

Antiviral activity of new flavonoids-containing phytopreparation against human alphaherpesvirus 2, hepatitis C virus, and coronavirus

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Abstract

The antiviral activity of the complex phytopreparation (alcohol tincture of *Filipendula vulgaris*, *Petroselinum crispum*, *Apium graveolens* radices; *Galium verum*, *Linaria vulgaris* herbs; *Calendula officinalis* flowers) was evaluated on the *in vitro* models of current uncontrolled viral infections: human alphaherpesvirus 2, hepatitis C (surrogate bovine diarrhea virus) and coronavirus of transmissible porcine gastroenteritis (TGEV) and demonstrated its high antiviral activity to all tested viruses. The phytopreparation solution is an active inhibitor of human alphaherpesvirus 2, hepatitis C surrogate virus (BVDV), and TGEV coronavirus reproduction with a selectivity index of 80, 320, and 320, respectively. In the guinea pig genital herpes *in vivo* experimental model, it was shown that phytopreparation solution (dilution of 1:100) in case of a combined treatment regimen (*per os* + application) delayed the onset of the infection's first symptoms and reduced the time of disease duration. The index of phytopreparation therapeutic action was at the same level as the Acyclovir (reference antiherpetic drug). The investigation of phytopreparation's mechanism of antiviral action allowed supposing that it could be realized via the induction of α IFN, inhibition of RNA, and DNA synthesis.

1. Introduction

The lack of wide-spectrum effective antivirals and the emergence of drug-resistant strains dictate the urgent need to find out new preparations that can alleviate viral infections. In this regard, herbal medicines are of great scientific interest and have significant research potential. Medicinal plants' antiviral action has been confirmed by many studies, particularly against human immunodeficiency virus [1, 2], influenza [3], herpes simplex [4], and hepatitis [2]. Plant biologically active compounds (BAC) summarized effect could be more effectively used to treat viral infections than single BAC. The advantage of herbal remedies compared with drugs of chemical origin may be considered the lower likelihood of causing resistant forms of virus occurrence [5].

Despite the availability of several effective vaccines, COVID-19 coronavirus infection and its treatment remain one of the world's top public health problems. At the same time, other viral infections continue to circulate in the population, although attention to them has somewhat declined due to pandemic COVID-19 [6]. Therefore, the search for antiviral drugs is relevant primarily for uncontrolled viral infections, such as herpes, hepatitis, coronaviruses, and influenza. It should be conducted both on models of viruses and their surrogate models.

Our herbal preparation is an alcoholic extract of six plants used in official and traditional medicine - *Filipendula vulgaris* roots, *Petroselinum crispum* roots, *Apium graveolens* roots, *Gallium verum* herb, *Linaria vulgaris* herb, and *Calendula officinalis* flowers [7].

Biologically active substances of this phytopreparation resemble in their structures endogenous estrogens but without estrogen-like activity. Therefore, phytopreparation has a selective antiestrogenic activity, which normalizes the imbalance of gonadotropic hormones and the menstrual cycle's second

(luteal) phase. Phytopreparation has a pronounced effect on glandular tissue and stromal elements of the mammary glands, ovaries, and uterus, exhibits antiproliferative and anti-inflammatory properties, prevents the development of dysplastic processes in these tissues [8]. It is used to treat menstrual disorders, fibrocystic mastopathy, retentional ovarian cysts. As part of complex therapy, phytopreparation is used to treat endometrial hyperplasia, uterine fibroids, endometriosis, and polycystic ovary syndrome [9].

The composition of the phytopreparation includes medicinal plants from different families, accumulating various groups of BAC, which have the potential for use not only in reproductive endocrinology.

The results of numerous scientific studies confirm that phenolic compounds contained in high concentrations in the raw material of this phytopreparation, especially phenolic acids (gallic, ellagic, salicylic acids), flavonoids (quercetin, rutin, apigenin kaempferol, catechin, epicatechin, and their derivatives) and other polyphenols are able to normalize a large number of enzymes and enzymatic systems, which are involved in the regulation of cell division and proliferation, platelet aggregation, detoxification, inflammation and the immune response development. These compounds have an antioxidant, anticancer, and immunomodulatory effect and cause a positive impact on hemostasis [9].

Scientific publications present a significant number of studies on the pronounced antiviral activity of these plant materials. Flavonoids (apigenin from *Petroselinum crispum* [10], apiin from *Petroselinum crispum* and *Apium graveolens*, pectolinarin, isolinariin, and pectolinarigenin from *Linaria vulgaris* [11–16]) are of particular interest among phytopreparation's phenolic active pharmaceutical ingredients. Flavonoids' antiviral activity has been actively studied for the last three decades. The first studies of apigenin and Acyclovir combined use demonstrated antiviral activities increase in experiments on cell culture with human alphaherpesviruses type 1 and 2 (HHV-1 and HHV-2) (formerly named herpes simplex viruses 1 and 2) [17]. This encouraged further studies of the antiviral activity of herbal remedies containing apigenin and its derivatives.

Flavonoids (quercetin from *Filipendula vulgaris* [16, 17], kaempferol from *Petroselinum crispum* [18], spireoside and isoquercitrin from *Filipendula vulgaris* [19]) are also promising active substances with antiviral activity. Selway J.W.T. [20] found out that quercetin, morin, rutin, dihydroquercetin, dihydrophysetin, leukocyanidin, pelargonidin and catechin have antiviral activity against seven types of viruses, including herpes simplex viruses, respiratory syncytial virus, poliovirus, Sindbis virus, etc. It is believed that the mechanism of such action is realized via viral polymerase activity inhibition. Quercetin and quercetin-3-rutinoside inhibited the reproduction of HHV-1 and HHV-2, adenoviruses types 3, 8, 11 [21].

According to Bogdanova N.S. et al. [22], *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 supposed to suppress H1N1 influenza viruses [23]. *In vitro* studies have shown that isorhamnetin inhibits the pseudotyped SARS-CoV-2 virus entering HEK293 (ACE2) cells [23, 24].

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* [25]. Xanthophyll lutein, which is contained in the raw material of *Calendula officinalis*, effectively inhibits the transcription of the hepatitis B virus [26]. *Petroselinum crispum* and *Apium graveolens* could be a promising source of furanocoumarins with antiviral activity through psoralen, bergapten, xanthotoxin, and coumarin - umbelliferon. It was shown, for example, that psoralen had antiviral activity against HHV-1 and influenza virus [27]. For substances of the furanocoumarin group, including bergapten and xanthotoxin, Ba Wool Lee et al. [28, 29] proved the presence of antiviral activity against influenza viruses H1N1 and H9N2 due to influenza virus, HHV-1, and Coxsackievirus B3 replication cycle's early phase inhibition.

Galium verum triterpene saponins, in particular, 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 [30]. The components of *Petroselinum crispum* and *Apium graveolens* essential oils (such as myristicin and apiol [31, 32]) have pronounced antifungal and antibacterial effects [33], which helps to alleviate concomitant disorders and to prevent the biofilms formation. Antiviral activity has been demonstrated for well-known flavonoids and other groups of biologically active substances [34]. Therefore, our complex phytopreparation antiviral activity confirmation is of scientific interest and may have practical significance as a new safe antiviral agent.

The aims of this study were:

- - *in vitro* viral models screening for searching phytopreparation's inhibitory effects on viruses' reproduction;
- - further in-depth *in vivo* antiviral effects studies.

As phytopreparation's antiviral action is supposed to involve the interferon system, its interferon-inducing activity was also evaluated when studying the mechanism of antiviral action.

2. Materials And Methods

2.1. Reagents

Purified water was obtained by the Milli-Q® Millipore system (Millipore, USA). All other chemicals had an analytical class of purity.

The transcriptional reaction mixture contains 0.5 µg of linearized DNA of plasmid *pTZ19R* with the promoter RNA polymerase T7, ribonucleoside triphosphates – each at a concentration of 2 mM, 20 U.a. of RNAase inhibitor RiboLock™ in the presence of 40 mM Tris-HCl pH 7,9, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol (DTT) and 12 U.a. T7 RNA polymerase.

2.2. Preparations

In this study, we used the phytopreparation Tazalok, a solution in drops for oral use (manufacturer Dr. Gustav Klein GmbH & Co. KG, Germany, declarant 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 total range of polyphenolic compounds in the phytopreparation was 2 mg/ml.

Tazalok's antiherpetic activity was studied compared to the acyclovir (abnormal nucleoside) effect. Acyclovir was selected for a reference as it has a precise antiviral mechanism of action - inhibits viral DNA polymerase, being phosphorylated by viral thymidine kinase [35].

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

2.3. Preparation of dilutions series

To determine phytopreparation cytotoxic concentration, a series of dilutions in the extractant from 1:5 to 1:2560 were prepared.

A Series of phytopreparation dilutions in purified water was prepared to determine the antiherpetic and anti-HCV (hepatitis C virus) activity *in vitro* – from 1:800 to 1:6400; for the anti-TGEV (transmissible gastroenteritis coronavirus) activity *in vitro* – from 1:100 to 1:3200.

Studies of antiherpetic activity and interferonogenicity *in vivo* were performed using phytopreparation in a dilution of 1:100 (in purified sterile water).

In vitro transcription reaction and PCR were performed after evaporation of the phytopreparation solution in a vacuum rotary evaporator at 40°C and further dissolution in DMSO (1 mg/ml).

2.4. Cell cultures

The original cell cultures were obtained from the Cell Cultures Museum of the Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine.

To determine the cytotoxic and antiviral effects of the phytopreparation, such cell cultures were used: PKE (transplanted pig embryonic kidney cell line) – for the coronavirus model of TGEV; BHK (transplanted culture of baby hamster kidney cells) – for HHV-2, MDBK (transplanted culture of the bovine kidney cell line) – for the model of bovine diarrhea virus.

Studies of mouse interferon activity were performed in a transplanted culture of OH-1 (transplanted mouse lymphoblastoid cells).

Cell cultures were grown in plastic plates in RPMI-1640 medium (Sigma-Aldrich, USA) containing 10% fetal calf serum (Nunc, Surface, Denmark). Cultures were incubated at 37°C in a humidified

atmosphere with a CO₂ supply in a thermostat.

2.5. Viruses

Four types of viral material were used in this study. HHV-2 and vesicular stomatitis virus (VSV) (synonym: *Indiana vesiculovirus*) were obtained from the Viruses Museum of the Ivanovsky Institute of Virology of the Russian Academy of Medical Sciences. The HHV-2 was maintained by serial passages in Vero cell culture. Infectious titer for cytopathogenic effect (CPE) in cell culture was $5.5 \div 9.0$ lg TCID₅₀ (tissue culture infectious dose) per 1 ml. In the case of the VSV, the infectious titer was $4.0 \div 5.0$ lg TCID₅₀ per 1 ml in the OH-1 cell culture.

The bovine diarrhea virus (BVDV) was provided by the Institute of Veterinary Medicine of the National Academy of Agrarian Sciences of Ukraine. BVDV is a small RNA virus belonging to the *Flaviviridae* family. BVDV is a surrogate system for studying the hepatitis C virus, which also belongs to the *Flaviviridae* family as well as the West Nile virus and Dengue virus [36].

Transmissible swine gastroenteritis virus (TGEV) is an etiological agent of transmissible swine gastroenteritis (TGP), a highly contagious intestinal disease of pigs. The infectious titer of the virus after ten passages in the culture of PKE cells was $6.0 \div 8.0$ lg ID₅₀. Strain D₅₂₋₅ (BRE₇₉) 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 Center in Jouan-en-Josas (France).

Before experimental studies, all viral material was stored at -70 °C.

2.6. Animals

Male outbred guinea pigs 250–300 g body weight (b. w.) and white nonlinear white-18 g b. w. from the experimental animals breeding vivarium of the National Academy of Sciences of Ukraine were used in the study. Animals were kept under a controlled temperature (from 22°C to 24°C), relative humidity of 40–70%, lighting (12 h light-dark cycle), and a standard pellet feed diet. The study was performed following the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and approved by the Institutional Animal Care and Use Committee.

2.7. Titration of infectivity

Titration of viral materials infectivity on cell cultures was performed by two methods:

- first – by cytopathogenic action, final dilutions with the infectivity titer determination by the Kerber method (Ashmarin's modification) [37] and presentation as TCID₅₀/ml;
- second – by the method of negative colonies (S-sign) under 1.35% agarose coating (Difco® Bacto® Agar, Carolina Biological Supply Company, USA) with the infectivity titer determination in plaque-forming units (PFU) per ml.

The results were calculated after 120 hours of cultivation at 38°C.

2.8. Determination of effective (EC₅₀) and cytotoxic (CC₅₀) concentration, selectivity index (SI)

The effective concentration (EC₅₀) is the drug's minimal dilution, which inhibits the development of virus-specific cytopathogenic action by 50% and infectious virus titer by at least 2 lg. To determine the EC₅₀, the test virus at a dose of 100 TCID₅₀/0.1 ml was added to the cell culture and incubated for 60 min at 37°C. After virus adsorption on the cells, the residues were removed; the cells were washed with a nutrient medium. Then phytopreparation in different concentrations was added to the maintenance medium (RPMI-1640 containing 2% fetal serum). The absence of cytopathogenic effect and infectious titer reduction in treated cultures with both these parameters simultaneous presence in control and the difference of infectious titers in the experiment compared to virus control by at least 2 lg allowed for establishing the phytopreparation EC₅₀.

Cytotoxic concentration (CC₅₀) – the concentration of the drug, which reduces the viability of cell culture by 50% – was determined by analyzing the cytotoxic effects of the test compounds. Different cell cultures were used to determine the CC₅₀ of the preparations. At least ten rows of wells in cell culture plates for each preparation dilution in a nutrient medium were used. Cell culture plates were incubated at 37 °C with 5% CO₂ for 5 days. Experimental and control cultures were monitored daily to determine the presence or absence of cytopathogenic effect.

The degree of CPE was determined by changes in cell morphology (rounding, shrinkage of cells, rejection from the surface of the holes “degeneratively” changed cells estimated by 4 + plus system from + to ++++):

“–” – a complete absence of cell degeneration;

“+” – affected no more than 25% (protection of cell monolayer from antiviral drugs by 75%);

“++” – affected no more than 50% of the cell monolayer;

“+++” – no more than 75% of the cell monolayer is affected;

“++++” – complete degeneration of the cell monolayer.

The CC₅₀ of the preparation was taken as its highest concentration, which did not cause degeneration of 50% of the cell monolayer.

The preparation selectivity index (SI) was determined as the ratio of CC₅₀ to EC₅₀. Test substances with an SI ≥ 16 *in vitro* were considered more active and promising for further animal studies.

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

To study the preparations for antiherpetic activity daily cultures of BHK cells were used. The growth medium was drained, then the cell monolayer was treated with the test preparations in different concentrations. After 1 hour of contact, herpes simplex virus HHV-2 at a dose of 100 TCID₅₀ was introduced. Cultures were incubated in a thermostat in CO₂ for 5 days, with daily monitoring under a microscope and noting the reproduction of the virus by the cytopathogenic effect of HHV-2 on BHK cells compared with control cultures, where the monolayer was not treated.

The anti-HCV activity was studied in MDBK culture, which was treated with different drug dilutions and added BVDV (contaminated with human hepatitis C virus) at a dose of 100 TCID₅₀/ml. Cultures were incubated in a thermostat for the manifestation of specific cytopathogenic effects in virus control, and then in the culture medium was determined the infectious titer of the virus.

The effect of phytopreparation on the reproduction of TGEV was studied using the same drug treatment regimen.

2.10. Study of antiviral activity *in vivo*. Infection model

We used a model of herpes infection in male guinea pigs [38]. The severity of the disease was recorded according to the indicators of edema, hyperemia, orchitis, and rash. A virus-containing fluid infected Guinea pigs with a 6.0 lg TCID₅₀/ml infectious titer. Virus-containing fluid was applied to the pre-scarified skin of the genitals. Scarification was performed using a surgical lancet on the ether-anesthetized animals. The scarification area size was 4 ÷ 7 mm². The virus-containing liquid was applied by a pipette immediately after scarification (followed by rubbing). Clinical symptoms of experimental genital herpes were recorded daily before treatment and observed throughout the disease period.

Treatment using only *per os* administration of the herbal medicine solution or a combined scheme with phytopreparation application on the infected wound surface and its solution *per os* for 5 days, began 24 hours after animals' infection. There were 4 groups of animals in the experiment:

- the 1st – animals infected only with the herpes virus;
- the 2nd – animals infected with the herpes virus and treated *per os* by a solution of phytopreparation (0.2 ml) at a dilution of 1:100;
- the 3rd – animals infected with the herpes virus and treated with a solution of herbal medicine with a combined scheme: applications + administration *per os* (0.2 ml + 0.2 ml) at a dilution of 1:100;
- the 4th – animals infected with the herpes virus and treated with acyclovir (application) of 0.2 ml at a 2.5 mg/ml concentration.

A 1:100 phytopreparation dilution was used because its concentrated alcoholic solution applied to the mucous and skin surfaces could cause burns and damage.

Criteria for infectious process severity assessment were: the area and degree of specific lesions, the presence of edema, redness, rash, and discharge (estimated by 4 points system). Observations of

animals were performed for 20 days. Each group included 7 animals.

The phytopreparation effectiveness was evaluated at the peak of the pathological process. By reducing the severity of clinical manifestations and reducing the duration of the disease, the index of therapeutic effect (TI) in the experimental groups compared with the control.

The TI% was defined as follows:

$$TI\% = (\Sigma_{\text{contrl}} - \Sigma_{\text{exp}}) / \Sigma_{\text{contrl}}$$

2.11. In vitro transcription reaction (T7RNA)

The effect of test preparations on RNA synthesis was studied in the T7 RNA transcription system using commercial reagents (Fermentas, Lithuania) [39]. Transcription was performed in 20 µl of the transcriptional reaction mixture. The substances were dissolved in DMSO (1 mg/ml). The concentration of DMSO in control and experimental samples was 2.5% (a concentration that did not affect the RNA synthesis productivity). The reaction mixture was kept for 45 min at 37 °C, and then the reaction was stopped by cooling (to -20 °C). The reaction products 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 the RNA bands in the images was measured by Scion Image densitometrical program.

2.12. Detection of strain D₅₂₋₅ (BRE₇₉) of TGEV RNA by reverse polymerase chain reaction (RT-PCR)

PCR was performed according to standard procedures using a kit for PCR (AmpliSens, Russian Federation) and a DNA recombinant plasmid based on the vector PUC 28 containing a sequence encoding the LIF gene (human leukemia inhibitory factor) as a matrix. The DNA concentration was 1–12 pg/100 µl of the reaction mixture. DNA amplification was performed in a thermostat for PCR analysis “Tertsyk” (DNA technology, Russian Federation). Test substances were tested in concentrations of 0.001–40 µg/ml.

Due to ethanol as an extractant that adversely affects the transcription reaction, the phytopreparation was evaporated to dryness on a vacuum rotor at 40°C and dissolved in the same volume of DMSO. As we used the phytopreparation dissolved in DMSO, the control was also with DMSO. For all experiments, all samples were 0.5 µl with a final volume of 20 µl.

According to the manufacturer's instructions, RNA isolation was performed using a “RIBO-sorb” kit (AmpliSens, Russia) and the reverse transcription reaction was performed using the “RevertAid™ H Minus First Strand cDNA Synthesis Kit” (Thermo Scientific, Lithuania). Nucleoprotein gene-specific oligonucleotide primers of the following sequence were used for PCR: direct Uni_1 (5'-TGCACTGATCAATGTGCTAG-3') and reverse Uni_2 (5'-TGAAAACACTGTGGCACCCCTT-3'). Amplified fragment – 309 base pairs. The set. “100 bp Plus DNA Ladder” (Thermo Fisher Scientific, Lithuania) was used as markers.

2.13. Induction of interferon (IFN) *in vivo*

The phytopreparation interferonogenicity was studied *in vivo* on mice with intraperitoneal administration of phytopreparation solution at a dilution of 1:100 (in sterile water).

As active interferon producers, studies were performed on nonlinear white mice ($14 \div 18$ g b.w.). The phytopreparation was administered intraperitoneally once at a dose of $\mu\text{l}/\text{mg}$ b.w. After 24 and 48 hours, mice were removed from the experiment via cervical dislocation, and collected blood samples were. Serum was obtained via centrifugation (1500 g, 10 min, and 25°C).

Each serum sample was divided into 2 equal parts. In the first part, serum was adjusted to pH 2.0; in the second – the pH was not changed, and serum was left at 4°C for 24 hours. Then the serum pH was restored 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).

2.14. Statistical analysis

The obtained data were expressed as the mean $\pm$ standard error of the mean ($M \pm \text{SEM}$) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test using OriginPro 7.5 Software. Differences were considered to be statistically significant at $p < 0.05$.

Nonlinear regression analysis was performed to calculate EC_{50} , CC_{50} , and SI.

3. Results And Discussion

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

The *in vitro* antiviral study of phytopreparation consisted of two stages – the initial determination of cytotoxicity in cell culture, i.e., its ability to inhibit virus reproduction due to irreversible effects on the morphology and metabolism virus-contained cells, and quantification of the phytopreparation effect on virus reproduction.

The most sensitive to selected viruses HHV-2, BVDV, TGEV, and universal cell cultures – PKE, BHK, and MDBK were used to study EC_{50} , CC_{50} , and SI. The phytopreparation antiviral action strength was assessed using EC_{50} and CC_{50} and their ratio through the calculation of SI (Table 1).

Table 1
The results of CC50 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
PKE	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

Analysis of test substances' cytotoxic effects results shows that the phytopreparation CC₅₀ in the culture of PKE cells sensitive to TGEV corresponds to 1:20 dilution, in the BHK culture – to 1:80, in the MDBK culture – to 1:40.

3.2. Study of phytopreparation antiviral activity on the models of human alphaherpesvirus 2, bovine diarrhea virus, and transmissible swine gastroenteritis virus *in vitro*

Results of phytopreparation antiherpetic activity (EC₅₀) determination in the culture of BHK cells are presented in Fig. 1.

Studies have shown that phytopreparation inhibits the herpes virus reproduction at a dilution of 1:6400.

Results of phytopreparation antiviral activity determination in the culture of MDBK cells with BVDV (the model of surrogate hepatitis C virus) are presented in Fig. 2.

According to the obtained results, the phytopreparation solution has antiviral activity at a dilution of 1:12800.

The results of determining the phytopreparation anticoronaviral activity (EC₅₀) in the PKE culture are presented in Fig. 3.

According to the results, it was found that phytopreparation inhibited TGEV reproduction (at a dilution of 1:6400).

The criteria for antiviral drug inhibitory activities assessment *in vitro* systems are their selectivity indices and reduction of infectious titer by 1.5 ÷ 2.0 lg TCID₅₀. Table 2 presents the summarized results of phytopreparation CC₅₀, EC₅₀, SI determination for HHV-2, surrogate hepatitis C virus (BVDV), and coronavirus TGEV.

Table 2
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	1:80	1:6400	80
BVDV	1:40	1:12800	320
TGEV	1:20	1:6400	320

Results on the antiviral activity of the phytopreparation solution in experimental models of human alphaherpesvirus 2, surrogate hepatitis C virus, and TGEV coronavirus (Table 2) indicate that the phytopreparation solution effectively inhibits the reproduction of all these viruses.

3.3. 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 reproduced via modeling at male guinea pigs' genital lesions with local administration of HHV-2. This model is quite close to genital herpes in humans, as the route of virus administration is as close as possible to natural human infection with HHV-2. The disease in experimental animals is also accompanied by central nervous system damage and latent virus preservation in the body. Among these, the possibility of multiple recurrences of the disease is also reproduced. The study results are presented in Table 3.

Table 3
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) <i>per os</i>	9,00 ± 2,30*	30,0	41,17
3	HHV-2 + Combined scheme (phytopreparation <i>per os</i> + application)	5,00 ± 1,50*	23,0	54,49
4	HHV-2 + Acyclovir, application	9,75 ± 2,86*	22,0	56,00
Note: *P < 0.05 (in comparison with group 1)				

It was shown that the use of phytopreparation solution *per os* one time per day for five days reduced the severity of symptoms to 30.0 points (which corresponds to the index of therapeutic effect – 41.17), significantly reduced the duration of the disease, and delayed the symptoms onset detection for three days (Fig. 4).

When using a combined treatment regimen (*per os* + application), the disease duration was five days, the index of therapeutic effect was 54.49%, and the delay in detecting symptoms was three days.

Thus, it was shown that the combined scheme of treatment by the phytopreparation was more effective in the experimental model of genital herpes than only *per os* administration.

3.4. Study of the mechanisms of phytopreparation antiviral effect

It is known that 3D structures of different DNA and RNA polymerases have a high degree of similarity and contain the exact structural domains and conservative motifs necessary for nucleic acid chain elongation [40]. Therefore, an easy-to-use and productive model system for transcription of RNA T7 have been successfully used for the selection of RNA synthesis inhibitors and the effective antimicrobial or antiviral agents - transcription inhibitors detection [41].

Phytopreparation inhibitory effects on RNA synthesis in the RNKPT7 system and DNA synthesis by PCR were determined. The study results are presented in Fig. 5.

As can be seen from the presented results of the study, the phytopreparation blocks both RNA synthesis and DNA synthesis *in vitro*, which is very characteristic of flavonoid-containing plant extracts [44].

3.5. Study of interferon induction by phytopreparation

The results of the phytopreparation interferonogenic activity study are shown in Table 4.

Table 4
Phytopreparation interferonogenic activity

Time, hours	Titers of IFN induced by phytopreparation, AU/ml		Titers of IFN induced by Poly(I)·Poly(C), AU/ml	
	ph+	ph-	ph+	ph-
24	320	320	2048	2048
48	1280	1280	1024	1024
Note: ph+ - with pH change, ph- - without pH change.				

The dynamics of interferon induction by the phytopreparation in mice were such that the maximum interferonogenic activity was recorded for a solution concentration of 1:100 after 48 hours. According to the acid resistance marker induced by the phytopreparation, IFN refers to αIFN.

4. Discussion

The creation of effective and safe antiviral drugs is associated with the need to combine viruses' reproduction-specific (selective) inhibition with no negative impact on the vital processes of macroorganism cells. The ability to influence specific enzymes involved in the virus's replication dramatically facilitates the development of etiological methods for viral infection treatment, one of the main areas of modern virology and pharmacology.

Substances of plant origin remain focused on developing antimicrobial and antiviral drugs [1–5]. A special place among them belongs to plant polyphenols and, in particular, flavonoids. The antiviral activity of natural flavonoids was first described in the mid-20th century. Currently, effective inhibition of different taxonomic groups of viruses' reproduction has been shown *in vitro* and *in vivo* experiments [10]. In recent decades, some flavonoid antiviral effects molecular mechanisms have been discovered. Much of the research in this area has focused on detecting flavonoid modifying impacts on cellular and viral enzymes. Flavonoids of different classes are inhibitors of many enzymes, including hydrolases, oxidoreductases, DNA synthases, RNA polymerases, phosphatases, protein phosphokinases, and oxygenases. Numerous effects of flavonoids in cells depend on the ability to modulate various components of intracellular signaling cascades actions, including tyrosine kinase, mitogen-activated protein kinase, protein kinase C and others [39, 42]. Such an inhibitory effect on enzymes can be competitive and non-competitive; most often, there is an allosteric mechanism of inhibition [43].

Phytopreparation Tazalok is a tincture of medicinal plant raw materials: *Filipendula vulgaris radices*, *Petroselinum crispum radices*, *Apium graveolens*, *Galium verum gramen*, *Linaria vulgaris gramen*, *Calendula officinalis flores* [7]. The drug is standardized for flavonoids, hydroxycinnamic acids, iridoids, and terpenoids [8]. These components have hormone-regulating, antiproliferative, anti-inflammatory, soothing, and tonic effects [8].

Given the composition of biologically active substances in the phytopreparation, it would be logical to assume the presence of antiviral activity. In our study, we pay special attention to the effect of the phytopreparation on *the in vitro* reproduction of RNA viruses (swine coronavirus and hepatitis C virus) and human alphaherpesvirus 2 and the effectiveness of the phytopreparation for genital herpes *in vitro*. It must be stressed that these viruses, on the one hand, are convenient models for the study of phytopreparations antiviral activities [39], and, on the other hand, the related viral diseases are significant medical and social problems for humanity.

As with any pharmacological and toxicological study of BAC in cell culture, we began by determining the effective (EC_{50}) and cytotoxic (CC_{50}) concentrations, as well as the selectivity index (SI). Without determining these indicators, further interpretation of the obtained experimental data was impossible.

In addition to BAC of plant origin (actually – the active ingredient), the drug contains an excipient with pronounced cytotoxic properties – ethanol, the effect of which in our experiments could be significant. In our experiments on the determination of CC_{50} , we used three cell lines (BHK, PKE, and MDBK) and

received data indicating significant differences in the cytotoxic effects of the phytopreparation (Table 2). In terms of the polyphenolic compounds' concentration CC_{50} was: 0.025 mg/ml for BHK, 0.05 mg/ml for PKE, and 0.1 mg/ml for MDBK.

The data of other authors on the determination of alcohol tinctures CC_{50} (with ethanol as an extractant) are comparable with our results. For example, in the experiments of Demir S. et al. [44], the CC_{50} of the alcohol extract of propolis was 0.375 mg/ml. The same parameter for the oil extract was 1.66 mg/ml (using human keratinocytes cell line). Data of Mattana C.M. et al. [45] showed that the values of CC_{50} for alcohol and aqueous *Acacia aroma* leaf extracts also differed 3.9 times: 0.465 and 1.8 mg/ml, respectively (using the Vero cell line).

Although various methods for determining CC_{50} polyphenol-containing phytoextracts have been used in the studies mentioned above (microscopy in [45] and MTS-assay in [46]) and biochemical tests are known to be more sensitive [46]), the use of more sensitive methods (MTT/MTS-assay, cellular ATP production test, etc.) cannot change the CC_{50} by order of magnitude. Differences in the research methods used cannot explain such significant differences in values between ethanol-containing preparations and preparations without ethanol. Therefore, the methods we have chosen for cytotoxicity evaluation are relevant, and the results obtained generally correlate with the data of other authors regarding polyphenol-containing herbal preparations.

The phytopreparation effective concentration (EC_{50}) results are equally exciting and significant. Given polyphenols content in the preparation (2 mg/ml), the EC_{50} in terms of this group of BAC was 0.31 μ g/ml in the case of HHV-2 and TGEV (considered as a model of SARS-CoV-2), and 0.155 μ g/ml in the case of BVDV (HCV model). The *in vitro* doses for the phytopreparation were significantly lower than similar effective (inhibitory) concentrations for other polyphenol-containing preparations or individual polyphenolic compounds. Thus, according to Demir et al. [44], the EC_{50} of the propolis alcohol extract relative to the HHV-2 was 2.9 μ g/ml (compared to acyclovir – 5.5 μ g/ml). The EC_{50} for the polyphenolic compounds epigallocatechin-3-gallate (EGCG) and delphinidin (anti-HCV activity) were 4.85 and 1.12 μ g/ml, respectively [49]. According to Nguyen et al. [50], the EC_{50} for EGCG and quercetin for severe acute respiratory syndrome-associated coronavirus (SARS-CoV) was about 22 μ g/ml. Thus, the complex of polyphenolic compounds of the studied phytopreparation inhibits *in vitro* reproduction of viruses (at least HHV-2, BVDV, and TGEV) in much lower concentrations than previously described polyphenolic compounds and their combinations (phytoextracts).

It should be noted that values of selectivity index (80 for BHK/HHV-2 and 320 for PKE/BVDV and MDBK/TGEV) for the studied phytopreparation look more promising compared to the other authors' data on polyphenol-containing phytoextracts: for example, in the determination of antiherpetic activity (anti-HHV-1) Pistachios Kernels (*Pistacia vera* L.) SI extract was only 3 [49], and in a similar study for *Euphorbia spinidens* SI was 15 [48]. Our data on the selectivity index of the studied phytopreparation are comparable to the similar parameter for acyclovir (179) [48].

The encouraging results of the *in vitro* experiments were further confirmed in the *in vivo* model. Our results *in vitro* fully correlate with data on the studied phytopreparation therapeutic efficacy in models of genital herpes *in vivo*. The use of phytopreparation *per os* had a therapeutic index equal to 41.17%. At the same time, the use of combined treatment regimens (*per os* + applications) increased the therapeutic index up to 54.49%, which was very close to the effectiveness of the traditional treatment regimen by acyclovir (applications) (therapeutic index = 56%).

The next stage of our research was to elucidate the possible mechanisms of the phytopreparation antiviral activity. As well as in the studies of antiviral activity, the experiments were carried out in parallel on *in vitro* and *in vivo* models.

In vitro studies in which the phytopreparation blocks both RNA synthesis and DNA synthesis confirmed the presence of at least one of the RNA and DNA virus replication inhibition molecular mechanisms (inhibition of viral nucleic acid synthesis). These data corresponded with other authors' results on flavonoid-containing plant extracts [42].

It should be noted that the promising *in vivo* therapeutic antiviral potential of the studied phytopreparation is associated with its' *in vivo* immunomodulatory effect realized via the induction of interferon biosynthesis. Moreover, the level of endogenous α IFN induction by the studied phytopreparation is similar to the effect mediated by the reference polynucleotides Poly (I) • Poly (C). These data corresponded with Rybalko S. et al. [42] results on other flavonoid-containing drugs.

5. Conclusion

Preclinical studies of phytopreparation include extracts of many herbs and plant roots, various groups of biologically active substances (mainly flavonoids) on experimental models of human alphaherpesvirus 2, hepatitis C surrogate virus (BVDV), and coronavirus demonstrate its high antiviral activity to all tested viruses.

The phytopreparation solution is an active inhibitor of human alphaherpesvirus 2, hepatitis C surrogate virus (BVDV), and TGEV coronavirus reproduction with a selectivity index of 80, 320, and 320, respectively. The phytopreparation effective concentrations (EC_{50}) in terms of the concentration of polyphenolic compounds were the following: 0.31 μ g/ml in the case of HHV-2 and TGEV and 0.155 μ g/ml in the case of BVDV.

As a result of experimental studies, the phytopreparation effectiveness in genital herpes treatment was demonstrated. In the guinea pigs' genital herpes model, it was shown that the phytopreparation in a dilution of 1:100 with a combined regimen (*per os* + application) delays the onset of the first symptoms and reduces the time of disease duration. The phytopreparation therapeutic index is at the level of the antiherpetic drug acyclovir. The mechanism of antiherpetic action of the phytopreparation could be realized due to the induction of α IFN, inhibition of RNA, and DNA synthesis.

Declarations

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Competing Interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Maryna Arkhypova, Daria Starosyla, Oleg Deriabin, Svitlana Rybalko and Alexander Vasylchenko.

The first draft of the manuscript was written by Alexander Galkin and Olena Golembiovaska and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Figures

Antiherpetic activity (BHK)

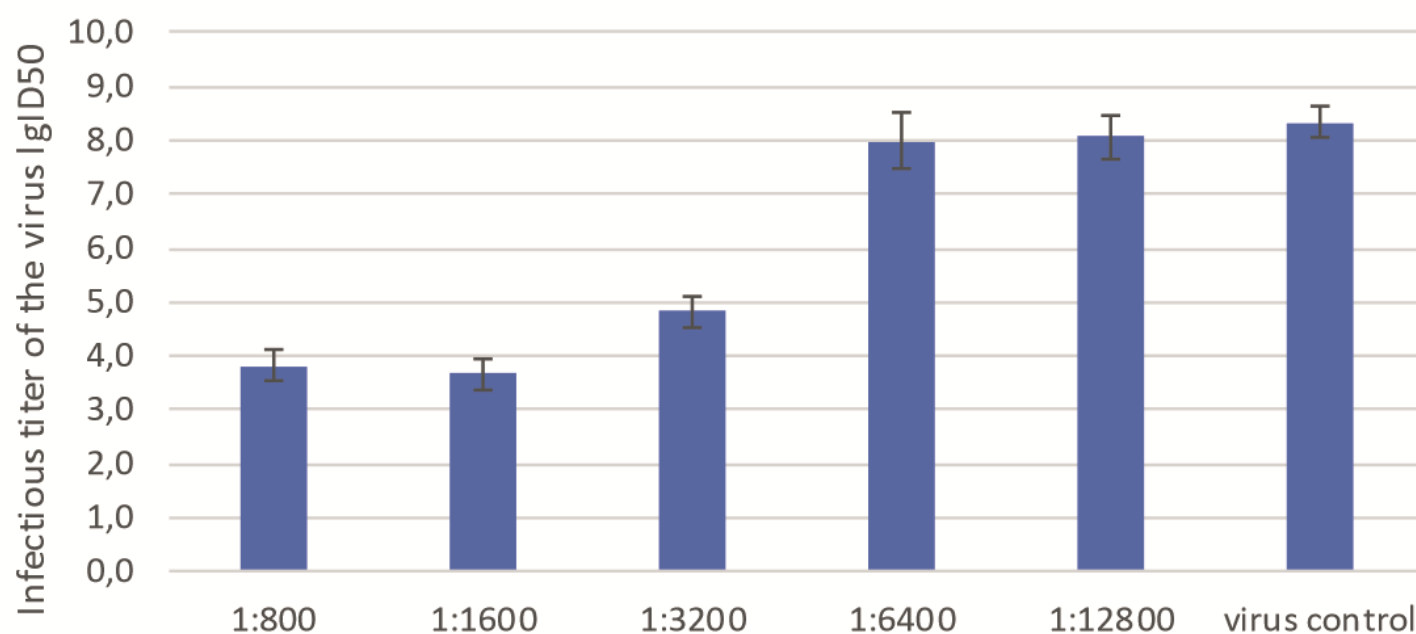


Figure 1

Phytopreparation antiherpetic activity in the culture of BHK cells.

Anti-HCV activity (MDBK)

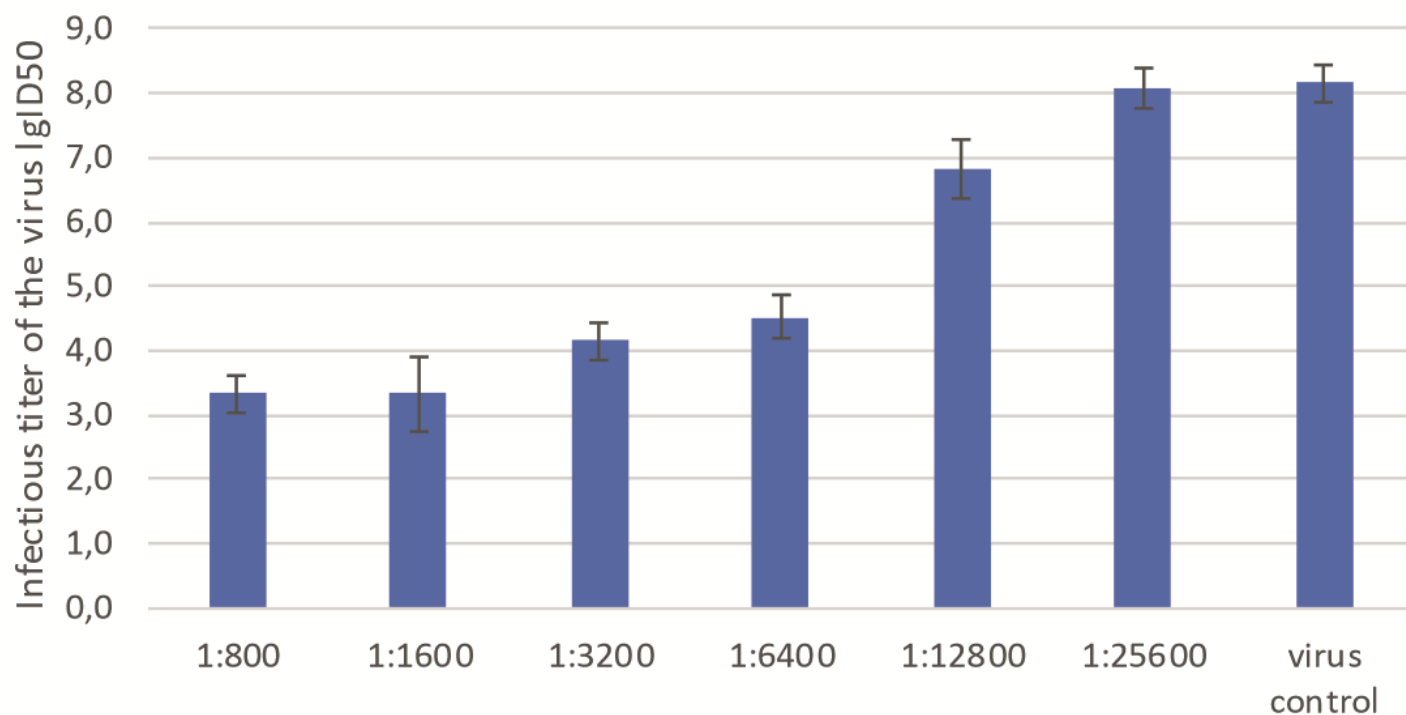


Figure 2

Phytopreparation antiviral activity in the culture of MDBK cells with BVDV.

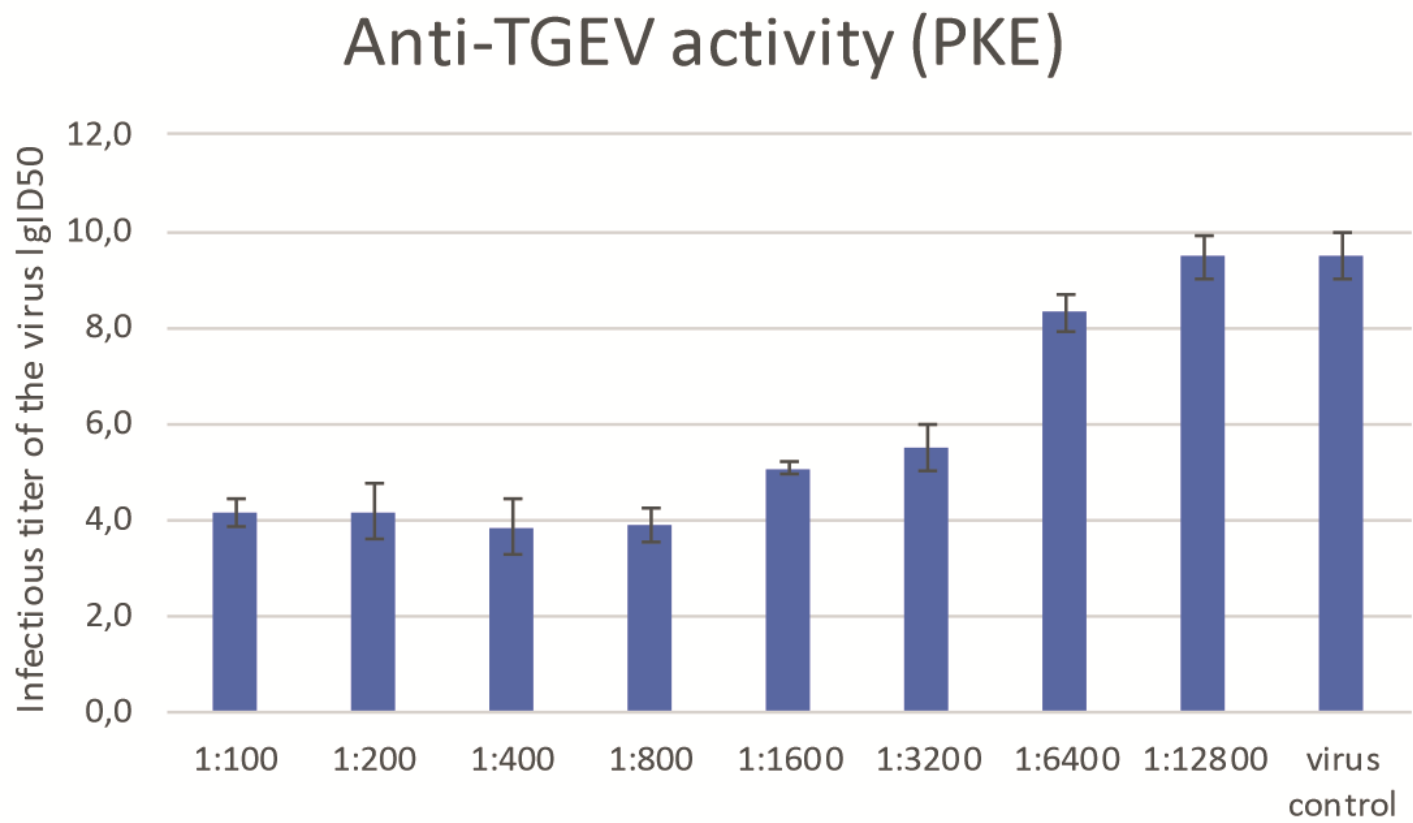


Figure 3

Infectious titer of TGEV in wells treated with different dilutions of phytopreparation and TGEV virus 100 ID₅₀.

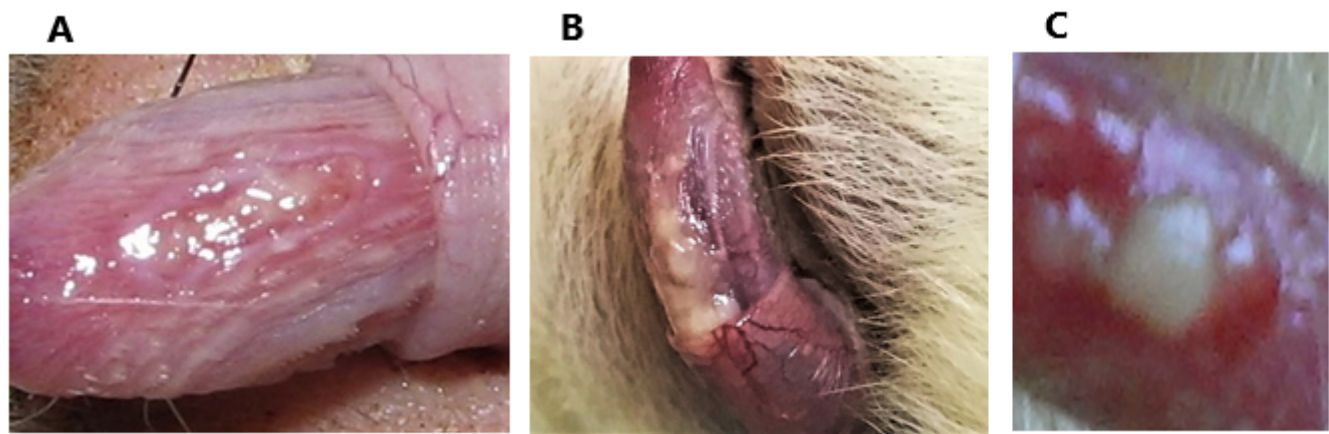


Figure 4

The severity of genital herpes symptoms: edema (A), redness (B), rash (C).

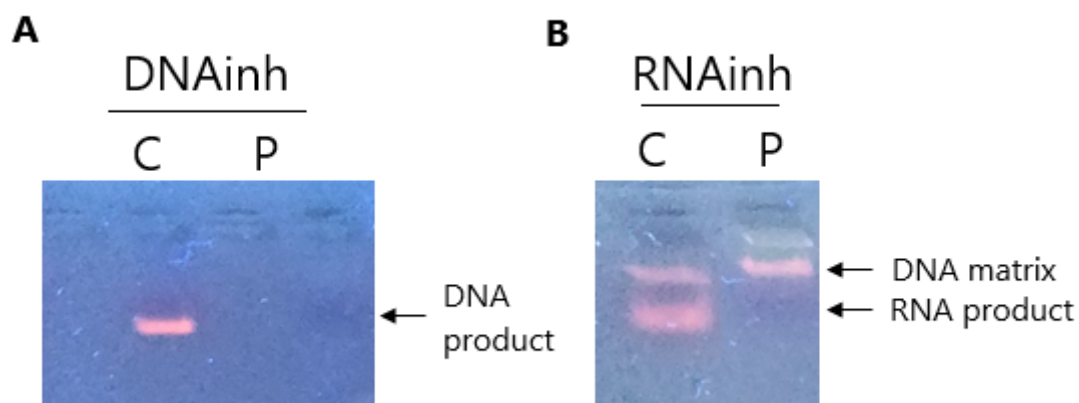


Figure 5

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 stripe) 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 stripe) (C: reaction control; P: phytopreparation).