To the honorable members of the Scientific Jury
Designated by Order No. РД-38-404/13.07.2022
of the Rector of Sofia University
"St Kliment Ohridski"
Prof. ANASTAS GERDJIKOV

Statement of Opinion

By **Assoc. Prof. Lubomira Nikolaeva-Glomb, MD, PhD**
Head of the Virology Department
of the National Center of Infectious and Parasitic Diseases (NCIPD), Sofia

On the PhD thesis in the field of higher education 4. Natural Sciences, Mathematics and Informatics, Professional Field 4.3. "Biological Sciences"
in the scientific specialty of Virology"

of Venelin Ventsislavov Tsvetkov,
BA in Molecular Biology and MA in Virology, full-time doctoral student in virology,
enrolled on July 15th, 2018 by order No. RD-20/1076 of 15.07.2018
of the Rector of Sofia University "St. Kliment Ohridski"

I hereby declare that I have no conflict of interest within the meaning of Art. 4, para. 5 of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB). I have no common publications with Venelin Tsvetkov.

The materials submitted for the defense meet the requirements of the Law and the Regulations for its implementation.

State-of-the-art, the Topic of the Dissertation and Relevance of the Problem

Human herpes viruses, some of which cause diseases known from antiquity and others that are more recently described, are ubiquitous. A characteristic feature of theirs is the fact that after the initial infection, they establish a lifelong latent infection with possible reactivations. Both primary infection and reactivations can be asymptomatic, but can also lead to clinical conditions of varying severity, including life-threatening ones. Although several highly effective and selectively acting antiherpetic drugs are available, the search for effective ways to contain, limit, and control herpes virus infection is a constantly evolving area of the science of viruology. The development of new anti herpes preparations continues with unceasing persistence, paying attention to alternative sources, such as natural products. To reveal the potential of Bulgarian medicinal plants would be a contribution to modern applied science. In this sense, the topic of the dissertation, dedicated to the search of active inhibitors of herpes

virus replication, is a relevant one, since part of it is dedicated to the antiviral effects of extracts from three Bulgarian plants.

The next part of the dissertation is devoted to physical factors affecting herpes viruses. In the context of the COVID-19 pandemic, it has become apparent that there is a need to expand our practical knowledge regarding the effectiveness of personal protective facemasks for everyday and non-professional use. Herpes virus is a convenient model because of the physical similarities between its virions and those of SARS-CoV-2 — both with an outer lipoprotein envelope, and both relatively spherical in shape and roughly the same size. In this sense, the next part of the dissertation work is extremely relevant and practically applicable.
Thirdly, the dissertation investigates the impact of low-temperature non-equilibrium gas discharge plasma on the replication of herpes viruses. Modern achievements of physics and chemistry are rapidly finding application in the field of human health. Plasma technologies are a relatively new "guest" of medicine, but they are already successfully applied in surgical endoscopic procedures and in the treatment of difficult-to-heal wounds. The inactivating effect of cold plasma on several dangerous pathogens has been established. And all this happened only in the last 10-20 years, which is a proof of the pioneering research for Bulgaria, laid down by this dissertation.

Nevertheless, I have some reserves about the wording of the title, namely ".....impact on the *realization* of herpes viruses". Despite the clarification of the use of the term realization in the course of the approbation that took place some time ago, I still think that the use of this term is inappropriate.

Literature Review

The presented literature review is divided into several parts, devoted successively to the classification, structure, and virus replication during lytic infection, the phenomenon of latent herpes virus infection, cytopathology and pathogenesis of the infection, characteristic features of the immune response, available and experimental therapy and immunoprophylaxis. Enough attention has been paid to natural products with an antiviral effect and to Bulgarian medicinal plants, in particular. An overview of the biological activities of the three plants, which are the subject of the current dissertation, is presented.

The literature review reveals the author knowledge on herpes viruses. It is spread over 42 pages and practically represents a little more than 1/3 of the dissertation work. 155 literature sources were cited, both classical and relatively recent publications on the considered

problems. However, a number of the facts described in the review are not supported by citation. Unfortunately, the review has not been updated since its last revision and the most recent source cited is from 2016. Some of the viral replication inhibitors listed in the review are no longer used in clinical practice, and others that are present on the pharmaceutical market, are not mentioned, for example, Vistide (cidofovir), the only preparation prescribed for the treatment of cytomegalovirus retinitis in HIV/AIDS patients.

The gap of more than 5 years for the rapidly developing science of virology, even more for the frontier area related to the application of plasma and non-pharmaceutical means of protection against human pathogenic viruses, is significant. The spelling and technical errors, especially those related to improper use of the articles, are annoying. Some of these remarks were also mentioned in my previous review. The indicated numbering of the citations in the text in a number of cases does not correspond to the list of references, which is a manifestation of an even more irritating carelessness. In addition, it would be much more convenient for the reader if the description of what is known so far about the three plants that are the subject of research in the dissertation, was distinguished from the rest of the overview, which refers to medicinal plants in general.

A concluding and, at the same time, a connecting paragraph with the following sections of the dissertation, which points out what studies have been conducted and what was the working hypothesis, is noticeably missing.

Purpose and Objectives of the Dissertation Research

The aim of the dissertation is practically in three directions: (i) to investigate the anti-herpes virus and virucidal effect of extracts from three Bulgarian medicinal plants, i.e. these are the biological factors referred to in the topic title. And the investigation of the physical factors accordingly includes: (ii) determining the effect on extracellular virions and on the replication of herpes viruses if the growth medium in which the herpes viruses are cultivated is treated with low-temperature non-equilibrium gas discharge plasma and (iii) determining the virus filtering effectiveness of personal protective face masks. Tasks to achieve the goal are set logically.

The Methodological Part

The methods used to determine the antiviral and virucidal activity of the studied biological and physical factors are from the arsenal of classical virology with relevant modern

modifications. These are the internationally recognized methods for testing antiviral and virucidal activity. They are adequate in relation to the answers sought. Nevertheless, most methods are described in unnecessary detail and are more didactic rather suitable for a methodical instruction, not for a scientific text. There are unnecessary repetitions of operating procedures. Numerous technical and spelling errors are made in this part of the dissertation, as well, that is especially irritating.

In several points in the "Materials and methods" section, it is mentioned that the so-called Mossman's MTT test is a test for determining antiviral activity. This is not so. The MTT test is a test to determine cell viability. In the dissertation, the test used to determine the antiviral activity was the CPE-inhibition test, and the Mossman test was used to objectively report the antiviral activity by distinguishing the preserved living cells as a result of the impact of the antiviral agent from the dead cells affected by the virus.

Results and Contributions of the Dissertation Work

Sufficient results have been obtained, corresponding to the set goals and objectives. The results are illustrated using numerous tables and figures. The Results section is divided into separate parts for each of the investigated physical or biological factors influencing the replication or the extracellular virus.

With regard to the studied plant extracts, the main chemotherapeutic indicators were determined for each of them - maximum non-toxic concentration, cytotoxic concentration 50, inhibitory concentration 50, selective index. The virucidal activity of the extracts was also investigated, but no such activity was found.

Plant extracts from three Bulgarian medicinal plants were studied - from the fruits of wild cranberry, Artemisia and Astrogallus. Cranberries were collected in two different geographical regions - in the mountain of Stara Planina and in Rhodope mountain. A total methanol extract, an extract containing mainly the flavonoids and phenolic acids, and an extract containing mainly the anthocyanins, were made from the fruits from each region, i.e. properties of 6 extracts were investigated. Astragalus was collected in the Vitosha mountain and a methanolic extract of its aerial parts was studied. Artemisia was collected in Ponor Mountain and two of its extracts, chloroform and aqueous ones, from its aerial parts were studied.

In summary, the antiviral and virucidal properties of a total of 9 extracts from 3 plants were investigated against two herpes viruses, i.e. human alphaherpesvirus 1 and 2. An antiviral

effect was found for the total extract of cranberries collected in Stara Planina, for the extract of Astrogalus and for the water extract from Artemisia. No virucidal effect was found for any of the tested extracts.

I would ask the author how he would explain the fact established by him that fraction B of the extract of cranberries collected in the area of Stara Planina had almost the same values of maximum non-toxic concentration and cytotoxic concentration 50, respectively 0.500 μg/mL and 0.556 μg/mL. A similar phenomenon, although with not so close values, is also observed with fraction B of the blueberry extract collected in the Rhodope – 0.75 and 0.84 mg/mL, respectively. What are the reasons for such a narrow range? The fact that the total extracts of cranberries collected in Stara Planina have an antiviral effect, while those in the Rhodope Mountains do not, is also a very interesting one. How could this be explained?

The second part of the dissertation is devoted to the study of the effect of physical factors and, in particular, to the determination of the virus-filtering efficiency of textile face masks and masks with a filtering face. The results obtained support the need for these personal protective equipment to limit the spread of respiratory pathogens the size of a medium-sized virus.

As for the study of the effect of treatment with low-temperature gas discharge plasma, I think that these studies are unique in the country and at all pioneering, since this field of biomedical sciences began to develop only in the last one or two decades. Developing a dissertation in this field is an outstanding contribution because it creates a specialist in an interdisciplinary field that certainly has a future ahead.

Based on the obtained results, the conclusions were formulated, with which I generally agree, but I think that nineteen conclusions are too many and unnecessarily dilute the achievements of the dissertation work. Conclusions could be much more concise. I fully support the contributions highlighted in the Statement of Originality.

Publications Connected with the Dissertation

In connection with the research in the dissertation, three papers have been published: two of them have an impact factor and a quartile. In one of them, Venelin Tsvetkov is the first author. In the third submitted article, published in the University's Annual Proceedings, he is also the first author. The presented papers are related to each of the three areas of inquiry in the dissertation.

A list of participations in international or national scientific events with presentations of the results of the dissertation is not presented.

Formatting of the dissertation and the Abstract

The submitted manuscript consists of 123 pages and follows the traditional presentation scheme: introduction – 2 pages, literature review – 42 pages (34% of the actual part of the dissertation) and personal research – 77 pages (62% of the actual part of the dissertation), the latter consisting of: aim and objectives – 2 pages, materials and methods – 28 pages, results and discussion – 42 pages, conclusions – 4 pages, and a Statement of Originality with contributions – 1 page. The list of cited literature covers 155 sources but the citation style is not uniform for individual sources. 12 figures and 6 tables illustrate the literature review, and the personal research is supported by 19 figures and 17 tables. Some of the figures are not numbered.

The abstract meets the requirements and correctly reflects the content and main achievements of the dissertation.

Conclusion

I believe that, regardless of my remarks, the presented dissertation is sufficient in terms of volume, methodical approaches, results obtained and contributions derived and represents a completed scientific work that meets the requirements for obtaining the educational and scientific degree "doctor" according to the Law and the Criteria of Sofia University "St. Kliment Ohridski". There are scientific and scientific-applied results representing an original contribution to science. There are also three papers on the topic of the dissertation, two of which are in specialized international journals with impact factor and quartile, as they are referenced in world-renowned databases.

I give my positive assessment and propose to the honorable Scientific Jury to award the educational and scientific degree "Doctor" to Venelin Ventsislavov Tsvetkov in the field of higher education 4. Natural sciences, professional direction 4.3. "Biological Sciences" in scientific specialty "Virusology".

Sofia, October 5th, 2022 Opinion prepared by:

Assoc. Prof. Lubomira Nikolaeva-Glomb, MD, PhD