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ANTIVIRAL ACTIVITY OF A METHANOL EXTRACT OF LYCORINE – RICH CLONE OF *LEUCOJUM AESTIVUM* L. AGAINST SUID HERPESVIRUS TYPE 1

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ABSTRACT

Suid herpesvirus type 1 (SHV-1) is one of the representatives of Herpesviridae with great importance in veterinary medicine and especially in pig industry and for that reason finding ways to prevent its transmission and spreading is crucial. Plants from A*maryllidaceae* family contain various bioactive compounds, some of which have antiviral potential. One of those plant species is *summer snowflake* (*Leucojum aestivum* L), a well-known source of the phytochemicals which have variety of medical uses. One of them is lycorine, an alkaloid which is known for its various biological activities like including antiviral. In this study we examine the *in vitro* antiviral activity of a methanol extract obtained from a lycorine-rich clone of *L. aestivum* against A2 strain of Suid herpesvirus type 1. The experiments were performed on MDBK cell line, using different dilutions of the extract ranging from 1:5 to 1:320, obtained after dissolving in cell culture media. The focus on our research was to define not only whether there is an antiviral activity but also the mode of action, so we apply several different models: direct treatment of the virus suspension, cell pre-treatment before infection, treatment of infected cells, and effect on virus adsorption. Results reveal that direct treatment of the virus, before spiking of the MDBK cell, leads to block of the viral activity at plant extract dilution of 1:10.

Key words: antiviral, summer snowflake, herpesvirus, pseudorabies, Suid herpesvirus.

Introduction

Aujeszky's disease (pseudorabies), is a contagious disease found predominantly in domestic pigs and wild boars but it can affect various other domestic and wild mammals, possibly even human (Zheng, et al.,2022; Wang et al.,2020). The disease is manifested mainly with nervous and respiratory symptoms and also reproductive failure in pregnant animals (Zheng, et al.,2022). The causative agent is Suid herpesvirus type 1 (SHV-1) which is enveloped virus with double strand DNA belongs to genus *Varicellovirus* of subfamily *Alphaherpesvirinae* in the family *Herpesviridae* (Murphy et al. 1999; Mettenleiter et al.,2019). Pseudorabies has a serious international socio-economic impact in pig industry and is put in the list of diseases of The World Organization for Animal Health (Díaz, et al., 2021; WOAH, 2023).

One of the possible approaches to reduce the risk of virus transmission in general is to use plant-derived biologically active substances applied as medicines or as part of the animal feed.

Leucojum aestivum L. (summer snowflake) is perennial bulbous geophyte, which belongs to *Amaryllidaceae* family (Parolo et al., 2011). Among the various biologically active substances found in the plant special attention deserve its alkaloids. They are proven to have various pharmacological activities like anticholinesterase, antiviral, antitumor, immunostimulant, antifungal, antiprotozoal, etc. (Bastida et al., 2011; Jin, 2011; Demir et al., 2022).

Lycorine is one of the most widespread and characteristic phenanthridine alkaloids and in *L. aestivum* it could be found as a dominant or minor alkaloid depending on the origin of the plant material (Bogdanova et al., 2008). Antiviral activity of lycorine has been confirmed in flaviviruses, Punta Toro and Rift Valley fever viruses and with less effectiveness in bunyaviruses (Ieven et al., 1982; Gabrielsen et al., 1992). Other viruses for which there are reports that lycorine or other alakaloids of the family. Herpes simplex virus type 1, poliovirus, Coxsackie and SARS-CoV, Zika virus (Ghosal et al., 1985; Renard-Nozaki et al. 1989; Li et al., 2005; Chen, et al., 2020).

The aim of our study was *in vitro* testing of the antiviral activity of the methanol extract of lycorine-rich clone of summer snowflake against SHV-1.

Materials and methods

Plant material and extraction

Leucojum aestivum herbage was harvested from the collection of the Institute of Biodiversity and Ecosystem Research (Bulgarian Academy of Sciences) during the flowering stage.

The extract was prepared by double extraction of the herbage with methanol in a 1:10 ratio for 24 hours at 25°C. During extraction, samples were sonicated 4 times in an ultrasonic bath for 10 min every 8 hours. After centrifugation for 10 min at 6000 rpm, extracts were filtered through a paper filter (FILTRAK 390, Munktell & Filtrak GmbH, Germany) and evaporated to dryness under vacuum at 40°C (Laborota 4003, Heidolph, Germany). 600 mg of the dry extract was diluted in 10 ml of phosphate buffered saline at pH 7.2, filtered through a 0.22 μm PES filter (Millipore, USA) and stored at -20°C in cryostat vials. The tested dilutions were: 1:5; 1:10; 1:20; 1:40; 1:80; 1:160; 1:320.

Cell cultures

For the experiments was used MDBK cell line (CCLV 1992, RIE 26), placed in 24-well plates, by 4×10^5 cells/ml.

MEM-Eagle and MEM-Hanks supplemented with antibiotics (penicillin 100 UI/ml, streptomycin 100 γ/ml), essential amino acids, 5% or 10% fetal calf serum (FTS) were used as growth and maintenance media. All the reagents were purchased by Sigma -Aldrich (St. Louis, MO, USA).

Determinations of the plant extract cytotoxicity

Cytotoxicity test was performed on a 24 well plates with a dense cell monolayer to which culture medium was added with the appropriate amount of stock solution of the tested extract until reaching the desired test concentration. The plates were placed at 37°C for 72 hours in a Binder CO2 incubator (Germany). The presence of cytotoxicity was monitored microscopically at the 24th, 48th and 72nd hour after addition of the extracts. Inside the 24 well plates, each tested dilutions as well as the control cells were made in duplicate. Each experiment was performed in three repetitions.

Virus titration

Pseudorabies virus strain A2 titer was determined by the end-point dilution method of Reed and Muench (1938) and was expressed as tissue-culture infectious doses 50%/ml ($TCID_{50}$/ml). At first we made ten-fold dilutions of the virus and transferred them into sterile flat-bottomed plates. Then cell culture MDBK was added and the plates and left for incubation at 37◦C for 72 h. The infectious titre of the stock virus was $10^{6.5}$ $TCID_{50}$/mL.

Antiviral activity tests

We followed the same experimental model that we use in a one of our previous research (Chervenkov et al.,2014). In short 4 treatment modes were used: cell pre-treatment with extract before

spiking with virus; direct treatment of the virus suspension with extract; treatment of infected cells with extract and plant extract and virus put together on the cell culture. The tested extract dilutions were chosen after determining the maximal non-cytotoxic concentration. The final virus concentration in the culture medium was 100 ID_{50}/well. The cell monolayer was inspected under invert microscope (Carl Zeiss, Jena, Germany) at the 24th, 48th and 72nd h after infection for presence of cytopathic effect (CPE). All experiments were done in triplicates with double wells for each extract dilution and the control of the cell monolayer.

Results

Dilutions of 1:5 of the methanol extract of *L. aestivum* cause a clearly visible cytotoxic effect. There was no observatory detrimental effect of the extract with the remaining dilutions of the extract. No cytotoxic effect was observed on cell culture controls untreated with the extract. The lowest dilution of the extract at which there was no cytotoxic effect on the MDBK cell line was 1:10, so it was determined as the maximum non-toxic concentration. Only dilutions of the extract greater than or equal to 1:10 were used to determine the type of antiviral activity against SHV-1.

To establish the presence of protective effect of the methanolic extract of *L. aestivum* against SHV-1, we performed treatments for 60 min at 37◦C on cell culture prior to virus infection. When MDBK cells were pretreated with the plant extract and subsequently infected with SHV-1, viral replication was not inhibited at any of the dilutions used.

When the methanolic extract of *L. aestivum* was added to SHV-1 immediately prior to infection of cell cultures, it was found that at dilution of 1:10, the viral replication was suppressed, manifested by the absence of CPE in all control days (Fig 1). Such effect was not found with the rest of the tested dilutions, where various degree of cytopathic changes typical of SHV-1 was observed, manifested by cell rounding, formation of cytoplasmic bridges and clustering of groups of cells, accompanied by detachment of most of the cells of the monolayer and by 72 h. post infection the entire cell monolayer was affected.

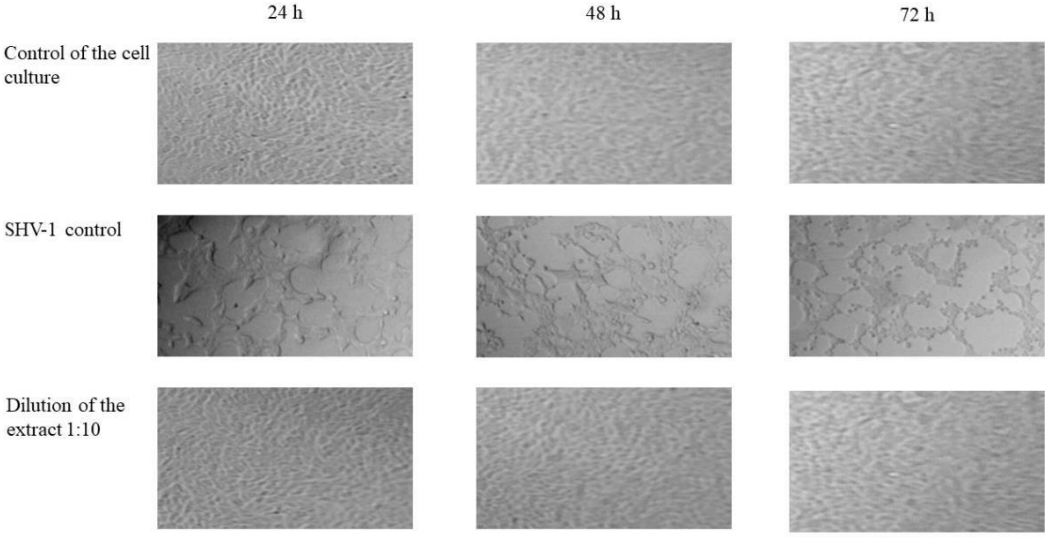

Figure 1: Results from pretreatment of the SHV-1 with methanol extract of *L. aestivum*, before infection of MDBK cell line (24, 48 and 72 h after viral absorption). Magnification x 20

When different dilutions of the extract were added to the virus infected cells, only dilution of 1:10 slowdown the development of CPE for the first 24 h post treatment, however at 72nd hour post treatment there was visible CPE in all tested dilutions.

To test the effect on viral adsorption, we add the virus and the plant extract together to the cells. Unfortunately, none of the tested dilutions proved effective in stopping the viral replication and CPE was observed even at the 24th hour post virus inoculation.

Discussion

The extract of *L. aestivum* used in our experiments was from a clone with biosynthetic profile dominated by the alkaloid lycorine (Bogdanova et al., 2008). Since lycorine has antiviral activity against some DNA and RNA viruses, we decided to test its effect against pseudorabies virus. Similar results of good tolerance after treatment with a plant extract rich in lycorine have also been found by other authors. Low cytotoxicity on Vero E6 and HepG2 cell lines was obtained when they were treated with *Lycoris radiata* extract, another plant containing high levels of lycorine (Li et al., 2005). In another study, Liu et al. (2011) found that lycorine at a concentration of 1.0 μg/ml significantly inhibited the cytopathic effect of EV71 (human enterovirus), on an RC cell line. Meanwhile, the cytotoxic concentration (CC50) of lycorine for this cell line was 48.5 μg/ml.

Our experiments showed a direct antiviral effect of L. aestivum extract against SHV-1 when the extract was added to the virus before infection of the cell culture, however, treatment of the cells before infection with the virus, addition of the extract after infection, and simultaneously addition of the extract with the virus to the cells did not stop viral replication, and the cytopathic effect characteristic of SHV-1, associated with rounding and detachment of infected cells was observed.

While in our study *L. aestivum* showed its antiviral action by blocking free virus before infecting the cells, in another study with other virus of the Herpesviridae family- HSV 1, Ieven et al. (1983) found no effect on extracellularly viral particles after treatment with lycorine, which is also the predominant alkaloid in the *L. aestivum* clone we used. However, in our experiments we used *L. aestivum* extract rather than pure lycorine which may account for the difference in results.

In the same study, lycorine slowed down the intracellular replication of HSV 1, in a dose-dependent manner and this was contributed to blocking of viral protein synthesis (Ieven et al.,1983). Blockade of viral synthesis by lycorine was also found by Liu et al. (2011) in EV71, a human enterovirus. Analysis of the inhibitory effect of lycorine reasserts blocking of viral polyprotein elongation during translation. In the same study, mice infected with a lethal dose of EV71 showed reduced mortality and pathological changes in muscle as a result of inhibition of viral replication by the lycorine.

Renard-Nozaki et al. (1989) also found antiviral activity of lycorine against HSV 1, which they attributed to inhibition of viral replication. In our studies, we didn't found suppressive effect on SHV-1 replication when the plant extract was applied to virus-infected cells. On the other hand, we found virus suppression after direct treatment with the extract. Again, these differences could be due to both the fact that we are working with a different virus and because we are using *L. aestivum* extract rather than pure lycorine.

Conclusion

We found that methanol extract of *L. aestivum* in dilutions from 1:10 to 1:320 does not caused visible cytotoxic effect on MDBK cell. The extract shows antiviral effect against SHV-1 when added to the virus before inoculation of the cell lines.

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