https://doi.org/10.31925/farmacia.2023.5.14 **ORIGINAL ARTICLE**

ANTIVIRAL ACTIVITY OF ORIGINAL FLAVONOIDS-CONTAINING PHYTOPREPARATION AGAINST HUMAN ALPHAHERPESVIRUS 2, HEPATITIS C SURROGATE VIRUS AND TRANSMISSIBLE GASTROENTERITIS CORONAVIRUS

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Abstract

This study evaluates the antiviral activity of a complex phytopreparation consisting of alcohol tinctures of *Filipendula vulgaris*, *Petroselinum crispum*, *Apium graveolens* radices, *Galium verum*, *Linaria vulgaris* herbs and *Calendula officinalis* flowers. The phytopreparation was tested on *in vitro* models of human alphaherpesvirus 2 (HHV-2), hepatitis C surrogate virus (bovine viral diarrhoea virus, BVDV) and transmissible gastroenteritis virus (TGEV). The phytopreparation solution demonstrated antiviral activity against all three viruses. The phytopreparation solution is an active inhibitor of HHV-2, BVDV and coronavirus (TGEV) replication with a selectivity index of 80, 320 and 160, respectively. Animal studies showed that the phytopreparation solution (1:100 dilution), when used as a combined treatment regimen (*per os* + application), delayed the onset of guinea pig genital herpes first symptoms and reduced the time of disease *in vivo*. The phytopreparation solution had an antiherpetic therapeutic action similar to that of acyclovir (a reference antiherpetic drug). The induction of αIFN and inhibition of RNA and DNA synthesis were suggested mechanisms of the antiviral action of the phytopreparation complex.

Rezumat

Studiul evaluează activitatea antivirală a unui fitopreparat complex format din tincturi alcoolice din rădăcini de *Filipendula vulgaris*, *Petroselinum crispum*, *Apium graveolens* radices, *Galium verum*, părți aeriene de *Linaria vulgaris* și inflorescențe de *Calendula officinalis*. Fitopreparatul a fost testat pe modele *in vitro* de alfaherpesvirus uman 2 (HHV-2), surogatul virusului hepatitei C umane (virusul diareii bovine, BVDV) și virusul gastroenteritei transmisibile (TGEV). Fitopreparatul a demonstrat activitate antivirală asupra celor trei virusuri. cu un indice de selectivitate de 80, 320 și, respectiv, 160. Studiile pe animale au arătat că fitopreparatul (diluție 1:100), atunci când este utilizată ca regim de tratament combinat (*per os* + aplicare), a întârziat apariția primelor simptome ale herpesului genital la cobai și a favorizat vindecarea, *in vivo*. Fitopreparatul a avut o acțiune terapeutică antiherpetică similară cu cea a aciclovirului (medicament antiherpetic de referință). Inducerea αIFN și inhibarea sintezei de ARN și ADN au fost sugerate mecanisme ale acțiunii antivirale a fitocomplexului.

Keywords: phytopreparation, human alphaherpesvirus 2, hepatitis C surrogate virus, transmissible gastroenteritis virus

Introduction

The need for more effective antivirals has become increasingly urgent due to the emergence of drug-resistant strains and the limited success of existing treatments. Herbal medicines have been identified as a promising area of research in this regard, owing to their biologically active compounds that may offer greater efficacy than single compounds. Compared to chemical drugs, herbal remedies have a lower likelihood of resulting in resistant forms of virus occurrence, making them a potentially safer option for long-term use [8, 22].

Medicinal herbs typically contain a variety of biologically active compounds (BAC) that can be isolated through extraction and formulated into an alcoholic tincture or a solid dosage form.

Tazalok, a commercially available alcoholic extract of six plants commonly used in official and traditional medicine [4, 5, 17, 30, 33, 42] - *Filipendula vulgaris* roots, *Petroselinum crispum* roots, *Apium graveolens* roots, *Gallium verum* herb, *Linaria vulgaris* herb and *Calendula officinalis* flowers [20], has been identified as a promising new safe antiviral agent. This phyto-preparation contains biologically active compounds similar to endogenous oestrogens, but without oestrogen-like effects [13, 39, 41]. It has pronounced effects on

glandular and stromal tissues in the mammary glands, ovaries and uterus. Additionally, it exhibits anti-inflammatory and antiproliferative properties and prevents the development of dysplastic processes in these tissues [19, 45]. The phytopreparation is composed of medicinal plants from different families that accumulate various groups of biologically active compounds with potential therapeutic uses in reproductive endocrinology and beyond.

Scientific publications present many studies on the pronounced antiviral activity of Tazalok plant materials [7, 9, 43]. Flavonoids such as apigenin from *Petroselinum crispum* [47], apiin from *Petroselinum crispum* and *Apium graveolens*, pectolinarin, isolinariin and pectolinarigenin from *Linaria vulgaris* [12, 24, 26, 30, 35, 49] are of particular interest among phytopreparation's phenolic active pharmaceutical ingredients.

Flavonoids (quercetin from *Filipendula vulgaris* [50], kaempferol from *Petroselinum crispum* [17], spireoside and isoquercitrin from *Filipendula vulgaris* [42] are also promising active substances with antiviral activity.

According to European Agency for the Evaluation of Medicinal Products (1999) [16], *Calendula officinalis* flowers tincture inhibits the replication of herpes simplex viruses, influenza A2 and influenza APR-8 viruses *in vitro*. Calendula's glycosylated flavonols rutin and isorhamnetin are used to suppress H1N1 influenza virus [2].

Hydroxycinnamic acids, which are present in significant quantities in *Galium verum*, *Filipendula vulgaris*, *Calendula officinalis* and *Linaria vulgaris*, have proven antiviral properties against infectious bronchitis virus (IBV) *in vivo* and *in vitro* [1]. Xanthophyll lutein, which is contained in the raw material of *Calendula officinalis*, effectively inhibits the transcription of the hepatitis B virus [30]. *Petroselinum crispum* and *Apium graveolens* could be a promising source of furanocoumarins with antiviral activity through psoralen, bergapten, xanthotoxin and umbelliferon with antiviral activity against HHV-1 and influenza viruses H1N1 and H9N2, Coxsackievirus B3 [3, 26, 38]. While furanocoumarins have the potential for antiviral effects, they also have a photosensitizing effect. However, taking the phytopreparation orally significantly lowers the risk of photosensitization that is linked to furanocoumarins.

Galium verum triterpene saponins, particularly lupeol and ursolic acid, have antiviral potential in the case of rotavirus infections, as ursolic acid inhibits the early stages of the rotavirus replication cycle [23, 49].

Confirmation of the antiviral activity of Tazalok complex phytopreparation is of scientific interest and may have practical significance as a new safe antiviral agent. The liquid dosage form is easier to implement and has better pharmacoeconomic benefits.

We utilized *in vitro* viral models to screen components of phytopreparation for inhibitory effects on virus replication. We then conducted in-depth *in vivo* antiviral effects studies. Additionally, we evaluated phytopreparation's interferon-inducing activity, as its antiviral action is proposed to involve the interferon system.

Materials and Methods

Reagents

Purified water was obtained using the Milli-Q® Millipore system (Millipore). All other chemicals were of analytical purity grade.

The transcriptional reaction mixture contained 0.5 μg of linearized DNA of plasmid *pTZ19R* with the promoter RNA polymerase T7, ribonucleoside tri-phosphates – each at a concentration of 2 mM, 20 Units of RNAase inhibitor activity RiboLock™ in the presence of 40 mM Tris-HCl pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM di-thiothreitol (DTT) and 12 Unit T7 RNA polymerase activity.

Herbal preparations

In this study, we used Tazalok, an oral drop solution produced by Dr. Gustav Klein GmbH & Co. KG, Germany and declared by UA Pro-Pharma LLC, Ukraine. Tazalok is an alcoholic tincture of medicinal plant raw materials mixture (1:10): dropwort (*Filipendula vulgaris*) roots – 0.28 g, parsley (*Petroselinum crispum*) roots – 0.225 g, celery (*Apium graveolens*) roots – 0.17 g, lady's bedstraw (*Galium verum*) herbs - 0.135 g, common toadflax (*Linaria vulgaris*) herbs – 0.11 g, common marigold (*Calendula officinalis*) flowers – 0.08 g (extractant: ethanol 40%). The content of extractives was not less than 1.5%. The drug was standardized for the presence and content of flavonoids, hydroxycinnamic acids, iridoids and terpenoids [45].

Tazalok's antiherpetic activity was compared to acyclovir *in vivo*. Acyclovir was chosen as a reference due to its precise antiviral mechanism of action - inhibiting viral DNA polymerase through phosphorylation by viral thymidine kinase [47].

To evaluate inductive effects, a standard inducer of α/β-IFN, double-stranded polyribonucleotide Poly(I)•Poly(C) (Calbiochem), was used.

Preparation of dilutions series

To determine the cytotoxic concentration of phyto-preparation, dilutions from 1:5 to 1:2560 in 40% ethanol were made. Dilutions from 1:800 to 1:6400 in purified water were prepared to determine anti-herpetic and anti-HCV activity *in vitro*, and from 1:100 to 1:3200 for anti-TGEV activity *in vitro*. Studies of antiherpetic activity and interferonogenicity *in vivo* were performed using phytopreparation in a 1:100 dilution in sterile water.

In vitro transcription and PCR were performed after evaporating the phytopreparation solution using a vacuum rotary evaporator at 40°C and dissolving it further in DMSO (1 mg/mL).

Cell cultures

The cell cultures used to determine cytotoxic and antiviral effects of the phytopreparation were obtained from the Cell Cultures Museum of "Kavetsky" Institute of Experimental Pathology, Oncology and Radiobiology in Ukraine.

To determine cytotoxic and antiviral effects of the phytopreparation, following cell cultures were used: PEK cell line (transplanted pig embryonic kidney cell line) was used for the TGEV coronavirus model; BHK cell line (transplanted culture of baby hamster kidney cells) was used for HHV-2 and MDBK cell line (transplanted culture of the Madin-Darby bovine kidney cell line) was used for the model of bovine viral diarrhoea virus (BVDV).

Mouse interferon activity was studied in transplanted OH-1 lymphoblastoid cells.

Cell cultures were grown in plastic plates and incubated in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% foetal calf serum (Nunclon, Surface) at 37°C in a 5% CO_2 humidified atmosphere.

Viruses

Four viruses were used in this study. HHV-2 and vesicular stomatitis virus (VSV) (also known as *Indiana vesiculovirus*) were obtained from the Viruses Museum of the "Ivanovsky" Institute of Virology of the Russian Academy of Medical Sciences. HHV-2 was maintained by serial passages in Vero cell culture. The infectious titre for cytopathogenic effect (CPE) in cell culture was 9.0 lg $TCID_{50}$ (tissue culture infectious dose) *per* 1 mL for HHV-2 and 5.0 lg $TCID_{50}$ *per* 1 mL for VSV in the OH-1 cell culture.

The bovine viral diarrhoea virus (BVDV) was provided by the Institute of Veterinary Medicine of the National Academy of Agricultural Sciences of Ukraine. BVDV is a small RNA virus belonging to *Flaviviridae* family. BVDV is a surrogate system for studying hepatitis C virus, as well as the West Nile virus and Dengue virus [10, 44], which also belongs to *Flaviviridae* family.

Transmissible gastroenteritis virus (TGEV) is an etiological agent of transmissible gastroenteritis of pigs (TGP), a highly contagious intestinal disease of pigs. The infectious titre of the virus after ten passages in the culture of PEK cells was 8.0 lg $TCID_{50}$. Strain D_{52-5} (BRE_{79}) of TGEV at fifth passage level monolayer culture of piglet's testicular cells (ST) was provided by Dr. Hubert Laude from the Laboratory of Molecular Virology and Immunology of the INRA Biotechnology Centre in Jouan-en-Josas (France).

All viral materials were stored at -70ºC prior to experimental studies.

Animals

Male outbred guinea pigs 250 - 300 g body weight (b.w.) and white nonlinear mice 14 - 18 g b.w., both sourced from the experimental animals breeding vivarium of the National Academy of Sciences of Ukraine, were utilized for the study. The animals were kept under controlled conditions of temperature (22°C to 24°C), relative humidity (40% to 70%), lighting (12-hour light-dark cycle) and fed a standard pellet diet. The study was performed following the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and was approved by the Institutional Animal Care and Use Committee. All animal-related procedures were performed in accordance with the ethical standards of the Animal Ethics Committee, "Gromashevsky" Institute of Epidemiology and Infectious Diseases of the National Academy of Medical Sciences of Ukraine (No.: AEC/25/2021).

Titration of infectivity

Two methods were used to titrate the infectivity of viral materials on cell cultures: the cytopathogenic method with titre determination by the Kerber method under Ashmarin's modification [6, 36] and presented as $TCID_{50}$/mL, and the method of negative colonies (S-sign) under 1.35% agarose coating (Difco® Bacto® Agar, Carolina Biological Supply Company) with titre determination in plaque-forming units (PFU) *per* mL. After 120 hours of cultivation at 38°C, the results were calculated.

Determination of effective (EC_{50}) and cytotoxic (CC_{50}) concentration, selectivity index (SI)

The effective concentration (EC_{50}) is the minimum drug dilution that reduces virus-specific cytopathogenic action and infectious virus titre by at least 50% and 2 lg, respectively. To determine the EC_{50}, 100 $TCID_{50}$/0.1 mL of the test virus was added to cell culture and incubated for 60 min at 37°C. After virus adsorption, the cells were washed, and phytopreparation in different concentrations was added to the maintenance medium (RPMI-1640 containing 2% foetal serum). The absence of cytopathogenic effect and infectious titre reduction in treated cultures with both these parameters and the difference of infectious titres in the experiment compared to virus control by at least 2 lg allowed for establishing the phytopreparation EC_{50}.

Cytotoxic concentration (CC_{50}) is the concentration of a drug that reduces the viability of a cell culture by 50%. It is determined by analysing the cytotoxic effects of test compounds using different cell cultures. At least ten rows of wells in cell culture plates for each preparation dilution in a nutrient medium are used. The cell culture plates are then incubated at 37°C with 5% CO_2 for 5 days, and the experimental and control cultures are monitored daily to determine the presence or absence of CPE.

The CPE degree was determined by changes in cell morphology, estimated by a 4+ system from - to ++++ indicating the extent of cell degeneration: "–" – a complete absence of cell degeneration; "+" – affected no more than 25% (protection of cell mono-layer from antiviral drugs by 75%); "++" – affected

no more than 50% of the cell monolayer; "+++" – affected no more than 75% of the cell monolayer and "++++" – complete degeneration of the cell monolayer.
The highest concentration of the preparation that did not cause 50% cell monolayer degeneration was used to determine the CC_{50}. The preparation selectivity index (SI) was calculated as the ratio of CC_{50} to EC_{50}. Test substances with an SI ≥ 16 *in vitro* were considered more promising for further animal studies [20].

Study of antiherpetic, anti-BVDV and anti-TGEV activity

To test antiherpetic activity, BHK cells were treated with test phytopreparations in varying concentrations. After 1 hour of contact, HHV-2 at a dose of 100 $TCID_{50}$ was introduced. Cultures were incubated in a thermostat in 5% CO_2 for 5 days, with daily monitoring under a microscope and noting the replication of the virus by the cytopathogenic effect of HHV-2 on BHK cells compared with control cultures, where the monolayer was not treated.
The study tested the anti-HCV activity using different phytopreparation dilutions on MDBK culture. BVDV contaminated with human hepatitis C virus was added at a dose of 100 $TCID_{50}$/mL. The cultures were then incubated in a thermostat to determine specific cytopathogenic effects in virus control followed by an estimation of infectious titre of the virus in the culture medium.
The study investigated the impact of phytopreparation on TGEV replication in the PEK cell line using the same drug treatment regimen.

Study of antiviral activity in vivo and establishment of infection model

We infected male guinea pigs weighing 250 - 300 g [27] with 6.0 lg $TCID_{50}$/mL of HHV-2 by applying it to pre-scarified skin of the genitals using a surgical lancet. The scarification area was 4 - 7 mm². Clinical symptoms of genital herpes were recorded daily before treatment and throughout the disease period, based on indicators of oedema, hyperaemia, orchitis and rash.
Treatment for animals infected with herpes virus involved *per os* administration of herbal medicine solution for 5 days, with a combined scheme of phytopreparation application on the infected wound surface and *per os* administration. Four groups of animals were tested, including a control group treated with acyclovir. The first group was infected only with the herpes virus, the second group was infected with the herpes virus and treated with a 0.2 mL solution of phytopreparation at a dilution of 1:100, the third group was infected with the herpes virus and treated with a combination of applications and oral administration of phytopreparation (0.2 mL + 0.2 mL) at a dilution of 1:100, and the fourth group was infected with the herpes virus and treated with acyclovir (control drug) through a 0.2 mL application at a concentration of 2.5 mg/mL.
A dilution of 1:100 of the phytopreparation was used because its concentrated alcoholic solution could cause burns and damage when applied to mucous and skin surfaces.
Criteria for assessing the severity of the infectious process included the area and degree of specific lesions, the presence of oedema, redness, rash and discharge (estimated by a 4-point system). Observations of animals were conducted for 20 days, with each group consisting of 7 animals.
The effectiveness of the phytopreparation was evaluated during the peak of the pathological process. The index of therapeutic effect (TI) was compared between the experimental and control groups by reducing the severity and duration of clinical manifestations. TI% was calculated using the following formula:

$$TI\% = (\Sigma contrl - \Sigma exp)/\Sigma contrl.$$

In vitro transcription reaction (T7RNA)

The study investigated the impact of test preparations on RNA synthesis in the transcription system, using commercial reagents from Fermentas [33]. The reaction mixture contained 20 μL of transcriptional reaction mixture, where substances were dissolved in DMSO (1 mg/mL). The control and experimental samples had 2.5% DMSO concentration, which had no effect on RNA synthesis productivity. The reaction mixture was kept at 37°C for 45 min and stopped by cooling (to -20°C). The RNA transcripts were detected by gel electrophoresis in 1% agarose with the addition of 0.3 μg/mL ethidium bromide. A UV-transilluminator visualized RNA transcripts. The intensity of RNA bands was measured by Scion Image densitometrical program.

Detection of strain D_{52-5} (BRE_{79}) of TGEV RNA by reverse polymerase chain reaction (RT-PCR)

PCR was performed using a kit for PCR (AmpliSens) and a DNA recombinant plasmid based on the vector PUC 28 containing a sequence encoding the LIF gene (human leukaemia inhibitory factor) as a matrix. DNA amplification was performed in a thermostat for PCR analysis "Tertsyk" (DNA technology). Test substances were tested in concentrations of 0.001 - 40 μg/mL.
Due to the negative effects of ethanol as an extractant on the transcription reaction, the phytopreparation was evaporated to dryness on a vacuum rotor at 40°C. The resulting residue was dissolved in the same volume of DMSO. As the phytopreparation was used in DMSO, the control was also mixed with DMSO. For all experiments, samples were 0.5 μL with a final volume of 20 μL.
RNA isolation was performed using a "RIBO-sorb" kit (AmpliSens) according to the manufacturer's instructions, and the reverse transcription reaction was performed using the "RevertAidTM H Minus First

Strand cDNA Synthesis Kit" (Thermo Scientific). Nucleoprotein gene-specific oligonucleotide primers with sequences forward Uni_1 (5'-TGCACTGATCAATGTGCTAG-3') and reverse Uni_2 (5'-TGAAAACACTGTGGCACCCTT-3') were used for PCR amplification of a 309 base pair fragment. The "100 bp Plus DNA Ladder" from Thermo Fisher Scientific was used as a marker.

Induction of interferon (IFN) in vivo

The interferonogenicity of the phytopreparation *in vivo* on non-linear white mice (14 - 18 g b.w.) *via* intraperitoneal administration at a 1:100 dilution in sterile water at a dose of µL/mg b.w. After 24 and 48 hours, mice were euthanized and blood samples were collected. Blood serum was separated by centrifugation (1500 g, 10 min and 25°C). Each serum sample was divided into two parts. In the first part, serum was adjusted to pH 2.0. In the second part, serum was kept at 4°C for 24 hours without changing the pH. After that, the pH of the serum was raised to 7.3. The interferon level was determined by the standard method of CPE inhibition of the vesicular stomatitis virus in the culture of transplanted cultures of OH-1 (transplanted mouse lymphoblastoid cells).

Statistical analysis

Obtained data were expressed as the mean ± standard error of the mean (M ± SEM) and analysed with one-way ANOVA followed by Tukey's test in OriginPro 7.5 Software. Differences were considered to be statistically significant at $p < 0.05$.

EC_{50}, CC_{50} and SI were calculated using nonlinear regression analysis.

Results and Discussion

Tazalok is a phytopreparation made up of a complex tincture from six plants that contain a variety of biologically active substances, with flavonoids being the most prominent. Flavonoids have been found to have antiviral properties against various types of viruses [7, 27, 31, 32, 35, 41, 48]. The Tazalok herbal preparation underwent comprehensive chemical analysis to determine the content of the dominant flavonoids in the herbal preparation – apigenin, quercetin, linarin and catechin. Quantification was performed using high-performance liquid chromatography coupled with UV-detection, according to a previously described method [18]. The content of apigenin was found to be 0.0072% (w/w). Apigenin, a flavone compound, is known for its antiviral, antioxidant and anti-inflammatory properties. Its presence in the Tazalok herbal preparation suggests a potential role in conferring antiviral activity against human alphaherpesvirus 2, hepatitis C surrogate virus and transmissible gastro-enteritis coronavirus, as investigated in this study. The content of apigenin correlates with the content of dropwort and plants of the *Apiaceae* family.

Quercetin, a flavonoid with antiviral, antioxidant and anti-inflammatory properties, is present in Tazalok herbal preparation at 0.48% (w/w), potentially contributing to its antiviral activity.

The Tazolok herbal preparation contained 0.0024% (w/w) of linarin, an iridoid glycoside known for its anti-inflammatory and neuroprotective effects.

The Tazalok herbal preparation contains catechin, a flavonoid with strong antioxidant and antiviral properties, at a concentration of 0.02% (w/w). The phytopreparation has a total range of polyphenolic compounds of 2 mg/mL.

Determination of effective (EC_{50}) and cytotoxic (CC_{50}) concentration, selectivity index (SI) in vitro

The antiviral study of phytopreparation consisted of two stages: assessing cytotoxicity in cell culture and quantifying its effect on virus replication. PEK, BHK and MDBK cell cultures were used to study EC_{50}, CC_{50} and SI (Table I).

According to the cytotoxicity test results, the phyto-preparation CC_{50} corresponds to a 1:20 dilution in PEK cells sensitive to TGEV, a 1:40 dilution in BHK culture and a 1:20 dilution in MDBK culture.

Table I

The results of CC_{50} determination in cell culture (% cell degeneration)

Cell culture	**% cell monolayer degeneration**								
	Preparation dilution								
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
PEK	100	100	50	0	0	0	0	0	0
BHK	100	100	100	0	0	0	0	0	0
MDBK	100	100	0	0	0	0	0	0	0

PEK – transplanted pig embryonic kidney cell line; BHK – transplanted culture of baby hamster kidney cells; MDBK – transplanted culture of the Madin-Darby bovine kidney cell line

Study of phytopreparation antiviral activity on the models of HHV-2, BVDV, TGEV and transmissible gastroenteritis virus in vitro

Results of phytopreparation antiherpetic activity (EC_{50}) in the culture of BHK cells are presented in Figure 1.

Studies have shown that a phytopreparation inhibits herpes virus replication at a 1:3200 dilution.

Figure 2 presents the antiviral activity of phyto-preparation on MDBK cells using BVDV as a surrogate for hepatitis C virus.

Based on the results, the phytopreparation solution shows antiviral activity when diluted to 1:6400.

The results of anti-coronavirus activity (EC_{50}) of phyto-preparations in PEK culture are shown in Figure 3.

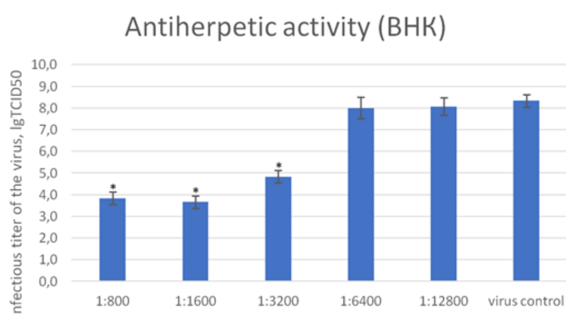

Figure 1.
Phytopreparation antiherpetic activity in the culture of BHK cells
(asterisk indicates $P < 0.05$ *vs*. virus control)

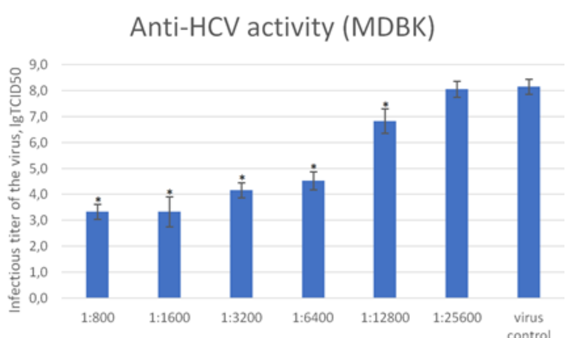

Figure 2.
Phytopreparation antiviral activity in the culture of MDBK cells with BVDV
(asterisk indicates $P < 0.05$ *vs*. virus control)

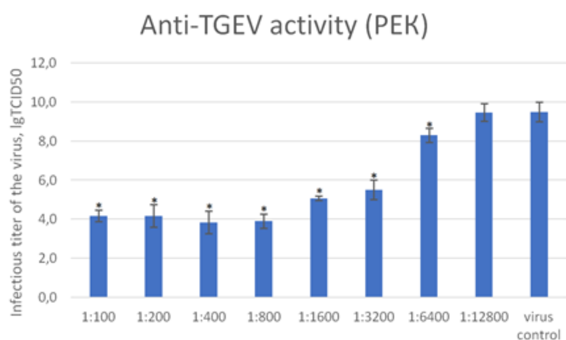

Figure 3.
Infectious titre of TGEV in wells treated with different dilutions of phytopreparation and TGEV virus 100 $TCID_{50}$
(asterisk indicates $P < 0.05$ *vs*. virus control)

According to the results, phytopreparation inhibited TGEV replication at a dilution of 1:3200.

The selectivity indices and reduction of infectious titre by 2.0 lg $TCID_{50}$ are criteria for antiviral drug inhibitory activities assessment *in vitro* systems. Table II presents the summarized results of phytopreparation CC_{50}, EC_{50} and SI for HHV-2, surrogate hepatitis C virus (BVDV) and coronavirus TGEV.

Table II

Phytopreparation selectivity indices for the human alphaherpesvirus 2 (HHV-2), the surrogate hepatitis C virus (BVDV) and the coronavirus (TGEV)

Virus model	CC_{50}	EC_{50}	SI
HHV-2	1:40	1:3200	80
BVDV	1:20	1:6400	320
TGEV	1:20	1:3200	160

HHV-2 – human alphaherpesvirus 2; BVDV – surrogate hepatitis C virus; TGEV – coronavirus

Table II shows that the phytopreparation solution effectively inhibits HHV-2, surrogate hepatitis C virus (BVDV) and coronavirus (TGEV) replication.

Study of phytopreparation antiherpetic activity for the treatment of human alphaherpesvirus 2 infection genital form in vivo

An experimental model of HHV-2 infection was created in male guinea pigs by locally administering HHV-2 to their genital lesions. This model closely mimics genital herpes in humans as the virus is administered in a manner similar to natural human infection with HHV-2. The disease in experimental animals also causes central nervous system damage and latent virus preservation in the body, and multiple recurrences of the disease are possible, as shown in Table III.

Phytopreparation solution taken orally once daily for five days reduced symptom severity by 30.0 points (index of therapeutic effect of 41.17%), shortened duration of disease and delayed symptom onset detection by three days. When combined with topical application, disease duration was five days, the index of therapeutic effect was 54.49%, and symptom detection was delayed by three days. Thus, the combined treatment was more effective in treating genital herpes than oral administration alone.

Table III

The preparation's effectiveness on the model of genital herpes in guinea pigs (n = 7)

Group number	Group	The duration of the disease, days, (M ± m)	Specific infectious process severity manifestation, points	Therapeutic index, %
1	HHV-2	15.00 ± 3.20	51.0	-
2	HHV-2 + phytopreparation solution (1:100) *per os*	9.00 ± 2.30*	30.0	41.17
3	HHV-2 + combined scheme (phytopreparation *per os* + application)	5.00 ± 1.50*	23.0	54.49
4	HHV-2 + acyclovir, application	9.75 ± 2.86*	22.0	56.00

Asterisk indicates $P < 0.05$ (vs. Group 1)

Study of the mechanisms of phytopreparation antiviral effect

It is known that the 3D structures of DNA and RNA polymerases are highly similar and contain the structural domains and conservative motifs necessary for nucleic acid chain elongation [45]. Thus, the RNA T7 model system has been successfully used to select RNA synthesis inhibitors and detect transcription inhibitors for effective antimicrobial or antiviral agents [33]. The inhibitory effects of phytopreparations on RNA synthesis in the RNKPT7 system and DNA synthesis by PCR were determined, and the study results are presented in Figure 4.

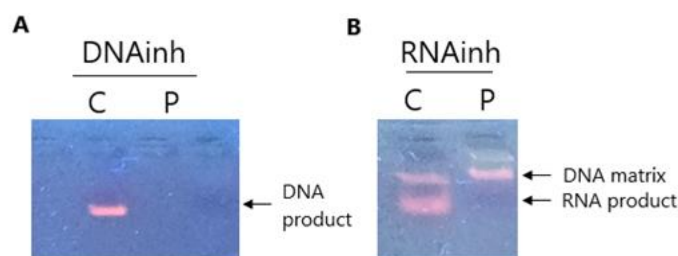

Figure 4.

Inhibition of DNA synthesis *in vitro* under the influence of phytopreparation: (**A**) - complete inhibition is determined by the absence of the DNA product in the agarose gel (the absence of a red band) and inhibition of RNA synthesis (transcription of TNK T7) *in vitro*; (**B**) - Complete inhibition is determined by the absence of RNA product in the agarose gel (the absence of a red band) (C: reaction control; P: phytopreparation)

The results of the study show that the phytopreparation, which contains flavonoids, inhibits both RNA and DNA synthesis *in vitro* [14].

Study of interferon induction by phytopreparation

The results of the phytopreparation interferonogenic activity study are shown in Table IV.

The maximum interferonogenic activity was induced by the phytopreparation at a solution concentration of 1:100 after 48 hours. The acid resistance marker induced by the phytopreparation showed that IFN refers to αIFN.

Table IV

Phytopreparation interferonogenic activity

Time, hours	Titres of IFN induced by phytopreparation, AU/mL		Titres of IFN induced by Poly(I)·Poly(C), AU/mL		Baseline titres of IFN in mice, AU/mL	
	ph+	ph-	ph+	ph-	ph+	ph-
24	320	320	2048	2048	10	10
48	1280	1280	1024	1024	-	-

ph+ – with pH change, ph- – without pH change

Given the composition of biologically active substances in the phytopreparation Tazalok, and as separated components have shown antiviral activity in the past [7, 9, 43], we studied the combined effect of the phytopreparation Tazalok on a number of viruses *in vitro* and *in vivo*, paying special attention to its effect on RNA viruses and the effectiveness of the phyto-preparation for genital herpes *in vitro*. These viruses are convenient models for the study of phytopreparations antiviral activities [35] and related viral diseases are significant medical and social problems for humanity.

Before conducting any pharmacological or toxicological study on BAC in cell culture, we determined the effective (EC_{50}) and cytotoxic (CC_{50}) concentrations, as well as the selectivity index (SI). Without establishing these indicators, interpreting experimental data was impossible.

To evaluate the toxicity of chemical compounds, enzymatic reactions are the preferred method (MTT/MTS-assay, cellular ATP production test, etc.), as they are more accurate than visual methods [15]. The accuracy of enzymatic methods makes it possible to compare different chemical substances for antiviral drugs and predict their safety profile. Since the toxicity profiles of individual plant components are well studied and indicate their extremely low toxicity, and the active pharmaceutical ingredient of the phyto-preparation is primarily composed of flavonoids, a semi-quantitative approach to cytotoxicity assessment is justified.

In our experiments, we found that the drug contains ethanol as an excipient, which has cytotoxic properties that can significantly affect the results. We used three cell lines (BHK, PEK and MDBK) and found significant differences in the cytotoxic effects of the phytopreparation (Table II). The CC_{50} concentration was 0.05 mg/mL for BHK, 0.1 mg/mL for PEK and 0.1 mg/mL for MDBK based on the concentration of polyphenolic compounds.

The CC_{50} of alcohol tinctures with ethanol as an extractant was determined by other authors. Demir *et al*. [14] found the CC_{50} of propolis alcohol extract was 0.375 mg/mL and 1.66 mg/mL for the oil extract using human keratinocytes cell line. Mattana *et al.*

[29] showed that the CC_{50} values for alcohol and aqueous *Acacia aroma* leaf extracts differed 3.9 times: 0.465 mg/mL and 1.8 mg/mL, respectively, using the Vero cell line [29].
Various methods have been used to determine CC_{50} polyphenol-containing phytoextracts in previous studies, including microscopy in Mattana *et al*. [29], MTS-assay in Dmytrenko *et al*. [15] and biochemical tests in Dmytrenko *et al*. [15].
However, more sensitive methods such as MTT/MTS-assay and cellular ATP production test cannot change the CC_{50} by an order of magnitude. The differences in research methods used cannot explain such significant differences in values between ethanol-containing preparations and preparations without ethanol. Therefore, the cytotoxicity evaluation methods chosen are relevant, and the results obtained generally correlate with the data of other authors regarding polyphenol-containing herbal preparations.
The EC_{50} results of our study for the phytopreparation were significant. The group of BAC had an EC_{50} of 0.625 μg/mL for HHV-2 and TGEV (model of SARS-CoV-2) and 0.312 μg/mL for BVDV (HCV model). *In vitro* doses were significantly lower than other polyphenol-containing preparations or individual polyphenolic compounds. Thus, Demir *et al*. [14] found that the EC_{50} of the propolis alcohol extract relative to the HHV-2 was 2.9 μg/mL (compared to acyclovir – 5.5 μg/mL). The EC_{50} for the polyphenolic compounds epigallocatechin-3-gallate (EGCG) and delphinidin (anti-HCV activity) were 4.85 and 1.12 μg/mL, respectively [11]. According to Nguyen *et al*. [32], the EC_{50} for EGCG and quercetin for severe acute respiratory syndrome-associated coronavirus (SARS-CoV) was about 22 μg/mL. Thus, the polyphenolic compounds in the studied phytopreparation inhibit the replication of viruses (HHV-2, BVDV and TGEV) in lower concentrations than previously described combinations of polyphenolic compounds and phyto-extracts.
Phytopreparation showed higher selectivity index values (80 for BHK/HHV-2, 320 for MDBK/BVDV and 160 for PEK/TGEV) compared to other polyphenol-containing phytoextracts studied. For example, in the determination of antiherpetic activity (anti-HHV-1) Pistachios Kernels (*Pistacia vera* L.) SI extract was 3 [11] and in a similar study for *Euphorbia spinidens* SI was 15 [24]. Our data is similar to acyclovir (179) from Karimi *et al.* [24] study.
Our *in vitro* results correlate with the phytopreparation's therapeutic efficacy *in vivo* models. The phytopreparation had a therapeutic index of 41.17% *per os*. Combined treatment regimens (*per os* + applications) increased the therapeutic index to 54.49%, close to acyclovir's effectiveness (therapeutic index = 56%).
In vitro studies confirmed the presence of RNA and DNA virus replication inhibition through phytopreparation's blockage of nucleic acid synthesis, aligning with other authors' findings on flavonoid-containing plant extracts [11, 35].
It is important to note that the phytopreparation studied has promising *in vivo* therapeutic antiviral potential due to its immunomodulatory effect, which induces interferon biosynthesis. Additionally, the level of endogenous αIFN induction is similar to the effect of the reference polynucleotides Poly (I) • Poly (C). These results are consistent with the findings of Rybalko *et al.* [40] regarding other flavonoid-containing drugs.

Conclusions

Phytopreparation with herb and plant root extracts containing mainly flavonoids showed high antiviral activity against human alphaherpesvirus 2 (HHV-2), hepatitis C surrogate virus (BVDV) and coronavirus (TGEV) in pre-clinical studies.
The phytopreparation solution is an active inhibitor of HHV-2, hepatitis C surrogate virus (BVDV) and coronavirus (TGEV) replication with a selectivity index of 80, 320 and 160, respectively. The phyto-preparation effective concentrations (EC_{50}) in terms of the concentration of polyphenolic compounds were the following: 0.625 μg/mL in the case of HHV-2 and TGEV and 0.312 μg/mL in the case of BVDV.
Experimental studies demonstrate the effectiveness of a phytopreparation in treating genital herpes. The phytopreparation, when administered with a combined regimen (*per os* + application) and in a dilution of 1:100, delays the onset of the first symptoms and reduces the duration of the disease. Its therapeutic index is equivalent to that of the antiherpetic drug acyclovir. The phytopreparation's antiherpetic action may be due to the induction of αIFN and inhibition of RNA and DNA synthesis.

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Conflict of interest

The authors declare no conflict of interest.

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