Research Article

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Antiviral Potential of Cistus L. against Infectious Bronchitis Virus and SARS-CoV-2

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ABSTRACT

Cistus L. (*Cistus*) is distributed in Türkiye by 5 species including *Cistus creticus* L. (*C. creticus*), *Cistus monspeliensis* L. (*C. monspeliensis*), *Cistus laurifolius* L. (*C. laurifolius*), *Cistus parviflorus* L. (*C. parviflorus*) and *Cistus salviifolius* L. (*C. salviifolius*). In this study, antiviral activity of 5 *Cistus* extracts were investigated against Infectious bronchitis virus (IBV) using the *in-ovo* antiviral activity assay, which measures both embryo viability and agglutination of chicken red blood cells. In further experiments, antiviral potential of *C. creticus* extract was also examined against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-infected Vero-E6 cells. *In-ovo* antiviral activity assays displayed that *C. parviflorus* and *C. monspeliensis* extracts caused more antiviral activity against IBV-D274 strain than other extracts. Further asssays showed that C. creticus extract led to dose and time dependent antiviral activity against SARS-CoV-2. The results proposed that *C. parviflorus* and *C. monspeliensis* extracts might have a potential for the treatment of virus-mediated diseases. Increasing incubation time and concentration of *C. creticus* extract led to increase of antiviral activity against SARS-CoV-2, which means that *C. creticus* extract had potent antiviral activity.

Keywords: *Cistus L.*, IBV, SARS-CoV-2, Antiviral

1. Introduction

Viral infections are one of the main reasons for human diseases as viruses target and weaken human immune systems. Synthetic antiviral drugs have been used to treat viral infections, however previous studies reported that antiviral drugs caused severe adverse effects such as hepatotoxicity, neurotoxicity, cardiovascular toxicity in addition to mild adverse effects [1-3]. Antiviral drug resistance is another critic issue, causing treatment failure and cost loss. Considering there is no existing effective and safe drug for the treatment of viral infections, virus mediated diseases possess global and vital risks for humankind [4].

Medicinal plants and food supplements protect human health and treat diseases owing to containing pharmacologically active compounds. According to a study conducted by the World Health Organization (WHO), approximately 80% of people trust traditional medicine based on medicinal plants to relieve their health problems [5,6]. Previous reports demonstrated that herbal products displayed potent antiviral activity against wide range of viruses with low adverse effects. Hence, medicinal plants and food supplements have become the focus of attention of researchers as alternatives to synthetic antiviral drugs due to the bioactive components they contain and the high side effects of synthetic drugs [7-10].

Cistus belongs to Cistaceae family with 21 species and is mainly distributed throughout in Anatolia and Mediterranean region [11]. *C. creticus, C. laurifolius, C. monspeliensis, C. parviflorus ve C. salviifolius* are the species located in Anatolia [12]. Ethnobotanical studies stated that different parts of *Cistus* species such as roots, flowers and leaves are used in folk medicine by local people to treat hyperglycemia, diabetes, peptic ulcer, diarrhea, inflammation, spasm, fever, infertility, urinary infection, snake poisoning, expectorant, hemostatic and edema in both Anatolia and Mediterranean area [13-16]. The basic reason of *Cistus* species showing a wide range of pharmacological activities is to possess many biologically active secondary metabolites (SMs) including flavonoids and polyphenolic compounds as well as terpenoids, including diterpenes, labdane-type diterpenes and chlerodanes [16-19].

Infectious bronchitis virus (IBV), the first coronavirus isolated in the 1930s, is one of the most causative agent of both highly contagious acute respiratory disease infectious bronchitis (IB) among poultry and economic losses in industrial production. IBV has a total of 32 lineages and 6 main genotypes based on the S gene [20]. D274 strain is named the European serotype possessing high spread and is potent a threat for poultry industry [21]. Mohajer Shojai et al. (2016) stated that IBV is a suitable model for other coronavirus diseases such as severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) [23]. Coronavirus disease 2019 (COVID-19), caused by the virus SARS-CoV-2, spread throughout the world in 2020 and was declared a pandemic [23]. Considering the fact that current SARS-CoV-2 epidemic, IBV model is significant and usefull for investigation of antiviral activity of molecules or extracts [22]. Limited effective antiviral drugs on virus mediated diseases lead to look for novel antiviral agents in pandemic situations such as SARS-CoV-2 to be obtained and used from plant sources that pollutes the environment less. For this purpose, in the current study firstly, determination of antiviral potential of *C. creticus, C. laurifolius, C. monspeliensis, C. parviflorus ve C. salviifolius* which are distrubuted in Izmir region against IBV-D274 strain was aimed. Secondly, considering the needs for new antiviral agents, *C. creticus* was selected for further studies, since the most abundant species with strong antiviral activity against IBV D274 was determined. From this point of view, the antiviral activity of *C. creticus* extract was investigated *in-vitro* against SARS-CoV-2 and chemical content of the extracts was determined by liquid chromatography and high-resolution mass spectrometry (LC-HRMS).

2. Material and Methods

Chemicals were purchased from Sigma-Aldrich (Darmstadt, Germany) except for cell culture reagents. Cell culture reagents were purchased from Thermo-Fisher Scientific (Loughborough, UK). Chicken IBV D274 strain (Netherlands) was kindly provided from Assoc. Prof. Fethiye ÇÖVEN. Specific pathogen-free embryo chicken eggs (SPF-ECEs) and chicken red blood cells were purchased from Izmir Bornova Veterinary Control Institute, Türkiye. The protocol for antiviral experiments was approved by the Ege University, Local Ethical Committee of Animal Experiment (approval number: 2020-051). SARS-CoV-2 studies were performed by *in-vitro* at BSL-III R&D facilities of Kocak Pharma Company (Kapaklı/Tekirdag).

2.1. Plant material

In this study, five *Cistus* L. species were collected from Izmir region to evaluate antiviral potential. *C. creticus, C. laurifolius, C. salvifolius, C. parviflorus, C. monspeliensi*s were collected and identified by Assoc. Prof. Hasan YILDIRIM and Dr. Erdinc OGUR in June 2021 with permission of Republic of Turkey Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks (approval number: 1037970339) from Izmir region. The locations of the plants and voucher numbers were shown in Table 1. Harvested leaves and shaws samples from each species were dried under open-air conditions.

2.2. Preparation of Cistus extracts

Dried and powdered leaves and shaws (10 g) *of C. creticus, C. salvifolius, C. monspeliensis, C. parviflorus* and *C. laurifolius* were extracted with 200 mL ethanol (100%), 200 mL methanol (100%), 200 mL ethanol:water (50%:50%), 200 mL methanol:water (50%:50%) using magnetic heater stirrer for 5 hours at 60°C. Then, extracts were filtered and evaporated to dryness at 50°C using a rotary evaporator. After evaporation, extracts were dissolved in 10 mL of methanol and phenol contents were measured. Extract with the highest amount of phenol compounds was dried using SpeedVac and all extracts were kept in dark conditions at +4°C until LC-HRMS analysis and antiviral experimental studies.

2.3. Total phenolic content assay

Total phenolic content assay (TPA) was carried out to determine the total phenolic component in *the C. creticus, C. salvifolius, C. monspeliensis, C. parviflorus* and *C. laurifolius* ethanol:water (50%:50%) extracts. Total phenolic contents were measured at 765 nm using modified Folin-Ciocalteu colorimetric method as described previously [24].

2.4. Determination of chemical contents of C. creticus by LC-HRMS

2.4.1. Phenolic standards

Ascorbic acid (≥99% Sigma-Aldrich), (-)-Epigallocatechin (>97% TRC Canada), (-)-Epigallocatechin gallate (>97% TRC Canada), Chlorogenic acid (≥98% In house isolated), Fumaric acid (≥99% Sigma-Aldrich), (-)-Epicatechin (≥90% Sigma-Aldrich), (-)-Epicatechin Gallate (>97% TRC Canada), Verbascoside (86.31% HWI ANALYTIK GMBH), Chicoric acid (>97% TRC Canada), Orientin (>97% TRC Canada), Caffeic acid (≥ 98% Sigma-Aldrich) Sinapinic acid (≥98% Carbosynth), Luteolin 7-glucoside (>97% TRC Canada), Rutin (≥94% Sigma-Aldrich), Hyperoside (>97% TRC Canada), Dihydrokaempferol (>97% Phytolab), Ellagic acid (>97% TRC Canada), Quercitrin (>97% TRC Canada), Myricetin (>95% Carl Roth GmbH + Co), Nepetin-7-glucoside (>97% Phytolab), Quercetin (≥95% Sigma-Aldrich), Salicylic acid (≥98% Sigma-Aldrich), Luteolin (95% Sigma-Aldrich), Nepetin (98% Supelco), Kaempferol (>90% Sigma-

Table 1. Locations of five collected *Cistus* L. species in Izmir

Taxon	Locality	Location-Elevation	Voucher Number*
C. creticus	Çeşme, Alaçatı, İzmir	N 38° 14' 00" E 25° 21' 48" 18 m	13052021 0101
C. laurifolius	Kemalpaşa, İzmir	N 38° 20' 44.5" E 27° 30' 16.6" 1050 m	13052021 0103
C. monspeliensis	Çeşme, Alaçatı, İzmir	N 38° 16' 304.0" E 026° 25' 03" 60 m	13052021 0102
C. parviflorus	Çeşme, Alaçatı, İzmir	N 38° 14' 00" E 25° 21' 48" 38 m	13052021 0104
C. salviifolius	Çeşme, Alaçatı, İzmir	N 38° 14' 00" E 25° 21' 48" 48 m	13052021 0105

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Aldrich), Apigenin (>97% TRC Canada), Hispidulin (>97% TRC Canada), Isosakuranetin (>97% Phytolab), Penduletin (>97% Phytolab), Rhamnocitrin (>97% Phytolab), Chrysin (≥96% Sigma-Aldrich), Acacetin (>97% TRC Canada).

2.4.2. Chromatographic conditions of chemical contents analyses of C. creticus

Approximately 100 mg of extract obtained from dried *C. creticus* L. species was dissolved in 3 mL of methanol:water (60:40) mixture in a 5 mL volumetric flask as described in our previous studies [25,26]. The volumetric flask was kept in an ultrasonic bath for approximately 20 minutes to obtain a clear solution. Then, 100 µL dihydrocapsaicin solution (100 mg/L stock solution) as an internal standard was added to the volumetric flask and the volumetric flask was filled with the mobile phase. The volumetric flask was mixed vigorously by hand and kept in an ultrasonic bath at 30°C for 10 minutes. After the resulting clear solution was filtered through a 0.45 µm Millipore Millex-HV filter, the sample was brought to room temperature and placed in the autosampler kept at 15°C for injection into the LC-HRMS device. Experiments were carried out by injecting 2 µL of solution from each vial into the LC-HRMS device. A Thermo OrbitrapQ-Exactive (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer was used as the LC-HRMS system, while Troyasil C18 LS (150 × 3 mm i.d., 5µm particle size) was used for chromatographic separation. For a good chromatographic separation, mobile phases consisting of water and methanol, which we optimized in our previous studies, were used [26,27]. Two different mobile phase systems consisting of 1% formic acid-water and 1% formic acid-methanol were used as Mobile Phase A and Mobile Phase B, respectively. The gradient program was started at 0-1.00 min at 50% B, 1.01–6.00 min at 100% B, and finally 6.01–15 min at 50% B. The flow rate of the mobile phase was set at 0.35 mL/min and the column temperature at 22°C. Environmental conditions were set as temperature 22.0±5.0°C and relative humidity (50±15)% relative humidity. In order to ionize small and relatively polar target compounds to a good degree in the mass spectrometer, Electrospray Ionization (ESI) source is used as an ionizing source. The device was programmed to scan ions between *m/z* 100–1100 at 70,000 resolution. The purities of the standard compounds used in the study are given in phenolic standards section. Compound identifications were determined according to the retention time values and mass spectra of each standard in the chromatographic system. Dihydrocapsaicin, which is 95% pure, was used as the internal standard in order to obtain results with low uncertainty by minimizing repeatability problems caused by external factors such as ionization in LC-HRMS measurements.

2.4.3. Method validation

Selectivity in LC-HRMS measurements is defined as monitoring only the mass pattern of the target analyte at a predetermined retention time value in the presence of other components or degradation products in the matrix [26,27]. In the applied method, it was determined that the target analytes were measured precisely and accurately identified in the matrix without any intervention. The specificity of the developed LC-ESI-HRMS method was determined by direct analysis (blind) of all the different solvents prepared, the *C. creticus* L. extract and the added target analytes.

Accuracy is a definition that expresses the closeness or best estimate of the measurement results to the true value of the amount of substance in the matrix. One of the methods used to determine accuracy is the recovery percentage of the method. This value was calculated based on the LC-ESI-HRMS data for each measurement according to the following formula:

% Recovery = Concentration recovered / concentration injected x 100.

In this study, a separate calibration curve was determined for each component to enable the quantitative determination of secondary metabolites and secondary metabolites in *C. creticus* extract by LC-ESI-HRMS. For this purpose, measurements of solutions at different concentrations were analyzed 6 times on the device and a calibration curve was obtained using values normalized with the internal standard against the nominal values of the nalites. The regression coefficient (R^2) and linear regression equation were obtained from the determined curve.

Limits of detection (LOD), limit of quantification (LOQ), were dertermined based on EURACHEM CITAC Guide and our previous studies [26-28]. Simply, it was determined as: LOD or LOQ = κSDa/b, where LOQ is 3 and κ = 3 for LOD. Since the measurmenet uncertainty was reported previously we have not discussed herein to avoid from reputation.

Detailed infromation can be found in our previous reports and international guides [26-28].

2.5. In-ovo antiviral activity against IBV

Antiviral activities of ethanol:water (50%:50%) extracts with high total phenolic content according to TPA results were performed as virucidal activity against IBV by *in-ovo.* Specific pathogen-free embryo chicken eggs (SPF-ECEs) were treated with 0.1 and 1 mg/mL of extract-IBV mixture at 37°C for 48 hours. DMSO and favipiravir were used as vehicle and positive controls, respectively as described previously [29,30]. After harvesting of allantoic fluid, hemagglutination assay was performed using 1% red blood cells (RBC) suspension by serial two-fold dilutions method to evaluate antiviral activities of plant extracts as described in previously [31]. Hemagglutination assay studies were carried out with the approval of the Ege University Animal Experiments Local Ethics Committee (EÜHADYEK) numbered 2020–051.

2.6. In-vitro antiviral activity against SARS-CoV-2

C. creticus L. ethanol:water (50%:50%) was selected for the investigation of antiviral activity against SARS-CoV-2 , since *C. creticus* have been frequently used product and commercially available due to having potent antiviral activity against IBV [31].

In this study, the African Green Monkey Kidney Epithelial Cell Line (Vero-E6) cell line was used for cytotoxicity and antiviral analysis. Vero-E6 was obtained from American Type Culture Collection (ATCC). Vero-E6 cells were cultured in Eagle's Minimum Essential Medium (EMEM) medium by adding 10% FBS at 37^0C in an incubator with 5% CO_2 [33]. Cells that were known to be in the logarithmic phase and were able to proliferate actively were used in experiments when they were observed to be 90% confluent on the surface to which they were attached. SARS-CoV-2 used in the study was provided by Kocak Pharmaceuticals (Vial no.31242/12.05.2020, isolated from throat swabs from a hospitalized patient in the recent COVID−19 outbreaks by the Turkish Ministry of Health). SARS-CoV-2 isolate used in this study correlates to SARS-COV-2 NC 045512 isolate at a ratio of 99.117% in the NCBI database [34]. SARS-CoV-2 was propagated in Vero-E6 cells and virus clarification was made when more than 65% cytopathic effect was observed in the cells.

Firstly, cytotoxicity assay was demonstrated on Vero E6 cells by MTT assay (0.5, 5 and 50 µg/mL) [35]. A 10-fold serial dilution of stock virus suspension was added to the plate containing *C. creticus* extract dissolved in DMSO, and the final concentrations of the extract were ensured to be 1 µg/ml and 10 µg/ml [36]. Two different incubation periods (10 and 60 minutes) were used. At the end of this period, the prepared virus extract suspension was added to Vero-E6 cell plates incubated for 24 hours at 37 ℃, 5% CO□. After 4 days of incubation, they were stained with 0.1% crystal violet solution and cytopathic effects (CPEs) in the cells were observed by inverted light microscopy. $TCID_{50}$ (Fifty percent tissue culture infective dose) was calculated by the Spearman-Karber Method [37,38].

2.7. Statistics

Data were expressed ± standart errors. The data obtained as a result of the experiments were made using the GraphPad Prism Software version 8.4.2 (San Diego, CA, USA) for Windows with a 95% confidence interval. Significance was accepted when $p \leq 0.05$.

3. Results and Discussion

The number of individuals with viral diseases is increasing every year due to resistant pathogens with high adaptation ability. Population growth accelerates the use of high-cost synthetic antiviral drugs. Considering the increasing drug costs and decreasing treatment effectiveness resulting from viral infections, it is essential to find cost-effective and effective antiviral solutions and develop new strategies [39].

Plant-derived drugs and medicinal plants have attracted attention in recent years due to their easy adaptability, low cost and less adverse effects compared to synthetic drugs [40]. Considering SARS-CoV-2 which emerged in 2019 and became a pandemic causing the death of many people, limited number of antiviral studies have existed for different *Cistus* species against viruses, especially SARS-CoV-2. Therefore, the main aim of the current study was to determine the major SMs of *Cistus* extracts and evaluate the antiviral potential of *Cistus* extracts against the IBV-D274 strain and SARS-CoV-2.

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After the different organic and water extract ratio, ethanol:water (50%:50%) extraction amounts and yields of *C. creticus, C. salvifolius, C. monspeliensis, C. parviflorus* and *C. laurifolius* were 22.35% (2.235 g), 24.46% (2.446 g), 30.23% (3.023 g), 21.32% (2.132 g) and 19,65% (1.965 g), respectively in Table 2.

TPA assay were appiled to determine total phonolic contents of each *Cistus* extracts. Total phenolic contents of *Cistus* extracts were listed in Table 3. TPA revealed that *C. creticus (*100% Ethanol), *C. salvifolius* (50% Ethanol), *C. monspeliensis* (50% Methanol), *C. parviflorus* (50% Methanol) and *C. laurifolius* (100% Ethanol) extracts had more total phenolic content than other exctracts (Table 3). Therefore, further antiviral experiments were performed in the presence of these selected extracts with high phenolic compounds.

C. creticus L. ethanol:water (50%:50%) extracts were analysed by LC-HRMS to detect major and minor compounds playing role in several biologic activities. Pharmacologically active phytochemicals of *C. creticus* L. extracts were shown in Table 4 and Figure 1-3. LC-HRMS analysis demonstrated that fumaric acid, acacetin, (-)-epicatechin, (-)-epigallocatechin and quercitrin were found as major compounds in *C. creticus* extracts. Further data about method validation and chromatographic results of chemical content analyses of *C. creticus* extracts by LC-HRMS might be seen in Figure 1-3.

HA assay were performed to determine virus presence in supernatant of whole groups. Titers from samples were showed in Table 5. The mean HA titer for the virus control group was 1024 after neglecting eggs due to manipulation and early mortality (Figure 4 and Table 5).

The antiviral effects of the *Cistus* extracts were evaluated by comparing with the virus control group whose virus titer was determined as 1024. All samples were labelled from L1 to L5 which indicates the plant species as describes in Table 5. Two different concentration (0.1 and 1 mg/kg *Cistus* extracts) were injected respectively, and the results are evaluated statistically. Significant activity was observed at 1 mg/kg concentrations of all extracts and no virus was detected at HA test. The extracts exhibited different results for a concentration of 0.1 mg/kg. The highest HA titers among 0.1 mg/kg samples were found in *C. monspeliensis* extract with virus titers of 1024, 4, 128 and 512, respectively ($p < 0.05$). HA titers in *C. parviflorus* extract were 512, 512, 256 and 512, while *C. laurifolius* extract was 512, 1024, 512 and 1024. *C. creticus* (having HA titers 1024, 1024, 512 and 1024) and *C. salviifolius* (having HA titers 2048, 1024, 2048 and 1024) extracts were similar to the virus control group. For these four groups, there was no significant relevance for 0.1 mg/kg compared with virus kontrol group. The most statistically significant result for 0.1 mg/kg was found between *C. monspeliensis* and *C. salviifolius* ($p < 0.001$).

After *in-ovo* antiviral activity, viability assay were performed to demonstrate that *C. creticus* extracts did not cytotoxic activity in the absence of virus in Vero cell line. Results indicated that *C. creticus* extracts did not show any cytototxic activity againt Vero cell line exposed to increased concentrations of *C. creticus* extract (Figure 5). Light microscopy image showed that morphological alterations were not observed for Vero cell line exposed to *C. creticus* extract at the concentrations of 0.5, 5 and 50 µg/mL (Figure 6).

$TCID_{50}$/mL values were shown in Figure 7. It has been determined that the infection dose decreased

Table 2. Extraction amounts and yields of *Cistus* L. species

Extract	Ethanol:water (50%:50%) Extract amount (g)	Extraction yield (%)
C. creticus	2,235	22,35
C. salviifolius	2.446	24,46
C. monspeliensis	3,023	30,23
C. parviflorus	2,132	21,32
C. laurifolius	1,965	19,65

Table 3. Total Phenolic Amounts in *Cistus* L. species

Experiment number	Plant	Solvent	mg GAE/gr plant
1	*C. creticus* L.	Ethanol	256.002
2	*C. creticus* L.	Methanol	170.748
3	*C. creticus* L.	50% ethanol	167.988
4	*C. creticus* L.	50% methanol	197.268
5	*C. salvifolius* L.	Ethanol	194.778
6	*C. salvifolius* L.	Methanol	18.051
7	*C. salvifolius* L.	50% ethanol	238.518
8	*C. salvifolius* L.	50% methanol	26.553
9	*C. monspeliensis* L.	Ethanol	163.674
10	*C. monspeliensis* L.	Methanol	171.906
11	*C. monspeliensis* L.	50% ethanol	168.324
12	*C. monspeliensis* L.	50% methanol	222.144
13	*C. parviflorus* L.	Ethanol	128.742
14	*C. parviflorus* L.	Methanol	190.242
15	*C. parviflorus* L.	50% ethanol	169.362
16	*C. parviflorus* L.	50% methanol	212.064
17	*C. laurifolius* L.	Ethanol	194.454
18	*C. laurifolius* L.	Methanol	176.604
19	*C. laurifolius* L.	50% ethanol	181.926
20	*C. laurifolius* L.	50% methanol	17.499

as the incubation period compared to SARS-CoV-2 group. Prolonging the incubation period reduced the infection dose at 1 µg/mL (60 min.) by approximately 20-fold compared to 1 µg/mL (10 min.), respectively. However, incubation period did not cause any alterations in infection dose for 10 µg/mL. In addition to the incubation period, increasing the concentration was also effective in reducing the infection dose. 10 µg/mL (10 min.) reduced infection dose by 100,000-fold compared to 1 µg/mL (10 min.). 10 µg/mL (60 min.) reduced infection dose by 1445-fold compared to 1 µg/mL (60 min.) (Figure 7).

In-ovo antiviral activity results revealed that *C. parviflorus* and *C. monspeliensis* species had more antiviral activity against the IBV-D274 strain than *C. creticus*, *C. salvifolius* and *C. laurifolius* (Figure 4 and Table 5). Different phenolic, flavonoid and terpenoid contents in each extract might be responsible for different biological activity (Table 3). Studies analyzing the efficacy of secondary metabolites of *Cistus* sp. *in-ovo* antiviral activity model are not reported. However, it is thought that *Cistus* sp. may exhibit *in-ovo* activity against IBV owing to the flavonoids, alkaloids, phenolics, terpenoids, and polysaccha-

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Table 4. Chemical contents of *C. creticus* L. (mg/kg ethanol:water (50%:50%) extract)

Molecule Name	*Cistus creticus*	U %(k=2)
Ascorbic acid	247.64	11.07
(-)-Epigallocatechin	2546.60	11.34
(-)-Epigallocatechin gallate	287.69	11.53
Chlorogenic acid	8.14	11.14
Fumaric acid	4676.82	11.14
(-)-Epicatechin	2576.96	11.91
(-)-Epicatechin gallate	<LOD	11.21
Verbascoside	<LOD	12.08
Chicoric acid	<LOD	10.97
Orientin	<LOD	11.47
Caffeic acid	<LOD	11.07
Sinapinic acid	<LOD	12.01
Luteolin 7-glucoside	<LOD	11.29
Rutin	492.67	11.79
Hyperoside	<LOD	11.5
Dihydrokaempferol	8.70	11.35
Ellagic acid	302.55	11.47
Quercitrin	1118.07	11.69
Myricetin	32.55	11.68
Nepetin-7-glucoside	<LOD	11.4
Quercetin	13.16	11.42
Salicylic acid	28.58	11.4
Luteolin	<LOD	12.41
Nepetin	<LOD	11.24
Kaempferol	45.42	11.9
Apigenin	116.53	11.54
Hispidulin	<LOD	11.23
Isosakuranetin	26.32	11.48
Penduletin	70.19	11.81
Rhamnocitrin	93.40	11.22
Chrysin	6.16	11.09
Acacetin	2739.27	11.36

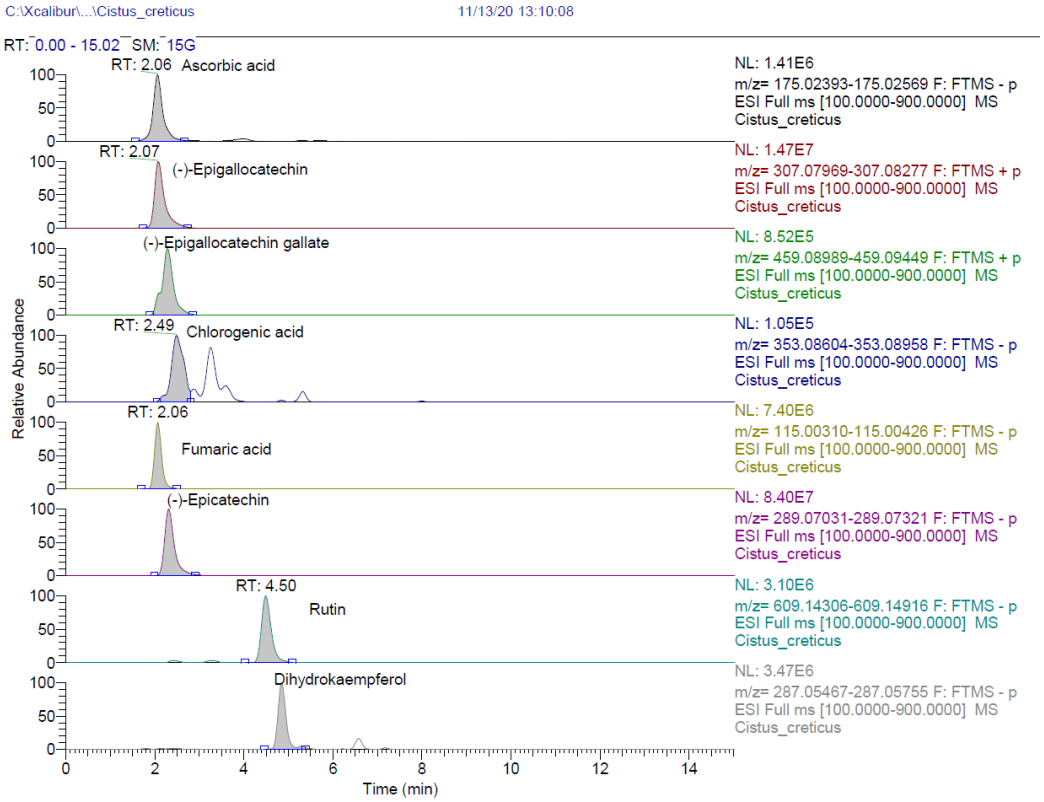

Figure 1. LC-HRMS chromatogram of *C. creticus* L.

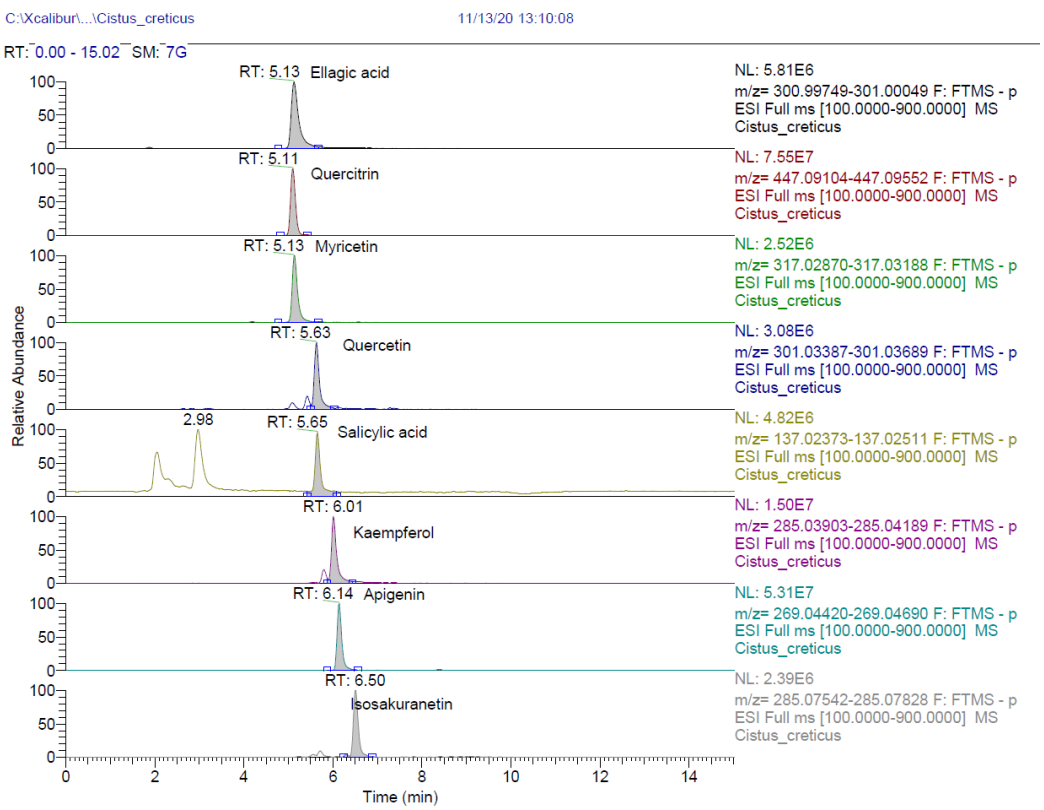

Figure 2. LC-HRMS chromatogram of *C. creticus* L. (continued)

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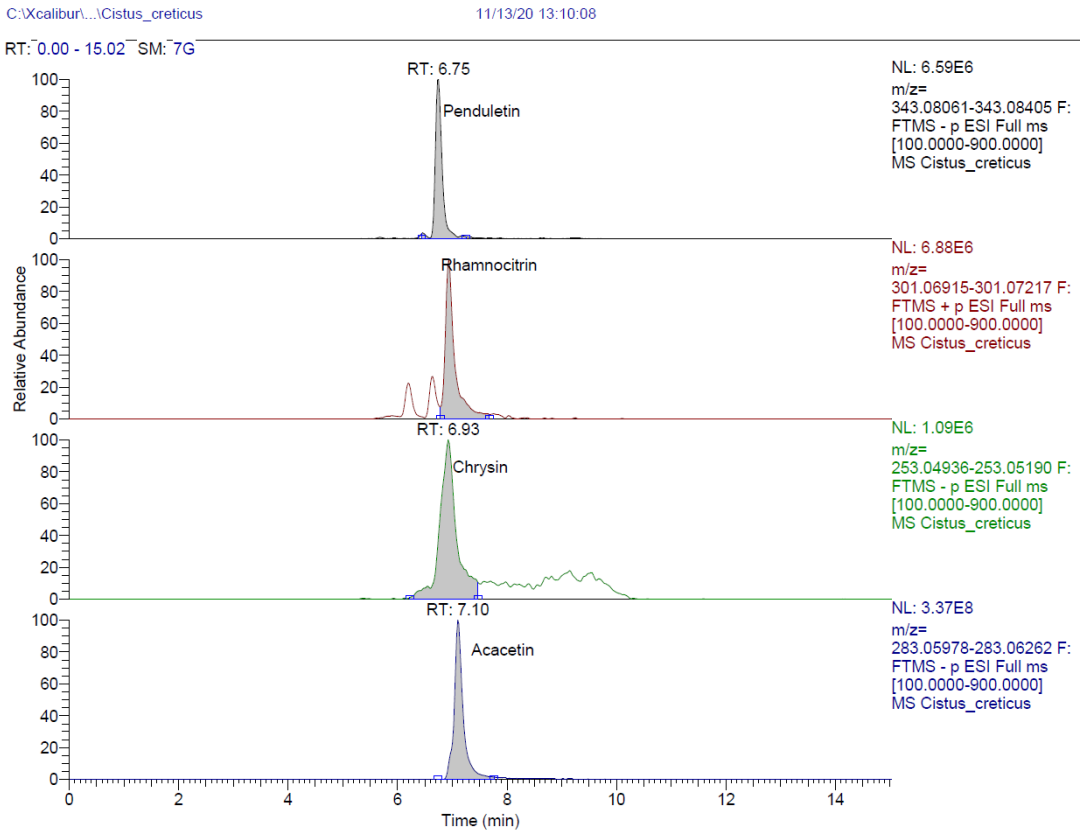

Figure 3. LC-HRMS chromatogram of *C. creticus* L. (continued)

rides they contain [41]. In addition to IBV model, antiviral potential of *C. creticus* extract, which is the most common in Türkiye and commercially available species in Europe, on SARS-CoV-2 was also examined. Increasing the incubation time (60 min.) and concentration of *C. creticus* extract (10 µg/mL) led to increase of antiviral activity, which means that *C. creticus* extract had dose-and time dependent antiviral activity against SARS-CoV-2 (Figure 7). Previous *in-vitro* studies reported that polyphenol-rich *C. creticus* extracts showed potent antiviral activity against influenza, dengue fever, HIV-1 and HIV-2 viruses at concentration ranges of 5.40 and 50 µg/ml, which means that the concentration of *C. creticus* extract with potent antiviral activity (10 µg/mL) in our study was consistent with previous *in-vitro* experiments [42-45].

Furthermore, *C. creticus* did not cause any cytotoxicity in Sars-CoV-2 infected Vero cells at 10 µg/ml (Figure 5-6). Cytotoxic activities were not also reported at 50 µg/ml of polyphenol-rich extract of *C. creticus* against influenza virus-infected human lung adenocarcinoma cell (A549) and Madin-Darby canine kidney (MDCK) [43]. Similarly, diethyl ether extract of *C. creticus* did not lead to cytotoxic activity at a concentration of 31.25 µg/ml in dengue virus-infected Vero cells [45]. Rebensburg et. al. (2016) mentioned that the polyphenolic compounds of *C. creticus* extract selectively targeted virus envelope proteins but did not penetrate into the host cell. This selectivity towards the virus rather than the host cell caused cell viability to be unaffected [44]. This might be the probable reason why *C. creticus* was not cytotoxic to Vero cell line in our study (Figure 5-6). Rebensburg et. al. (2016) also reported that *C. creticus* extract did not cause any viral resistance for 168 days of incubation [44]. The possible explanation for overcoming viral resistance might be the fact that it was the plant containing the most phenolic compounds in its leaves and there is a large amount of secondary metabolites in the aerial parts. These different components eliminate viral resistance through different mechanisms. Considering this information, it becomes clear that it is more advantageous to use medicinal plants and food supplements

Table 5. Hemagglutination assay results of *Cistus* L. Extracts

Sample	Conc. (µg/g)	No of Total Eggs	HA titer (log_2)				HA titer (log_2) (Mean)	HA titer (Mean)
			Egg 1	Egg 2	Egg 3	Egg 4		
Positive control		4	11	11	11	11	11	2048
(Only virus)								
Vehicle control		4	11	11	11	11	11	2048
(DMSO)								
Favipiravir	10 µg/g	4	9	8	10	9	9	512
(Antiviral drug)	25 µg/g	4	9	8	8	7	8	256
Negative control		4	0	0	0	0	0	0
(Untreated ECE)								
C. creticus	0.1 µg/g	4	10	10	9	10	9.75	890
	1 µg/g	4	1	1	1	1	2	2
C. salviifolius	0.1 µg/g	4	11	10	11	10	10.50	1450
	1 µg/g	4	1	1	1	1	2	2
C. parviflorus	0.1 µg/g	4	9	9	8	9	8.75	430
	1 µg/g	4	1	1	1	1	2	2
C. monspeliensis	0.1 µg/g	4	10	2	7	9	7	128
	1 µg/g	4	1	1	1	1	2	2
C. laurifolius	0.1 µg/g	4	9	10	9	10	9.50	725
	1 µg/g	4	1	1	1	1	2	2

as extracts rather than isolated molecules to increase treatment success [44-46].

SMs are natural products responsible for pharmacologic activities of medicinal plants and food supplements [47]. The reason why *Cistus* species had different ratio of antiviral activities (Figure 4 and Table 5) might be due to the difference in the diversity and amounts of SMs such as phenolic molecules (Table 3). Advanced chromatographic techniques such as LC-MS or LC-MS/MS have been used to isolate and identify SMs including phenolic and flavonoid compounds in the extract of several *Cistus* species [46-48]. In our study, LC-HRMS analysis displayed that fumaric acid, acacetin, (-)-epicatechin, (-)-epigallocatechin, quercitrin were the most abundant SMs in *C. creticus* extract (Table 4 and Figure 1-3). Former study indicated that while the essential oils of *C. villosus* and *C. salviifolius* were found as non-terpene structure, the essential oils of *C. creticus* and *C. monspeliensis* were monoterpene and diterpene structures [49]. *C. creticus* extracts of different organic solvents such as petroleum ether, dichloromethane and methanol revealed that the chemical compositions of the extracts were rich in diterpenes, labdane-type diterpenes, triterpenes, tannins, heterosides, flavonoids and saponosides [17,50]. While trans-tiliroside was determined as the most abundant flavonoids in methanol extracts of *C. salviifolius* and *C. creticus*, hyperin and myrcetin flavonoids

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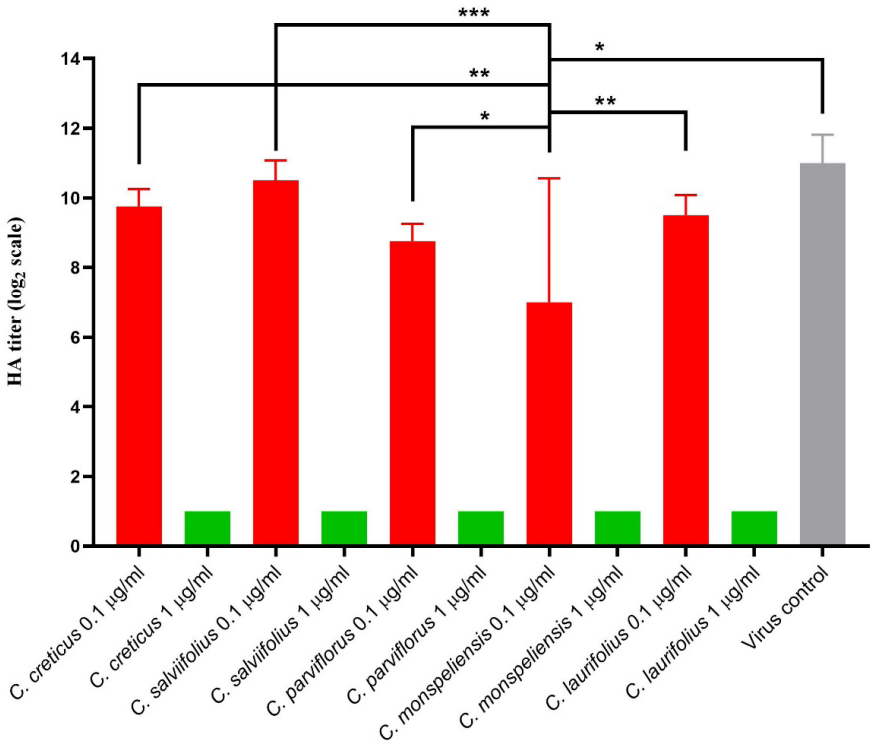

Figure 4. Hemagglutination titre of *Cistus* L. extracts against IBV D274 after *in ovo* treatment in chorioallantoic fludics.

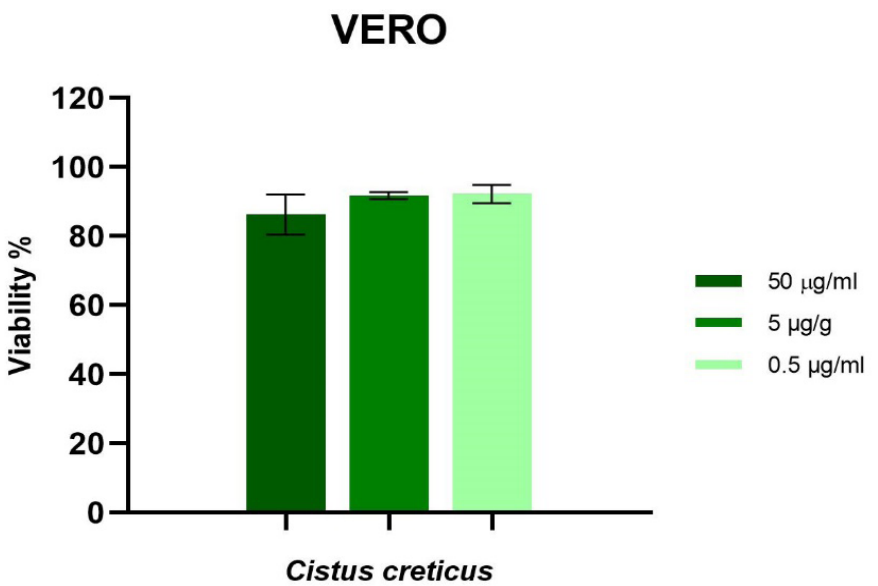

Figure 5. Viability of Vero cell line against *C. creticus* L. extract samples.

were determined as the most abundant flavonoids in methanol extracts of *C. laurifolius* [51]. In another study, chemical components of *Cistus* species were detected as scopoletin, methyl flavogallonate, hydroquinone, arbutin, methyl β-glucopyranoside and glucopyranoside along with kaempferol and quercetin derivatives [17,18]. Carev et. al. (2020) reported the major and minor compounds in aqueous extracts of

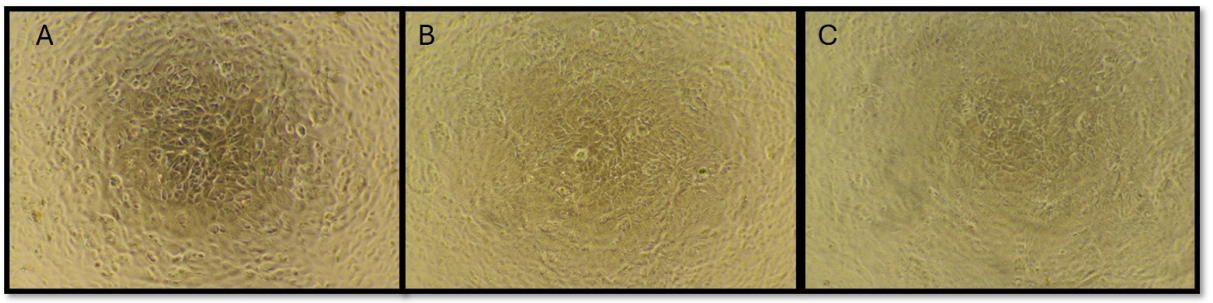

Figure 6. Light microscopy image (10x) of Vero cell line exposed to increasing concentrations (A:0.5, B:5 and C:50 µg/mL) of *C. creticus* L. extract.

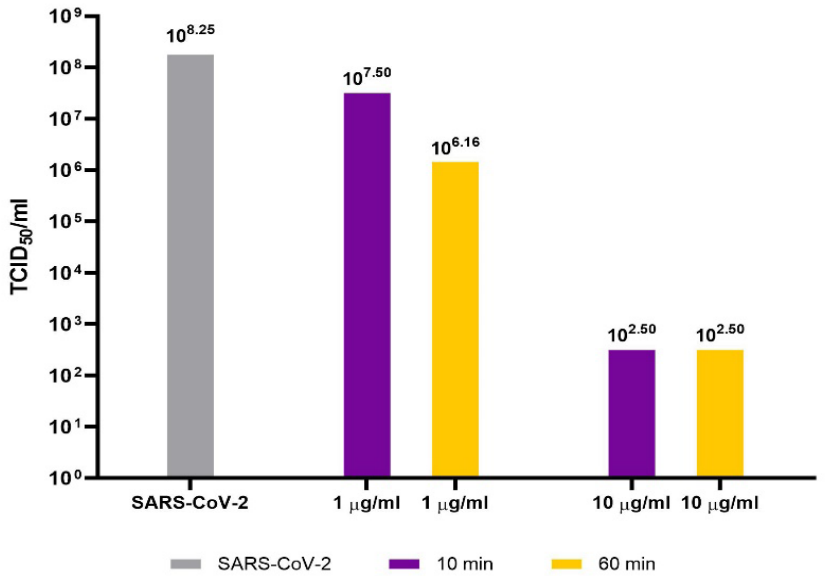

Figure 7. $TCID_{50}$/mL of SARS-CoV-2 incubated in the presence of *C. creticus* L. extact (1 and 10 µg/mL) for 10 and 60 minutes.

C. creticus and *C. salviifolius*. Rutin was the only compound which was similar to our study as a major compound [48]. It is not surprise that the major and minor components of our study and previous *Cistus* L. studies are different, as the amount and diversity of SMs in plants might vary depending on the season of collection, altitude, amount of moisture or soil structure. In addition, the solvents with which *Cistus* L. species are extracted and the chromatography conditions also affect the major and minor components of *Cistus* L. species [47].

As mentioned above, fumaric acid, acacetin, (-)-epicatechin, (-)-epigallocatechin, quercitrin were found as the most abundant SMs in *C. creticus* L. extract (Table 4 and Figure 1-3). Fumaric acid is not only an intermediate product used in the Krebs cycle, but also provides great benefits in the treatment of cancer, cardiovascular and immunology diseases with its ester structure. Antiviral contribution of fumaric acid might be activation of the Krebs cycle instead of the synthesis of nucleotides necessary for the proliferation of the virus [52,53]. Acetatine, the second most abundant SMs in our study, has been found to have antiviral activity (IC_{50}: 1.20 µg/mL to 2.50 µg/mL) against influenza virus and HIV in previous studies [54,55]. Another major compound (-)-epicatechin has been shown to protect Mayaro virus-infected cells by 100% against infection at a concentration of 124 µg/mL [56]. In studies conducted with (-)-epi-

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gallocatechin gallate and (-)-epigallocatechin, it was stated that polyphenols showed antiviral activity not only by interacting with the hemagglutinin of the influenza virus but also by altering the physical properties of the virus membrane structure [57,58]. The antiviral activity of quercitrin, another major compound in our study, against dengue virus was investigated and the results showed that it caused moderate antiviral activity (IC_{50}: 467.27 µg/mL) [59]. Considering the data available to date, two main antiviral activity mechanisms of *C. creticus* L. extract have been suggested. Schwerdtfeger et. al. (2008) reported that aqueous extract of *C. creticus* L. extract caused strong inhibition of neuraminidase, which allows newly replicated viruses to separate from the host cell [60]. Rebensburg et. al. (2016) reported that *C. creticus* extract prevented host cell infection by breaking down the viral envelope [44]. Apart from both mechanisms, it has been also stated that herbal components might exhibit biological activity through multiple mechanisms rather than a single mechanism [45,61]. Although the antiviral mechanisms of the major compounds detected in our results are not fully clear, considering the results of previous experiments, it is thought that the relevant compounds which were found as the major SMs might contribute to antiviral activity in our study.

4. Conclusion

Our study aimed to investigate antiviral potential of *Cistus* L. against IBV and SARS-CoV-2. *In-ovo* antiviral activity assays indicated that extracts of *C. parviflorus* and *C. monspeliensis* demonstrated effective and strong antiviral activity against IBV-D274 strain. Furthermore, *C. creticus* extract, which is the most used commercially extract for influenza, led to potent antiviral activity against SARS-CoV-2. In the light of the above information and results of current study, further experiments need to perform with *C. parviflorus, C. monspeliensis* and *C. creticus* to isolate and identify the active molecules responsible for antiviral activity against IBV and SARS-CoV-2. Further studies also must be carried out to illuminate the pharmacological mechanisms of the extracts or isolated compounds of *Cistus* L. species causing antiviral activity. Further studies with an extract containing a combination of effective antiviral compounds instead of a single effective compound might contribute to both increasing treatment success and reducing viral resistance. Finally, it is envisaged to conduct clinical studies to report pharmacological and toxicological effects of *Cistus* L. species on human health to develop novel products that might be used in the pharmaceutical industry and as food supplements.

In this study, *Cistus* L. species were collected with the permission of Republic of Turkey Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks (approval number: 1037970339). In addition, the protocol for antiviral experiments was approved by the Ege University, Local Ethical Committee of Animal Experiments (approval number: 2020-051).

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Conflict of Interest

The authors declare that they have no conflict of interest.

Statement of Contribution of Researchers

Design and Concept – F.B., A.E., F.O.C., G.G.E., E.A.O., A.C.G, A.N.; Data Collection/ Processing – F.B., F.O.C., G.G.E., E.A.O., A.C.G, A.N.; Analysis – F.B., F.O.C., G.G.E., E.A.O., A.C.G, A.N; Writing – F.B., A.E., F.O.C., G.G.E., E.A.O., A.C.G, A.N.;

Critical Reviews – F.B., A.E., F.O.C., G.G.E., E.A.O., A.C.G, A.N.

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