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Research Article

Evaluation of Antiviral Effects and Toxicity of Herbal Medicine Vipdervir Capsules

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ABSTRACT

Background: Antiviral vaccine is not effective, synthetic antiviral drugs are highly toxic, leading to increased interest in herbal medicines as promising antiviral drugs. Recently, Vipdervir has been developed from medicinal herbs with the aim to support and treat diseases caused by viruses such as H5N1 and SARS-CoV-2. In the present study, we assessed Vipdervir's antiviral activity against H5N1 and SARS-CoV-2. In addition, we also evaluated the acute toxicity and repeated dose toxicity of Vipdervir in mice and rabbits, respectively. **Methods**: H5N1 inhibitory effect of Vipdervir was assessed using hemagglutination inhibition assay. Vipdervir's SARS-CoV-2 inhibitory effect was evaluated by Plaque Reduction Neutralization assay. Acute and repeated dose oral toxicities of Vipdervir were determined according to OECD 423 and OECD 407 guidelines, respectively. **Results:** Data show that Vipdervir is effective against both H5N1 and SARS-CoV-2. At concentrations of 3 mg/mL and 5 mg/mL Vipdervir completely inhibits H5N1. At a concentration of 50 µg/mL Vipdervir showed an inhibitory effect on SARS-CoV-2. Acute toxicity data revealed that the LD_{50} of Vipdervir is greater than 35200 mg/kg, b.wt. in mice. Repeated toxicity data indicated that Vipdervir did not induce significant differences in body weight gain, hematology and clinical biochemistry in compared to the control group. The No Observed Adverse Effect Level of Vipdervir is greater than 613.8 mg/kg b.wt./day in rabbits. No delayed toxicity effects of Vipdervir were observed. **Conclusion:** Vipdervir capsules were found to be antiviral effective and relatively safe in the tested doses and experimental conditions.

Key words: Antiviral, COVID-19, Herbal, H5N1, SARS-CoV-2.

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INTRODUCTION

Viral respiratory infections cause life-threatening illness in many people worldwide and affect the lives of millions worldwide each year. Global concern about respiratory viruses has recently increased dramatically due to the emergence of some new viruses, especially avian influenza A, H5N1 and SARS-CoV-2.[1] H5N1 was first discovered and caused a global flu pandemic in 1997. Influenza A/H5N1 is considered a dangerous infectious disease, causing high mortality to humans and animals.[2] SARS-CoV-2 causes COVID-19 (Coronavirus Disease 2019) and was discovered in December 2019 in Wuhan, China.[3] Its alarmingly rapid transmission from country to country across the world with the high level of morbidity and mortality.[4,5] The disease has led to disruptions in medical services of many countries in the world.[6] The global outbreak of the pandemic has disturbed social, religious, economic, financial and political structures across the world.[7]

Influenza virus belongs to the Orthomyxoviridae family, which contains influenza A, B and C viruses. Types A and B are responsible for seasonal flu epidemics each year. Infection of influenza virus remains one of the most serious threats to human health and causes epidemics or pandemics. Influenza viruses are responsible for acute contagious respiratory infections.[8] Influenza A viruses cause the most virulent disease among the three influenza types and, based on the antibody responses to these viruses, they may be divided into different serotypes.[9] They contain seven or eight pieces of single stranded, segmented negative-sense RNA. Their genomes encode eleven proteins including HA, NA, NP, PB1, PB1-F2, PB2, NS1, NEP, PA, M1 and M2. The main antigenic factors of influenza A and B viruses are the hemagglutinin (HA) and neuraminidase (NA) transmembrane glycoprotein knobs.[10] Recently, many combinations of 16 HA (H1-16) and 9 NA (N1-9) subtypes have been identified in wild birds, while two additional subtypes (H17N10 and H18N11) have been isolated in bats.[11,12] Influenza infection results in the uncontrolled increase of pro-inflammatory cytokines, which makes this infection a strong risk factor for severe complications which may be terminal.[13] It can induce a cytokine storm. The cytokine storm is a systemic expression of a healthy and strong immune system, and is potentially lethal, consisting of positive feedback between cytokines and immune cells with high levels of various cytokiness.[14]

SARS-CoV-2 is an enveloped RNA virus possessing a positive sense, single-stranded genome around 29.9 Kb. A large part at the 5′ end of viral genome encodes the pp1ab and pp1a polyproteins, fractured into 15 non-structural proteins which consist of nsp1-16. In the 3′ end, the virus genome encodes four structural proteins including surface spike (S), matrix (M), envelope (E), nucleocapsid (N) and eight accessory proteins. Structural and accessory proteins participate in virus morphogenesis and immune system interference, while non-structural proteins are involved in virus replication. The spike

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glycoprotein is essential for the attachment to the host cell receptors and determines tissue tropism.[15]

The failure of many conventional drugs against viral infections combined with the onset of specific viral resistance has led to an increasing interest in plants as promising antiviral agents.[16] Nature provides an immense library of novel chemicals to explore for the development of drugs to treat various ailments including viral diseases.[17] Since natural compounds, including phenolic acids, terpenes, flavonoids, coumarins, lignans, alkaloids and proteins play an essential role in inhibiting viruses and acting as complementary therapies against viral infections,[18] we developed a herbal product named Vipdervir containing of natural antivirus ingredients that against H5N1 and SARS-CoV-2 viruses, the cause of the current pandemic of COVID-19. In present study, we investigated antiviral effects of herbal product, Vipdervir, on H5N1 and SARS-CoV-2 viruses *in vitro*. We also assessment acute and repeated dose toxicity studies of Vipdervir in mice and rabbits, respectively.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's Modified Eagle's Media, fetal bovine serum, trypsin/EDTA solution, penicillin-streptomycin solution, and phosphate-buffered saline were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Clinical diagnostic kits were purchased from Sysmex Corporation (Kobe, Japan), Beckman Coulter (Brea, CA, USA), and Sigma-Aldrich Corporation (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

Cells: Vero cells (African green monkey kidney) were obtained from the American Type Culture Collection (ATCC; CRL-1587; lot #59168527; Manassas, VA, USA) and cultured at low passage at 37 °C/5 % CO2 in Dulbecco's Modified Eagle's Media (DMEM) with or without 10 % heat-inactivated fetal bovine serum (FBS). The creation of a master cell line bank ensured that cells with low passage numbers were used for all experiments.

Animals

Swiss mice (18 - 20 g) were provided by the National Institute of Hygiene and Epidemiology (Ha Noi, Viet Nam). Eight to ten mice were housed in each cage. New Zealand White Rabbits (1.8 - 2.2 kg) were provided by the National Institute of Drug Quality Control (Ha Noi, Viet Nam). Rabbits were individually housed in stainless steel cages. Animals were acclimatized in an animal room under a 12/12 h light/dark cycle at 22 °C ± 2 °C for one week before the experiment onset. All animal care procedures were conducted in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Preparation of medicine

Using AutoDock 4.1 software[19] for molecular docking to find active ingredients from the library of active ingredients that prevent virus interactions with host cells, as well as viral replication in host cells, through blockade of key molecules involved in viral replication, transcription and genetic translation. Based on the screening results (Table 1) to select active ingredients with high binding affinity to target molecules that are present abundantly in Vietnamese herbs. Herbs containing active ingredients with high binding affinity to target molecules selected for the formulation of Vipdervir include: *herba Andrographis* (andrographolide), *radix Bupleuri* (saikosaponin), *Folium Artemisiae annuae* (artemisinin), *herba Houttuyniae cordatae* (rutin and quercetin), *radix Zingiber* (gingerol), *radix et rhizoma Glycyrrhizae* (glycyrrhizic acid), *Flos Styphnolobium japonici* (rutin and quercetin), *herba et fructus Silybi* (sylymarin), *radix Scutellariae* (baicalein). These herbs were extracted and purified to create herbal extracts containing the selected active ingredients with high content. Herbal extracts are combined with necessary excipients to form Vipdevir capsules.

Evaluation of the inhibitory effects of Vipdervir on H5N1

Vipdervir was mixed in PBS 1X and combined with H5N1 virus to obtain a mixture with Vipdervir concentrations of 5 mg/mL, 3 mg/mL, 1.5 mg/mL, 0.5 mg/mL, 0.3 mg/mL, 0.15 mg/mL, 0.06 mg/mL and 0.03 mg/mL. Viral concentrations were similar at all drug concentrations. Inject each concentration into 6 eggs. Incubate eggs for 3 days at 33.5 °C. The control is the virus solution mixed in 1X PBS without drugs. Then collect the egg fluid to check for erythrocyte agglutination. Specifically, 10 µL of egg solution was diluted with 90 µL of 1X PBS solution (denoted as TR solution). The TR solution was mixed well, then diluted in a second order series. On a 96-well plate, 50 µL of TR solution was added 50 µL of 0.5% chicken red blood cells and shake gently. Incubate at 4 °C for 30 minutes. Each of TR solution was done in triplecate. Read the results when the control erythrocyte sedimentation. The agglutination titre (HAU) is the highest dilution of antigen that causes erythrocyte agglutination.

Evaluation of the inhibitory effects of Vipdervir on SARS-CoV-2

Evaluation of the ability to inhibit the growth of SARS-CoV-2 virus of Vipdervir was carried out by the method of Plaque Reduction Neutralization Test (PRNT) - PRNT50.

SARS-CoV-2 virus with defined concentrations (1 PFU, 10 PFU, 50 PFU and 100 PFU) was interacted with Vipdervir at non-cytotoxic concentration of 50 µL/mL at 37 °C/60 min. After incubation, the virus-preparation suspension was infected on the infectious cells (Vero E6). The active ingredients in Vipdervir have the ability to inhibit the infection of the SARS-CoV-2 virus, the remaining viruses that are not inhibited by the preparation will infect cells and create necrotic clusters. The total number of necrotic clusters appearing after 96 hours of infection was determined, the number of necrotic clusters was reduced by 50% compared to the positive control that was determined to be able to inhibit SARS-CoV-2.

Acute oral toxicity study

The acute toxicity of Vipdervir was conducted in mice following the guidelines of Organization for Economic Co-operation and Development (OECD) for testing of chemicals, TG 423 with minor modification. Mice were randomised divided into 4 groups, including 1 control group and three experimental groups (10 mice/group). The test groups were received doses of 21, 43 and 64 capsules/kg b.wt of Vipdervir (20 mL/kg b.wt in water) *via* gastric intubation whilst the control group received water (20 mL/kg b.wt). Average weight per vipdervir capsule is 550 mg. The toxic signs were monitored after administration, including physiological signs, behavior, movement, changes in skin, fur texture, eating, drinking, stool and urine every 15 min within the first hour and gradually decreased in frequency within the first 24 h. Mice were observed once a day for a period of 7 days. The numbers of dead mice in the test and control groups were recorded. Body weights of mice were measured at the time just before administration (day 0), day 4 and day 7 after administration to compare with the control group. On day 8, mice were euthanized to gross pathological examination of all the major internal organs such as heart, lung, liver, kidney, spleen, stomach, intestine.

Repeated dose 28-day oral toxicity study

A 28-day repeated oral toxicity study was conduct in rabbits according to the OECD guide lines, TG 407 with minor modification. Rabbit were randomised divided into three groups, including 1 control and 2 test

Table 1: List of active ingredients screened on target molecules.

Active ingredients	Binding affinity to target molecules									
	Virus SARS-CoV-2									H5N1
	Spike S	ACE2	3CLpro (NSP5)	PLpro (NSP3)	Helicase (NSP13)	Endoribo-nuclease (NSP15)	Exoribo-nuclease (NSP10)	Methyl-transferase (NSP16)	RdRp (NSP12)	HA (H5)
Chloroquine	-6.5	-5.9	-5.7	-6.5	-6.9	-6.5	-7.2	-6.6	-6.1	-6.5
Hydroxy-chloroquine	-7.4	-6.2	-5.6	-6.4	-6.3	-6.6	-7.2	-6.6	-6.8	-6.4
Lopinavir	-7.2	-7.6	-6.8	-8.8	-7.1	-8.7	-9.1	-7.7	-7.3	-7
Remdesivir	-7.3	-7.7	-7.4	-8.5	-8.1	-9	-8.8	-8.1	-8.6	-7.1
Niclosamide	-7	-7	-6.6	-7.7	-7.7	-8.6	-7.9	-7.3	-8.4	-7.2
Favipiravir	-5.9	-5.7	-5.9	-6.3	-6	-6.5	-5.3	-5.7	-6	-5.9
Indinavir	-8.7	-9.3	-7.9	-9	-7.4	-8.7	-10	-8.4	-7.8	-7.2
Atazanavir	-7.6	-8.4	-6.7	-8	-7.6	-9	-8	-7.5	-7.4	-6.5
Ciclesonide	-9	-9	-7.9	-9.8	-9.1	-9.6	-8.9	-8.4	-9.5	-8.5
6-shogaol	-5.6	-6.1	-6.1	-6.4	-6.9	-7.2	-6.9	-6.7	-7.2	-5.9
6-gingerol	-5.8	-6.2	-5.4	-6.5	-6.4	-6.4	-6.9	-6.8	-8.9	-5.4
Absithin	-10	-9.7	-8.6	-6	-9.9	-10.5	-11.8	-9.1	-10.5	-8
Artemisinin	-7.2	-7.2	-7.2	-7.9	-7.7	-9	-8.1	-7.6	-7.9	-7.4
Baicalein	-8.7	-9.3	-8.8	-9	-8.9	-6.3	-7.7	-7.9	-9.6	-6.8
EGCG	-7.2	-7.4	-5.8	-8	-8.4	-8.9	-8.4	-9.1	-7.1	-8.6
Emodin	-7.3	-7.8	-8.4	-7.8	-7.9	-8.6	-8.5	-8	-8.6	-7.8
Eugenol	-5.8	-5.2	-5.5	-5.5	-5.9	-6.3	-5.7	-5.7	-6.3	-5.1
Glycyrrhizic acid	-8.7	-9	-7.5	-9.7	-9.5	-10.7	-10.5	-9	-9.1	-9.1
Hesperidin	-9.6	-9.8	-8.6	-9.4	-9.4	-9.8	-9.2	-9	-9.5	-8.5
Kaempferol	-8.8	-7.4	-7.9	-7.7	-8.2	-8.8	-7.6	-7.9	-8.2	-8.1
Lecithin	-8.2	-8.7	-8.3	-9.1	-9	-4.9	-5.6	-5.7	-8.8	-7.8
Myricerin	-7.7	-7.9	-8	-8.1	-8.6	-8.8	-7.6	-8.5	-8.8	-7.6
Licorine	-7.3	-7.5	-6.9	-7.7	-9.2	-7.6	-7	-8.1	-8.5	-7.4
Oroxindin	-8.9	-9.1	-8.1	-8.5	-9.1	-8.8	-8.7	-8.9	-9.4	-7.8
Quercetin	-8.9	-9.8	-9.2	-9.3	-10.3	-8.2	-8.9	-8.7	-9	-8.1
Rutin	-9.2	-8.9	-7.8	-9.7	-10	-9.6	-10	-8.6	-9	-8.6
Resveratrol	-7.3	-7	-7	-7.1	-10	-7.5	-7.7	-7.2	-7.5	-6.8
Saikosaponin A	-9.5	-9.6	-8.9	-10.8	-10.1	-10.2	-9	-10.2	-10.5	-9.2
Curcumin	-7.3	-7.4	-7.1	-7.4	-7.5	-8.6	-7.7	-7.4	-8.4	-6.5
Andrographolide	-8.8	-7.4	-7.5	-7.6	-8	-7.3	-7.8	-7.2	-7.3	-6.9
14-deoxy-andrographolide	-7.8	-7.3	-7.4	-7.5	-7.3	-7.3	-7.5	-7	-7.3	-7.2
Pyrazofurin	-7.1	-7.3	-7.1	-6.9	-7.4	-7.6	-7.6	-7.1	-7.3	-6.8
Rabivirin	-7.2	-7	-7	-6.7	-6.9	-7.1	-7.7	-6.8	-7.1	-6.5
Anabsinthin	-11	-9.9	-8.7	-9.3	-9.2	-9.9	-11.7	-9.6	-9.8	-8.9

Note: ACE2 (Angiotensin-converting enzyme 2), 3Clpro (3C-like protease), PLpro (papain-like protease)

Table 2: The results of the assessment of the red blood cell agglutination (HA).

Concentration of Vipdervir (mg/mL)	5	3	1.5	0.5	0.3	0.15	0.06	0.03	0.00
HAU (mean)	0	0	13	17	20	20	26	33	320

groups (8 rabbits/group). The intended human dose of Vipdervir is 6 capsules/person (50 kg)/day. The rabbit dose of 0.372 capsules/kg/day was arrived from human dose based on body surface area conversion. In the present study, Vipdervir were administered at two dose levels i.e., 0.372 and 1.116 capsules/kg/day for 28 days. The control group rabbits were given distilled water. The rabbits were checked daily for the food and water consumption, physical condition, behavior, stool and urine. The weight of rabbits at day 0, 7, 14, 21, and 28 and 42 were recorded. The hematological parameters (number of red blood cells, white blood cells, platelets, hemoglobin, hematocrit) and biochemical parameters (aspartate transaminase - AST, Alanine transaminase - ALT, total protein, total bilirubin, cholesterol, creatinine, urea and glucose) at day 0, 14, 28 and 42 were measured. At day 29, three rabbits in each group were randomly euthanized for macroscopic and histological evaluation. At the end of the study, remain animals were dissected to observe the general organs of the heart, liver, kidney, lung, stomach and intestine. The organs (liver, kidney and lung) were fixed in 10% neutral buffered formalin, trimmed and a 10 µm thickness of tissue sections were stained with hematoxylin and eosin (HE) for histopathological examination.

Statistical analyses

Data were expressed as mean ± standard deviation of the mean (S.D). Experimental data were analyzed by a one-way ANOVA followed by the Bonferroni's multiple comparison tests and a two-tailed Student's t test in the Prism program version 8.0 (Graph Pad Software, Inc., San. Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

The inhibitory effects of Vipdervir on H5N1

The results of the assessment of the red blood cell agglutination (HA) was presented in Table 2.

According to the results in Table 3: Red blood cells in the wells with Vipdervir concentrations of 5 mg/mL and 3 mg/mL are completely settled. That means the virus no longer replicates in the eggs. Thus, Vipdervir completely inhibited the growth of H5N1 virus at concentrations of 3 mg/mL or more. Inhibition of virus growth was dependent on drug concentration, in the control group (injection of virus without drug), the virus was not inhibited.

The inhibitory effects of Vipdervir on SARS-CoV-2

Evaluation of the inhibitory ability of Vipdervir on the growth of SARS-CoV-2 virus at a viral concentration range of 1 PFU to 100 PFU equivalent to the amount of SARS-CoV-2 virus in biological samples determined by real -time PCR kit currently has C_t = 24 and C_t = 20.5. Accordingly, if the RT-PCR test sample has a C_t < 37, the sample is positive for SARS-CoV-2. The test results showed that Vipdervir 50 µg/ml was able to inhibit the growth of SARS-CoV-2 virus at viral concentrations of 1 PFU to 10 PFU (Figure 1).

Acute oral toxicity study

There were no abnormal signs or dead mice were found in control group and 2 the test groups dosed 21 and 43 capsules/kg. In the test group of 64 capsules/kg, 2 out of 10 mice had seizures, and 10 out of 10 mice had sweating. The average weight of mice during the 7-day test did not show a statistically significant difference between the test groups (T) and the control group (C) (previous $P_{ANOVA\ before} > 0.05$; P (TC); $P_{(T\text{-}C)\ before} > 0.05$; $P_{(T\text{-}T)\ before} > 0.05$). After seven days, the mice in both control and test groups steadily gained weight compared to day 0 in each group ($P_{before\text{-}after} < 0.001$). The difference in average weight among the test groups compared to the control group at day seven was not statistically significant ($P_{ANOVA\ after} > 0.05$; $P_{(T\text{-}C)\ after} > 0.05$; $P_{(T\text{-}T)\ after} > 0.05$) (Table 3). In this experiment, the LD_{50} was > 64 capsules/kg b.wt. (equivalent to 35200 mg/kg b.wt.). According to the toxicity classification of Globally Harmonized System (GHS, 2021), Vipdervir was considered acute toxicity below the GHS threshold and was not classified by GHS.

Table 3: Effects of Vipdervir on body weight in mice.

Group	Before test		After test		Gained weight (%)	$P_{before\text{-}after}$
	Weight of mice (g)	P_{before}	Weight of mice (g)	P_{after}		
Control	19.79 ± 0.85	$P_{ANOVA} > 0.05$	30.09 ± 1.00	$P_{ANOVA} > 0.05$	152.4	P < 0.001
Test 1	19.82 ± 0.78	$P_{T1\text{-}C} > 0.05$	30.01 ± 1.19	$P_{T1\text{-}C} > 0.05$	151.5	P < 0.001
Test 2	19.58 ± 0.86	$P_{T2\text{-}C} > 0.05$	29.82 ± 1.03	$P_{T2\text{-}C} > 0.05$	152.5	P < 0.001
Test 3	19.90 ± 0.81	$P_{T3\text{-}C} > 0.05$	29.71 ± 1.52	$P_{T3\text{-}C} > 0.05$	149.6	P < 0.001

Table 4: Effects of Vipdervir on body weight in rabbits.

Group (n = 8)	Body Weight (kg)						P
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 42	
Control	2.03 ± 0.14	2.23 ± 0.15	2.34 ± 0.13	2.40 ± 0.14	2.50 ± 0.19	2.58 ± 0.16	$P_{before\text{-}after} < 0.001$
Test 1 (low dose)	2.03 ± 0.18	2.23 ± 0.24	2.36 ± 0.22	2.43 ± 0.22	2.53 ± 0.23	2.63 ± 0.24	$P_{before\text{-}after} < 0.001$ $P_{before(T1\text{-}C)} > 0.05$ $P_{after(T1\text{-}C)} > 0.05$
Test 2 (high dose)	2.03 ± 0.14	2.15 ± 0.13	2.24 ± 0.14	2.34 ± 0.13	2.44 ± 0.17	2.59 ± 0.17	$P_{before\text{-}after} < 0.001$ $P_{before(T2\text{-}C)} > 0.05$ $P_{after(T2\text{-}C)} > 0.05$

Table 5: Effects of Vipdervir on hematological parameters in rabbits.

Group	RBC (x 10^{12}/l)	WBC (x 10^9/l)	PLT (x 10^9/l)	HCT (%)	HGB (g/dl)
Day 0 (n = 8)					
Control	5.7 ± 0.5	9.3 ± 2.2	521.5 ± 119.6	38.0 ± 3.7	11.6 ± 1.2
Test 1	5.9 ± 0.4	8.3 ± 1.1	528.1 ± 99.2	39.0 ± 2.9	12.2 ± 0.8
Test 2	5.7 ± 0.6	8.4 ± 1.4	511.0 ± 133.9	37.8 ± 2.8	11.8 ± 0.9
Day 14 (n = 8)					
Control	5.8 ± 0.6	9.3 ± 0.7	444.0 ± 72.7	38.6 ± 2.6	12.1 ± 1.0
Test 1	5.7 ± 0.3	8.6 ± 1.4	502.9 ± 92.7	38.8 ± 1.9	11.8 ± 0.4
Test 2	5.6 ± 0.4	8.8 ± 1.2	436.1 ± 94.7	37.8 ± 1.3	11.8 ± 0.5
Day 28 (n = 8)					
Control	6.1 ± 0.4	8.7 ± 1.5	503.1 ± 153.6	39.5 ± 3.0	12.6 ± 0.9
Test 1	6.0 ± 0.4	7.5 ± 0.8	437.4 ± 64.0	39.4 ± 2.1	12.6 ± 0.6
Test 2	5.9 ± 0.5	8.2 ± 1.2	461.9 ± 73.7	38.6 ± 3.5	12.3 ± 1.0
Day 42 (n = 5)					
Group	RBC (x 10^{12}/l)	WBC (x 10^9/l)	PLT (x 10^9/l)	HCT (%)	HGB (g/dl)
Control	6.0 ± 0.6	8.7 ± 1.4	413.2 ± 75.2	38.8 ± 4.1	12.1 ± 1.1
Test 1	5.8 ± 0.4	8.1 ± 0.5	363.2 ± 28.8	39.3 ± 3.0	12.5 ± 0.8
Test 2	6.0 ± 0.2	10.2 ± 2.1	452.4 ± 91.0	40.0 ± 1.3	12.4 ± 0.4

Repeated oral toxicity study

In the repeated toxicity study, no abnormality in the eating or movement of rabbits was found in all test groups. Before the experiment, the average weights of rabbits in the test groups (T) were not different from the control group (C) ($P_{before(T1-C)} > 0.05$; $P_{before(T2-C)} > 0.05$). After 14 and 28 days, rabbits gained weight steadily in both control and test groups ($P_{before-after} < 0.05$) compared to day 0, while no significant difference in the average weights was determined between the test group and the control group ($P_{(T1-C)\ after} > 0.05$; $P_{(T2-C)\ after} > 0.05$). After 42 days, the rabbits were still healthy and gained weight well. No statistically difference in average weight between the test groups and the control group was found ($P_{(T1-C)\ after} > 0.05$; $P_{(T2-C)\ after} > 0.05$) (Table 4).

At day 14, 28 and 42, the hematological and biochemical indices were not statically significant difference between the control and the test groups ($P_{(T-C)\ day\ 0} > 0.05$); ($P_{(T-C)\ day\ 14} > 0.05$; $P_{(T-C)\ day\ 28} > 0.05$); $P_{(T-C)\ day\ 42} > 0.05$) (Table 5 and 6).

HE stained liver, kidney and lung specimens were observed under an optical microscope. Macroscopic observation revealed the normal appearance and color of the heart, liver, kidney, lung, stomach, intestines of rabbits in the test groups compared to the control group after the experiment (data not shown). The histopathology observation showed that the liver, kidney and lung tissues of rabbits in the test groups were not abnormal compared with the control group (Figure 2).

DISCUSSION

Although there are many vaccines against COVID-19 as well as many drugs licensed to treat this disease. The COVID-19 pandemic is still happening very complicatedly. Chemical drugs to treat COVID-19 are not very effective against SARS-CoV-2, but the side effects are quite serious and have not been fully studied due to the short time of drug development.[20] Therefore, the need for drugs of medicinal origin to treat COVID-19 is urgent.

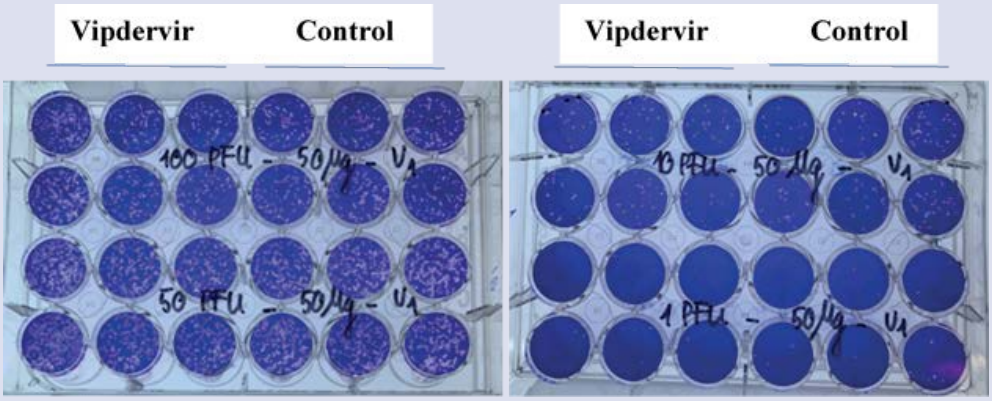

Figure 1: Effects of Vipdervir on SARS-CoV- 2.

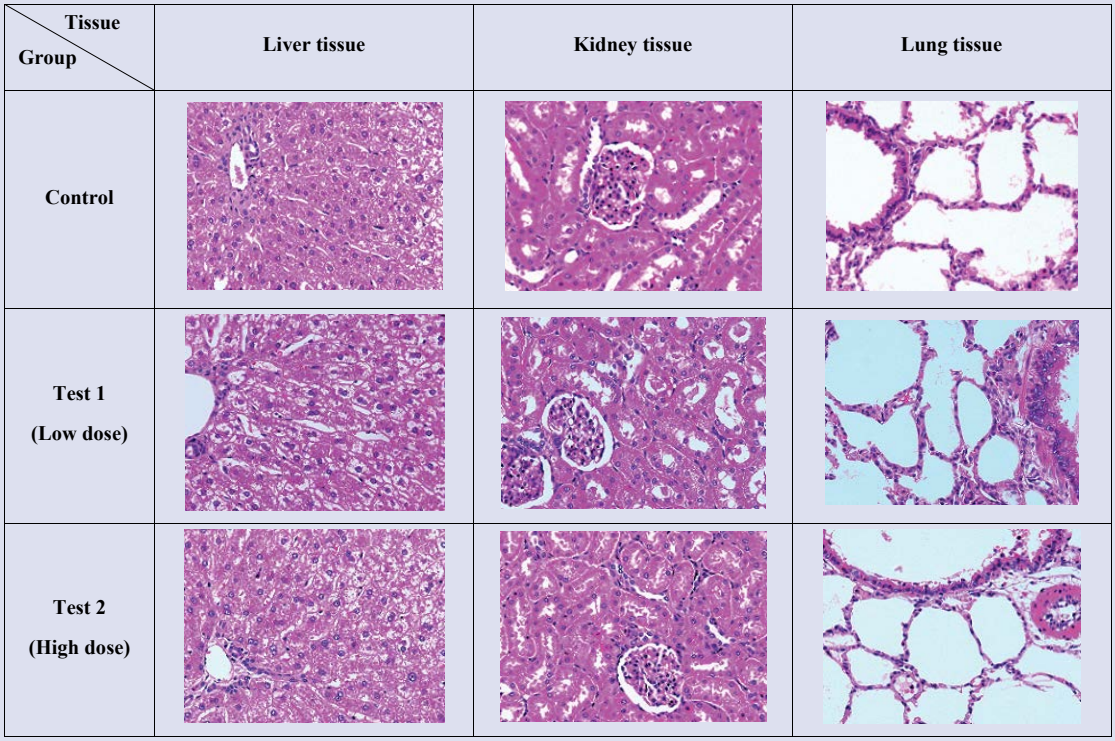

Figure 2: The histopathology observation of control and Vipdervir treated animals for 28 days.

Table 6: Effects of Vipdervir on biochemical parameters in rabbits.

Group	AST (U/l)	ALT (U/l)	T.Bilirubin (µmol/l)	T.Protein (g/l)	Cholesterol (mmol/l)	Urea (mmol/l)	Creatinin (µmol/l)	Glucose (mmol/l)
Day 0 (n = 8)								
Control	47.4 ± 19.0	71.8 ± 11.9	2.5 ± 1.1	56.8 ± 5.3	2.2 ± 0.9	7.2 ± 1.6	91.5 ± 19.2	6.4 ± 0.5
Test 1	40.9 ± 16.1	68.0 ± 17.9	1.8 ± 0.9	57.9 ± 3.4	1.7 ± 0.4	6.3 ± 1.3	86.5 ± 12.1	6.6 ± 0.5
Test 2	38.3 ± 12.1	64.3 ± 8.5	2.1 ± 1.1	54.2 ± 5.3	1.9 ± 0.6	6.7 ± 0.5	89.6 ± 12.2	6.5 ± 0.7
Day 14 (n = 8)								
Control	36.7 ± 6.9	74.1 ± 13.7	1.9 ± 0.8	54.5 ± 3.1	1.4 ± 0.7	6.6 ± 0.9	93.5 ± 7.6	6.6 ± 0.7
Test 1	38.3 ± 6.6	83.7 ± 10.9	1.7 ± 0.6	54.0 ± 2.0	1.6 ± 0.5	5.9 ± 1.0	92.0 ± 7.0	6.5 ± 0.5
Test 2	35.4 ± 12.8	71.6 ± 12.1	1.7 ± 0.9	52.7 ± 3.0	1.7 ± 0.5	6.3 ± 1.0	86.2 ± 8.8	6.7 ± 0.8
Day 28 (n = 8)								
Control	32.4 ± 9.4	76.5 ± 16.5	1.8 ± 0.8	64.8 ± 3.7	1.5 ± 0.8	6.5 ± 1.6	90.3± 8.0	6.9 ± 0.6
Test 1	31.7 ± 10.1	72.0 ± 16.0	2.0 ± 1.1	63.3 ± 3.3	1.4 ± 0.5	6.3 ± 1.0	99.2 ± 11.9	7.2 ± 0.6
Test 2	30.5 ± 9.0	63.1 ± 8.2	1.8 ± 1.0	64.8 ± 5.2	1.5 ± 0.7	6.0 ± 1.3	93.1 ± 12.8	7.4 ± 0.5
Day 42 (n = 5)								
Control	41.8 ± 15.5	78.3 ± 12.2	1.7 ± 0.8	66.1 ± 1.9	1.5 ± 0.7	5.9 ± 1.0	102.4 ± 16.9	7.0 ± 1.0
Test 1	54.5 ± 24.2	88.5 ± 17.8	1.4 ± 0.4	64.5 ± 3.8	1.3 ± 0.4	5.9 ± 0.6	111.6 ± 4.7	7.0 ± 1.0
Test 2	37.1 ± 11.2	73.4 ± 4.8	1.5 ± 0.7	65.6 ± 2.8	1.5 ± 0.7	5.5 ± 0.6	100.2 ± 12.6	7.3 ± 0.9

Ancient remedies in traditional medicine in China as well as other countries have been issued for the prevention and treatment of COVID-19.[21] Recent clinical data also show the effectiveness of traditional medicine in the treatment of COVID-19.[22-25] Inheriting the knowledge of traditional medicine combined with modern medicine on the pathogenic mechanism of SARS-CoV-2 virus, scientists of the Vietnam Academy of Sciences have developed the herbal medicine Vipdervir used to prevent and support the treatment of COVID-19. Vipdervir contains medicinal herbs with the main active ingredients: andrographolide, saikosaponin, rutin, quercetin, gingerol, glycyrrhizic acid, and baicalein that have high affinity for target molecules involved in the pathogenic mechanism of SARS-CoV-2 including molecular targets on the viral surface and molecular targets on host cells. The target molecule on the surface of the SARS-CoV-2 virus 2, the Spike (S) protein. In the early stages of infection, the S protein binds to angiotensin-converting enzyme 2 (ACE2) host receptor and adheres to the cell membrane. Spike is essential for the virus to enter the host cells.[26] S Protein consists of two subunits: the S1 and S2 subunits. S1 has a role in receptor binding, while S2 has a role in membrane synthesis. More specifically, S1 combines with the cognate receptor to induce a dramatic structural change in S2, leading to fusion of the viral envelope and host cell membrane, followed by release of the nucleocapsid into the cytoplasm.[27] By this mechanism, targeting the S protein has the potential to block the entry of SARS-CoV-2 virus into host cells thereby stopping the infection process in the first step. Molecular targets on host cells include: ACE2 receptor, papain-like protease (PLpro), chymotrypsin-lile protease (3CLpro), RNA-dependent RNA polymerase (RdRp), Helicase, Endoribonuclease. SARS-CoV-2 can enter ACE2-expressing cells but not ACE2-expressing cells or cells expressing other coronavirus receptors, such as aminopeptidase N and dipeptidyl peptidase 4, indicating that ACE2 is the cell receptor for SARS-CoV-2.[28] PLpro is a protease responsible for the translation of viral genetic material into polypeptides of structural and non-structural proteins, which play an important role in replication, assembly, and generation new viruses.[29] 3CLpro is one of the important proteases for RdRP generation, replication and viral infection. It is among popular target against SARS-CoV-2 that has received major attention from researchers.[30] RdRp is an essential enzyme for viral replication because it catalyzes viral RNA synthesis.[31] During viral RNA replication, RdRP determines the fidelity and the rate of replication and mutation of the virus to facilitate adaptation of virus to the environment and even to the new host, thereby influencing the evolution of the virus.[32] Because it plays an important role in the virus life cycle, it has long been a target of interest to many researchers.[31,33,34] Helicase (nsp 13) is another important enzyme for viral replication that has been identified as a potential therapeutic target.[35] Heliscase of SARS-CoV-2 has NTPase and RNA helicase activity and can hydrolyze all NTPs and unwinding of double stranded RNA in an NTP-dependent manner. In agreement with the drug development theory of Vipdevir, experiment data on Vipdervir's ability to inhibit H5N1 and SARS-CoV-2 viruses show that Vipdervir at concentrations from 3 mg/mL is capable of completely inhibiting H5N1 and Vipdervir at a dose of 50 µg/mL has the ability to inhibit the development of SARS-CoV-2 virus at viral concentrations 1 PFU to 100 PFU. Our data are consistent with previous research data showing that herbal medicines may has antiviral activity[36,37] that are effective in the prevention and treatment of Covid 19.[24,38-40] However, Vipdervir's mechanism of inhibiting H5N1 and SARS-CoV-2 viruses is still unknown. Further studies are needed to clarify the mechanisms underlying Vipdervir's inhibition of H5N1 and SARS-CoV-2 viruses.

In the present study, in addition to evaluating the antiviral effect of Vipdervir, we also evaluated the toxicity of the drug by testing acute toxicity and repeated dose toxicity. The results of the acute toxicity test of Vipdervir showed that at oral doses up to 43 capsules/kg (equivalent to 23650 mg/kg b.wt.) no mice died, no signs of toxicity were detected. The maximum oral dose of 64 capsules/kg (equivalent to 35200 mg/kg b.wt.) all mice sweated, 2 out of 10 test mice had seizures and no mice died. Based on the Globally Harmonised System of Classification and Labelling of chemical, with an LD_{50} greater than 35200 mg/kg Vipdervir can be classified under category-5. Therefore, it can be concluded that Vipdervir can be safely used in oral formulations. The results of repeated dose toxicity test of Vipdervir show ed that Vipdervir did not produce any alteration in the hematological parameters, clinical biochemistry and normal weight gain of rabbits. Hematological and biochemical indicators play an important role in determining drug-induced toxicity.[41] Change in hematological and biochemical indicators warns of adverse effects of the drug on the relevant organs in the body. Consistent with the biochemical and hematological results, the histopathological results also showed that Vipdervir did not induce adverse effects on the structure of liver and kidney tissues. There were no signs of toxicity with respect to hematology, clinical chemistry, gross and histopathological examinations noted in Vipdervir group in day 42, it indicated that Vipdervir did not produce delayed onset of toxicity. Based on repeated dose oral toxicity results, the No Observed Adverse Effect Level (NOAEL) of Vipdervir is greater than 1.116 capsules/kg/day (equivalent to 613.8 mg/kg/day).

CONCLUSION

Vipdervir capsules, herbal medicine, showed antiviral activities against H5N1 and SARS-CoV-2. Oral toxicity data indicated that Vipdervir was relatively safe in the experimental conditions. Present study suggest that Vipdervir capsules might be potential candidate for supporting or treatment of viral diseases.

CONFLICTS OF INTEREST STATEMENT

All authors declare they have no conflicts of interest in the conduct and reporting of research.

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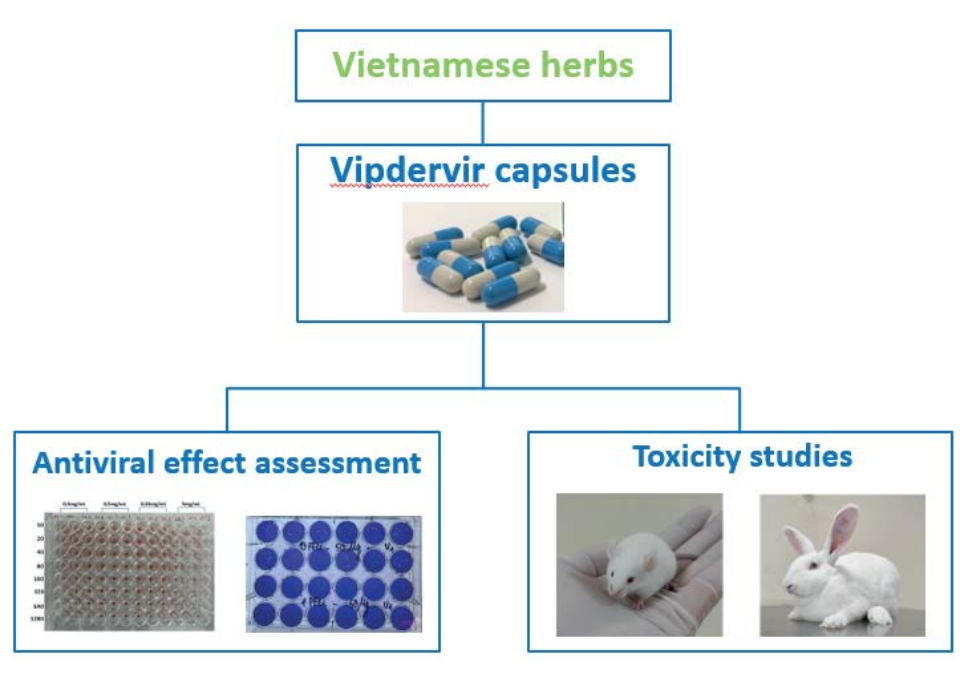
GRAPHICAL ABSTRACT



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