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Effect of extracts of some species from Phylum Mollusca against the replication of Human Alphaherpesviruses types

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In the present study, hemolymph from *Rapana venosa* (hRv), *Helix lucorum* (Hl) and *Eriphia verrucosa* (hEv), mucus from *Helix aspersa* (Ha) and structural subunit α-HaH from hemocyanin of *H. aspersa* (sHa) were tested against replication of antiviral drugs acyclovir (ACV) sensitive strain F and BA of human alphaherpesvirus type 1 and type 2 *in vitro*. All six extracts showed no anti-herpesvirus activity using an MTT-based colorimetric assay to detect inhibition of Human Alphaherpesviruses (HHV) replication. In a virus analysis, the six extracts tested reduced the infectivity of both viruses from the two strains used to varying degrees and applied at maximum non-toxic concentrations. Fractions from hemolymph from *R. venosa* (MW 30-100 kDa) and from *E. verrucosa* (MW 3-100 kDa) showed the highest activity (over 99% inhibition of extracellular virions infectivity by first and second type of viruses respectively), sufficient to be considered pharmacologically significant. Hemolymph from *R. venosa* and *E. verrucosa* and mucus from *H. aspersa* have little effect on the adsorption of BA strain of human alpha herpesvirus type 2, and strain F of the HHV 1. The effect on the first type being more pronounced.

Key words: hemolymph from *R. venosa*, *H. lucorum* and *E. verrucosa*; mucus from *H. aspersa*; human alphaherpesviruses

INTRODUCTION

"Herpes" is a medical condition caused by members of the genus Simplexvirus (Human Alphaherpesviruses (HHV) types 1 and 2). According to the WHO, worldwide 3.7 billion people under age 50 (67%) and 491 million people aged 15-49 (13%) are infected with HHV-1 and HHV-2, respectively [1]. Infection with both types of HHV may be asymptomatic (but the virus can still be transmitted to others). In the presence of symptoms, painful sores and blisters around the mouth and lips (when a person is infected with HHV-1) and on the genitals and anus (genital herpes) (when a person is infected with HHV-2) are observed. This causes in the infected individual psychological distress very often and forces him to adjust to live with the infection. The risk group is immunocompromised patients and neonates in whom the lack of an adequate immune response is the cause of encephalitis, meningitis, neonatal herpes and even death. All Herpesviridae family representatives establish a latent infection that results in recurrence of symptoms. As the latent virus cannot be affected, modern therapy is aimed at controlling the symptoms of the primary and recurrent HHV infections [2].

Nucleoside analogues such as ACV and its derivatives are most commonly used for treatment. Although this group of substances are very effective selective inhibitors, their improper use leads to the development of resistant viral mutants [3]. An effective alternative is therefore needed. Such are, for example, substances of natural origin.

In invertebrates lack a highly specific adaptive immune system and they use their innate and non-adaptive immune system to resist pathogen invasions [4]. Many gastropods and arthropods are subject of scientific studies with aim discovery of novel antimicrobial and antiviral compounds. Cellular immunity of these species predominantly involves the phagocytic activity of hemocytes, whereas humoral immunity requires the release of antimicrobial factors [5, 6]. The structural subunit βc-HaH of hemocyanins of Helix aspersa and two peptides isolated from the hemolymph of the molluscan garden snail Helix lucorum shows antimicrobial activities against Staphylococcus aureus, Streptococcus epidermidis and also against

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Escherichia coli [7, 8]. The fraction with peptides (produced by the mucus of garden snail Cornu aspersum) with molecular masses below 3 kDa exhibited antibacterial activity against Gram-negative Pseudomonas aeruginosa AP9 and Gram-positive Brevibacillus laterosporus BT271 bacteria. The inhibition effects of the peptides can be explained with the amino acid residues [9].

Hemocyanins from *R. venosa, H. lucorum* and marine cancer *C. aestuarii* and their glycosylated derivatives have been shown a promising antiviral effect against a number of viruses from different groups, such as human respiratory syncytial virus (hRSV), influenza virus A (H3N2 type), herpes 1 and type 2 (HSV-1 and HSV-2), Epstein-Barr virus (EBV) It has been established a relationship between carbohydrate structures and the antiviral properties of hemocyanin derivatives, and a new mechanism has been proposed for explain the antiviral effect of hemocyanin by molluscs [10-12]. Haemolymph and lipophilic extract of the digestive gland (from abalone Haliotis laevigata (Haliotidae)) both showed antiviral activity against the human herpesvirus HSV-1 *in vitro*. The haemolymph inhibited viral infection at an early stage. In contrast, the antiviral effect of the lipophilic extract may act at an intracellular stage of infection [13]. A modified peptide derived from myticin C (an antimicrobial peptide from the Mediterranean mussel (Mytilus galloprovincialis)) or the nanoencapsulated normal peptide also showed antiviral activity against the human herpesviruses HSV-1 and HSV-2 *in vitro* [14]. One of the fractions of mucus of land slug Phyllocaulis boraceiensis with the main constituents hydroxy-tritriacontapentaenoic acid and hydroxy-pentatriacontapentaenoic acid, exhibited antiviral activity against measles virus *in vitro* [15].

And last but not the least. The human bladder cancer permanent cell line CAL-29 is sensitive to the action of the hemocyanins from the molluscs Helix lucorum, Rapana venosa and their functional units [16].

EXPERIMENTAL

Materials and Extracts

The hemolymph from garden snails *H. lucorum* and marine snail *R. venosa* were obtained as described previously [17, 18]. Two fractions were isolated from hemolymph of *R. venosa*, containing compounds with MW 1-10 kDa, and with MW 30-100 kDa.

Two protein fractions were also isolated from hemolymph of *H. lucorum* (with MW under 100 kDa) and from crab *E. verrucosa* (with MW 3-100 kDa). The hemolymph from *E. verrucosa* was extracted from 10 crabs (3-5 mL per crab) living in area near to Kamchia region of the Black Sea and homogenized in 0.01 mol/L Tris-buffer (Sigma-Aldrich, Steinheim, Germany) [19].

Two fractions we purified from snail *H. aspersa*, structural subunit α-HaH from the hemolymph [20] and a protein fraction with MW over 50 kDa from mucus [21].

All fractions were obtained from purified hemolymph from *R. venosa*, *H. lucorum* and *E. verrucosa* and *H. aspersa* mucus by ultrafiltration using membrane (Millipore™ Ultrafiltration Membrane Filters) with different size pores – MWCO from 100 kDa, 50 kDa, 30 kDa, 3 kDa and 1 kDa.

SDS-PAGE Electrophoresis

Protein fractions were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli method with modifications [22]. Equal volumes containing approximately 15 μg/lane of the samples dissolved in Laemmli sample buffer and protein standard mixture (Precision Plus Protein., All Blue, Bio-Rad, Feldkirchen, Germany) were separated by 12.0% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue G-250.

Cells and Viruses

MDBK (Madine and Darby bovine kidney) cell line, grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 8% (growth medium) and 4% (maintenance medium) fetal calf serum (FCS) (with Gentamycin 8 μg/ml and 10 mM HEPES buffer), were used in the experiments. This study employed the F strain of Human alphaherpesvirus type 1 and Ba strain of Human alphaherpesvirus type 2. The virus was propagated in MDBK cells and stored at −70 °C until used.

Determining the effect of extracts on cell culture

The cytotoxicity (cell viability) was determined by colorimetric MTT assay [23]. Confluent monolayers of MDBK cells in 96-well plates were overlaid with 0.1 ml/well maintenance medium, 0.1 ml/well of serial two-fold dilutions of the extracts (in maintenance medium) or 0.1 ml/well only maintenance medium (in cell controls) and were

incubated at 37 °C for 48 h. On the second day 0.020 ml of MTT (Sigma-Aldrich) – (5 mg/ml in PBS) was added to each well and the plates were incubated for 2 h at 37 °C. The optical densities (OD) were determined by plate reader at λ = 540 nm. The percentage of viable treated cells was calculated by the following formula [(ODexp.)/(ODcell control)]*100, where (ODexp.) and (ODcell control) indicate the absorbencies of the test sample and the cell control, respectively. The 50% cytotoxicity concentration (CC50) was calculated by regression analysis of the dose – response curves generated from the data. MTC was determined microscopically on the 48th hour.

Survival study of virus-infected and extract-treated cells. MTT-based colorimetric assay for detection of HHV replication inhibition

We used a modification of an MTT assay developed for screening of anti-HHV compounds by Takeuchi *et al.* [24]. Confluent monolayers in 96-well plates were overlaid with 0.1 ml/well of virus suspension – MOI = 100 CCID50/well. The plates were incubated for 1 h at 37 °C and dilutions of the extracts or only maintenance medium (for virus control) were added after that. Uninoculated cells were used for cell control. On day 5, the plates were treated in the same way described in the method for measuring cell viability. The percentage of protection was calculated by the following formula [(ODexp.) – (ODvirus control)/(ODcell control) – (ODvirus control)]*100, where (ODexp.), (ODvirus control), and (ODcell control) indicate the absorbencies of the test sample, the virus control and the cell control, respectively. The 50% effective concentration (EC50) was calculated by regression analysis of the dose – response curves generated from the data. Selectivity index (SI) was calculated as CC50/EC50.

Study of the effect of the extracts on the extracellular virions of HHV 1 and HHV 2

The direct virus inactivating effect of the plant extracts was tested by direct contact assay. Undiluted stock virus suspensions were treated with equal volumes of the compounds in MNC (maximal nontoxic concentration), prepared in maintenance medium and incubated at 37 °C for 5′, 15′, 30′, 60′, 120′, 240′ and 360′. Undiluted stock virus suspensions were treated with equal volumes of compound free maintenance medium for control. At the end of each time interval, the control and the treated viruses were freezed, and the difference in the biological activities between them was determined on the base of infectivity. The surviving infectious virus titres were determined in CPE assay using the method of Reed and Muench [25].

Activity against viral adsorption

The effect of the extracts over viral adsorbtion was tested by direct contact assay. Undiluted stock virus suspensions were treated with equal volumes of the compounds in MTC, prepared in maintenance medium and incubated at 4 °C for 15′, 30′, 60′, 120′ over cell culture. Undiluted stock virus suspensions were treated with equal volumes of compound free maintenance medium for control. At the end of each time interval, the control and the treated cell layers were flushed twice with PBS and then fresh medium were put over the cells. After 24 hours both arrays were freezed, and the difference in the biological activities between them was determined on the base of infectivity. The surviving infectious virus titres were determined in CPE assay using the method of Reed and Muench [21].

RESULTS AND DISCUSSION

Protein fractions were characterized by 12.5% SDS-PAGE analysis. The analysis on Fig. 1 shows the difference in the performance of proteins in them.

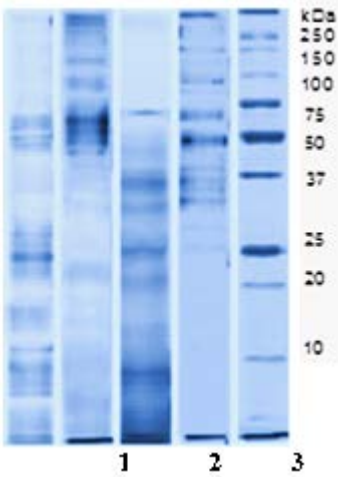

Fig. 1. 12.5% SDS-PAGE analysis, positions: 1) Fraction from *H. lucorum* hemolymph with Mw <100 kDa; 2) Fraction from *H. aspersa* mucus with MW >50 kDa, 3) Fraction from hemolymph of *E. verrucosa* with MW 3-100 kDa; 4) Fraction from hemolymph of *R. venosa* with Mw 30-100 kDa; 5) standard proteins from Bio-rad.

Analysis of the *H. lucorum* hemolymph fraction with Mw <100 kDa (Fig. 1, position 1) confirmed

the presence of various proteins and peptides with molecular weights between 2 and 6.8 kDa, recently determined by mass spectrometry [26]. Several proteins were identified in the protein fraction of *H. aspersa* mucus with MW above 50 kDa (Fig. 1, position 2) [27, 28]. As shown on Fig. 1 position 3, peptides with MW<10 kDa and proteins in a wide range of ~ 20 kDa, ~ 25 kDa, between 30-40 kDa and ~ 75 kDa were found in the fraction of hemolymph from sea crabs *E. verrucosa*. Several proteins with MW 35-45 kDa, ~ 50 kDa, ~ 65 kDa and ~ 100 kDa were also found in hemolymph of *R. venosa* with Mw 30-100 kDa.

Determination of MTC and CC50 values and the effect of extracts on cell culture

We initially examined the cytotoxicity of each of the extracts we used. We applied the MTT test to determine live and early apoptotic cells [23].

In the used experimental setup, concentrations of the extracts in the range from 1 µg/ml to 20 µg/ml were tested. The values obtained for each extract are presented (Table 1).

Table 1. Data on the cytotoxicity of the studied extracts.

Extract	**MTC [µg/ml]**	**CC_{50} [µg/ml]**
Hemolymph from *Rapana venosa* with MW 1-10 kDa	10	>10
Hemolymph from *Rapana venosa* with MW 30-100 kDa	2	2.36
Hemolymph from *Helix lucorum* with MW under 100 kDa	>20	>20
Hemolymph from *Eriphia verrucosa* with MW 3-100 kDa	>20	>20
Mucus from *Helix aspersa* with MW over 50 kDa	>20	>20
Structural subunit α-HaH from hemocyanin of *Helix aspersa*	10	>10

After graphical expression of the obtained values, MTCs and CC_{50} were determined. When comparing the experimental data (values for CC_{50}) it is seen that hemolymph from *R. venosa* 30-100 kDa have the highest cytotoxicity – 2.36 µg/ml. The fractions from hemolymph of *H. lucorum* with MW under 100 kDa, from hemolymph of *E. verrucosa* with MW 3-100 kDa and mucus from *H. aspersa* with MW over 50 kDa have the lowest respectively over 20 µg/ml.

The extract of hemolymph from *R. venosa* with MW 30-100 kDa was found to have the highest toxicity, followed by the extract with MW 1-10 kDa, which is equivalent to the toxicity of the α-NaH structural subunit from hemocyanin of Helix aspersa. With the same toxicity are three extracts of hemolymph from *H. lucorum* with MW below 100 kDa, extract of hemolymph from *E. verrucosa* with MW 3-100 kDa and mucus from *H. aspersa* with MW over 50 kDa. However, they show lower toxicity than other extracts.

Survival study of virus-infected and extract-treated cells

All the extracts were tested for concentration depended activity over viral replication with maximal concentration tested – equal to the MTC concentration equal to the determined to one from the cell toxicity experiment. All six extracts were tested against both HHV-1 and HHV-2 viruses with no effect except the extract from hemolymph of *R. venosa* with MW 1-10kDa – with inhibition up to 20% when used in MTC. All other extracts showed no activity against the viral replication.

Study of the effect of the extracts on the extracellular virions of HHV 1 and HHV 2

For a more complete study of the antiherpes activity of the extracts, their effect on extracellular virions was studied by direct contact method. The extracts were administered at a concentration corresponding to their MTC. The change in the infectivity of the virus was observed at different duration of contact with the studied extract (5, 10, 15, 30, 60, 120, 240 and 360 minutes).

The results obtained for the virucidal action of the extracts are presented in Table 2. Data on the effect of the extracts on the extracellular virions of HHV 1 and HHV 2, after 360 min. of exposure

Table 2. Data on the Virucidal activity of the studied extracts.

Extract	**MTC µg/ml**	**Virucidal effect [Δlog]**	
		HHV-1	**HHV-2**
Hemolymph from *Rapana venosa* with MW 1-10 kDa	10	1.33	0.66
Hemolymph from *Rapana venosa* with MW 30-100 kDa	2	2.33	0.83
Hemolymph from *Helix lucorum* with MW under 100 kDa	>20	0.67	0.33
Hemolymph from *Eriphia verrucosa* with MW 3-100 kDa	>20	1.67	2.66
Mucus from *Helix aspersa* with MW over 50 kDa	>20	2	1.83
Subunit from hemocyanin of α-HaH from *Helix aspersa*	10	1.55	1.83

showed some virucidal activity against both types of viruses.

Virucidal action against HHV 1 strain F

The most pronounced effect on extracellular virions was shown by extract from hemolymph from *R. venosa* with MW 30-100 kDa and extract from mucus from *H. aspersa* with MW over 50 kDa. The present results in Table 2 show that the virus titter in the samples decreased by two or more logarithms (99.99 and more percent viral inhibition) compared to the virus titter in the control samples.

When examining hemolymph from *R. venosa* with MW 30-100 kDa, a reduction in viral infectivity was observed in the initial stages of exposure (at the fifth minute of treatment). At the thirtieth minute of treatment, the viral titter decreased by 1.33 log (95.32% inhibition). The effect is enhanced by increasing the treatment time of the samples. At the end of the study interval, a decrease in viral titter by more than 2 logs (99.53% inhibition of viral infectivity) was found (Table 2).

The study of *H. aspersa* mucus also showed a reduction in viral infectivity. The effect remains relatively constant with increasing sample treatment time. At the fifteenth minute of treatment, the viral titter decreased by 1.33 log (95.32% inhibition) At the end of the study interval (360 minutes), a decrease in viral titter by 2 logs (99%) was reported (Table 2).

The remaining extracts - hemolymph from *R. venosa* 1 with MW 1-10 kDa, from *H. lucorum* with MW under 100 kDa and hemolymph from *E. verrucosa* with MW 3-100 kDa, Structural subunit α-HaH from hemocyanin of *H. aspersa*, show some weak virucidal action in the initial stages of exposure. The decrease in viral infectivity at the end of the study interval and the decrease in viral titter in the samples was about 1 log (90%) compared to the virus titter in the control samples (Table 2).

Virucidal action against HHV 2 strain BA

In the study of the extracts provided to us against the extracellular virions of HHV 2, we found that Hemolymph from *Eriphia verrucosa* with MW 3-100 kDa has the strongest effect on the infectivity of the virus. The decrease in viral titter in the sample at the fifth minute was 0.66%, and at the 360th minute it reached 2.66 log or 99.78% viral inhibition (Table 2).

The virucidal effect is enhanced by increasing the treatment time, i. e. the virucidal activity of the extract against the HHV2 is 1 logarithm higher than that observed in the treatment of the HHV1. When studying the activity of the shown good effect against the first type of virus, Mucus from *H. aspersa* with MW over 50 kDa, we found a relatively good effect on HHV 2 (Table 2). The decrease in viral titter in the samples was 1.83 log compared to the viral control. A decrease in viral titter is reported as early as the 15th minute - 1 log. The tendency of decrease of the viral titter is preserved and at the end of the treatment the decrease is by 1.83 log. or 98.52% viral inhibition.

Hemolymph from *R. venosa* with MW 30-100 kDa, which has a significant effect on the first type of virus, has a significantly weaker effect on the HHV 2, at maximum exposure time Δlog is 0.83.

Activity against viral adsorption

Of all the extracts studied, hemolymph from *R. venosa* with MW 30-100 kDa, Hemolymph from *Eriphia verrucosa* with MW 3-100 kDa and mucus from *H. aspersa* with MW over 50 kDa showed the highest activity (Fig. 2 and Fig. 3). The remaining extracts showed no effect on the adsorption of the two types of viruses.

Activity of the extracts on the adsorption of the HHV-1 strain F

Already in the initial stages of treatment (15th minute) a decrease in the viral titter in the treated samples is reported in contrast to the virus titter in the untreated samples with extracts (Fig. 2). At the 120th minute from the beginning of the treatment, the hemolymph from *R. venosa* with MW 30-100 kDa showed the strongest effect on the adsorption.

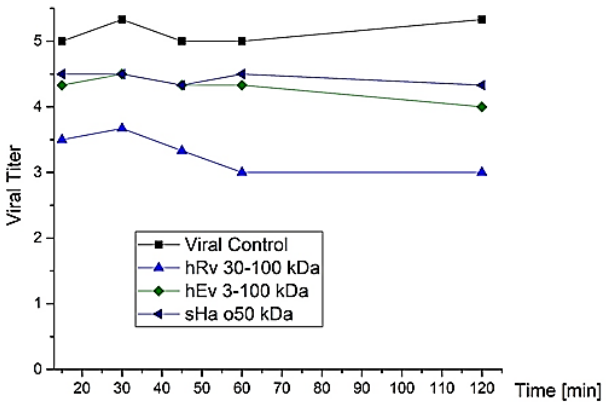

Fig. 2. Activity of the extracts on the adsorption of the HHV-1.

The decrease in viral titter compared to viral control was 2.33 log or 99.53% viral inhibition. When treated with other two extracts, hemolymph from *E. verrucosa* with MW 3-100 kDa and mucus from *H. aspersa* with MW over 50 kDa, a decrease in viral titter of about 1 log was also recorded. In hemolymph from *E. verrucosa* with MW 3-100 kDa - 1.33 log and in mucus from *H. aspersa* over 50 kDa 1 log. The percentage of viral inhibition was 95.32 and 90, respectively. These results were reported when treating the samples for 120 minutes. The remaining extracts had no effect on the adsorption of the HHV 1 strain F.

Activity of the extracts on the adsorption of the HHV-2 strain BA

Only three of the six extracts studied, hemolymph from *R. venosa* 30-100 kDa, hemolymph from *E. verrucosa* 3-100 kDa and mucus from *H. aspersa* over 50 kDa, had an effect on the adsorption of the two types of virus (Fig. 3).

Compared to the effect on the adsorption of the HHV 1, the three extracts showed a weaker effect. Again, the strongest effect of hemolymph from *R. venosa* with MW 30-100 kDa is reported. As early as the 15th minute of exposure, the viral titter decreased by 1.17 log, reaching 1-66 log or 97.81% viral inhibition at the end of the test period.

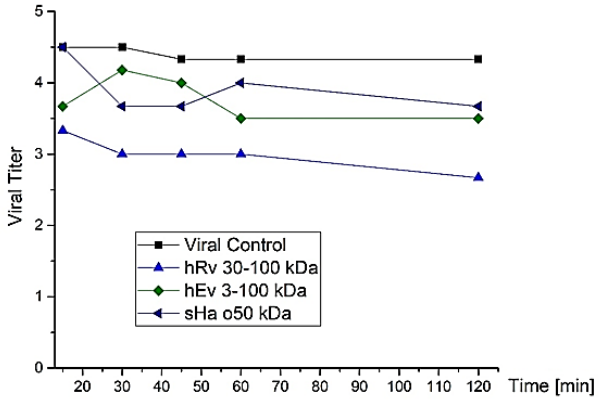

Fig. 3. Activity of the extracts on the adsorption of the HHV-2.

In the other two extracts, hemolymph from *E. verrucosa* with MW 3-100 kDa and mucus from *H. aspersa* with MW over 50 kDa, Δlog reached respectively 0.83 (85.21% viral inhibition) and 0.66 (78.12% viral inhibition).

CONCLUSION

Contrary to the reports of other author teams, the effect of various extracts of invertebrates on the intracellular stages of replication of some viruses [13, 14, 29], our team did not find significant antiviral activity of the studied extracts. Only the extract of hemolymph of R. venosa with MW 1-10 kDa showed a weak effect - inhibition of up to 20%.

The antiviral activity of the studied extracts is manifested as activity against extracellular virions of HHV-1 and extracellular virions of HHV-2. The highest virucidal activity on the HHV 1 shows hemolymph from *R. venosa* with MW 30-100 kDa. Hemolymph from *E. verrucosa* with MW 3-100 kDa has the best virucidal effect in relation to the second type of virus and relatively good in relation to the first type. Mucus from *H. aspersa* with MW over 50 kDa also shows good virucidal activity against HHV type 1 and relatively good against the second type. Clarification of the mechanism of action of active extracts is the focus of future developments. Because the experiments involved an effect on extracellular virions, we assume that the extracts acted at the level of virus addressable proteins. The existence of some differences in the glycoproteins of the viral envelopes of the two types of viruses is the probable reason for the different activity of the extracts with respect to HHV-1 and HHV-2. Similar results for the virucidal action of biological mollusc material on a number of viruses, including HSV-1, have been reported by other scientific teams [15, 29].

Given the low cytotoxicity and the fact that virucidal activity of hemolymph from *E. verrucosa* with MW 3-100 kDa and mucus from *H. aspersa* with MW over 50 kDa against both types of viruses are strong and nearly 99%, that is sufficient to be considered pharmacologically significant.

It should be noted that the hemolymph of *R. venosa* with MW 30-100 kDa has a strong virucidal action against the first type of virus and a clear effect on the adsorption of both types of viruses. Similar activity against viruses with this type of pathophysiology can be considered a product for human use. The effect of some of the extracts on viral adsorption should not be underestimated, as the virus detects chronic infection in some of the infected tissues.

From the obtained results for the effect of the studied extracts on the adsorption of both types of viruses, as well as for the effect on the extracellular virions of both types of viruses, it can be concluded that the three extracts, hemolymph from *R. venosa* 30-100 kDa, hemolymph from *E. verrucosa* with MW 3-100 kDa and mucucs from *H. aspersa* with MW over 50 kDa are suitable for pharmacological studies and use as products. for local application.

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