REVIEW

by Prof. Ludmil Penuv Kirazov, Doctor of science
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of a dissertation work for obtaining an educational and scientific degree "doctor" in the field of higher education: 4. Natural sciences, mathematics and informatics, professional direction: 4.3. Biological Sciences scientific specialty Biochemistry, code 06.01.10.

with author Inna Aleksandrovna Sulikovska, free doctoral student at IEMPAM-BAS

on the topic **"Optimization and adaptation of the method for determining phototoxicity *in vitro* 3T3 NRU phototoxicity test, to LED - solar simulator Helios-iO"**

Scientific consultant: Doz. Ivan Iliev, PhD

General presentation of the procedure and the doctoral student

By order No. RD-15-54 dated 06/03/2022, I have been appointed as an internal member of the scientific jury for the defense of the dissertation work by the director of IEMPAM, and at the first meeting of the jury I was selected as a reviewer. I have received all the documents required by the Regulations for the conditions and procedures for acquiring scientific degrees and holding academic positions in IEMPAM and in accordance with the rules for application of ZRAS in the Republic of Bulgaria and BAS.

The author of the dissertation, Inna Aleksandrovna Sulikovska, is a doctoral student of independent training at IEMPAM-BAS. In the period 2011 - 2017, she obtained a master's degree in the specialty of biomedical engineer-researcher at the National Technical University of Ukraine "Igor Sikorsky Polytechnic Institute", Faculty of Biomedical Engineering. In 2017, she was on a three-month specialization under the Erasmus+ program at the University of Patras, Greece, Biomedical Electronics Laboratory. After graduating from higher education, she worked for half a year as an X-ray laboratory assistant, cone-beam computed tomography. In 2017, he began doctoral studies at the Institute of Molecular Biology and Genetics at the National Academy of Sciences of Ukraine, and since 2019 he has been a biologist in the "Pathology" section at IEMPAM - BAS.

Actuality of the topic

The dissertation is aimed at an actual and significant problem – the phototoxicity. This term describes the property of natural and synthetic substances to change their structure under the

influence of sunlight (primarily under ultraviolet radiation), which results in the formation of derivatives having a toxic effect in the organism. Hundreds of drugs have been identified that exhibit phototoxicity. Among them are antibiotics, diuretics, nonsteroidal anti-inflammatory drugs, retinoids, neuroleptics and others. Phototoxicity is also exhibited by a number of substances of natural origin. It is therefore recommended that all substances used in medicine and cosmetics to be tested to avoid a risk of unwanted effects. The dissertation characterizes and adapts a method for a quick and relatively easy test of substances for their photosensitivity, which can find application in practice.

Knowledge of the state of research on the problem

The introduction, the literature review and the use of the cited literature show a good knowledge of the state of research on the problem treated in the dissertation.

Characterization and evaluation of the dissertation work and contributions

The dissertation is written on 147 pages and illustrated with 16 tables and 60 figures. It contains the usual sections for a similar scientific work: introduction (2 pages), literature review (39 pages), aim and objectives (1 page), materials and methods (22 pages), results and discussion (45 pages), conclusion (1 page), inference (1 page), original contributions (1 page), publications related to the dissertation (1 page) and references (26 pages). 322 sources are used in the bibliographic reference. This structure represents a well-balanced content of the dissertation, dominated by the results and their discussion.

The literature review is thorough and comprehensive. It has three main sections. The first section examines the role of sunlight in biological processes, its characteristics and the mechanisms of its absorption by the skin. The different types of sunlight simulators are presented, and their advantages and disadvantages are discussed in detail.

The second section is devoted to phototoxicity. The development of knowledge about phototoxicity over the centuries and its application in medicine and pharmacy is traced. The mechanisms through which phototoxicity manifests are examined in detail, illustrating them with the reactions of specific substances. The role of reactive oxygen forms in damage to biological molecules and in various forms of cancer is shown. Various classes of drugs causing photosensitivity are comprehensively discussed. The advantages and disadvantages of different types of tests (*in chemico, in silico, in vitro and in vivo*) for evaluating phototoxicity are described, and *in vitro* tests are discussed in great detail with an emphasis on the *in vitro* BALB/c 3T3 NRU test used in the thesis.

In the third section, the application of the phenomenon of phototoxicity is considered, and here the development of the regulation of the photosafety tests of substances used in medicine is followed. The method of photodynamic therapy in which selective destruction of pathological areas is caused by a photochemical reaction and its use in oncology is explained.

The purpose of the present study is formulated precisely and clearly, and the fulfillment of the set tasks guarantee its achievement.

Materials and methods adequately used in the dissertation to achieve the set tasks are described in detail. Methods include cell cultures, treatment with test substances, determination of cell viability, determination of cytotoxicity and phototoxicity, determination of cell proliferation, light and fluorescence microscopy and flow cytometric analysis. The software products GraphPad Prism 4 and Origin8 were used for statistical analysis, which convinced the credibility of the reported results.

The research presented in **Results and Discussion** (45 p.) is divided into four main sections.

Characterization of solar simulator LE-9ND55-H.

The author has done the necessary research to determine the characteristics of the solar simulator chosen for the experiments, which are not announced by the manufacturer, but are of crucial importance for the device's suitability for solving the tasks set.

A comparison was made between the spectrum of the solar simulator and the spectrum of natural sunlight. The light output of the simulator and the radiation power density distribution were determined on a two-dimensional model of a 96-well plate. The results make it possible to determine which wells can be used so that the maximum difference in the power of the radiation falling into them is 5%. The simulator has also been validated under laboratory conditions for determining phototoxicity according to a standard protocol using substances that are known from literature to have strong, medium and those that do not exhibit phototoxicity. For completeness of characterization, three different cell lines were used.

To validate the simulator further, a cytochemical analysis with two-step May-Grunwald Giemsa staining was performed, as well as a fluorescence analysis of apoptosis at different concentrations of the phototoxic substance.

Phototoxicity study of natural products.

This section is devoted to proving the effectiveness and practical applicability of the solar simulator in the phototoxicity study of plant extracts. Extracts from 4 plants prepared with different organic solvents were used and their lack of phototoxicity was shown.

The same extracts were tested for antitumor activity on 8 tumor cell lines. Antiproliferative activity was measuared with MTT assay. Original data were obtained on the effects of different extracts on different cell lines.

Experiments were performed to determine the mechanism of antitumor action of plant extracts. Using flow cytometric analysis, the distribution of cells in the different phases of the cell cycle was investigated. For this purpose, extracts and cell lines with the strongest antiproliferative activity were selected and original results were obtained, which were discussed and compared with literature data.

Studies were conducted on 4 tumor cell lines to determine the pro-apoptotic potential of selected extracts. The results show selectivity of the effects of the different extracts on different cell lines.

Safety test of newly synthesized peptides.

Within the thesis, these studies are included to show the practical application of the solar simulator and the adapted *in vitro* 3T3 NRU test for determining phototoxicity. 8 analogs of a natural antimicrobial peptide (KLAKLAK)2 were investigated and a high level of their photosafety was demonstrated.

Safety test of chemically synthesized compounds.

Derivatives of thienopyrimidines, which are a promising class of synthetic compounds with a wide range of biological actions including antitumor and antimicrobial, were also tested for phototoxicity. 6 newly synthesized compounds were used, which showed different characteristics after irradiation, but all were non-phototoxic.

In the dissertation, it is proposed that in addition to the regulated test for phototoxicity, a test using a *in vitro* model of human skin should also be done, for which keratinocytes are most suitable. Their applicability is proven in the conducted experiments.

This section also shows the results of studies on potential antitumor activity of chemically synthesized compounds. 6 thieno[3,2-d]pyrimidine derivatives and breast cancer cell lines were used. The effects of the compounds on the two tumor cell lines and the control were characterized.

The conclusions and contributions are clearly defined by the author and accurately reflect the results of her research.

The abstract is a shortened version of the dissertation and reflects the important parts of it.

Assessment of the publications and personal contribution of the PhD student

The doctoral student has published part of the obtained results in two articles. One in which she is a co-author has a high rank in the scientific field (impact factor 4.412, Q1). I am convinced of Inna Sulikovska's significant contribution. The other, in which she is the first author, also has an impact factor (0.198, Q3) and her contribution is fundamental.

The report on the fulfillment of the minimum requirements for the acquisition of the ONS "doctor" according to ZRASRB and the Regulations of IEMPAM shows the fulfillment of the requirements (90 points for a requirement of 80). The report on the received credits according to the credit system indicates fulfillment of the mandatory minimum (130 out of 130 required).

Recommendations

To work actively to apply the obtained results in practice.

CONCLUSION

The problem treated in the dissertation is relevant and significant. The objective is formulated precisely and clearly, which shows a good knowledge of the problem. A wide range of properly selected methods was used. As a result of the conducted research, data were obtained that have scientific, scientific-applied and applicable significance.

The dissertation and the abstract are very well designed. The results are discussed comprehensively, which shows a good knowledge of the literature on the problem. The doctoral student proves that she has theoretical knowledge and skills for conducting independent scientific research. The submitted materials and documents for the procedure meet the requirements in the Republic of Bulgaria and the Rules for its implementation of IEMPAM.

All this gives me reason to confidently recommend to the respected scientific jury to vote positively to award Inna Alesksandrovna Sulikovska the educational and scientific degree "doctor" in the field of higher education 4. Natural sciences, mathematics and informatics, professional direction 4.3. Biological sciences, scientific specialty Biochemistry (code 01.06.10.).

Sofia, 14.07.2022

Prof. Ludmil Penuv Kirazov, Doctor of sciences