NEW FORMULATION EFFECTIVE AGAINST SARS CoV-2 AND H1N1

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People apply a wide variety of over-the-counter treatments as food supplements against respiratory tract infections. In this study, we investigated the antiviral efficacy of piperine, capsaicin, and glycyrrhetinic acid (GA) against coronaviruses (SARS-CoV-2) and Influenza viruses (H1N1), which are associated with acute respiratory infections. TCID50 concentrations of SARS-CoV-2 and H1N1 isolates were determined. Then, the effectiveness of phytochemicals against these viral isolates at non-toxic concentrations was investigated. For this purpose, the MTT method and RT-PCR techniques were used. Non-toxic concentrations of Capsaicin, Piperine, and GA on Vero cells were determined as 0.625 µg/ml, and Ceramide at 1.25 µg/ml and lower concentrations. It was determined that Capsaicin and Piperine showed very significant antiviral activity against SARS CoV-2 when compared to standard drugs at concentrations of 0.312 µg/ml. The effectiveness of GA was determined to be lower (0.625 µg/ml) than these two phytochemicals. Capsaicin, piperine, and GA exhibited antiviral activity on SARS-CoV-2 and H1N1 viral replication, especially the activity of the combination of capsaicin plus piperine against SARS CoV-2 was stronger than the activity against N1N1 than against H1N1. In addition, it was determined that the triple combination of these components exhibited stronger antiviral activity than single and double combinations, resulting in a significant decrease in the number of viral copies. We think that this new formulation containing three different phytochemical compounds is effective against SARS-CoV-2 and influenza viruses, which are the most important causes of morbidity and mortality from respiratory tract viruses, and can be effective, especially for prophylactic purposes.

Keywords: SARS CoV-2, phytochemicals, influenza virus.

INTRODUCTION

Piperine is a phytochemical found in the fruits and roots of plants such as *Piper nigrum* L. and *Piper longum*. It has very rich pharmacological activities such as antimicrobial, anti-inflammatory, immunosuppressive, anti-cancer, neuroprotective, and antioxidant effects (Derosa *et al*., 2016; Haq *et al*., 2021; Imran *et al*., 2022).

Capsaicin is a compound found in chili peppers that has burning and irritating properties. Capsaicin is the phenolic responsible for hot peppers' characteristic taste and pungency (8-methyl-N-vanillyl-6-none amid). Capsaicin (N-vanillyl-8-methyl-6-(E)-none amide) is a unique and important compound from the group component of capsaicinoids. This component is found only in plants of the genus Capsicum (Srinivasan, 2016; Wang *et al*., 2022).

It is the primary source of chili pepper's pungency or bitterness. Traditionally, capsaicin has been used to relieve pain. Recently, some studies have shown that capsaicin has important therapeutic effects on many diseases such as diabetes, hypertension, cancer, and obesity (Wang *et al*., 2022).

The use of natural products is increasing due to their low toxicity on healthy cells, and natural products have been used frequently in research against many infectious agents all over the world (Rodrigues *et al*., 2016).

One of the natural products used in folk medicine for many years is the licorice root plant. Licorice root has been used to relieve stomach ailments due to its widespread pharmacological effects. One of licorice root's most important active components (Glycyrrhiza glabra) is Glycyrrhetinic acid. Glycyrrhetinic acid is a hydrolytic product of Glycyrrhizic acid, a component of licorice root. Glycyrrhetinic acid is a compound

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obtained from licorice root extract and is a bioactive triterpene glycoside with anti-inflammatory, anti-ulcer, anti-allergic, antidote, anti-oxidant, anti-tumor, and antiviral and anti-bacterial activity (Pastorino *et al*., 2018).

Influenza viruses and coronaviruses, which have an important place in respiratory tract viruses, are important respiratory tract pathogens causing morbidity and mortality in every flu season due to the rapidity of viral replication and easy transmission. These viruses have created endemics and pandemics throughout human history. Humanity has experienced a SARS-CoV-2 pandemic, whose impact is still felt, and has been exposed to similar or even more serious pandemic and endemic influenza epidemics (Kim *et al*., 2018).

The SARS-CoV-2 pandemic is an important viral agent with very serious consequences all over the world. Although there are many drug studies today, the search for a wide variety of active molecules continues to prevent mucosal transmission against SASR CoV-2 and other respiratory pathogens due to the lack of an effective molecule and insufficient protection of vaccines (Kirtipal *et al*., 2020).

In this study, we investigated the antiviral effectiveness of a new formulation containing various phytochemicals against SARS CoV-2 and H1N1.

MATERIALS AND METHODS

Cell Culture Studies

The Vero cell line was used as cell culture in the present study. Cell cultures were incubated in RPMI-1640 broth containing 10% fetal calf serum, 10 mM HEPES, 100 IU/ml penicillin/streptomycin with 4mM glutamine, in an incubator with 5% carbon dioxide at 37 °C. The cultivation of cells was adjusted to $1x10^5$ cells per ml. Cell culture studies were carried out in 100, 200, and 500 ml culture dishes, in containers containing 10 percent of the culture vessel.

Cell cultures were incubated by waiting until cell growth covered the culture dish surface.

During this period, the medium was changed when a change in the pH of the medium was observed. Cells were removed from the culture dish surface with 0.25% trypsinization solution and incubated at 1250 rpm for 10 min. collected by centrifugation. Cell viability and number were determined by hemocytometer after staining with 1% trypan blue dye prepared in 0.9% NaCl.

Proliferation Assays

Proliferation experiments in cell culture were performed in 96-well flat-bottomed microplates. Cells were inoculated into the wells with RPMI-1640 medium containing 10% fetal calf serum, with $1x10^5$ cells per ml.

ACTIVITY STUDIES

Preparation of Cell Cultures

Firstly, non-toxic concentrations of compounds (piperine, capsaicin, and glycyrrhetinic acid) were determined in Vero cell cultures. For this purpose, the concentrations of the compounds containing different concentrations were added to the

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medium and their non-toxic concentrations on the cells were determined. Dimethyl sulfoxide (DMSO) was used to dissolve chemical compounds. Effects on cell growth were performed by evaluating both morphologically and in terms of cell viability with an inverted microscope. Antiviral activity studies were performed on non-toxic concentrations to be determined.

In cytotoxicity tests, cell density was set to $1x10^5$ cells/ml. After the cells were inoculated into the culture dishes, they were incubated for 6 hours for the cells to adhere to the culture dish surface, then the chemical compounds were added to the cells. Cultures containing only the amount of DMSO used as the solvent were selected as the control group. In addition, cultures without chemicals were evaluated as a negative control. All experiments were performed in triplicate and repeated 3 times.

DETECTION OF SARS COV-2 AND H1N1 BY SYBR GREEN RT-PCR METHOD

The activity of the compounds against SARS CoV-2 was determined by RT-PCR demonstration of viral replication in Vero cells treated with these three compounds. For this purpose, viral RNA isolations were performed by taking 300 ml of culture filtrates treated with compounds. For this, a commercially available viral RNA isolation kit was used. Subsequently, viral copy numbers were determined using primers specific for SARS-CoV-2.

(hCOVassay1 primer: Forward: 5'GCCTCTTCTCGTTCCTCATCAC 3'Reverse: 5'AGCAGCATCACCGCCATTG 3', hCOVassay2 primer: Forward: 5' AGCCTCTTCTCGTTCCTCATCAC 3' Reverse: 5' CCGCCATTGCCAGCCATTC 3'). For viral copy number determination, 500 ng, 100 ng, and 50 ng dilutions of the SARS-CoV-2 genome were made and the viral load in each sample was determined in terms of copy number (Marinowic *et al*., 2021).

The replication level of H1N1 was determined according to the method described by (Yang *et al*., 2009).

3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE (MTT) METHOD

The MTT method is a frequently used practical method for determining cell viability. MTT is a substance actively absorbed into cells and is reduced to colored, water-insoluble formazan by a mitochondrial-dependent reaction (Mosmann *et al*., 1983). The MTT-reducing property of cells is considered a measure of cell viability. The dye density obtained as a result of MTT analysis correlates with the number of viable cells. In the MTT method, the decrease in cell viability following viral inoculation was evaluated in favor of viral replication, while high cell viability was evaluated as an antiviral effect.

In the study, the highest dosage of chemical compounds determined as non-toxic was used in antiviral effectiveness tests. After adding the compounds to the cultures inoculated with SARS CoV-2, they were incubated for 96 hours at 37°C in a 5% carbon dioxide incubator. Then, 10 µl of MTT was added to each well and the plates were incubated for 4 hours under the same conditions, and absorbance measurements were made at a 570 nm spectrophotometer.

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Titrating the Virus Strains

Viral strains extracted from -80 ºC were rapidly thawed in a double boiler at 37 ºC and then incubated in cell lines for 96 hours. Then, the viral culture vessel was freeze-thawed and the cells were blasted. The cells were collected from the culture dish and centrifuged at 4000 rpm for 20 minutes and the supernatant was collected as a virus solution.

Then, the virus suspension was infected with Vero cells in 96-well flat-bottom microplates, and the infectious dose calculation was performed as stated in the literature. Vero cells were produced by inoculating ($1x10^5$ cells/ml) into 96-well microplates. When cell growth covered the surface of the wells, the medium was taken from each well and 50 µl of 10-fold diluted stock virus solution (from $10^{-1}$to 10^{-6}), prepared in Serum-free RPMI-1640 medium, was added to the cells. For viral adsorption, after incubation for 2 hours at 37 ºC, 50 µl of the medium was added to each well and incubated for 7 days. At the end of the incubation, viral dilutions were examined for the presence of CPE and noted. Viral titer was calculated by Reed and Muench method.

All experiments were performed independently of each other in triplicate (Reed and Muench, 1938; Allahverdiyev *et al*., 2004).

RESULTS

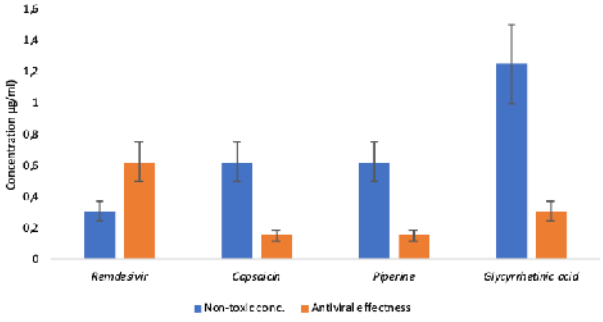

Figure 1. Concentrations of non-toxic and antiviral activity of Capsaicin, Piperine, and glycyrrhetinic acid against SARS CoV-2

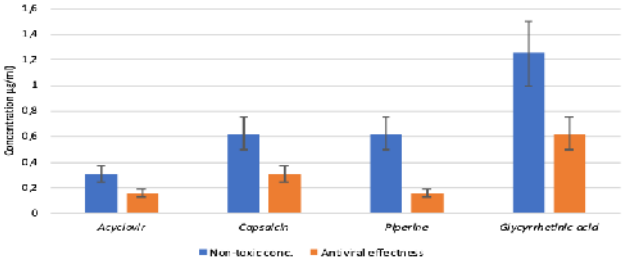

Figure 2. Concentrations of non-toxic and antiviral activity of Capsaicin, Piperine, and glycyrrhetinic acid against H1N1

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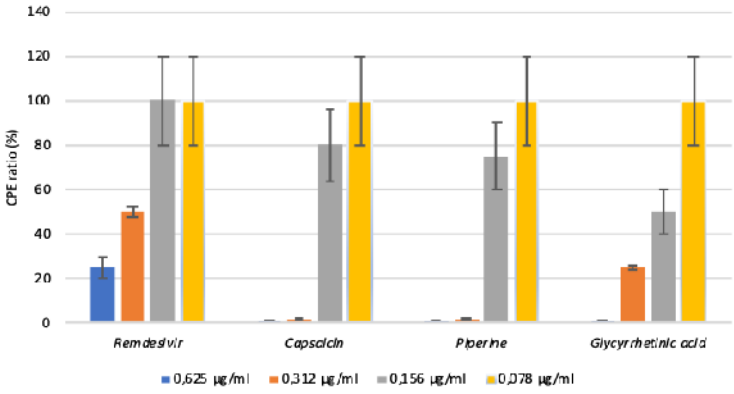

Figure 3. Evaluation of the efficacy of Capsaicin, Piperine and Glycyrrhetinic acid on SARS CoV-2 in terms of the presence of CPE

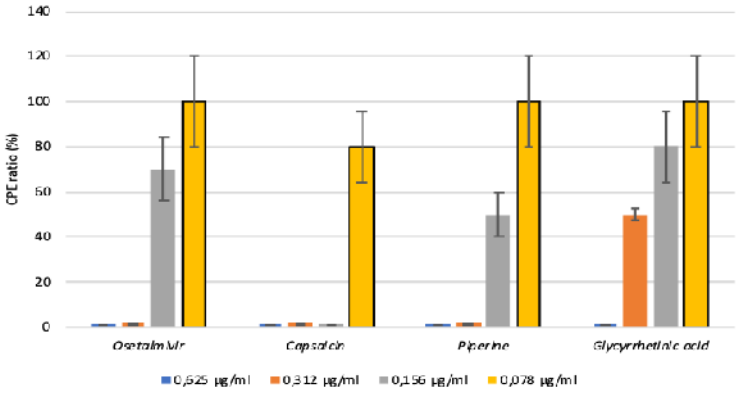

Figure 4. Evaluation of the efficacy of Capsaicin, Piperine and Glycyrrhetinic acid on H1N1 in terms of the presence of CPE

Non-toxic concentrations of Capsaicin, Piperine, and GA on Vero cells were determined as 0.625 µg/ml and 0.625 µg/ml 1.25 µg/ml, respectively. It was determined that Capsaicin and Piperine showed very significant antiviral activity against SARS CoV-2 when compared to standard drugs at concentrations of 0.312 µg/ml. The effectiveness of GA was determined to be lower (0.625 µg/ml) than these two phytochemicals.

In the study, capsaicin, piperine, and GA exhibited antiviral activity on SARS CoV-2 and H1N1 viral replication, especially the activity of the combination of capsaicin plus piperine against SARS CoV-2 was stronger than the activity against N1N1 than against H1N1. In addition, it was determined that the triple combination of these components (capsaicin, piperine, and GA) exhibited stronger antiviral activity than single and double combinations, resulting in a significant decrease in the number of viral copies.

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CONCLUSION

Capsaicin, piperine, and GA are important plant phytochemicals. These phytochemicals were found to be particularly effective against respiratory viral agents. Important respiratory pathogens such as coronaviruses and influenza viruses are viruses with a broad host spectrum that can mutate frequently. Over the years, such viral agents can lead to severe epidemics with endemics and pandemics for humans. Our study findings show that these phytochemicals, effective against these pathogens, can be used both for synthesizing new drugs and prophylaxis during epidemics in winter.

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