Antiviral Potential of Specially Selected Bulgarian Propolis Ex-Tracts: In Vitro Mechanism of Action Against Structurally Different Viruses

Neli Milenova Vilhelmova-Ilieva *, Ivanka Nikolova Nikolova, Nadya Yordanova Nikolova, Zdravka Dimitrova Petrova, Madlen Stephanova Trepechova, Dora Ilieva Holechek, Mina Mihaylova Todorova, Mariana Georgieva Topuzova, Yulian Dimitrov Tumbarski *

Posted Date: 15 June 2023

doi: 10.20944/preprints202306.1120.v1

Keywords: propolis extracts; antiviral activity; virucidal activity; viral adsorption; Human coronavirus; Human respiratory syncytial virus; Herpes simplex virus; Human rhinovirus; Human adenovirus

Article

Antiviral Potential of Specially Selected Bulgarian Propolis Ex-Tracts: In Vitro Mechanism of Action Against Structurally Different Viruses

Neli Milenova Vilhelmova-Ilieva [1,*], Ivanka Nikolova Nikolova [1], Nadya Yordanova Nikolova [1], Zdravka Dimitrova Petrova [1,2], Madlena Stephanova Trepechova [1], Dora Ilieva Holechek [1], Mina Mihaylova Todorova [3], Mariyana Georgieva Topuzova [4], Ivan Georgiev Ivanov [4] and Yulian Dimitrov Tumbarski [5,*]

[1] The Stephan Angeloff Institute of Microbiology - Bulgarian Academy of Sciences, 26 Georgi Bonchev str., 1113 Sofia, Bulgaria
[2] Institute of Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, 25 Georgi Bonchev, 1113, Sofia, Bulgari
[3] Department of Organic Chemistry, Paisii Hilendarski University of Plovdiv, 24 Tsar Asen str., 4000 Plovdiv, Bulgaria
[4] Department of Organic Chemistry and Inorganic Chemistry, University of Food Technologies, 26 Maritsa blvd., 4002 Plovdiv, Bulgaria
[5] Department of Microbiology, University of Food Technologies, 26 Maritsa blvd., 4002 Plovdiv, Bulgaria
* Correspondence: nelivili@gmail.com; tumbarski@abv.bg

Abstract: Propolis is a natural mixture of resins, wax and pollen from plant buds and flowers, enriched with enzymes and bee saliva. It also contains various essential oils, vitamins, mineral salts, trace elements, hormones and ferments. It has been found that propolis possesses antimicrobial, antiviral and anti-inflammatory properties. We have studied the antiviral activity of six extracts of Bulgarian propolis collected from six districts of Bulgaria. The study was conducted against structurally different viruses: Human coronavirus strain OC-43 (HCoV OC-43) and Human respiratory syncytial virus type 2 (HRSV-2) (enveloped RNA viruses), Human herpes simplex virus type 1 (HSV-1) (enveloped DNA virus), Human rhinovirus type 14 (HRV-14) (non-enveloped RNA virus) and Human adenovirus type 5 (HadV-5) (non-enveloped DNA virus). The influence of the extracts on the internal replicative cycle of viruses was determined using the cytopathic effect (CPE) inhibition test. Of interest were the virucidal activity, the influence on the stage of viral adsorption to the host cell and the protective effect on healthy cells, which were determined by the final dilution method. The change in viral infectivity under the action of propolis extracts was compared with untreated controls and Δlgs were determined. Most propolis samples administered during the viral replicative cycle demonstrated the strongest activity against HCoV OC-43 replication. The influence of propolis extracts on the viability of extracellular virions was expressed to a different degree in the various viruses studied, and the effect was significantly stronger in those with an envelope. Almost all extracts significantly inhibited the adsorption step of the herpes virus and, to a less extent, of the coronavirus to the host cell, and some of them applied before viral infection demonstrated a protective effect on healthy cells. Our results enlarge the knowledge about the action of propolis and could open new perspectives for its application in viral infections treatment.

Keywords: propolis extracts; antiviral activity; virucidal activity; viral adsorption, Human coronavirus; Human respiratory syncytial virus; Herpes simplex virus; Human rhinovirus; Human adenovirus

1. Introduction

Propolis (bee glue) is a resinous substance produced by honeybees (*Apis mellifera* L.) after collecting exudates from flowers and leaf buds of various tree species. It serves as a building and defensive material – bees use it to fill up cracks, smooth the internal hive walls, repair and seal up the honeycomb cells, and embalm dead invaders inside the hive, thus removing the unpleasant smell

 doi:10.20944/preprints202306.1120.v1

and the microflora accompanying their decomposition, and protecting the bee colony from infections [1, 2]. In order to produce propolis, the worker bees transport the plant material to the hive and mix it with beeswax and saliva secreted by their salivary glands, thereby obtaining a hydrophobic substance with sticky consistency. Some recent studies have revealed that the number of the chemical compounds identified in propolis has reached 850. They form a heterogeneous mixture, which includes polyphenolic compounds, like flavonoids (quercetin, galangin, chrysin), followed by aromatic acids and esters, aliphatic acids and esters, volatile compounds, hydrocarbons, steroids, enzymes, waxy acids, alcohols, aldehydes, ketones, amino acids, micro- and macronutrients, vitamins, essential oils, sugars, pollen and other organic matter [3]. The colour (green, brown, red or yellow), the chemical composition and biological properties of propolis are highly variable, depending on the plant source and the characteristics of the geographic region from which it is collected [4].

Propolis is one of the most valuable bee products, which has been used by humans for thousands of years. As a non-toxic product and a valuable source of many biologically active compounds, propolis has been successfully applied as a remedy in the folk and traditional medicine. The rich chemical composition of propolis determines its diverse pharmacological activities. It has been found that propolis demonstrate antibacterial, antiviral, antiparasitic, antioxidant, immunomodulatory, anti-inflammatory, anticarcinogenic, hepatoprotective, anti-ulcerogenic, anti-allergic, antidiabetic, astringent, anaesthetic and other health beneficial effects [5, 6].

Viral outbreaks are widely spread and represent an important problem for the health sector. The application of non-toxic natural products without adverse effects as alternative of the chemotherapeutics is essential for the therapy of viral infections. In this respect, propolis has shown a great promise to be used as a potentially effective antiviral agent [7]. It has been reported to possess inhibitory activity against both DNA and RNA viruses. Amoros et al. (1992) [8] investigated the *in vitro* antiviral effect of propolis on herpes simplex virus type 1 and 2 (HSV-1 and HSV-2), adenovirus type 2, vesicular stomatitis virus and poliovirus type 2. The obtained results demonstrated that propolis had remarkable activity against poliovirus and herpes viruses, while vesicular stomatitis virus and adenovirus were less susceptible. Serkedjieva et al. (1992) [9] evaluated the *in vitro* antiviral activity of Bulgarian propolis on H3N2 and H1N1 influenza viruses, and stated that propolis inhibited the viral replication. Years later, the anti-influenza virus activity has been confirmed by Kujumgiev et al. (1999) [10], who investigated the inhibitory effect of propolis extracts on avian influenza virus A/strain Weybridge (H7N7). Other studies reveal the antiviral potential of propolis against herpes simplex virus (HSV-1 and HSV-2) [11], coronavirus 2 (SARS-CoV-2) [12], varicella zoster virus [13], infectious bursal disease virus (IBDV), Reovirus [14] and canine distemper virus [15].

In the present study, the antiviral activity against structurally different viruses of six Bulgarian propolis extracts collected from six districts of Bulgaria was determined.

2. Materials and Methods

2.1. Host Cell Lines

Human colon carcinoma (HCT-8) cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The cell line HCT-8 [HRT-18] (ATCC-CCL-244, LGC Standards) was maintained at 37 °C and a constant supply of 5% CO_2 using growth medium RPMI 1640 (Roswell Park Memorial Institute Medium, ATCC -30-2001) containing 10% horse serum (ATCC-30-2021), supplemented with 0.3 g/L L-glutamine (Sigma-Aldrich, Darmstadt, Germany), and an antibiotic mixture of 100 UI penicillin and 0, 1 mg streptomycin/mL (both Sigma-Aldrich).

Madin-Darbey bovine kidney (MDBK) cells were provided by the National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria. Cells were grown in DMEM growth medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco BRL, USA), supplemented with 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin 100

 doi:10.20944/preprints202306.1120.v1

IU/ml, streptomycin 100 μg/ml). Incubation was performed in incubator (HERA cell 150, Heraeus, Hanau, Germany) at 37 °C and a 5% CO_2 atmosphere.

A cell line derived from human laryngeal carcinoma - Human epithelial type 2 (HEp-2) cells (National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria) were cultured in DMEM growth medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco, BRL, USA), 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml). It was maintained in a humidified atmosphere of 5% CO_2 at 37 °C (Heraeus, Hanau, Germany).

Human cervical epithelioid carcinoma cells (HeLa Ohio-I) were kindly provided by Dr. D. Barnard (Utah State University, Logan, USA). The cell line was grown in DMEM growth medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, BRL, USA), 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin 100 IU/ ml, streptomycin 100 μg/ml) at 37 °C and 5% CO_2 in a HERA cell 150 incubator (Heraeus, Hanau, Germany).

2.2. Viruses

Human Coronavirus OC-43 (HCoV-OC43) (ATCC: VR-1558) strain was propagated in HCT-8 cells in RPMI 1640 medium supplemented with 2% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were lysed 5 days after infection by 2 freeze and thaw cycles and virus was titrated according to the Reed and Muench formula. Virus and mock aliquots were stored at −80 °C, as described in our previous study [16]. The infectious titer of stock virus was $10^{6.5}$CCID$_{50}$/ml.

Herpes simplex virus type 1, Victoria strain (HSV-1) was received from Prof. S. Dundarov, National Center of Infectious and Parasitic Diseases, Sofia. The virus was replicated in confluent monolayer of MDBK cells in a maintenance solution Dulbecco's modified Eagles's medium (DMEM) Gibco BRL, Paisley, Scotland, UK, plus 0.5% fetal bovine serum (Gibco BRL, Scotland, UK) and antibiotics (penicillin 100 IU/ ml, streptomycin 100 μg/ml). After incubation at 37°C in a 5% CO_2 incubator, the viral yield was frozen at -80°C [16]. The infectious titer of stock virus was $10^{8.5}$ CCID$_{50}$/ml.

Human rhinovirus type 14 (strain 1059) (HRV-14) was used for the experiments described. The virus was purchased from the American Type Culture Collection (Manassas, VA, USA). HRV-14 stocks were prepared in HeLa Ohio-I cells in a maintenance DMEM medium with 2 % fetal bovine serum and antibiotics (penicillin 100 IU/ ml, streptomycin 100 μg/ml). After incubation at 33°C in a 5% CO_2 incubator, the recovered virus was frozen at -80°C. Stock virus titer was $10^{3.5}$ CCID$_{50}$/ml.

Human respiratory syncytial virus type 2 (Long; HRSV-2), kindly provided by the Regional Center for Hygiene and Epidemiology, Plovdiv, Bulgaria. Virus was grown in HEp-2 cells (DMEM maintenance medium (Gibco, BRL) containing 10 mmol/l HEPES buffer (Gibco, BRL), 0.5% fetal calf serum (Gibco BRL) and antibiotics (penicillin 100 IU/ ml, streptomycin 100 μg/ml). After incubation at 37° C in a 5% CO2 incubator, the viral yield was frozen at -80 °C. The infectious viral titer was determined as 104.5 CCID$_{50}$/ml.

Human adenovirus type 5 (HadV-5), kindly provided by the District Center for Hygiene and Epidemiology, Plovdiv, Bulgaria. The virus was replicated in HEp-2 cells in the presence of DMEM (Gibco, BRL) maintenance medium containing 10 mmol/l HEPES buffer (Gibco, BRL), 0.5% fetal calf serum (Gibco BRL) and antibiotics antibiotics (penicillin 100 IU/ ml, streptomycin 100 μg/ml). The resulting amount of virus was frozen at -80°C. Infectious viral titer 105.0 CCID$_{50}$/ml.

2.3. Raw Propolis Material

Six fresh propolis samples collected from beekeepers at the end of the active beekeeping season (August–October) in six locations of Bulgaria were used in the study (Table 1). The samples were stored in plastic containers at room temperature in darkness until analysis.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 June 2023 doi:10.20944/preprints202306.1120.v1

Table 1. Origin of the propolis samples.

Propolis sample (PS)	Town/village	Municipality	District	GPS Coordinates
PS1	Silistra	Silistra	Silistra	44°07′N 27°17′E
PS2	Simitli	Simitli	Blagoevgrad	41°54′N 23°08′E
PS3	Gorna Malina	Gorna Malina	Sofia	42°41′N 23°42′E
PS4	Shumen	Shumen	Shumen	43°16′N 26°55′E
PS5	Vladimir	Radomir	Pernik	42°26'N 23°05'E
PS6	Cherven breg	Dupnitsa	Kyustendil	42°18′N 23°10′E

2.4. Reference Compound

Remdesivir `(GS-5734, RDV, REM, Veklury®)(Gilead Science Ireland UC) was initially dissolved in double distilled water to a concentration of 150 mg/ml and then diluted in RPMI nutrient medium to the required concentrations.

Acyclovir {ACV, [9-(2-hydroxyethoxymethyl)-guanine]} was kindly provided by the Deutsches Kresforschung Zentrum, Heidelberg, with a stock concentration of 3 mM solution in DMSO. Then, falling dilutions were made in DMEM medium to the required concentration.

Ribavirin (1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide), kindly provided by Prof. R. W. Sidwell, Utah State University, Logan, USA). Ribavirin was dissolved directly into the DMEM medium.

2.5. Preparation of Propolis Extracts

The propolis samples were finely chopped by a blender (Bosch, Germany). In order to prepare propolis extracts, a mass of 1 g of each sample was weighed, placed in a plastic tube and macerated with 10 ml of 70% ethanol (Sigma-Aldrich, Merck, Germany). Next, the samples were vigorously shaken on vortex V-1 (Biosan, Latvia) for 15-20 s and placed at room temperature for 72 h in darkness. During the extraction, the samples were periodically vortexed. The obtained extracts were filtered through filter paper and then stored at 4°C for further analyses [17].

2.6. Total Phenolic Content of Propolis Extracts

The total phenolic content (TPC) was assessed using a Folin-Ciocalteu reagent. The reaction mixture contained 1 mL of Folin-Ciocalteu reagent (Sigma-Aldrich, Merck), 0.8 mL of 7.5% sodium carbonate (Sigma-Aldrich, Merck), and 0.2 mL of the tested propolis extract. The absorbance was measured spectrophotometrically (Camspec M107, Spectronic-Camspec Ltd., UK) at 765 nm after incubation at room temperature for 20 min (in darkness), against a blank (distilled water). The results were presented as mg equivalent of gallic acid (GAE)/g propolis [18].

2.7. Total Flavonoid Content of Propolis Extracts

The total flavonoid content (TFC) was determined according to the method described by Ivanov et al. (2014). An aliquot of 1 mL of the propolis extract was added to 0.1 mL of 10% $Al(NO_3)_3$, 0.1 mL of 1 M CH_3COOK, and 3.8 mL of distilled water. The absorbance was measured at 415 nm after incubation at room temperature for 40 min, using a quercetin as a standard. The results are expressed as mg quercetin equivalents (QE)/g propolis.

2.8. Antioxidant Activity of Propolis Extracts

DPPH radical scavenging assay. The reaction mixture containing 2.85 mL of DPPH reagent (2,2-diphenyl-1-picrylhydrazyl) and 0.15 mL of the tested propolis extract was kept at 37 °C for 15 min. The reduction of absorbance was measured at 517 nm against a blank (methanol). The antioxidant activity was expressed as mM Trolox® equivalents (TE)/g propolis [18].

 doi:10.20944/preprints202306.1120.v1

Ferric-reducing antioxidant power (FRAP) assay. The FRAP reagent was freshly prepared with 300 mM acetate buffer with pH 3.6, 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM hydrochloric acid, and 20 mM Iron (III) chloride hexahydrate in distilled water in a ratio of 10:1:1. The reaction mixture (3 mL of FRAP reagent and 0.1 mL of the propolis extract) was incubated at 37 °C for 10 min, in darkness. The absorbance was measured at 593 nm against a blank (distilled water). The antioxidant activity was expressed as mM TE/g propolis [18].

2.9. Cytotoxicity Assay

A confluent monolayer cell culture in 96-well plates (Costar®, Corning Inc., Kennebunk, ME, USA) was treated with 0.1 mL/well containing support medium that did not contain/or contained decreasing concentrations of the tested propolis extracts. Cells are incubated under the characteristic conditions under which subsequent virus experiments will be performed. At 33°C and 5% CO_2 for 5 days (for HCT-8); 33 °C and 5% CO_2 for 2 days (for HeLa Ohio-I) and 2 days at 37 °C and 5% CO_2 for MDBK and HEp-2 cells. After the given period of time, the tested extracts were removed, the cells were washed and incubated with neutral red (NR) dye at 37°C for 3 hours. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test sample that reduced cell viability by 50% compared to untreated controls. Each sample was tested in triplicate with four wells per replicate.

The maximally tolerated concentration (MTC) of the extracts, which is the concentration at which they do not affect the cell monolayer, was also determined. The methodology is described in more detail in our previous study [16].

2.10. Antiviral Activity Assay

The cytopathic effect inhibition (CPE) test was used to determine the antiviral activity of propolis extracts. A confluent cell monolayer in 96-well plates was infected with 100 cell culture infectious doses of 50% ($CCID_{50}$) in 0.1 ml. After 2 hours of adsorption at 33°C (for HCoV OC-43 and HRV-14), 2 hours of adsorption at 37°C (for HRSV-2) and 1 hour of adsorption at 37°C (for HSV-1 and HadV-5) unattached virus was removed and the tested extract was added at different concentrations and the cells were incubated for 5 days at 33 °C (for HCoV OC-43); 2 days at 33 °C (for HRV-14) or 2 days at 37 °C (for HRSV-2, HSV-1 and HadV-5) and in the presence of 5% CO_2. The cytopathic effect was determined using a neutral red uptake assay and the percentage of CPE inhibition for each test sample concentration was calculated using the following formula:

% CPE = [OD test sample – OD virus control]/[OD toxicity control – OD virus control]x100

where ODtest sample is the mean of the ODs of the wells inoculated with virus and treated with the test sample at the corresponding concentration, ODs virus control is the mean of the ODs of the virus control wells (no compound in the medium), and OD control for toxicity is the mean of the ODs of the wells not inoculated with virus but treated with the corresponding concentration of the test compound. The 50% inhibitory concentration (IC_{50}) is defined as the concentration of the test substance that inhibits 50% of viral replication compared to the viral control. The selectivity index (SI) is calculated from the CC_{50}/IC_{50} ratio [16].

2.11. Virucidal Assay

Samples with a total volume of 1 ml containing virus (104 $CCID_{50}$) and tested propolis extract at its maximum permissible concentration (MTC) in a 1:1 ratio were prepared. A sample containing untreated virus diluted 1:1 with DMEM medium was incubated in parallel. The control and experimental samples were incubated at room temperature for different time intervals (15, 30, 60, 90 and 120) min. Then, by the endpoint dilution method of Reed and Muench (1938) [19], the content of residual infectious virus in each sample and Δlgs compared to untreated controls were determined.

 doi:10.20944/preprints202306.1120.v1

2.12. Effect on Viral Adsorption

Twenty-four well plates containing a monolayer of HCT-8 or MDBK monolayers were pre-chilled to 4 °C and inoculated with 10^4 $CCID_{50}$ of HCoV OC-43 or HSV-1, respectively. Along with the virus, the monolayer was also treated with the tested propolis extracts in their MTC and incubated at 4 °C for the time of virus adsorption. At different time intervals different for the two types of virus (15, 30, 45 and 60 min for HSV-1 or 15, 30, 60, 90 and 120 min for HCoV OC-43), the virus and the propolis extract were removed, the cells were washed with PBS, then the cells were covered with maintenance medium and incubated at 37 °C (HSV-1) or at 33 °C (for HCoV OC-43) in the presence of 5% CO_2 for 24 h. After freezing and thawing three times, the infectious viral titer of each sample was determined, compared to the viral titer of the control for the given time interval, and Δlgs were determined. Each sample was prepared in quadruplicate.

2.13. Pre-Treatment of Healthy Cells

A monolayer of MDBK or HCT-8 cells previously grown in 24-well cell culture plates (CELLSTAR, Greiner Bio-One) were treated with the propolis extracts in their MTC. The samples were incubated for different time intervals of 15, 30, 60, 90 and 120 minutes at 37 °C. After the given time interval, the extracts were removed and the cells were washed with PBS and inoculated with the respective virus strain (1000 $CCID_{50}$ in 1 ml / well). After 120 min (for HCoV OC-43) and 60 min (for HSV-1) of adsorption, unadsorbed virus was removed and the cells were covered with support medium. Samples were incubated at 33 °C (for HCoV OC-43) and 37 °C (HSV-1) in the presence of 5% CO_2 for 24 h. This was followed by triplicate freezing and thawing of samples, and determination of infectious virus titers. Δlg compared to the viral titer of the control (untreated with extract) for the given time interval was determined. Each sample was prepared in quadruplicate.

2.14. Statistical Analysis

Data on cytotoxicity and antiviral effects were analysed statistically. The values of CC_{50} and IC_{50} were presented as means ± SD. The differences' significance between the cytotoxicity values of propolis extracts and the reference substances, as well as between the effects of the test products on the viral replication was done by Student's t-test, p-values of < 0.05 were considered significant. The final data sets were analyzed with the Graph Pad Prism 4 software.

3. Results

Recent studies show that the number of chemical compounds identified as constituents of propolis has reached 850, as the polyphenols (especially flavonoids) are the main compounds, which contribute to its biological activity [3], we stopped our attention on the content of these ingredients in the individual propolis extracts. The results obtained for the total phenolic content (TPC) and total flavonoid content (TFC) of the propolis extracts used in the study are summarized in Table 2. The highest TPC value was found in PS6 (256.07 ± 0.56 mg GAE/g propolis), followed by PS3, PS4, PS1 and PS2. PS5 showed the lowest TPC value (151 .66 ± 0.32 mg GAE/g propolis). The highest TFC value was determined for PS2 (124.07 ± 2.23 mg QE/g propolis), followed by PS3, PS5, PS1 and PS4. PS6 showed the lowest TFC value (74.55 ± 0.15 mg QE/g propolis), respectively.

Polyphenols and flavonoids have been shown to exhibit good antioxidant activity. Due to their high content in the propolis samples we studied, we made the assumption that the extracts should also exhibit such activity. The results of the antioxidant activity assessed by the DPPH method showed that PS2 possessed the highest antioxidant activity, while PS1 had the lowest antioxidant potential. In contrast, PS6 possessed the highest antioxidant activity determined by the FRAP method, while PS5 showed the lowest value by the same method (Table 2).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 June 2023 doi:10.20944/preprints202306.1120.v1

Table 2. Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity of the ethanolic propolis extracts.

Propolis sample	TPC, mg GAE/g	TFC, mg QE/g	Antioxidant activity	
			DPPH, mM TE/g	FRAP, mM TE/g
PS1	**216.85 ± 0.28**	78.30 ± 0.38	873.20 ± 14.50	867.52 ± 60.00
PS2*	168.01 ± 0.63	124.07 ± 2.23	1201.37 ± 26.23	715.28 ± 6.12
PS3	243.13 ± 0.28	87.96 ± 0.11	1106.88 ± 43.50	1000.14 ± 37.50
PS4	230.79 ± 0.30	77.04 ± 0.50	1083.34 ± 34.80	978.92 ± 17.50
PS5*	151.66 ± 0.32	87.04 ± 2.01	1016.26 ± 22.20	645.34 ± 11.25
PS6	256.07 ± 0.56	74.55 ± 0.15	1133.53 ± 23.22	1085.02 ± 22.50

* The result is presented in our previous study [20].

For the correct conduct of the antiviral experiments with non-toxic concentrations of the propolis extracts, their cytotoxicity was previously determined. The effect of the extracts on the cells was determined against the four cell lines (HCT-8, MDBK, HEp-2 and HeLa Ohio) on which the antiviral experiments were subsequently performed. Against MDBK, HEp-2 and HeLa Ohio cell lines, cytotoxicity was measured after 2 days of treatment with the extracts, as this is the time interval at which the antiviral experiments for the respective viruses were also determined. To obtain a good cytopathic effect with HCoV OC-43, a longer time is needed - 5 days, therefore the cytotoxicity with the HCT-8 cell line also occurs on the 5th day.

From the experiments performed, it can be seen that, in general, the highest cytotoxicity was reported for the HCT-8 cell line, most likely because the exposure time was the longest. From the rest of the cell lines, where the effect was measured for the same time interval, the extracts showed the weakest cytotoxicity against the HeLa Ohio cell line, close but slightly higher toxicity in MDBK cells, and the most sensitive to the action of propolis extracts from the cells tested turned out to be HEp-2. The weakest cytotoxicity was shown by the extracts PS4, PS3 and PS1 against HeLa Ohio cells, as well as PS 1 and PS3 against the MDBK cell line. As a general effect on the four cell lines, PS6 showed the highest toxicity. When comparing the cytotoxicity of the tested samples to the reference substances used, it is noticed that they are less toxic than Ribavirin, but demonstrate several times higher cytotoxicity compared to Acyclovir and Remdesivir (Table 3).

Table 3. *In vitro* assessment of cytotoxicity of the propolis extracts.

Propolis sample	Cytotoxicity (μg/ml)							
	HCT-8		MDBK		HEp-2		HeLa Ohio	
	CC_{50}	MTC	CC_{50}	MTC	CC_{50}	MTC	CC_{50}	MTC
PS1	58.0±8.2**	10.0	120.0±6.7**	32.0	56.5±2.1*	30.0	158.0±7.2**	70.0
PS2	48.0±4.5**	10.0	72.5±3.8**	10.0	68.2±3.3*	30.0	77.7±2.9*	35.0
PS3	62.6±5.2**	10.0	104.7±7.3**	32.0	57.0±2.5*	30.0	174.0±6.5**	70.0
PS4	52.0±3.6**	10.0	73.5±3.2**	10.0	67.7±2.9*	30.0	190.0±7.0**	70.0
PS5	59.8±7.3**	10.0	71.8±4.7**	10.0	62.5±2.4*	30.0	77.0±2,5*	35.0
PS6	57.0±6.5**	10.0	66.2±5.7**	10.0	59.4±1.5*	20.0	58.0±2.0*	30.0
Acyclovir	nd	nd	291.0±9.4**	nd	nd	nd	nd	nd
Remdesivir	250.0±4.3	nd	nd	nd	nd	nd	nd	nd
Ribavirin	nd	nd	nd	nd	14.0±0.5	nd	34.0±0.5	nd

nd - no data;. * $p < 0.05$; when comparing the value of each propolis extract with the corresponding reference substance for the given cell line;. ** $p < 0.001$, when comparing the value of each propolis extract with the corresponding reference substance for the given cell line.

After determining the range of non-toxic concentrations of the propolis samples, their influence on the internal replicative cycle of the virus at concentrations lower than the CC_{50} was investigated.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 June 2023 doi:10.20944/preprints202306.1120.v1

In general, the influence of the extracts is strongest for the herpes virus, and to a slightly lesser extent for the coronavirus and the rhinovirus. The effect was weakest with adenovirus and almost in the same range was the inhibition reported with RSV-2. The highest selective index (SI) of all propolis samples showed PS4 against HSV -1 (SI = 45.3) and HCoV OC-43 (SI = 43.3). PS2 also showed significant activity against HSV-1 replication (SI = 32.9). Distinct activity was also demonstrated by PS1 (SI = 28.7) applied to the replication of HRV-14 and PS2 (SI = 26.6) affecting HCoV OC-43. The substances PS4 (SI = 22.3) for HRV-14 and PS6 (SI = 21.7) for HSV-1 have similar activity. Significantly lower was the influence of PS2 (SI = 10.0) on HRV-14; PS5 (SI = 8.9) in HSV-1; and PS 1 and PS2 with SI between 7.5 - 8.6 for RSV-2 and HAdV-5 (Table 4).

Table 4. *In vitro* antiviral activity of the propolis extracts.

Propolis sample	Antivirus activity (μg/ml)									
	HCoV OC-43		HSV-1		HAdV-5		RSV-2		HRV-14	
	IC_{50} (μg/ml)	SI	IC_{50} (μg/ml)	SI	IC_{50} (μg/ml)	SI	IC_{50} (μg/ml)	SI	IC_{50} (μg/ml)	SI
PS1	-	-	-	-	7.5±0.2**	7.5	6.7±0.3**	8.4	5.5±0.2**	28.7
PS2	1.8±0.3*	26.6	2.2±0.3*	32.9	8.2±0.3**	8.3	7.9±0.3**	8.6	7.8±0.2**	10.0
PS3	-	-	-	-	-	-	47.0±2.2**	1.2	7.0±0.1**	2.8
PS4	1.2±0.1*	43.3	1.4±0.02*	45.3	-	-	44.0±2.4**	1.5	8.5±0.3**	22.3
PS5	10.2±0.9	5.8	8.2±1.2**	8.9	-	-	46.0±1.8**	1.4	-	-
PS6	10.8±0.7	5.2	3.3±0.8**	21.7	-	-	-	-	-	-
Acyclovir	nd	nd	0.33±0.03	881.8	nd	nd	nd	nd	nd	nd
Remdesivir	12.5±0.9	200.0	nd	nd	nd	nd	nd	nd	nd	nd
Ribavirin	nd	nd	nd	nd	0.2±0.01	70.0	0.3±0.01	46.6	0.5±0.02	68.0

-, lack of inhibition of viral replication;. nd - no data;. * $p < 0.05$; when comparing value of the each propolis extract with the corresponding reference substance for the given virus strain;. ** $p < 0.001$, when comparing value of the each propolis extract with the corresponding reference substance for the given virus strain.

After determining the influence of the studied propolis samples on the replication of the structurally different viruses, the next step in our research was to determine the influence of the extracts on the vitality of the extracellular virions. The results from the experiments showed a stronger effect on enveloped viruses compared to non-enveloped ones. The effect was monitored at different time intervals and, in general, a dependence of the effect on the exposure time was noticed - with longer exposure, the inhibition of virus particles increased. The most significant was the effect of PS5 (Δlg = 2.25) at 90 and 120 min on HCoV OC-43 virions, with a similar effect on HSV-1 and to a less extent on HRSV-2. A distinct effect of Δlg = 2.0 in HCoV OC-43 was also demonstrated by PS4 and PS6, whose influence on HRSV-2 was less pronounced, respectively Δlg = 1.6 and Δlg = 2.0 at the longest interval of 120 min. PS6 was the only one of the six investigated extracts, which exhibited a significant effect (Δlg = 1=8) at 120 min on HRV-14. PS1 also exerted a suppressive effect on extracellular HSV-1, even at a contact duration interval of 30 min, compared to HCoV OC-43 virions, the effect was weaker (Δlg = 1.75) (Tables 5 and 6).

Table 5. Virucidal activity of enveloped viruses.

Propolis sample	Δlg														
	HCoV OC-43					HRSV-2					HSV-1				
	15 min	30 min	60 min	90 min	120 min	15 min	30 min	60 min	90 min	120 min	15 min	30 min	60 min	90 min	120 min
PS1	1.25	1.25	1.25	1.75	1.75	0.0	0.0	0.5	1.0	1.0	1.0	1.75	1.75	2.0	2.0
PS2	1.0	1.25	1.5	1.5	1.5	0.2	0.2	0.6	1.5	1.5	1.0	1.0	1.5	1.5	1.5
PS3	0.5	0.5	1.0	1.5	1.5	0.2	0.2	0.7	1.2	1.6	1.0	1.0	1.25	1.25	1.25
PS4	0.5	1.0	1.5	2.0	2.0	0.3	0.3	0.7	1.4	1.6	1.0	1.0	1.5	1.5	1.5

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 June 2023 doi:10.20944/preprints202306.1120.v1

PS5	1.25	1.25	1.25	2.25	2.25	0.3	0.3	0.9	1.5	2.0	1.0	1.0	1.5	1.75	2.25
PS6	1.0	1.25	1.25	2.0	2.0	0.2	0.2	0.9	1.7	2.0	1.0	1.0	1.5	1.5	1.75
70% etanol	5.75	5.75	5.75	5.75	5.75	4.5	4.5	4.5	4.5	4.5	4.75	4.75	4.74	4.74	4.74

Table 6. Virucidal activity of non-enveloped viruses.

Propolis sample	**Δlg**									
	HAdV-5					**HRV-14**				
	15 min	30 min	60 min	90 min	120 min	15 min	30 min	60 min	90 min	120 min
PS1	0.0	0.0	0.5	1.0	1.0	0.0	0.0	0.1	1.4	1.6
PS2	0.0	0.0	0.7	1.0	1.0	0.0	0.0	0.0	1.2	1.5
PS3	0.0	0.0	0.5	1.0	1.0	0.0	0.0	0.3	1.2	1.6
PS4	0.0	0.0	0.8	0.8	1.2	0.0	0.0	0.2	1.3	1.5
PS5	0.0	0.0	0.4	0.8	0.8	0.0	0.0	0.2	1.3	1.3
PS6	0.0	0.0	0.6	0.8	1.0	0.0	0.0	0.0	1.5	1.8
70% etanol	5.0	5.0	5.0	5.0	5.0	3.2	3.2	3.2	3.2	3.2

Having determined the effect of propolis extracts on extracellular virions and virus replication in the cell, the next step in our research was to follow the effect of the extracts on the adsorption step of the virus to the cell. The experiments were carried out with the two viruses for which we obtained the most distinct activity so far - HSV-1 and HCoV OC-43. The influence was again followed at different time intervals depending on the duration of viral adsorption (up to 60 min for the herpes virus and up to 120 min for the coronavirus). All extracts demonstrated varying degrees of influence on this stage of the viral reproduction. The inhibition of the process was more pronounced in HSV-1 compared to HCoV OC-43. The strongest effect on HSV-1 was shown by PS5 and PS6 (Δlg = 2.25) at 30 min after exposure, and the influence of PS6 was significant as early as 15 min (Δlg = 1.75). The effect of PS2, PS3 and PS4 (Δlg = 2.0) was also significant at 30 min and remained unchanged until the last investigated time interval of 60 min. PS1, although to a less extent, also affected the adsorption stage of the virus with a decrease of the viral titer with Δlg = 1.75.

When tracking the adsorption of HCoV OC-43 to the HCT-8 cells, a significantly weaker influence of the extracts was observed, and at a slightly longer contact period - at 90 or 120 min. Here, the influence of PS4 is most significant per 120 min (Δlg = 2.25). Also a distinct effect at 120 min of Δlg = 1.75 was demonstrated by PS1, PS2 and PS5. PS3 and PS6 showed weak activity towards the adsorption of HCoV OC-43 (Table 7).

Table 7. Influence of the extracts on the stage of adsorption of HSV-1 and HCoV OC-43 to sensitive cells.

Propolis sample	**Δlg**								
	HSV-1				**HCoV OC-43**				
	15 min	**30 min**	**45 min**	**60 min**	**15 min**	**30 min**	**60 min**	**90 min**	**120 min**
PS1	1.5	1.75	1.75	1.75	1.0	1.0	1.0	1.5	1.75
PS2	1.5	2.0	2.0	2.0	1.0	1.0	1.0	1.25	1.75
PS3	1.5	1.75	2.0	2.0	0.75	1.0	1.0	1.25	1.5
PS4	1.5	2.0	2.0	2.0	0.75	0.75	1.0	1.75	2.25
PS5	1.25	2.25	2.25	2.25	1.5	1.5	1.5	1.75	1.75
PS6	1.75	2.25	2.25	2.25	1.5	1.5	1.5	1.5	1.5

After tracking the influence of propolis extracts on different stages of viral reproduction and vitality, we investigated whether the studied substances have a protective effect on healthy cells and protect them from a subsequent viral infection. Again, the experiments were performed with MDBK and HCT-8 cell lines and the viral strains HSV-1 and HCoV OC-43. The study showed that none of the extracts significantly protected HCT-8 cells from HCoV OC-43 infection. The maximum decrease

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 June 2023 doi:10.20944/preprints202306.1120.v1

in viral titer was Δlg = 1.0. In MDBK cells the effect was significantly stronger. The most clear protection was demonstrated by PS3 from 15 min of treatment (Δlg = 2.0), and the effect increased at 120 min to Δlg = 2.25. Similar protection was shown by PS4, but the effect was significant at 30 min. PS2 also has a strong influence, which maintains the same activity during all monitored time intervals (Δlg = 2.0). The other three propolis extracts: PS1, PS5 and PS6 showed weak protective effect on sensitive healthy cells (Table 8).

Table 8. Protective effect of pre-treatment of extracts on healthy cells and subsequent virus infection.

Propolis sample	Δlg									
	HSV-1					HCoV OC-43				
	15 min	30 min	60 min	90 min	120 min	15 min	30 min	60 min	90 min	120 min
PS1	1.25	1.25	1.25	1.25	1.25	0.5	0.5	0.5	1.0	1.0
PS2	2.0	2.0	2.0	2.0	2.0	0.5	0.5	0.5	1.0	1.0
PS3	2.0	2.0	2.0	2.0	2.25	0.5	0.5	0.5	1.0	1.0
PS4	1.0	2.0	2.0	2.0	2.25	0.5	0.5	0.5	1.0	1.0
PS5	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
PS6	1.0	1.0	1.0	1.0	1.25	0.5	0.5	0.5	0.5	0.5

4. Discussion

In some viruses that cause respiratory, intestinal, skin, sexually transmitted infections in humans, antiviral agents have been developed that significantly reduce the severity of symptoms and shorten the recovery period. However, therapy failure is increasingly observed due to the selection of therapy-resistant mutants [21]. This is a major reason for the intensive search for new, unconventional antiviral agents that are closer to the cell components, low toxic, cause fewer side effects, and can serve as an alternative to the currently used antiviral therapeutics.

In recent decades, more and more data have been accumulated from the study of the various biological activities of propolis. One of its benefits is the impact on developing viral infections [22, 23]. The exact mechanisms by which its influence is carried out are still not sufficiently studied. Two main directions of its action have been established: (1) direct interaction on the virus or on stages of its replication [23, 24, 25, 26, 27, 28] and (2) stimulation of the immune system to overcome infection [29, 30, 31].

The mechanism and strength of the antiviral effect of propolis is determined by the multitude of substances that are included in its composition, which is determined by the geographical location and the season of the year in which it was obtained [31]. In the temperate zones of Europe, North America and Asia, where the predominant source is poplar tree species, most often black poplar (*Populus nigra*), propolis contains mainly flavanones and flavones and smaller amounts of phenolic acids and their esters [32]. Propolis flavonoids show antiviral activity against DNA and RNA viruses [33] as well as immunomodulatory action [30]. Over 150 types of flavonoids have been found in different types of propolis [34]. Propolis from tropical countries contains mainly complex phenolic compounds such as prenylated para-coumaric acids, prenylated flavonoids, caffeoylquinic acid derivatives and lignans [35].

Some studies show that propolis can affect virus replication [29], by reducing the synthesis of viral RNA transcripts in cells and thus reducing the number of coronavirus particles [28] or by inhibiting of Varicella zoster virus DNA polymerase [27]. Another potential mechanism of inhibition of viral replication is the proven inhibitory activity of Sulawesi propolis compounds against the enzymatic activity of SARS-CoV-2 main protease [25].

Many research have shown that in contact with the viral particle, propolis destroys the ability of the pathogen to enter the cell [29, 36, 37]. Virus particles with altered morphology were observed, suggesting possible damage to viral envelope proteins. Virions were also found in an electrodense

layer formed around the cell membrane. This has been suggested to affect the entry of the virus into the host cell and disrupt its replication cycle [38].

Our results were in agreement with other scientific publication on this topic. We used different experimental setups, each adding the propolis extracts at different stages of the viral infection cycle. In a similar way, the data obtained by other researchers were close to ours - proving a significant influence of propolis on extracellular virions, especially in enveloped viruses, as well as on the stage of viral adsorption on susceptible cells [36, 37, 39].

We obtained similar results in our previous study of Canadian propolis [40], where we demonstrated an effect on virions and the adsorption stage of Herpes simplex virus types 1 and 2 to MDBK cells. In the present study with the Bulgarian propolises, an influence on the viral replicative cycle was also found in some of the samples, which is clearly due to the differences in the composition of the propolises due to their different geographical origin.

We also introduced a new research setup where we treated the still healthy cells with the propolis extracts and determined the degree of protection that propolis shows on the cell membrane from subsequent viral infection. Our results reconfirm the data obtained by other teams who used a similar experimental methodology and found that the application of 0.5 mg/ml EEP two hours before infection in MDBK cells caused a reduction in the number of Aujeszky's disease virus formed plaque compared to the other treatments used or to the infected and untreated culture [38].

5. Conclusions

The present study once again confirms our previously reported data on the antiviral activity of propolis. The study of the activity against the replication of structurally distinct viruses showed a different degree of inhibition in individual propolis samples. In direct contact of the propolis extracts with the virus particles, it was found that the effect was stronger for enveloped viruses, which is most likely the result of interaction of the components included in the composition of propolis with the viral proteins of the envelope, necessary for attachment and entry into the cell, resulting in inactivation of the virus. The data accumulated so far on the activity of propolis, present it as a promising candidate for inclusion in the prevention and treatment of many infectious diseases, but it is necessary to expand the knowledge of its mechanism of action for its more complete application.

Funding: This research was funded by the National Science Fund at the Ministry of Education and Science, Bulgaria, approved by Research Grant № КП-06-ДК1/3 "Biopolymer-based functional platforms for advanced *in vitro* target and co-delivery of therapeutic payloads for the treatment of coronavirus infection".

Author Contributions: Conceptualization, N.M.V-I., I.N.N. and Y.D.T.; Propolis samples providing – Y.D.T.; Methodology, N.M.V-I., I.N.N., N.Y.N., Z.D.P., I.G.I., M.M.T., and M.G.T.; Formal Analysis, N.M.V-I., I.G.I., Y.D.T. and I.N.N.; Investigation, N.M.V-I., I.N.N., N.Y.N., Z.D.P., M.S.T., D.I.H., I.G.I., Y.D.T., M.M.T., and M.G.T.; Resources, N.M.V-I., I.G.I., Y.D.T., and I.N.N.; Data Curation, N.M.V-I., I.G.I., Y.D.T., and I.N.N.; Writing—Original Draft Preparation, N.N.V-I., Y.D.T., M.S.T. and Z.D.P..; Writing—Review & Editing, N.M.V-I., I.N., and Y.D.T.; Software, N.M.V-I. and I.N.N., Visualization, N.M.V-I., I.G.I., Y.D.T., and I.N.N.; Project Administration, N.M.V-I.; Funding Acquisition, N.M.V-I.; Supervision, N.M.V-I. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest. The sponsors had no role in the design, execution, interpretation, or writing of the study.

References

1. Sforcin: J. M. Propolis and the immune system: a review. Journal of Ethnopharmacology **2007**, 113(1), 1–14. https://doi.org/10.1016/j.jep.2007.05.012
2. Wagh, V. D. Propolis: A wonder bees product and its pharmacological potentials. Advances in Pharmacological Sciences, **2013**, 308249. https://doi.org/10.1155/2013/308249
3. Bouchelaghem, S. Propolis characterization and antimicrobial activities against Staphylococcus aureus and Candida albicans: A review. Saudi Journal of Biological Sciences, **2022**, 29, 1936-1946. http://doi.org/10.1016/j.sjbs.2021.11.063
4. Bankova, V., Popova, M., Trusheva, B. New emerging fields of application of propolis. Macedonian Journal of Chemistry and Chemical Engineering, **2016**, 35(1), 1-11. http://doi.org/10.20450/mjcce.2016.864
5. Salomao, K., Dantas, A. P., Borba, C. M., Campos, L. C., Machado, D. G., Aquino Neto, F, R., de Castro, S. L. Chemical composition and microbicidal activity of extracts from Brazilian and Bulgarian propolis. Letters in Applied Microbiology, **2004**, 38(2), 87-92. https://doi.org/10.1111/j.1472-765x.2003.01458.x
6. Cauich-Kumul, R. and Campos, M. R. S. Bee propolis: properties, chemical composition, applications, and potential health effects. In Bioactive Compounds. **2019**, (pp. 227-243). Elsevier. https://doi.org/10.1016/B978-0-12-814774-0.00012-8
7. Ripari N., Sartori A.A., da Silva Honorio M., Conte F.L., Tasca K.I., Santiago K.B., Sforcin J.M. Propolis antiviral and immunomodulatory activity: a review and perspectives for COVID-19 treatment. Journal of Pharmacy and Pharmacology, **2021**, 73, 281–299. doi:10.1093/jpp/rgaa067.
8. Amoros M., Sauvager F., Girre L., Cormier M. *In vitro* antiviral activity of propolis. Apidologie, **1992**, 23, 231-240.
9. Serkedjieva J., Manolova N., Bankova V. Anti-influenza virus effect of some propolis constituents and their analogues (esters of substituted cinnamic acids). J Nat Prod **1992**; 55(3): 294–7. https://doi.org/10.1021/np50081a003
10. Kujumgiev A., Tsvetkova I., Serkedjieva Y., Bankova V., Christov R., Popov S. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. Journal of Ethnopharmacology, **1999**, 64(3), 235–240. doi:10.1016/s0378-8741(98)00131-7.
11. Demir S., Atayoglu A.T., Galeotti F., Garzarella E.U., Zaccaria V., Volpi N., Karagoz A., Sahin F. Antiviral activity of different extracts of standardized propolis preparations against HSV. Antiviral Therapy, **2020**, 25(7), 353-363. doi: 10.3851/IMP3383.
12. Fiorini AC, Scorza CA, de Almeida ACG, Fonseca MCM, Finsterer J, Fonseca FLA, Scorza CA. Antiviral activity of Brazilian Green Propolis extract against SARSCoV-2 (Severe Acute Respiratory Syndrome - Coronavirus 2) infection: case report and review. Clinics, **2021**, 76:e2357.
13. Labská, K.; Plodková, H.; Pumannová, M.; Sensch, K.H. Antiviral activity of propolis special extract GH 2002 against Varicella zoster virus *in vitro*. Die Pharmazie - An International Journal of Pharmaceutical Sciences, **2018**, 73 (12), 733-736.
14. El Hady, F. K. A. and Hegazi, A. G. Egyptian Propolis: 2. Chemical Composition, Antiviral And Antimicrobial Activities Of East Nile Delta Propolis. Zeitschrift für Naturforschung C, **2002**, 57(3-4), 386–394. doi:10.1515/znc-2002-3-431
15. González-Búrquez, M.J., González-Díaz, F.R., García-Tovar, C.G., Carrillo-Miranda, L., Soto-Zárate, C.I., Canales-Martínez, M.M., Penieres-Carrillo, J.G., Crúz-Sánchez, T.A., Fonseca-Coronado, S. Comparison between In Vitro Antiviral Effect of Mexican Propolis and Three Commercial Flavonoids against Canine Distemper Virus. Evidence-Based Complementary and Alternative Medicine, **2018**, vol. 2018, Article ID 7092416. https://doi.org/10.1155/2018/7092416
16. Nikolova, I., Paunova-Krasteva, T., Petrova, Z., Grozdanov, P., Nikolova, N., Tsonev, G., Triantafyllidis, A., Andreev, S., Trepechova, M., Milkova, V., Vilhelmova-Ilieva, N., Bulgarian medicinal extracts as natural inhibitors with antiviral and antibacterial activity. Plants, **2022**, 11(13): DOI: 10.3390/plants11131666.
17. Tumbarski, Y. D, Todorova, M. M., Topuzova, M. G., Georgieva, P. I., Ganeva, Z. A., Mihov, R. B., Yanakieva, V. B. Antifungal Activity of Carboxymethyl Cellulose Edible Films Enriched with Propolis Extracts and Their Role in Improvement of the Storage Life of Kashkaval Cheese. Current Research in Nutrition and Food Science, **2021**, 9(2), 487-499. https://doi.org/10.12944/CRNFSJ.9.2.12
18. Ivanov I. G., Vrancheva R. Z., Marchev A. S., Petkova N. T., Aneva I. Y., Denev P. P., Georgiev V. G., Pavlov A. I. Antioxidant activities and phenolic compounds in Bulgarian Fumaria species. International Journal of Current Microbiology and Applied Sciences, **2014**, 3 (2), 296-306.
19. Reed, L.J. and Muench, H. "A simple method of estimating fifty percent endpoints". *The American Journal of Hygiene* **1938**, 27, 493–497.
20. Tumbarski Y., Ilieva V., Yanakieva V., Topuzova M., Todorova M., Merdzhanov P. Application of propolis extracts as biopreservatives in a cosmetic product (hand cream). Journal of Hygienic Engineering and Design, **2023**, 43, in press.

21. Sargsyan, K., Mazmanian, K. & Lim, C. A strategy for evaluating potential antiviral resistance to small molecule drugs and application to SARS-CoV-2. *Sci Rep* **2023**, 13, 502 https://doi.org/10.1038/s41598-023-27649-6
22. Lima, W.G.; Brito, J.C.M.; da Cruz Nizer, W.S. Bee products as a source of promising therapeutic and chemoprophylaxis strategies against COVID-19 (SARS-CoV-2). Phyther. Res. **2020**, 35, 743–750.
23. Refaat, H.; Mady, F.M.; Sarhan, H.A.; Rateb, H.S.; Alaaeldin, E. Optimization and evaluation of propolis liposomes as a promising therapeutic approach for COVID-19. Int. J. Pharm. **2020**, 120028.
24. Josè M. Alvarez-Suarez (Ed.) Bee products – chemical and biological properties. Springer International Publishing AG, **2017**.
25. Silva-Beltrán, N.P.; Balderrama-Carmona, A.P.; Umsza-Guez, M.A.; Souza Machado, B.A. Antiviral effects of Brazilian green and red propolis extracts on Enterovirus surrogates. Environ. Sci. Pollut. Res. **2020**, 27, 28510–28517.
26. Sahlan, M.; Irdiani, R.; Flamandita, D.; Aditama, R.; Alfarraj, S.; Javed Ansari, M.; Cahya Khayrani, A.; Kartika Pratami, D.; Lischer, K. Molecular interaction analysis of Sulawesi propolis compounds with SARS-CoV-2 main protease as preliminary study for COVID-19 drug discovery. J. King Saud Univ. Sci. **2020**, 101234.
27. Kumar, V.; Dhanjal, J.K.; Bhargava, P.; Kaul, A.; Wang, J.; Zhang, H.; Kaul, S.C.; Wadhwa, R.; Sundar, D. Withanone and Withaferin-A are predicted to interact with transmembrane protease serine 2 (TMPRSS2) and block entry of SARS-CoV-2 into cells. J. Biomol. Struct. Dyn. **2020**.
28. Labská, K.; Plodková, H.; Pumannová, M.; Sensch, K.H. Antiviral activity of propolis special extract GH 2002 against Varicella zoster virus in vitro. Die Pharm. Int. J. Pharm. Sci. **2018**, 73, 733–736.
29. Ożarowski, M.; Karpiński, T.M. The Effects of Propolis on Viral Respiratory Diseases. Molecules **2023**, 28, 359. https://doi.org/10.3390/molecules28010359.
30. Zulhendri, F., Chandrasekaran, K., Kowacz, M., Ravalia, M., Kripal, K., Fearnley, J., Perera, C.O. Antiviral, Antibacterial, Antifungal, and Antiparasitic Properties of Propolis: A Review. *FOODS*. **2021**,10 (6); DOI: 10.3390/foods10061360.
31. Ma, X., Guo, Z.H., Li, Y.A., Yang, K., Li, X.H., Liu, Y.L., Shen, Z.Q., Zhao, L., Zhang, Z.Q. Phytochemical Constituents of Propolis Flavonoid, Immunological Enhancement, and Anti-porcine Parvovirus Activities Isolated From Propolis. Frontiers in Veterinary Science. **2022**, 9; DOI: 10.3389/fvets.2022.857183.
32. Mendonça, R.Z., Nascimento, R.M., Fernandes, A.C.O., Silva Jr. P.L. Antiviral action of aqueous extracts of propolis from Scaptotrigona aff. Postica against Zica, Chikungunya, and Mayaro virus. bioRxiv **2022**. 11.03.515030; doi: https://doi.org/10.1101/2022.11.03.515030.
33. Bankova, V. Recent trends and important developments in propolis research. Evid Based Complement Altern Med **2005** March; 2(1): 29-32.
34. Debiaggi, M.; Tateo, F.; Pagani, L.; Luini, M.; Romero, E. Effects of propolis flavonoids on virus infectivity and replication. Microbiologica **1990**, 13, 207–213.
35. Huang, S., Zhang, C.P., Wang, K., Li, G.Q., and Hu, F.L. Recent Advances in the Chemical Composition of Propolis. Molecules, **2014**, 19, 19610-19632.
36. Schnitzler, P.; Neuner, A.; Nolkemper, S.; Zundel, C.; Nowack, H.; Sensch, K.H.; Reichling, J. Antiviral activity and mode of action of propolis extracts and selected compounds. Phyther. Res. **2010**, 24, S20–S28.
37. Nolkemper, S.; Reichling, J.; Sensch, K.H.; Schnitzler, P. Mechanism of herpes simplex virus type 2 suppression by propolis extracts. Phytomedicine **2010**, 17, 132–138.
38. Búrquez, M.d.J.G.; Mosqueda, M.d.L.J.; Mendoza, H.R.; Zárate, C.I.S.; Miranda, L.C.; Sánchez, T.A.C. Protective effect of a Mexican propolis on MDBK cells exposed to aujeszky's disease virus (pseudorabies virus). African J. Tradit. Complement. Altern. Med. **2015**, 12, 106–111.
39. Búfalo, M.C.; Figueiredo, A.S.; De Sousa, J.P.B.; Candeias, J.M.G.; Bastos, J.K.; Sforcin, J.M. Anti-poliovirus activity of Baccharis dracunculifolia and propolis by cell viability determination and real-time PCR. J. Appl. Microbiol. **2009**, 107, 1669–1680.
40. Bankova, V.; Galabov, A.S.; Antonova, D.; Vilhelmova, N.; Di Perri, B. Chemical composition of propolis extract ACF® and activity against herpes simplex virus. Phytomedicine **2014**, 21, 1432–1438.