Investigating the Effect of Ethanol Extract of Satureja khuzestanica Jamzad on patients with COVID-19 using Real-Time PCR

Abstract

In recent years, a new variant of Betacoronavirus has been spread globally called COVID-19. It can cause severe respiratory disease or, in some cases, even death. Traditional medicines to treat this disease are not so promising. Thus, the lack of a specific treatment for COVID-19 has encouraged researchers in most countries to use medicinal plants known as antivirus medicines. In the present study, the effect of ethanol extract of Satureja khuzestanica Jamzad on patients with COVID-19 has been investigated. For this purpose, 40 patients with COVID-19 were recruited. After RNA was extracted, the samples with low CT were used to infect the Vero cell line. The infected cells were treated with different concentrations of the extract of Satureja khuzestanica Jamzad, and the cellular viability was checked using the MTT test. Moreover, the level of gene expression was evaluated using Real-Time PCR. As the results showed, Satureja khuzestanica Jamzad can decrease the number of coronaviruses, and the expression of corona genes under the effect of it has a significant decrease compared to that of the control group. It was concluded that Satureja khuzestanica Jamzad could kill the coronavirus, and consequently, its compounds are of antiviral properties.

Keywords: *coronavirus, COVID-19, Satureja khuzestanica Jamzad, medicinal plants*

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Introduction

Coronavirus was first identified in patients with pneumonia symptoms in December 2019 in Wuhan, China(Tan & Aboulhosn, 2020). As several studies show, it appears that bats were the original host of the new coronavirus but passed through an intermediary animal, likely an anteater to humans. Then the epidemic spread among humans Zhang, Tian, Lou, & Chen, 2020).

COVID-19 can infect all people of different ages**.** The droplets coming out through sneezing/coughing, propelled from an infected person, transmit the infection (Zou et al., 2020). The symptoms of this disease are different from one person to another, so some people may have no symptoms, but some others may experience acute respiratory syndrome and disorders. Some common symptoms include cough, fever, headache, sore throat, difficulty breathing or shortness of breath, conjunctivitis, etc., which are not recognizable from other chronic respiratory infections (Chen et al., 2020b). Besides the involvement of the respiratory system in COVID-19, other complications are digestive system problems (Wang, Gao, Lou, & Zhang, 2020), loss of taste (Machado & Gutierrez, 2020), anosmia (loss of the sense of smell), kidney disorders, diabetes complications (Chen et al., 2020), cerebrovascular complications (Altable & de la Serna, 2020), cardiac complications (Driggin et al., 2020), liver complications (Liu et al., 2017) and nervous system complications (Liu et al., 2017).

The mortality rate due to COVID-19 occurs largely in the elderly and those with underlying conditions (50% to 75% of deaths). CFR of COVID-19 ranges between 4 and 11% among hospitalized adult patients, and the fatality rate, in general, is about 2-3%. The severity of the disease in children and infants is significantly less common than in adults (Li & Fan, 2020).

When a person is diagnosed with COVID-19, antiviral drugs are widely prescribed. Antiviral drugs such as interferon-alpha and ritonavir have been recommended, which are useful according to previous research. Several traditional drugs, such as Chloroquine and Hydroxychloroquine, with certain beneficial effects for COVID-19, have been tested in the laboratory. However, the clinical drug response is not very promising, and toxicity is an unavoidable problem that can cause serious side effects. The lack of a specific treatment for COVID-19 has encouraged many researchers all over the world to use medicinal plants known as antivirals in ethnopharmacology.

Alkaloids are a rich source of important chemical compounds, which can be used for the development of new antiviral agents. Alkaloids are widely present in plants, especially in seed plants (Kittakoop *et al*., 2014). Some viral diseases can be treated using these approved antiviral compounds. Most of the approved antiviral drugs are somehow directly or indirectly associated with side effects, which eventually raises the need for the development of antivirals based on natural phytochemicals. Globally, the development of antivirals is shifting toward plant-derived products as they are less toxic and have less chance of developing resistance (Alrasheid, Babiker, & Awad, 2021).

Satureja khuzestanica Jamzad belongs to the Lamiaceae family, which produces compounds called Terpenes and other types of compounds. These produced compounds are stored in the epidermal glands, leaves, stems, and reproductive organs of the plant. The chemical and physical properties of the extract of Satureja khuzestanica Jamzad are different depending on the number of its constituent compounds and phenolic compound. Eighteen compounds have been identified in this plant, including Carvacrol (29.6%), para-cymene (39.6%), Linalool (2.4%), and Gamma-Terpine (18.9%) (VA., 1999).

Some medicinal compounds extracted from Satureja khuzestanica Jamzad are Flavonoids, Rosmarinic acid (RosA), Sterols, Tannins, and Terpenoids. The main compounds from the essential oil of Satureja khuzestanica Jamzad are Myrcene, Carvacrol, Gamma-Terpinene, Terpinen-4-ol, and para-cymene. Iranian researchers have investigated the compounds from the essential oil of this plant in different regions. They found that in each of the habitats of this plant species, carvacrol is the most abundant constituent of the essential oils, and it exists in up to 90% of all of them(Farsam *et al*., 2004; Oliveira *et al*., 2012). carried out a study to investigate the antifungal and antibacterial effects of different compounds of Summer savory and Satureja khuzestanica Jamzad. They identified 22 and 21 compounds in summer savory and Satureja khuzestanica Jamzad, respectively. The main constituents of essential oil of summer savory were carvacrol (36.56%), gamma-terpinene (75.24%), and para-cymene (81.5%), and the main constituents of essential oil of Satureja khuzestanica Jamzad were carvacrol (69.62%), gamma-terpinene (25.9%) and para-cymene (36.8 percent). Therefore, they reported that the essential oils of both plants are antimicrobial. Sultanzadeh and colleagues (2018) investigated the antimicrobial, antioxidant, cytotoxic, and apoptosis activity of Satureja khuzestanica Jamzad. They showed that the methanol and ethanol extracts of this plant are of good inhibitory activity against gram-negative (Escherichia coli ATCC 35218) and gram-positive (*Bacillus subtilis* ATCC 6633). The antiviral effect of this plant has been investigated in different countries. On the one hand, COVID-19 is an epidemic, and the rate of its death is high, but there is no proper drug with high efficacy to treat it. On the other hand, the number of research on plant extracts and reports on the antiviral properties of some plants, such as savory plants, is increasing. Considering these facts, this research is to investigate the effect of the ethanol extract of Satureja khuzestanica Jamzad on COVID-19 using Real-Time PCR.

Method

In the present study, 40 samples of COVID-19 were collected from the corona section of Amir Al-Momenin, Sina, and Beheshti hospitals located in Maragheh. The samples were transferred to the laboratory for viral RNA extraction, and then Real-Time PCR was done. In addition, in this study, the Vero cell line - established from the kidney of an African green monkey -was used. It was prepared from the cell bank of the Research Institute of Genetic Engineering and Biotechnology.

Viral cell culture

Vero cells were used to culture the COVID- 19 virus. These cells were prepared from the Laboratory of Virology of Health University, Tehran University of Medical Sciences. RPMI1640 medium (10% FBS) was used to culture cells. First, the cells were incubated at 37°C with 5% CO2, and a cell monolayer was generated. The Covid-19 virus was inoculated. The infected flask was incubated for 2 hours at 37°C until the viruses were completely absorbed by the cells. Then, 10 ml of complete culture medium was added, and incubation was repeated. Once more than 80% of the cells were infected by the cells (after 24 h), the viruses were harvested and titrated using $TCID_{50}$.

Preparation of essential oil of Satureja khuzestanica Jamzad

The Satureja khuzestanica Jamzad was collected from the khorramabad and Khuzestan regions from June to August. After washing, it was dried and ground. The powder was incubated in alcohol for 15 days. Then mixing was done using a shaker at 25°C for 48 hours at 120 rpm. After passing through the filter, the extract was transferred into a rotary device at 40°C so that the excellent solvents and concentration were removed. 400 µl of the extract was filtered using a 0.22 µm filter to prepare 1 mg of the dry matter of the resulting extract. Then, 100 µl of 1% dimethyl sulfite was added to dissolve the extract completely. The needed patches were prepared based on the original stocks.

MTT test to measure the cytotoxic effect of the extract of Satureja khuzestanica Jamzad

18 mg of MTT powder was dissolved in 5 ml of RPMI medium, and it was used for the test. The cells were transferred to the wells of the 96-well plate containing 100 µL complete cell culture media. They, then, were incubated for 24 hours at 37°C. Different concentrations (125, 250, 500, 1000, 2000, and 3000 µg/ml) of the extract of Satureja khuzestanica Jamzad were added to the wells. An hour after the incubation, 48 µL of MTT solution was added to the wells and incubated for 3 hours. In the end, 100 µL of DMSO was added to each of the wells to dissolve the formazan completely. The optical absorption of each well was checked using ELIZA Reader at a wavelength of 570 nm.

RNA extraction and Real-Time PCR

Viral RNA extraction was done using the CIVID-19 One-step RT-PCR kit (Pishtaz Teb, Iran) according to its protocol. The overall quality of an RNA extracted was determined using electrophoresis on Agarose gel. The probe and primer mix of this kit targets the conserved genomic sequences of the RdRp region and the nucleocapsid (N) protein simultaneously.

Once the liquid enzyme mixture was prepared according to the instructions of the kit, 9 µL of the enzyme mixture and 1 µL of the primer-probe RdRp/N/ICON/FAM/HEX/ROX were mixed. Then 5 µL of the RNA template sample was added. The final volume of the reaction increased to 20 µL per vial using water. The obtained result was placed inside a Real-Time PCR machine, and the channel (region RdRp) FAM and (gene N) HEX were selected to detect COVID-19. The channel ROX was set to detect the internal control (RNasep Gene).). The test was performed according to the temperature schedule below:

Table 1: Proper temperature and time conditions for Real-Time PCR

Phase	Temperature (°C)	Time(Min.)	Cycle
Reverse transcription	50	20	1
cDNA Initial denaturation	95	3	1
Denaturation	94	10	45
Annealing, Extention and fluorescence	55	40	45
Cooling	25	10	1

Statistical data were analyzed using SPSS PASW Statistic22 software and student's t-test. Graph drawing was done using GraphPad PRISM Version 6.01 software. P value less than 0.05 was considered to be significant ($P<0.05$).

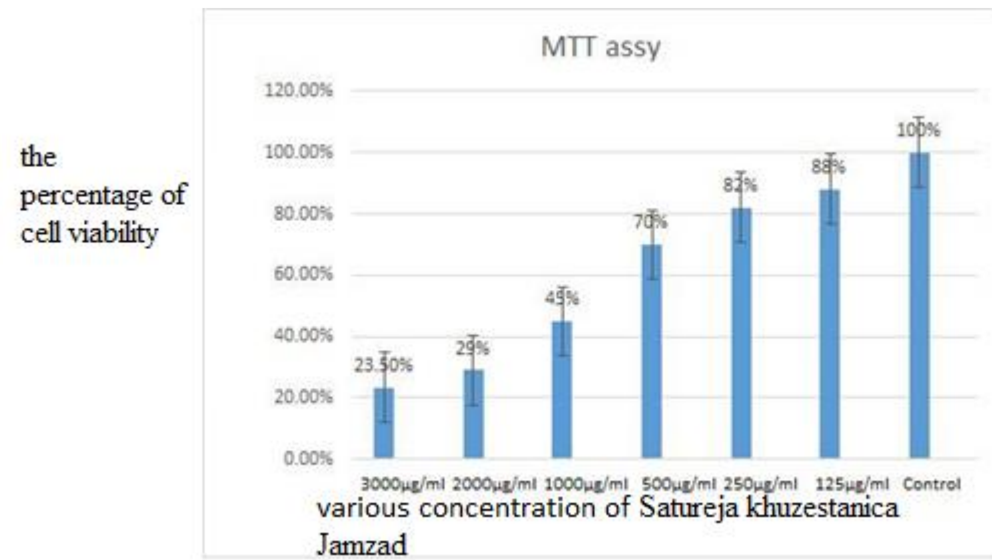
Results

Once the extract of Satureja khuzestanica Jamzad was prepared, the following formula was used to calculate the percentage of the obtained extract, which showed an amount equal to 7.5%.

The percentage of the obtained extract= $\frac{\textit{The weight of the obtained extract per gram}}{\textit{the weight of the used plant per gram}} \times 100$

The results of the MTT test

The percentage of cell viability obtained from the MTT test is shown in Figure 1. 3 independent values ± SD were used for this aim. It was found that the extract of the Satureja khuzestanica Jamzad had a better performance at a concentration of 1 mg/ml and the percentage of cell viability at this concentration was equal to 45%, which indicated the better effect of this concentration on the treatment of coronavirus.



Graph 1: the percentage of cell viability after treatment with different concentrations of Satureja khuzestanica Jamzad

Electrophoresis for PCR product

Agarose gel electrophoresis was used to determine the quality of RNA extracted from the samples, and the specific band related to the understudied gene was observed (Figure 1).

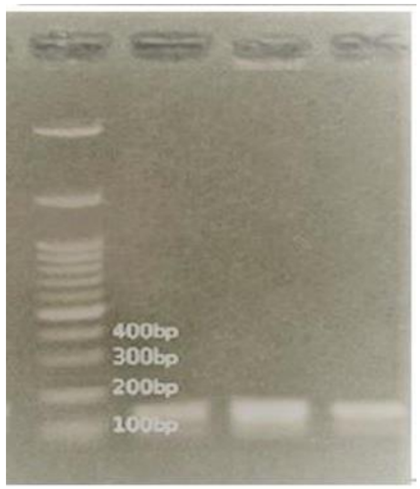


Figure 1: Generated bands from PCR product on Agarose gel

The results of Real-Time PCR

The level of Rdrp and N gene expression was evaluated using Real-Time PCR in the samples treated with the extract of Satureja khuzestanica Jamzad and the samples of the control group in order to investigate the effect of the extract of this plant on the treatment of COVID-19. According to the proliferation curves obtained after the effect of the extract on the expression of 2 genes, at CT<25, the samples had entered the log phase in lower cycles, which reflected the high level of these genes' expression (Graph 2). In CT>25, the samples entered the log phase in higher cycles, which reflected that the level of gene expression in this CT was low (Graph 3).

The results of investigating changes in the expression of RDRP and N genes in the COVID-19 virus treated with the extract of Satureja khuzestanica Jamzad

As the results from the samples treated with the extract of Satureja khuzestanica Jamzad showed, the expression level of RDRP and N genes in the COVID-19 virus at a concentration of 1 mg/ml had a significant decrease compared to the control group (P< 0.0001). According to Tables 1 and 2, the average Cts of the treated group was higher than that of the control group, which showed a decrease in the number of viruses in the first group. In the treatment using a concentration of 1 mg/ml of the plant, a double expression reduction in the understudied genes was observed, which was a sign of the death of the COVID-19 virus. Graph 4 shows the level of changes in the expression of the genes of COVID-19 in the control and groups treated with Satureja khuzestanica Jamzad.

Table 1: Changes in the expression of understudied genes after treatment with the extract of Satureja khuzestanica Jamzad (samples with Ct less than 25)

Group Statistics					
	Group	N	Mean	Std. Deviation	Std. Error Mean
Ct	Treated	21	30.048	2.9954	.6537
	Control	21	21.738	2.3907	.5217

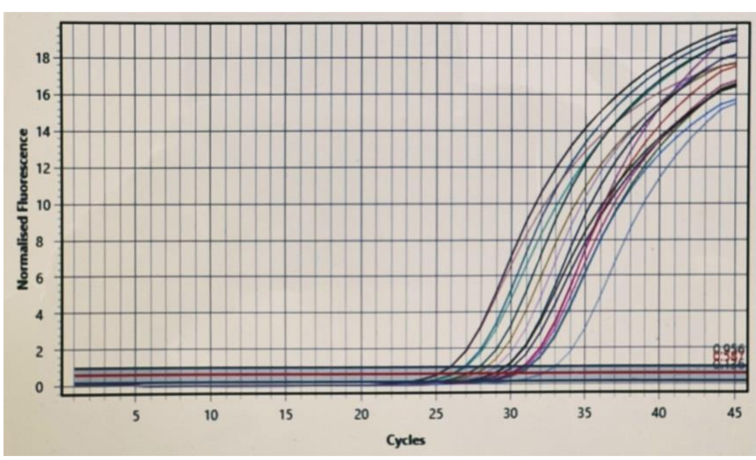
		t-test for equality of means			**Levene's test for equality of variances**					
		F	Sig.	T	df	Sig.	Mean difference	Std. error difference	95% confidence interval of the difference	
									Lower	upper
ct	**Equal variances**	.572	.454	9.936	40	.000	8.3095	.8363	6.6193	9.9998

	assumed/or assumed			9.936	38.125	.000	8.3095	.8363	6.6193	10.0024

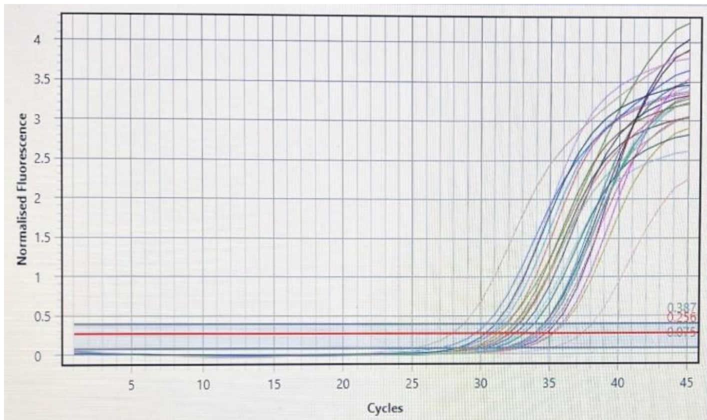
Table 2: Changes in the expression of understudied genes after treatment with the extract of Satureja khuzestanica Jamzad (samples with Ct more than 25)

Group Statistics					
	Group	N	Mean	Std. Deviation	Std. Error Mean
Ct	Treated	18	33.889	1.8986	.4475
	Control	18	28.167	1.7405	.4102

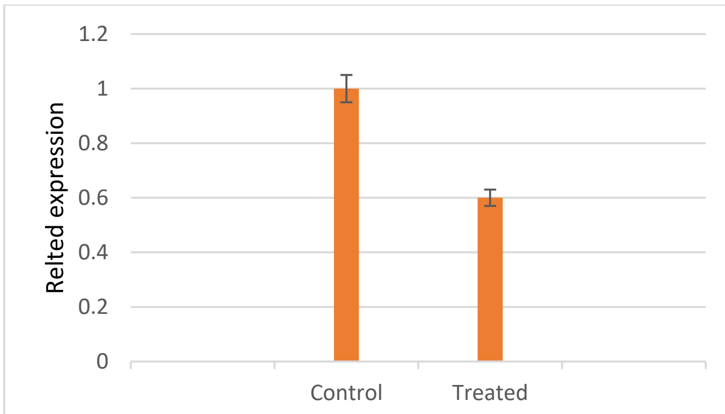
		t-test for equality of means			**Levenes test for equality of variances**					
		F	Sig.	T	df	Sig.	Mean difference	Std. error difference	95% confidence interval of the difference	
									Lower	upper
ct	**Equal variances assumed/or assumed**	1.281	0.266	9.426	34	.000	5.7222	.6071	4.4885	6.9560
				6.426	33.746	.000	5.7222	.6071	4.4881	6.9563



Graph 2: Exponential amplification diagram in Real-time PCR in samples with CT<25 related to Rdrp gene



Graph 3: Exponential amplification curve in Real-time PCR in samples with CT>25 related to Rdrp gene



Graph 4:

The level of changes in the expression of COVID-19 genes in the control and groups treated with Satureja khuzestanica Jamzad

Discussion and Conclusion

To now, millions of people around the world have been infected with the coronavirus, and the case fatality rate is estimated at around 7% in hospitalized patients [4] and 2.3% overall (Richardson *et al*., 2020). Since this disease has a high prevalence, the health of the community is difficult to improve. Thus, maintaining the health of the immune system and finding effective treatments to boost the immune system are very important (Cunningham-Rundles *et al*., 2011). The drugs used to treat covid-19 infection are not very promising regarding their clinical response, and toxicity is an unavoidable problem that may cause serious complications (Otitoloju et al., 2020). Moreover, the development of antiviral drug resistance against present antiviral agents necessitates using new effective compounds against viral infections (Chattopadhyay *et al*., 2009). In the past two decades, the modern pharmaceutical policy has dominantly moved towards using medicinal plants and treating diseases with natural compounds (Dai & Mumper, 2010). Using such medicines to treat diseases is increasing because of their better efficacy, fewer side effects, relatively low cost compared to chemical medicines as well as their ability to target different cell signaling pathways (Hasanpourghadi, Pandurangan, & Mustafa, 2017).

The essential oils extracted from some species of the savory plant show a wide range of biological antibacterial, antifungal, and antiviral activities (Behravan et al., 2004). Thus, this study investigated the antiviral activity of the ethanol extract of Satureja khuzestanica Jamzad and its effect on coronavirus in 40 samples of patient**s** with COVID-19; as results showed, in samples with Ct<25, the level of viral gene expression in cases treated with the ethanol extract of Satureja khuzestanica Jamzad, a significant decrease was observed compared to the control samples ($p<0.05$) so that the value of Cts of the treated samples was more than that of the samples in the control group. Moreover, examining samples with Ct>25 in the group treated with the ethanol extract of the same plant showed that the level of gene expression had a significant decrease compared to the control group ($p<0.05$). In this case, the level of Cts in the treated group was higher compared to the control group, which reflected the death of the COVID-19 virus in the samples treated with the ethanol extract of Satureja khuzestanica Jamzad. Therefore, as the results of the MTT test and the results from examining the level of the gene expression of the COVID-19 virus showed, the extract of Satureja khuzestanica Jamzad in a concentration equal to 1 mg/ml killed coronavirus and had antiviral properties. These results are similar to the results from the studies investigating the antiviral effects of the extract of savory on the COVID-19 virus. For example, in their study on the effect of Satureja on patients with COVID-19, Ezaouine and colleagues found that this plant was of the potential to modulate microbiota. They reported that this extract could be used as a food supplement to modulate the body's immune system to prevent and fight against viral infections (Ezaouine et al., 2022). In another study carried out, it was shown that L.Satureja acts as a protease inhibitor in COVID-19. Thus, various phenolic compounds present in this plant could reduce the activity of the SARS-Cov-2 protease enzyme significantly and have an antiviral function. In the present study, the MTT test was used to show that the extract of Satureja khuzestanica Jamzad at a concentration equal to 1 mg/ml could kill the COVID-19 virus probably due to the effect on the protease enzyme of this virus, which inhibits its function. Qamar and colleagues used a molecular docking approach in their study to investigate the structures with high potential in coronavirus. They reported that the structures of myristin, isoflavone, and methyl rosmarinate are very effective in curing patients with COVID-19 (ul Qamar, Alqahtani, Alamri, & Chen, 2020). In a study carried out using the same approach, it was found that Stilbene compounds were effective in treating coronavirus. did a study on the effect of 3 plant extracts (Garden Thyme, fennel, and Hypericum perforatum) on pulmonary complications caused by the coronavirus. They found that in patients who used the extracts of these 3 plants, the speed of recovery increased, and the duration of hospitalization decreased compared to the control groups. Moreover, CRP changes were significant in both the control and experimental groups. Since the ethanol extract of Satureja khuzestanica Jamzad kills the COVID-19 virus, using it by patients may increase recovery and reduce the duration of hospitalization.

Khaerunnisa and colleagues (2020) investigated potential inhibitors of COVID-19 main protease (Mpro) from several medicinal plant compounds. They showed that nelfinavir and lopinavir were potential treatment options and kaempferol, quercetin, cynaroside, DMC, naringenin, apigenin 7-glucoside, oleuropein, curcumin, catechin, and epicatechin-gallate were the most recommended compounds, which act as potential inhibitors of COVID-19 in medicinal plants.

In a sample, the effect of adding Satureja hortensis L. (Savory) to the diet of broiler chickens to fight against the Newcastle virus and influenza virus has been investigated. These results showed that adding 5 g of savory in one kg of food ration generated a significant antiviral effect. showed the antiviral activity of the extract of savory in laboratory conditions against the bronchitis virus and its inhibitory effect on the proliferation of the same virus (Lelešius et al., 2019). In addition, the properties of this plant to inhabit gene expression were investigated. In one case, reported the inhibitory effect of Satureja khuzestanica Jamzad on the exoenzyme S, exotoxin A gene expression, secretion system, and antibiotic efflux pumps of Pseudomonas aneruginosa using Real-time PCR.

Utomo and colleagues (2020) investigated the effect of several medicinal plants and showed that citrus had the best potential as an inhibitor for developing SARS-CoV-2. Galangal, sappan wood and Curcuma came second. They all were useful in preventing COVID-19. Murugesan and colleagues (2021) examined the potential of Emblica Officinalis (amla), Phyllanthus niruri Linn. (bhumi amla), and Tinospora cordifolia to treat COVID-19 and showed that (bhumi amla) and Tinospora cordifolia (giloy) were important bioactive compounds to inhibit the Mpro enzyme activity of COVID-19. They reported that other compounds, including amritoseide, apigenin-6-C-glucosyl7-O-glucoside, pectolinarin, and astragalin, had the ability to inhibit the Mpro enzyme in COVID-19.

There are various antiviral compounds in Satureja khuzestanica Jamzad, and much research has been done on it, which shows the beneficial properties of the plant in killing different viruses. Thus, in this study, its antiviral properties on the COVID-19 virus were investigated. The results of MTT studies and gene expression analysis showed that Satureja khuzestanica Jamzad killed the COVID-19 virus, so the extract of this plant can be used as an effective compound to treat or reduce the severity of corona disease. For future studies, it is suggested to investigate the effect of other plant compounds and their synergistic effect on inhibiting the proliferation of the COVID-19 virus and treating the infectious disease caused by this virus.

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