

SARS-CoV-2 CORONAVIRUS REPLICATION INHIBITOR BASED ON HUMIC SUBSTANCES

Abstract

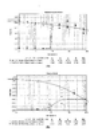
FIELD: pharmaceutical industry.

SUBSTANCE: group of inventions relates to the pharmaceutical industry, namely to the use of water-soluble humic substances from brown coal or from chaga to inhibit the replication of the SARS-CoV-2 coronavirus. The use of water-soluble humic substances from brown coal obtained as a result of the exothermic reaction of crushed brown coal in a mixture of 2% ammonia and hydrogen peroxide, followed by precipitation and re-precipitation of humic substances with hydrochloric acid, dilution in water at pH 7.0-8.0, drying, with or without trypsin treatment, to inhibit the replication of the SARS-CoV-2 coronavirus, which, at concentrations in the range of 14.34-23.1 mcg/ml, exhibit a 50% antiviral dose-dependent activity against SARS-CoV-2 in tests on Vero cell cultures. The use of water-soluble humic substances from the chaga, basid fungus *Inonotus obliquus*, obtained as a result of the exothermic reaction of crushed chaga in a mixture of 2% ammonia and hydrogen peroxide, followed by precipitation and re-precipitation of humic substances with hydrochloric acid, dilution in water at pH 7.0-8.0, drying, treatment with trypsin, to inhibit the replication of the SARS-CoV-2 coronavirus, which at a concentration of 23.447 mcg/ml exhibit 50% antiviral dose-dependent inhibitory activity against SARS-CoV-2 in Vero cell culture tests.

EFFECT: above-described use of humic substances is effective for inhibiting the replication of the SARS-CoV-2 coronavirus.

2 cl, 1 dwg, 1 tbl, 8 ex

Images (1)



Classifications

■ **A61K35/10** Peat; Amber; Turf; Humus

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Claims (2)

Hide Dependent ^
translated from Russian

1. The use of water-soluble humic substances from brown coal, obtained as a result of the exothermic reaction of crushed brown coal in a mixture of 2% ammonia and hydrogen peroxide, followed by precipitation and re-precipitation of humic substances with hydrochloric acid, dilution in water at pH 7.0-8.0, drying, with or without trypsinization, to inhibit the replication of SARS-CoV-2 coronavirus, which at a concentration in the range of 14.34-23.1 $\mu\text{g} / \text{ml}$ exhibit 50% antiviral dose-dependent activity against SARS-CoV-2 in tests for cell cultures Vero. 2. The use of water-soluble humic substances from chaga - the basid fungus *Inonotus obliquus*, obtained as a result of the exothermic reaction of crushed chaga in a mixture of 2% ammonia and hydrogen peroxide, followed by precipitation and re-precipitation of humic substances with hydrochloric acid, dilution in water at pH 7.0-8, 0, drying, treatment with trypsin to inhibit the replication of SARS-CoV-2 coronavirus, which at a concentration of 23.447 $\mu\text{g} / \text{ml}$ exhibit 50% antiviral inhibitory dose-dependent activity against SARS-CoV-2 in tests on Vero cell cultures.

Description

translated from Russian

The present invention relates to a replication inhibitor of the novel SARS-CoV-2 coronavirus based on humic substances of natural origin.

The outbreak of the Covid-19 pandemic, which began at the end of 2019 in China and spread throughout the world, poses a threat to humanity, so the search for substances that inhibit the replication of the virus is relevant for the creation of new drugs.

Humic substances (HS) are a necessary and indispensable part of all metabolic processes in the biosphere; they are high molecular weight heteropolymers and are part of natural objects. All humic substances are formed from organic residues. This process is called humification. One of the most probable mechanisms of humification in the biosphere can be recognized as the biosynthesis of melanins by bacteria and fungi - precursors of humic substances [Popov A.I. Humic substances: properties, structure, education / Ed. E.I. Ermakova. - SPb.: Publishing house of St. Petersburg. University, 2004. - 248 p.].

It is known that the chaga mushroom *Inonotus obliquus*, growing on live birches, contains not only melanin, but also humic substances in black growths (sclerotia), which largely determine the medicinal properties of this mushroom [Shivrina A.N. Chemical characteristics of the active principles of chaga // Products of biosynthesis of higher fungi and their use. M-L., Science, 1966. - S. 49-56; Shashkina N.Ya., Shashkin P.N., Sergeev A.V. Chemical and medico-biological properties of chaga (review) // Chemical and pharmaceutical journal. - 2006. - T. 40. - No. 10. - S. 37-44].

The main part of humic substances is resistant to biochemical degradation, so they accumulate in the soil, and are also part of peat, brown coal, sapropel. Humic substances are found almost everywhere in nature. Their content in sea waters is 0.1-3 mg / l, in river waters - 20 mg / l, and in swamps - up to 200 mg / l. In soils, humic substances are 1-12%, while most of them are in chernozems. The leaders in the content of these compounds are organogenic rocks, which include coal, peat, sapropel, and oil shale. Usually humates are obtained from oxidized brown coal (it is also called leonardite), because it contains up to 85% of humic substances. This coal is also convenient because it has a low calorific value, so it is usually raked into dumps. It turns out that the main source of humic substances is waste from brown coal mining,

RU2752872C1

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Inventor: [Тамара Владимировна Теплякова, Олег Викторович Пьянков, Максим Олегович Скарнович, Николай Иванович Бормотов, Алевтина Леонидовна Потешкина, Алена Сергеевна Овчинникова, Анастасия Викторовна Магеррамова, Екатерина Игоревна Филиппова, Анастасия Алексеевна Черемискина](#)

Worldwide applications

2020 [RU](#)

Application RU2020136343A events 

2020-11-03	Application filed by Федеральное бюджетное учреждение науки "Государственный научный центр вирусологии и биотехнологии "Вектор" Федеральной службы по надзору в сфере защиты прав потребителей и благополучия человека (ФБУН ГНЦ ВБ "Вектор" Роспотребнадзора)
2020-11-03	Priority to RU2020136343A
2021-08-11	Application granted
2021-08-11	Publication of RU2752872C1

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and this fully corresponds to the basic principles of "green chemistry". The reserves of brown coal in the world exceed 1 trillion tons [Perminova I.V. Humic substances - a challenge to chemists of the XXI century // Chemistry and Life. - 2008. - 1. - S. 50-55].

Humification is the process of converting organic residues into humic substances, scientists consider one of the greatest inventions of nature. The nature of the mineralization is partially residues, CO₂ is returned to the atmosphere, water - plants. The multiplicity of functions of humic substances is due to their diversity in composition, molecular weights, and properties. The presence of various functional groups in HS determines, firstly, the participation of these compounds in chemical oxidation-reduction reactions, and, secondly, the reactivity in general, including ion exchange and the formation of chelate compounds. As colloids, HS exhibit surface-active and electro-surface properties [Orlov D.S. Humic substances in the biosphere. Soros Educational Journal. - 1997. - No. 2 - S. 56-63].

Currently, both in Russia and abroad, dietary supplements based on humic substances are widely used: "Gumivit", "Purified Mumiyo Altai", "Gimilan", "Fulvomil" (Russia), "Oxyhumatek" (South Africa), "Avirol"™ (USA), Humic & Fulvic Acid (USA), VitalityBoostHA (USA), ColloidalMineralsXLwithFulvicAcid (Australia), RF2 LiquidPlantMinerals (Canada). Scientists are studying the pharmacological properties of humic compounds in order to develop drugs based on them. The development of methodological foundations for the standardization of humic substances in the row "raw material - substance - preparation" is an urgent task. [Savchenko I.A. Dornieva I.N., Luksha E.A., Pasechnik K.K. Biological activity of humic substances: prospects and problems of their application in medicine (review). // Medial, 2019. - №1 (23). - S. 54-60].

Several groups of humic substances are known: humic acids, which are soluble only in alkaline solutions; humatmelanic acids extracted from the crude residue (gel) of humic acids with ethyl alcohol; fulvic acids, soluble in water, alkaline and acidic solutions; humin is a practically insoluble and non-recoverable substance.

Irregular structure, heterogeneity of structural elements and polydispersity are characteristic of humic acids. The structural units of humic acids are aromatic condensed systems with side chains and heterocycles. Functional groups are carboxyl, carbonyl, phenolic and alcohol hydroxides, quinoid groups, methoxyls, amino and amido groups, mono-, di-, polysaccharides, peptides, mineral components. The biological activity of humic acids may be due to the presence of various functional groups in these compounds; colloidal properties and component composition. Humic acids bind metal ions and organic toxicants in water and soil into strong complexes. The related substances are not as dangerous as they lose their bioavailability. Humic acids have a high sorption capacity. Adsorption by humic acids includes not only physical, but also chemical interactions.

Humic substances from sea and lake bottom sediments and water were fractionated into humic acids and fulvic acids. The antimicrobial effect of 12 preparations of these compounds was established against 11 strains of anaerobic bacteria and 8 strains of aerobic bacteria, as well as 2 strains of yeast-like fungi. This indicates the participation of humic compounds in the self-purification of sea and lake waters [Kupryszewski G., Pempkowiak J., Kedzia A. The effect of humic substances isolated from a variety of marine and lacustrine environments on different microorganisms // Oceanologia, 43 (2), 2001. - 3. pp. 257-261].

The American company LaubBioChemicalsCorp. (Newport Beach, California) was founded in 1996 by Dr. R. J. Laub, primarily to develop synthetic and natural humic compounds of the HEPsYL trademark for the treatment of HIV infection and other viral diseases. A high degree of inhibition of viruses such as HSV-1 and HSV-2 (herpes virus types 1 and 2, US patent 6,524,566, 2003), influenza viruses (type A and B, patent No. US 6,576,229, 2003) has been shown.), hemorrhagic fever virus (US patent 6,524,567, 2003), HIV virus - human immunodeficiency virus (US patent 6,534,049, 2003). Known antiviral agent based on humic acids obtained from medical lignin (brand "Polypepan", reg. N 80/1211/3 - nonspecific enteral sorbent) by oxidation at 150-170 ° C for 0.5-1.5 hours and a pressure of 2.0-2.5 MPa [RF patent No. 2172176, IPC A61K 35/78, publ. 08/20/2001]. The drug is effective against herpes simplex virus type 2, influenza type A virus (PR-8), and Rous sarcoma virus BH-RSV (RAV-I) serological subgroup A.

Humic substances, being heteropolymers, can serve as a source of structural fragments of organic macromolecules in biosynthesis occurring in living organisms [Al Popov, V.N. Zelenkov, T.V. Teplyakova. Biological activity and biochemistry of humic substances. Part 1. Biochemical aspect (literature review) // Bulletin of the Russian Academy of Natural Sciences, 2016. - №1. - S. 11-18].

It is known that humic substances have a wide spectrum of therapeutic action: adaptogenic, antitoxic, hepatoprotective, antimicrobial, diuretic and anti-inflammatory action [Popov A.I., Zelenkov V.N., Teplyakova T.V. Biological activity and biochemistry of humic substances. Part 2. Biomedical aspect. Literature review) // Bulletin of the Russian Academy of Natural Sciences, 2016. - No. 5. - S. 9-15.

It has been shown that humic acids have antiviral activity against human immunodeficiency viruses [Kornilaeva G.V., Perminova I.V., Gilyazova A.V. et al. Humic substances as promising compounds for the creation of microbicidal preparations // Russian Journal of Immunology. 2010. T. 4 (13), No. 3. S. 255-260]. The most active fractions of humic compounds had a pronounced antiviral activity against the pandemic influenza virus A / California / 04/09 (H1N1 pdm09): ID50 = 3.75 Mg / ml, IS = 266; highly pathogenic avian influenza virus A / Commongull / Chany / 06 (H5N1): ID50 = 9.5 Mg / ml, IS = 105; against Herpes simplex virus (HSVII): ID50 = 3.5 Mg / ml, IS = 285; against Human immunodeficiency virus I (HIVI): ID50 = 2.7 Mg / ml, IS = 370. [Ilycheva T.N., Balakhnin S.M., Gashnikova N.M., Durymanov A.G., Ananko G.G., Kosogova T.A., Miloshenko T.P., Redkin B.A., Teplyakova T.V. Antiviral Activity of Humic Substances // Third International Conference of CISIHSS on Humic Innovative Technologies Tenth International Conference da Rostim "Humic Substances and Other Biologically Active Compounds in Agriculture) EQT-daRostim-2014 November 19-23, 2014, Lomonosov Moscow State University, Moscow, Russia. P. 42].

Known use of natural humic acids isolated from the soil for the treatment of HIV infection [US application No. 20040137085, IPC A61K 35/78, publ. July 15, 2004]. The drug is manufactured by the American company Aldrich Chemical Company and is referred to in the application as AVC. Suppression of HIV infection occurs when the leukocytes of an HIV-infected person come into contact with a preparation of humic acid. Also claimed is a method of immunostimulation of interleukin-2, a method of enhancing the immune response to vaccination using humic acid as an adjuvant when vaccinating a patient with HIV infection.

However, this US application considers the use of humic acids only for the treatment of HIV infection and there is no data on their use for the treatment of other viral diseases.

Known agent against sexual transmission of HIV / AIDS, made in the form of a suppository containing a pharmacologically acceptable base and an active substance (RF patent No. 2531945, IPC A61K 35/02, publ. 27.07.2014). A fraction of humic acids isolated from oxidized brown coal is used as an active substance with anti-HIV activity, and cocoa butter or hard confectionery fat and an emulsifier are used as a base. Isolation of the fraction of humic acids (HAC-1) is carried out by treating brown coal with a 1% sodium hydroxide solution (NaOH) and heating the mixture for 2 hours in a boiling water bath (at a temperature of 80 ° C). After cooling, the reaction products are centrifuged, the solution is decanted, the insoluble residue is washed once or twice with 100 ml of 1% sodium hydroxide solution and centrifuged again, collecting the main extract and washings in one receiver. To the resulting solution is added a solution of concentrated hydrochloric acid to pH 2 to precipitate humic acids. The formed precipitate of humic acids is separated by centrifugation and desalted by dialysis. The resulting preparation of humic acids is dried at 80 ° C in a drying oven.

Known patent for an antiviral agent based on humic acids obtained from oxidized brown coal [Teplyakova T.V., Ananko G.G., Kazachinskaya E.I., Nosik N.N., Nosik D.N., Lobach O.A., Kiseleva I.A., Antiviral agent based on humic acids: Pat. 2678986 C1 Rus. Federation. No. 20118110484, app. 03/23/2018; publ. 02/05/2019, Bul. No. 4]. The agent has activity against the influenza A / H1N1 / California / 2009 virus (at a concentration of 3-4 µg / ml); against herpesvirus type 1 (at a concentration of 45-55 µg / ml); herpes virus type 2 (at a concentration of 100-150 µg / ml); West Nile virus (at a concentration of 4.2-5.4 µg / ml); human immunodeficiency virus (at a concentration of 45-55 µg / ml). The technical result of this invention is to obtain a preparation of humic acids from oxidized brown coal with a lower content of toxic substances and a wider spectrum of antiviral action against viruses pathogenic for humans.

However, the activity against the SARS-CoV-2 coronavirus of this agent based on humic acids has not been tested.

Natural compounds are known such as flavonoids [https://bioflavit.ru/wp-content/uploads/Taxifolin-basel-rus2.pdf] that inhibit the main protease SARS-CoV-1, and their effectiveness has been demonstrated using resonance transfer fluorescence energy (FRET) [Pillaiyar, T.; Manickam, M.; Namasivayam, V.; Hayashi, Y.; Jung, S. H. An

over view of severe acute respiratory syndrome - coronavirus (SARS-CoV) 3CL protease inhibitors: Peptidomimetic and small molecule chemotherapy. Journal of Medicinal Chemistry 2016, 59, 6595-6628.].

In addition, in vivo experiments, the bioflavonoid (-) taxifolin (dihydroquercetin) is active against other viruses, such as the Coxsackie B4 virus. It is known that Siberian larch (*Larix sibirica*) produces (-) - taxifolin, which is a natural resource for its extraction. In addition, food preparations containing (-) - taxifolin are readily available from pharmacies and some companies, providing direct and quick access to a potential antiviral drug. It is noteworthy that (-) - taxifolin carries seven hydrogen bonds, which is the highest in the choice of antiviral compounds. Since hydrogen bonds are a key factor determining the specificity of a drug (-) - taxifolin can be a natural alternative to the proposed CP-1-CP-11 inhibitors [Wade, R.C.; Goodford, P.J. The role of hydrogen-bonds in drug binding. Progress in clinical and biological research. 1989, 289, P. 433-444].

It is also known that the heparin contained in algae is able to block the SARS-CoV-2 coronavirus better than, for example, the drug Remdesivir, which is actively used in the United States to treat COVID-19 [Sulfated polysaccharides effectively inhibit SARS-CoV-2 in vitro / Paul S. Kwon, Hanseul Oh, Seok-Joon Kwon, Weihua Jin, Fuming Zhang, Keith Fraser, Jung Joo Hong, Robert J. Linhardt & Jonathans. Dordick. Cell Discovery, volume 6, Article number: 50 (2020)]. Scientists have purposefully investigated the properties of heparin, an anticoagulant known to all. This substance has been proven to have exceptional binding properties to the spike protein (S-protein) of SARS-CoV-2 and reduces the activity of this virus.

The closest analogue for the intended purpose (prototype) is a SARS-CoV coronavirus replication inhibitor based on an aqueous plant extract exhibiting dose-dependent activity against SARS-CoV in cell culture tests [US application No. 20080038382, IPC A61P 31/12, publ. February 14, 2008]. As an aqueous plant extract, it contains an aqueous extract of the perennial herb *Scutellaria* spp. (a species of the *Shlemnik* genus) effective for treating a patient with SARS-CoV infection. This agent inhibits the infectivity of SARS-CoV by about 50% at the highest concentration used (200 µg / ml). The effect is dose dependent, given that less inhibition is observed at the lower dose tested. The significantly higher inhibition was greater than ribavirin (100 µg / ml).

However, this closest analogue, the inhibitor of the SARS-CoV coronavirus, has not been tested for activity against the novel SARS-CoV-2 coronavirus.

The technical result of the claimed invention is to obtain a new natural inhibitor of the replication of the coronavirus SARS-CoV-2 based on humic substances of natural origin and to expand the range of drugs against said coronavirus.

The specified technical result is achieved by the fact that in the inhibitor of coronavirus replication based on water-soluble substances of natural origin, exhibiting dose-dependent antiviral activity in tests on cell cultures, according to the invention, as water-soluble substances of natural origin, it contains water-soluble humic substances obtained from brown coal, or the basinal fungus *Inonotus obliquus*, or a 1: 1 mixture of humic substances from brown coal and melanin obtained from the basinal fungus *Inonotus obliquus*, the inhibitory aqueous solution of which in the range 8.279-23.447 µg / ml exhibits 50% antiviral dose-dependent activity against SARS-CoV-2 in Vero cell culture tests.

The invention is illustrated by the drawings shown in FIG. 1, A, B - OD measurement results (respectively for samples 20-51, 20-55 of the inhibitor), depending on the concentration of the drug, are presented in a semi-logarithmic coordinate system. The abscissa (X) shows the concentration of drugs in a logarithmic scale, and the ordinate (Y) shows OD in a linear scale.

Below are the options for obtaining the claimed inhibitor of replication of the coronavirus SARS-CoV-2 based on a water-soluble fraction of humic substances of natural origin.

Example 1 Obtaining a water-soluble fraction of humic substances from brown coal, sample 20-50.

The sample was prepared according to the patent of Teplyakova T.V. et al. (RU 2678986 C1, 2019), but without trypsin treatment.

Brown coal was taken from the TPP-5 in Novosibirsk, brought from the Borodinsky mine named after M.I. Shchadova, supplier - Joint Stock Company SUEK-Krasnoyarsk.

To 25 g of brown coal (grinding 0.25 mm on a universal mill MF-10 basik IKA) add 50 ml of 2% ammonia and 40 ml of hydrogen peroxide (29-32% GOST 10929-76, rev. No. 1.2.). The mixture is stirred until the exothermic reaction is complete and the foam is completely precipitated, centrifuged in a Centra CL3 centrifuge for 20 min at 4000 rpm, humic substances are precipitated with hydrochloric acid, successively re-precipitated five times. The precipitate is diluted in a small amount of distilled water and the pH is adjusted to a value of 7-8 units. Then it is dried at + 30 ° C. The sample for testing contains humic substances in an amount of 2 mg in 1 ml of sterile water.

An inhibitor was obtained containing an aqueous solution of humic substances, having an inhibitory dose at a concentration of 23.1 µg / ml, providing 50% antiviral protection in tests on Vero cell culture against SARS-CoV-2 coronavirus.

Example 2. Obtaining a mixture of humic acid from brown coal and melanin from natural raw chaga, sample 20-51.

For this, 10 mg of humic acid (IC₅₀ = 14.34 µg / ml) and 10 mg of melanin from chaga (IC₅₀ = 8.188 µg / ml) are mixed in a 1: 1 ratio and dissolved in 10 ml of sterile water. The sample for testing contains a mixture of humic substances (humic acid and melanin) in the amount of 2 mg in 1 ml of sterile water.

An inhibitor was obtained containing an aqueous solution of a mixture in a 1: 1 ratio of humic acid from brown coal and melanin from chaga, having an inhibitory dose at a concentration of 8.279 µg / ml, providing 50% antiviral protection in Vero cell culture tests against SARS-CoV coronavirus -2.

Example 3. Biologically active food additive (BAA) Gimilan, sample 20-53.

Biologically active food additive (BAA) Gimilan is produced in Biotechnology LLC (660052 Krasnoyarsk, Zatonskaya str. 46 g). Registration certificate No. RU.77.99.11.003.E.003421.03.14; composition: a complex of humic and fulvic acids from brown coal. Produced on the basis of technical specifications TU-9197-003-38605220-14. Date of registration: 26.03.2014.

The drug Gimilan in liquid form in an amount of 40 ml is dried at + 30 ° C. The test specimen contains 2 mg in 1 ml of sterile water.

An inhibitor was obtained containing an aqueous solution of humic substances, having an inhibitory dose at a concentration of 23.292 µg / ml, providing 50% antiviral protection in tests on Vero cell culture against SARS-CoV-2 coronavirus.

Example 4. Obtaining a water-soluble fraction of humic acids from brown coal with trypsin treatment to reduce toxic substances, sample 20-55.

The sample was prepared according to the patent of Teplyakova T.V. et al. (RU 2678986 C1, 2019). Brown coal was delivered from the Krasnoyarsk Territory from the Borodinsky open-pit mine. M.I. Shchadova, supplier - Joint Stock Company SU EK-Krasnoyarsk.

To 25 g of brown coal (grinding 0.25 mm on a universal mill MF-10 basik IKA) add 50 ml of 2% ammonia and 40 ml of hydrogen peroxide (29-32% GOST 10929-76, rev. No. 1.2.). The mixture is stirred until the exothermic reaction is complete and the foam has completely precipitated. The product is centrifuged in a Centra CL3 centrifuge for 20 min at 4000 rpm, humic substances are precipitated with hydrochloric acid, followed by reprecipitation five times. The precipitate is diluted in a small amount of distilled water and the pH is adjusted to a value of 7-8 units. The drug is dried at + 30 ° C.

In order to reduce toxic substances, the drug is further treated with trypsin. For this, 1 g of dry humic acids is dissolved in 20 ml of sterile water, 1 ml of Tris base (TU 6.09-42-92-76 XY 1M 1.21 g in 10 ml) and 30 µl of trypsin (Trypsin-EDTA (0.05% o) in DPBS (Ix) produced by CAPRICORN). The mixture is kept for 5 hours in a thermostat

at + 37°C. Then humic substances are precipitated with hydrochloric acid, centrifuged in a centrifuge (tabletop, Centra CL3) for 20 minutes at 4000 rpm, pH is adjusted to 7-8 units. and dried at + 30 ° C. The test specimen contains 2 mg in 1 ml of sterile water.

An inhibitor was obtained containing an aqueous solution of humic substances, having an inhibitory dose at a concentration of 14.34 µg / ml, providing 50% antiviral protection in tests on Vero cell culture against SARS-CoV-2 coronavirus.

Example 5. Obtaining humic acids from pharmacy chaga, sample

20-61. (The sample was prepared according to the patent of Teplyakova T.V. et al., RU 2678986 C1, 2019). Add 100 ml of 2% ammonia and 80 ml of hydrogen peroxide (29-32%) GOST 10929-76, rev. No. 1.2.). After the completion of the exothermic reaction and complete precipitation of the foam, the mixture is filtered through two nylon filters, humic acids are precipitated with hydrochloric acid, and successively re-precipitated five times. The precipitate is diluted in a small amount of distilled water and the pH is adjusted to a value of 7-8 units. followed by drying at + 30 ° C.

Next, take 2.5 g of the obtained dry humic acids from chaga, dissolve in 50 ml of sterile water, add 2.5 ml of Tris base (TU 6.09-42-92-76 XY 1M 1.21 g in 10 ml) and 75 µl of trypsin (Trypsin-EDTA (0.05% o) in DPBS (ix) manufactured by CAPRICORN). The mixture is kept for 5 hours in a thermostat at + 37°C and humic compounds are precipitated with hydrochloric acid. The precipitate is centrifuged in a centrifuge (benchtop Centra CL3) for 20 min at 4000 rpm, the pH is adjusted to 7-8 units. and dried at + 30 ° C. The test specimen contains 2 mg in 1 ml of sterile water.

An inhibitor was obtained containing an aqueous solution of humic substances in the form of acids, having an inhibitory dose at a concentration of 23.447 µg / ml, providing 50% antiviral protection in tests on Vero cell culture against SARS-CoV-2 coronavirus.

Example 6. Determination of the antiviral activity of samples against SARS-Cov-2 coronavirus in Vero cell cultures.

Cell cultures. In the work, we used transplantable cultures of kidney cells of the African green monkey Vero, obtained from the collection of cell cultures of the FBSI SSC VB Vector. A monolayer of cells was grown in 96-well plates (0.1-0.15 ml / well of cell suspension with a concentration of $1.0-1.5 \times 10^5$ cells / ml) in DMEM medium (OOO Biolot, Russia) in the presence of 10% fetal bovine serum (Gibco, USA) with the addition of penicillin (100 U / ml), streptomycin (100 µg / ml) and amphotericin B (0.25 µg / ml) (Antibiotic-Antimycotic (100X) (Gibco, USA) When cells with a virus were cultured, DMEM culture medium with antibiotics, without serum, was used as a maintenance medium.

The SARS-CoV-2 virus strain nCov / Victoria / 1/2020 with an infectious titer of 5.0 ± 0.29 lg TCD₅₀ / ML was obtained from the State collection of causative agents of viral infections and rickettsioses of the FBSI SSC VB "Vector".

The virus concentration was determined by titration in a Vero cell culture during cultivation for 3 days at 37 ° C, 5% CO₂ and humidity 85-90%. Virus titers were calculated by the Spearman-Kärber method, expressed in decimal logarithms of 50% tissue cytopathic doses in ml (lg TCD₅₀ / ML) and presented as $M \pm m$ (± 195) for a 95% confidence level (195) [Sachs L. Statistical estimation. M.: Statistics. 1976; 598 s; Virology Methods Manual. Edited by: Brian WJ Mahy and Hillar O. Kangro. Academic Press. 1996; 374 p.].

Colorimetric method for in vitro determination of cytotoxicity and antiviral activity of drugs.

The study of cytotoxicity and antiviral efficacy of drugs was carried out by the colorimetric method by changing the optical density (OD) of a dye solution absorbed by living cells in a monolayer [Oestereich L., Ltadtke A., Wurr S., Rieger T., Munoz-Fontela C, Gtinter S. Successful treatment of advanced Ebola virus infection with T-705 (favipiravir) in a small animal model // Antiviral Research, 2014., 105: 17-21; Baker R.O., Bray M., Huggins J.W. Potential antiviral therapeutics for smallpox, monkey pox and other orthopox virus infections // Antiviral Research., 2003., 57: 13-23; Paragas J., Whitehouse C. A., Endy T. P., Bray M. A simple assay for determining antiviral activity against Crimean-Congo hemorrhagic fever virus // Antiviral Research. 2004, 62: 21-25].

To evaluate the effectiveness of each, 8 consecutive 3-fold dilutions of the preparations were used. The initial concentration of extracts on the plates when assessing the cytotoxicity and antiviral activity of the preparations was 300 µg / ml.

When evaluating the antiviral activity of the preparations to the wells of 96-well plates with a monolayer of Vero cells was added 0.1 ml of a dilution of extracts in DMEM medium (OOO "Biolot", Russia), and after 2 hours of incubation at 37 ° C, 5% CO₂ was introduced 0.1 ml of virus dilution in serum-free DMEM medium with a multiplicity of infection (MOI) 0.1 TCD₅₀ / cell. With such a multiplicity of infection, the cytopathic effect of the virus on the cell monolayer after 3 days of incubation reaches at least 90% (control of the virus without adding the drug). In addition, an intact monolayer (without virus and drugs) was used as a "cell control".

After 3 days of incubation in the wells of the plate with a monolayer of Vero cells in the culture medium was added a vital (intravital) dye neutral red 0.05 ml for 1.5 hours at 37 ° C. After that, the cell monolayer was washed twice with saline solution, lysis buffer was added, and after 30 min the optical density (OD) was determined.

Optical density was measured using a Multiskan FC plate reader (ThermoScientific, USA). The results of measuring OD depending on the concentration of the drug are presented in a semilogarithmic coordinate system (Fig. 1, A, B). In this case, the abscissa axis (X) shows the concentration of drugs in a logarithmic measurement scale, and the ordinate axis (Y) - OD in a linear measurement scale. The OD values were used to calculate the 50% toxic concentration (TC₅₀ in µg / ml) and 50% inhibitory (effective) concentration (IC₅₀ in µg / ml) of the preparation using the SoftMaxPro-4.0 software. TC₅₀ is the value of the concentration of the drug in the well of the plate, destroying 50% of the cells in the monolayer. IC₅₀ is the concentration of drug that inhibits viral replication and keeps 50% of cells viable.

Based on these indicators, the selectivity index (SI) of the drug was calculated: $SI = TC_{50} : IC_{50}$ [Guidelines for experimental (preclinical) study of new pharmacological substances. Ed. Khabrieva R.U. M.: JSC "Publishing House" Medicine ". 2005; 832 s; Methodical recommendations for the study of the specific activity of interferon inducers. Guidelines for conducting clinical trials of medicinal products. Part One / Ed. A.N. Mironov. - M.: Grif and K, 2012. - 944 s].

Example 8. Results of determining the antiviral activity of water-soluble fractions of humic substances against SARS-Cov-2 coronavirus on Vero cells.

Table 1 shows the results of evaluating the antiviral activity of humic substances against coronavirus, strain nCov / Victoria / 1/2020.

The best samples for inhibition of coronavirus replication, strain nCov / Victoria / 1/2020, in Vero cell culture are samples: 20-51 and 0-55. Sample 20-51 is a mixture of humic acid from brown coal with melanin from chaga. Perhaps there is a synergistic effect here.

Samples 20-50, 20-53 and 20-61 have similar results (IC₅₀ is 23.1; 23.292 and 23.447 µg / ml, respectively). These drugs have low toxicity and high activity against the SARS-Cov-2 coronavirus.

Таблица 1 – Противовирусная активность гуминовых веществ в отношении коронавируса SARS-CoV-2 (штаммnCov/Victoria/1/2020)

№ образца	Код образца	Масса сухого вещества, мг/мл	Вещество	Схема применения/культура клеток	TC ₅₀ , мкг/мл	IC ₅₀ , мкг/мл	SI (TC ₅₀ /IC ₅₀)
1	20-50	2,0	Гуминовая кислота по патенту RU 2678986 (без трипсина)	07.09.2020 Профилактическая Vero,3 сут.	>666,7	23,1	28,86
2	20-51	2,0	Гуминовая кислота из бурых углей с меланином из чаги	07.09.2020 Профилактическая Vero,3 сут	>666,7	8,279	80,53
3	20-53	2,0	Гуминовое вещество, БАД Гимилан	15.09.2020 Профилактическая Vero,4сут	495,0	23,292	21,3
4	20-55	2,0	Гуминовая кислота по патенту RU 2678986	15.09.2020 Профилактическая Vero,4сут	589,6	14,34	41,11
5	20-61	2,0	Гуминовое кислота по патенту RU 2678986	15.09.2020 Профилактическая Vero,4 сут	618,0	23,447	26,36

Таблица 1 – Противовирусная активность гуминовых веществ в отношении коронавируса SARS-CoV-2 (штаммnCov/Victoria/1/2020)

№ образца	Код образца	Масса сухого вещества, мг/мл	Вещество	Схема применения/культура клеток	TC ₅₀ , мкг/мл	IC ₅₀ , мкг/мл	SI (TC ₅₀ /IC ₅₀)
1	20-50	2,0	Гуминовая кислота по патенту RU 2678986 (без трипсина)	07.09.2020 Профилактическая Vero,3 сут.	>666,7	23,1	28,86
2	20-51	2,0	Гуминовая кислота из бурых углей с меланином из чаги	07.09.2020 Профилактическая Vero,3 сут	>666,7	8,279	80,53
3	20-53	2,0	Гуминовое вещество, БАД Гимилан	15.09.2020 Профилактическая Vero,4сут	495,0	23,292	21,3
4	20-55	2,0	Гуминовая кислота по патенту RU 2678986	15.09.2020 Профилактическая Vero,4сут	589,6	14,34	41,11
5	20-61	2,0	Гуминовое кислота по патенту RU 2678986	15.09.2020 Профилактическая Vero,4 сут	618,0	23,447	26,36

Patent Citations (4)

Publication number	Priority date	Publication date	Assignee	Title
US20080038382A1 *	2004-02-27	2008-02-14	Shouming Zhong	Extracts of Scutellaria for the Treatment of Sars
RU2531945C2 *	2013-01-25	2014-10-27	Федеральное государственное бюджетное учреждение "Государственный научный центр "Институт иммунологии" Федерального медико-биологического агентства	Medication against hiv/aids transmission through sexual contact
RU2618398C1 *	2016-04-19	2017-05-03	Федеральное государственное бюджетное образовательное учреждение высшего образования "Волгоградский государственный технический	Method of producing melanins from chaga

университет" (ВолгГТУ)				
RU2678986C1 *	2018-03-23	2019-02-05	Федеральное бюджетное учреждение науки "Государственный научный центр вирусологии и биотехнологии "Вектор" Федеральной службы по надзору в сфере защиты прав потребителей и благополучия человека (ФБУН ГНЦ ВБ "Вектор" Роспотребнадзора)	Antiviral agent based on humic acids
Family To Family Citations				

* Cited by examiner, † Cited by third party

Non-Patent Citations (4)
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Title
GUANQUM GONG et al. Extraction of Fulvic Acid from Lignite and Characterization of Its Functional Groups // ACS Omega 2020, 5, 27953–27961, от 22.10.2020, найдено в интернет 22.01.2021, https://pubs.acs.org/doi/pdf/10.1021/acsomega.0c03388 . *
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Publication	Publication Date	Title
Nakashima et al.	1987	Purification and characterization of an avian myeloblastosis and human immunodeficiency virus reverse transcriptase inhibitor, sulfated polysaccharides extracted from sea algae
Zandi et al.	2007	Antiviral activity of Aloe vera against herpes simplex virus type 2: An in vitro study
Lau et al.	2015	Ethnomedicinal uses, pharmacological activities, and cultivation of Lignosus spp.(tiger' s milk mushrooms) in Malaysia—A review
CN107441501A	2017-12-08	Drug-loaded liposome of antibacterial peptide modification and its production and use
Serkedjieva	2004	Antiviral activity of the red marine alga Ceramium rubrum
WO2005067955A1	2005-07-28	Antimicrobial activity from medicinal mushrooms
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CN103599071A	2014-02-26	Preparation method of polyinosinic-polycytidylic acid dry powder
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CN106727623B	2020-03-17	Application of seaweed oligosaccharide in preparation of anti-avian leukosis virus preparation
CN101596246B	2011-10-26	Smoked plum extractive and wild jujube seed extractive compound preparation as well as preparation method and application thereof
VA et al.	2020	Antiviral activity of total polysaccharide fraction of water and ethanol extracts of Pleurotus pulmonarius against the influenza A virus
US7399825B2	2008-07-15	Synthetic peptide, inhibitor to DNA viruses
CN1548056A	2004-11-24	Application of chitin, chitosan and their derivatives in preparing antiviral
RU2752872C1	2021-08-11	SARS-CoV-2 CORONAVIRUS REPLICATION INHIBITOR BASED ON HUMIC SUBSTANCES
RU2678986C1	2019-02-05	Antiviral agent based on humic acids
Zime-Diawara et al.	2015	The antimalarial action of aqueous and hydro alcoholic extracts of Artemisia annua L. cultivated in Benin: In vitro and in vivo studies
Amouroux et al.	1998	Antiviral activity in vitro of Cupressus sempervirens on two human retroviruses HIV and HTLV
Ogura et al.	2010	Evaluation of an edible blue-green alga, Aphanothece sacrum, for its inhibitory effect on replication of herpes simplex virus type 2 and influenza virus type A
WO2010040254A1	2010-04-15	The use of flavones from radix scutellariae in manufacture of medicaments for treating enterovirus infection
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Bajrai et al.	2021	In vitro screening of anti-viral and virucidal effects against SARS-CoV-2 by Hypericum perforatum and Echinacea
Hafez et al.	2020	Humic substances as an environmental-friendly organic wastes potentially help as natural anti-virus to inhibit COVID-19

Priority And Related Applications

Priority Applications (1) ▲

Application	Priority date	Filing date	Title
RU2020136343A	2020-11-03	2020-11-03	SARS-CoV-2 CORONAVIRUS REPLICATION INHIBITOR BASED ON HUMIC SUBSTANCES

Applications Claiming Priority (1) ▲

Application	Filing date	Title
RU2020136343A	2020-11-03	SARS-CoV-2 CORONAVIRUS REPLICATION INHIBITOR BASED ON HUMIC SUBSTANCES

Concepts ▲

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■ inhibitor		title,description	14	0.000
■ anti-viral		claims,abstract,description	28	0.000
■ lignite		claims,abstract,description	25	0.000
■ HCl		claims,abstract,description	20	0.000
■ effects		claims,abstract,description	19	0.000
■ mixture		claims,abstract,description	18	0.000
■ cell culture		claims,abstract,description	17	0.000
■ water		claims,abstract,description	17	0.000
■ Vero Cells		claims,abstract,description	15	0.000
■ hydrogen peroxide		claims,abstract,description	14	0.000
■ Trypsin		claims,abstract,description	9	0.000
■ Trypsin		claims,abstract,description	9	0.000
■ trypsin		claims,abstract,description	9	0.000
■ trypsin		claims,abstract,description	9	0.000
■ dose-response relationship		claims,abstract,description	8	0.000
■ ammonia		claims,abstract,description	7	0.000
■ chemical reaction		claims,abstract,description	7	0.000
■ dilution		claims,abstract,description	6	0.000
■ drying		claims,abstract,description	6	0.000
■ Inonotus obliquus		claims,abstract,description	5	0.000
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■ reprecipitation		claims,abstract,description	5	0.000
■ pharmaceutical industry		abstract	2	0.000
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