INTRODUCTION

In the last decade, there has been an increase in the prevalence of viral infections worldwide, one of which is the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection [1]. The SARS-CoV-2 pandemic was officially announced by the World Health Organization (WHO) on March 11, 2020 [2].

Clinical research has focused on several approved antiviral drugs, including remdesivir [4], favipiravir [5], and molnupiravir [6]. Several medicinal plants have been shown to have an inhibitory effect on viral infections in vitro and in vivo, thus making them candidate for aid in the treatment of SARS-CoV-2 [7, 8]. Plants have a variety of secondary metabolites such as flavonoids, terpenoids, lignans, tannins, and alkaloids that have a range of anti-infectious activities and antioxidants [9]. Several secondary metabolites are used as antivirals, including phenolates, carotenoids, terpenoids, and alkaloids [10].

Sausurea lappa is a plant that is being considered as a potential antiviral candidate. According to literature studies, the analysis of S. lappa shows the presence of several metabolites, including sesquiterpenes, flavonoids, phytosterols, lignans, and terpenes. Some of the most important chemicals that have been isolated are costunolide lappidactone, dihydrocostunolide, and dehydrocostus lactone [11, 12]. Myrcene is a chemical in S. lappa that can interact with the angiotensin-converting enzyme 2 (ACE2) receptor. This receptor is responsible for allowing the entry of SARS-CoV-2 into host cells. The anti-inflammatory capabilities of S. lappa can be utilized in treating COVID-19 by mitigating the levels of inflammatory cytokines such as TNF-α and IL-1β [13]. Exploration of a plant metabolite’s potential in revolutionizing COVID-19 treatment offers a promising avenue for therapeutic development. Previous research focused on investigating the effects of S. lappa on SARS-CoV-2, but it was still unable to explain the mechanisms of the extract as antiviral. This study investigates the novel impact of this natural compound on SARS-CoV-2, which has not been previously explored.

MATERIALS AND METHODS

S. lappa root extraction

Sausurea lappa root was obtained as a sample in the pharmaceutical biology laboratory at the Department of Pharmacy, Faculty of Medicine and Health Sciences, UIN Maulana Malik Ibrahim, Malang, with reference number 075/212/10220. Simplicia S. lappa is extracted using the UAE (Ultrasound-Assisted Extraction) method with a 96% ethanol solvent. The extraction results are filtered using filtered paper and dialized with nitrogen gas, thus obtaining a concentrated extract. The extract is collected, applied in a rotary evaporator to obtain a thick extract, and then dried in the oven at a temperature of 40 °C [14].

Cells and virus

Vero E6 cells cultivated in Dulbeco’s modified Eagle’s medium (DMEM, Gibco-INVICENTRON) containing 100 g/ml penicillin, 100 g/ml streptomycin (Sigma) and 5% Fetal Bovine Serum (FBS, Gibco-Invitrogen), at 37 °C in a CO2 incubator of 5%. Bovine serum albumin (BSA, Roche), TritonX-100 (Promega), Formaldehyde...
the mobile phase (A) CHCl3:MeOH; (B) N-hexane: ethyl acetate, and quercetine as control. (A) and (C) observed under UV 254 nm; (B) and (D) observed under UV 366 nm. Q1-Q2 = quercetin, S1-S2 = S. lappa ethanol extract

Fig. 1: Thin layer chromatography (TLC) test results on 96% ethanol extract of S. lappa. GF254 silica gel stationary phase, the mobile phase (A) CHCl3:MeOH; (B) N-hexane: ethyl acetate, and quercetine as control. (A) and (C) observed under UV 254 nm; (B) and (D) observed under UV 366 nm. Q1-Q2 = quercetin, S1-S2 = S. lappa ethanol extract

Time addition experiment
To determine the mechanism of the extract's inhibition against SARS-CoV-2, a time addition experiment was conducted with three mechanisms: (1) Entry-post entry mechanism is achieved by adding S. lappa extract along with viral inoculation for 1 hour and then for a 24-hour period of infection, (2) Entry step inhibition, in which the virus and test material are inoculated on the cell for 2 h, then incubated for 24 h with the added medium, (3) Post-entry inhibition is the way the virus is inoculated into the cell, then incubated for 24 h with the addition of S. lappa extract [19]. Supernatants of each treatment are harvested on days 1; 2; 3; and 6, followed by qRT-PCR and Western blot analysis [19, 20].

Immunoblotting
The infected Vero cells are injected with a RIPA buffer, then run with SDS-polyacrylamide gel electrophoresis (SDS page) and transferred to a polyvinilidene membrane (PVDF) (Millipore, Bedford, MA, USA). The membrane was then incubated with a primary monoclonal anti-spike mouse (GTX632604; GeneTex, 1:5000) and an anti-nucleocapsid monoclonal mouse. (GTX632269, GeneTex, 1:2000) [20].

Statistical analysis
Analysis of data from the cytotoxic test with the MTT method obtained absorbance data from each concentration of the test substance. Cytotoxicity concentration of 50% (CC50) is determined by probit analysis using SPSS software. Antiviral test data is obtained from the Ct value, which is analyzed with Probit using SPSS software to obtain the IC50 value. Analysis of the barrier mechanism by calculating the Ct values of each entry stage, post-entry, and entry-post-entry at a specific concentration of substance compared to the control data were obtained in the form of means and standard deviations. Statistically significant differences between groups are determined using one-way analysis of variance (ANOVA) with a p-value of less than 0.05. The expression of S and N proteins is calculated using ImageJ software.

RESULTS
Thin layer chromatography (TLC) analysis of extracts of Saussurea lappa
The results of the characterization of chemical compounds with TLC show that 96% of the ethanol extract from the S. lappa root contains terpenoid compounds, specifically triterpenoids, that are shown with a change in stain color to purple after heating, as shown in fig. 1.
Cytotoxic test
Toxicity testing of the test material was performed on Vero E6 cells without SARS-CoV-2 administration, and then absorption measurements were performed at wavelengths of 560 nm and 750 nm. In this test, a cytotoxicity concentration of 50 (CC50) of 131.4 μg/ml was obtained.

Anti-SARS-CoV-2 activity ethanol extract (96% S. lappa)
Antiviral activity tests of 96% S. lappa ethanol extract were conducted using Vero E6 cells and SARS-CoV-2 virus variant B.1.470. Supernatants from each treatment were collected on days 1, 2, 3, and 6, then a polymerase chain reaction test with target gene E and ORF1ab was conducted to determine the Ct value. This test resulted in a 50% inhibition concentration of 40 μg/ml. The selectivity index (SI) describes the ratio of the cytotoxicity (CC50) to the antiviral activity (IC50) of a drug. A higher SI indicates how effective a drug is for a specific virus infection [21]. This means that the higher the SI, the greater the chance that the test material will develop into a product. The SI value of ethanol extract 96% root S. lappa is obtained at 3.51.

Based on fig. 2, the results of the Ct value can be seen on each day of observation in different test groups. Different test results show that the variable Ct values between the test groups have significant differences (p<0.05).

### Table 1: Results of the Ct value of S. lappa ethanol extract activity against SARS-CoV-2 with target gene E

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ct values (Average)±SD*</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μg/ml</td>
<td></td>
<td>28.90±0.035</td>
<td>26.38±0.021</td>
<td>26.15±0.078</td>
<td>26.54±0.042</td>
</tr>
<tr>
<td>75 μg/ml</td>
<td></td>
<td>26.03±0.219</td>
<td>18.95±0.989</td>
<td>17.30±0.002</td>
<td>16.93±0.028</td>
</tr>
<tr>
<td>50 μg/ml</td>
<td></td>
<td>26.70±0.085</td>
<td>17.00±0.020</td>
<td>16.93±0.028</td>
<td>14.80±0.021</td>
</tr>
<tr>
<td>25 μg/ml</td>
<td></td>
<td>25.15±0.169</td>
<td>16.73±0.035</td>
<td>13.48±0.078</td>
<td>10.80±0.007</td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td>27.57±0.332</td>
<td>16.21±0.014</td>
<td>12.19±0.002</td>
<td>7.29±0.113</td>
</tr>
<tr>
<td>Remdesivir</td>
<td></td>
<td>28.18±0.057</td>
<td>20.56±0.071</td>
<td>18.86±0.063</td>
<td>17.95±0.092</td>
</tr>
</tbody>
</table>

*SD value (Standard Deviation) 3 times replication

### Table 2: Results of the Ct value of S. lappa ethanol extract activity against SARS-CoV-2 with target gene ORF1ab

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ct values (Average)±SD*</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μg/ml</td>
<td></td>
<td>28.24±0.08</td>
<td>25.94±0.01</td>
<td>25.41±0.17</td>
<td>26.54±0.04</td>
</tr>
<tr>
<td>75 μg/ml</td>
<td></td>
<td>25.47±0.07</td>
<td>18.95±0.10</td>
<td>18.95±0.09</td>
<td>16.93±0.03</td>
</tr>
<tr>
<td>50 μg/ml</td>
<td></td>
<td>26.24±0.08</td>
<td>18.95±0.09</td>
<td>15.71±0.17</td>
<td>14.80±0.02</td>
</tr>
<tr>
<td>25 μg/ml</td>
<td></td>
<td>24.85±0.19</td>
<td>16.73±0.04</td>
<td>12.62±0.00</td>
<td>10.80±0.01</td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td>26.95±0.12</td>
<td>15.86±0.05</td>
<td>11.16±0.13</td>
<td>7.29±0.11</td>
</tr>
<tr>
<td>Remdesivir</td>
<td></td>
<td>27.59±0.20</td>
<td>20.57±0.05</td>
<td>19.13±0.05</td>
<td>17.95±0.09</td>
</tr>
</tbody>
</table>

*SD value (Standard Deviation) 3 times replication
Fig. 3: Ct value of *S. lappa* ethanol extract against SARS-CoV-2 on days 1, 2, 3, and 6 of observations with target gene ORF1ab

The results demonstrated that *S. lappa* ethanol extract has activity as an antiviral for SARS-CoV-2 by assessing the Ct value with target Gen E and ORF1ab (fig. 2 and 3). Compared to virus control, *S. lappa* ethanol extracts can increase Ct values significantly depending on the dose and day of observation ($p$-value<0.05). However, at a dose of 100 μg/ml, the extract produces toxic effects on Vero E6 cells, so from the RT-PCR results, a higher Ct value than the positive control is obtained (Remdesivir).

Fig. 4: Observation results on day 3, with the microscope inverted to see cells experiencing cytopathic effects. (A) shows the presence of toxic effects of extracts on E6 Vero cells; (B), (C), (D), and (E) show cytopathic effects characterized by cell rounding and detachment of cells; (F) shows no occurrence of cytopathic effects

**Time addition experiment**

The results of the time addition experiment, as shown in fig. 5, show a difference between the result of the Ct value on each day of observation and the stage of the working mechanism of the test material. On the observation of the first day, there are significant differences between the results of Ct values in each treatment group ($p$<0.05), where the entry-post entry mechanism obtains the highest value. The same results were found on days 2, 3, and 6.

**Table 3: Ct value of time addition experiment based on day observation with target Gene E**

<table>
<thead>
<tr>
<th>Day</th>
<th>Entry-post entry (++)</th>
<th>Entry (+)</th>
<th>Post entry (+)</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30.62±0.205</td>
<td>28.96±0.042</td>
<td>26.03±0.219</td>
<td>27.77±0.002</td>
</tr>
<tr>
<td>2</td>
<td>26.70±0.085</td>
<td>24.85±0.198</td>
<td>20.56±0.070</td>
<td>17.39±0.028</td>
</tr>
<tr>
<td>3</td>
<td>20.57±0.070</td>
<td>19.16±0.049</td>
<td>18.25±0.002</td>
<td>11.53±0.177</td>
</tr>
<tr>
<td>6</td>
<td>16.68±0.023</td>
<td>13.00±0.318</td>
<td>11.53±0.177</td>
<td>6.97±0.127</td>
</tr>
</tbody>
</table>

*p*SD value (Standard Deviation) 3 times replication
Table 4: Ct value of time addition experiment based on day observation with target Gene ORF1ab

<table>
<thead>
<tr>
<th>Day</th>
<th>Entry-post entry (++)</th>
<th>Entry (+)</th>
<th>Post Entry (+)</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30.36±0.45</td>
<td>28.33±0.05</td>
<td>25.56±0.01</td>
<td>27.41±0.04</td>
</tr>
<tr>
<td>2</td>
<td>26.24±0.08</td>
<td>24.85±0.19</td>
<td>20.57±0.07</td>
<td>16.91±0.02</td>
</tr>
<tr>
<td>3</td>
<td>20.56±0.07</td>
<td>19.16±0.05</td>
<td>18.25±0.04</td>
<td>10.61±0.09</td>
</tr>
<tr>
<td>6</td>
<td>16.68±0.23</td>
<td>12.93±0.08</td>
<td>10.61±0.09</td>
<td>6.03±0.16</td>
</tr>
</tbody>
</table>

*SD value (Standard Deviation) 3 times replication

Fig. 5: Time addition experiment with target gene E

Fig. 6: Time addition experiment with target gene ORF1ab
Time addition experiments with target genes based on correlation tests (fig. 7) show strong negative correlations between Ct value results and observation days, both on entry-post entry and post-entry mechanisms. This suggests that the longer the observation day, the lower the Ct values, both in the treatment and control groups.

**Fig. 7: Correlation of Ct value result with observation day**

Based on the correlation test (fig. 8), a strong positive correlation exists between the dose of *S. lappa* extract and the Ct value on day two observation, with a degree of significance of 0.001. Similarly, the results of Ct values on days 3 and 6 of observation show a strongly positive correlation between the extract dose and the result of the Ct value, with a degree of significance of 0.000. This indicates that the higher the dose, the higher the Ct value.

However, on day one observations, it showed a weak positive correlation, with a degree of significance of 0.272 (target gene E) and 0.267 (target gene ORF1ab).

**Fig. 8: The dose correlation of the extract with the resulting Ct value on observation days 1, 2, 3, and 6**

**Fig. 9: Protein expression in S and N SARS-CoV-2.** (A) The result of a western blot with the antibody Protein S; (1) 50 µg/ml; (2) remdesivir; (3) negative control; (B) The result of a western blot with the antibody Protein N; (1) 75 µg/ml; (2) 50 µg/ml; (3) 25 µg/ml; (4) Remdesivir; (5) negative control; (C) Protein S expression; (D) Protein N expression
Immunoblotting

In the time addition experiment, it is known that S. lappa ethanol extract is likely to work at the entry post-entry stage, so it is necessary to test the expression of proteins produced by viruses, especially proteins related to the identification stage, such as protein S, and proteins associated with virus replication, namely protein N. The result of the protein expressions S and N is shown in Fig. 9.

Quantitative analysis of protein expression using ImageJ software showed a decrease in S protein expression by 52.1% in the positive control group (Remdesivir) and by 47.1% in extract concentrations of 50 μg/ml compared to the control group. Decreases in N protein expression were also shown in the remdesivir group as well as the extract groups of 25, 50, and 75 μg/ml, respectively, by 24.1%, 11.8%, 22.7%, and 10.9%.

DISCUSSION

The bioactivity of medicinal plants is affected by the chemical content. In this study, the TLC results showed that S. lappa ethanol extract includes terpenoid components. This aligns with the literature study of S. lappa, which contains various variable metabolites such as sesquiterpenes, flavonoids, phytosterols, lignans, and terpenes. Sesquiterpenes are the most abundant compounds in S. lappa, with costulolide and dehydrocostus lactone being the main active ingredients [22, 23]. Multiple investigations have demonstrated that terpenoids tend to function as inhibitors of viral proteins and enzymes, such as protein S, and proteins associated with virus replication, namely protein N. The myrcene compound present in S. lappa impacts the ACE-2 receptor, hence impeding the entry of SARS-CoV-2 into the host cells [25].

The antiviral activity was analyzed to see how well the ethanol extract of S. lappa protected Vero E6 cells from infection with SARS-CoV-2. The results showed that the extract can inhibit SARS-CoV-2, with IC50 values of 40 μg/ml. Other studies have indicated that S. lappa extract has partial antiviral activity with effectiveness of 75%, 65%, and 13% against the SARS-CoV-2/Muhan strain at concentrations of 1/64, 1/128, and 1/256 [26]. While the toxicity test also showed that S. lappa ethanol extract had a CC50 of 131.4 μg/ml, this result was similar to previous reports that showed S. lappa ethanol extracts had a CC50 of 162.9 μg/ml [27] and 70.36±0.8 μg/ml [28]. The selectivity index (SI) for this study was 3.51 points. An ideal substance as an antiviral is one whose cytotoxic concentration is high and the inhibitory concentration of the antiviral is low [29].

This study also explains a positive correlation between the extract dose and the Ct value, where the higher the extraction dose, the higher the Ct value when compared to the control group. This study aligns with the research conducted by Ulbegi Polat et al. (2023), which discovered a positive correlation between the concentration of extracts and the percentage of antiviral activity. In other words, as the concentration of the extracts increased, the level of antiviral activity also increased [26]. An experiment was conducted to discover the specific stage of the virus cycle in which the extract exhibits antiviral action. The extract is introduced at various stages throughout the infection to achieve this objective. Fig. 5 and 6 demonstrate that the ethanol extract of S. lappa exhibits both virucidal action, preventing virus entry, and inhibitory effects on the virus once it has entered host cells. This is demonstrated by obtaining a higher Ct value at the entry-post entry level compared to the entry and post-entry levels alone. Other research suggests that a water extract from Saussurea costus can inhibit the virus once it enters the host cells [30]. There was a strong negative correlation between the results of the Ct value and the day of observation, suggesting that the longer the observation day, the lower the Ct value, which means that the extract activity against the virus will decrease.

The Western blot analysis of immunoblotting revealed a reduction in the expression of S and N proteins in the treatment group compared to the negative controls. Protein S is linked to virus recognition, whereas protein N is a structural protein (SARS-CoV-2) that facilitates the packing of genetic material, assists in the folding of RNA, transports proteins inside cells, breaks down DNA, and restricts the host’s immunological response [31, 32]. Protein N, the most abundant protein in virus-infected cells, is characterized by its genetic stability, making it a promising candidate for therapeutic targeting [33].

CONCLUSION

Thin-layer chromatography results obtained terpenoid compounds from S. lappa ethanol extract. The ethanol extracts of S. lappa demonstrate antiviral properties against SARS-CoV-2, with a cytotoxic concentration (CC50) of 131.4 μg/ml and an inhibitory concentration (IC50) of 40 μg/ml. This results in a selectivity index of 3.51. It is possible that S. lappa extracts work at the entry-post entry stage against SARS-CoV-2, as well as a decrease in the expression of proteins S and N in the treatment group compared to the negative control.

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AUTHORS CONTRIBUTIONS

AAF: concept and design, literature search, experimental studies, data analysis, data analysis, manuscript preparation, manuscript editing; DS: concept and design, data acquisition, data analysis, manuscript preparation; MD: concept and design, data acquisition, data analysis, manuscript preparation; SK: concept and design, data acquisition, data analysis, manuscript preparation; RM: concept and design, experimental studies, data acquisition, data analysis, manuscript preparation, manuscript editing; MLC: concept and design, experimental studies, data acquisition, data analysis, manuscript preparation; SRP: concept and design, data acquisition, data analysis, manuscript preparation

CONFLICT OF INTEREST

There is no conflict of interest among all the authors.

REFERENCES


