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In Vitro Assessment of a Natural Monoterpene as an Antiviral Compound against Low Pathogenic Human Coronavirus 229E

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Abstract

The emergence of novel viruses, including SARS-CoV-2, has highlighted the need for effective antiviral treatments. This study aimed to evaluate the antiviral potential of pine oil and α-terpineol against low pathogenic corona virus 229E (HCoV-229E), which serves as an alternative model for COVID-19. The study investigated the virucidal, adsorption, and multiplication inhibitory effects of pine oil and α-terpineol on HCoV-229E, as well as their impact on proinflammatory gene expression in an infected human colorectal adenocarcinoma cell line. Pine oil and α-terpineol exhibited high CC50 values and moderate in vitro inhibitory activity against HCoV-229E, with selectivity indices of 8.23 and 7.7, respectively. Plaque reduction tests demonstrated that these compounds effectively suppressed HCoV-229E during the adsorption and replication stages, and they were found to reduce the expression of proinflammatory genes associated with the development of a cytokine storm. The findings of this study suggest that pine oil and α-terpineol possess significant antiviral properties against HCoV-229E and warrant further investigation as potential therapeutic agents for viral infections, including COVID-19.

Keywords: *Pine oil; α-Terpineol compound; Antiviral activity; Low pathogenic coronavirus 229E*

1. Introduction

Viral infections around the world have led to significant disease, mortality, and economic impacts. In recent years, new viruses like SARS-CoV-2 and monkeypox have emerged. Antiviral therapy is a crucial approach for preventing viral infections. Currently, only one antiviral drug, remdesivir, has been approved to treat COVID-19 [1, 2]. The impact of remdesivir on COVID-19 mortality rates is controversial [3]. As a result, antiviral drugs to treat this infection are in scarcity. Terpinols are tertiary monocyclic monoterpene alcohols found in the essential oils of numerous plants, including pine oil, cajeput oil, and petitgrain oil. Terpinols are divided into five isomers: alpha-, beta-, gamma-, delta-, and terpinen-4-ol, the last of which is the most frequent in nature. α-Terpineol is the main component of the terpineol isomer combination. In the industrial sector, alpha-terpineol is particularly useful. It has a nice lilac-like odor and is commonly used in perfumes, cosmetics, and aromatic smells [4, 5]. α-Terpineol is in high demand due to its several biological applications as an antioxidant [6, 7], anticancer [8, 9], antidepressant [10], and antifungal agent [11]. It is also insecticidal and can be used to increase skin penetration [12]. α-Terpineol was active also as an antipathogen against a variety of bacterial microbes, including *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Proteus vulgaris*, and *Escherichia coli* [13, 15]. Furthermore, it displayed biological pest control and antifungal properties such as *Aspergillus niger* [16, 17]. There are relatively few articles demonstrating the antiviral efficacy of pine oil against viral diseases such as the Influenza A Virus [18]. Therefore, the goal of this study was to investigate the possible inhibitory effects of pine oil and α-Terpineol as an antiviral therapeutic option for inactivating HCoV-229E. The mechanism of viral infectivity inhibition was also

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explored. Finally, we examined the impact of induced proinflammatory gene expression in response to pine oil or α-Terpineol exposure in infected cells.

2. Methods

2.1. Pine oil and alpha Terpineol compounds

Pine oil (*Pinus Palustrus*) is a clear, colorless to light amber liquid rich in tertiary and secondary terpen alcohols. It was originally used as a solvent and disinfectant. Pine oil is derived by distilling small pine branches, and it is also extracted or steam distilled from pine tree wood. (-) α-terpineol (C10 H18 O) is the alpha form of terpineol, which is a naturally monoterpene alcohol derived from cajuput oil, pine oil, and petit green oil. It is also a solvent that is used to extract natural spices and as an acetate fiber solvent. Pharmalog GmbH (Bonen, Germany) provided the pine oil and α-terpineol utilized in this study, which were diluted using PBS to stock solutions and kept at -80° C for all subsequent assays.

2.2. GCMS analysis of pine oil and α-terpineol

The GC-MS analysis was performed with the following gas chromatography-mass spectrometry instrument stands: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a thermo mass spectrometer detector (ISQ Single Quadruple Mass Spectrometer). A TR-5 MS column (30 m x 0.32 mm i.d., 0.25 m film thickness) was used in the GC-MS system. The following temperature program was used to conduct the analyses with helium as the carrier gas at a flow rate of 1.0 mL/min and a split ratio of 1:10: 60ºC for 1 minute, then 4ºC/min to 240ºC and hold for 1 minute. The injector and detector were kept at 210 degrees Celsius. 1 L of the mixes was always injected with diluted samples (1:10 hexane, v/v). Electron ionization (EI) at 70 eV yielded mass spectra with a spectrum range of m/z 40-450. The chemical constituents of the essential oil were identified by their retention indices (relative to n-alkanes C8-C22), mass spectrum matching to (authentic standards, Wiley spectral library collection, and NSIT library database) using AMDIS software (www.amdis.net).

2.3. Cell culture, and virus

The Caco-2 cell line (Human colorectal adenocarcinoma cells) was donated by Nawah Scientific Inc. (Mokatam, Cairo, Egypt) and cultivated in DMEM (Dulbecco's Modified Eagle's Medium), Gibco, USA. 10% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin were added to the culture medium. The cells were incubated at 37 degrees Celsius in a humid environment with 5% CO_2. For *in vitro* viral challenge, Egypt's Nawah Scientific Inc. for Scientific Research Services provided HCoV-229E virus which was obtained from Egypt's Centre of Scientific Excellence for Influenza Viral Collections, National Research Centre in Egypt. Virus titers were quantified using the limit-dilution technique and expressed as a 50% cell culture infective dose ($TCID_{50}$) of 0.9 x 10^5. Virus stocks were kept at -80°C until needed.

2.4. Determination of half-maximal cytotoxic concentration (CC_{50}) using Caco-2 Cell line

The crystal violet technique, as previously reported [19, 20], was used to assess the half-maximal cytotoxic concentration (CC_{50}) in Caco-2 cells. To begin, stocks were prepared by dissolving compounds in the required medium to acquire various working concentrations. In 96-well plates, cells were grown at a density of 1 x 10^5 cells/mL in 100 µL medium each well. Following a 24-hour incubation at 37ºC in 5% CO_2, cells were treated in triplicate with varied doses of the candidate compounds. After 72 hours of treatment, cells were washed with PBS and fixed for 1 hour at room temperature with 10% formaldehyde after discarding supernatant material. 0.1% crystal violet was applied to the plates for 20 minutes before washing and drying. Following that, 200 µL of methanol was poured to each well to dissolve the crystal violet. Finally, absorbance at λ max 570 nm was measured using a multi-well plate reader. GraphPad prism program v.5 was used to do non-linear regression analysis on the cytotoxicity of different concentrations relative to untreated cells.

2.5. Determination of half-maximal inhibitory concentration against HCoV-229E challenge

The previously determined approach was applied with slight modifications to estimate the half-maximal inhibitory concentration for candidate compounds [21, 22]. Caco-2 cells were sown on 96-well plates and cultured in a humidified 37ºC incubator with 5% CO_2 for 24 hours. Caco-2 confluent 96-well plats were infected with 100 µl of stock HCoV-229E virus for 60 minutes at 37ºC. For 1 hour, the cell monolayers were exposed to 100 µL of media containing different concentrations of the candidate compounds. Cells were then kept at 37ºC in a 5% CO_2 incubator. The supernatant was removed after 72 hours, and cell monolayers were rinsed with sterile PBS before adding MTT solution (20 µl of 5 mg/ml stock solution) to each well and incubating at 37ºC for 4 hours. The produced formazan crystals were dissolved in each well with 200 l of acidified isopropanol. The absorbance of formazan solutions was measured using a multi-well

plate reader (BMGLABTECH®FLUOstar Omega, Germany) at λ max 540 nm using 620 nm as a reference wavelength. The Reed & Munech algorithm was used to calculate CPE inhibition compared to control [23, 24].

2.6. Determination of the inhibition mechanism against HCoV-229E

Plaque reduction assay was used as previously described [24, 25] to assess if the candidate compounds have a "virucidal impact" on the viral particle and/or interfere with viral adsorption and/or viral reproduction during the virus life cycle.

1. ***Mechanism of Viral Adsorption***: Caco-2 cells (10^4 cells/mL) were cultured in a 6-well plate for 24 hours at 37ºC to test the viral adsorption process. Compounds were co-incubated with cells in 200 µL media at 4°C without supplementation. Three washes with supplement-free media were used to eliminate the non-absorbed compounds. Pretreated cells were co-incubated for 1 hour in DMEM media with HCoV-229E virus diluted to 10^3 PFU/well and 2% agarose. Plates were hardened and incubated at 37ºC until viral plaques formed. For 1 hour, cells were fixed in 10% formalin solution and stained with crystal violet. In contrast to control wells, the relative percentage of plaque development decrease was determined.

2. ***ViralReplication Mechanism***: Cells sown in a 6-well plate at a concentration of (10^4 cell/mL) were infected for 1 hour with virus after a 24-hour incubation period at 37ºC. The non-absorbed virus particles were cleaned three times with supplement-free media. Candidate compounds were given to infected cells at various doses for another 1 hour. The inoculum was withdrawn and replenished with 3 mL of DMEM containing 2% agarose. Soft agar was allowed to firm until viral plaques formed. Following that, plaques were fixed, stained, and computed as previously stated.

3. ***Virucidal Mechanism***: In a 6-well plate, cells were seeded at a concentration of (10^4 cells/mL) for 24 hours at 37ºC. Following that, 200 µL of serum-free DMEM containing HCoV-229E virus was introduced to each compound sample. After 1 hour of incubation, this combination was diluted two-fold three times with serum-free media. Following that, 100 µL of each dilution was applied to the cell monolayer. After 1 hour of interaction with the cells, medium was introduced. Soft agar was allowed to firm until viral plaques formed. The plaques were fixed, stained, and computed as previously reported.

2.7. Candidate pro-inflammatory markers gene expression examined using RT-PCR

Caco-2 cells were sown in 6-well plates and incubated at 37ºC with 5% CO_2 for 24 hours. After 24 hours, the cells were given a 50 µg/ml dosage of each compound, followed by HCoV-229E infection. Cells were extracted in triplicate after 3, 6, 12, and 24 hours of incubation for the expression assay. Total RNA was extracted from the final sample combination using a QIAamp RNA Mini kit (Qiagen, Hilden, Germany), as directed by the manufacturer. To summaries, RNA was extracted from 140 µL of each sample to yield 40 µL of RNA solution. The quality of the RNA was determined using a Nanodrop Spectrophotometer (A260/280 ratio). The purified RNA was immediately used to synthesis cDNA. The ReverAid RT Kit (ThermoFisher Scientific, Waltham, USA) was used to reverse transcribe 5 µL of RNA according to the manufacturer's procedure. Real-Time PCR Amplification on the Rotor Gene Q platform (Qiagen, Hilden, Germany) was used to measure TNF-α and IL-6 expression using the primer sets listed in (Table 1). As a control, the housekeeping β-actin gene was employed. In a final volume of 20 µl, the cDNA was mixed with SYBER Green Master Mix (Qiagen, Hilden, Germany). Real-time PCR reactions were conducted for 5 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 70°C. All tests were performed in triplicates. The cycle threshold (CT) values were determined and used to report the expression, which was computed by subtracting the CT values of housekeeping from the CT values of the targets. To assess the relative quantitative levels of target expression, the $2^{-\Delta\Delta (Ct)}$ method was applied. GraphPad prism v.5 was used to calculate the relative expression in control and treated samples [26-29].

2.8. Statistical analysis

All experiments were run in triplicate, and all computations were performed with GraphPad PRISM and linear regression analysis (Version 5.0.1, GraphPad Software, San Diego, CA, USA). CC50/IC50 was used to calculate the selective index (SI).

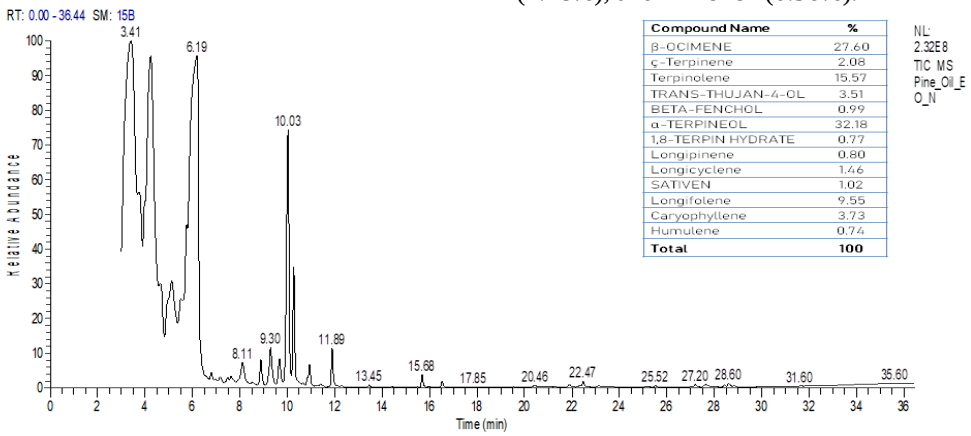
Table 1: Primer sequences used in this study

Gene	Name	Sequence	Reference
β-Actin	Forward Primer Reverse Primer	CATGAAGTGTGACGTGGACATCC GCTGATCCACATCTGCTGGAAGG	[28]
TNF-α	Forward Primer Reverse Primer	ACCCTCTCTCCCCTGGAAAGGACA TGAGGAACAAGCACCGCCTGGA	[28]
IL-6	Forward Primer Reverse Primer	ACCTGAACCTTCCAAAGATG GCTTGTTCCTCACTACTCTC	[29]

3. Results

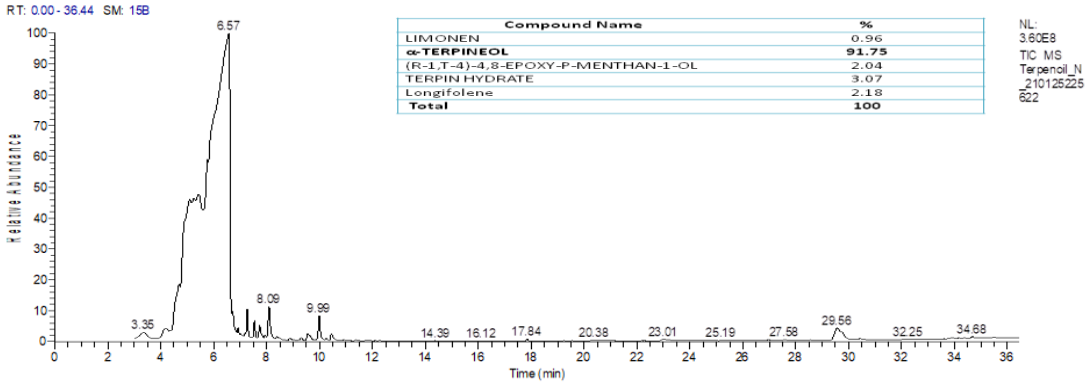
3.1. Chemical Composition of tested compounds

The chemical descriptions of pine oil and α-terpineol were originally completed by using GCMS. Pine oil's main components have been found as α-Terpineol (32.18%), β-Ocimene (27.6%), and Terpineol (15.57%). Other ingredients such as Logifolene (9.55%), caryophyllene (3.73%), trans-thujan-4-ol (3.51%), and ç-terpinene (2.08%) are also found. As shown in (Figure 1), traces of additional components ranging from (1.4-0.74%) have also been identified. As shown in (Figure 2), GCMS analysis of α-terpineol, on the other hand, revealed that it comprises (91.75%), with a trace of Terpin hydrate (3.07%), followed by longifolene (2.18%), and Limonen (0.96%).



Compound Name	%
β-OCIMENE	27.60
ç-Terpinene	2.08
Terpinolene	15.57
TRANS-THUJAN-4-OL	3.51
BETA-FENCHOL	0.99
α-TERPINEOL	32.18
1,8-TERPIN HYDRATE	0.77
Longipinene	0.80
Longicyclene	1.46
SATIVEN	1.02
Longifolene	9.55
Caryophyllene	3.73
Humulene	0.74
Total	**100**

Figure 1: GCMS analysis of pine oil used in this study; α-Terpineol (32.18%), β-Ocimene (27.6%), and Terpineol (15.57%) are the main components have been identified.



Compound Name	%
LIMONEN	0.96
α-TERPINEOL	**91.75**
(R-1,T-4)-4,8-EPOXY-P-MENTHAN-1-OL	2.04
TERPIN HYDRATE	3.07
Longifolene	2.18
Total	**100**

Figure 2: GCMS analysis of α-Terpineol used in this study; the purity of α-Terpineol up to 91.75%,

3.2. Cytotoxicity evaluation

To assess the cytotoxicity (CC_{50}) of pine oil and α-terpineol on the Caco-2 cell line, the candidate compounds were serially diluted (two-fold) and added to cell culture to examine how they influenced the growth and viability of the Caco-2 cell line. Crystal violet was used to measure cell viability after three days of incubation. The highest non-cytotoxic concentrations of pine oil and α-terpineol were determined to be 30.108 and 19.788 µM, respectively. The 50% cytotoxic and growth inhibition doses were calculated using the mean dose-response curves of three separate studies (Figure 3). Oil-treated and α-terpineol-treated cells remained morphologically viable after 24 hours. After 27 hours of treatment, Caco-2 cells remained adhered to the bottom of the plates and showed no morphological alterations at any dosage.

3.3. Antiviral evaluation using MTT assay.

The cytopathic inhibition assay was used to assess the antiviral effectiveness of the pine oil- and α-terpineol against HCoV-229E. As a control, untreated virus-infected cells were employed in the assay. Our data reveal that pine oil- and α-terpineol have a remarkable antiviral activity, with the 50% inhibitory concentration (IC_{50}) for HCoV-229E being 3.658 and 2.558 µM, respectively (Figure 3). Table (2) displays the results as a selective index (SI) of 8.23 and 7.7, respectively, based on the average of three separate trails.

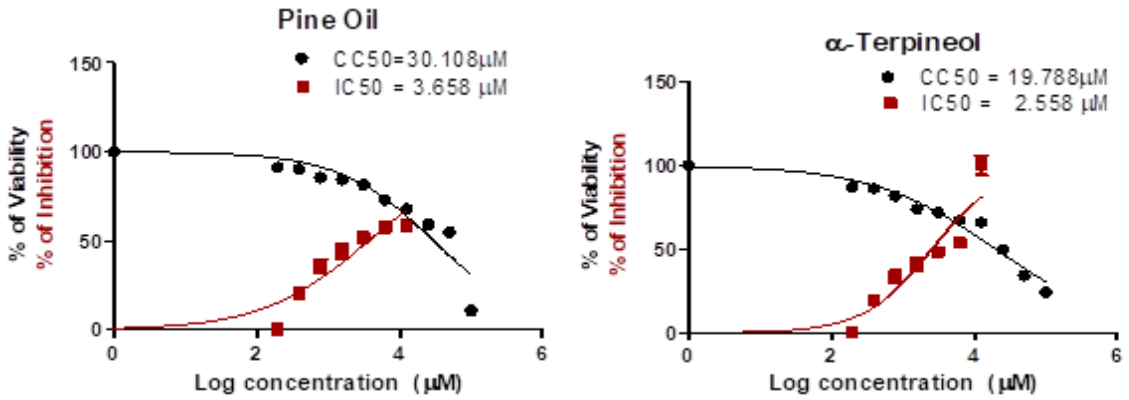


Figure 3: Cytotoxicity and antiviral activity of pine oil and α-Terpineol against HCoV-229E virus. The cytotoxicity for pine oıl and terpineol were 30.1 and 19.7 µM respectively. While the inhibitory effect for both were 3.6 and 2.5 µM respectively.

Table 2: Inhibitory Concentration (IC_{50}) and selective index (SI) forpine oil and α-Terpineol against HCoV-229E viral infection.

Sample	IC_{50} (µM)	SI
Pine Oil	3.658 ± 1.12	8.23
α-Terpineol	2.558 ± 0.83	7.7

3.4. Mode of action evaluation

To investigate the mode of action, a plaque reduction assay was done to investigate if pine oil and α-Terpineol interfere with the viral replication directly by having a virucidal impact, or indirectly by preventing viral adsorption into host cell receptors or hindering intracellular viral replication. Surprisingly, pine oil and α-Terpineol had a virucidal impact on HCoV-229E, with >79% and 86% viral suppression, respectively (Figure 4). Furthermore, interfering with viral adsorption resulted in a considerable viral decrease (10.5% and 13.8%) but had no effect on replication (1%, and 2.4%).

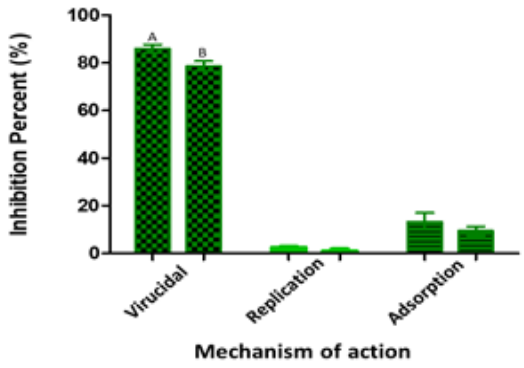


Figure 4: Mode of inhibitory mechanism for α- α-Terpineol (**A**) and pine oil (**B**) against low pathogenic coronavirus 229E. By using the plaque reduction assay, the three principal mechanisms were evaluated: virucidal, adsorption, and replication.

3.5. Inflammatory markers expression

The effects of pine oil and α-terpineol on the gene expression of two proinflammatory markers were investigated. Consequently, treating CaCo-2 cells infected with HCoV-229E with pine oil or α-Terpineol dramatically decreased the production of proinflammatory markers including TNF-α and IL-6 over time (Figure 5).

4. Discussion

The recent SARS-CoV-2 coronavirus pandemic (2019) demonstrates how viruses may wreak havoc on health-care systems throughout the world. Coronaviruses transmit quickly from person to person, causing severe morbidity and mortality. Unfortunately, the antiviral arsenal is inefficient against this virus, demanding the development of novel antiviral agents. Natural terpinols are present in the essential oils of many plants, including pine oil, and exhibit a variety of biological functions, including antiviral action against influenza virus as previously described [18]. As a result, the purpose of this study was to investigate into the antiviral activity of pine oil and α-terpineol against HCoV-229E.

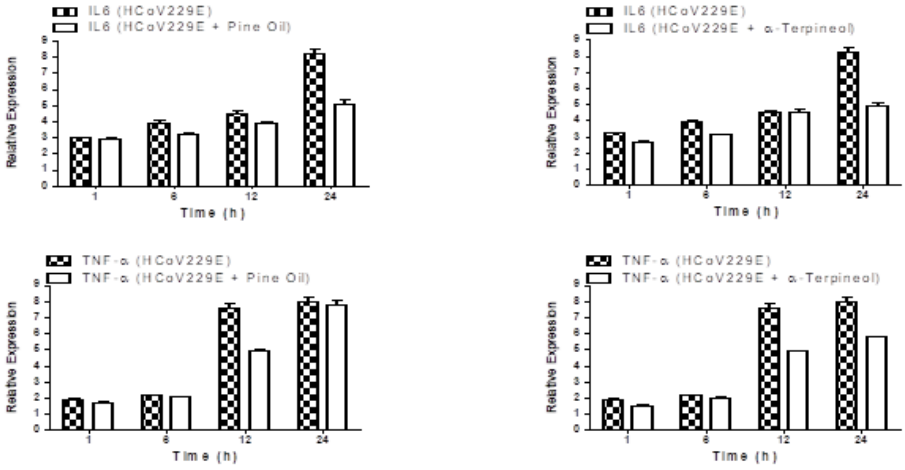


Figure 5: The anti-inflammatory *in vitro* activity of pine oil and α-Terpineol. Relative gene expression levels of IL6, and TNF-α in HCoV229E infected Caco-2 cells with or without-treatment with pine oil or α-Terpineol. Data are shown as the mean ± SD

Our findings are almost similar to the toxicity's results of the previous report conduct on HeLa cell line [30], which reported the CC_{50} of α-terpineol is 13.12 µg/ml. In order to investigate the antiviral activity, the cytopathic inhibition assay was used to assess the antiviral effectiveness of the pine oil- and α-terpineol against HCoV-229E. Our findings revealed that the pine oil- and α-terpineol have a remarkable antiviral activity, with the 50% inhibitory concentration (IC_{50}) for HCoV-229E being 3.658 and 2.558 µM, respectively (Figure 3). Table (2) displays the results as a selective index (SI) of 8.23 and 7.7, respectively, based on the average of three separate trails. In contrast, pine oil exhibits antiviral action against Influenza A virus [18] with reduction percentage ranged 40 % to 90% due to a reduction in HA activity. According to Astani*et al.* [31], monoterpene compounds extracted from different sources of essential oils had previously been able also to reduce viral infectivity of herpes simplex virus type-1 by 80%. From the preceding, it is obvious that our findings are broadly compatible with previously published articles on antiviral action against a variety of viral infections, including Influenza and herpes simplex viral infections.

More details, Terpineol (32.18% in *Pinus Palustris* essential oil) and Terpinolene (15.57% in the essential oil) were revealed to have antiviral effects. They were also found in the essential oil of tea tree, which has potent anti-influenza. The essential oil was extracted from *Melaluca alternifolia* leaves and contains (1.5-0.8 %) Terpineol and (1.5-5 %) Terpinolne [32]. The components of tea tree oil, terpinen-4-ol, trepinolene, and α-Terpineol, have anti-influenza virus action (H1N1), with IC_{50} values of 25, 12, and 250 µg/ml [18, 33]. The α-Terpineol was shown to be one of the principal monoterpene components in essential oils from eucalyptus, tea tree, and thyme, with antiviral activity against herps simplex type 1 (HSV1) *in vitro*. These essential oils reduced viral infectivity by more than 96%, while the monoterpenes suppressed HSV by more than 80% [31]. By plaque reduction assay, α-Terpineol and terpinolene were identified in *Melaluca altefornia* essential oil (TTO) and exhibit antiviral activity against poliovirus type 1, coxsackie virus B1, adenovirus type 2, herps simplex (HSV) type 1 and 2 [34]. Furthermore, the essential oil of *Fortunella margarita* fresh leaves and fruits demonstrated the presence of α-terpineol, one of the principal components of the fruit's essential oil, at 55, 5%. The antiviral activity of essential oils from both leaves and fruits was tested against the avian influenza-A virus (H1N1), and the findings revealed that the oil from the fruits had a high potency, while the oil from the leaves had a stronger antimicrobial activity than the oil from the fruits [34]. On the other hand, to investigate the mode of action, a plaque reduction assay was performed to investigate if pine oil and α-Terpineol interfere with the viral replication directly by having a virucidal impact, or indirectly by preventing viral adsorption into host cell receptors or hindering intracellular viral

replication [24, 25]. Our findings are consistent with prior research that revealed a strong antiviral impact through direct inactivation of free viral particles of herpes simplex virus when treated with monoterpenes or some essential oils including pine oil. However, when these compounds were introduced to host cells before or after infection, they had a low impact [31]. In most cases, respiratory viral infections are associated with an increase in pro-inflammatory markers such as IL-6, which contribute to viral immunopathology [33]. In this regard, the effects of pine oil and α-terpineol on the gene expression of some inflammatory indicators were investigated. As a consequence, treating CaCo-2 cells infected with HCoV-229E with pine oil or α-Terpineol dramatically decreased the production of proinflammatory markers including TNF-α and IL-6 over time (Figure 5). The WHO has recommended that monoclonal antibodies, such as IL-6 blockers, be used to treat SARS-CoV-2 [35]. In this regard, pine oil and α-Terpineol are promising compounds since it not only suppresses HCoV-229E replication but also reduces the expression of proinflammatory indicators generated by viral infection. Our findings are consistent with prior report that established α-terpineol treatment reduces IL-6 and IL-10 production in epithelial buccal cells [6]. These encouraging findings promote the idea of additional research into pine oil and α-terpineol as potential lead antiviral drugs for treating coronavirus infections.

Conclusion

In conclusion, our investigation indicated that pine oil and -terpineol are effective HCoV-229E inhibitors. Cell-based tests and molecular expression analyses validated this conclusion. Pine oil and α-terpineol inhibit HCoV-229E reproduction by virucidal and adsorption mechanisms. Interestingly, pine oil and α-terpineol promote cellular gene expression by inhibiting the production of pro-inflammatory markers TNF-α and Il-6.

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