

To **Prof. Dr. Hristo Gagov**,
Chairman of the Scientific Jury,
determined by Order No. RD-38-404 of 13.07.2022
of the Rector of the Sofia University "St. Kliment Ohridski"
Prof. Dr. Anastas Gerdzikov

R E V I E W

by Prof. **Neli Stoyanova Korsun**, MD, DSc
of the dissertation work of **Venelin Ventsislavov Tsvetkov** -
full-time doctoral student at the Faculty of Biology of the SU "St. Kliment Ohridski"
on the topic "Study the impact of physical and biological factors on the implementation of herpes
viruses" for awarding the educational and scientific degree "doctor"
in professional direction 4.3 Biological sciences (Virology)

As a member of the scientific jury, I declare that I do not have any joint publications and participation in scientific forums and projects with the doctoral student.

Career development of the Ph.D. student

Venelin Tsvetkov was born in 1993. He graduated from the "Geo Milev" Mathematics High School in Pleven in 2012. From 10.2012 to 07.2016 he was a bachelor's student in molecular biology at the Faculty of Biology of the Sofia University "St. Kliment Ohridski", after which he completed a master's degree in virology at the same faculty in 02.2018. His GPA during the master's degree was an excellent 6.00. Since 17.07.2018, he has been a full-time doctoral student in the Virology Laboratory of the Faculty of Biology at the SU. During his doctoral studies, he passed all five exams of his individual plan with excellent results. From 07.2017 to 12.2019, he worked as a biologist at the "Ramus" Medical Diagnostic Laboratory, Sofia, and from 12.2019 to the present moment, he is a clinical research monitor. He speaks English at the B2 level.

Relevance of the topic of the dissertation

The high incidence of herpes viral infections occurring in several cases with severe clinical manifestations (encephalitis, meningitis, keratitis, etc.), the emergence of resistance of the causative viruses to the existing antiviral drugs, and the significant toxicity of some of these drugs support the relevance of scientific problematic, aimed at the discovery of new antiviral agents with a good therapeutic effect. The study of plant-based natural products would lead to the identification of biologically active substances with a desirable antiherpetic effect, but with lower toxicity compared to synthetic preparations. Investigating the impact of physical factors such as low-temperature non-equilibrium plasma on the replication of herpes viruses would offer an alternative approach to the treatment of these infections.

General characteristics of the dissertation work

The dissertation is structured following the requirements of the Regulations for the Implementation of the Law for the Development of the Academic Staff in the Republic of Bulgaria (LDASRB). It has a volume of 123 pages and contains: title page; table of contents (3 pages); abbreviations used (3 pages); introduction (2 pages); literature review (42 pages); purpose and tasks (2 pages), materials and methods (19 pages); results and discussion (31 pages); conclusion (2 pages); conclusions (4 pages); declaration of originality (contributions) (1 page); publications related to the dissertation work (1 page); references (12 pages). The ratio between the individual sections of the dissertation meets the accepted requirements. The exposition is illustrated by 23 tables and 31 figures.

Dissertation Review and Evaluation

In the *introduction*, the classification of herpes viruses and data on the widespread prevalence of herpes simplex viruses (HSV), as well as the high incidence of these infections are presented. It is emphasized the need to develop new preparations with an antiviral activity that are not dependent on the thymidine kinase enzyme and are less toxic.

The *literature review* is detailed, informative, and well-structured. In the beginning, historical information about herpes viruses and a detailed classification of the 3 subfamilies of herpes viruses are presented. The morphology, genome, and capsid of these viruses are reviewed in detail. Considerable space is devoted to HSV replication and its latency. An important section of the review is devoted to the immune response of the human body to HSV infection and the prevention and therapy of these infections. Directly related to the topic of the dissertation is the section «Inhibitors of the viral replicative cycle», in which anti-herpes inhibitors of synthetic origin (pyrimidine and purine analogs) and inhibitors of natural origin are discussed. Numerous examples of established anti-herpetic activity of extracts from several plants are cited, which speaks to the good knowledge of the Ph.D. student on this topic. Low-temperature non-equilibrium plasma is presented as an innovative approach in anti-herpes therapy, the effect of which has been studied against various viruses: noroviruses, adenoviruses, influenza and parainfluenza viruses, HSV, etc. In this section of the review, not all reported data is supported by citations, and some citations do not correspond to the text to which they are referenced. References to literary sources are also missing in the section on the application of personal protective equipment (masks). The mechanisms of the virucidal action of the low-temperature non-equilibrium plasma have not been specified. Excluding these omissions, it can be concluded that the review is competently written and testifies to the doctoral student's excellent literary awareness, and his ability to systematize, summarize and interpret the existing scientific information on the problem.

The doctoral student's research begins with Chapter 3. *Aim and objectives*. The dissertation work aims to investigate the effect of plant extracts and solutions treated by low-temperature non-equilibrium gas-discharge plasma (LNGP) on the replication and extracellular virions of human herpes viruses. Determining the degree of protection of personal protective equipment. Three tasks with 7 sub-tasks which include the main directions in the research process are precisely formulated.

To achieve the set goals, the Ph.D. student mainly uses virological methods based on cell culture. The methods are selected correctly given the tasks set. For each investigated biological and physical factor, its possible toxic effect on cell culture (survival) is initially investigated, after which its antiviral effect is evaluated. The virucidal activity of three plant species and LNGP against two representatives of herpes viruses – HSV-1 (strain F) and HSV-2 (strain DD which is acyclovir-resistant) is studied. Acyclovir is used as a reference inhibitor. The methods are presented in great detail, which allows their reproducibility. The need to study the active forms of oxygen produced under the influence of LNGP is not well explained. Such an explanation should be presented in the literature review.

In the "*Results and Discussion*" section, all experimental setups and their results are presented sequentially. The graphical representations of the obtained data illustrate the text very well and support the analyzes made.

When examining 6 extracts from the fruits of wild cranberry *Vaccinium Vitis-idea L.* (family *Ericaceae*), collected in Stara Planina and the Rhodopes, it is found that the two total extracts have the lowest cytotoxicity. Inhibitory concentration (IC_{50}), which inhibits HSV-1 (F) replication by 50%, is reached only with the total extract from Stara Planina (1.27 mg/ml), where the maximum non-toxic concentration (MNC) is 6 mg/ml. When this extract affects the replication of HSV-2 (DD), an IC_{50} of 2.09 mg/ml is reached. Stara Planina total extract provides 74.4% and 98% protection against the first and second strains of HSV, respectively, when administered in MNC. For comparison, acyclovir in MNC provides protection of 100% and 10%, respectively.

When studying the plant *Astragalus glycyphyllos L.* (methanolic defatted extract), an IC_{50} of 0.671 mg/ml is reached against HSV-1 (F) and 0.378 mg/ml against HSV-2 (DD). In an MNC of 1 mg/ml, the extract provides 74.49% and about 70% protection against the first and the second model strain, respectively, i.e. the extract did not exhibit a strict selective action against the two strains used.

Aqueous extract and chloroform soxhlet extract of the plant *A. chamaemelifolia Vill.* are studied. Only with the aqueous extract, when it is administered in the MNC, relatively good protective effects are found: 69.32% and 67.72% against HSV-1 (F) and HSV-2 (DD), respectively. An IC_{50} of 0.58 mg/ml and 0.62 mg/ml is achieved against the two strains of HSV-1 and HSV-2, respectively, and the MNC is 1 mg/ml. In contrast to the aqueous extract, the chloroform soxhlet extract exhibits very weak activity against the two strains of HSV-1 and HSV-2 tested.

Analyzes and discussion of own results are present in the given section. However, there is a lack of comparison with the results of other researchers who have conducted similar studies to establish antiviral activity against HSV in other plant extracts. The reference list contains several such literature sources with whose data the doctoral student could compare his results. Such comparative analyzes would help to evaluate the levels of protection obtained in the investigated plants in the present study. In addition, in several places in the exposition, there is a repetition of the same text paragraphs (pages 10, 32, 69, and 71).

Comparative analyzes with literature data are also missing in the next study aimed at determining the degree of protection of personal protective equipment (face masks). Research on the protective properties of face masks about SARS-CoV-2 was of utmost importance at the beginning of the

COVID-19 pandemic, when there were many conflicting opinions on this issue. From this point of view, the conducted research is up-to-date and of great practical importance. From the results shown in Table 15, it is not clear what the difference is between masks CFC1-10. In the Conclusion section of the dissertation work, it is shown that more than 90 face masks have been studied, and in Table 15 (indicators are in English), data for 15 masks are given. The term "community face covering" used is not very appropriate - probably referring to fabric (cloth) face masks, as opposed to medical (surgical) and filtering ones.

The established antiviral effects of LNHP offer an innovative approach to the treatment of herpes diseases (for example, herpes keratitis), which has not yet been applied in Bulgaria. As a partially ionized gas composed mainly of photons, ions, and free electrons, plasma also contains biologically active atoms (O and N), molecules (e.g. O₃, H₂O₂, and HNO₂), and radicals {OH, NO, and [O₂(a1Δg)]}, with which its antibacterial and antiviral effects are associated.

The Ph.D. student has developed a suitable algorithm to study the effect of LNGP on HSV replication. Initially, the cytotoxic effect of a cell culture medium treated with such plasma is examined, i.e. the exposure time at which there is 50% cell viability, assessing the importance of plasma source power and the need to dilute the treated cell culture medium with untreated one. After determining the appropriate conditions, the effect of LNGP-treated cell culture medium on HSV-1 (F) replication is examined. No significant percentage of protection on the cell monolayer is observed in any of the 5 experimental setups. It is concluded that the treatment of cell culture medium with LNGP does not affect the replication and extracellular virions of HSV-1 (F). The Ph.D. student carries out similar studies by diluting the HSV-1 (F) viral suspension with distilled water in a ratio of 1:1 and 1:2, then treating it in the active zone of the flame for different time intervals. When treating the viral suspension, diluted with distilled H₂O in a ratio of 1:1 and 1:2 at a plasma power of 13 W, a maximum reduction of the viral titer is established by 1.23 log and 1.67 log, respectively, at an exposure time of 300 sec. The doctoral student determines the role of the reactive oxygen species produced under the influence of LNGP for the antiviral effects, as well as their type and amount. Based on the conducted research, V.Tsvetkov concludes that at early exposure intervals (5-10 sec.), the amount of active forms of oxygen is the highest, then it decreases during longer treatment due to interaction with nitrogen from the atmosphere and the formation of nitrates and nitrites. However, this explanation contradicts the fact that the strongest virucidal effect is found when the virus suspension is diluted 1:2 with distilled H₂O at an exposure time of 300 sec. How can this fact be explained?

At the end of the dissertation, the doctoral student presents a *Conclusion* summarizing the main results achieved in the research. Nineteen *conclusions* are made, which in a synthesized form repeat the results obtained in all the conducted experiments. In my opinion, it could be stated a conclusion that the total extract from the fruits of the wild cranberry *Vaccinium Vitis-idea L.* (family *Ericaceae*) collected in the Stara Planina and to a lesser extent the extracts of the other investigated plants are promising in the treatment of diseases caused by acyclovir-resistant strains of HSV-2.

I accept the 3 *contributions* mentioned in the dissertation. In my opinion, the first two contributions are original, and the third is scientific-applied.

The *literary sources* used are 155, 16 of which are by Bulgarian authors, and 4 are in Bulgarian. Only 7 (4.5%) publications are from the last 10 years (2012-2021).

Evaluation of the abstract of the dissertation

The abstract has a volume of 62 pages. Its content presents in a systematized form the main results of the dissertation work.

Publication activity and compliance with minimum national requirements

In connection with the topic of the dissertation, 3 full-text scientific publications have been published. Two of the publications are in journals with an impact factor: *Compt. Rend. l'Acad. Bulgaria Sci.* with IF_{2018} 0.251 (Q2) and *Plasma Medicine* with IF_{2020} 0.828 (Q3). The third article is published in the *Annuaire de l'Universite de Sofia "St. Kliment Ohridski". Faculte de Biologie* (ISSN 0204-9902), which is included in the National Reference List of contemporary Bulgarian scientific publications with peer review. In two of the publications, the doctoral student is the first author. It covers and exceeds the minimum national requirements for the acquisition of the educational and scientific degree "doctor", specified in the Regulations for the implementation of the LDASRB (amended and supplemented, SG No. 15 of 19.02.2019): group A (dissertation – 50 points) and group G (required 30 points). The Ph.D. student has one publication in a Q2 journal (20 points) and one publication in a Q3 journal (15 points). Thus, he collects 50 points according to indicator A and 35 points according to indicator G. He also meets the specific minimum requirements for candidates to acquire the Doctoral degree at the SU "St. Kl. Ohridski": a minimum of 2 publications, of which at least 1 publication from group I (publications in journals Q1 and Q2) or group II (publications in journals Q3 and Q4).

CONCLUSION

The dissertation work of Venelin Ventsislavov Tsvetkov is up-to-date and excellently executed with some remarks on the layout. Labor-intensive scientific research is performed, requiring great precision and experience, including using an innovative method applied for the first time in our country. The results obtained by the doctoral student represent a significant contribution to the development efforts of new antiviral products in the field of herpes diseases. The dissertation shows that the doctoral student has in-depth theoretical knowledge of the subject of the dissertation and the ability for independent research. The dissertation complies with the requirements of the Law for the development of the academic staff in the Republic of Bulgaria (LDASRB), the Regulations for its implementation (amended and supplemented in SG No. 15 of February 19, 2019), and the Regulations of the SU "St. Kl. Ohridski". I give a positive assessment and propose to the respected scientific jury to award Venelin Ventsislavov Tsvetkov the educational and scientific degree "Doctor" in professional direction 4.3. Biological Sciences (Virology).

30.09.2022 г.

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/Prof. Neli Korsun, MD, DSc/

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