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In-vitro Antiviral Activity of ZingiVir-H, a Novel Herbomineral Drug against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

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Abstract

The COVID-19 pandemic has diseased several million people around the globe, with a death toll of over one million. To prevent the severity of this disease and death, there is a pressing need to find novel therapeutic agents to overcome this deadly disease. Even though several clinical trials are now underway to test potential therapies, the global response to the COVID-19 outbreak has largely been limited to monitoring/ containment. We report here that ZingiVir-H, a herb mineral antiviral drug with significant antiviral activity in *in vitro* conditions. The efficacy of ZingiVir-H in inhibiting the replication of SARS-CoV-2 was determined in a screening assay using cultured Vero cells. The cytotoxicity of ZingiVir-H in uninfected cells was determined using the MTT assay. The results showed that ZingiVir-H inhibits viral replication in a dose-dependent manner with an EC50 of 33.75 µg/ml. The results demonstrate that ZingiVir-H has high antiviral potential and may lead to the development of novel pharmaceutical agents to cure COVID-19 and the diseases caused by other emerging coronaviruses in the future. Interestingly, cytotoxicity was not observed at concentrations up to 500 µg/ml. Therefore, ZingiVir-H warrants further investigation for possible benefits in humans.

Keywords: Antiviral activity; Covid-19; Herbomineral drug; SARS-CoV-2; ZingiVir-H

Introduction

A case of pneumonia caused by a viral infection was diagnosed in Wuhan, China, at the end of 2019 [1]. The pathogen was identified as a novel enveloped RNA betacoronavirus2, now known as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which shares a phylogenetic relationship with SARS-CoV. Since then, the Coronavirus SARS-CoV-2, also known as Covid-19, has caused a global pandemic [2,3]. The Coronavirus Disease 2019 (Covid-19) has led to more than 100 million infections and over 2.1 million deaths worldwide, and more than 25 million infections and over 4,29,000 deaths in the US alone as of 28 January in the year, rendering it one of the most life-threatening infectious disease outbreaks in human history.

Covid-19 infection causes severe pneumonia, characterized by symptoms such as fever, a persistent cough, and progressive breathing failure associated with respiratory complications. The high hospitalization rate, risk of mortality, and lack of a specific established treatment made it critical to develop an effective Covid-19 therapy [4]. Since there is no specific Covid-19 medication available in clinics, supportive care and on occasion, combination therapy with wide-spectrum antiviral drugs and corticosteroids remains the mainstay of standard clinical treatment to manage this illness [5]. Hence, it's far urgent to identify more effective therapeutic options in response to the quick propagation of SARS-CoV-2. As future coronavirus outbreak is highly possible, it is desired to develop broad-spectrum antivirals suitable for the prevention and treatment of current circulating CoVs and future emerging CoVs. As a result, it is critical to find novel medications, particularly antivirals that can effectively treat this prevalent SARS-CoV-2 outbreak [6].

Natural-derived metabolites constantly become a worthy therapeutic alternative against several upcoming ailments, including viral infections, because they are innately better tolerated in the human body. According to a study, natural products or their derivatives accounted for 49% of all small molecules approved by the US Food and Drug Administration (FDA) between 1940 and 2014 [7]. Recently Pankajakasthuri herbals India has formulated ZingiVir-H drug. Furthermore, a pilot clinical trial of ZingiVir-H demonstrated significant efficacy and safety in hospitalized adults diagnosed with viral infection [8]. Two different toxicity studies on ZingiVir-H and its ingredients revealed that the drug is safe and free from any toxic effects [9,10]. The ingredients used for the formulation of ZingiVir-H are provided in [Table 1].

Sl. No	Scientific name	Form used	Quantity/
			500 mg
1	Eugenia caryophyllus	Dried clove bud powder	55 mg
2	Zingiber officinale	Fresh rhizome juice and aqueous extract	200 mg

3	Cyperus rotundus	Dried roots and rhizomes powder	35 mg
4	Hedyotis corymbosa	Dried whole plant powder	30 mg
5	Trachyspermum ammi	Dried fruit powder	60 mg
6	HgS (Mercuric sulphide)	Purified and processed as per texts.	20 mg
7	As2S3 (Arsenic trisulphide)	Purified and processed as per texts	10 mg
8	Starch	-	90 mg

Table 1: Composition of ZingiVir-H tablets.

In this context, we sought to assess the *in vitro* antiviral activity of ZingiVir-H against SARS-CoV-2 and, on top of that, could be readily available for further pre-clinical and clinical evaluation.

Materials and Methods

Materials

The known antiviral drug, remdesivir was purchased from Med-Chem Express and dissolved in DMSO before use. All other reagents and solvents used in this study were of the highest quality available. Milli-Q water was used in all experiments.

ZingiVir-H-study drug

The study drug is ZingiVir-H, a herbomineral Ayurvedic preparation in tablet form. ZingiVir-H (500 mg) tablets are produced at a Good Manufacturing Practices (GMP) approved production line at Pankajakasthuri Herbals India Pvt. Ltd. situated at Poovachal, Thiruvananthapuram, and Kerala, India. The ingredients used for formulating ZingiVir-H were provided in Table 1.

Virus and cell collection

The clinically isolated SARS-CoV-2 strain was maintained in production in Vero E6 cells (American type culture collection ATCC CRL-1586.) in Dulbecco's Modified Eagle's Medium (DMEM) with 4% of fetal bovine serum and 1% glutamine (complete medium). Vero cells were used to inoculate the virus against the SARS-CoV-2 strain. Cells were seeded in a 12-well microplate at a density of 5 cells/well × 104 cells/well and cultured in DMEM (Gibco, USA) containing 10% foetal bovine serum (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA), and 1% amphotericin-B (Gibco, USA) (Gibco, USA). Cells were cultured for 24 hours in a CO_2 incubator at 37°C in a humidified atmosphere of 5% CO_2 in order to achieve 80%-90% confluence.

Preparation of ZingiVir-H solution for the study

Each tablet containing active drugs was triturated and mixed until homogenous. Approximately 50 mg equivalent mass of drugs was weighed and added to dimethyl sulfoxide to solubilize the drugs. The suspension was sonicated for 15 minutes in a water bath before being added to Rosewell Park Memorial Institute (RPMI) media, sonicated again, and vortexed to mix until homogeneous. Under aseptic conditions, the suspension was filtered through a polycarbonate membrane with a pore size of 0.45 m and then a pore size of 0.22 m. The filtrate was vortexed with 10% foetal bovine serum and penicillin-streptomycin to create a homogeneous mixture for use as a stock solution. The samples were made by diluting each drug's stock solution with RPMI complete media at an appropriate level of dilution to produce a determined concentration.

Cytotoxicity assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay is a reliable measure of cell viability, was used to determine the cytotoxicity of the test drug. The basic idea behind this assay is that mitochondrial succinate dehydrogenase reduces yellow MTT dye to an insoluble, coloured (dark purple) formazan product. The purple formazan-containing cells are then solubilized in an organic solvent such as DMSO, releasing the solubilized formazan reagent, which is then measured using spectrophotometry. The assay was carried out based on the following protocol. In brief, ten-fold serial dilutions (1000 µg/ml to 50 µg/ml) of the ZingiVir-H were made with the maintenance. Monolayers of Vero cells were seeded into each well of a 96-well microplate and treated with various concentrations of ZingiVir-H. Plates were incubated at 37°C in 5% CO2 humidified incubator for 72 h. The plates were then examined under a microscope for signs of cell death Cyto Pathic Effect (CPE). The maximum non-toxic concentration was defined as the lowest dilution of ZingiVir-H that had no toxic effect on cells. Following the incubation period, the old medium was removed and 25 µl of MTT solution (2 mg/ml) was added to each well before incubating for 2 hours at 37°C. After that, the MTT solution was removed from the wells and DMSO (75 µl) was added to dissolve formazan crystal. Optical Density (OD) was measured spectrophotometrically at 490 nm. The experiment was conducted in a triplicate set and the 50% Cytotoxic Concentration (CC50) was calculated based on the reading obtained.

Antiviral activity assay of ZingiVir-H

The antiviral activity assay was performed as per the protocol routinely followed for determining antiviral activity against SARS-CoV-2. A known inhibitor of SARS-CoV-2 (Remdesivir) was used as a positive control in the assay. To check the antiviral activity, Vero E6 cells were seeded in 96-well plates at 80% confluency and then infected with the SARS-CoV-2 isolate at a Multiplicity of Infection (MOI) of 0.1 h for 2 h. Subsequently, the inoculum was aspirated and fresh media containing different concentrations of the ZingiVir-H (25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml and 400 µg/ml) was added to the cells. Each concentration was assayed in triplicates. ZingiVir-H showing more than 50% anti-SARS-CoV2 activity was considered for EC50 determination. At 24 h post-infection, the supernatant and cells were subjected to viral RNA isolation followed by qRT-PCR to determine the SARS-CoV-2 viral load in the cells (cell associated) and culture supernatants (released virus particles), qRT-PCR was performed using primers specific for

the viral spike, nucleocapsid and ORF1a for both released and cell-associated viruses.

Statistical Analysis

All experiments were performed in triplicate sets. Data are shown as mean ± S.D. all the statistical analysis for this study was conducted using Graph Pad Prism Software 8.0 (Graph Pad Software Inc., La Jolla, CA, USA).

Results

Before conducting the antiviral assay, we first conduct the cytotoxicity of ZingiVir-H in Vero cell lines by MTT assay. The ZingiVir-H was not toxic to the cells at concentrations upto at least 500 µg/ml. The cell viability at this concentration was 98.1% ± 3.1% relative to untreated cells (Figure 1). In our study ZingiVir-H produced dose-dependent inhibition of viral replication, with an EC50 (concentration producing 50% inhibition of viral replication) of about 33.75 µg/ml) (Figure 2).

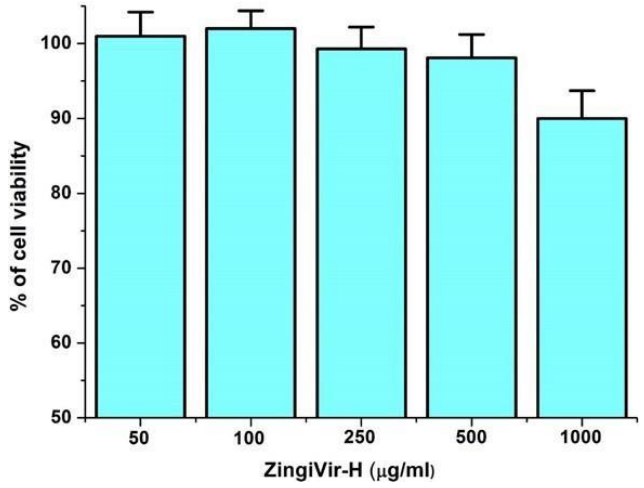

Figure 1: Viability of Vero cells at several concentrations of ZingiVir-H.

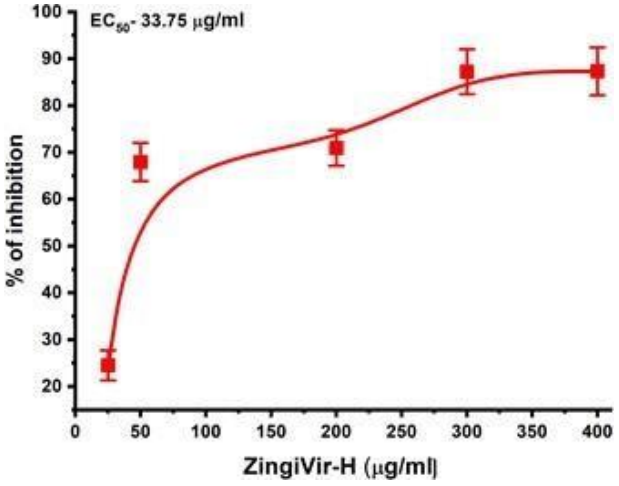

Figure 2: Inhibition of SARS-CoV-2 replication in Vero cells by ZingiVir-H at several concentrations.

Discussion

The Covid-19 pandemic has gravely illustrated the need for counter measures against emerging epidemic and pandemic CoVs. Broad-spectrum antiviral drugs, antibodies, and vaccines are needed to combat the current pandemic and those that will emerge in the future. Given the magnitude and devastation of the current Covid-19 outbreak, as well as the ongoing threat of CoVs causing human disease, there is an urgent need to develop effective and safe therapies to treat these patients. There are currently no approved therapies for CoVs, including SARS-CoV-2. The experimental therapies used in conjunction with known antiviral agents have either limited efficacy (remdesivir) or high systemic toxicity (hydroxychloroquine), limiting their usefulness [11]. Finding new therapies that are both effective and safe is critical.

Currently, no specific therapy such as relevant antiviral drugs is available for COVID-19. A variety of traditional medicinal plants and herbs have been shown to have antiviral activity against various viruses. Herbs can provide valuable sources of components with immune modulatory, anti-inflammatory, anti-oxidative, and antiviral properties, resulting in positive effects on virus-affected systems [12].

Ginger one of the major components of ZingiVir-H displays direct antiviral effects and can have a protective role against ARDS [13, 14], which is the major cause of mortality in patients with severe COVID-19.

Thus, ginger can have several advantageous effects on many organs that are known to affect by the COVID-19 virus. *S.* Aromaticum another ingredient used for formulating ZingiVir-H is rich with eugeniin. At a concentration of 5 g/mL, this compound was identified as an anti-herpes simplex virus compound. Eugeniin inhibits viral DNA synthesis by acting as a selective inhibitor of the HSV1 DNA polymerase, and eugenol inhibits viral replication and infection by acting as a selective inhibitor of the HSV1 DNA polymerase [15,16].

A report on the modulation of CYP3A4 enzyme by the rhizome fractions of C. Rotundus suggests its safe consumption concerning drug metabolism and efficacy. The study also provided the basis for the hepatoprotective and Hepatitis B Virus (HBV) inhibitory activity of *C.* Rotundus [17]. *C.* Rotundus was rich in β-amyrin and stigmasta-5, 22-dien-3-ol. β-amyrin and stigmasta-5, 22-dien-3-ol-exhibited the best binding interactions and stability.

Finally, Absorption Distribution Metabolism Excretion and Toxicity (ADMET) studies were carried out to better understand the compounds' pharmacokinetic properties and safety profile. Overall, the results show that phytochemicals derived from Cyperus rotundus Linn, specifically amyrin and stigmasta-5, 22- dien-3-ol, can be tested as potential inhibitors of SARS-CoV-2 Mpro [18].

Trachyspermum ammi, another ingredient inhibited viral protease enzymes in Hepatitis C Virus (HCV) infection. In addition to this Roy et al. also reported the antiviral activity of essential oil of Trachyspermum Ammi against Japanese encephalitis virus. From all these, it is clear that potent herbs with strong antiviral principles formulated ZingiVir-H. The results of our also clearly revealed that ZingiVir-H has strong *in-vitro* antiviral activity.

Conclusion

To summarize our findings, we demonstrated ZingiVir-antiviral H's activity against SARS-CoV-2. More research is being conducted to isolate and elucidate the bioactive components responsible for ZingiVir-antiviral H's activity, as well as to determine their mechanism of action. To the best of our knowledge, this is the first report of antiviral activity of ZingiVir-H, a herb mineral ayurvedic drug, and it suggests that ZingiVir-H may have some benefits in the treatment of infectious diseases, warranting further research.

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