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Synergistic Antiviral Effects of Combined Fractions from *Eucalyptus camaldulensis* Against SARS-CoV-2

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused substantial global issues that significantly impacted public health. The emergence of SARS-CoV-2 variants poses a great challenge for pandemic control efforts, including developing vaccines and antiviral therapeutics. Natural remedies derived from various aromatic and medicinal plants have been used for centuries in traditional medicine systems for their potential antiviral properties. Here, we examined the *invitro* antiviral activity of *Eucalyptus camaldulensis* extract and its different fractions of leaves and bark against SARS-CoV-2.The cytotoxicity concentration (CC_{50}) for these fractions ranged between ~105.7 and 586 µg/mL. Methanol extract of eucalyptus and its fractions were shown to inhibit SARS-CoV-2at half-maximal inhibitory concentrations (IC_{50}) of ~ 3 to 134µg/mL. The inhibitory effect of the dichloromethane fraction from the leaves and bark of the eucalyptus plant was moderate for each, reaching 61.1% and 56.3%, respectively. However, after combining both fractions, the inhibition percentage reached 73.7% using the highest safe concentration, indicating that the inhibitory effect changed from moderate to promising due to the synergy between the components of the monoterpenes and sesquiterpenes compounds. The major phytochemical constituents were analyzed using GC-MS analysis. The bark fraction showed the presence of monoterpenes and sesquiterpenes as major components, while the leaves showed sesquiterpenes only. GC-MS of the highly antiviral dichloromethane fraction for the combined leaves and bark revealed the presence of spathulenol and pinanediol as main components. These findings indicate that *Eucalyptus camaldulensis* compounds are potentially useful as highly effective antiviral agents against SARS-CoV-2, and further preclinical studies are recommended.

Keywords: *Eucalyptus camaldulensis*; Antiviral; SARS-CoV-2; and GC-MS.

1. Introduction

Emerging respiratory infectious diseases pose a substantial risk to humans due to their extremely high potential to spread from person to person. Corona viruses are the main cause of respiratory tract diseases in the human population. Severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) is the agent that caused the COVID-19 pandemic. SARS-CoV-2 continues to pose a threat to global health. As of 16 July 2024, the SARS-CoV-2 virus has affected more than 776 million patients and caused around 7.1 million deaths worldwide [1].

Despite the continuous efforts to develop new and highly effective treatment and control platforms for SARS-CoV-2, the virus continues to circulate, and new variants continue to emerge [2]. Several vaccine platforms have been introduced worldwide, and specific antiviral drugs played an important role in the early stages of the pandemic. In 2020, several studies were conducted to determine which approved drugs can be repurposed and assess their effectiveness against SARS-CoV-2. A synergistic antiviral-based strategy is required since, in addition to the production rate, pharmaceutical companies are currently dealing with the recently disclosed SARS-CoV-2 variations, against which not all vaccines have demonstrated sustained efficacy [3]. The FDA in the United States has not yet approved any medicine as a definitive cure for the infection, although certain recent treatments have been authorized for use in hospital settings [4].

Virus-related chronic respiratory illness is currently a global health issue related to COVID-19. While research is being conducted globally to develop appropriate drugs and vaccines for disease control, there is, till now, no specific treatment or appropriate therapy available. The main protease (M^{pro}) is a crucial enzyme responsible for converting polypeptides into functional proteins,

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which demonstrates how coronavirus replication occurs. Finding natural and efficient M^{pro} inhibitors maybe a secure and successful strategy for COVID-19 management [5].
Essential oils can be extracted from plants through mechanical extraction, dry distillation, or water vapor distillation[6]. More than 100 distinct chemical components, mostly odorant ones like sesquiterpenes, monoterpenes, and terpenoids, are present in these oils [7]. A variety of bacteria's metabolic processes can be inhibited by essential oils, depending on their exact composition [8].
One of the most important plants is eucalyptus, *Myrtaceae* family, and ismost commonly found in tropical and subtropical regions. The *Myrtaceae* family has 140 genera and over 3800 species [9]. Many Myrtaceae species have substantial antibacterial properties, and their volatile oils are added to lotions, soaps, and toothpastes as antibacterial and antifungal ingredients [10]. The extract of eucalyptus leaves retained the highest concentration of phytochemicals, including quinones, saponins, carbohydrates, tannins, phenols, flavonoids, and fats[11, 12]. The DPPH scavenging activity of the methanolic extract, measured in terms of ascorbic acid, indicated a noteworthy level of antioxidant activity. The phenolic content was found to be 11.41%, and the total flavonoid content was 35.88% [11].
Molecular docking was used to conduct an *in-silico* study to examine the effect of eucalyptus biocompounds on M^{pro} [5] and showed that eucalyptol exhibited the least binding energy to M^{pro} without causing any toxicity. This suggests that eucalyptol has the potential to be used as an inhibitor against COVID-19 and for treating the virus infection [13].
The biological benefits of the eucalyptus plant are quite diverse, including antibacterial, antiseptic, antioxidant, anti-inflammatory, and anticancer properties. The major ingredient that gives eucalyptus oils their therapeutic properties, including prevention of COVID-19, is eucalyptol (1,8-cineole) [14].
Eucalyptus oil showed antiviral properties against a variety of viruses, such as the coxsackie B, poliovirus, echovirus 6, influenza A (H1N1) virus, rotavirus, and herpes simplex viruses HSV-I and HSV-II. Furthermore, eucalyptus oil demonstrated antiseptic qualities and prevented the spread of viruses on various filters and cutlery [15].
Although the evergreen scent and several medicinal benefits of eucalyptus oils are attributed to a component known as eucalyptol, eucalyptus oils are full of multiple natural compounds that work in harmony to provide a variety of health-promoting effects [16]. Eucalyptol has also shown a promising therapeutic role in the management and prevention of COVID-19, acting as a potential inhibitor of the SARS-CoV-2 main protease[17].

In the present study, the methanol extract of leaves and bark of the eucalyptus plant and their corresponding fractions were evaluated against SARS-CoV-2. Subsequently, the combined fractions of bark and leaves were studied to determine their combined activity contributing to the overall synergism between eucalyptus leaves and bark. The phytochemical compounds of the leaves and bark were identified separately, and then the total extract of the combined leaves and bark and their corresponding fractions were identified through GC-MS analysis.

2. Materials and methods

Cells and virus

The African green monkey kidney epithelial Vero-E6 (ATCC No CRL-1586) cells were kindly provided by Dr. Richard Webby, St. Jude Children's Research Hospital, Department of Host-Microbe Interactions, Division of Virology, USA. Vero-E6 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented with 5-7% for cell growth, 1% penicillin-streptomycin (Sigma-Aldrich), and 2 mM L-glutamine (Sigma-Aldrich) at 37 °C with 5% CO_2. SARS-CoV-2 strain hCoV-19/Egypt/NRC-03/2020 (GISAID accession number: EPI_ISL_430819) was isolated and propagated in Vero-E6 cells.

Plant materials and extracts preparation

The leaves and bark parts of the eucalyptus tree (*Eucalyptuscamaldulensis*) were collected from Gharbia Governorate, Egypt. The plants were kindly identified by Prof. Dr. Ibrahim Abd El-Rahim Mashaly, Professor of Plant Ecology and Flora, Botany Department, Faculty of Science, Mansoura University, Egypt.The dry leaves and bark parts (~15g) of the eucalyptus tree were soaked in aqueous methanol (3×150ml methanol (70%)) for 3~4 days at ambient temperature using dark glass bottles. Then, the obtained methanol extract was applied to evaporation in vacuo (under vacuum) with the aid of a rotary evaporator. The afforded residue of the extract was partitioned in distilled water (100 mL), followed by subsequent extraction with *n*-hexane, dichloromethane, and ethyl acetate. In accordance, five groups of extracts were obtained: a) the original methanol extract, b) *n*-hexane fraction, c) dichloromethane fraction, d) ethyl acetate fraction, and e) the water residue extract.

Cytotoxicity assay

The cytotoxicity of plant extracts was evaluated on Vero-E6 cells by the Crystal Violet assay [18]. Briefly, Vero-E6 cells were seeded in 96-well plates at a cell density of $1.0x10^4$ cells/well in DMEM supplemented with 5% FBS. Then, the plates were incubated for 24 h at 37 ºC with 5% CO_2. After incubation, plant extract dilutions were prepared in separate 96-well plates, and 50 µl of each dilution was added to each well in quadruplicate and incubated for 72 hrs at 37 ºC with 5% CO_2. After incubation, cells were treated with 10% formaldehyde for fixation and incubated for 3h at room temperature. After washing, cells were stained with 50 µl/well of 0.5% crystal violet solution, and the monolayers were then washed and dried. The stain was then dissolved with 200 µl of absolute methanol. Absorbance was measured at λ max 570 nm using a multi-well plate reader. The cytotoxicity% compared to the untreated cells was determined according to the following equation. The plot of % cytotoxicity versus tested sample concentrations was used to calculatethe concentration that exhibited CC_{50} (50% cytotoxicity).

$$\%\ \text{Cytotoxicity} = ((\text{OD. of cells without treatment} - \text{OD of cells with treatment}) / (\text{OD of cells without treatment}) \text{ X } 100).$$

Evaluation of the antiviral activity against SARS-CoV-2

Titration of the virus with tissue culture infectious dose ($TCID_{50}$)

To determine the viral dilutions, the tissue culture infectious dose 50 ($TCID_{50}$) was conducted [18]. Briefly, Vero-E6 cells were seeded in 96-well plates at a cell density of $1.0x10^4$ cells/well in DMEM supplemented with 5% FBS. Then, the plates were incubated for 24 h at 37 ºC with 5% CO_2. After incubation, cells were infected with ten-fold serial dilutions of the virus in triplicate and incubated for 72 hours at 37 °C in a humidified 5% CO_2 incubator. The $TCID_{50}$ value was calculated according to the method of Reed and Muench [19].

Inhibitory Concentration 50 (IC_{50}) determination

To determine the antiviral activity of the compounds, the Inhibitory Concentration 50 (IC_{50}) was performed as previously described [20]. Briefly, Vero-E6 cells were seeded in 96-well plates at a cell density of $1.0x10^4$ cells/well in DMEM and incubated overnight in a humidified 37 °C incubator in a 5% CO_2atmosphere.After incubation, cell monolayers were washed once with 1X PBS solution, then treated with different serial dilutions of the examined compounds together with 100 $TCID_{50}$/well from the virus (hCoV-19/Egypt/NRC-03/2020 (GSAID: EPI_ISL_430820)) and kept at room temperature (RT) for 1 hour, before starting incubation. After the incubation in 5% CO_2 at 37 °C for 3 days post-infection, the cells were fixed with 4% *p*-formaldehyde (100 µL) for 20 min, then stained with crystal violet (0.1%) in distilled water at RT for 15 min. Absolute methanol (100 µL) was added to dissolve the crystal violet dye per well, and the optical density of the color was measured at $\lambda = 570$ nm using an Anthos Zenyth 200rt plate reader (Anthos Labtec Instruments, Heerhugowaard, Netherlands). The IC_{50} of the tested sample is the concentration required to decrease the virus-induced cytopathic effect by 50%, relative to the virus control.

Viral replication inhibition by Plaque Reduction Assay

The viral replication inhibition of plant extracts was determined by reducing the infectious particles of SARS-CoV-2 by plaque reduction assay [20]. Briefly, $3.0x10^5$ Vero-E6 cells/well were seeded in 6-well plates for 24 hours at 37 °C, with 5% CO_2. Then, the viral inoculum was removed, and Vero-E6 cells were cultivated in a 6-well plate (10^5cells/mL) for 24-36 hours at 37 °C. The virus was inoculated directly into the cells and incubated for 1 hour at 37 °C. The inocula containing the non-adsorbed viral particles were removed by washing the cells three times with supplement-free medium. The test compound was added in varying concentrations to infected cells for another 1-hour contact time. After removing the inocula containing the tested extracts and different fractions, 3 mL of DMEM supplemented with 2% agarose was added to the cell monolayer. Plates were left to solidify and incubated at 37 °C with 5% $CO_2$for 72 hours until the appearance of viral plaques. Cell monolayers were fixed in 10% formaldehyde for 6 hours and stained with crystal violet. Control wells contained Vero-E6 cells incubated with the virus. Plaques were counted, and the percentage reduction in plaque formation was compared to the control wells and according to the following equation.

$$\textbf{Viral inhibition\% } \% = \frac{\textbf{Viral count control cells} - \textbf{viral count treated cells}}{\textbf{Viral count control cells}} \times \mathbf{100}$$

Gas chromatography–mass spectrometry (GC-MS) analysis

The chemical composition of the samples was identified using a Trace GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS(30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was initially held at 50 °C and then increased to 250 °C (rate 5 °C/min, hold 2 min), then increased to the final temperature of 300 °C (rate 30 °C /min, hold 2 min). The injector and MS transfer line temperatures were kept at 270 and 260 °C, respectively; Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 4 min, and diluted samples of 1 µL were injected automatically using Autosampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 50–650 in full scan mode. The ion source temperature was set at 200 °C. The components were identified by comparison of their mass spectra with those of WILEY 09 and NIST 14 mass spectral databases[21].

Statistical Analyses

All experiments were performed in three biological repeats.Statistical tests and graphical data presentation were carried out using GraphPad Prism 8.01 software.CC_{50}, IC_{50}, and SI variance among extract types within leaves, bark, and combination were tested with two-way ANOVA; the row factor represents the different plant parts,and the column factor represents the extraction solution. Variance in viral inhibition was tested for Remdesivir in a two-way ANOVA with Bonferroni testing for multiple comparison correction.

3. Results

Cytotoxicity and antiviral activity of eucalyptus leaf extracts

For the cytotoxic assay, the methanol extract, *n*-hexane, dichloromethane, ethyl acetate fractions, and water residue of eucalyptus leaves extracts were evaluated from 10 mg/mL to 2 mg/mL using double serial dilutions. After 48 h treatment, the extracts from leaves of eucalyptus showed a broad range of cytotoxicity concentration 50 (CC_{50}) that ranged from 105.7 µg/mL to 586.1 µg/mL (Figure 1). Based on the results of the cytotoxic assay, the half-maximal inhibitory concentration 50 (IC_{50}) for SARS-CoV-2 was determined, the extracts of leaves eucalyptus showed a broad range from 47.96, 12.2, 2.99, 14.39, and 94.3 µg/mL for methanol extract, *n*-hexane, dichloromethane, ethyl acetate fractions and water residue, respectively (Table 1, and Figure 1).

The selectivity index SI was calculated according to CC_{50} and IC_{50}results. The extracts showed a range from 4 to 72.7, where dichloromethane fraction showed highest selectivity index that reached 72.7, while water residue showed a selectivity index of 4, which was the lowest among all the fractions, as shown in Figure 1 and Table 1.

Table 1. The half-maximal cytotoxic concentration (CC_{50}) in µg, half-maximal inhibitory concentration (IC_{50}) in µg, and selectivity safety index (SI) values of the tested eucalyptus leaves and bark extracts against SARS-CoV-2.

Fractions Name Eucalyptus	CC_{50} µg/mL			P value (column, row factor)	IC_{50} µg/mL			P value (column, row factor)	SI (CC_{50} / IC_{50})			P value (column, row factor)
	Leaves	Bark	Leaves + Bark		Leaves	Bark	Leaves + Bark		Leaves	Bark	Leaves + Bark	
Methanol extract	586.1	570.1	567.9	<0.0001, 0.0431	47.96	133.9	19.98	0.0414, 0.0355	12.22	4.26	28.42	<0.0001, 0.1006
***n*-Hexane Fr.**	105.7	165.8	154.6		12.2	30.34	8.487		8.66	5.46	18.22	
Dichloromethane Fr.	217.3	253.7	402.6		2.991	50.16	5.12		72.65	5.1	78.63	
Ethyl acetate Fr.	161.3	255.8	368.9		14.39	67.17	50.39		11.2	3.8	7.32	
Water residue	378.2	585.7	581		94.3	95.34	25.5		4	6.14	22.78	
Remdesivir	351.8				2.345				150			

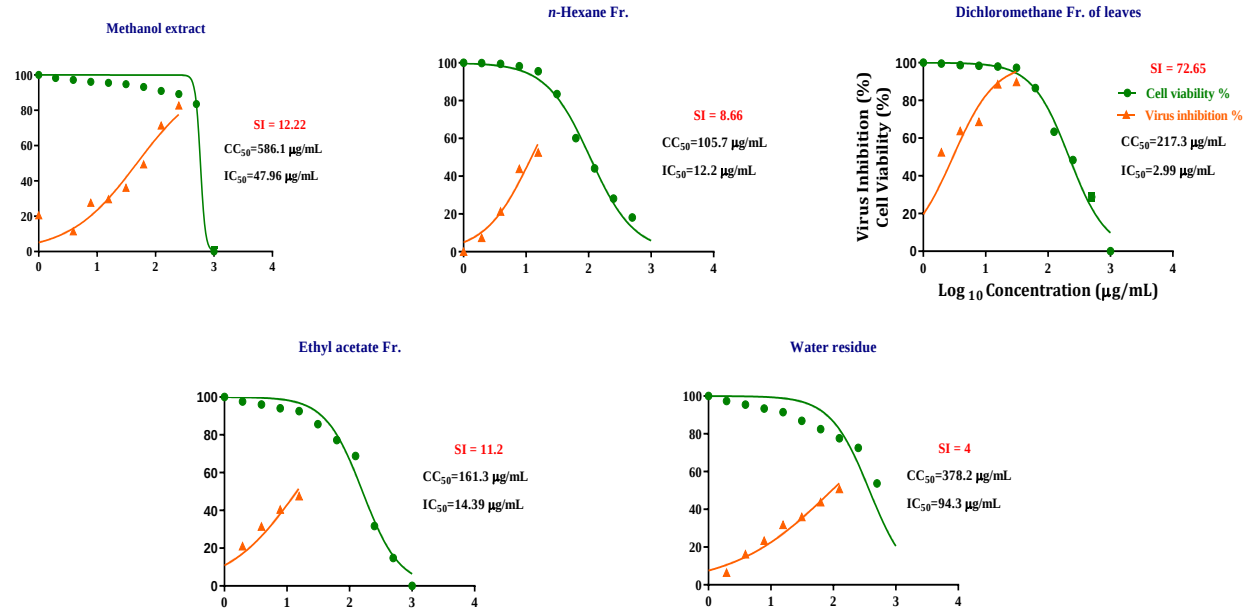

Figure 1. Determination of CC_{50} and IC_{50} of the tested eucalyptus leaves extracts in Vero-E6 cells against SARS-CoV-2.

The cytotoxicity concentration 50 (CC_{50}) of the methanol extract, *n*-hexane, dichloromethane, ethyl acetate fractions and water residue of eucalyptus leaves were 586.1, 105.7, 217.3, 161.3 and 378.2 µg/mL, respectively, while the inhibitory concentration 50 (IC_{50}) of the SARS-CoV-2 virus was 47.96, 12.2, 2.99, 14.39 and 94.3 µg/mL, respectively, and the selectivity index (SI) was 12.22, 8.66, 72.65, 11.2 and 4, respectively.

Cytotoxicity and antiviral activity of eucalyptus bark extracts

The cytotoxicity concentration 50 (CC_{50}) of the methanol extract, *n*-hexane, dichloromethane, ethyl acetate fractions and water residue of eucalyptus bark were 570.1, 165.8, 253.7, 255.8 and 585.7 µg/mL, respectively,while the inhibitory concentration 50 (IC_{50}) of the SARS-CoV-2 virus was 133.9, 30.34, 50.16, 67.17 and 95.34 µg/mL, respectively, and the selectivity index (SI) was 4.26, 5.46, 5.1, 3.8 and 6.14, respectively (Table 1, and Figure 2).

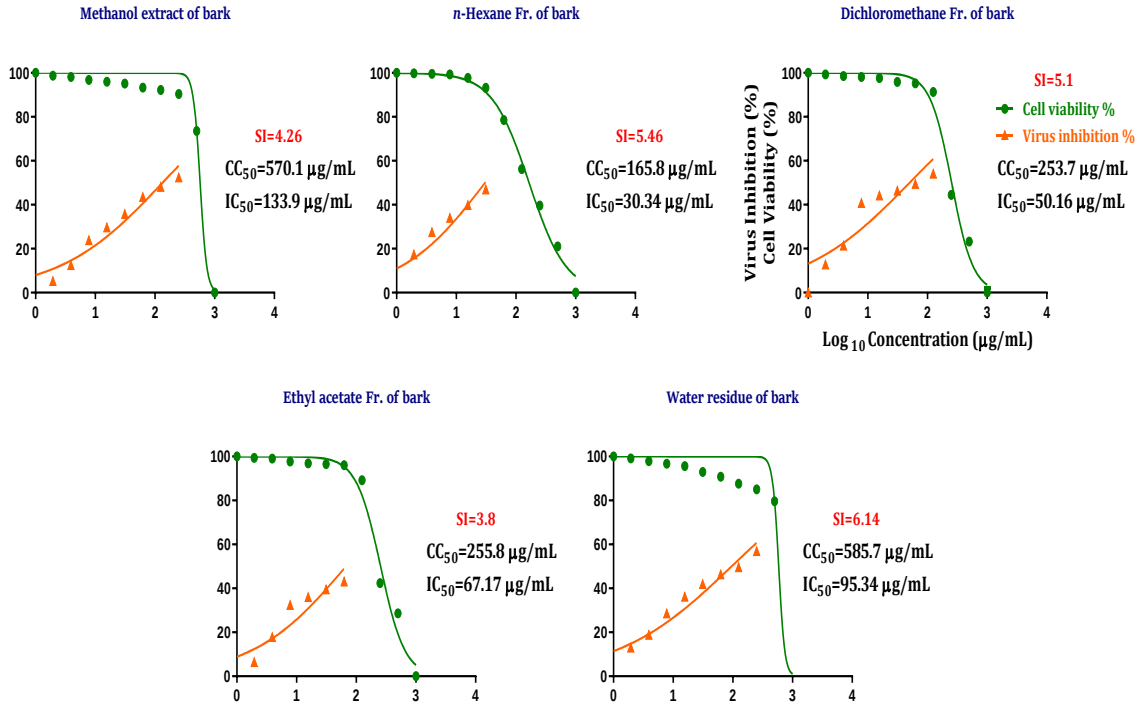

Figure 2. Determination of CC_{50} and IC_{50} of the tested eucalyptus bark extracts in Vero-E6 cells against SARS-CoV-2.

Synergistic effect of leaves and bark of eucalyptus on antiviral activity

According to the results of the antiviral activity of leaves and bark of eucalyptus, we aimed to determine the effect of a combination of extracts against SARS-CoV-2. The $CC_{50}$of the methanol extract, *n*-hexane, dichloromethane, ethyl acetate fractions, and water residue of eucalyptus leaves and bark together were determined (Table 1 and Figure 3). Dichloromethane fraction and methanol extracts showed strong antiviral activity against SARS-CoV-2, with SI78.63 and 28.24, respectively, while water residue and *n*-hexane fractionshowed high antiviral activity against SARS-CoV-2, with SI 22.7 and 18.22, respectively.

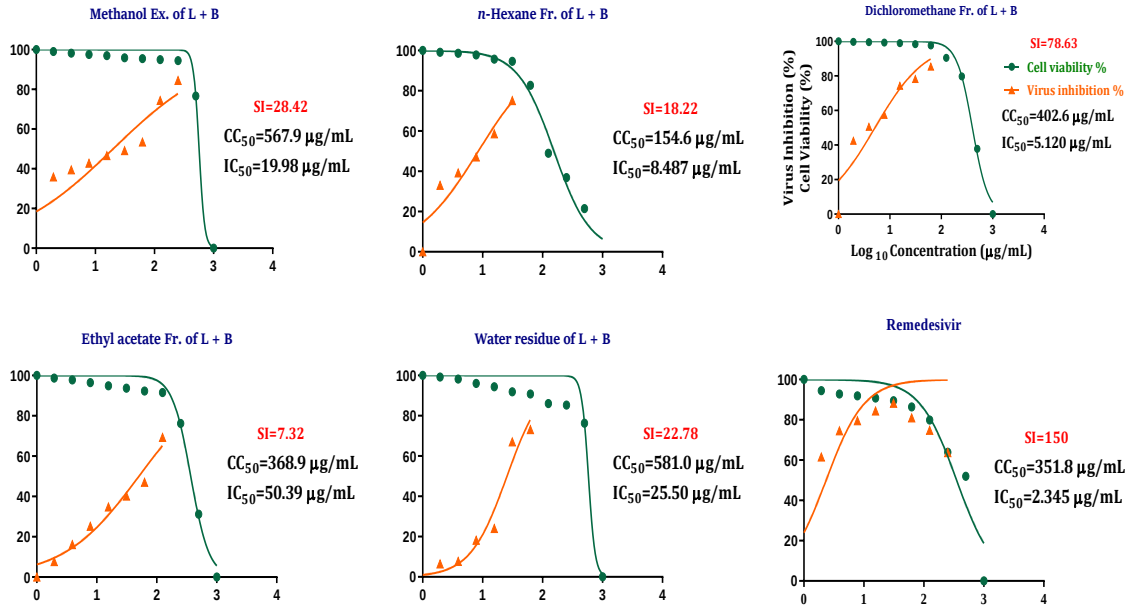

Figure 3. Determination of CC_{50} and IC_{50} of the tested combined eucalyptus leaves, bark methanol extracts, and corresponding fractions and Remdesivir in Vero-E6 cells against SARS-CoV-2.

The cytotoxicity concentration 50 (CC_{50}) of the methanol extract, *n*-hexane, dichloromethane, ethyl acetate fractions and water residue of eucalyptus leaves and bark together were 567.9, 154.6, 402.6, 368.9 and 581 µg/mL, respectively, while the inhibitory concentration 50 (IC_{50}) of the SARS-CoV-2 virus was 19.98, 8.5, 5.12, 50.39 and 25.5 µg/mL, respectively, and the selectivity index was 28.42, 18.22, 78.63, 7.32 and 22.78, respectively. Also, the cytotoxicity of the synthetic compound, Remdesivir, as a control drug, was evaluated in Vero-E6 cells by EC_{50}(crystal violet assay). The compound was essentially non-toxic for Vero-E6 cells up to a dose of 351.8 µg/mL, with aninhibitory concentration (IC_{50}) of 2.345 µg/mL, and the SI equal to 150.

Effect of leaves and bark of eucalyptus on viral replication

Based on the CC_{50} of each fraction, three different concentrations of methanol extract and different fractions of eucalyptus leaves were examined using the plaque reduction assay to determine the inhibition of viral replication. The extracts and fractions of eucalyptus showed a slightly to moderately high range of antiviral activity (from 36.8 to 61.1%). The methanol extract of eucalyptus leaves showed inhibition ranging from 26.3% to 36.8%. The fractions of eucalyptus leaves (*n*-hexane, ethyl acetate fractions, and water residue) showed moderate inhibition, ranging from 31.6% to 47.4%. In contrast, the dichloromethane fraction of eucalyptus leaves showed higher inhibition compared to other fractions (Table 2, Figure 4). The eucalyptus bark fractions were examined for antiviral efficacy against the SARS-CoV-2 virus (Table 2, Figure 5); the methanol extract of eucalyptus bark showed low inhibition of viral replication compared with the leaf extract, ranging from 2.6% to 13.2%. In contrast, the *n*-hexane fraction, ethyl acetate, and dichloromethane fraction of eucalyptus bark showed higher inhibition of viral replication than water residue (Figure 5).

Table 2: SARS-CoV-2 inhibition by different concentrations of eucalyptus fractions of leaves, bark, and leaves-bark combination.

Fraction Name Eucalyptus	Conc.µg/mL			Viral Inhibition%, (P value)		
	Leaves	Bark	Leaves + Bark	Leaves	Bark	Leaves + Bark
Methanol Ex.	575	570	550	36.8, (0.0001)	13.2, (0.0001)	53.2, (0.0001)
	525	520	500	30, (0.0001)	7.9, (0.0001)	41.6, (0.0001)
	475	470	450	26.3, (0.0001)	2.6, (0.0001)	35.8, (0.0001)
***n*-Hexane Fr.**	100	150	150	37.9, (0.0001)	55.3, (0.0001)	47.9, (0.0001)
	75	125	125	34.2, (0.0001)	52.6, (0.0001)	38.9, (0.0001)
	50	100	100	31.6, (0.0001)	50, (0.0001)	36.8, (0.0001)
Dichloromethane Fr.	200	250	400	61.1, (0.0001)	56.3, (0.0001)	73.7, (0.1491)
	175	225	350	59.5, (0.0001)	50, (0.0001)	71.1, (0.0001)
	150	200	300	57.9, (0.0001)	48.9, (0.0001)	70, (0.0001)
Ethyl acetate Fr.	150	250	370	47.4, (0.0001)	53.2, (0.0001)	54.2, (0.0001)
	125	225	320	42.1, (0.0001)	51.1, (0.0001)	48.4, (0.0001)
	100	200	270	39.5, (0.0001)	47.9, (0.0001)	43.2, (0.0001)
Water residue	375	575	575	44.7, (0.0001)	31.6, (0.0001)	41.1, (0.0001)
	325	525	525	42.1, (0.0001)	26.3, (0.0001)	35.3, (0.0001)
	275	475	475	36.8, (0.0001)	21.1, (0.0001)	32.1, (0.0001)
Remdesivir	350			91.6		
	300			87.3		
	250			83.5		

Viral inhibition for each concentration was compared to Remdesivir in a two-way ANOVA with a Bonferroni test for multiple comparisons.

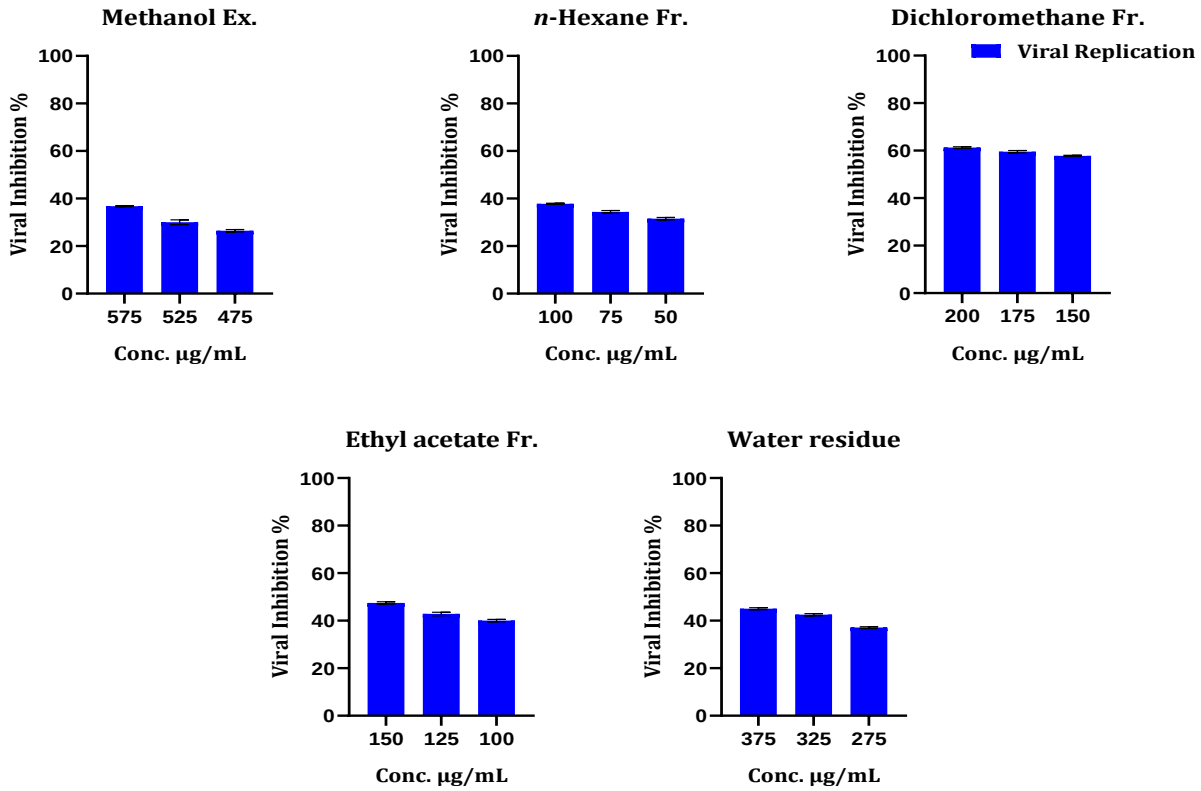

Figure 4: Effect of eucalyptus leaves extract and its fractions on SARS-CoV-2 viral replication.

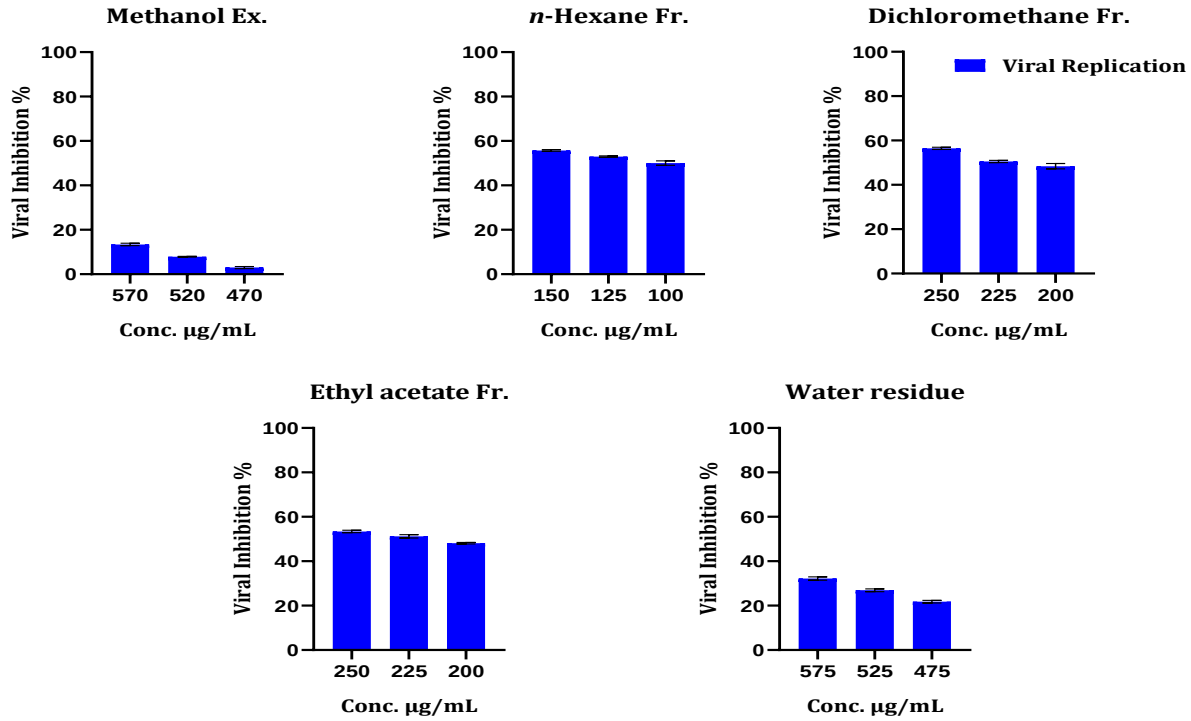

Figure 5: Effect of eucalyptus bark extract and its fractions on SARS-CoV-2 viral replication.

Synergistic effect of leaves and bark of eucalyptus on viral replication

To determine the synergistic effectof methanol extract and different fractions of eucalyptus leaves and bark fractions combined in a 1:1 ratio against the SARS-CoV-2 virus, we used three different concentrations of extracts to examine the inhibition of viral replication using the plaque reduction assay. The results showed that only the dichloromethane fraction had a promising effect of antiviral activity against the SARS-CoV-2 virus, while the rest of the eucalyptus extract fractions showed moderate inhibition (Figure 6). The different concentrations of dichloromethane fractions 300, 350, and 400µg/mL, showed inhibition of 70%, 71.1%, and 73.7%, respectively, while the Remdesivir, as a control drug at different concentrations of 250, 300, and 350 µg/mL, showed viral inhibition of 83.5%, 87.3%, and 91.6%, respectively (Figure 6).

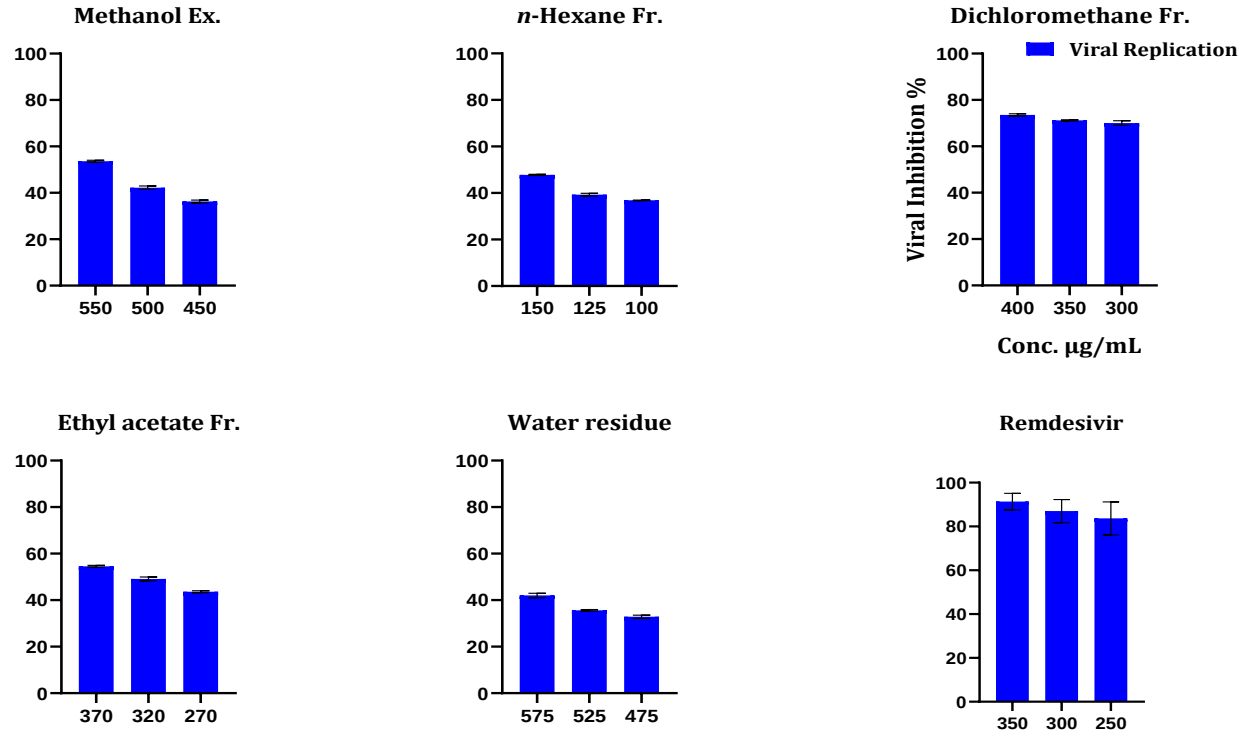

Figure 6:Effect of combined eucalyptus leaves, bark methanol extracts, and corresponding fractions,and compare with Remdesivir on SARS-CoV-2viral replication.

The above results showed a noticeable synergy between the dichloromethane fraction of leaves and bark. The rate of inhibition of the dichloromethane fraction from leaves reached 61.1% viral inhibition (using the highest safe concentration), and the rate of inhibition of the dichloromethane fraction from bark reached 56.3% viral inhibition (using the highest safe concentration). Each fraction had moderate or above moderate inhibition, but when the two fractions were combined (leaves plus bark in a 1:1 ratio), the rate of viral inhibition increased to 73.7% viral inhibition (using the highest safe concentration), which represents a promising result.

Overall,no significant differencein SARS-CoV-2 inhibition between Remdesivir and the eucalyptus leaves-bark dichloromethane fraction (p value, 0.1491).By the way, all other eucalyptus extracts had a notable low to moderate antiviral activity against SARS-CoV-2 compared to Remdesivir.

Gas chromatography–mass spectrometry (GC-MS) analysis of Eucalyptus

GC-MS analysis was focused on the comparison of the major identified compounds of leaves, bark (individually), and the combined fractions of leaves and bark, with a percentage of more than 1% for the dichloromethane fraction (see supplementary file for each GC-MS analysis).

GC-MS analysis of the bark (Figure 7) showed the presence of several monoterpenes and sesquiterpenes as major components. The major identified monoterpenes were pinanediol (6.42%), followed by cyclohexanebutanal (3.80%) and cyclohexene-1-methanol-a,a'-4-trimethyl(3.43%), while the most identified sesquiterpenes were spathulenol (9.36%), followed by ledene oxide

(3.39%) and isoaromadendrene epoxide (3.08%). Further sesquiterpenes and monoterpenes were tentatively identified (Figure 8),and fatty acids such as pentadecanoic acid(3.21%).

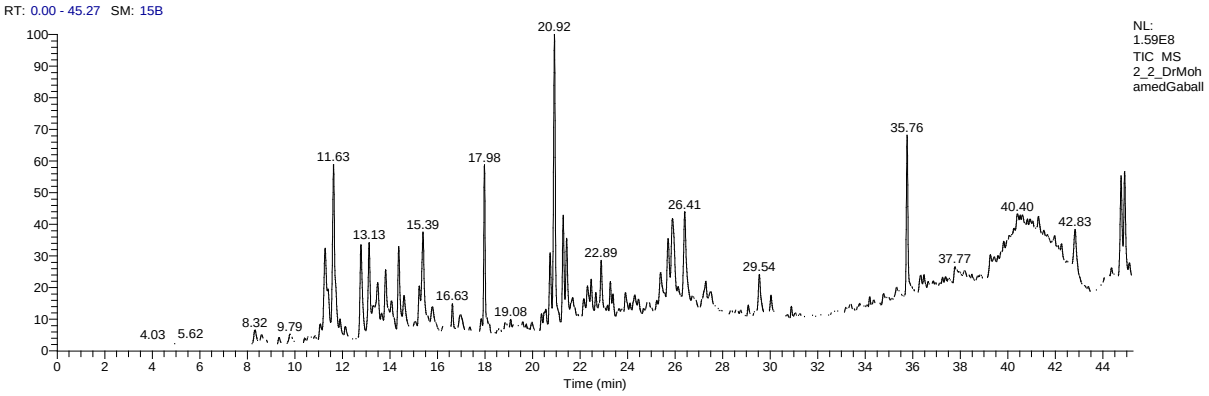

Figure 7. GC-MS chromatogram of the eucalyptus bark.

GC-MS of the leaves (Figure 8) showed the presence of sesquiterpenes only as major components, with the highest percentage for zerumbone (6.82%), followed by germacrenone (3.86%) and curcumenol (3.82%). pentadecanoic acid was identified at 4.2% (Table 3).

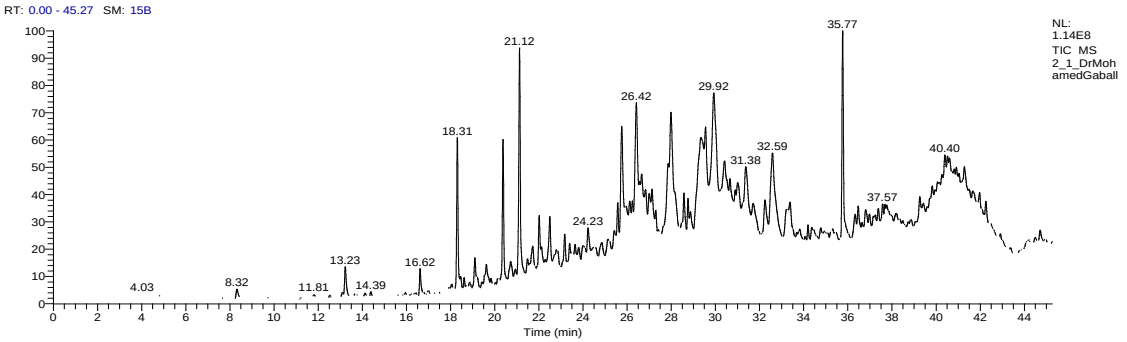

Figure 8. GC-MS chromatogram of the eucalyptus leaves.

GC-MS of the combined dichloromethane fraction of leaves and bark (Figure 9) revealed that spathulenol and pinanediol were the dominant sesquiand monoterpenes, with 8.04% and 4.88%, respectively, followed by cyclopropazulenol sesquiterpene (4.69%) and cyclohexanebutanal monoterpene (3.22%). Pentadecanoic acid (fatty acid) was observed in the combined fractions at 4.43% (Table 3). Further compounds, including mono- and sesquiterpenes, were tentatively identified with a lower percentage. Figure 10 shows the chemical structure of potential major compounds identified by GC-MS.

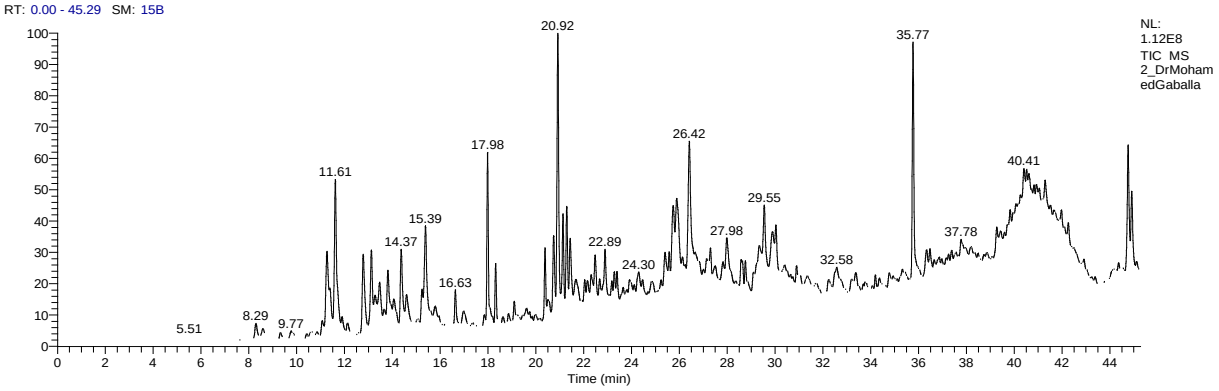

Figure 9. GC-MS chromatogram of combined eucalyptus leaves and bark.

Table 3. Compounds identified by GC-MS of Eucalyptus bark and leaves (individually) and the combined fractions (see supplementary files).

Compound	*RT	Molecular Formula	Bark %	Leaves %	Total %	Chemical class
Cyclohexanebutanal, 2-methyl-3-oxo	11.27	$C_{11}H_{18}O_2$	3.80	-	3.22	Monoterpenes
Pinanediol	11.63	$C_{10}H_{18}O_2$	6.42	-	4.88	
Cyclohexene-1-methanol-a,a'-4-trimethyl	12.78	$C_{10}H_{18}O$	3.43	-	2.63	
1H-Cycloprop[e]azulen-7-ol	17.98	$C_{15}H_{24}O$	4.82	-	4.69	Sesquiterpenes
Germacra-3,7(11),9-trien-6-one (3,7-Cyclodecadien-1-one, 3,7-dimethyl-10-(1-methylethylidene)-, (E,E)-)	20.38	$C_{15}H_{22}O$	-	3.86	1.86	
(-)-Spathulenol	20.93	$C_{15}H_{26}O_2$	9.36	-	8.04	
Zerumbone	21.13	$C_{15}H_{22}O$	-	6.82	2.32	
Isoaromadendrene epoxide	21.29	$C_{15}H_{24}O$	3.08	-	2.53	
Curcumenol	25.76	$C_{15}H_{22}O_2$	-	3.82	1.78	
Pentadecanoic acid	26.42	$C_{15}H_{30}O_2$	3.21	4.2	4.43	Fatty acids

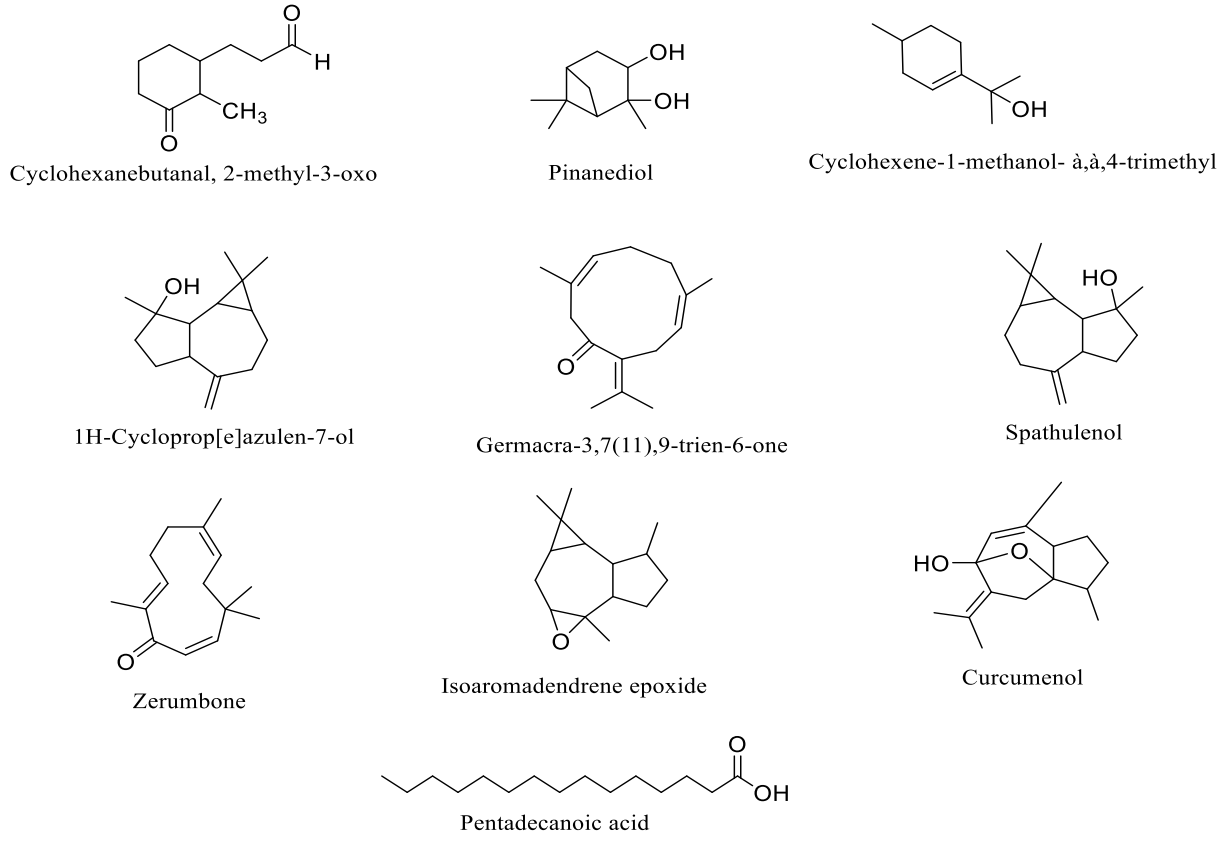

Figure 10. Chemical Structure of potential major compounds identified by GC-MS.

4. Discussion

Corona viruses are respiratory pathogens that affect humans and are responsible for significant rates of morbidity and mortality worldwide. Recently, after the emergence of the new corona virus SARS-CoV-2, concerns have become greater as the virus spreads through human-to-human transmission, posing a significant public health issue. Although vaccination provides primary protection against SARS-CoV-2 infection, persistent antigenic variation and unexpected mutations in the SARS-CoV-2 virus make vaccine strains relatively or completely ineffective and must be tested annually or semi-annually. Therefore, vaccine production may not meet the need during the SARS-CoV-2 pandemic. Traditional plants are used in alternative medicine. The

objective of this study was to identify novel anti-SARS-CoV-2 inhibitors using plant extracts and different fractions of *Eucalyptus camaldulensis*.

In this study, a preliminary screening was conducted to estimate the antiviral effect of *Eucalyptuscamaldulensis* against the SARS-CoV-2 virus. Our results showed that the dichloromethane fraction of the combined leaves and bark is effective in targeting and tracking the replication process of SARS-CoV-2 virus, highlighting its antiviral activity, with phytochemicals having a potential inhibitory effect on SARS-CoV-2 virus.

The significant antiviral activity of the dichloromethane fraction of the combined fractions of leaves and bark was higher than the activity of each fraction individually. This might be attributed to the presence of monoterpenes and sesquiterpenes together, as well as the combined number of sesquiterpenes in the combined fractions[22]. Monoterpenes can inhibit viral replication by interfering with viral enzymes or by affecting host cell processes [23-25]. Sesquiterpenes also exhibit antiviral effects; they may inhibit viralentry, replication, or assembly[25, 26].When monoterpenes and sesquiterpenes are used together, they may synergistically enhance the antiviral activity due to the combination of their different mechanisms; thus,the combination of monoterpenes and sesquiterpenes could be used as a promising natural antiviral strategy [27, 28]. GC-MS results identified cyclohexanebutanal,2-methyl-3-oxo, and cyclohexene-1-methanol-a,a'-4-trimethylwith 3.22% and2.63%, respectively. The literature reported that SARS-CoV-2 protease has been inhibited by a range of inhibitors that contain a conformationally-constrained cyclohexane moiety in their structure. This moiety is intended to exploit new chemical space and engage in optimally favorable binding interactions with the protease's active site [29]. In addition, the high percentage of pinanediol (4.88%) might contribute positively as a new antiviral agent, This may be due to the role that pinanediol plays as one of the most important anti-inflammatory molecules[30].

The antiviral activities of the major detected sesquiterpenes by GC-MS are shown herein. Spathulenol is the major identified sesquiterpene produced by the oxidative cyclization of bicyclogermacrene [31]. Spathulenol is a sesquiterpene component of essential oils in several aromatic species [32] and reported with antimicrobial [33]antiproliferative, anti-inflammatory, and immunomodulatory [34] activities.Spathulenol has a high hydrophobicity structure, thereby allowing easier penetration across the plasma membrane and interaction with intracellular proteins and/or intra-organelle sites [35]. Different species of eucalyptus from Tunisia and Egypt showed the presence of spathulenol sesquiterpene [36, 37].

The second major sesquiterpene was cycloprop-azulen-7-ol, reported with antimicrobial, anti-proliferative, anti-inflammatory, and immunomodulatory activities in addition to application in neurodegenerative diseases as an alarm pheromone [33, 34, 38, 39].

Further sesquiterpenes with interesting activities were identified, including germacrane-type,whichwas shown topossess antiviral and anticancer properties [40]. Chromatographic fractionation of the essential oils of *Salvia desoleana* identified germacrene D, which was probably responsible for the overall antiviral effect, as it effectively suppresses acyclovir-sensitive and acyclovir-resistant HSV-2 strains [41]. Zerumbone sesquiterpene has attracted considerable attention due to its potential therapeutic effects, particularly in the treatment of cancer [42] in addition to antimicrobial, antiviral, hepatoprotective, and neuroprotective effects [43].

The reported literature indicated that curcumin and curcuminoids may be effective therapeutic agents against a variety of viral zoonoses by targeting various proteins and signalingpathways [44]. Therefore, curcumenol sesquiterpene might have participated in the dichloromethane fraction activity.

Several studies, including molecular docking, showed that the active component of the eucalyptus plant is cineol, a monoterpene [5, 13, 16, 17].In addition to beta-caryophyllene [23, 45], while the above results revealed that the main components were spathulenol and pinanediol. The outcome of this study reported that the dichloromethane fraction of leaves and bark of eucalyptus acts as a potential inhibitor against the SARS-CoV-2 virus.Limitations of this study include that the antiviral activity of Eucalyptus camaldulensis extract against SARS-CoV-2was examined *in vitro.* So,further studies are needed to determine the efficacy, safety, and therapeutic potential in animal models.

Conclusions

Natural compounds are an important source of biologically active compounds with antiviral effects. They can provide an alternative treatment to traditionally known antiviral drugs and have significant antiviral activity against SARS-CoV-2. In this study, the antiviral activity of the dichloromethane fraction from the combined eucalyptus leaf and bark fractions was significantly higher than the antiviral activity of either of them individually. This can be attributed to the synergistic action of monoterpenes and sesquiterpenes in inhibiting SARS-CoV-2.

Conflicts of interest

The authors declare no conflict of interest.

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