

## REVIEW

By Prof. Rayko Dimitrov Peshev, Ph.D., head of Dept. "Epizootology and Infectious Diseases of Animals" in National Diagnostic and Research Veterinary Medical Institute, Sofia, according to the announced competition for "Docent" in professional direction 4.3. Biological sciences, scientific specialty Virology (Molecular Virology), announced in State newspaper, no. 65 of 28.07.2023 for the needs of the Faculty of Biology at the "St. Kl. Ohridski" University.

In connection with the competition announced by the Faculty of Biology of the SU "St. Kl. Ohridski" Professional direction 4.3 Biological sciences, scientific specialty Virology (Molecular virology), announced in State Gaset, no. 65 of 28.07.2023 for the occupation of the academic position "Docent", the documents were submitted by a single candidate, Chief assistant, Ph.D., Kalina August Shishkova. The necessary documents for the competition are presented, namely: curriculum vitae, diploma for completed higher education, diploma for educational and scientific degree doctor, certificate for the occupied academic positions of assistant, senior assistant and chief assistant, list of scientific publications, list of scientific publications for participation in the competition for "Docent", list of scientific contributions, minimum required points by groups of indicators, noticed citations, summary of the articles submitted for participation in the competition for "Docent".

Autobiographical data for the candidate: Head assistant, Ph.D. Kalina Shishkova was born on 10.10.1970 in the city of Sofia. He completed his secondary education in 1988 at 29 ESPU "G. Bakalov", Sofia, from 1992 to 1997 she completed his master's degree with honors at the Faculty of Biology, Sofia University "St. Kliment Ohridski", specialty "Molecular Biology". In 2014, he successfully defended a dissertation to obtain a PhD degree, and the topic of the dissertation was "Distribution and genetic diversity of Torque teno viruses (family Anelloviridae) in Bulgaria". From 1997 to 2002, he was a specialist Virologist at the Institute of Microbiology, BAS, from 2002 to 2004, he was a part-time assistant at the Faculty of Biology of Sofia University, from 2004 to 2010, she was an assistant at the Biological Faculty, from 2010 to 2011 senior assistant, and from 2011 until now she is the chief assistant in the "Virusology" Laboratory of the Faculty of Biology at the Sofia University. Or, for 25 years, the candidate has been engaged in virological studies.

### **Scientific publication activity**

A list with a total number of 33 scientific articles is presented, of which the first three are related to the dissertation work, and No. 2 is the abstract of the dissertation work. These articles have already been used to acquire the educational and scientific degree "Doctor" and will not be reviewed. In connection with the announced competition for associate professor, 21 publications were presented, which were printed in journals with an impact factor and with reviewers. The results in the presented articles have a scientific-fundamental and applied nature.

Scientific Communication No. 1 reports the results of the synthesis of new hydroxycinnamic acid amides of thiazole containing TFA. The antioxidant and antiviral activities of these amides against the replication of influenza virus H<sub>3</sub>N<sub>2</sub> and human herpesvirus

1 and 2 (HHV 1 and HHV 2) were investigated. Compounds 2a-c were found to be ineffective against influenza A virus. All of the tested amides showed no antiviral effect against HSV 1 and 2 in comparison with natural and unnatural C-protected amino acids.

In Scientific Communication No. 2, the antiviral activity of native mollusc hemocyanin molecules *Rapana venosa* hemocyanin (RVH 2) was investigated. A heterogeneous mixture of glycans was detected by MALDI TOF-MS. Native molecules as well as glycosylated and non-glycosylated units were investigated and only the glycosylated unit was found to have antiviral activity.

In Scientific Communication No. 3, the data from the synthesis of new esters of acyclovir with peptidomimetics were reflected and the antiviral activity against HSV 1 and 2 was evaluated in vitro. The authors conclude that pseudomimetics enhance the antiviral activity of acyclovir.

In Scientific Communication No. 4, chloroform, methanolic, ethanolic and aqueous extracts were obtained from wild and in vitro derived plants *Lamium album* and *Leonorus cardiaca* and the significant blocking of in vitro replication of HSV 1 and 2 in MDBK cells was found. It was found that the chloroform extracts have the strongest antiviral effect. Other extracts have similar or weaker effects against viruses.

In Scientific Communication No. 5 Chloroform extracts were obtained by Soxhlet extraction from in vitro and in vivo harvested plants of the *Lamium album* family, which were tested for antiviral activity in MDBK cells against HHV 1 and HHV 2. It was found that extracts obtained from wild plants have a stronger inhibitory and virucidal effect.

Scientific communication No. 6 is a review of medicinal plants in Bulgaria. The article describes the phytochemical composition and its influence on the specific stages of virus replication. The effect of 9 plant species on the life cycle of viruses is discussed.

From the *Nepeta nuda* plant, 4 extracts were obtained by extraction with chloroform and methanol, which were tested for cytotoxicity on MDBK cell cultures and the activity of the extracts against HSV 1 and 2 was determined. The methanol extract was found to be the most active against the viruses. It has been proven that 4 extracts inactivate the extracellular forms of HSV 2. From a native plant, the chloroform extract has the strongest effect, which causes 99% inactivation and slightly affects the entry of the virus into cells (scientific communication no. 7).

Scientific communication No. 8 reflects the data from the preparation of chloroform and methanol extracts from the plant *Teuricum chamaedris* by Soxhlet extraction and methanol extracts by thermostat. The extracts were tested for antiviral activity against HSV 2 in MDBK cells and the thermostated methanol extract was found to have a weaker antiviral effect. Two of the extracts tested inhibited HSV 2 replication. The extracts were found to inactivate extracellular virus, adsorption and penetration. Anti-HSV 2 activity of crude extracts of *Teuricum chamaedris* has been demonstrated for the first time.

In Scientific Paper No. 9, the antiviral activity of an aqueous extract obtained from in vivo cultured *Nepeta nuda* plant was reported, the maximum non-toxic concentration was determined and it was found that the extract significantly inhibited the replication of HSV 2. It was found that the infectious viral progeny was inhibited to 98%.

Article No. 10 reports the results of the study of an aqueous and chloroform extract of the plant *Artemisia chamaemelifolia* vill (Asteracea) in relation to the replication of two strains of herpesvirus sensitive - BA and resistant to acyclovir - DD. A dose-dependent effect was found. At the maximum non-toxic concentration, the aqueous extract exhibits weak virucidal activity. The chloroform Soxhlet extract did not affect the replication of both strains and the infectivity of the extracellular virions.

Communication No. 11 reported the effect of an aqueous extract of the *Nepeta nuda* plant on the replication of acyclovir-sensitive and -resistant human alpha herpesvirus 1 and demonstrated potent antiherpesviral activity against both species. The action of the extract was found not to be due to inactivation of extracellular virions, but rather to block adsorption but not penetration. It was found that the aqueous extract continued to exhibit antiviral activity even when added 10 h after infection. The authors believe that the extract acts on both the early and late stages of HHV 1 replication.

Communication No. 12 reflects the data from the studies of esters of anti-herpesvirus agents - genciclovir and penciclovir with bile acids and amino acid esters of acyclovir. The in vitro amino acid activity against HHV 1 and HHV 2 was evaluated. Antiviral assays demonstrated that the modified analogs of ACV and PCV were less active than the generics against HHV 1 and HHV 2.

Communication No. 13 presents data from studies of Torgo tenovirus members of the Alenoviridae family. Genetic sequences were found to be related and fall into one cluster, some of the sequences can be considered as a separate cluster, or to be the start of a new branch within this cluster, together with the reference sequences grouped in genotype 1.

Paper No. 14 reported the results of the effect of physical factors on the replication of extracellular HSV 1 virions on surface-wave gas discharge plasma-treated culture medium and distilled water and found a reduction in virus titer compared to the control.

Communication No. 15 tested hemolymph from *Rapana Venosa*, *Erifia verucosa* and mucus from *Helix aspersa* against acyclovir-susceptible strains of HHV 1 and HHV 2. All six extracts showed no antiherpesviral activity. Hemolymph fractions from *Rapana Venosa* and *Erifia verucosa* showed the highest activity - 99% inactivation of infectivity, with the effect on HSV 1 being more pronounced.

Scientific Communication No. 16 reported data from research on the *Nepeta nuda* plant and found that the wild plant contains different amounts of phenolic compounds, which are higher in wild compared to cultivated plants and exhibit higher antioxidant and anti-herpesvirus activity.

Scientific Communication No.17 found that soil composition is one of the most important factors for the amount of active molecules for the antiviral activity of the extracts. It was established that the most active is the chloroform Soxhlet extract of *Teuricum chamaedris* from the area of the village of Dobromirski. It inactivated the extracellular virions of HSV 2 up to 99.5%, and the methanol thermostated extract was 99.9%. The authors believe that the soil may influence the antiviral activity of the plant.

In article No. 18, the data from the studies of newly isolated strains of lactic acid bacteria are reflected. No direct virucidal activity was found in the tested supernatants, but 5 of the

strains had an effect on the inhibition of viral replication. Two of the strains have strong activity against HHV 2.

Communication No. 19 gives the studies on extracts of *Astragalus glycyphyllos*, a dry extract being obtained. Between 60-70% protection was found against both strains of HSV when added simultaneously and 1 h after infection of MDBK cells.

Scientific Communication No. 20 found a statistically significant association between the presence of periodontitis-causing bacteria and high-risk human papillomaviruses. The most common human papillomavirus (HPV) that gives a positive result for bacteria has been shown to be HPV 58.

In Scientific Communication No. 21, human coronavirus 229E was investigated for the evaluation of some analogues of the ion channel inhibitors amantadine and rimantadine. Amantadine derivatives show a slightly weaker inhibitory effect. The 4 R derivatives of rimantadine had the same effect as the control and inhibited viral replication by 37%. The other 2 derivatives of rimantadine have lower activity. Two of the compounds 2A and 4A showed promising binding affinity to SARS Cov 2 RNA polymerase site and SARS Cov 2 NSP 3.

From the results of the presented scientific reports of a candidate, I can summarize the important contributions in the field of experimental and medical virology can be divided thematically into several more important directions, namely:

- I. Screening of natural products for antiviral activity
  - II. Investigation of new synthetic compounds for the presence of antiviral effect
  - III. Medical virology
  - IV. Study of the antiherpetic effect of physical factors.
- I. Screening of natural products for antiviral activity

Investigation of natural products for antiviral activity, which are presented in scientific reports (No 4, 5, 6, 7, 8, 9, 10, 11, 16, 17, 19). They reflect the main scientific activity of the candidate and are a priority for the research work of the Virology Laboratory of the Faculty of Biology. The action of natural products has been studied on the HHV 1 and HHV 2 model. In recent years, quite a few drug-resistant herpes virus strains have been selected and appeared, which is also the reason for the study of new natural products for antiviral activity. The contributions relate to the study of the antiherpetic activity of extracts isolated from in vitro grown plants. Therefore, almost half of the works of the candidate are of such a focus, as a large number of extracts, their fractions and active substances isolated from plants have been studied. Through the conducted studies, the probable mechanisms of the antiviral effect have been clarified and the potential opportunities for the development of anti-herpes preparations for use in human medicine have been established.

The most important contributions related to the screening of natural products for antiviral activity: Demonstration of the inhibitory and inactivating effect against the strains of both types of HSV, including the acyclovir resistant strain DD of HSV-2 by chloroform, methanol, water and ethanol extracts obtained by different methods from native, laboratory-cultivated and reintroduced specimens of plants from the Lamiaceae family - *Lamium album* L. and *Leonorus cardiaca* L. A strong antiherpetic effect of the methanolic and aqueous extract

has been proven. Since medicinal plants are rich in secondary metabolites contributing to their adaptive immune response, the metabolic activity of *N. nuda* operating under variable environments was investigated. Through a comparative analysis of wild-grown and in vitro cultivated plants, the change in phenolic and iridoid compounds and their associated immune effects were evaluated.

The studies of the antiviral activity of Bulgarian medicinal plants from the last thirty years was summarized in the review. The effect of various extracts obtained from wild and in vitro propagated plants was also investigated. In a review article (No. 6) the phytochemical composition and its influence on specific stages of the viral life cycle are discussed in which the families: Amaryllidaceae, Fabaceae, Geraniaceae, Lamiaceae, Onagraceae, Ranunculaceae, Rosaceae, Crophulariaceae and Rhodophyta are considered as most potent.

Together with researchers from IOHCF and abroad, the antiviral action of hemocyanins (both whole molecules and structural and functional subunits) isolated from the Black Sea rapana (*Rapana hemocyanin*) (No2) and representatives of the genus Mollusca, *Eriphia verrucosa* (hEv), mucus from *Helix aspersa* (Ha) and structural subunit  $\alpha$ -HaH from hemocyanin from *H. aspersa* (sHa) relative to BHC-1 replication (No15). The antiviral effects of native RvH and *Helix lucorum* hemocyanin (HIH) molecules, their structural subunits and the glycosylated functional unit RvH2-e and the non-glycosylated unit RvH2 against HSV virus type 1 were investigated.

Secondary metabolites were obtained from fermented products of lactic acid bacteria (No18), which were investigated for antiherpetic activity. The mechanisms of action of probiotic strains have been studied and found to exert antiviral effects through various mechanisms, including direct interaction with viruses, production of antiviral compounds, or modulation of the immune system. It was found that two of the five strains studied - *Lactobacillus delbrueckii* subsp. *Bulgaricus* KZM 2-11-3 and *Lactiplantibacillus plantarum* KC 5-12 have strong activity against HHV-2 with a selectivity index (SI) above 45, which is a good premise for further research.

## II. Research of new synthetic compounds for the presence of an antiviral effect

Another direction in which the candidate has contributions is the research of newly synthetic compounds for the presence of an anti-herpes effect. On the basis of the knowledge of the structure and functions of antiviral preparations used for treatment, new substances have been developed and applied through directed synthesis. These are thiazole hydroxycinnamic acid amides containing TFA, valine-4-carboxylic acid ethyl ester.

They were tested against the in vitro replication of influenza virus A (H3N2) and human herpesvirus 1 and 2. Also new esters (No3 and No12) of acyclovir with peptidomimetics were synthesized and their antiviral activity on herpes simplex replication was evaluated virus type 1 (HSV-1) and type 2 (HSV-2) in vitro. Peptidomimetics containing oxazole and thiazolyl-thiazole moieties have been found to enhance antiviral activity. Esters of the antiherpetic drugs ganciclovir and penciclovir by bile acids (cholic, chenodeoxycholic and deoxycholic) and amino acid esters of acyclovir were obtained, and their in vitro antiviral activity against HHV 1 viruses was determined, and it was shown that the modified analogues of ACV and PCV were less active than generic agents against HSV-1 and HSV-2. Studies of the human coronavirus 229E (HCoV-229E) with some analogues of the ion channel inhibitors amantadine and

rimantadine are interesting, and the derivative A 4 has been shown to have higher antiviral activity than Amantadine (No21). The molecular structures of the newly synthesized compounds were investigated by single crystal X-ray analysis. Molecular docking studies showed that two of the investigated compounds 2A and 4A have good binding affinity to SARS-CoV-2 RNA-dependent RNA polymerase site and SARS-CoV-2 Nsp3 site).

### III. Medical virology

In the field of medical virology, one of the most important contributions was the demonstration for the first time in the country of Torque Teno viruses (No13) by adapting different primer systems. In samples from blood donors, patients with registered viral hepatitis, with a primary brain tumor, with respiratory diseases, kidney transplant and patients with unknown etiology, the Torque Teno virus has been proven. During genomic analysis, it was found that most of the sequences of the Bulgarian isolates are genetically related, highly correlated and fall into one cluster. The analysis shows that they are closely related and most likely have a common ancestor. Some of the sequences can also be considered as a separate cluster or the start of a new branch within this cluster, together with the reference sequences grouped by literature data into genotype 1. In patients with periodontitis (No. 20), a relationship was established between the bacteria causing the disease and high-risk human papillomaviruses (HPV), and a statistically significant relationship was demonstrated between the presence of periodontitis-causing bacteria and high-risk strains of HPV, as the most common papilloma genotype is HPV58.

### IV. Study of the antiherpetic effect of physical factors.

Another important direction in the studies is the study of the impact of physical factors on the replication and extracellular virions of HHV 1 (No14). Surface wave gas discharge plasma treated culture media and distilled water were tested for antiviral and virucidal activity and reduction with 1.67 log<sub>10</sub> in the titer of the virus sample was found compared to the control. The impact of physical factors on VHS 1 has been established.

### **Fulfillment of minimum national requirements**

From the reference for the minimum national requirements under indicator - 1 A for a defended dissertation the candidate has 50 points, under point B indicator 2 – no points, under point B indicators 3 and 4 there is a total of 100 points with 2 articles in Q 1 – 50 points, 1 article in Q 2 – 20 points and 2 articles in Q 3 – 30 points. According to indicator D - a sum of indicators from 5 to 10 according to the requirements of the Law, there should be 200 points, and she exceeds this indicator and collects 258 points as follows: of 2 articles in Q 1 are 50 points, from 5 articles in Q 2 are 100 points, from 4 articles in Q 3 are 60 points and from 4 articles in Q 4 are 48 points. In indicator group D, the sum of the points for indicator 11 should be 50, and it presents 30 citations, which bring it 60 points, that is, it exceeds the requirements for this indicator as well. From the presented scientific reports and citations, it can be seen that the candidate fulfills the minimum national requirements for docent.

### Additional evidence for the doctoral research activity

From the report on the participation in scientific projects the candidate is participated in 20 scientific research projects, as a leading contractor. She was the supervisor of 9 graduates who successfully defended their theses. As a result of the research carried out since 2009, 25

articles in scientific journals, 1 article in a series and 7 articles in collections have been published. The candidate has 48 participations in international conferences and symposia with 25 posters, 1 plenary report and 20 sectional reports. From the reference on the citations, it can be seen that until now the chief assistant dr. Shishkova has 137 citations of her scientific articles, which speaks of their quality and that she is recognizable by the scientific community. The candidate, in co-authorship with A. Hinkov, has written and published a manual for practical classes in virology, according to which students and graduates of the Faculty of Biology of the SU are studied. From the reference made from the individual study plans, it is also visible the huge study workload, as for the last 6 years there are 3181.5 hours of study work, of which 1803.9 hours are classroom work.

### **Conclusion**

The scientific - research and applied achievements presented to me by the chief assistant d-r. Kalina August Shishkova, and the received results in the field of virology give me the full right to conclude that she meets the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria, the Rules for its Application in the Biological Faculty of Sofia University. The candidate has been working for more than 25 years in the field of virology, during which time significant research and applied results have been obtained, which have been presented in front of scientific community. The participation in the education of students in Biology is tangible, which can be seen from both classroom and study hours. The scientific metrics and indicators have been fulfilled and meet the requirements for acquiring the academic position "Docent". I will vote positively and allow myself to recommend to the members of the Scientific Jury and to the members of the Scientific Council of the Faculty of Biology at Sofia University to vote positively for awarding of academic position "Docent" to the chief assistant d-r Kalina August Shishkova in professional field 4.3 Biological Sciences.

Sofia 8.11.2023

Reviewer:

Prof. Rayko Peshev, DSc