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Investigation of Cytotoxic Effects and Antiviral Efficacy of Six Medicinal Plants against SARS-CoV-2

Altı Tıbbi Bitkinin Sitotoksik Etkileri ve SARS-CoV-2'ye Karşı Antiviral Etkinliğinin Araştırılması

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Abstract

Aim: Today, the COVID-19 pandemic, which causes deaths in 224 countries around the world, continues to show its effect all over the world. However, unfortunately, there are few studies that determine the effect of natural products derived from plants on COVID-19. However, as it is known, the source of most drugs is plants and medicinal aromatic plants have been used frequently for therapeutic purposes since the existence of humanity. The aim of this study is to investigate the cytotoxic effects of six medicinal plants such as Licorice (*Glycyrrhiza glabra*), Saffron (*Crocus sativus L.*), Black Cumin (*Nigella sativa L.*), Laurel (*Lauris nobilis*), Buckwheat (*Lavandula stoechas*) and Zahter (*Thymbra spicata L. var. spicata*) and their antiviral activities against SARS-CoV-2 in vitro conditions.

Material and Method: This study was carried out in two stages. In the first stage, plants were collected and extracts were obtained. At the beginning of the second stage, cytotoxic effects on vero cells at non-cytotoxic broad-spectrum concentrations against SARS-CoV-2 in cell culture of six medicinal plants were investigated. In this step, the concentration of six ethnobotanically important medicinal plants that were not cytotoxic to SARS-CoV-2 was determined. In the continuation of the second stage, the plants were evaluated for the determination of viral replication inhibition and their antiviral effectiveness against SARS-CoV-2. In this step, in vitro antiviral effects of plants against SARS-CoV-2 were determined at a concentration that did not show cytotoxic effects..

Results: The concentration of six plants used in the study without cytotoxic effects was determined.. Among the plants examined, it was determined that the only plant that was effective against SARS-CoV-2 in vitro conditions was the licorice plant (*Glycyrrhiza glabra*). The licorice plant was found to inhibit SARS-CoV-2 in vitro at the 2nd dilution (1:4) after the initial concentration.

Conclusion: According to the findings obtained from our study, it was determined that the licorice plant was effective against the SARS-CoV-2 in vitro conditions. Supported by further studies, it can be thought that our findings may contribute to the fight against the COVID-19 pandemic.

Keywords: SARS-CoV-2, Antiviral Efficacy, Plant Extract, Licorice (*Glycyrrhiza glabra*), Saffron (*Crocus sativus L.*), Black Cumin (*Nigella sativa L.*), Laurel (*Lauris nobilis*), Buckwheat (*Lavandula stoechas*), Zahter (*Thymbra spicata L. var. spicata*).

Öz

Amaç: Bugün dünya genelinde 224 ülkede ölümlere neden olan COVID-19 salgını tüm dünyada etkisini göstermeye devam etmektedir. Ancak bitkilerden elde edilen doğal ürünlerin COVID-19 üzerindeki etkisini belirleyen malesef az sayıda çalışma bulunmaktadır. Ancak bilindiği üzere çoğu ilaçların kaynağı bitkilerdir ve tıbbi aromatik bitkiler insanlığın varoluşundan bu yana tedavi amaçlı sıkça kullanılmıştır. Bu çalışmanın amacı, Meyan (*Glycyrrhiza glabra*), Safran (*Crocus sativus L.*), Çörek otu (*Nigella sativa L.*), Defne (*Lauris nobilis*), Karabaş (*Lavandula stoechas*) ve Zahter (*Thymbra spicata L. var. spicata*) gibi altı tibbi bitkinin sitotoksik etkileri ve SARS-CoV-2' ye karşı antiviral etkinliklerini in vitro koşullarda araştırmaktır.

Gereç ve Yöntem: Bu çalışma, iki aşamada gerçekleştirildi. İlk aşamada bitkiler toplandı ve ekstraktlar elde edildi. İkinci aşamanın başlangıcında, altı tıbbi bitkinin hücre kültüründe SARS-CoV-2'ye karşı geniş spektrumlu konsantrasyonlarda Vero hücreleri üzerindeki sitotoksik etkinlikleri incelendi. Bu aşamada, etnobotanik açıdan önemli altı tıbbi bitkinin SARS-CoV-2 üzerine sitotoksik olmadığı konsantrasyon belirlenmiştir. İkinci aşamanın devamında, bitkilerin viral replikasyon inhibisyonunun belirlenmesi ve SARS-CoV-2' ye karşı antiviral etkinlikleri değerlendirildi. Bu aşamada, bitkilerin sitotoksik etki göstermeyen konsantrasyonda SARS-CoV-2' ye karşı in vitro koşullarda antiviral etkileri belirlenmiştir.

Bulgular: Çalışmada kullanılan altı bitkinin sitotoksik etkisi olmayan konsantrasyonu belirlendi. İncelenen bitkiler içerisinde SARS-CoV-2' ye karşı in vitro koşullarda etkili olan tek bitkinin meyan bitkisi (*Glycyrrhiza glabra*) olduğu belirlendi. Meyan bitkisinin, başlangıç konsantrasyonunun ardından 2. seyreltmede (1:4) SARS-CoV-2' yi in vitro koşullarda inhibe ettiği tespit edilmiştir.

Sonuç; Çalışmamızdan elde edilen bulgulara göre, Meyan bitkisinin in vitro şartlarda SARS-CoV-2' ye karşı etkili olduğu belirlenmiştir. Bulgularımızın daha ileri çalışmalar ile desteklenerek COVID-19 pandemisi ile mücadeleye katkısı olabileceği düşünülebilir.

Anahtar Sözcükler: SARS-CoV-2, Antiviral Etkinlik, Bitki Ekstraktı, Meyan (*Glycyrrhiza glabra*), Safran (*Crocus sativus L.*), Çörek Otu (*Nigella sativa L.*), Defne (*Lauris nobilis*), Karabaş (*Lavandula stoechas*), Zahter (*Thymbra spicata L. var. spicata*).

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INTRODUCTION

COVID-19 is a new strain of coronavirus, and this virus family is zoonotic and can infect humans from animals. Besides, the contamination is very high and spread rapidly over the world. It is seen that this virus can use human angiotensin-converting enzyme II (ACE2) effectively and can multiply in human respiratory tract cells. The virus (2019-nCoV) first appeared in the Hubei Province of China in late 2019. The agent was defined as a coronavirus that was not previously detected in humans in a group of patients presenting with pneumonia, and the name of the disease was accepted as COVID-19.[1-3] Today, the coronavirus epidemic, which has killed 481 million cases and 6.4 million deaths in 224 countries around the world, continues to increase its impact all over the world.[4]

COVID-19 treatment is primarily supportive, and the role of antiviral agents has not yet been determined. Promising approaches to COVID-19 focus on traditional medicine such as medicinal plant extracts. Traditional medicine of many countries of the world recommends some herbs for the prevention, treatment, and rehabilitation of diseases, including COVID-19.[5] Herbal remedies for various diseases have been prescribed by mankind for thousands of years. According to numerous articles, some herbs have antiviral activities and have shown positive effects in practically treating many viral infections. Herbs that act as antiviral agents to treat viral infections have been applied or prescribed as supportive therapy.[6]

Herbal and traditional medicines have been used since the first days of the COVID-19 epidemic in China. These traditional medicines have been shown to result in 90% recovery of 214 treated patients. Besides, some traditional herbal medicines have been reported to prevent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in healthy people and improve the health status of patients with mild, or severe symptoms.[1,7]

Medicinal and aromatic plants are very important as they contain bioactive compounds that can be used in the development of official medicines against various diseases with little or no side effects.

The purpose of current study is to investigate the cytotoxic effects of *Glycyrrhiza glabra*, *Crocus sativus*, *Nigella sativa*, *Lauris nobilis*, *Lavandula stoechas* and *Thymbra spicata var. spicata* which are medicinal plants and their antiviral activities against SARS-CoV-2 in vitro conditions.

MATERIAL AND METHOD

The study was performed in two stages. In the first stage, initially, the plant samples were collected. Then, plant extracts were obtained. The first stage of the study was performed Altınözü Vocational School of Agricultural Sciences Medicinal and Aromatic Plants Laboratory in Hatay Mustafa Kemal University. In the second stage of the study, the cytotoxic effect of the examined plant extracts and theirs effect against SARS-CoV-2 were determined in Ankara University Biotechnology Institute Laboratory and BSL3 laboratory.

Ethics Statement

The cytotoxic effect and antiviral efficacy research part of the study was carried out in a biotechnology institute laboratory with a 3rd degree biosafety level. In addition, the ethics of this study approval was obtained from the Karamanoğlu Mehmetbey University Faculty of Medicine Clinical Research Ethics Committee (Date: 26.07.2022, Decision No: 07-2022/4).

Plant Materials

The plant materials were collected from Hatay/Turkey by a botanical academic expert (HA). Then, their genus and species (scientific name) were identified and registered in the University of Hatay Mustafa Kemal, Herbarium of Centre for Implementation and Research of Plant Health Clinic (MKUBK). The assigned voucher codes for *Glycyrrhiza glabra* (MKUBK-H-0271), *Crocus sativus L.* (MKUBK-H-0276), *Nigella sativa L.* (MKUBK-H-0274), *Lauris nobilis* (MKUBK-H-0273), *Lavandula stoechas* (MKUBK-H-0275), and *Thymbra spicata L. var. spicata* (MKUBK-H-0272), respectively. The plants used in the study are in **Table 1**.

Table 1. Areas where plant material is collected in Hatay province

No	Plant name	Drog	Location of the plant
1	Licorice	root	Kumlu
2	Saffron	stigma	Kırıkhan
3	Nigella	seed	Antakya
4	Laurel	fruits	Defne
5	Laurel	leaf	Defne
6	Buckwheat	flowers	Yayladağı
7	Zahter	herbaceous herb	Altınözü

Water Extraction

The water extraction method was applied to the root of the licorice plant and the stigma of the Saffron plant. Samples were prepared as 10 g of the root in 100 ml of distilled water, and 1 g of stigma in 100 ml of distilled water. Obtained mixtures were extracted in an ultrasonic bath (ALEX MACHİNE, İstanbul/Turkey) at 28°C for 15 mi.[8,9]

Cold Pressed Extraction

The seeds of the Nigella and the fruit of the Laurel plant were extracted by the cold press method. Oil extraction was carried out using a propeller (Kocmaksan, İzmir, Turkey). A screw expeller powered by a 10 kW electric motor was used to extract oil from the seeds of the plants tested. The expellers were cleaned after each extraction. Obtained oils were filtered and stored 4°C.[10,11,12]

Essential Oil Extraction

The plants collected from nature were dried at room temperature without washing. To obtain essential oil, distillation was carried out in a Clevenger-type hydrodistillation device in Hatay Mustafa Kemal University Altınözü Agricultural Sciences Laboratory for 3 hours. The essential oil obtained was measured in ml and % ratios (v/w) were determined. It was stored in the refrigerator by putting it in sealed storage bottles.[13-15]

Cell Culture

Vero E6 (ATCC: CRL-1586) cells used in the study were maintained in DMEM medium (Sigma, USA) containing 10% FBS (Biological Industries, Israel), and 1% penicillin/streptomycin (Biowest, USA) at 37°C in a humidified incubator in an atmosphere of 5% CO_2. In vitro assay was carried to determine the cytotoxic effects of six plant samples. Vero cells were seeded into a 96-well plate at a density of 1×104 cell and incubated at 37°C. Vero cells were treated with plant samples at a concentration of 25, 50, 75, 100, 150, 200, 250 µl concentrations for 24 and 48 h. The cell viability was evaluated by following the MTT (3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide) reduction assay. MTT-solution (5 mg/mL in PBS) was added into each well and then incubated for 4 h at 37°C. The 100 µl DMSO was added into each well to extract the insoluble formazan crystals within the cells. The absorbance was measured at 540 nm using a microplate reader. The results represent the average values of six experiments.

Viral Infection

Vero E6 cells (ATCC: CRL-1586) were used to propagate SARS-CoV-2 and performed all cytotoxicity and antiviral tests. Infectivity tests related to antiviral assays were performed in BSL-3 facility of the Department of Virology, Faculty of Veterinary Medicine, Ankara University. To evaluate the effect of the extracts on Vero E6 cells viability, 10-fold dilutions of plant extracts were added on 90% confluent monolayers in 96-well culture plates. After 72 hours of incubation, the maximum noncytotoxic concentration (MNCC) for all extracts was determined by microscopic observation and CC50 (extract concentration that is toxic for at least half of the cells) was determined by the crystal violet uptake method. Briefly, cell monolayers were fixed and stained with a crystal violet 0.75% in 40% methanol solution and incubated for 15 minutes at 37°C.

A primary antiviral screening test was conducted by cytopathic effect (CPE) reduction assay, which involves the protection of SARS-CoV-2 caused lysis of Vero E6 cells by extracts. Briefly, in a 96-well culture plate, 0.1 moi of the virus was inoculated on Vero E6 cells. After 1 hour of adsorption at 37°C in a 5% CO_2 humidified atmosphere, cells were washed with phosphate buffer saline (PBS) and the highest non-toxic dilution (in DMEM High glucose, Gibco, Germany) detected before were added. Cytotoxicity and cell controls were included. After 72 hours of incubation at 37°C in a 5% CO_2 humidified atmosphere, cells were fixed and stained as described above. The SARS-CoV-2 activity of the extracts was evaluated by direct observation of CPE reduction.[16,17]

Statistical

The effective concentration-lethal dose relationship of plant extracts on virus infectivity was evaluated statistically by one-way analysis of variance. The SPSS Software (version 24.0, IBM Corp., Chicago, IL, USA) program was utilized for data analysis. The confidence interval was determined as $p<0.05$.

RESULTS

Primarily, it was determined that six plants whose effects were examined were not cytotoxic in various concentrations between 25 µl and 250 µl is shown as following figures [Licorice (**Figure 1**), Saffron (**Figure 2**), Black Cumin (Nigella) seed (**Figure 3**), Laurel seed (**Figure 4**), Laurel leaf (**Figure 5**), Buckwheat (**Figure 6**) and Zahter (**Figure 7**) plants], respectively. Secondly, the effects of plants against SARS-CoV-2 under in vitro conditions were investigated. Accordingly, it was determined that licorice plant (*Glycyrrhiza glabra*) was the only plant that was effective against SARS-CoV-2 only in vitro, among the plants mentioned, and it inhibited SARS-CoV-2 in vitro at the 2nd dilution (1:4) after the initial concentration (**Table 2**). This shows that it is a phytotherapy drug candidate for the safe use of its phytotoxic effect in terms of effectiveness. However, other plants were found to be ineffective against SARS-CoV-2 (**Table 2**).

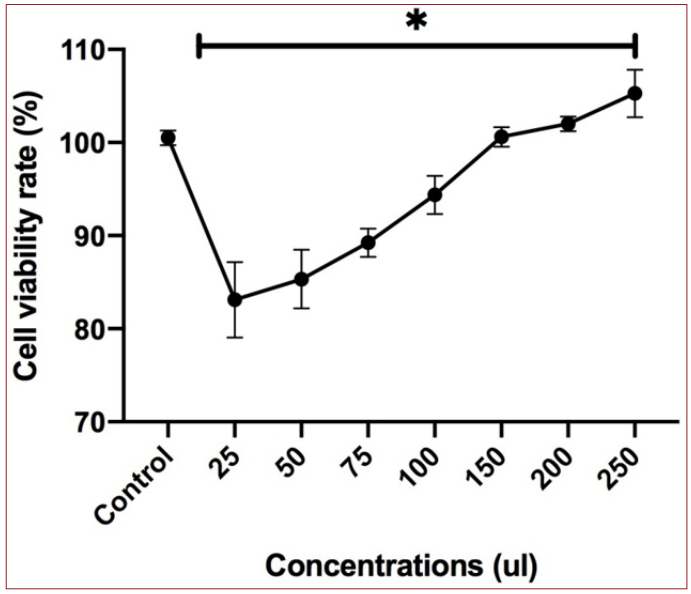

Figure 1. It shows that the Licorice plant (*Glycyrrhiza glabra* water extract) is non-cytotoxic (between concentrations 25- 250 µl, in 48th hour). * $p<0.05$.

Table 2. Antiviral (Anti SARS-CoV-2) activity screening test results for the Licorice, Saffron, Black Cumin, Laurel, Buckwheat and Zahter plants in Vero E6.

Dilutions	Antiviral Activity			Licorice[a]	Saffron[b]	Black Cumin[c]	Laurel[d]	Buckwheat[e]	Zahter[f]
	1	2	3	4	4	4	4	4	4
-1				Tox	Tox	Tox	Tox	Tox	Tox
-2 (1:2)				Tox	Tox	Tox	Tox	Tox	Tox
-3 (1:4)				**0/4***	4/4	4/4	4/4	4/4	4/4
-4 (1:8)					4/4	4/4	4/4	4/4	4/4
-5					4/4	4/4	4/4	4/4	4/4
-6					4/4	4/4	4/4	4/4	4/4
-7					4/4	4/4	4/4	4/4	4/4
-8					4/4	4/4	4/4	4/4	4/4
-9					4/4	4/4	4/4	4/4	4/4
-10					4/4	4/4	4/4	4/4	4/4
Viral Concentration	0/4	1/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4
Cell Concentration	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

Tox: Toxicity
[a]: Antiviral activity screening test results for the Licorice plant (*Glycyrrhiza glabra* water extract) in Vero E6
[b]: Antiviral activity screening test results for the Saffron plant (*Crocus sativus L.* water extract) in Vero E6.
[c]: Antiviral activity screening test results for the Black Cumin plant seeds ((*Nigella sativa L.* cold press essential oil) in Vero E6.
[d]: Antiviral activity screening test results for the Laurel plant (*Laurus nobilis* leaf essential oil and *Laurus nobilis* seeds cold press essential oil) in Vero E6.
[e]: Antiviral activity screening test results for the Buckwheat plant (*Lavandula stoechas* essential oil) in Vero E6.
[f]: Antiviral activity screening test results for the Zahter plant (*Thymbra spicata L. var. spicata* essential oil) in Vero E6.
*: The non-cytotoxic dose was determined at 75 µl. Therefore, 75 µl was determined as the initial concentration.
In the ¼ dilution of the 3rd dilution, it was determined that the Licorice plant inhibited the viral activity in vitro conditions. It was observed that other plants did not show any antiviral activity on SARS CoV-2 under in vitro conditions.

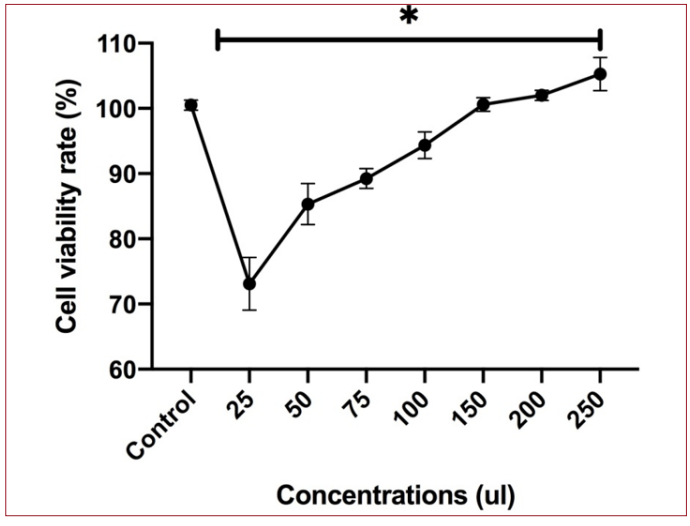

Figure 2. It shows that the *Crocus sativus L.* water extract is non-cytotoxic (between concentrations 25- 250 µl, in 48th hour). * p<0.05.

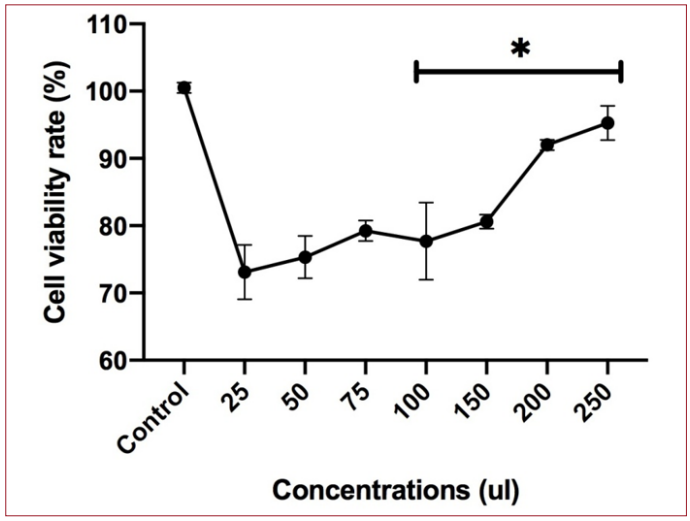

Figure 3. It shows that the *Nigella sativa L.* seed cold press essential oil is non-cytotoxic (between concentrations 25- 250 µl, in 48th hour). * p<0.05.

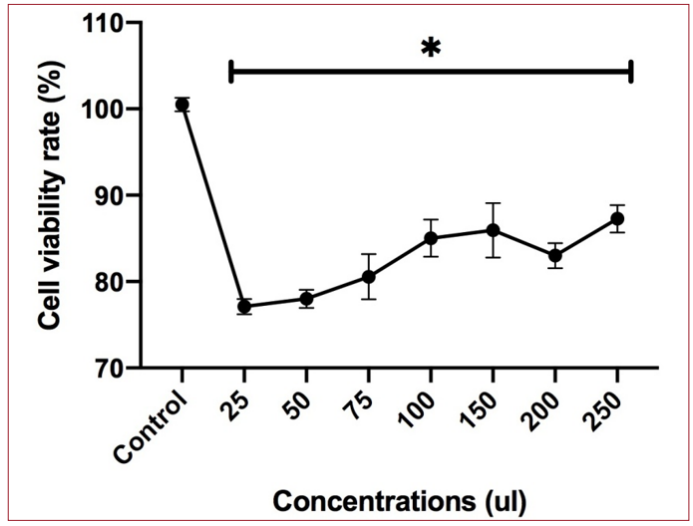

Figure 4. It shows that the *Laurus nobilis* seed cold press essential oil is non-cytotoxic (between concentrations 25- 250 µl, in 48th hour). * p<0.05.

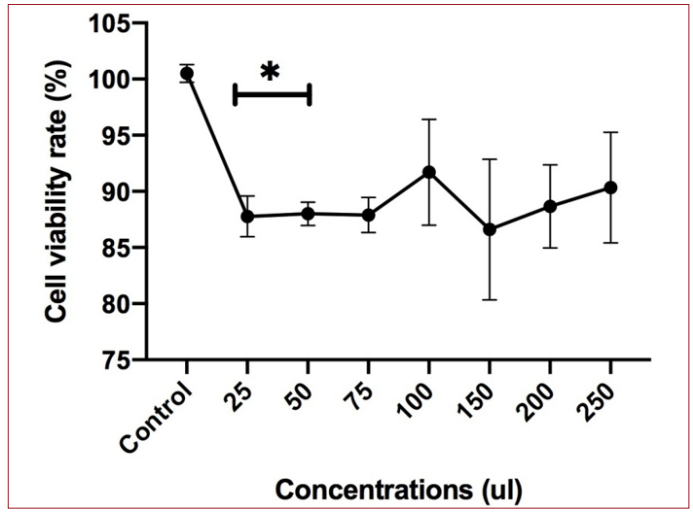

Figure 5. It shows that the *Laurus nobilis* leaf essential oil is non-cytotoxic (between concentrations 25- 250 µl, in 48th hour). * p<0.05.

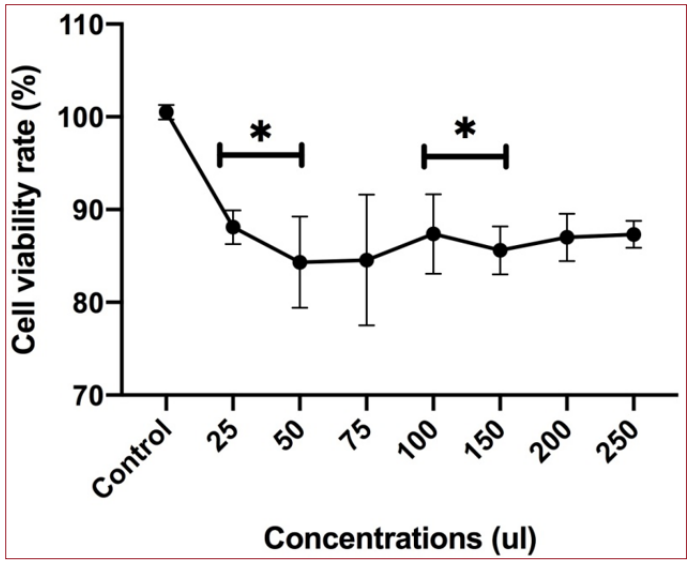

Figure 6. It shows that the *Lavandula stoechas* essential oil is non-cytotoxic (between concentrations 25- 250 µl, in 48th hour). * p<0.05.

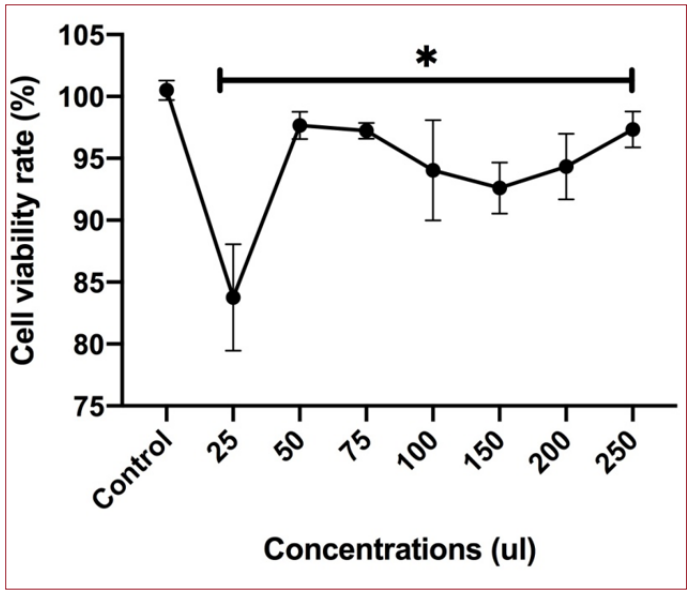

Figure 7. It shows that the *Thymbra spicata L. var. spicata* essential oil is non-cytotoxic (between concentrations 25- 250 µl, in 48th hour). * p<0.05.

DISCUSSION

In literature studies and consuming medicinal herbs such as *Allium sativum*, *Camellia sinensis*, *Zingiber officinale*, *Nigella sativa*, *Echina cea* spp. They reported that immune-enhancing herbs such as *Hypericum perforatum* and *Glycyrrhiza glabra* could be effective against COVID-19. [18] However, these plants have not been studied in vitro. [18] Among these plants, the antiviral activity of the *Nigella sativa* plant included in our study against SARS-CoV-2 has not been found. Again, from these plants, *Glycyrrhiza glabra* emerged as an antiviral agent. Although there are many studies on the antiviral effectiveness of licorice and glycyrrhizicacid, the fact that there are no widely in vitro studies on the effectiveness of SARS-CoV-2 shows the importance of this study.[19-21]

There is much literature about the use of the saffron plant as an important antiviral agent.[22-24] None of these studies, the antiviral activities for saffron extract and its main components affected, and Crocin and picrocrocin could be promising anti-HSV and anti-HIV agents for herbal treatment against vira infections.[25] In this study, the saffron plant with proven antiviral activity did not show antiviral effects against SARS-CoV-2.

In our study, the cold press essential oil obtained from these ed of the Nigella plant (*Nigella sativa L.*) was ineffective in vitro conditions against SARS-CoV-2 at all concentrations following the initial concentration (**Table 2**). Antiviral activity of *Nigella sativa* alcoholic extracts against PPRV was investigated in vitro. It showed an antiviral effect in Verocellline and at the prepared dose of 50 µg/ml. *Nigella sativa* did not show an antiviral effect against SARS-CoV-2 in this study. Also, the cold press essential oil obtained from these ed of the laurel plant (*Lauris nobilis*) was found to be ineffective in vitro conditions against SARS-CoV-2 at all concentrations following the initial concentration (**Table 2**).

The essential oil obtained from the leaf of the Laurel plant (*Lauris nobilis*) was ineffective in vitro conditions against SARS-CoV-2 at all concentrations following the initial concentration (**Table 2**). Leaf essential oil has a wide range of bioactive properties due to its bioactive function such as antimicrobial, antifungal, antioxidant, antiviral, pesticide and food applications.[26] However, in this study, *Laurus nobilis* did not show an antiviral effect against SARS-CoV-2 (**Table 2**).

The essential oil obtained from the flower of the Buckwheat plant (*Lavandula stoechas*) was ineffective in vitro conditions against SARS-CoV-2 at all concentrations following the initial concentration. Moreover, the essential oil obtained from the herb of the Zahter (*Thymbra spicata L. var. spicata*) was ineffective in vitro conditions against SARS-CoV-2 at all concentrations (**Table 2**).

The findings of this study showed that Licorice plant inhibited SARS-CoV-2 by its antiviral effect in vitro (**Table 2**). In the literature, it is stated that the Licorice plant (*Glyrrhiza glabra L.*) which has antiviral activity, can support the immune system in the treatment of COVID-19. [27] Antiviral activity has ben showed in Vero cells and in patients.[28] In a study, it has been reported that the active ingredients of Licorice root, Glycyrrhizin and Glycyrrhetinic Acid, are directly effective in reducing the spread of COVID-19. Also, it was stated that glycyrrhizin performed the effect of reducing the severity of the COVID-19 by reducing the entry points of SARS-CoV-2 into the cell and with an antiinflammatory effect. Morever, this effect is dependent[28] or independent[29] of Angiotensin Converting Enzyme-2.

CONCLUSION

Previously, medicinal and aromatic plants have been successfully used to treat many viral diseases.[30] Although many plants have been proposed that are effective against COVID-19, no in vitro studies have been found on the effectiveness of these plants to date. According to the findings obtained in this study, Licorice plant (*Glycyrrhiza glabra*) was discovered to be effective against SARS-CoV-2 in vitro conditions. It is easy to use, rapid results can be obtained in combating the epidemic. We hope that speeding up studies on other medicinal and aromatic plants to achieve faster results after this study can be valuable in combating the epidemic.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out with the permission of Karamanoğlu Mehmetbey University Faculty of Medicine Clinical Research Ethics Committee (Date: 26.07.2022, Decision No: 07-2022/4). In addition, the cytotoxic effect and antiviral efficacy research part of the study was carried out in a biotechnology institute laboratory with a 3rd degree biosafety level.

Informed Consent: No written informed consent form was required in this study.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The author has no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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