SOFIS UNIVERCITY „ST. KLIMENT OHRIDSKI“

FACULTY OF BIOLOGY

LABORATORY „VIROLOGY“

Venelin Ventsislavov Tsvetkov

# Study the impact of physical and biological factors on the implementation of herpes viruses

Abstract
of a dissertation
for awarding the educational and scientific degree "doctor"
Professional direction 4.3 Biological sciences
(Virusology)

**Supervisor:**
Prof. Stoyan Angelov Shishkov

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Given the emerging COVID-19 pandemic, there is a global need for effective, inexpensive, easy-to-produce, and accessible personal protective equipment (face masks) to reduce the transmission of aerosol-borne pathogens. In this regard, some research laboratories focused their efforts on determining the degree of effectiveness of protective face masks. The Virology laboratory team joined the joint effort to determine the effectiveness of manufactured and marketed face masks with filtering efficiency. Out of more than 90 filtering efficiency face masks provided to us for research, after conducting the experiments to determine the degree of protection by applying a modified VFE method, the results showed that the FFP type filtering face masks provide the greatest degree of protection compared to the Public Face Masks masks, with a filtering efficiency of 99%. ........................................ 47

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# Introduction

Members of the Herpesviridae family are one of the most widespread viruses in nature. Isolated from all vertebrates, they cause diseases with different severity and clinical picture - from asymptomatic infections and self-limited skin lesions, to severe generalized infections, meningitis, encephalitis and malignant neoplasms. Among the more than 90 members of the Herpesviridae family, there are nine human herpes virus pathogens: Human alphaherpesvirus 1 (HSV-1); Human alphaherpesvirus 2 (HSV-2); Human alphaherpesvirus 3 (VZV); Human betaherpesvirus 5 (CMV); Human betaherpesvirus 6A; Human betaherpesvirus 6B; Human betaherpesvirus 7 (HHV-7); Human gammaherpesvirus 4 (EBV) and Human gammaherpesvirus 8 (KSAV) divided into three subfamilies: Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae. Essential for human pathology are HSV-1 and HSV-2, which are also the subject of research in this dissertation.

HSV-1 and HSV-2 have plagued humanity for centuries and are the cause of hidden pandemics of labial and genital herpes. Primary HSV infection occurs by age 5 and is usually asymptomatic. According to the latest data, more than 536 million people in the world are infected with HSV-2 (genital herpes), with more than 23.6 million newly infected people annually. Globally, the region most affected by HSV-2 is sub-Saharan Africa. Seroprevalence in these regions reaches over 80% of the population over 35 years of age. In some parts of the world, the seroprevalence of HSV-1 is over 90% [8]. Even more disturbing is the fact that in 80% of cases the virus is transmitted in the absence of skin lesions or other symptoms. This phenomenon is known as "asymptomatic viral transmission". A risk contingent for new infection are newborns, in which the infection can be fatal, and according to WHO data for 2011, such risky births were 1 in every 3,000 cases in the United States. Another socially significant disease associated with HSV 1 is herpes simplex keratitis. HSV-1 is the most common cause of blindness in the Western world. In the USA alone, 300,000 new cases of ocular infection caused by HSV 1 are reported annually[134].

In the fight against herpes infections, various medications are used, the most widely used of which are nucleoside analogs, which cause defects in the replication of the viral nucleic acid. A significant problem associated with the effects of this class of drugs is their dependence on the viral enzyme thymidine kinase (TK). Viral strains with a mutant TC or lacking one were not affected by the treatment. Among the large number of highly active medicinal preparations that have been created, there are those that have many side, unwanted effects on the body. That is why phytoproducts are of increasing interest, i.e. biologically active substances contained in plants. They are very well tolerated by the human body and are often less toxic. Medicinal plants have by no means lost their importance, despite the increase in the relative share of synthetic preparations. Total plant extracts, due to the fact that they contain more than one biologically active substance, mimic combination therapy with several synthetic preparations (a major approach to overcome the emergence of drug resistance).

The development of technologies makes it possible to study the body's reactions to the application of various innovative therapies and to follow the impact of technologies on viral and bacterial infections in vitro and in vivo. Plasma-based technologies occupy an increasingly large

place in innovative treatment methods. One of the fastest growing areas in plasma technology is so-called plasma medicine, an emerging field of medicine that exploits the ability of non-equilibrium plasmas to initiate, control and catalyze various complex behaviors and reactions in biological systems. Non-equilibrium plasmas are non-destructive to tissues, safe and effective against bacterial and viral pathogens.

# Purpose and tasks

1.1 Purpose

The aim of this dissertation is to investigate the effect of plant extracts and low-temperature non-equilibrium gas discharge plasma solutions on the replication and extracellular virions of herpes viruses. Determining the degree of protection of personal protective equipment.

2.2 Tasks

To fulfill the set goal, the following tasks were identified:

2.2.1 Investigating the effect of plant extracts on the replication and extracellular virions of human herpesvirus type 1, strain F and acyclovir-resistant herpesvirus type 2, strain DD

2.2.1.1. Establishing the survival of cells from cell line MDBK when exposed to plant extracts. Determination of the values of MNK and CK50.

2.2.1.2. Determination of the effect of the studied extracts on the replication of HSV-1 and HSV-2. Investigation of the survival of cells infected with the respective virus strains and treated with the investigated extracts.

2.2.1.3. Determination of the effects of the extracts on extracellular virions.

2.2.2. Determining the degree of protection of personal protective equipment by applying a modified VFE method.

2.2.2.1. Determination of virus filtering efficiency.

2.2.3. Effect of plasma-source activated culture medium and water on replication and extracellular virions of HSV-1 strain F

2.2.3.1. Investigation of the cytotoxic effect of a culture medium treated with the used low-temperature non-equilibrium gas-discharge plasma.

2.2.3.2. Determination of survival of virus-infected cells and treated with low-temperature non-equilibrium gas-discharge plasma-activated culture medium, by MTT assay.

2.2.3.3. Investigating the effects of low-temperature non-equilibrium gas-discharge plasma on extracellular virions.

2.2.3.4. Determination of type and amount of reactive oxygen species (RONS) produced under the influence of low-temperature non-equilibrium gas discharge plasma

## Materials and methodology

A. Investigating the effect of plant extracts on the replication and extracellular virions of human herpesvirus type 1, strain F and acyclovir-resistant herpesvirus type 2, strain DD

### 1. Materials

### 1.1. Cell line

All experiments were performed with the Madin-Darby Bovine Kidney (MDBK) monolayer epithelial-like cell line. The origin of the mentioned cell line is from the kidney of a bull (Bos. taurus.). The cell line sustains productive infection at high infectious doses of HSV, making it suitable for processing and analyzing experimental data.

### 1.2. Medium

MDBK cells were cultured in Eagle's medium (selected from a catalog for the appropriate cell line). MEM (Dulbecco modified) - DMEM (Biochrom), enriched with FBS - 8% for the growth and 4% for the maintenance medium, antibiotic (Gentamycin 0.008 mg/ml) and 10 mM HEPES. The acidity of the medium was adjusted with 7% NaHCO3 solution and 1% HCL solution.

### 1.3 Viral model

In our experiments, the first type of Herpes Simplex Virus (HSV-1), strain F, and the second type of Herpes Simplex Virus (HSV-2), strain DD, which is acyclovir resistant, were used. Viruses were cultured in a 24-hour monolayer culture of the MDBK cell line with DMEM (with 4% FBS.) maintenance medium. For this purpose, 15 ml of cell suspension with a cell density of 1.5x105 cells/ml was poured into plastic mattresses for cell cultivation (Orange Scientific) with an area of

25 cm2. After the formation of a dense monolayer (usually after 24 hours), 0.2 were inoculated into the culture ml undiluted virus suspension. After 1 hour of adsorption at room temperature, 15 ml of DMEM medium (with 4% FBS.) was added. The mattresses were incubated in a thermostat (Memmert) at 37°C. Virus-induced cytopathic effect (CPE) was reported daily. At 90-100% coverage of the cell monolayer—most often after 24 hours—mattresses were frozen. After freezing and thawing once, the resulting viral stock was titrated and dispensed into epenfrots stored at -70°C in a cold room.

1.3. **Solution of MTT [(3-4,5 dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide] working solution (Sigma).**

*The solution (with a concentration of 5 mg/ml) was prepared by dissolving 5 mg of dry MTT in 1 ml of PBS, then filtered through a Millipore filter (pore size 0.22m) and stored at -20C until its use. Lysing solution for the MTT test – DMSO (dimethylsulfoxide).*1.4 Растителни екстракти

### 1.4.1. Plant extracts from *in vivo* cultivated *Vacinium vitis-idaea L.*

In the current dissertation, extracts from the fruits of the wild cranberry Vacinium vitis-idaea L. (family Ericaceae) were studied. The fruits used were collected in September 2018 from: Stara Planina (M. Beklemeto) and Rhodopi (H. Perelik) . Fruits were collected from pre-marked plants in the middle of the harvesting period characteristic of the respective region and were transported to the laboratory within 24h. After sorting to remove unripe, overripe or injured specimens, fruits were frozen in liquid nitrogen, ground in a grinder (TussueLiser, QIAGEN) and lyophilized (CHRIST, ALPHA 1-2 LD plus).

A total of six extracts were obtained - total methanol extract (base extract), fraction B (phenolic acids, flavonoids) and fraction C (anthocyanins). The type of extracts is presented in Table 1.

**Table 1. Description of the used extracts**.

| Plant | Family | № | Solvent | Volume(ml) | (g) | Conc. (mg/ml) | Съкратено наименование |
|---|---|---|---|---|---|---|---|
| *Vaccinium Vitis-idaea* | *Ericaceae* | 1 | DMSO | 1.8 | 1.824 | 1013.3 | S.t.pl. Total |
| | | 2 | DMSO | 1.9 | 1.969 | 1036.3 | Rod. Total |
| | | 3 | DMSO+MET | 1.1+1.1 | 1.027 | 466.8 | S.t.pl.B |

| *L.* | | 4 | DMSO+MET | 0.9+0.9 | 0.934 | 518.9 | Rod B |
|---|---|---|---|---|---|---|---|
| | | 5 | DMSO+MET | 1.3+1.3 | 1.298 | 499.2 | S.t.pl.C |
| | | 6 | DMSO+MET | 1.4+1.4 | 1.402 | 500.7 | Rod C |

Extracts of *Vaccinium vitis-idaea L.* were made by assoc. prof. Ivayla Dincheva - ABI, Sofia.

### 1.4.2. Extracts from *in vivo* cultivated plants *Astragalus glycyphyllos L.*

In the current dissertation, a methanolic defatted extract from the aerial part of Astragalus glycyphyllos L. was investigated. The plant material was collected in the month of June 2017 from a site in Vitosha during the flowering phase. A specimen with the number SOM-1400 has been deposited in the IBEI Herbarium, BAS.

The extract was provided by Prof. I. Krasteva from the Faculty of Pharmacy at the Medical University - Sofia.

## 2 Methodology

### 2.1. Subcultivating of the used cell line

The medium in which the cells are cultured is poured off (withdrawn) and discarded. The monolayer was washed three times with phosphate buffered saline (PBS) or Ca 2+ and Mg 2+ - free saline or EDTA for 1-2 min, shaking the dish, then discarding the solution. Trypsin-versin dissociating solution pre-warmed to 37°C was added. After 3-10 min, when the cells are completely detached, the dissociating solution is withdrawn. A small volume of DMEM growth medium was added and cells were resuspended. Cells from the suspension were counted and brought to a defined volume at a density of 1.5 x 10 5 cells/ml, then plated onto mats (Orange Scientific) or cell culture plates. The cells were incubated in a thermostat at 37°C.

### 2.2. Establishing the survival of cells from cell line MDBK when exposed to plant extracts. Determination of the values of MNK and CK50

Cytotoxic concentration 50 (CC50) is defined as the concentration of the tested substance at which 50% destruction of cells occurs as a result of the toxic action of the substance.

The maximum non-toxic concentration (MNC) is defined as the highest concentration of the test substance that does not cause damage or death to the treated cells.

The MTT method described by Mosmann (Mosmann, T., 1983) was used to determine CC50 and MNC. 20 years ago Tim Mos.mann proposed to use the tetrazolium salt 3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyltetrazolium bromide (MTT) to determine cell viability. MTT is a water-soluble tetrazolium salt, imparting a yellowish color to the solution. Placed in the cellular environment, MTT passes into the cellular cytosol, reaches the mitochondria, and with the help of mitochondrial dehydrogenases, the tetrazolium ring is torn off and the soluble yellow salt is converted into a water-insoluble formazan product with a blue color. The ability of cells to reduce MTT is an indicator of mitochondrial integrity and activity, which could be interpreted as a measure of viability and/or cell number. To measure the amount of formazan, the cells are treated with a solution that lyses them and simultaneously dissolves the blue crystals. The amount of the latter is measured spectrophotometrically at a wavelength of 540 nm. Dead cells do not participate in this conversion, due to the non-functioning of their mitochondria. The amount of transformed MTT to insoluble formazan crystals is directly proportional to the number of living cells and provides information on cell viability. To determine the toxicity of the extracts we studied, the following working protocol was used:

> After counting the cells, the latter were resuspended in DMEM growth medium (with 8% FBS), followed by plating the cell suspensions, at a concentration of 1.5 x 10 5 cl/ml, in sterile 96-well plates (Orange Scientific), per 0.2 ml per well. End rows and columns sometimes showed volume reduction with longer culture and were not seeded with cells. Only saline was instilled there. When the cell monolayer reached between 90 – 100% confluency (typically after 24 h), the supernatant was decanted and 0.1 ml of DMEM maintenance medium (with 4% FBS) and 0.1 ml of the previously prepared dilutions for each substance were added. At least 3 wells were instilled with each dilution of the substance. Only culture medium without substance (0.2 ml each) is dripped into several wells and serve as cell control. Since the used volume of substance (of a given concentration) when dripped into the well is diluted twice, the actual concentration of each added dilution is twice lower than the previously prepared one. The plate thus treated was incubated in a thermostat (Memmert) at a temperature of 37°C for 72 hours. At the end of the third day, 0.02 ml of MTT working solution was dripped into each well (except for the end rows and columns) (making its final concentration in the well 0.5 mg/ml), after which the plate was incubated in a thermostat ( Memmert) at 37oC for 30 min. The nutrient medium, with MTT dissolved in it, is removed after incubation, after which 0.2 ml of lysing solution is added. The plate thus treated was read spectrophotometrically at 540 nm using

an ELISA reader Multiscan MX. Cell survival was determined as the percentage of live cells in wells treated with various concentrations of the test substance compared to control untreated cells. For this purpose, the following formula is used:

$$\% \text{ cell vitality} = \frac{\text{A540 optic density of treated cells}}{\text{A540 optic density of contorll}} \times 100$$

From the constructed curve "dose (concentration) of the substance - cell survival" the CC50 and MNC are calculated.

## 2.3. Determination of infectious virus titer

### 2.3.1. Reed-Muench finite dilution method (Reed & Muench, 1938):

After counting the cells, the latter were resuspended in growth medium DMEM (with 8% FBS). The cell suspensions were then plated at a concentration of 1.5 x 10 5 cl/ml in sterile 96-well plates (Orange Scientific) (0.2 ml per well). Dropping tenfold dilutions of the virus suspension in DMEM (with 4% FBS) medium were prepared immediately before use. Upon reaching 90 – 100% confluency of the monolayer (typically after 24 hours), the supernatant in each well was decanted. The cell monolayer in the wells was infected with the previously prepared tenfold dilutions of the virus. The volume of the inoculum for one well is 0.1 ml. With each dilution of the virus, 4 wells are infected. Four wells were instilled with culture medium alone and served as cell controls. The virus was adsorbed for one hour in a thermostat (Memmert) at a temperature of 37°C, after which 0.1 ml of DMEM medium (with 4% FBS) was added to each well. The plate treated in this way is incubated in a thermostat at a temperature of 37°C. The results are recorded microscopically at 48 hours after infecting the cells. The infectious viral titer is determined by the presence or absence of a viral cytopathic effect and is calculated according to the following formula:

T = (log of dilution where % infected is above 50%) + (proportionality factor x log of dilution factor)

Proportionality factor = [(% infected above 50%) – 50%]/[(% infected above 50%) – (% infected below 50%)]

## 2.4. Determination of the effect of the studied extracts on the replication of HSV-1 and HSV-2. Investigation of the survival of cells infected with the respective virus strains and treated with the investigated extracts.

The antiviral activity of the studied extracts was determined by the MTT test developed by Mosmann (Mosmann, T., 1983) and modified by Pauwels, Takeuchi, Sudo for rapid screening of compounds for antiviral activity (Pauwels, R. et al., 1988; Takeuchi, H. et al., 1991; Sudo, K. et al., 1994).

Under the influence of the cytopathic action of the virus, the cells die. Determination of cell viability, by means of the MTT test of virus-infected and substance-treated cells, is indicative of the antiviral effect of the substance used. From the data presented in the literature, the antiviral effect should be considered when the ratio of the absorbance of the viral control to the absorbance of the cell control is less than or equal to 0.2 (80% inhibition of the formation of formazan crystals in the respective cell line infected with virus). To adapt the method to the cell line we used, the time for which the values described above are reached when infecting the cells with a different viral dose was determined in advance. The following work protocol is used to determine the antiviral activity of the extracts studied by us:

After counting the cells, they were resuspended in DMEM growth medium (with 8% FBS). The cell suspensions were then plated in sterile 96-well plates (Orange Scientific) at a cell concentration of 1.5 x 105 cl/ml, 0.2 ml per well. In the end rows and columns, only physiological solution is dripped. When the cell monolayer reaches between 90 – 100% confluency (typically after 24 hours), the supernatant in each well is decanted. Infection of the cell monolayer follows. The inoculum volume per well is 0.1 ml, at a working dose of 100 TKID50. In several wells (at least three), 0.1 ml of DMEM medium (with 4% FBS.) serve as a cell control is dripped. The virus adsorbs for one hour in a thermostat at a temperature of 37oC. During virus adsorption, dilutions of the investigated substances are prepared. After the virus adsorption time has elapsed, the plate is processed as follows:

- Control cells (non-infected with virus and non-treated cells) - 0.1 ml of DMEM medium (with 4% FBS) is added to the wells designated for cell control (at least three).
- Virus control (virus-infected and substance-untreated cells) – 0.1 ml of DMEM medium (with 4% FBS) is added to the wells designated for virus control (at least 3).

- Virus-infected and substance-treated cells – 0.1 ml of the previously prepared dilutions of the tested extracts are instilled into the wells, with each dilution instilling at least three wells.

When interpreting the results, it should be borne in mind that the concentration of each added dilution is two times lower than the previously prepared one.

The plate treated in this way is incubated in a thermostat at a temperature of 37°C for 5-6 days (depending on the development of the cytopathic effect in the viral control).

0.02 ml of MTT working solution (making its final concentration in the well 0.5 mg/ml) was dropped into each well (except for the end rows and columns), after which the plate was incubated in a thermostat (Memmert) at 37℃ in for 30 minutes. After the incubation, the nutrient medium, with MTT dissolved in it, is removed and 0.2 ml of the lysing solution is added. The plate thus treated was read spectrophotometrically at 540 nm using an ELISA reader Multiscan MX.

The activity of the substance is expressed as a percentage of protection (survival) of the cells and is determined by the following formula:

[ODv – ODVC]

---------------------- X 100 (%), where:

[ODCC. – ODVC]

ODV – absorption of infected and substance-treated cells

ODVC – uptake of virus-infected cells (viral control without substance)

ODCC. – absorption of uninfected and untreated cells (cell control).

*For the reliability of the experiment, it is important that there is no cytopathic effect in the cell control and that the ratio of the absorbance of the viral control to the absorbance of the cell control is less than or equal to 0.2. Acyclovir was used as the reference inhibitor.*

## 2.5. Evaluating $IC_{50}$ and SI

The inhibitory concentration 50 (IC50) is the concentration of the test substance that inhibits viral replication by 50%. The IC50 calculation takes into account the data from the microtiter MTT test. The IC50 is determined directly from the dose-response curve. Selective index for the samples was evaluated by the formula:

IC50/CC50 = where:

IC50 – Inhibitory concentration -

CC50 – Cytotoxic concentration

## 2.6. Determination of the effects of the extracts on extracellular virions

Impact on extracellular virions was determined by a direct contact method. Equal volumes (0.25 ml each) of undiluted virus suspension and maintenance medium with the extract in MNK, in the control of equal volumes of undiluted virus suspension and maintenance medium (without extract), were incubated at 37°C for 5 min., 15 min., 30 min., 60 min., 120 min. and 240 min. At the end of each time interval, samples and controls were frozen, thawed, and their titers determined according to the method of Reed and Muench (Reed and Muench, 1938).

B. Determining the degree of protection of personal protective equipment by applying a modified VFE method.

## 1. Materials

## 1.1 Six stage impactor of Andersen

A six stage Andersen cascade impactor, model FSC-A6 manufactured by Honri Airclean Technology Co., LTD, with an additional modified first segment to better seal the edges of the test material. Each of the impactor segments has progressively decreasing openings (from 1.18mm to 0.25mm), simulating the size of the alveoli in the human lung.

## 2. Methodology

### 2.1 Virus Filtering Efficiency (VFE)

The method is based on EN 14683:2019 + AC:2019. Filtering efficiency was measured on subject HSV 1 and cell line MDBK. The virus suspension was aerosolized by the MAD nebulizer at each test. The concentration used was 3x106 infectious doses 50 (CCDI50) in 3ml DMEM for 120 seconds. The resulting aerosol is sucked into the system through an opening in the uppermost segment of the six-stage Andersen cascade impactor, with a test petri placed on the entire segment. The air flow rate is maintained at 28.3 l/min. Samples as well as positive controls were obtained by the VFE method. A negative control was performed without the presence of virus in the aerated stream to determine if additional elements affecting the performance of the staging were present. The filtering efficiency was calculated by determining the viral titer of the entire segment of the Andersen imapactor using the method of Reed and Mench.

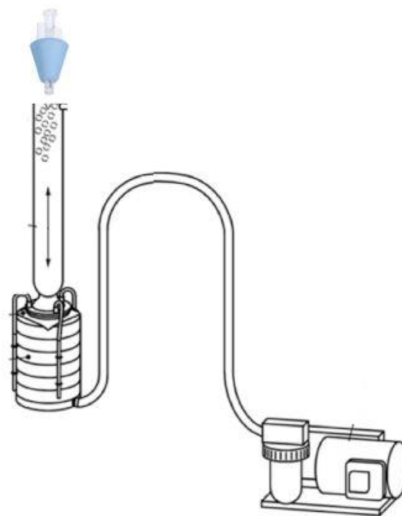


**Figure 1.** Schematic representation of an experimental setup for virus filtering efficiency

C. Effect of plasma-source activated culture medium and distilled water on HSV-1 replication and extracellular virions

## 1. Materials

### 1.1 Plasma source:

A surface wave discharge in argon at atmospheric pressure was used in all experiments. The plasma is created by a surface electromagnetic wave with a frequency of 2.45 GHz, a gas flow of 5 l/min in a quartz tube with an outer diameter of 8 mm and an inner diameter of 4 mm (tube 8/4). When conducting the experiments, three powers were used - 13W, 15W and 20W, in which the wave creates an argon plasma torch outside the quartz tube in the air. This allows direct treatment of the samples in the active zone of the discharge. The gas discharge conditions are selected so that the gas temperature of the plasma does not exceed 40°C. Characteristic of the plasma installation with which we work in this thesis is that the treatment of the samples is carried out in the active zone in which the plasma is created. In the case of dielectric barrier discharge and plasma jet, the treatment is carried out by afterglow outside the active zone of the plasma discharge. An advantage of the surface wave discharge is the absence of electrodes that dust and contaminate the plasma. Another feature of the available plasma installation is that a higher plasma density is obtained compared to other electrodeless discharges at the same power. Thanks to the high wave frequency and the small power at which it works, the results are achieved in a short time and with cold plasma – the plasma torch does not exceed a temperature of 40 °C, (figure 2). Thus we eliminate the thermal effect and the results are due to the other factors produced by the plasma discharge

Plasma is a multicomponent system of electrons, ions and neutral particles. Each type of particle is characterized by a different average energy. The temperature of the corresponding component can be defined when the system is in thermodynamic equilibrium.

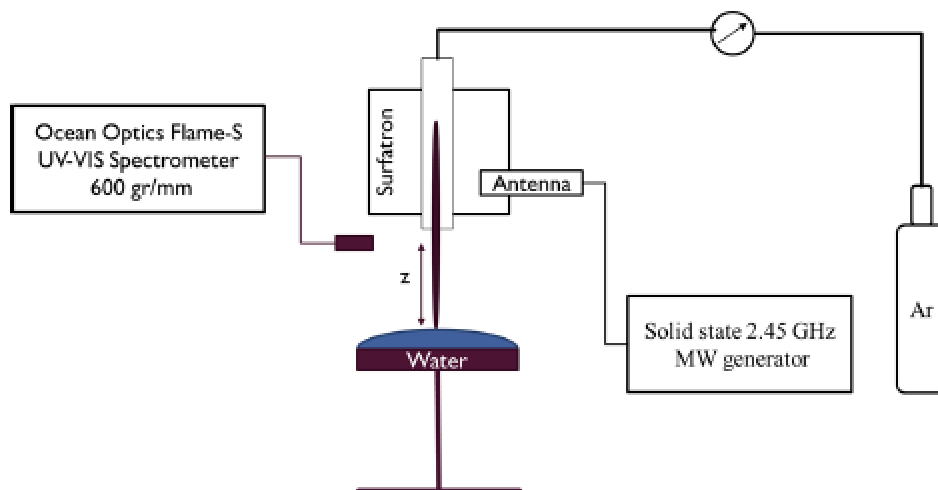
The ion temperature, or plasma temperature, can be expressed in energy units. According to her, plasma is divided into:

- Low temperature – at $T_i < 10^5$ K
- High temperature – at $T_i > 10^5$ K

Based on the average kinetic energy of the particles in the plasma, it can be divided into:

- equilibrium - at thermodynamic equilibrium, the electron, ion and neutral particle temperatures are equal;

- non-equilibrium – any of the components is not an equilibrium function; plasma in local thermodynamic equilibrium - parameters change in space and time, but due to the high rate of change at a given point, we can assume that we have thermodynamic equilibrium.




**Фигура 2.** Schematic representation of an experimental setup of a surface wave discharge in argon at atmospheric pressure (Tsvetkov V., et all; Effect of Plasma-Activated Medium and Water on Replication and Extracellular Virions of Herpes Simplex Virus-1; 2020; Plasma Medicine, 10(1):15-26 (2020)

## 1.2. PEG 8000 (polyethyleneglycoll 8000)

A 60% solution is prepared as 0.6 g of PEG dry substance is dissolved in 1 ml of PBS, then filtered through a millipore filter (pore size 0.22 µm) and stored at a temperature of 4°C until use. It is used in the purification of viral particles.

## 1.3. Chemiluminescent system for the detection of reactive oxygen species (ROS.)

Lucigenin (3mg/ml), NaOH (100mM). Buffer – to 10 ml of 100 mM Tris (pH 9.4) add 0.2 ml of hemolysate (10 ml of $H_2O$ + 0.02 ml of total blood).

Luminol 10 mM, 50 mM borate buffer, horseradish hperoxidase.

## 2. Methodology:

### 2.1. Isolation and purification of HSV-1, strain F

After obtaining a viral suspension, following infection of a confluent monolayer and subsequent freezing and thawing, the supernatant is collected. For this purpose, the virus suspension was centrifuged at 5000 revolutions per minute (RPM) for 20 minutes. Polyethylene glycol (PEG 8000) with a concentration of 60%, dissolved in PBS, filtered through a sterile filter was added to the collected supernatant. The ratio of supernatant to 60% PEG 8000 is 3:1. After 24 hours of residence at a temperature of 4 °C, the mixture was centrifuged at 5000 revolutions per minute (RPM) for 40 minutes. The pellet was resuspended in 1.2 ml of PBS. Isolated HSV-1 (F) was further purified using a 20% to 60% density sucrose gradient. To obtain all one of the layers of the gradient, equal amounts of PBS are mixed. (10x) and 30% sucrose solution. Increasing amounts of Percoll (pH 8.5–9.5), 1 ml for a 20% gradient to 3 ml for a 60% gradient, were added to the mixture. The solution is brought to a final volume of 5 ml with distilled water. All one of the layers is created by layering. Pour 2 ml of each layer into a centrifuge tube. After centrifugation at 6000 revolutions per minute (RPM) for 90 minutes, the band between 20% and 30% sucrose solution is collected.

### 2.2 Investigation of the cytotoxic effect of a culture medium treated with the used low-temperature non-equilibrium gas-discharge plasma

The MTT method described by Mosmann was used to study the cytotoxic effect (CV) of plasma and determine cell viability.

The following working protocol was used to determine the toxic effect of the treated solutions:

After counting the cells, the latter were resuspended in the appropriate volume of growth medium DMEM (with 8% FBS). The cell suspension was then plated, with a cell concentration of 1.5x10 5 cells/ml, in sterile 96-well plates (Orange Scientific) at 0.2 ml of suspension per well. Because of data obtained experimentally that in the end rows and columns sometimes a decrease in volume occurs during longer cultivation, they are not seeded with cells, but only serum-free culture medium is instilled. When the cell monolayer reached between 90 and 100% confluency (typically after 24 hours), the supernatant was decanted and gas-discharge plasma-treated DMEM (4% FBS) was added. The following experimental setups were applied:

- Treated medium at a plasma power of 20 W. The medium is treated in the active zone of the discharge, with the treatment duration being 30 sec., 60 sec., 90 sec., 120 sec., 240 sec., 300 sec.. To each well, adds the treated medium in a volume of 0.1 ml, after which 0.1 ml of untreated maintenance medium is added. At least three wells are instilled for each of the treatment times.
- Plasma parameters are the same as described above. The medium is treated for the same time intervals. The volume of instilled treated culture medium was 0.2 ml without adding untreated one.
- The medium is treated at a plasma power of 15 W. 0.2 ml of treated support medium are dripped. The time intervals of medium treatment are the same (30 sec., 60 sec., 90 sec., 120 sec., 240 sec., 300 sec.).
- The medium is treated at a plasma power of 13 W. 0.2 ml of treated supporting nutrient medium are instilled. The time intervals for treatment are the same.

In all experimental setups, the treatment of DMEM support medium was performed immediately before its addition to the cell monolayer, being treated in a volume of 5 ml. In several wells of each plate, only nutrient medium is dripped without treatment of the plasma torch (0.2 ml) and serve as cell control. The plates thus treated were incubated in a thermostat (Memmert) at a temperature of 37 °C for 72 hours.

After the specified incubation period of the plates, 0.02 ml of MTT working solution (with its final concentration in the well being 0.5 mg/ml) was dripped into each well (except the end rows and columns), after which the plate was incubated in a thermostat ( Memmert) at a temperature of 37 °C for 30 min. After incubation, the culture medium, with MTT dissolved in it, was removed, after which 0.2 ml of the lysis solution was added. The plate thus treated was read spectrophotometrically at a wavelength of 540 nm using an ELISA reader Multiscan MX. Cell survival was determined as the percentage of viable cells in wells treated with plasma-treated medium at the various exposure times and plasma source powers used (described above) compared to untreated control culture cells. For this purpose, we applied the following formula:

$$\%\ \text{cell vitality} = \frac{\text{A540 optic density of treated cells}}{\text{A540 optic density of contorll}} \times 100$$

From the constructed "dose - cell survival" curve, the conditions of treatment of the environment are calculated, in which 50% cell viability is taken into account, as well as the maximally tolerable impact of the environment treated under different conditions on cell survival.

## 2.3. Determination of the survival of virus-infected cells and treated with activated low-temperature non-equilibrium gas-discharge plasma medium, by MTT test.

Antiviral activity of media activated by low-temperature non-equilibrium gas discharge plasma was determined by the MTT test developed by Mosmann and modified by Pauwels, Takeuchi, Sudo for rapid screening of compounds for antiherpetic activity. Determination of cell viability by MMT assay of cells infected with virus and treated with plasma-source activated media is indicative of antiviral activity.

The following working protocol was used to determine the antiviral activity of the low-temperature non-equilibrium gas discharge plasma media:

After counting, the cells were resuspended in DMEM growth medium (with 8% FBS). The cell suspension was dispersed in sterile 96-well plates (Orange S. Scientific) in a volume of 0.2 ml per well and at a concentration of 1.5x105 cells/ml. Experiments were performed in a fully confluent cell monolayer. One hour before the treatment of the nutrient medium, the cell monolayer was infected with the used viral strain 0.1 ml per well, at a concentration of 10,000 TKID50/0.2 ml. This is followed by one hour of adsorption of the virus in a thermostat at a temperature of 37 °C. Maintenance medium DMEM (with 4% FBS) was treated on the plasma source in a volume of 5 ml and source parameters: 13 W plasma power, gas flow 5 L/min and treatment time intervals: 30 sec, 60 sec, 90 sec., 120 sec., 240 sec., 300 sec. After treatment of the medium, virus-infected cells were treated according to the following methods:

- Sequential treatment with removal of unadsorbed virus. After 1 hour of virus adsorption, the virus suspension was withdrawn. This is followed by a triple wash with physiological solution. In each well, add 0.2 ml of maintenance nutrient medium treated as described above. After waiting 5 min and 20 min, the treated medium was removed and replaced with 0.2 ml of untreated medium.
- Sequential treatment with removal of unadsorbed virus. After 1 hour of virus adsorption, the virus suspension was withdrawn. This is followed by a triple wash with physiological solution.

In each well, add 0.2 ml of maintenance medium treated as described above without subsequent removal and replacement with untreated maintenance medium.

- Sequential treatment without removal of unadsorbed virus. After 1 hour of virus adsorption, 0.1 ml of maintenance medium treated as described above was added to each well.
- Parallel treatment of samples. After removal of the growth medium above the cell monolayer, equal volumes (0.1 ml each) of virus suspension and treated support medium were instilled into each well.

In all work protocols for each plate, the corresponding controls are also processed:

Control cells (non-infected with virus and cells not treated with activated medium) – 0.2 ml of untreated DMEM support medium is added to the wells designated for cell control (at least three).

Virus control (virus-infected and untreated cells with activated medium) – 0.1 ml of DMEM support medium is added to the wells designated for virus control (at least 3).

Plates treated in this way were incubated in a thermostat (Memmert) at a temperature of 37°C for 4–5 days (depending on the development of the cytopathic effect in the viral control).

After the respective incubation period, 0.02 ml of MTT working solution (with its final concentration in the respective well being 0.5 mg/ml) was dripped into each well (except for the end rows and columns), after which the plates were incubated in a thermostat ( Memmert) at 37°C for 30 min. After the incubation, the nutrient medium, with the MTT dissolved in it, is removed and 0.2 ml of the lysis solution is added. Plates thus treated are read spectrophotometrically at a wavelength of 540 nm using an ELISA reader Multiscan MX.

The presence or absence of antiviral activity was determined as % cell survival under the conditions of the respective experiment and was calculated by the following formula:

$$\frac{[OD_v - ODVC]}{[ODCC. - ODVC]} \times 100\ (\%)$$

where:

ODV – absorption of infected and substance-treated cells

ODVC – uptake of virus-infected cells (viral control without substance)

ODCC. – absorption of uninfected and untreated cells (cell control).

For the validity of the experiment, it is important that there is no cytopathic effect in the cell control and that the ratio of the absorbance of the virus control to the absorbance of the cell control is less than or equal to 0.2.

## 2.4 Investigating the effects of low-temperature non-equilibrium gas-discharge plasma on extracellular virions

To determine the effect of low temperature non-equilibrium gas discharge plasma on the infectivity of HSV-1 strain F virus, a direct contact method was used. For this purpose, the following working protocols have been applied:

- After determining the viral titer (10-6.50 TKID50), the viral suspension was diluted 1:1 with dH2O with a final volume of 3 ml. The thus diluted suspension was treated with the plasma source at a source power of 13 W and a gas flow rate of 5 l/min for treatment time intervals 30 s., 60 s., 90 s., 120 s., 180 s., 240 s. After infecting a cell monolayer with the treated virus suspension, the virus titer was determined by the method described above (Determination of infectious virus titer by the Reed-Muench final dilution method.
- After determining the viral titer (10-6.50 TKID50), the viral suspension was diluted 1:2 with dH2O with a final volume of 3 ml. The thus diluted suspension was treated with the plasma source at a source power of 13 W and a gas flow rate of 5 l/min for treatment time intervals of 30 sec., 60 sec., 90 sec., 120 sec., 180 sec., 240 sec.. After infection of a cell monolayer with the treated virus suspension, the virus titer is determined according to the method described above (Determination of infectious virus titer by the Reed-Muench final dilution method (Reed & Muench, 1938).

## 2.5. Determination of type and amount of reactive oxygen species (ROS) produced under the influence of low-temperature non-equilibrium gas discharge plasma.

The amount of hydrogen peroxide was investigated in two different experimental setups:

- With the participation of lucigenin: registers active forms of oxygen. 0.03 ml of lucigenin (3 mg/ml) and 0.01 ml of NaOH (100 mM) were added to 5 µl of buffer,

mixed in cuvettes, and 0.05 ml of the treated sample was added to the thus obtained mixture. The buffer was prepared by adding 0.2 ml hemolysate (10 ml $H_2O$ + 0.02 ml whole blood) to 10 ml 100 mM Tris (pH 9.4).

- With the participation of luminol: registers hydrogen peroxide – $H_2O_2$. 50 mM borate buffer and 10 mM luminol were used. Mix 10 ml borate buffer and 0.1 ml luminol. 0.5 ml of the thus obtained solution are dripped into cuvettes. 0.005 ml of horseradish peroxidase is added to each, as well as 0.005 ml of the corresponding sample.

The effect of plasma-treated PBS in the presence of $H_2O_2$ (0.0011 mL; 50 mM), as well as the effect of PBS, $dH_2O$, and saline without addition of $H_2O_2$, on the chemiluminescent signal upon plasma treatment for the respective time intervals from 10 to 60 seconds and at 13 W and 20 W plasma power, applying the respective experimental setups.

- Influence of DMEM on the chemiluminescence signal in the presence of $H_2O_2$ (0.001 ml; 50 mM)
- effect of 4% DMEM on the chemiluminescence signal in the presence of $H_2O_2$ (0.001 ml; 50 mM)
- effect of saline on the chemiluminescent signal.
- effect of $dH_2O$ on the chemiluminescent signal.
- effect of PBS on the chemiluminescence signal in the presence of $H_2O_2$ (0.001 ml; 50 mM)
- effect of PBS on chemiluminescence signal

As a control, start signals were measured in which only $H_2O_2$ (0.001 ml; 50 mM) was present in the system. The quenching effect of DMEM was used to control relative to the radical forms obtained. After the addition of the test solution, the start signal and the output signals of the respective solutions were measured (one of the two experimental setups).

# 4. Results

## A. Investigating the effect of plant extracts on the replication and extracellular virions of human herpesvirus type 1, strain F and acyclovir-resistant herpesvirus type 2, strain DD

### 1. Investigation of the antiviral activity of extracts obtained from in vivo cultivated representatives of Vacinium vitis-idaea L.

#### 1.1 Establishing the cytotoxic effect of the extracts on the cells of the used cell culture. Determination of the values of MNK and CK50

According to the aim and objectives of this thesis, the cytotoxic effect of each of the extracts we used was initially determined. We applied the MTT assay to determine live and dead cells.

In the experimental set-up used, concentrations ranging from 0.187 mg/ml to 20 mg/ml of the extracts from Vaccinium vitis - idaea L were tested.

The values obtained for each extract are presented in Table 2 and plotted graphically in Figure 3.

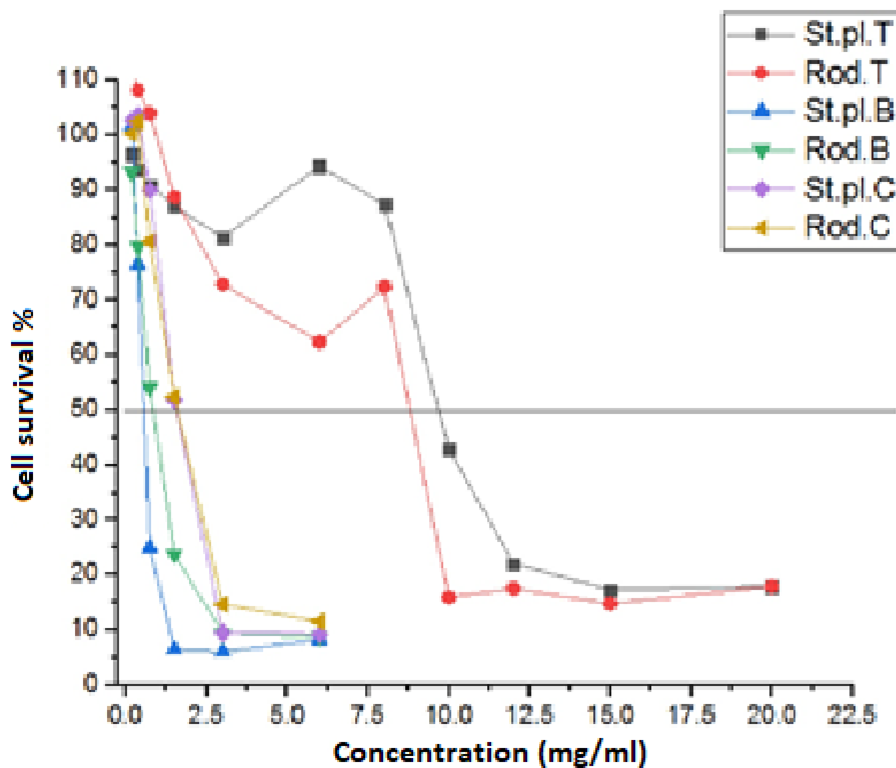



**Figure 3.** Cell survival of cell line MDBK challenged with extracts of Vaccinium vitis-idaea L.

**Table 2.** Cytotoxicity data (MNC and CC50) of extracts from Vaccinium vitis - idaea L.

| **Extracts** | **MNC (mg/ml)** | **$CK_{50}$ (mg/ml)** |
|---|---|---|
| **St.pl. Total** | 6 | 9.63 |
| **Rod. Total** | 3 | 8.81 |
| **St.pl.B** | 0.5 | 0.556 |
| **Rod. B** | 0.75 | 0.84 |
| **St.pl.C** | 1 | 1.56 |
| **Rod. C** | 1 | 1.61 |

From the obtained data, it was found that for the total extract S.t.Pl.T, CC50 is 9.63 mg/ml, and MNC is 6 mg/ml. The second total extract, Rod.T, exhibits an MNC of 3 mg/ml, and its CC50 value is 8.81 mg/ml. The maximum non-toxic concentration found in the extracts from fraction B is respectively: in the Stara planina extract, it is 0.5 mg/ml, and for the Rodopi extract – 0.75 mg/ml The corresponding CC50 values for these extracts are: for St.pl.B – 0.556 mg/ml, and for Rod.B e 0.84 mg/ml Extracts from fraction C have the same values for MNK (1mg/ml), and the reported CC50 of the two extracts is respectively for St.pl.C - 1.56 mg/ml, and for Rod.C is 1.61 mg/ml. When comparing the experimental data (values for CC50), it was established that the extracts from fraction B had the greatest cytotoxicity, and the total extracts had the least.

The hierarchical order looks like this (from higher to lower toxicity):

**S.t.pl.B >Rod.B >S.t.pl.C >Rod.C >Rod.T >S.t.pl.T**

## 1.2. Study of the survival of cells infected with HSV-1 (F) and treated with the tested extracts.

The effect of plant extracts on the replication of HSV-1 strain (F) and of HSV-2 strain (DD-acyclovir resistant) was determined by the MTT test developed by Mos.mann (Mosmann, T., 1983) and modified by Pauwels, Takeuchi, Sudo for rapid screening of compounds for antiviral activity (Pauwels, R. et al., 1988; Takeuchi, H. et al., 1991; Sudo, K. et al., 1994). In the experiments, we used a working dose of 100 TKID50 (at a virus titer of 10-6.33 TKID50). Acyclovir was used as a positive control in each experiment.

The obtained results of the conducted experiments are presented graphically in **figure 3**, **figure 4**, **figure 5** and tabularly in **table 3**.

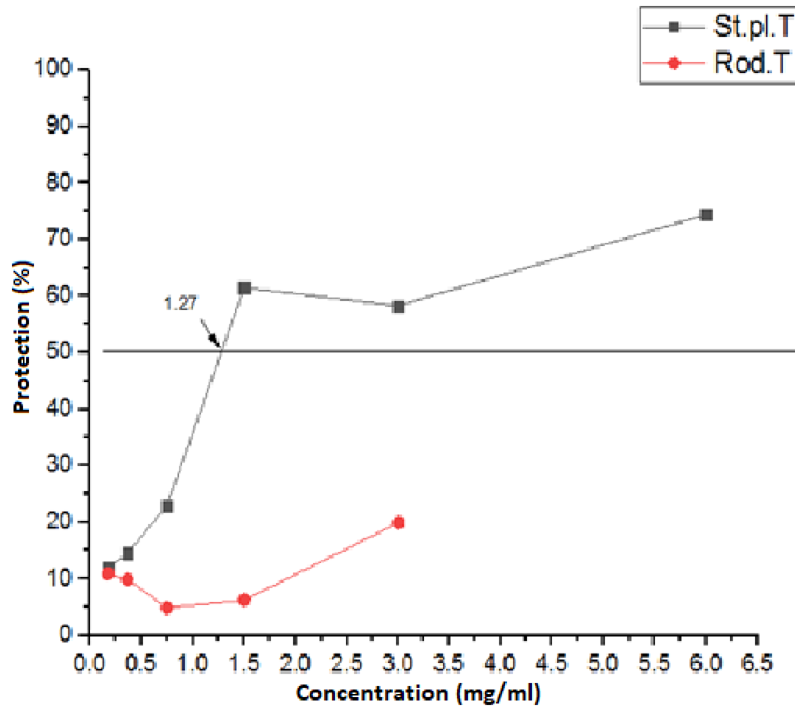
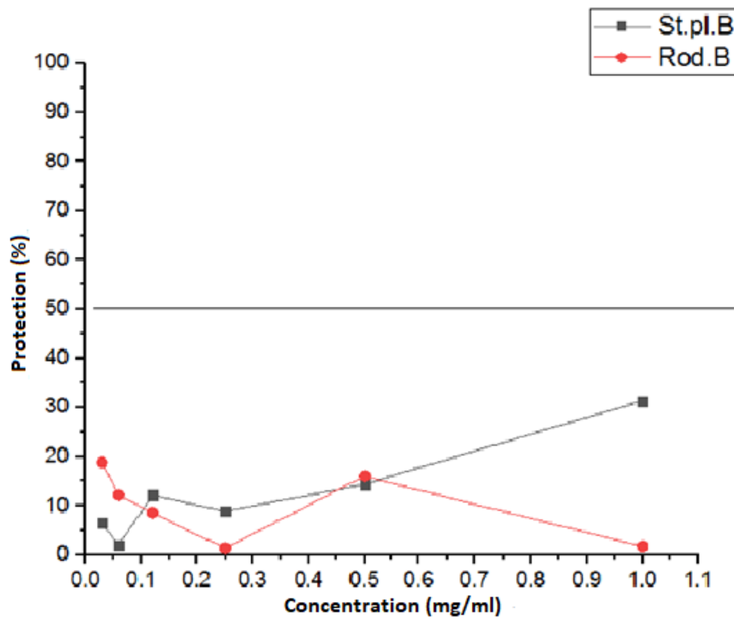


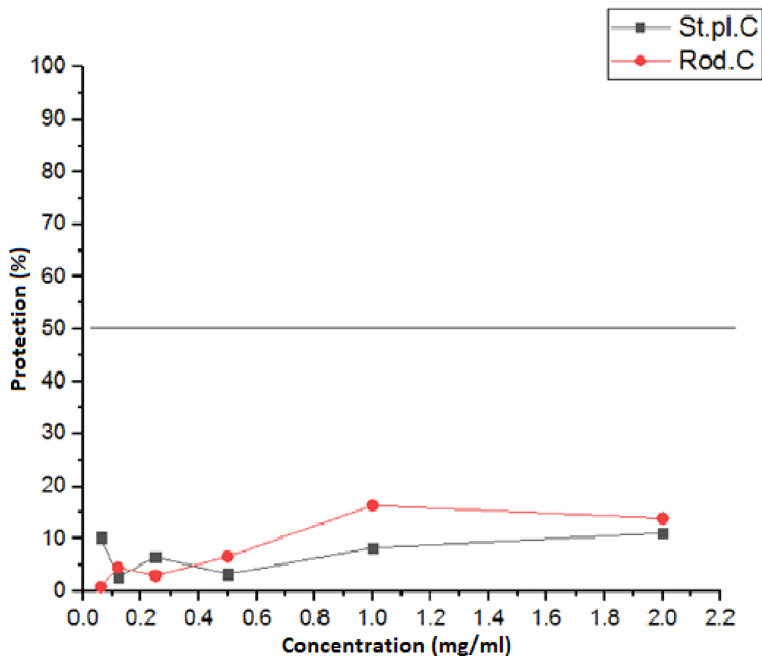

**Figure 4.** Effect on HSV-1 replication (F) of treatment with total extracts (St.pl.T and Rod.T) of Vaccinium vitis -idaea L.

From the graph in Figure 14 it is clear that the IC50 (1.27 mg/ml) was reached with the extract St.pl.T but not with Rod.T, both extracts being tested at concentrations starting from 6 mg/ml (MNC).



**Фигура 5.** Effect on HSV-1(F) replication by treatment with extracts of Vaccinium vitis -idaea L, fraction B (St.pl.B and Rod.B)

With the extracts from fraction B, as can be seen from the graph, IC50 is not reached. They have also been studied in descending two-fold concentrations starting at 1 mg/ml.




**Фигура 6.** Effect on HSV-1 replication (F) upon treatment with extracts of Vaccinium vitis-idaea L., fraction C (St.pl.C and Rod.C)

The data presented graphically in **Figure 4**, **Figure 5** and **Figure 6** are combined in **Table 3.**

**Таблица 3.** Data on percent protection and IC50 of extracts from Vaccinium vitis-idaea L., on HSV-1 replication (F).

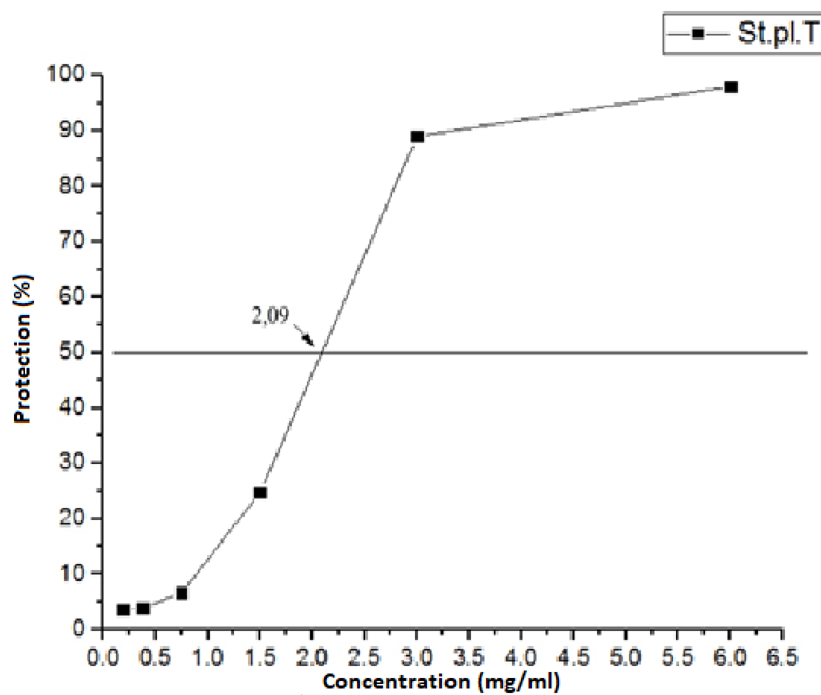
| **Екстракт** | **% протекция на клетките в МНК** | **ИК$_{50}$ мг/мл** | **ЦК$_{50}$ мг/мл** | **Селективен индекс** |
|---|---|---|---|---|
| **St.pl.T** | 74.44 | 1.27 | 9.63 | 0.13 |
| **Rod. T** | 19.96 | н.д. | 8.81 | н.д. |
| **St.pl.B** | 23,5 | н.д | 0.556 | н.д |
| **Rod. B** | 9.2 | н.д | 0.84 | н.д |

| **St.pl.C** | 8.3 | н.д | 1.56 | н.д |
|---|---|---|---|---|
| **Rod.C** | 16.41 | н.д | 1.61 | н.д |
| **АЦВ** | 100 | 0.000430 | 0.218 | 452.08 |

*н.д. – няма данни

## 1.3. Study of the survival of cells infected with HSV-2, strain DD and treated with the total extract of Stara planina.

To study the survival of cells infected with HSV-2 strain (DD), only one of the total extracts was used - St.pl.T, which showed the highest values for cell protection in the study of HSV-1q strain F. The obtained results of the conducted experiment are presented in **table 4** and depicted graphically in **figure 7**.




**Фигура 7.** Effect of total extract (St.pl.T) of Vaccinium vitis-idaea L. on HSV-2 (DD) replication.

**Table 4.** Data on percentage protection and IC50 of total extract of Vaccinium vitis-idaea L., against HSV-2 (DD).

| Extract | % protection in MNC | $IC_{50}$ mg/ml | $CC_{50}$ mg/ml | SI |
|---|---|---|---|---|
| **St.pl.T** | 98 | 2,09 | 9.63 | 0.22 |
| **ACV** | 10 | N/A | 0.218 | N/A |

*N/A not applicable

The extract (St.pl.T) was tested at concentrations from 0.187 mg/ml to 6 mg/ml, and at its MNC (6mg/ml), the reported percentage of cell protection was 98%. The achieved IC50 of the extract, in this experiment, was 2.09 mg/ml, and the calculated selective index was 0.22. In comparison, the same extract reached an IC50 of 1.27 mg/ml administered in the experiments with strain F

**1.4. Determination of the effects of the extracts on extracellular virions**

For a more complete study of the antiherpetic effect of Vaccinium vitis-idaea L. extracts, their effect on extracellular virions was also investigated by direct contact method. The extracts were applied at a concentration corresponding to their MNC. The change in infectivity of the virus at different durations of contact with the studied extract (5, 15, 30, 60, 120, and 240 minutes) was followed. When conducting the described experiment, no inactivation of the virions was reported as a result of the treatment with the different extracts. When reporting the results, no difference in the titer of the treated wells and the untreated viral control was observed.

## 2. Establishing the effectiveness of extracts obtained from in vivo cultivated representatives of the species Astragalus glycyphyllos L.

### 2.1. Determination of the survival of treated with methanol defatted extract of Astragalus glycyphyllos L., cells of cell line MDBK. Determination of the values of MNK and CK50

The concentrations of the methanolic defatted extract of Astragalus glycyphyllos L. used in the experiment ranged from 0.0625 mg/ml to 8 mg/ml. The results obtained during the conducted experiments are presented in **table 5** and depicted graphically in **figure 8**.

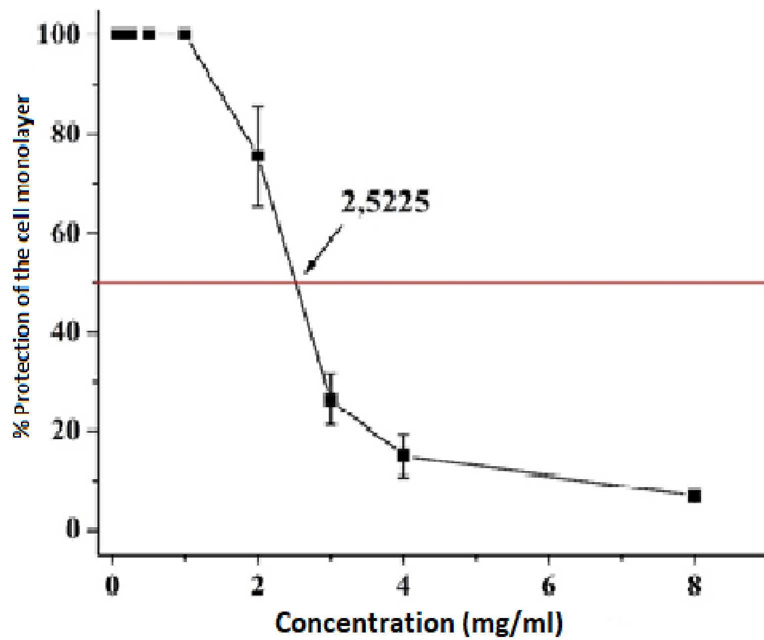



**Figure 8.** Cell survival of MDBK cell line treated with defatted methanolic extract of Astragalus glycyphyllos L.

**Table 5.** Cytotoxicity data of a methanolic defatted extract of Astragalus glycyphyllos L.

| MNC (mg/ml) | $CK_{50}$ (mg/ml) |
|---|---|
| 1 | 2.5225 |

It is evident from the obtained results that the maximum concentration at which 100% cell survival is reached is 1 mg/ml. At a substance concentration of 2.5225 mg/ml, survival is 50%. A dose-dependent reduction in the toxicity of the extract was observed.

## 2.2. Study of the survival of HSV-1 (F) and HSV-2 (DD) infected and treated with methanolic defatted extract of Astragalus glycyphyllos L cells.

In the applied experimental setup, CAA was used as a reference inhibitor to confirm the accuracy of the experimental results.

The results are summarized in **Table 6** and graphically depicted in **Figure 8**, for HSV-1 (F) and **Figure 9** for HSV-2 (DD).

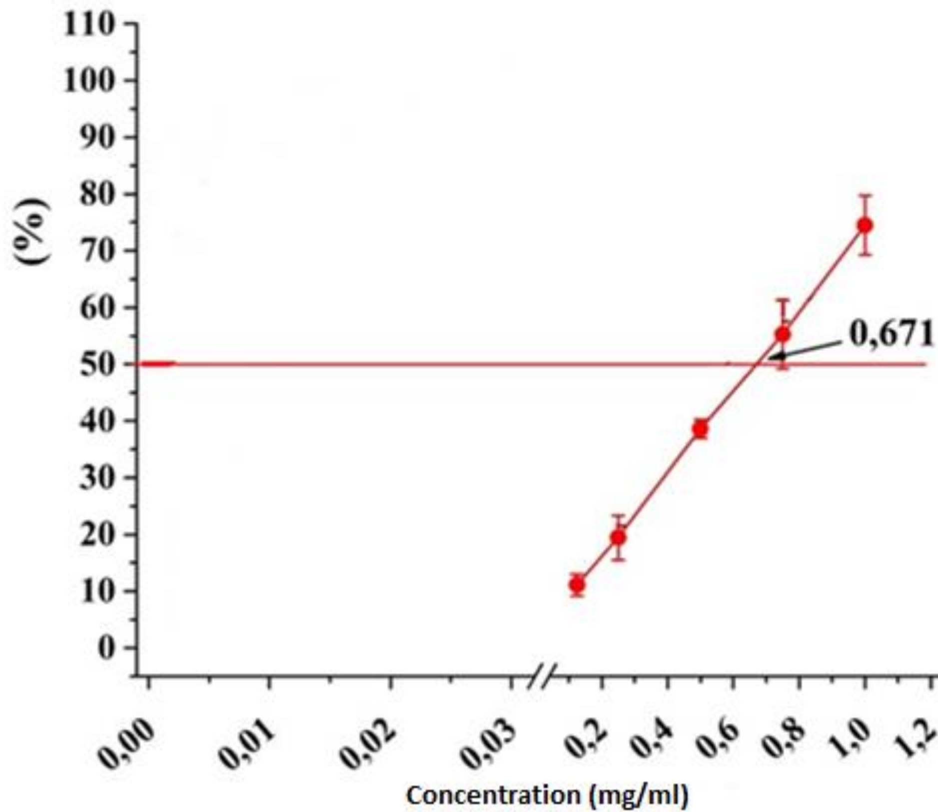



**Figure 9.** Effect of methanolic defatted extract of Astragalus glycyphyllos L. ( ) on the replication of HSV-1 strain F.

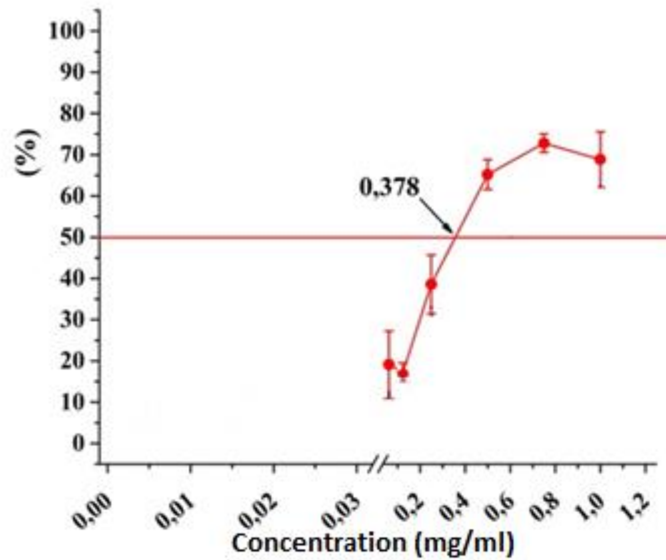



**Figure 10.** Effect of methanolic defatted extract of Astragalus glycyphyllos L. ( ) on the replication of HSV-2 strain DD.

From the data in Figure 19, it is clear that the methanolic defatted extract of Astragalus glycyphyllos L. shows a protective effect against HSV-1 (F). In MNK, the protection reaches 74.49 % (IC50 = 0.672 mg/ml) protection, respectively, in the experimental setting. With a decrease in the dose, the antiviral effect of the substance decreases.

**Table 8.** Data on the antiviral activity of a methanolic defatted extract of Astragalus glycyphyllos L. on the replication of HSV-1 strain F and HSV-2 strain DD.

| **Extracts** | | | | |
|---|---|---|---|---|
| | | | | |
| | **HSV-1 (F)** | | **HSV-2 (DD)** | |
| | **$IC_{50}$ (mg/ml)** | **SI** | **$IC_{50}$ (mg/ml)** | **SI** |
| **Methanol extract** | **0,671** | **0.26** | **0,378** | **0.15** |

The methanolic defatted extract of Astragalus glycyphyllos L. protected in MNK (**Table 8, Figure 10 and Figure 11**) the cells of the cell line infected with HSV-1 strain F and HSV-2 strain DD. The IC50 data are relatively close, indicating that the extract does not exhibit a strictly selective action against one of the two strains used.

## 2.3. Effect of methanolic defatted extract of Astragalus glycyphyllos L on extracellular virions of HSV-1 strain F and HSV-2 strain DD.

The concentration used in the conducted experiments corresponds to the MNC of the methanolic defatted extract. No difference was found in the titer of the affected virus suspension and the untreated one at any of the time intervals. From the obtained experimental data, it was concluded that the extract does not exhibit virucidal activity.

# B. Determining the degree of protection of personal protective equipment by applying a modified VFE method.

Widespread Community face coverings (community face covering) or CFC, as well as filtering facepieces (filtering facepiece) or FFP level 2 and 3 according to EN149:2001. The differences between the different types of FFP 2 and 3 masks come from the number of layers of non-woven fabric used to make them. Masks of the CFC category, unlike the multi-layered FFP types of masks, have only one layer of synthetic material. Placed in the viral flow path, they reduced the titer of the sample by 0.17 log compared to the viral control, which equates to 33.00% efficiency. When increasing the number of layers, either cotton or non-woven, the titer of the virus used decreases by between 0.34 – 0.67 logarithms. The filtering efficiency varies between 55.00% and 79.00%. When treating CFCs made of three layers, the viral titer is reduced by 2 logarithms, the filtering efficiency equals 99.00%.

All standardized FFP2 and 3 masks are composed of three layers. Placed in the path of the viral flow, masks of this type reduce the viral titer by two logarithms (filtering efficiency – 99.00%)

## C. Effect of plasma-source activated culture media and distilled water on replication and extracellular virions of HSV-1 strain F

### 1. Investigation of the cytotoxic effect of a culture medium treated with the used low-temperature non-equilibrium gas-discharge plasma.

First, the cytotoxic effect of plasma was investigated and cell viability was determined. An MTT assay was applied to determine live and dead cells (Mosmann, T., 1983).

In the experimental setups, DMEM (4% FBS) culture media treated with low-temperature non-equilibrium gas discharge plasma were tested at different powers, as well as different volumes of instilled treated and untreated media.

The values obtained when applying the different experimental setups are presented in table 15 and are graphically depicted in figure 23 and . The cytotoxic effect (CV50) of the medium treated at different time intervals and plasma parameters, where 50% cell viability is considered, was determined.

**Table 9.** CV50 data of low-temperature non-equilibrium gas-discharge plasma treated nutrient medium.

| **Staging** | **$CC_{50}$ (сек)** |
|---|---|
| **20 W 0.1 ml Treated DMEM + 0.1 ml untreated DMEM** | N/A* |
| **20 W 0.2 ml treated DMEM** | 232 |
| **15 W 0.2 ml treated DMEM** | 258 |
| **13 W 0.2 ml treated DMEM** | N/A* |

*N/A – not applicable

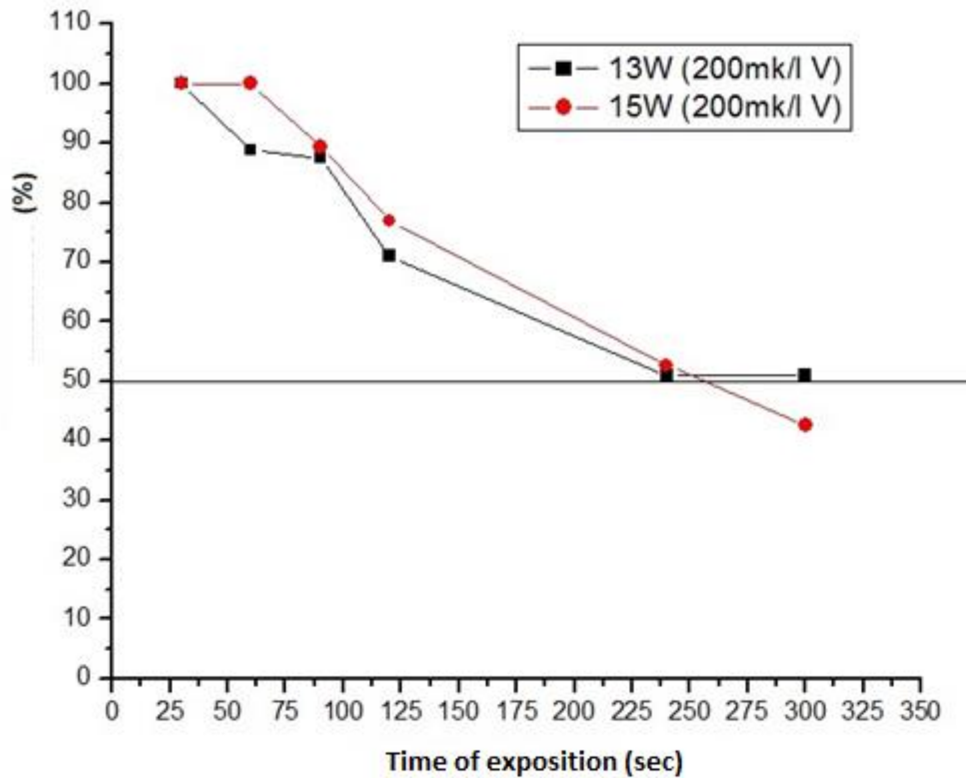



**Figure 9.** Survival of MDBK cells when treated with plasma source powers of 15 W (•) and 13 W (■) and added volumes of 0.2 ml of treated medium without addition of untreated medium

The obtained results show that only in two of the experimental setups results reaching below 50% survival of the cells from the cell culture are obtained - when maintaining the cells with 0.2 ml of treated medium and power of the plasma source 20 W and with 0.2 ml of treated medium at power 15 W. Using 20 W power of the plasma source, 50 % cell survival was reported at 232 s, and using 0.2 ml of treated medium at 15 W power, 50 % cell survival was reported at 258 s.

When treating the cell monolayer with 0.1 ml of plasma-treated medium and adding 0.1 ml of untreated medium, cell survival reaches 70% when the sample is treated for 300 seconds.

When cells were treated with 0.2 ml of plasma-treated medium without addition of untreated cell survival approached 50%.

## 2. Investigating the effect of low-temperature non-equilibrium gas discharge plasma treatment on HSV–1 (strain F) replication

The survival of cells infected with virus and treated with medium treated with low-temperature non-equilibrium gas discharge plasma was determined by MTT assay. The obtained experimental values are presented in **Table 10**.

**Table 11.** Antiviral effect of plasma-treated nutrient medium in the different experimental setups

| Staging | Protection(%) | $CC_{50}$ |
|---|---|---|
| **13 W 0.2 ml treated DMEM (5 min)** | 33,63 | N/A* |
| **13 W 0.2 ml treated DMED (20 min)** | 30,60 | N/A* |
| **13 W 0.2 ml Treated DMEM paralel** | 50,24 | N/A* |
| **13 W 0.1 ml Treated DMED concecutive** | 37,95 | N/A* |
| **13 W 0.1 ml Treated DMEM paralel** | н.д* | N/A* |

*N/A – not applicable

No significant percentage of protection on the cell monolayer was observed in any of the experimental setups. The highest percentage of protection is achieved with a plasma power of 13 W and consecutive treatment of the environment without subsequent removal of the same. The lowest percentage of cell protection was found at 13 W plasma power and removal of the treated medium after 5 min and replacement with untreated.

## 3. Investigating the effect of low-temperature non-equilibrium gas discharge plasma-treated distilled water on extracellular HSV-1 strain F virions

A direct contact method was used to determine the effect of low-temperature non-equilibrium gas discharge plasma on the infectivity of HSV-1 strain F virus. The virus used was titrated and experiments were performed with 10-6.50 TKID50.

To conduct the experiments, two-fold and three-fold dilutions of the virus suspension were prepared with final volumes of 3 ml, in accordance with the working protocols described in the materials and methods section. The discharges thus made were directly treated in the active zone of

the flame at a plasma source power of 13 W for time intervals of 60 sec., 120 sec., 240 sec. and 300 sec.

The results obtained in our experiments are presented in **Tables 11, 12** and **Figures 13** and 14

**Table 11.** Change in the titer of the virus sample diluted with distilled water in a ratio of 1:1 and treated for the specified time intervals at a plasma source power of 13 W

| **Time** | **Viral control** | **Sample** | **Δ log** |
|---|---|---|---|
| 60 | 6.23 | 6.23 | 0 |
| 120 | 6.23 | 6.23 | 0 |
| 240 | 6.23 | 5.75 | 0.48 |
| 300 | 6.23 | 5.00 | 1.23 |

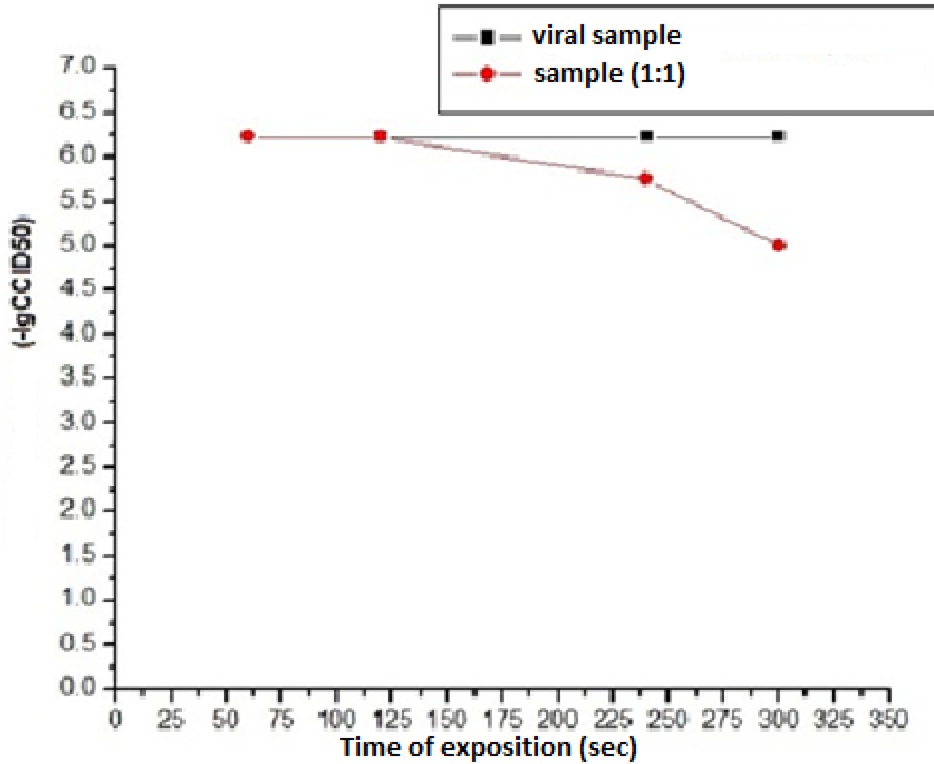



**Figure 12.** Change in titer of virus sample (•) diluted 1:1 with distilled water and treated for the specified time intervals at 13 W plasma source power versus viral control titer (■)

From the obtained data, it can be concluded that when treating the viral suspension diluted in dH2O in a ratio of 1:1 and treated at a plasma power of 13 W, the first results appear after the 240th second, with a decrease in the viral titer by 0.48 log , at an exposure time of 300 seconds, the viral titer decreases by 1.23 log. The results obtained when treating a virus suspension diluted in a ratio of 1:2 at a plasma power of 13 W are presented in Table 12 and graphically depicted in **Figure 12**.

**Table. 12** Change in the titer of the virus sample diluted with distilled water in a ratio of 1:2 and treated for the specified time intervals at a plasma source power of 13 W

| Time | Viral control | Viral sample | Δ log |
|---|---|---|---|

| 60 | 6.00 | 6.00 | 0 |
| --- | --- | --- | --- |
| 120 | 6.00 | 5.50 | 0.50 |
| 240 | 6.00 | 5.23 | 0.77 |
| 300 | 6.00 | 4.33 | 1.67 |

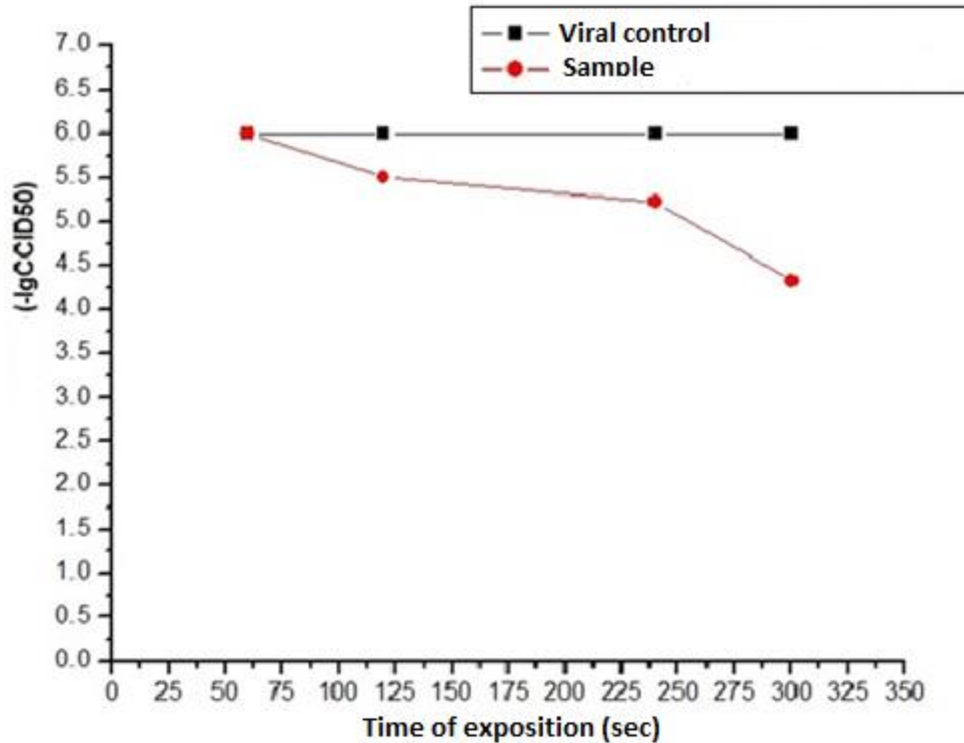



**Figure 14.** Change in titer of viral sample (•) diluted 1:2 with distilled water and treated for the specified time intervals at 13 W plasma source power versus viral control titer (■)

From the obtained data, it can be seen that the first results are established after the 120th second of treatment at a dilution of 1:2, reducing the viral titer by 0.5 log. When the plasma treatment lasts 300 s, the viral titer decreases by 1.67 log

## 4. Determination of type and amount of reactive oxygen species (ROS) produced under the influence of low-temperature non-equilibrium gas discharge plasma

Applying the first experimental setup described in the materials and methods section, the start signals were measured when only $H_2O_2$ (0.001 mL; 50 mM) was present in the system. After adding 0.05 ml of the test solution, the output signal was also measured. The reported results are presented in **Table 14**.

**Table 14.** Comparison of chemiluminescent signal before and after addition of DMEM, 4% DMEM and PBS in the presence of $H_2O_2$

| Staging | Signal at the start | Signal at the end |
| --- | --- | --- |
| DMEM | 1700 mV | 80 mV |
| 4% DMEM | 1700 mV | 200 mV |
| PBS. | 1700 mV | 470 mV |

The obtained results show that all three investigated solutions have a strong extinguishing effect compared to hydrogen peroxide, showing that DMEM has the strongest effect and PBS the weakest. A possible reason for this strong quenching activity is the rich component composition of each of the solutions. Based on the obtained data, we moved on to the study of solutions poorer in terms of their component composition.

Comparing the obtained data with the results obtained from the experiments on the cytotoxic effect of the plasma and the low percentage of cell protection when studying the influence of the plasma-treated medium on the replication of HSV-1, we judge that the nutrient medium used in the working protocols has a high extinguishing effect on the obtained radicals, which is most likely due to the rich component composition and the presence of vitamins in the medium used.

The effect on the chemiluminescent signal of surface wave treated, low-temperature non-equilibrium gas-discharge plasma for different time intervals (from 10 to 60 seconds) at two powers of 13

W and 20 W, saline was investigated. The first experimental setup described in the materials and methods section was applied.

The obtained results are tabulated in **table 15** and graphically depicted in **figure 15**

**Table 15.** Effect of plasma-treated saline on the chemiluminescent signal

| **Time of exposition (sec)** | **Signal 13W (in mV)** | **Signal 20W (in mV)** |
|---|---|---|
| 10 | 140 | 170 |
| 20 | 136 | 163 |
| 30 | 133 | 158 |
| 40 | 131 | 154 |
| 60 | 128 | 147 |

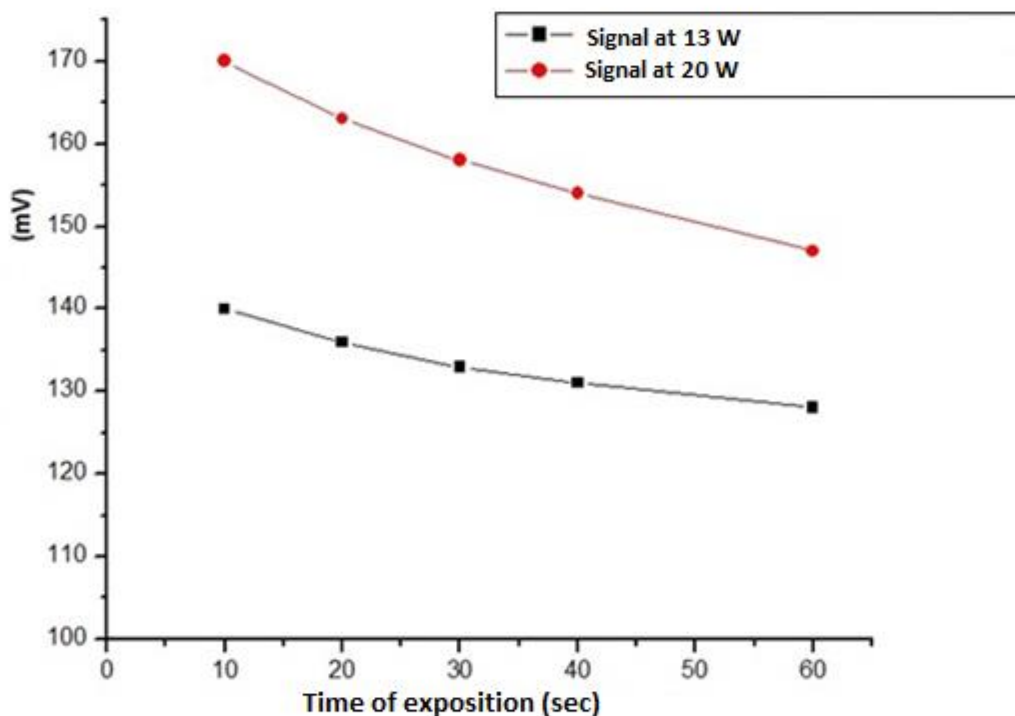



**Figure 16.** Effect of plasma-treated saline on the chemiluminescent signal

The obtained results support the theory of the quenching effect of multicomponent solutions. The data indicate that at early exposure intervals the levels of reactive oxygen species (in particular H2O2) are higher. The subsequent decrease in signal can be explained by the interaction of the peroxide with the chloride ions in the solution and the formation of hypochloride ions.

After studies focused on the emission spectrum of the resulting plasma, it became clear that RONS (Reactive Oxygen and Nitrogen Species) were present in the system. It also becomes clear that OH radicals are observed with great intensity at the water-plasma contact point, due to the dissociation of water molecules. The concentration of OH depends on the contact surface area of plasma and water, as well as on the applied power used to produce the plasma. Similar studies conducted in the absence of water show no change in temperature or radicals. The nuclear reactor does not contribute to change the output plasma.

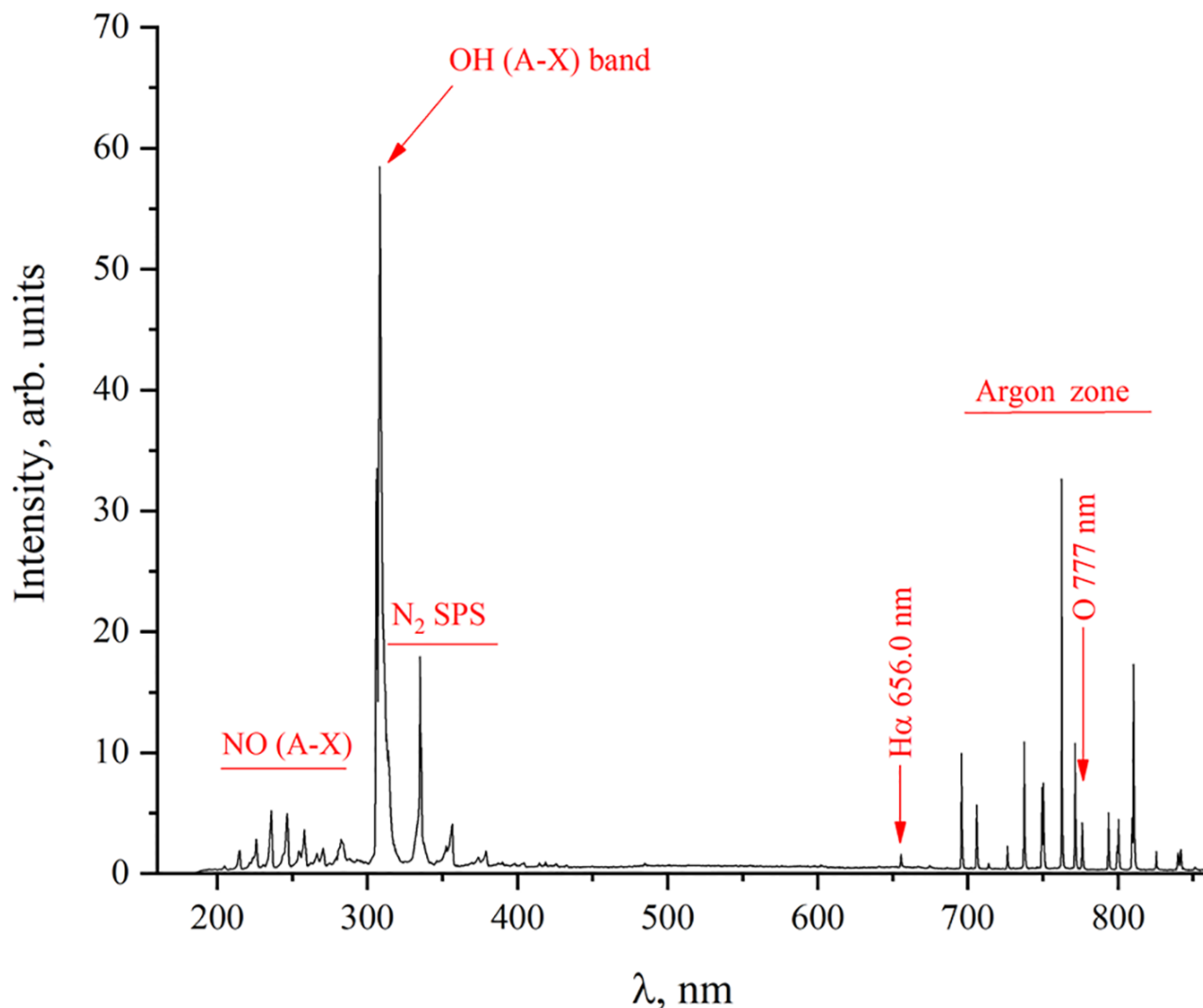



**Figure 17.** Optical emission spectrum at contact of the plasma-water surface (Tsvetkov V., et all; Effect of Plasma-Activated Medium and Water on Replication and Extracellular Virions of Herpes Simplex Virus-1; 2020; Plasma Medicine, 10(1) :15-26 (2020)

In our experiments, the effect of cold plasma on the type and amount of reactive oxygen species (ROS) in the treatment of distilled water was determined by using the chemiluminescence system described in the materials and methods section. The output signal was measured every 10 seconds after being treated with a low-temperature non-equilibrium gas discharge plasma for different time intervals (from 10 to 60 seconds) at two plasma powers – 13 W and 20 W, applying the first experimental setup described in the Materials and Methods section . The results are presented in Table 21 and plotted graphically in Figure 29

**Table 15.** Effect of dH2O on the chemiluminescent signal

| Time of exposition (sec) | Signal at 20W (in mV) | Signal at 13W (in mV) |
|---|---|---|
| 0 | 50 | 50 |
| 5 | 208 | 298 |
| 10 | 110 | 164 |
| 20 | 90 | 120 |
| 30 | 90 | 113 |
| 40 | 87 | 110 |
| 60 | 85 | 105 |

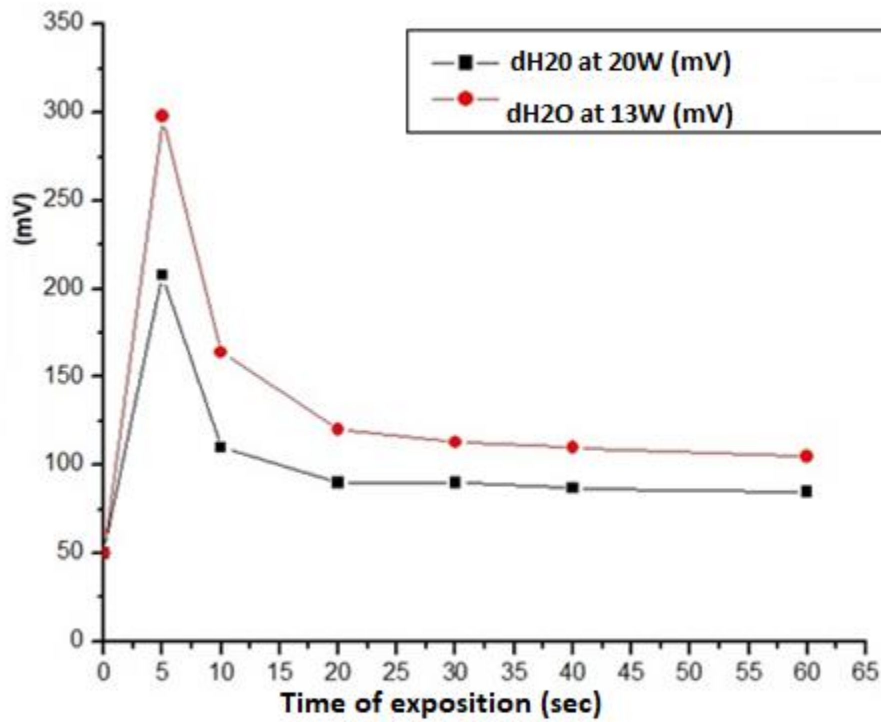



**Figure 18.** Effect of dH2O on the chemiluminescent signal

From the results, it can be seen that with the small time intervals of treatment, the amount of active forms of oxygen is the highest. This is probably due to the stronger virucidal effect when diluting the virus suspension in a ratio of 1:2 with dH2O. The decrease in the amount of active forms of oxygen during longer treatment could be due to interaction with nitrogen from the atmosphere and the formation of nitrates and nitrites.

To investigate the effect of PBS on the chemiluminescent signal. we used enhanced chemiluminescence to obtain a clearer signal. The solution was treated for 30 sec., 180 sec. and 300 sec. at a plasma power of 13 W. The method is described as a second experimental setup in the materials and methods section.

The results are presented in **Table 16** and illustrated in **Figure 18.**

**Table 16.** Effect of PBS on chemiluminescence signal

| **Time of exposition (sec)** | **PBS signal at 13W (в mV)** |
|---|---|
| 30 | 25 |
| 180 | 44 |
| 300 | 18,5 |

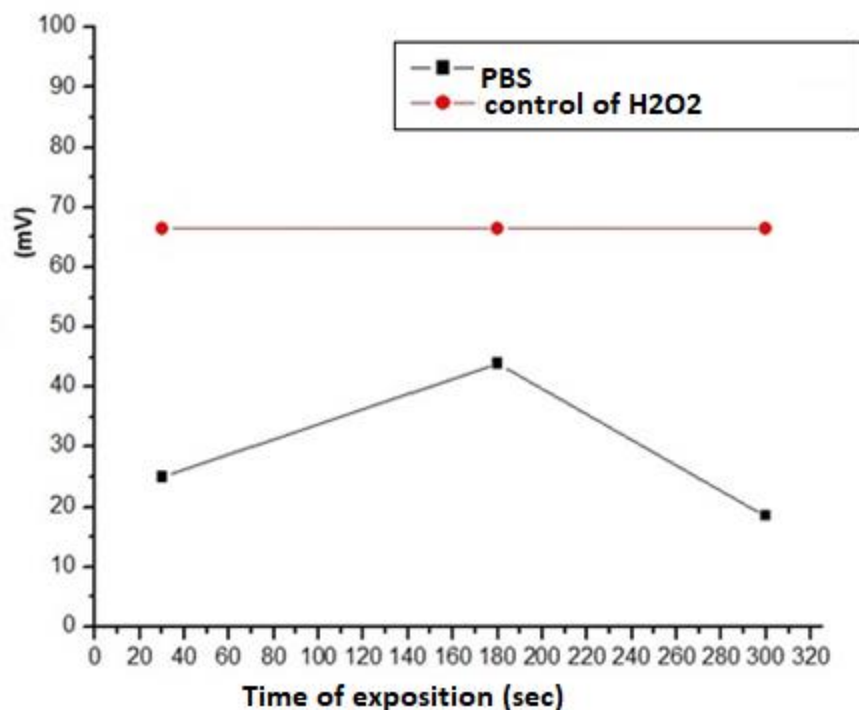



**Figure 19.** Effect of PBS on chemiluminescence signal

One of the possible reasons for the change in the amount of active forms of oxygen at the 180th sund and the sharp decrease thereafter.

# 5. Conclusion

The members of the Herpesviridae family are one of the most widespread viruses in nature. Isolated from all vertebrates, they cause diseases with different severity and clinical picture - from asymptomatic infections and self-limited skin lesions, to severe generalized infections, meningitis, encephalitis and malignant neoplasms.

In the fight against herpes infections, various medications are used, the most widely used of which are nucleoside analogs, which cause defects in the replication of the viral nucleic acid. A significant problem associated with the effects of this class of drugs is their dependence on the viral enzyme thymidine kinase. Another disadvantage of the mentioned medicinal preparations is the induction of drug resistance. Phytoproducts are of increasing interest, i.e. biologically active substances contained in plants Total plant extracts, due to the fact that they contain more than one biologically active substance, mimic combination

therapy, which is also the main approach to overcome the emergence of drug resistance. Given the above, we aimed to study the antiherpetic effect of extracts from Vaccinium vitis-idaea L., Astragalus glycyphyllos L. and Artemisia chamaemelifolia Vill. The strongest protective effect was reported in the exposure of cells inoculated with HSV 2 (DD) and treated with the total extract of Vaccinium vitis-idaea L., from plants collected from Stara planina – 98%. The extracts obtained from the other plants we studied showed about 70% protection against HSV-1 (F) and HSV-2 (DD).

The development of technologies makes it possible to study and apply different methods to monitor their impact on viral and bacterial infections in vitro and in vivo. Plasma-based technologies occupy an increasingly large place in innovative treatment methods. One of the fastest growing areas in plasma technology is so-called plasma medicine, an emerging field of medicine that exploits the ability of non-equilibrium plasmas to initiate, control and catalyze various complex behaviors and reactions in biological systems. Non-equilibrium plasmas are tissue-friendly, safe and effective against bacterial and viral pathogens.

When investigating the effect of a plasma-source activated medium (developed by a team of Bulgarian scientists) on the replication and extracellular virions of HSV-1 strain F, it was found that the treated medium did not protect the cells inoculated with the virus. We assume that this is due to the high quenching effect of the multicomponent solutions on the presence and lifetime of the oxygen radicals generated by the plasma source.

When studying the virucidal effect of a plasma-treated virus suspension diluted in a ratio of 1:2 with dH2O, a decrease in the titer of the virus sample was found compared to the control by 1.67 log. This again confirms our assumption that monocomponent solutions generate oxygen radicals to a greater extent, which are the basis of virucidal action.

Given the emerging COVID-19 pandemic, there is a global need for effective, inexpensive, easy-to-produce, and accessible personal protective equipment (face masks) to reduce the transmission of aerosol-borne pathogens. In this regard, some research laboratories focused their efforts on determining the degree of effectiveness of protective face masks. The Virology laboratory team joined the joint effort to determine the effectiveness of manufactured and marketed face masks with filtering efficiency. Out of more than 90 filtering efficiency face masks provided to us for research, after conducting the experiments to determine the degree of protection by applying a modified VFE method, the results showed that the FFP type filtering face masks provide the greatest degree of protection compared to the Public Face Masks masks, with a filtering efficiency of 99%.

# 6. CONCLUSIONS

The following conclusions can be drawn from the results obtained when investigating the effect of plant extracts on the replication and extracellular virions of human herpesvirus type 1, strain F and acyclovir-resistant herpesvirus type 2, strain DD:

1. When comparing the experimental data obtained for the kd CC50 values in the study of extracts from Vaccinium vitis-idaea L., it was found that the extracts from fraction B (St.pl.B) exhibited the greatest cytotoxicity, and the total extract ( St.pl.T) . The constructed hierarchical order of the studied extracts obtained from Vaccinium vitis-idaea L. looks as follows:
    i. St.pl.B >Rod.B >St.pl.C >Rod.C >Rod.T >St.pl.T

2. After conducting experimental procedures to determine the antiviral activity of extracts from Vaccinium vitis-idaea L., it was found that one of them showed the highest percentage of cell protection. The total extract from Stara planina in MNK showed 74.44% protection of the cell monolayer infected with HSV-1 (F). An IC50 of 1.27 mg/ml was reached and a selectivity index of 0.13 was determined. In the second total extract Rod.T, the percentage of protection in its MNC was 19.96%, which could mean that the soil composition has an influence on the composition of BAV contained in the plant. In the experiments performed with HSV-2 (DD), the extract showed 98% protection and reached an IC50 of 2.09 mg/ml. The selective index is 0.22

3. When considering the results obtained for the effect of the extracts on the extracellular virions of HSV-1 (F), no difference was observed in the titers of the treated virus and the viral control.

4. When determining the survival of treated with methanol defatted extract from aerial parts of Astragalus glycyphyllos L., cells of cell line MDBK, the maximum non-toxic concentration was determined - 1mg/ml. CC50 is 2.52 mg/ml

5. After conducting experimental setups to determine the antiviral activity of the Astragalus glycyphyllos L. extract, the manifestation of a certain cellular protection was established. The extract in MNK exhibited 74.49% protection of the cell monolayer infected with HSV-1 (F). The IC50 was reached, 0.6721 mg/ml and a selectivity index of 0.26 was determined. When using HSV-2 (DD), the extract showed protection and reached an IC50 of 0.378 mg/µ. The selective index is 0.15. Against the same strain, ACV did not show a protective effect. From the selective index data obtained, it can be concluded that the extract does not show a strict selective action with respect to one of the two strains used.

6. When reporting the results for the effect of Astragalus glycyphyllos L. extract on extracellular HSV-1 (F) and HSV-2 (DD) virions, no difference was observed in the titers of the treated virus and the viral control.

7. After carrying out the experiments to determine the degree of protection of personal protective equipment by applying a modified VFE method, the results show that the filtering face masks type (FFP) provide a greater degree of protection compared to the Public face masks, and the filtering efficiency is 99%.

8. When studying the cytotoxic effect of the low-temperature non-equilibrium gas discharge plasma used, only two of the experimental setups reached below 50% survival of the cell monolayer - when maintaining the cells with 0.2 ml of treated medium and a plasma power of 20 W and with 0.2 ml of treated medium and plasma power 15 W. These values are reached respectively at 232 sec. per treatment and at 258 sec. of treatment.

9. No significant percentage of protection on the cell monolayer was observed in any of the experimental setups. The highest percentage of protection is achieved with a plasma power of 13 W and consecutive treatment of the environment without subsequent removal of the same. The lowest percentage of cell protection was found at 13 W plasma power and removal of the treated medium after 5 min and replacement with untreated.

10. From the obtained results on the virucidal effect of a plasma-treated virus suspension, it can be concluded that when treating the virus suspension diluted in dH2O in a ratio of 1:1 and treated at a plasma power of 13 W, the first results appear after the 240th second, while the decrease in viral titer is 0.48 log, at an exposure time of 300 seconds, the viral titer decreases by 1.23 log.

11. When studying the virucidal effect of a plasma-treated virus suspension diluted in a ratio of 1:2 with dH2O, a decrease in the titer of the virus sample was found compared to the control by 1.67 log.

12. A strong quenching effect of DMEM medium and DMEM medium (with 4% FBS) was found when examining the type and amount of reactive oxygen species produced by treatment with low-temperature non-equilibrium gas discharge plasma in the presence of $H_2O_2$ and using an experimental setup including lucigenin.

13. The results of investigating the effect of plasma-treated saline on the chemiluminescent signal, when applying the first experimental setup in the presence of lucigenin, showed that at the early measurement intervals the levels of reactive oxygen species (in particular $H_2O_2$) were higher.

14. When investigating the effect of plasma-treated $dH_2O$ on the chemiluminescence signal, when applying an experimental setup in the presence of lucigenin, a strong decrease of reactive oxygen species was found after the short time intervals of treatment, which could be explained by the interaction of the formed radicals with nitrogen from the atmosphere and the formation of nitrates and nitrites.

15. When investigating the effect of PBS on the chemiluminescence signal and using enhanced chemiluminescence with the participation of luminol, a decrease in the output signal was found when using 13 W plasma power.

16. Comparing the obtained data with the results obtained from the experiments on the cytotoxic effect of the plasma and the low percentage of cell protection when studying the influence of the plasma-treated medium on the replication of HSV-1, we judge that the nutrient medium used in the working protocols has a high extinguishing effect on the obtained radicals, which is most likely due to the rich component composition and the presence of vitamins in the medium used

# 7. Declaration of Originality (Contributions)

1. A relatively strong protective effect was reported under the influence of cells inoculated with HSV 2 (DD) and treated with the total extract of Vaccinium vitis-idaea L., from plants collected from Stara Planina - 98%. E xtracts obtained from the other plants we studied showed about 70% protection against HSV-1 (F) and HSV-2 (DD).
2. For the first time worldwide, the impact of food and water treated with surface wave non-equilibrium gas discharge plasma (constructed by a Bulgarian team of scientists) for antiviral and virucidal action has been studied. When studying the virucidal effect of a plasma-treated virus suspension diluted in a ratio of 1:2 with dH2O, a decrease in the titer of the virus in the virus sample was found compared to the control by 1.67 log.
3. The Virology laboratory team joined the joint effort to determine the effectiveness of manufactured and marketed face masks with filtering efficiency. From over 90 face masks with filtering efficiency provided to us for research, after conducting the experiments to determine the degree of protection by applying a modified VFE method, the results showed that the filtering face masks of the FFP type provide the greatest degree of protection compared to the Public Face Masks masks, with a filtering efficiency of 99%. The reliability of the results obtained by this method has been confirmed by a certified European laboratory.

## 8. Publications related to the dissertation work

1. Angelova, P., A. Hinkov, V. Tsvetkov, D.Todorov, K. Shishkova, D. Dragolova, S. Shishkov, V.Kapchina-Toteva. 2018. Antiherpes virus activity of extracts from Artemisia chamaemelifolia Vill. Compt. Rend. l’Acad. Bulg. Sci., Vol 72, No11, pp.1475-1483. IF2017 = 0.251; SJR2017 = 0.21 Q2

2. Tsvetkov V., A. Hinkov, D. Todorov, E. Benova, I. Tsonev, T. Bogdanov, S. Shishkov, K. Shishkova 2020. Effect of plasma-activated medium and water on replication and extracellular virions of Herpes Simplex Virus-1. Plasma Medicine, Vol 10, pp.15-26 IF2022=0.83; SJR2022=0.264 Q3

3. Tsvetkov V., D. Todorov, A. Hinkov, K. Shishkova, S. Shishkov 2022. Study of community face coverings and commercially available face masks using a modified viral filtration method. Annual of Sofia University “St. Kliment Ohridski” Faculty of Biology, Book 4, volum 107, pp. 124-131