Water Extract of the Chaga Medicinal Mushroom, *Inonotus obliquus* (Agaricomycetes), Inhibits SARS-CoV-2 Replication in Vero E6 and Vero Cell Culture Experiments

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**ABSTRACT:** The antiviral properties of water extracts from pharmaceutical raw materials of the chaga mushroom, *Inonotus obliquus*, were studied against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). All studies with infectious materials were carried out in an isolated virological laboratory of the State Research Center of Virology and Biotechnology Vector of Rospotrebnadzor, which has a sanitary and epidemiological conclusion for the right to work with pathogenic biological agents of I–II pathogenicity groups. Antiviral activity was determined by the ability of *I. obliquus* water extracts to inhibit the replication of SARS-CoV-2 (nCoV/Victoria/1/2020 strain) in Vero E6 and Vero cell cultures. The results of these studies showed that water extracts of *I. obliquus* are characterized by low toxicity in Vero and Vero E6 cell cultures and have antiviral activity against SARS-CoV-2. The 50% inhibitory concentration ranged from 0.75 to 11.6 μg/mL. A patent for the invention was received (Patent RU, 2741714 C 1, 2021).

**KEY WORDS:** SARS-CoV-2, COVID-19 pandemic, basidiomycetous fungi, chaga mushroom, *Inonotus obliquus*, antiviral activity, medicinal mushrooms

**ABBREVIATIONS:** COVID-19, coronavirus disease 2019; DMEM, Dulbecco’s modified Eagle’s medium; IC₅₀, 50% inhibitory (effective) concentration; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SI, selectivity index; SRC VB, State Research Center of Virology and Biotechnology; TC₅₀, 50% toxic concentration; TCD₅₀, 50% tissue cytopathic dose

**I. INTRODUCTION**

The new human influenza virus that caused the coronavirus disease 2019 (COVID-19) pandemic first appeared in late 2019 in Wuhan, China, and has since spread to all countries of the world. In addition to creating vaccines, the search for antiviral compounds of natural origin against the new coronavirus that causes COVID-19 (severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]) is also relevant.

Higher Basidiomycetes represent an unlimited source of polysaccharides with antitumor and immunomodulatory properties. The first medicinal preparations from mushrooms were polysaccharides, including krestin from *Trametes versicolor*, lentinan from *Lentinus edodes*, schizophyllan from *Schizophyllum commune*, and ganoderan from *Ganoderma lucidum*.¹⁻² Later, preparations and many extracts from fungi were found to exhibit not only antitumor properties but also antiviral activity.³⁻⁹ Undoubtedly, many medicinal mushrooms can be useful in the complex treatment and prevention of COVID-19 as well as in recovery from the disease.¹⁰

Since 2008, the State Research Center of Virology and Biotechnology (SRC VB) Vector of the Federal Service for Surveillance in Consumer Rights Protection and Human Wellbeing has been studying the antiviral activity of fruit bodies of fungi from forests of Southwestern Siberia as well as those of 60 strains isolated from them in a pure culture. Based on screening the polysaccharides and melanin extracts from fungi, the broadest spectrum of antiviral activity is found in the chaga mushroom, *Inonotus obliquus* (Ach.
ex Pers.) Pilát (Hymenochaetales, Agaricomycetes, Basidiomycota). Water extracts from natural *I. obliquus* inhibit the replication of different strains of influenza virus, herpes virus types 1 and 2, West Nile virus, immunodeficiency virus type 1, and some orthopoxviruses, including variola virus.\(^\text{11-20}\)

Natural substances of *I. obliquus* contain a very wide range of different compounds that are formed in the close interaction of birch and mushroom.\(^\text{21}\) These are pigments that form a chromogenic polyphenol-carboxylic complex exhibiting antitumor activity caused by the fact that phenolic compounds regulate the activity of cytoplasmic and mitochondrial ATPases and reduce the formation of ADP. Since magnified cells are more dependent on glycolysis than normal cells, the violation of this process negatively affects their development. Examples include pterins (pteridine derivatives, whose presence is due to the cytostatic effect of *I. obliquus*); polysaccharides (6–8%), agaricic and humin-like *I. obliquus* acids (up to 60%), organic acids (the total content of which is 0.5–1.3%; oxalic, acetic, formic, vanillic, lilac, n-oxybenzoic, as well as two triterpene acids from the group of tetracyclic triterpenes—inonotic and obliquinic), lipids (di- and triglycerides), steroid substances (sterols, ergosterol, as well as tetracyclic triterpenes such as lanosterol and inotodiol, which exhibits antiblastic activity), lignin, fiber, free phenols, flavonoids, coumarin peucedanin, cellulose, resins, trace amounts of alkaloids of unknown structure, ash (12.3%; which is rich in manganese and may be important for the therapeutic effect of *I. obliquus* as an enzyme activator), and other trace elements in the form of oxides such as copper, barium, zinc, iron, silicon, aluminum, calcium, and magnesium. Preparations of *I. obliquus* are widely used in medicine.\(^\text{22}\)

After extensive clinical and chemical studies, *I. obliquus* was approved for use by the Pharmacological Committee of the Ministry of Health of the USSR in 1955. Based on Pharmacopoeia Article FS 42-53-72, the finished raw material of the fungus *I. obliquus* has the following characteristics: 1) extractive substances not < 20%, 2) chromogenic complex not < 50% by weight of the total dry residue of extractive substances, 3) moisture not < 14%; and 4) ash not > 14%.

*I. obliquus* is available in the Russian pharmacy network in the form of crushed natural raw materials. Pieces of raw material should pass through a sieve with holes with a 7-mm diameter. The material color is dark brown and has no smell. Crushed *I. obliquus* is intended for use in the form of decoctions as a symptomatic remedy for chronic gastritis and dyskinesia of the gastrointestinal tract with the phenomena of atony, stomach ulcer, and oncological diseases.

Pharmacies also offer the drug Befungin, which presents a concentrated water extract of *I. obliquus* (1000 g) supplemented with cobalt sulfate heptahydrate (2 g), 95% ethyl alcohol (100 g), and purified water to obtain 1 liter of the drug. The pharmacological action of Befungin is determined by the effect of its constituent biologically active substances (polysaccharides, humin-like chagic acid, organic acids, trace elements, steroids, and other compounds). It regulates metabolic processes, increases the body’s defenses, and acts as a strengthening agent. Indications for use are as described above, including as a symptomatic remedy for chronic gastritis and dyskinesia of the gastrointestinal tract with the phenomena of atony, stomach ulcer, and oncological diseases.

In other countries, active studies of various *I. obliquus* compounds for anticancer, antioxidant, antiallergic, anti-inflammatory, immunomodulatory, and antiviral activities are also being conducted.\(^\text{23-25}\)

In connection with the current situation with COVID-19 worldwide, studies of biologically active compounds from fungi that are most effective against influenza viruses will be particularly relevant. Thus, our task was to study the antiviral properties of water extracts from pharmaceutical raw materials of *I. obliquus* against SARS-CoV-2 in cell culture experiments.

**II. MATERIALS AND METHODS**

**A. Preparation of Water Extracts from Pharmaceutical Raw Materials of *I. obliquus***

*I. obliquus* collected by procurers in western Siberia and Altai was used. Pharmaceutical raw materials of *I. obliquus* and distilled water were used. Five samples were obtained by processing the suspension under
different temperature modes (Table 1) and during different heating periods (from 1 to 72 h) as well as drying modes. In addition, three samples were ground to 1 mm in a MF-10 laboratory mill (IKA-WERKE, Germany), and one sample was ground in the RM-1 roller mill developed at the Institute of Solid-State Chemistry and Mechanochemistry of the Siberian Branch of the Russian Academy of Sciences. One sample was not crushed; it contained particles from 0.1 to 7.0 mm according to Pharmacopoeia Article FS 42-53-72. After cooling, the amount of dry matter was measured in 1 mL of the liquid sample with the conventional weight method.26

B. Determination of Antiviral Activity of Extracts against the SARS-CoV-2 nCoV/Victoria/1/2020 Strain in Vero E6 and Vero Cell Cultures

In this work, we used the continuous cultures of Vero E6 and Vero African green monkey kidney cells obtained from the cell culture collection of the FBRI SRC VB Vector of Rospotrebnadzor. The cell monolayer was grown in 96-well plates (0.1–0.15 mL/well of cell suspension with a concentration of 1.0–1.5 × 10⁴ cells/mL) in Dulbecco’s modified Eagle’s medium (DMEM) (BioloT, Russia) in the presence of 10% bovine embryonic serum (Gibco, USA) supplemented with penicillin (100 U/mL), streptomycin (100 U/mL), and amphotericin B (0.25 U/mL) (antibiotic-antimycotic, 100×; Gibco). The serum-free DMEM culture medium with antibiotics was used as a supporting medium to cultivate cells with the virus.

SARS-CoV-2 virus nCoV/Victoria/1/2020 strain with an infectious titer of 5.0 ± 0.29 (± 0.57) lg 50% tissue cytopathic dose (TCD₅₀)/mL was obtained from the State Collection of Pathogens of Viral Infections and rickettsioses of the Federal State Budgetary Institution SRC VB Vector of Rospotrebnadzor.

The virus concentration was determined by titration in Vero E6 or Vero cell culture incubated for 3 days at 37°C, 5% CO₂, and 85–90% humidity according to the cytopathic effect of the virus on the cell monolayer. The infectious titers were calculated with the Spearman–Kerber method, expressed in decimal logarithms of 50% TCD₅₀ in milliliters (lg TCD₅₀/mL) and presented as M ± m (± I₉₅) for the 95% confidence level.27,28

The colorimetric method was used to determine the cytotoxicity and antiviral activities of the preparations in vitro. Changes in the optical density (OD) of the dye solution absorbed by living cells in the monolayer fold dilutions of the preparations were used to evaluate the effectiveness of each preparation.29–31 The maximum concentration of extracts when assessing the cytotoxicity and antiviral activity of the preparations was 300 µg/mL.

### TABLE 1: Samples from the pharmaceutic chaga, Inonotus obliquus, prepared for study of its antiviral activity against the SARS-CoV-2 nCoV/Victoria/1/2020 strain

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Chaga particle size (mm)</th>
<th>Ratio of dry chaga to water (g:mL)</th>
<th>Chaga suspension treatment mode</th>
<th>Drying mode, temperature (°C)</th>
<th>Amount of dry matter in the test sample (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–13</td>
<td>1.0</td>
<td>20:200</td>
<td>50</td>
<td>50 ± 2</td>
<td>2</td>
</tr>
<tr>
<td>20–15</td>
<td>1.0</td>
<td>20:200</td>
<td>50</td>
<td>50 ± 2</td>
<td>2</td>
</tr>
<tr>
<td>20–16</td>
<td>70.0–80.0 µm</td>
<td>20:200</td>
<td>50</td>
<td>50 ± 2</td>
<td>2</td>
</tr>
<tr>
<td>20–17</td>
<td>0.1–7.0</td>
<td>200:1000</td>
<td>95</td>
<td>50 ± 2</td>
<td>2</td>
</tr>
<tr>
<td>20–26</td>
<td>1.0</td>
<td>20:200</td>
<td>Poured and kept at room temperature for 1 h</td>
<td>9.4</td>
<td></td>
</tr>
</tbody>
</table>

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To evaluate the antiviral activity of the preparations, 0.1 mL of dilutions of the extracts in DMEM (BioloT) was added into each well of 96-well plates with a monolayer of Vero E6 or Vero cells. After 2 h of incubation at 37°C and 5% CO₂, 0.1 mL of the virus dilution was added in serum-free DMEM with a multiplicity of infection (MOI) of 0.1 TCD₅₀/cell. With such an MOI, the cytopathic effect of the virus on the cell monolayer after 3 days of incubation reaches at least 90% (control of the virus without the preparation added). In addition, wells with an intact monolayer of cells (without the virus and preparations) were used as a “cell control.”

After 72 h of incubation after infection, 0.075 mL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, USA] in a phosphate-salt buffer with a concentration of 1 mg/mL was added to the wells of the tablet. After 1.5 h of incubation at 37°C, the dye was removed, the cells were lysed with dimethyl sulfoxide, and the OD was measured.

The OD was measured using a Multiskan FC platereader (ThermoScientific, USA). The results of OD measurement depending on the concentration of the preparation are presented in a semilogarithmic coordinate system (Fig. 1). The abscissa (X) axis shows the concentration of preparations in the logarithmic measurement scale, and the ordinate (Y) axis shows the OD in the linear measurement scale. Based on the OD values, the 50% toxic concentration (TC₅₀ in µg/mL) and the 50% inhibitory (effective) concentration (IC₅₀ in µg/mL) of the preparation were calculated using SoftMaxPro-4.0 software. TC₅₀ is the concentration of the preparation in the plate well which destroys 50% of cells in the monolayer. IC₅₀ is the concentration of the preparation that inhibits the virus replication and keeps 50% of the cells viable.

Based on these parameters, the selectivity index (SI) of the preparation was calculated as follows: SI = TC₅₀ / IC₅₀.

III. RESULTS

A. Samples from the Pharmaceutic of I. obliquus

Extracts for the study of antiviral activity against SARS-CoV-2, treatment modes, and concentrations are presented in Table 1.

FIG. 1: Antiviral activity of water extract 20–13 obtained from Inonotus obliquus against SARS-CoV-2 nCoV/Victoria/1/2020 strain (square) and its toxicity (circle) in Vero cell culture (SoftMax 4.0 program) when the extract was applied 2 h before infecting the monolayer. The abscissa axis is the concentration in micrograms per milliliter, and the ordinate axis is the optical density (OD).
B. Activity of *I. obliquus* Water Extracts against SARS-CoV-2

Table 2 shows the results on the effectiveness of the antiviral activity of *I. obliquus* water extracts against the coronavirus nCoV/Victoria/1/2020 strain. As can be seen from Table 2, sample 20–13 showed the best result on Vero cells, both in terms IC₅₀ (0.759 µg/mL) and the SI (155.5) values (Fig. 1). The sample was prepared by heating the aqueous suspension of *I. obliquus* at 50°C for 48 h, which corresponded to previous recommendations.

Samples 20–15, 20–16, and 20–17 also showed an antiviral effect. At the same time, in the Vero E6 cell line, we noted a tendency toward higher inhibition efficiency for samples 20–15 and 20–16 compared to that in the Vero cell line, with IC₅₀ values of 2.766 and 3.70 versus 5.979 and 5.578, respectively. The SIs for samples 20–15, 20–16, and 20–17 were also higher in the Vero E6 cell line than in the Vero cell line, with values of 108.5, 81.1, and 120 versus 16.73, 17.93, and 41.67, respectively.

Thus, the best samples to inhibit the replication of SARS-CoV-2 *in vitro* are *I. obliquus* samples in the form of aqueous solutions that have not been subjected to prolonged temperature exposure. This is sample 20–13, which was heated for 48 h at 50°C in the form of an aqueous suspension. The other is sample 20–17 whose aqueous suspension was heated on a water bath at 95°C for 3 h. This sample is a variant of the extract, which can be obtained even at home, without additional grinding of pharmaceutic natural raw materials of chaga for 3 h in a water bath.

Samples that were heated longer did not show any advantage (samples 20–15 and 20–16 were prepared for 72 h at 50°C). Befungin obtained by extraction with heated water (68–72°C) in three reactors and mixing with cobalt salts and alcohol showed weak or no activity.

IV. DISCUSSION

The studies conducted according to the preventive scheme show that water extracts of the mushroom *I. obliquus* are characterized by low toxicity in Vero and Vero E6 cell cultures and have antiviral activity against SARS-CoV-2.

Water extracts of *I. obliquus* obtained by processing at a temperature of 50–95°C for 1–72 h had an inhibitory concentration, which in the range of 0.75–11.6 µg/mL shows 50% antiviral activity against SARS-CoV-2.

### TABLE 2: Toxicity and antiviral activity of water extracts of *Inonotus obliquus* against SARS-CoV-2 using the “preventive” scheme

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample code</th>
<th>Dry matter mass (mg/mL)</th>
<th>Substance</th>
<th>Application scheme/3-day cell culture</th>
<th>TC₅₀ (µg/mL)</th>
<th>IC₅₀ (µg/mL)</th>
<th>SI (TC₅₀/IC₅₀)</th>
</tr>
</thead>
</table>
One of the properties of *I. obliquus* is its ability to improve blood rheology. This is especially important for the treatment of COVID-19 infection because of the threat of thrombosis. A study conducted by Korean scientists described the extraction and characterization of a peptide from *I. obliquus* that inhibits platelet aggregation, and the results showed that the ethanol extract of mycelium *I. obliquus* AS1 74006 exhibited the highest inhibitory activity against platelet aggregation (81.2%). The purified platelet aggregation inhibitor presents a novel tripeptide with a molecular weight of 365 Da, which carries the Trp-Gly-Cys sequence and high inhibitory activity against platelet aggregation in mice.

Since *I. obliquus* contains humic substances and melanin involved in their creation, the mechanism of action of *I. obliquus* extracts may be related to their sorption capacity and direct effect on pathogenic organisms, including viruses, bacteria, and fungi.

### V. CONCLUSIONS

As a result of the *in vitro* studies conducted, the aqueous extracts of *I. obliquus* were shown to have high antiviral activity against SARS-CoV-2. Under optimal preparation conditions, water extracts of chaga showed an IC₅₀ of 0.75 μg/mL for SARS-CoV-2 replication with low toxicity and a high SI of 155.5.

*I. obliquus* contains melanin and humic substances. Perhaps this is a key factor in the effectiveness of aqueous chaga extracts against coronavirus.

Considering the results obtained on antiviral activity, low toxicity, the ability to improve blood rheology in relation to platelet aggregation, and its natural origin, aqueous extracts of the chaga mushroom, *I. obliquus*, can be considered for use as a therapeutic and preventive drug against COVID-19.

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