochemical composition and pharmacological effects are described. The next chapter is dedicated to lipid nanoparticles—their principle composition and structure, methods of their preparation (including 14 methods with their advantages and limitations), characterization, and areas of application with a focus on their utilization in the treatment of wounds. The fifth chapter discusses semi-solid dosage forms focusing on gels and bigels. The review concludes with the conclusions drawn by the PhD candidate based on the literature review, which logically leads to the formulation of the purpose of the reviewed dissertation work.

Aim and objectives of the thesis

Based on the literature review, the thesis author formulated the aim of this dissertation, namely the development of nanostructured lipid carriers with St. John's wort extract for dermal application and accelerated wound healing. Four main objectives have been defined, and their logical sequence and solution enable the achievement of the ultimate goal.

Research methodology

The overall methodology of the study follows a logical course aimed at solving the objectives set in relation to the aim of the dissertation. The PhD candidate has chosen modern methods, which she skillfully applies, demonstrating in-depth theoretical knowledge of their essence. The large number of methodologies utilized in the thesis emphasizes the broad multidisciplinary nature of the study and a comprehensive approach - starting from obtaining a St. John's wort extract rich in hyperforin (proven by HPLC-UV/Vis analysis) through the preparation, physicochemical characterization of nanostructured lipid carriers, and the study of their antiviral and antimicrobial effects to the preparation and characterization of the bigels and *in vivo* monitoring of their wound-healing activity and determination of their antioxidant properties.

Analysis of results

The conducted own research is presented on 34 pages and is illustrated clearly and understandably with 22 figures and 12 tables. The investigations were executed in the following main directions, which logically follow the set objectives:

1. Preparation of St. John's wort extract rich in hyperforin: A modified method of maceration of the plant with limited influence of light and atmospheric oxygen was developed, as a result of which an extract with a content of the target BAS higher than 40% (determined by a developed HPLC-UV/Vis protocol) was obtained.

2. Development and characterization of nanostructured lipid carriers loaded with hyperforin-rich St. John's wort extract. Preliminary studies were conducted to obtain "blank" nanostructured lipid carriers containing solid lipids, liquid oils, and a nonionic surfactant couple (polysorbate 80 and sorbitan monooleate). High-shear homogenization with subsequent ultrasonication under varying technological conditions was applied. Twenty models were obtained and characterized in terms of visual appearance, average particle size and size distribution, zeta potential, degree of crystallinity, and physical stability during storage. Based on the results, two optimal samples were selected (NLC3 and NLC4 – containing glyceryl behenate and borage oil or almond oil). Further, they were loaded with hyperforin-rich St. John's wort extract (HP-NLC3 and HP-NLC4). The above-mentioned physicochemical characteristics were determined for the two selected models, along with their entrapment efficiency (EE), and monitored during one-year storage at 4°C. The data from the conducted studies show that HP-NLC4 has a higher EE and better ability to preserve the hyperforin-rich extract incorporated in them. Infrared spectroscopy has established the successful encapsulation of the hyperforin-rich St. John's wort extract in HP-NLC3 and HP-NLC4 (1.25%) and the lack of extract-lipid interactions. The *in vitro* studies show a microbicidal effect of the HP-NLC dispersions against *K. pneumoniae*, *S. aureus*, and *C. albicans*. Their antiviral effect against HSV-1 is weak but nevertheless present. A more pronounced activity against the studied

pathogens is observed with HP-NLC4. The absence of such in the "blank" nanoparticles proves the activity of the extract rich in hyperforin. For further research and inclusion in a biphasic gel as a semi-solid vehicle, sample HP-NLC4 (containing glyceryl behenate, borage oil, polysorbate 80, and sorbitan monooleate) was logically chosen.

3. Development and characterization of a semi-solid dosage form (biphasic gel) incorporating nanostructured lipid carriers loaded with hyperforin-rich St. John's wort extract. The low viscosity of the lipid nanodispersion necessitates its incorporation into a semi-solid dermal form suitable for application. For this purpose, eight biphasic gels (bigels)—"blank" and loaded with HP-NLC4, were prepared and characterized. The results show that the prepared bigels ("blank" and loaded with HP-NLC4) possess the desired physical stability and skin pH tolerance, and the compositions containing 20% oil phase stand out with better uniformity. Increasing the oleogel content has a significant effect on the spreadability and hardness of the "blank" bigels, as well as on the cohesiveness and adhesiveness of the nanodispersion-loaded semi-solid dosage forms. Their rheological characterization shows that they all exhibit pseudoplastic properties. Vehicles with a hydrogel-to-oleogel ratio of 80:20 possess optimal consistency and structural homogeneity. Based on the obtained results and conclusions drawn for further research, the HP-NLC-BG2 model (with a hydrogel-to-oleogel ratio of 80:20) was logically chosen as a carrier for the nanoencapsulated hyperforin-rich St. John's wort extract, with which to conduct *in vivo* investigation of its wound healing and antioxidant properties on experimental animals.

4. An *in vivo* study of the wound-healing potential of the selected semi-solid dosage form was conducted on an excision wound model in experimental animals. The tissue regeneration was monitored based on the changes in the wound size over time. It was found that the HP-NLC-BG2 had the most pronounced wound-healing effect. The plasma antioxidant status was determined by assessing the antioxidant capacity and the degree of oxidative stress. According to the obtained results, the antioxidant effect of HP-NLC-BG2 is mainly achieved by free radical scavenging.

All the research results are thoroughly analyzed and discussed. The logical presentation of the results is particularly impressive, especially the fact that at the end of each subsection of the Results and Discussion chapter, Yoana Sotirova has drawn conclusions that guide the course of further research.

Ultimately, the conclusions that arise from the results obtained from each objective set in the dissertation are clearly and precisely formulated.

Contributions and significance of the development of science and practice

A detailed acquaintance with the PhD thesis of Yoana Nikolova Sotirova showed that it contains original scientific-theoretical and scientific-applied contributions, defended in the presented work. Five contributions of a scientific-theoretical nature and two of a scientific-applied nature have been made, which can be summarized as follows:

1. An original protocol for obtaining a St. John's wort extract with a high hyperforin content (> 40%), including 48-h maceration in the dark under argon ambiance at a plant material-to-extractant (anhydrous dichloromethane) ratio of 1:3.33, was developed.

2. An innovative semi-solid dosage form was prepared—a combination of biphasic gel and nanostructured lipid carriers (gelled with poloxamer 407 HP-NLC4 lipid nanodispersion and

borage oil structured with sorbitan monostearate), which allows the dermal application of hyperforin-rich St. John's wort extract and provides accelerated wound healing compared to a reference herbal product.

Assessment of thesis-related publications

In connection with the thesis, two publications have been attached, in which the PhD student is the first author. One of the publications is in the authoritative periodical *Gels* with IF 5.0. According to Google Scholar, this article already has seven citations without auto-citations. This is evidence of the interest of the scientific community in the conducted research and the obtained results.

Yoana Sotirova has participated in two research projects and three scientific forums in our country and abroad. The presented materials are related to the thesis and have been introduced to a larger circle of researchers.

Personal participation of the PhD student in the conducted research

A significant volume of experimental work has been carried out. The obtained results are of a scientific and applied nature. There is no doubt that the PhD student personally participated in the review of the problem, formulation of the aim and objectives, conducting the experimental studies, analyzing the results and the contributions made, and with the support of scientific supervisors.

Thesis summary

The thesis summary is prepared in accordance with the requirements. It includes an introduction, aim and objectives, materials and methods, and results and discussion. The presented research and discussion fully reflect the main results achieved in the thesis. The obtained results are illustrated with a sufficient number of figures and tables. The conclusions coincide with those in the dissertation. The scientific contributions are included, as well as a list of scientific publications and participation in scientific forums in connection with the thesis. Familiarization with the thesis summary allows one to fully understand the problem being developed, the conducted research, and the interpretation of the obtained results. In conclusion, the thesis summary fully reflects the studies carried out in the thesis and shows the obtained results and their interpretation, conclusions, and generalizations.

Critical remarks and recommendations

I have no critical remarks or recommendations.

CONCLUSION

The PhD thesis ***contains original scientific results that represent a contribution to science*** and **meets all the requirements** of the Law on Academic Staff Development in the Republic of Bulgaria, the Regulations for the Implementation of the Law on Academic Staff Development in the

Republic of Bulgaria, and the Regulations on Academic Staff Development at the Medical University of Varna.

The thesis shows that the PhD student **Yoana Nikolova Sotirova possesses** theoretical knowledge and professional skills in the scientific specialty "Pharmacology (incl. pharmacokinetics and chemotherapy)". She has mastered many methods, conducted extended experimental studies, demonstrated the ability to interpret the obtained results, and logically planned and conducted subsequent experiments. The latter proves the qualities and skills for independent conduct of scientific research.

Due to the above, I express my ***positive assessment*** of the conducted research, presented by the above-reviewed thesis, thesis summary, achieved results, and contributions, and I ***propose to the esteemed scientific jury to award the educational and scientific degree "Doctor of Philosophy"*** to ***Yoana Nikolova Sotirova*** in the doctoral programme ***"Pharmacology (including pharmacokinetics and chemotherapy)" for the developed thesis entitled "Nanostructured Lipid Carriers Loaded with Hypericum perforatum L. Extract for Dermal Application and Accelerated Wound Healing"***.

17.01.2025
Sofia

Reviewer: .....
(Prof. Margarita Traikova, PhD)

REVIEW

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Relevance of the topic and appropriateness of the set aim and objectives

The thesis is dedicated to a current problem related to introducing effective dosage forms comprising new therapeutic agents to solve unresolved therapeutic practice issues. Wound healing is a complex, dynamic physiological process. Its course is influenced by several exogenous and endogenous factors. The currently used therapeutic agents and approaches do not always manage to balance the necessary microenvironment in the damaged skin tissue in time. The latter necessitates the search and introduction of new medicinal substances with improved activity and broad-spectrum action, such as hyperforin—a biologically active substance (BAS) that is responsible for the wound-healing properties of St. John's wort (*Hypericum perforatum* L.). The medicinal plant is used both in cases of mental disorders (depression, anxiety, and insomnia) and in the therapy of skin pathological conditions, including wounds of various natures. Hyperforin is characterized by high photosensitivity and low water solubility, which is the basis for searching for an appropriate technological approach to improve these physicochemical characteristics. Nanostructured lipid carriers have been used to solve these issues. They can potentially improve chemical stability and provide an extended release of BAS. Bigels, as a semi-solid dosage form for application to the skin, were chosen as a suitable carrier for hyperforin-rich extract-loaded lipid nanoparticles. This choice is logical, considering that bigels combine the advantages while limiting the disadvantages of hydro- and oleogels. In this sense, the topic of the PhD thesis of Yoana Sotirova, namely the possibility of including a hyperforin-rich St. John's wort extract in nanostructured lipid carriers as a general strategy for accelerated wound healing, is particularly relevant.

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Aim and objectives of the thesis

Based on the literature review, the thesis author formulated the aim of this dissertation, namely the development of nanostructured lipid carriers with St. John's wort extract for dermal application and accelerated wound healing. Four main objectives have been defined, and their logical sequence and solution enable the achievement of the ultimate goal.

Research methodology

The overall methodology of the study follows a logical course aimed at solving the objectives set in relation to the aim of the dissertation. The PhD candidate has chosen modern methods, which she skillfully applies, demonstrating in-depth theoretical knowledge of their essence. The large number of methodologies utilized in the thesis emphasizes the broad multidisciplinary nature of the study and a comprehensive approach - starting from obtaining a St. John's wort extract rich in hyperforin (proven by HPLC-UV/Vis analysis) through the preparation, physicochemical characterization of nanostructured lipid carriers, and the study of their antiviral and antimicrobial effects to the preparation and characterization of the bigels and *in vivo* monitoring of their wound-healing activity and determination of their antioxidant properties.

Analysis of results

The conducted own research is presented on 34 pages and is illustrated clearly and understandably with 22 figures and 12 tables. The investigations were executed in the following main directions, which logically follow the set objectives:

1. Preparation of St. John's wort extract rich in hyperforin: A modified method of maceration of the plant with limited influence of light and atmospheric oxygen was developed, as a result of which an extract with a content of the target BAS higher than 40% (determined by a developed HPLC-UV/Vis protocol) was obtained.

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Ultimately, the conclusions that arise from the results obtained from each objective set in the dissertation are clearly and precisely formulated.

Contributions and significance of the development of science and practice

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Yoana Sotirova has participated in two research projects and three scientific forums in our country and abroad. The presented materials are related to the thesis and have been introduced to a larger circle of researchers.

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A significant volume of experimental work has been carried out. The obtained results are of a scientific and applied nature. There is no doubt that the PhD student personally participated in the review of the problem, formulation of the aim and objectives, conducting the experimental studies, analyzing the results and the contributions made, and with the support of scientific supervisors.

Thesis summary

The thesis summary is prepared in accordance with the requirements. It includes an introduction, aim and objectives, materials and methods, and results and discussion. The presented research and discussion fully reflect the main results achieved in the thesis. The obtained results are illustrated with a sufficient number of figures and tables. The conclusions coincide with those in the dissertation. The scientific contributions are included, as well as a list of scientific publications and participation in scientific forums in connection with the thesis. Familiarization with the thesis summary allows one to fully understand the problem being developed, the conducted research, and the interpretation of the obtained results. In conclusion, the thesis summary fully reflects the studies carried out in the thesis and shows the obtained results and their interpretation, conclusions, and generalizations.

Critical remarks and recommendations

I have no critical remarks or recommendations.

CONCLUSION

The PhD thesis ***contains original scientific results that represent a contribution to science*** and **meets all the requirements** of the Law on Academic Staff Development in the Republic of Bulgaria, the Regulations for the Implementation of the Law on Academic Staff Development in the

Republic of Bulgaria, and the Regulations on Academic Staff Development at the Medical University of Varna.

The thesis shows that the PhD student **Yoana Nikolova Sotirova possesses** theoretical knowledge and professional skills in the scientific specialty "Pharmacology (incl. pharmacokinetics and chemotherapy)". She has mastered many methods, conducted extended experimental studies, demonstrated the ability to interpret the obtained results, and logically planned and conducted subsequent experiments. The latter proves the qualities and skills for independent conduct of scientific research.

Due to the above, I express my ***positive assessment*** of the conducted research, presented by the above-reviewed thesis, thesis summary, achieved results, and contributions, and I ***propose to the esteemed scientific jury to award the educational and scientific degree "Doctor of Philosophy"*** to ***Yoana Nikolova Sotirova*** in the doctoral programme ***"Pharmacology (including pharmacokinetics and chemotherapy)" for the developed thesis entitled "Nanostructured Lipid Carriers Loaded with Hypericum perforatum L. Extract for Dermal Application and Accelerated Wound Healing"***.

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