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Investigation of the Antiviral Effects of *Saussurea lappa* Root Extract against SARS-CoV-2 Virus

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Scope: The purpose of this study was to look into the antiviral activity of a plant extract derived from the roots of the *Saussurea lappa* as a food supplement against SARS-CoV-2 infection.
Methods and results: Vero E6 cells are employed in the study to test the neutralizing effect of *Saussurea lappa* extract against the SARS-CoV-2 virus. For anti-viral activity detection, a sensitive real-time cell analyzer (xCELLigence RTCA) with a high repetition rate is used. A challenge experiment in mice is planned as a result of the in vitro analysis. A challenge test against SARS-CoV-2 is performed with 10 adult female K18-hACE2 transgenic mice in each group for this purpose. The mice in the *S. lappa* Group are gavaged 2 days before the virus is administered intranasally (i.n.). The control group received PBS instead of the extract. SARS-CoV-2 virus is administered i.n. under anesthesia for the first 3 days of the experiment, and *S. lappa* extract was administered by gavage in the afternoon. On the 10th day, mice in the *S. lappa* group survived the study, whereas animals in the control group grew ill and/or died. In this study, the extract protects the mice against the SARS-CoV-2 virus in 90% of the cases.
Conclusions: This study demonstrates that the Saussurea plant has antiviral effects against SARS-CoV-2 in vitro and in animal models.

1. Introduction

A group of pneumonia patients was reported in Wuhan, Hubei Province, China, in December 2019. Later, the disease quickly spread throughout the world, becoming a pandemic[1–3] SARS-CoV, a Coronavirus family virus that caused severe acute respiratory syndrome in 2002, MERS-CoV virus (Middle East respiratory syndrome coronavirus), another coronavirus discovered in Saudi Arabia in 2012, and finally severe acute respiratory syndrome SARS-CoV-2, a third Coronavirinae subfamily member that infects humans, has led to a new lethal disease.[4]

In March 2020, the World Health Organization (WHO) said that the SARS-CoV-2 virus was a global pandemic. As of March 29, 2023, there were about 761 402 282 confirmed cases and 6 887 000 deaths around the world.[54]

An excessive immune response develops as a result of viral infection in COVID-19 (SARS-CoV-2 infection) disease. Inflammatory responses and acute respiratory disease syndrome (ARDS) can have serious consequences, including multiple organ failure. This situation is known as "cytokine storm."[5] To stop the spread of SARS-CoV-2, it is important to control the immune response and lessen the effects of the cytokine storm.[6,7]

Favipiravir or Remdesivir, drugs that were developed to treat viral infections such as Ebola or influenza, were initially used as standard therapy for COVID-19 treatment.[8–11] Recent clinical studies have revealed that these treatments have little or no effect, especially in patients with severe symptoms.[12,13]

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Molnupiravir, a recently developed influenza treatment, has been shown to have more promising effects in reducing hospitalization and death rates in mild to moderate COVID-19 cases.[52] Even though there are a lot of ongoing scientific and clinical studies, no drugs have been found or approved to treat SARS-CoV-2.

Even though patients with SARS-CoV-2 infection were given non-specific treatments to ease their symptoms and improve their prognosis, their immune systems were mostly responsible for keeping the infection from getting worse.

So far, new therapeutic approaches such as vaccine development (AstraZeneca, Moderna Inc., Johnson & Johnson/Jannsen, and Pfizer-BioNTech), antiviral drug discovery (lopinavir-ritonavir, remdesivir, favipiravir, interferon-alfa [IFN-α], ribavirin, and arbidol), and combination therapy methods have piqued the interest of researchers.[14]

Synthetic antivirals, in particular, were used to prevent virus replication, entry, and transmission. These antiviral agents have been the basis for drug discovery research studies against COVID-19. Numerous studies have shown that using herbal medicines alone or in combination with conventional medicines to treat people infected with COVID-19 can be beneficial.[15,17]

Many symptoms can be significantly improved and mortality reduced by using herbal remedies. These herbs have a variety of antiviral, antioxidant, anti-inflammatory, and immune-boosting properties, which make them potential COVID-19 treatment options.[18] Herbal medicines may have benefits such as reducing acidity in endosomes and inhibiting the production of proinflammatory cytokines to prevent virus replication, according to recent research.[19,20] Furthermore, the antioxidant properties of certain medicinal plants have been discovered. Plant-derived antioxidants are important in reducing the effects of oxidative stress.

The Saussurea plant, which belongs to the Asteraceae family, is found in India. One of the most well-known species in this genus is *Saussurea costus*, a synonym of *Saussurea lappa*. *S. costus* (root oil and roots) has emerged as a significant pharmaceutical raw material on the global market.[21] *S. lappa* is an aromatic and medicinal plant that grows in the Himalayan region at an average altitude of about 3500 m.[22] Dried *S. costus* (SC) roots have been used to treat a variety of diseases and ailments since ancient times, including indigestion, stomach problems, ulcers, upper respiratory tract diseases, asthma, cough, tuberculosis, and rheumatism.[21,23]

According to Zahara et al.,[24] this herb has the potential to be used in the development of potent antiviral drugs. Also, Deabes et al.[25] showed that *S. costus* extracts can help to protect people from multiple diseases that are resistant to antibiotics and they can be used to treat certain infections instead of antibiotics. As a result, we demonstrated the feasibility of using an extract obtained from the root of *S. lappa* to treat COVID-19 with in vitro and in vivo models by utilizing its antiviral properties in this study.

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2. Experimental Section

2.1. *S. lappa* Root Identification and Registration

S. lappa grew in three areas within India's borders. These were the regions of Jammu and Kashmir, Himachal Pradesh, and Uttaranchal. IPMC GROUP Pharmaceutical Industry funds imported the *S. lappa* plant used in this study from Pakistan due to its therapeutic effect. Al Saudia Enterprise, which was based in Pakistan, was an international company that sold the *S. lappa* plant.

2.2. Preparation of Plant Extracts

The *S. lappa* plant was ground in a laboratory blender. To improve extraction efficiency, it was sieved through a 50 µm stainless steel sieve. At room temperature (20 °C), 1 kg of prepared plant powder was mixed with 10 L of distilled water. The prepared mixture was incubated in a 70 °C water bath for 24 h. It was boiled at 100 °C for 4 h and then cooled to room temperature. Then it was filtered through filter paper 595 Whatman. The aqueous plant extract was stored in opaque amber glass bottles in the refrigerator at +4 °C.

2.3. Evaluation of In Vitro the Anti-Viral Potential of a Sample of *S. lappa* Extracts Using a Real-Time Cell Analyzer

Experiments were done with the SARS-CoV-2 virus strain (Wuhan B.1.36) to determine how effectively the *S. lappa* extract sample worked against viruses. The Ministry of Health's Directorate of Public Health supplied the SARS-CoV-2 virus strain Wuhan B.1.36. All virus growth and anti-virus tests were done in biosafety level 3 (BSL-3) labs at the TUBITAK, Marmara Research Center (TUBITAK MRC), Life Sciences, Medical Biotechnology Unit in Gebze, Turkey. This laboratory had all the international certificates needed to work with SARS-CoV-2.

The Vero E6 (ATCC CRL-1586TM, Manassas, VA, USA) cell line was provided by ATCC, the American Type Culture Collection. The cells were cultured in a low-glucose Dulbecco's modified Eagle's medium (DMEM, HyClone, Cat # SH30021.01, Gibco, NY, USA) containing 10% fetal bovine serum (FBS; HyClone Laboratories, Cat # SV30160.03, Gibco, NY, USA) and 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin (HyClone, Cat # SV30010, Gibco, NY, USA) at 37 °C and 5% CO_2.

To evaluate the effect of *S. lappa* extract on the virus, a high-throughput, quantitative assay protocol was performed using a real-time cell analyzer (RTCA) instrument called the xCELLigence MP (Agilent Technologies, CA, USA). This protocol was based on examining the course of infected cells and evaluating the cytopathic effect of the virus.[26] The RTCA MP system analyzed the impedance of cells and examined how viruses affected cells by measuring the viability of cells. The cytopathic effect could decrease the number of cells in an infected condition. By measuring cell viability with cell index (CI) and electronic impedance, the RTCA method made it easier to find and observe cellular death caused by viruses or anti-viral treatments. In the

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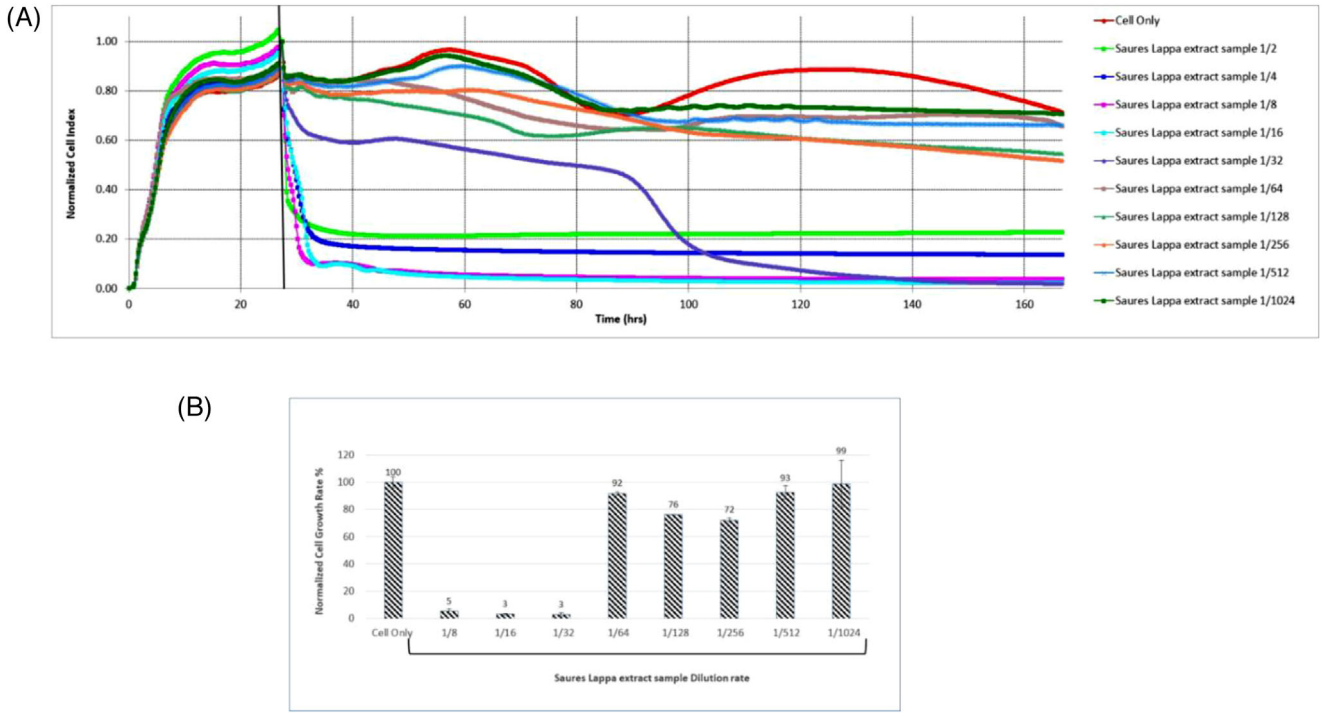


Figure 1. Using the xCELLigence RTCA MP real-time cell analysis equipment, the sample of Saussurea lappa extract was tested to understand its cytotoxic effect on the Vero E6 cell line. A) The xCELLigence system's normalized cell index graph with different dilution rates of Saussurea lappa extract sample. B) The bar graph shows the effect of *S. lappa* extract samples at different concentrations on Vero E6 cells as normalized cell growth %. The graph depicts the influence of various doses of *S. lappa* extract samples on the growth of Vero E6 cells as a percentage of normalized cell growth. The material is more cytotoxic as the normalized cell growth percentage decreases. Cell index data were normalized and renamed NCI based on the time the virus was introduced into the experiment.

sample, the cytopathic effect caused by SARS-CoV-2 was blocked completely or partially. If the SARS-CoV-2-induced cytopathic effect was inhibited at a tested concentration, the test sample was considered "completely" anti-viral for this concentration. In contrast, it was labeled "partially" anti-viral if its effect was delayed, and it was accepted that it did not prevent the cytopathic effect entirely.[27,28]

One hundred sixty hours of data collection were done at 15-min intervals. The (CI) was a measurement of the relative change in electrical impedance that revealed the condition of the cell at the moment. A normalized cell index (NCI) data were generated after all samples from the experiment were normalized to the period when the virus was injected.

To determine the percentage anti-viral effect of the sample, the time point at which the NCI value of the virus control sample was closest to zero was used. Using this time point to compare the NCI values of the other samples, the anti-viral activity rate was found.

2.4. Preparation of *S. lappa* Extract Working Samples

The working samples were prepared from a stock solution via serial dilution. At a 1/2 dilution in 2× Dulbecco's Modified Eagle's Medium (DMEM-low glucose, Gibco) with 4% FBS solution, the first sample dilution (1/2) was made to adjust the final content of 1× media with 2% FBS.

2.5. Cytotoxicity of *S. lappa* Extract

In this study, Vero E6 (ATCC CRL-1586TM) cells were cultured in 1× DMEM supplemented with 10% FBS. The cells were seeded (25 000 cells per well) in the xCELLigence RTCA MP device's 96-well E-plate to determine the cytotoxicity of *S. lappa* extract samples. The device was placed at 37 °C with a 5% CO_2 humidity incubator throughout the procedure. After 24 h, samples that had been prepared for serial dilution (from 1/2 to 1/1024) were placed in the 96-well E-plate of the xCELLigence RTCA MP device. Cells were incubated with samples for 160 h at 37 °C with 5% CO_2 (**Figure 1**A,B).

During this time, the xCELLigence RTCA MP instrument converted electrical impedance into CI at 15-min intervals. Higher CIvalues indicated increased cell viability, whereas lower CI values indicated poor cell health and decreased cell viability. Using Agilent Technologies' RTCA Pro 2.6.0 (Basic) software, the acquired data were examined using the NCI value of each well versus controls after the test was completed (Agilent Technologies Inc., Santa Clara, CA, USA).

2.6. Analyses of Anti-Viral Activities Using RTCA

The study used a high-throughput and quantitative real-time cell analysis protocol developed with the help of the xCELLigence RTCA MP (Agilent Technologies, CA, USA) to detect the

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anti-viral action of a *S. lappa* extract sample by measuring the virus's cytopathic effect and the progression of the virus's infection in infected cells (CPE). Vero E6 cells were seeded (25 000 cells per well) in sterile, disposable wells of the xCELLigence gold-coated E-plate. Throughout the procedure, the instrument was placed in an incubator. Seeded cells were observed for settling and proliferation for 24 h. The samples were prepared by following the instructions in the section "Preparation of *S. lappa* extract samples." They were incubated for 1 h at 37 °C with 5% CO_2 saturation with 3.5×10^5 PFU mL^{-1} of the Wuhan strain SARS-CoV-2 virus. After removing the media from the wells of a 96-well E-plate, the samples were placed in the wells and incubated at 37 °C with 5% CO_2 for 160 h. The cell-only (state of no infection) control sample was prepared using untreated wells containing just cells and media containing 2% FBS. The wells containing only cells and viruses in medium containing 2% FBS served as a control sample for viruses only (state of infection). Wells, having just cells and sample dilutions, were utilized as a sample cytotoxicity control. The device converted electrical impedance into cell-index data at 15-min intervals throughout the procedure.[29–32] After the experiment, the test data were analyzed with Agilent Technologies, Inc.'s RTCA Software Pro 2.6.0 and a CI value plot of each sample wells versus control wells (Basic). When determining what percentage of a sample at different concentrations worked against viruses, virus-free controls at the same concentrations were used to compare.

2.7. Ethics Statement

All experimental procedures with animals were approved by TUBITAK MRC, Life Sciences, Medical Biotechnology Unit in Gebze, Turkey. All procedures in this study involving animals were reviewed and approved by the Institutional Biosafety Committee and Institutional Animal Care and Use Committee (HADYEK-16563500-111-3026); all the experiments were conducted in compliance with all relevant ethical regulations. The experiments were conducted in BSL3 and animal BSL3 (ABSL3) facilities at TUBITAK MRC Life Sciences.

2.8. Mice

The K18-hACE2 [B6.Cg-Tg(K18-hACE2)2Prlmn/J] transgenic mice used in this study were provided by the Jackson Laboratory in the United States. TUBITAK MRC Life Sciences Experimental Animals Unit had rights for the production of K18-hACE2 transgenic mice. All of the experiments were conducted in a biocontainment isocage, which was part of ABSL 3. 8–10-week-old female K18-hACE2 transgenic mice were used, and 10 mice were used for each group. There were two groups in this study: the *S. lappa* treatment group and the placebo group (PBS).

2.9. Determination of *S. lappa* Dosage

Humans could take *S. lappa* extract twice a day as a food supplement. Besides that, because the extract was administered orally, the challenge experiment in mice was designed accordingly. Before starting the challenge study, the OECD 423 standard was used to test 4 K18-hACE2 transgenic mice for acute oral toxicity. This was done to find out if *S. lappa* extract was harmful to mice. According to the standard, a single oral dose of 2000 mg kg^{-1} was given to each mouse. At the end of 7 days, gross pathology showed no signs of toxicity or death.[22,33] In the challenge experiment, mice were given 100 mg kg^{-1} body weight of *S. lappa* extract orally once a day.[24,33]

2.10. Intranasal Delivery Method for SARS-CoV-2 Infection in Mouse Models

Ten mice each were separated into two groups. Group one mice received the extract as a gavage (100 mg kg^{-1} body weight) 2 days before the virus was administered intranasally (in). Instead of the extract, the control group (Group two) was given PBS. After 2 days, the animals were taken to a BSL 3 laboratory for a challenge experiment with the B.1.36 strain (Wuhan) of SARS-CoV-2 virus, which had 10^5 $TCID_{50}$ values. During the first 3 days of the experiment, 50 µL of 10^5 $TCID_{50}$ SARS-CoV-2 virus was intranasally administered under anesthesia, and *S. lappa* extract was administered as gavage in the afternoon.[37] Drugs were applied to the virus in the SARS-CoV-2 challenge experiment for 4 days in treatment trials. *S. lappa* was applied 4 days after the last virus application in the study. *S. lappa* was used in this study for a total of 8 days. The experiment lasted a total of 10 days. After being infected with the virus, mice were monitored on a daily basis for morbidity (body weight) and mortality. Mice that lost more than 25% of their baseline body weight were considered to have reached the experimental endpoint and were exterminated. Pathological examinations were performed after the animals were sacrificed. All abdominal organs and thoracic cavity organs were examined with the naked eye during gross pathology. One half of each mouse lung was used to compare viral load by Real Time PCR, and the other half for histopathological analysis.

2.11. Histopathological Analysis

Tissue samples were fixed in 10% buffered formalin, dehydrated in a series of alcohol solutions of ascending concentration, and embedded in paraffin wax. Sections were cut to a 5 µm thickness. Before staining, sections were deparaffinized with xylene and rehydrated in a series of alcohol solutions of decreasing concentration. Sections were stained with hematoxylin for 5 min and then rinsed in running tap water. After washing, they were stained with eosin for 5 min and rinsed in running tap water. The stained sections were dehydrated, cleared, and mounted. The slides were viewed under the Zeiss Axio Vert A1 microscope (Zeiss, Germany) and zen software (Zeiss, Germany).

2.12. Tissue Homogenization

After the mice' lungs were harvested, half of the lungs were separated for Real Time PCR analysis. Lung tissues were homogenized separately in 2 mL of PBS using an ultrasonic homogenizer

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at 70% amplitude for 90 s (Bandelin HD2200.2, Germany) for viral isolation. Tissue homogenates were centrifuged at 21 500 × *g* for 10 min and supernatants were collected into 15 mL falcon tubes.

2.13. Viral RNA Isolation and RT-PCR

Viral RNA was extracted with the QIAamp Viral RNA Mini kit Cat: 52906 (QIAGEN, USA) according to the instructions of the manufacturer. The viral RNA detection was performed using SARS-CoV-2 nucleocapsid specific primer and probes detailed below with One Step PrimeScript III RT-PCR Kit (Takara, Japan). All reactions were performed on a CFX96 Touch instrument (Bio-Rad, USA) with the following Real-Time PCR conditions: 52 °C for 5 min, 95 °C for 10 s, then 44 cycles of 95 °C for 5 s and 55 °C for 30 s. The primer and probe sequences that were used for RT-PCR were CDC recommended and FDA (EUA) approved NC1 and NC2 primer-probe sets whose target region was the Nucleocapsid (NC) gene of SARS-CoV-2. (Primer and probe sequences are: N1 Forward: 5'-GAC CCC AAA ATC AGC GAA AT-3', N1 Reverse: 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3' N1 Probe: 5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3 N2 Forward: 5'-TTA CAA ACA TTG GCC GCA AA-3' N2 Reverse: 5'-GCG CGA CAT TCC GAA GAA-3' N2 Probe: 5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3).[55]

2.14. Statistical Analysis

GraphPad Prism version 9.4.1 (GraphPad Software, San Diego, CA, USA, www.graphpad.com), was used to analyze all the data. The data were presented as mean ± standard deviation. An unpaired student's *t*-test was used for comparison between two groups, while one way ANOVA was used for comparisons including multiple parameters. A two-sided p value <0.05 was considered statistically significant. Details of each analysis were indicated in the figure legends.

3. Results

3.1. The Efficiency of Antiviral Activity of *S. lappa* Extract Using an In Vitro Real-Time Cell Analyzer

With real-time, unlabeled analytical method protocol, we could study the anti-viral effect of our sample on the virus for a long time. The data collected showed that the *S. lappa* extract sample was not cytotoxic to the VERO E6 cell line at concentrations between 1/64 and 1/1024. Sample dilutions between 1/2 and 1/32 were cytotoxic (Figure 1A,B). For this reason, anti-viral activity evaluations were made at 1/64 and above *S. lappa* extract sample dilutions.

During the experiments in the RTCA device, it was determined that the virus given to the cells at the 24th h caused the infected cells to die after 70 h and decreased the NCI value to zero. The cells that received only medium at the end of the 24th h could live until the end of the 160th h. However, it was observed that the growth curve dropped slightly during the late hours due to nutrient deficiency. The "only virus" sample that killed all the cells was used as a reference point for assessing the anti-viral action of the sample on the virus. It was noticed that when the virus was added to the cells at the 24th h, cell death did not begin until the 70th h, and by the 160th h, there were no viable cells left.

Based on the results, the samples were able to inactivate the SARS-CoV-2/Wuhan strain B.1.36 at different ratios of 1/64, 1/128, and 1/256. Compared to the controls, it was observed that the samples with dilution rates at 1/64, 1/128, and 1/256 have partial anti-viral activity with efficiencies of 75%, 65%, and 13%, respectively. Extended data **Figure 2**A,B illustrate representative graphs for partial anti-viral samples.

3.2. In vivo Experiments with *S. lappa* Extracts

After discovering that *S. lappa* extract could neutralize the SARS-CoV-2 (B.1.36 Wuhan) virus in vitro, it was decided to continue the experiment with an in vivo challenge. We investigated the clinical potential of *S. lappa* extraction in the COVID-19 infected mouse model. We made mice to drink the extract orally for a total of 8 days, beginning 2 days before the disease. The placebo group received PBS containing the virus (**Figure 3**A).

An average of more than 10% weight loss was observed in the control group at 7 days post-infection (dpi), but there was no significant reduction in the mean weight of the treated group given the *S. lappa* extract (Figure 3B). At 8 dpi, 6 of 10 animals in the control group had significant weight loss (>15%) and were sacrificed because they were on the verge of death. Only one of the 10 animals in the treated group given *S. lappa* extract showed this condition (Figure 3B).[34–37]

One out of 10 animals in the group 1 that was treated with *S. lappa* extract developed disease at 9 dpi, while nine animals (90%) were completely healthy and survived until the end of the experiment. Gross pathology showed that most of the lungs in the control group had pneumonic and hyperemic areas, while the lungs in the treated group were clean and showed no signs of infection.

Ct values obtained from PCR revealed that nine out of 10 animals in the control group had a high viral load (Ct values <20), compared to 1 in 10 animals in the *S. lappa* extract treated group. Ct values were not read in the Real Time PCR device because viral load was not detected in nine healthy animals in the treated group for which extract was given. Ct value of 16 was found in only one animal in the treated group. In the treated group, histological examination of lung tissues revealed healing and restoration of normal tissue components (Figure 3C).[35–37]

Lung sections were analyzed after staining with hematoxylin and eosin and lung inflammation was semi-quantitatively scored between 0 and 3 (0: no inflammation, 1: low, 2: medium, 3: high). Control mice exhibited significantly higher histopathology scores than extract treated mice. In control group's lungs interstitial inflammatory cell infiltration, alveolar septal thickening and more parenchymal infiltration at peribronchiolar regions were observed compared to extract treated animals which had relatively healthy lungs (**Figure 4**)[35–39]

4. Discussion

Sun et al. investigated at the cytotoxic effects of *S. costus* extraction in human cancer cell lines.[35] They demonstrated that,

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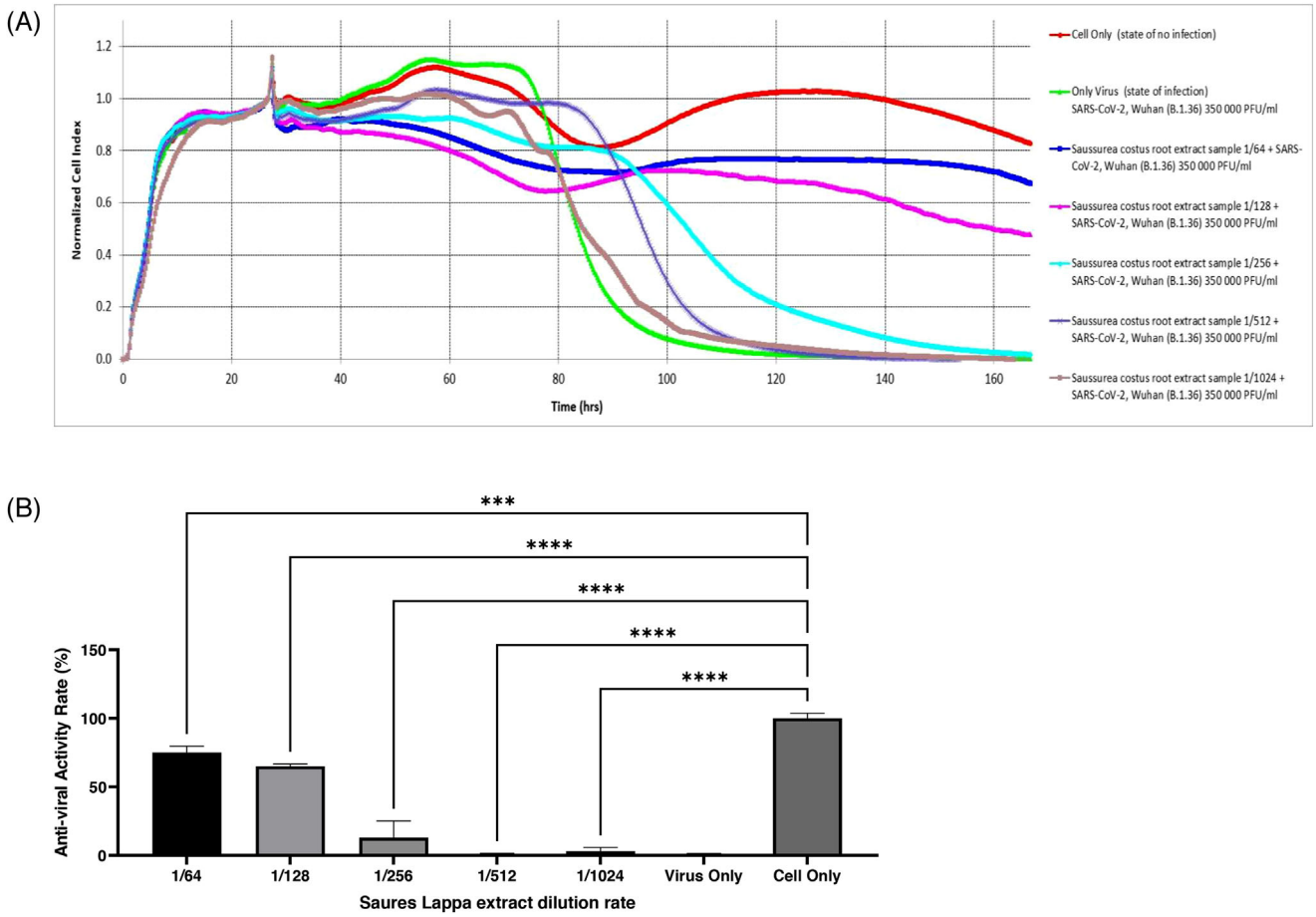


Figure 2. Using the xCELLigence RTCA MP real-time cell analysis equipment, the sample of *S. lappa* extract was tested to understand its anti-viral efficiency against the SARS-CoV-2 Wuhan (B.1.36) strain. A) The xCELLigence system's normalized cell index graph. Virus alone; Vero E_6 cells infected with 3.5×10^5 PFU mL^{-1} SARS-CoV-2 (B.1.36) (state of infection/green line); cell alone; Vero E6 cells with no infection (state of no infection/red line). B) The bar graph shows the anti-viral activity rate of *S. lappa* extract samples. Cell Index data were normalized and renamed NCI based on the time the virus was introduced into the experiment. In (B) four asterisks (****) indicate *p* value less than 0.0001 and three asterisks (***) indicate *p* value less than 0.001 by one-way ANOVA with Dunnet's multiple comparisons. The test was performed using GRAPhPad Prism version 9.4.1 (681) for Windows, GraphPad Software, San Diego, CA, USA.

depending on the dose and time, *S. costus* extraction exhibited strong cytotoxicity, with CD_{50} values in the range from 1.6 to 3.5 μg mL^{-1}. In another study, human hepatoma Hep3B cells were used to test the effectiveness of *S. lappa* root extract against HBV. Costunolide and dehydrocostus lactone, two active compounds isolated from *S. lappa*, showed inhibition of HBsAg expression with IC_{50} values of 1.0 and 2.0 mmol L^{-1}, respectively. They reported that the components in *S. lappa* root extract have potent anti-HBV activities,[41,42] demonstrated that *S. costus* ethanol extract inhibited (TNF)-alpha production in RAW 264.7 cells stimulated with LPS by more than 50%.

To accurately investigate the anti-viral activity of *S. lappa* extract, it is required to determine the concentrations at which the sample is non-cytotoxic. If the sample is toxic, it would be difficult to understand whether the virus or the sample caused the lethal effect on the cell. Similarly, dilutions of *S. lappa* extract ranging from 1/2 to 1/32 were cytotoxic to the VERO E6 cell line. When the anti-viral activity efficiency of the extract was examined at levels where the extract was not cytotoxic, the results indicated that the extract exhibited the maximum anti-viral activity against the SARS-CoV-2 virus when diluted 1/64 times. In contrast, when the dilution coefficient of the *S. lappa* extract sample becomes 1/256, the effect of multiple dilutions of the sample on the virus diminishes, and the sample loses its anti-viral action.

Replication of the SARS-CoV-2 virus occurs mostly in the throat and salivary glands, and transmission of the virus occurs through infected saliva, respiratory secretions, or droplets spat from the respiratory tract.[3,43,44] Based on the results presented here, the sample, when used in the throat and mouth, will make virus particles on the mucous membranes inactive, lowering the risk of SARS-CoV-2 transmission and the likelihood of it spreading further.

With the pandemic, much research on K18-ACE2 transgenic mouse model studies against COVID-19 disease has been performed. A comparison was made to fit the model by following the virus load in animals with intranasal virus administration on different days. Winkler et al. discovered that after virus administration at 4 and 7 dpi, K18-ACE2 transgenic mice lost significant

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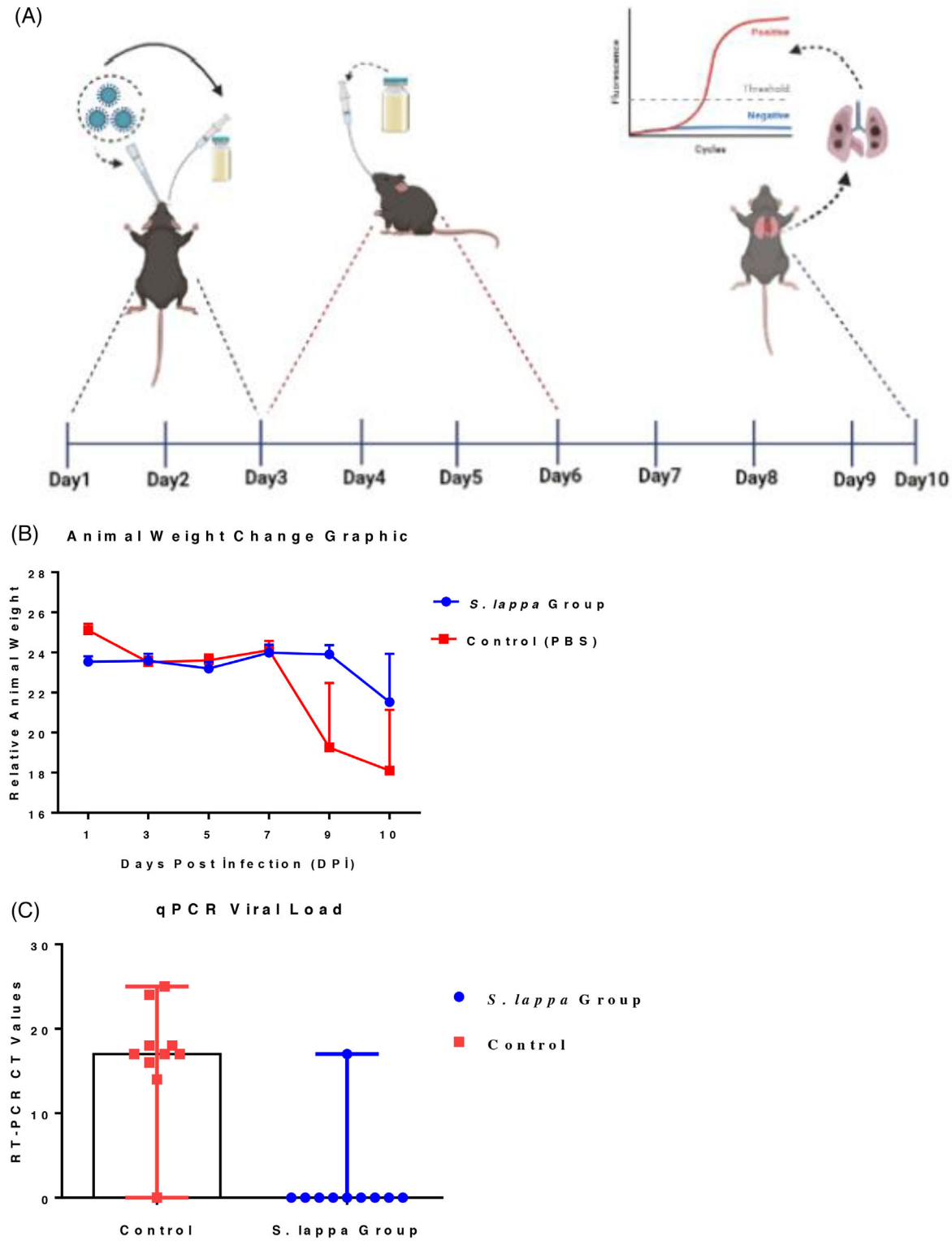


Figure 3. Neutralization effect of *S. lappa* extraction in vivo. A) Schematic representation of in vivo experiment to test efficacy of *S. lappa* extraction in vivo. Human ACE2 (hACE2)-expressing mice were infected with SARS-CoV-2 (Wuhan) isolates for 3 days and treated with *S. lappa* extract for 8 days, starting from the 3rd day after infection ($n = 10$/group). B) Average change in body weight by time in control and *S. lappa* extract treated mice. The weight graph was applied to a two-way ANNOVA test. The graph's p value is 0.0295. During the experimental period, there was no significant variance between the *S. lappa* treated groups and control groups. C) Viral loads in lungs from control and *S. lappa* treated groups ($n = 10$), measured via real-time PCR targeting two different regions of SARS-CoV-2 Nucleocapsid gene. The t test was used in this graph. The p value is $p < 0.0001$, and it was determined to be significant because $p < 0.05$.

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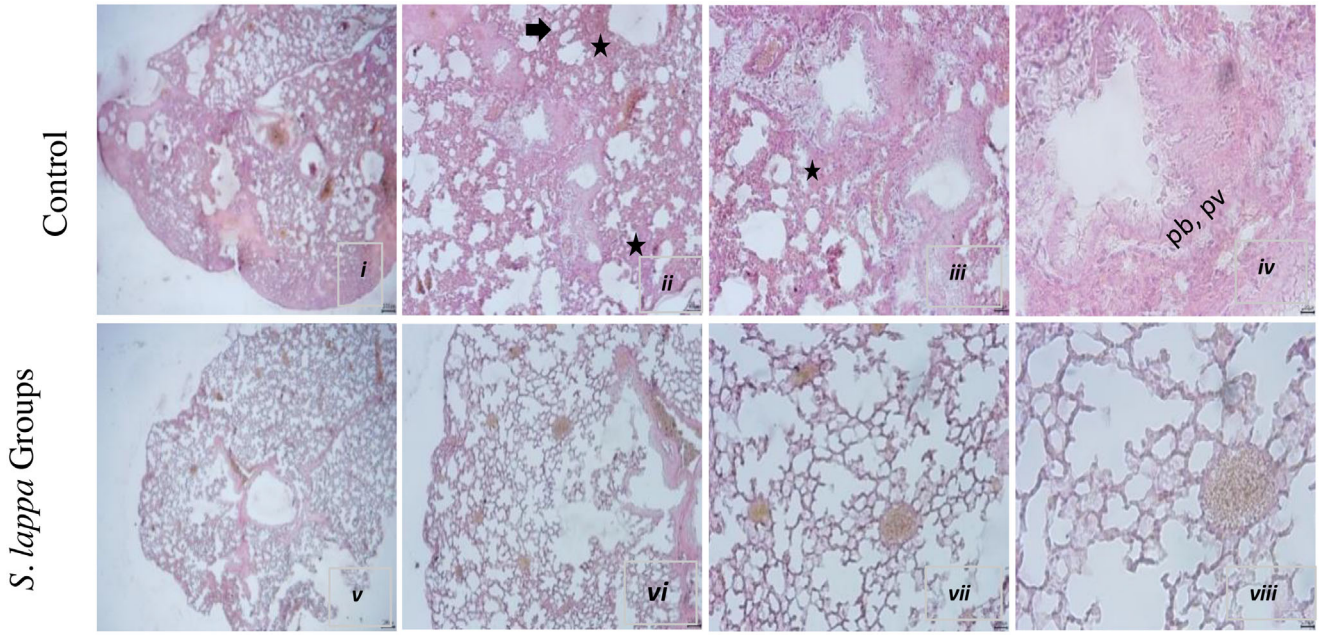


Figure 4. In vivo neutralization effect of *S. lappa* extract representative IHC images of lung tissues obtained from control (A–D) and extract treated (E–H) groups. The tissue architecture of control group shows interstitial inflammatory cell infiltration (asterisk, B), alveolar septal thickening (arrow, B) and more parenchymal infiltration at peribronchiolar (pb, D) and perivascular (pv, D) regions. *S. lappa* extract treated group shows decreased amount of interstitial inflammatory cell infiltration and normal alveolar morphology (arrowhead, G, H).

weight and had high viral loads at 2, 4, and 7 dpi.[45] Bao et al., the amount of virus detected in the lungs of hACE2 mice was highest at 3 dpi, decreased at 5 dpi, and continued to decrease at 7 dpi.[46] According to the studies, if K18-ACE2 transgenic mice are exposed to the virus every other day, the viral load begins to decrease after the fifth day, drops to an insignificant level after the seventh day, and the virus vanishes within a few days. Finally, in this study, intranasal administration of B.1.36 virus with a high $TCID_{50}$ value was performed on consecutive days to determine *S. lappa* protection in our control animals and to establish a full disease.[34,36,38,39]

S. costus synonym *S. lappa* has been shown to exhibit anti-inflammatory, anticancer/antitumor, anti-ulcer and cholagogic, antimicrobial, antiparasitic, hepatoprotective, and immunomodulator properties in both in vitro and in vivo experiments,[41,47–50] Elshaer et al. investigated the effects of removing sodium nitrite damage from *S. lappa* using three different solutions: ethanol extraction, methanol extraction, and direct aqueous extraction. These extracts have been used to study *S. lappa*'s various anti-inflammatory activities.[22]

To investigate the effects of *S. lappa*, various in vivo animal studies have been conducted. Mitra et al.[47] investigated the antiulcer activity of *S. lappa* herbal formulation on Wistar rats and guinea pigs. The formulation was given to the animals orally at a dose of 600 mg kg^{-1}. The herbal formulation of *S. lappa* was found to be effective in gastric ulcer, cold-resistant ulceration, and duodenal ulcer models caused by alcohol and aspirin in this study. Another study looked at the effect of 50, 100, and 200 mg kg^{-1} doses of ethanol extract of *S. costus* on acute and chronic inflammation in mice and rats. The study concluded that it exhibited anti-inflammatory and anti-arthritic activity.[51] Furthermore, its curative effects on acute oral toxicity and myocardial infarction were investigated in vivo.[22,23]

Although *S. lappa* has been used to treat bronchitis since many years, its effect on upper respiratory tract infections caused by coronaviruses was studied for the first time using in vitro and in vivo studies. In our study, we discovered that using *S. lappa* aqueous extract on a regular basis during the COVID-19 disease protects and heals animals. Furthermore, no pathological lesions were found in the lungs or other organs.

The next study should look at the total substance content of *S. lappa* extract. Following this stage, a human dose research study will take place, and the product will be ready for human consumption as supplementary food.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Keywords

antiviral, COVID-19, herbal medicine, neutralization, root extracts, SARS-CoV-2, *Saussurea lappa*

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