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Research Article

Terpenes and cannabidiol against human corona and influenza viruses–Anti-inflammatory and antiviral in vitro evaluation

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ABSTRACT

The activity of the terpenes and Cannabidiol (CBD) against human coronavirus (HCoV) strain OC43 and influenza A (H1N1) was evaluated in human lung fibroblasts (MRC-5 cells). Also, we examined whether these ingredients inhibit pro-inflammatory cytokines in peripheral blood mononuclear cells (PBMC). The tested preparations exhibited both anti-inflammatory and antiviral effects. The combination of terpenes was effective against both HCoV-OC43 and influenza A (H1N1) virus. The addition of CBD improved the antiviral activity in some, but not all cases. This variation in activity may suggest an antiviral mechanism. In addition, there was a strong correlation between the quantitative results from a cell-viability assay and the cytopathic effect after 72 h, as observed under a microscope. The anti-inflammatory properties of terpenes were demonstrated using a pro-inflammatory cytokine-inhibition assay, which revealed significant cytokine inhibition and enhanced by the addition of CBD.

1. Introduction

Infectious diseases have had a significant impact on human existence from ancient times. The introduction of naturally occurring bioactive compounds, as well as nature-inspired, synthetic compounds into drug-research programs has increased human life spans considerably since the 1940s. Phytochemicals, such as terpenes, have demonstrated therapeutic potential. Terpenes are natural, isoprene-based molecules that are characterized by unique aromas, a high degree of chemical diversity, and high volatility. In vitro studies have confirmed the antiviral potential of terpenes against both RNA and DNA viruses, such as herpes simplex virus [1,2], bronchitis virus [3], West Nile virus [4], HIV-1 [5] and others [6]. Those studies and a study previously published by our team [7] support the efficacy of terpenes against human coronaviruses and influenza viruses.

Coronaviruses are enveloped, non-segmented, positive-strand RNA viruses of the family *Coronaviridae*. They cause a wide spectrum of illnesses, including respiratory and gastrointestinal diseases in humans [8]. Currently, seven human coronaviruses (HCoVs) have been identified. Four of those, HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1, are non-zoonotic and cause worldwide outbreaks of upper respiratory tract infections, predominantly in the winter [9]. SARS-CoV-2 is the seventh coronavirus known to infect humans and the third zoonotic virus after SARS-CoV and MERS-CoV.

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the cause of the coronavirus disease 2019 (COVID-19) pandemic that continues to pose a threat to public health and economies around the world. As of 8 May 2022, over 514 million cases and over six million deaths have been reported globally, as reported by the World Health Organization [10]. This febrile respiratory and systemic illness is highly contagious and potentially life-threatening. The pandemic has led to a search for active antiviral compounds to treat the disease. Recently, the potential of phytochemicals, such as terpenes, for use as potent antiviral agents has received considerable attention, encouraged by the fact that they are plentiful, naturally occurring substances that are cost-effective and have nearly zero toxicity.

Phytochemicals that have previously been reported to have antiviral effects against HCoVs can be considered as emerging drug candidates for

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the treatment of COVID-19. Glycyrrhizin, a triterpene naturally occurring in licorice (*Glycyrrhiza glabra*) roots, was one of the first compounds found to be active against SARS coronavirus (SARS-CoV) in vitro, where it was shown to inhibit SARS-CoV replication with an EC_{50} of 365 μM [11]. Glycyrrhizin has been used to effectively treat SARS patients [12]. *Laurus nobilis* essential oil, with beta-ocimene, 1,8-cineole, alpha-pinene, and beta-pinene as its main constituents, has also been shown to exert antiviral activity against SARS-CoV, with an IC_{50} value of 120 mg/mL [13]. More recent works reviewed the antiviral activity of plant secondary metabolites as a possible SARS-CoV-2 Treatment [14], assessed the Antiviral activity of *Humulus lupulus* against MERS-CoV using in-vitro and in-silico tools [15] and investigated the antiviral effects of *Saussurea lappa* root extract against SARS-CoV-2 in vitro and in animal models [16].

A proprietary terpene formulation, named NT-VRL®, has been shown to have antiviral properties against HCoV-229E [7]. The tested formulation exhibited an antiviral effect when it was pre-incubated with host cells prior to virus infection. The addition of cannabidiol (CBD) potentiated the antiviral effect and was more effective than the positive controls pyrazofurin and glycyrrhizin. The terpene formulation prevented cell death, an observation that was confirmed by microscopic evaluation of cell morphology.

NT-VRL® was developed using a proprietary phytochemical database for optimized antiviral and anti-inflammatory activity. Thirty compounds were selected from Eybna's library of 350 compounds. These 30 volatile compounds exhibited the necessary biological activity and met safety standards for inhalation. Multiple combinations and concentrations of these 30 compounds were tested against HCoV-229E and the treatment code-named NT-VRL® was found to be the most potent formulation (Fig. 1). The main constituents of the NT-VRL® formulation are beta caryophyllene, eucalyptol, and citral.

While SARS-CoV-2 is highly contagious and requires a Level 4 biosafety facility, working with a less virulent strain, such as HCoV-229E or HCoV-OC43, is considered a safe alternative [9,17,18].

Influenza viruses are negative single-stranded RNA viruses that are members of the *Orthomyxoviridae* family [19]. Influenza viruses cause emerging and re-emerging respiratory illnesses in humans, due to the continuous evolution of their genomes. The three main types are alpha influenza, beta influenza, and gamma influenza. Alpha influenza is the most significant in terms of human morbidity and mortality [20]; whereas gamma influenza is responsible for mild symptoms [21]. The influenza A viruses that frequently infect people are the H1N1 virus and the H3N2 virus [22].

Many strains of influenza virus have developed resistance to commonly used treatments, demanding the development of genome-specific therapies. Natural phytochemicals are a diverse group of bioactive molecules with the potential to serve as novel antiviral treatments.

The objective of the present study was to test the formulation NT-VRL® with and without CBD at different concentrations against two coronaviruses: HCoV-OC43 and HCoV-229E, and influenza A virus in human lung fibroblasts (MRC-5 cells) and Peripheral blood mononuclear cells (PBMC), to evaluate its potential antiviral and anti-inflammatory activity in vitro. In addition, to determine morphologic changes of MRC-5 cells on electron microscopy, before and after treatment with NT-VRL®. Those cells were exposed to either HCoV-OC43, influenza A (H1N1), or HCoV-229E. To the best of our knowledge, this is the first study to test a designated terpene formulation with and without CBD for anti-inflammatory and antiviral activity against both a HCoV and an influenza virus.

2. Material and methods

2.1. Materials and reagents

MRC-5 cells, HCoV-229E strain HCoV-OC43, and influenza A (H1N1) virus were purchased from the American Type Culture Collection (ATCC; Manassas, VA). All media ingredients and cell-proliferation kits (XTT) were purchased from Biological Industries (Israel). CBD was purchased from Recipharm Israel Ltd. (Israel). NT-VRL® was obtained from Eybna Technologies Ltd. (Israel). Glycyrrhizin was obtained from Penta International Corporation (NJ, USA) and pyrazofurin was purchased from Sigma Corporation (Israel). Commercial frozen human peripheral blood mononuclear cells (PBMCs) for the cytotoxicity and

A

Terpenes classification	Number of Compounds
Natural Terpenes	10,0000 - 50,000
GRAS	100 - 2,500
Food Grade	10 - 1,000
Safe via Inhalation	50 - 100
Antiviral Anti-inflammatory	30 - 70
NT-VRL® Formulation	30

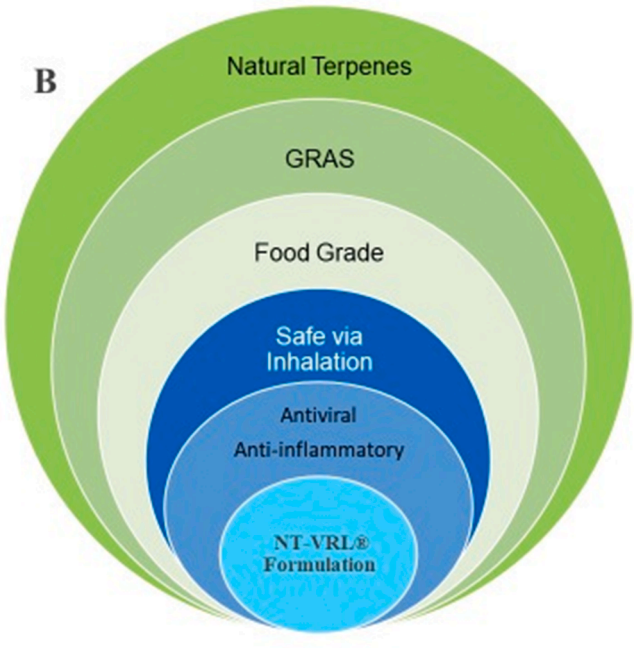

Fig. 1. Process of terpene selection for NT-VRL® development. A. Terpenes range number of compounds per classification. B. Terpenes that were used for NT-VRL® are generally recognized as safe (GRAS), food-grade (FEMA), safe to inhale, and possess antiviral and anti-inflammatory properties.

cytokine secretion assays were purchased from Lonza (Switzerland).

2.2. Testing the efficacy of pre-treatment and post-adsorption

MRC-5 cells were plated at 1×10^4 cells/well in 96-well plates, in Eagle's Minimum Essential Medium (EMEM) supplemented with 10 % fetal calf serum as was previously described [23] with minor modifications. The plates were then incubated at 37 °C in 5 % CO_2. For the Pre-Treatment, After 24 h, the medium was discarded, and 100 μL of EMEM supplemented with 1 % fetal calf serum was added to the cells, supplemented with the treatments (NT-VRL®ℊ and/or CBD) at a previously tested nontoxic concentration [7]. The cells were incubated for 1 h at 34 °C in 5 % CO_2. Next, 1 μL of medium (used for control) or virus (HCoV-OC43 1.45×10^{16} PFU/mL, influenza H1N1 5.91×10^{10} PFU/mL) was added to the cells. For the Post-Adsorption, After 24 h, the medium was discarded and EMEM supplemented with 1 % fetal calf serum was added to the cells with or without 1 μL of virus (HCoV-OC43 1.45×10^{16} PFU/mL, influenza H1N1 5.91×10^{10} PFU/mL). The cells were incubated for 1 h at 34 °C in 5 % CO_2. The medium was discarded and 100 μL of EMEM supplemented with 1 % fetal calf serum was added to the cells, supplemented with the treatments (NT-VRL®ℊ and/or CBD) according to each nontoxic concentration and 1 μL of media or virus (HCoV-OC43 1.45×10^{16} PFU/mL, influenza H1N1 5.91×10^{10} PFU/mL).The cells were incubated for an additional 72 ± 2 h at 34 °C in 5 % CO_2. Using an inverted microscope, a representative photo was taken of the cells in each treatment at 24, 48, and 72 h post-infection. The virus-induced cytopathic effect was observed in comparison with the parallel virus control and cell control. Finally, cells were subjected to an XTT assay.

2.3. XTT-based viability assay

XTT is a colorimetric assay for the nonradioactive quantification of cellular proliferation, viability, and cytotoxicity. Assay was performed as previously described [24]. At the end of each incubation period, media was discarded from all wells and 100 μL of fresh culture medium was added to the cells together with 50 μL of XTT reagent. Optical density (OD) was measured at a wavelength of 450 nm (after subtraction of the non-specific OD at 620 nm).

2.4. Preparation of samples for microscopy

MRC-5 cells were plated on one 96-well plate and on two 96-well plates with polylysine-coated 0.5-mm coverslips, as previously described [25] in their culture medium, at 1×10^4 cells/well. Cells were allowed to attach for 16–24 h at 37 °C, in 5 % CO_2. Then, the culture medium was discarded and 100 μL of assay media was added to the cells, supplemented with treatments (NT-VRL®ℊ and/or CBD). The cells were incubated for 1 h at 37 °C, in 5 % CO_2. Finally, 1 μL of assay media or virus (HCoV-OC43 1.45×10^{16} PFU/mL, HCoV-229E 7.24×10^8 PFU/mL, influenza H1N1 5.91×10^{10} PFU/mL) was added to the cells and the plates were incubated for 72 h. In addition, 10 μL of virus only was added to the plates for 30 min just before fixation. For the fixation, the medium was discarded, and the plates were washed once with 150 μL phosphate-buffered saline (PBS) per well. The cells were fixed with 2 % paraformaldehyde, 3 % glutaraldehyde, and 1 % sucrose in PBS for 1 h at room temperature (at 150 μL per well). The cells were then washed three times with 150 μL PBS per well (by leaving PBS on the cells for 5 min each wash). Next, the cells were dehydrated with increasing concentrations of filtered (0.22 μm filter) ethanol. The drying process proceeded as follows: 20 % ethanol for 20 min, 50 % ethanol for 20 min, 70 % ethanol for 20 min, 90 % ethanol for 20 min, 95 % ethanol for 20 min, and, finally, 100 % ethanol for 20 min using a Critical Point Dryer (Quorum K850; Quorum Technologies, Laughton, UK).

2.5. Scanning electron microscopy

After drying, the samples were arranged on an aluminum stub and coated with 1 nm of gold-palladium (Quorum Q150T ES Spatter Coater). Microphotographs were recorded using the scanning electron microscope JEOL model, 7800f. The images were taken with an accelerating voltage of 2 kV and a secondary electron detector.

2.6. Cytotoxicity assay

Frozen human PBMCs were thawed and suspended in a suitable culture media at a final concentration of 1×10^6 cells per mL and 100 μL/well of cell suspension were cultured in triplicates in a flat-bottom, 96-well plate to achieve a final number of 1×10^5 cells per well. Cells were incubated with each treatment for 24 h. Cell viability was assayed by using alamarBlue™ reagent [26]. The control compounds included dexamethasone alone as a non-toxic solution and Triton (1 %) as a cytotoxic reagent.

2.7. Cytokine-expression assay

Frozen human PBMCs were thawed and suspended in a designated culture media at a final concentration of 1×10^6 cells per mL and 500 μL/well of cell suspension were cultured in triplicate in 24-well plates, to achieve a final number of 5×10^5 cells per well. The controls and treatments were diluted in culture media to achieve the final assay concentration. Cells were incubated with the treatments and controls for 1 h, treated with 100 ng lipopolysaccharides and incubated for 24 h. One μg/mL dexamethasone served as positive control. Following incubation, the supernatants were collected, ELISA (R&D Systems; Minneapolis, MN) was used to measure the levels of a panel of secreted cytokines [27], and the optical density was determined by 'CLARIOStar-plus' plate-reader (BMG Labtech; Ortenberg, Germany). The concentration of cytokines (pg/mL) was calculated using a standard curve.

2.8. Statistical analysis

All the data was analyzed using GraphPad Prism 8.0.1 software (GraphPad Software, San Diego, CA, USA). Normality of data distribution was assessed using the Shapiro–Wilk test and for the homogeneity of variance with Bartlett's test. All analysis compared the control group results with those of the different groups using two-way analysis of variance (ANOVA) multiple-comparison and Dunnett post hoc tests. Results were considered statistically significant where p-value < 0.05.

3. Results

3.1. Antiviral effect

3.1.1. Cytotoxicity of compounds

MRC-5 cells were screened against a range of concentrations of CBD (2–10 μg/mL) and NT-VRL®ℊ (5–100 μg/mL), as shown in Fig. 2. The chosen concentrations for the efficacy test did not affect cell viability and were considered non-toxic. Those concentrations were: CBD ≤ 1 μg/mL and NT-VRL®ℊ ≤ 10 μg/mL.

3.1.2. Efficacy of compounds against HCoV-OC43: cell pre-treatment

MRC-5 cells were pre-treated with the compounds prior to inoculation with HCoV-OC43. As shown in Fig. 3, the viability of cells that were infected with HCoV-OC43, but otherwise untreated, was reduced to ~24 % of the viability of the uninfected control cells. Pre-incubation of the cells with all compounds prior to virus inoculation increased cell viability. The combination of 10 μg/mL NT-VRL®ℊ and 1 μg/mL CBD was the most effective treatment associated with the highest level of cell viability ($p < 0.05$).

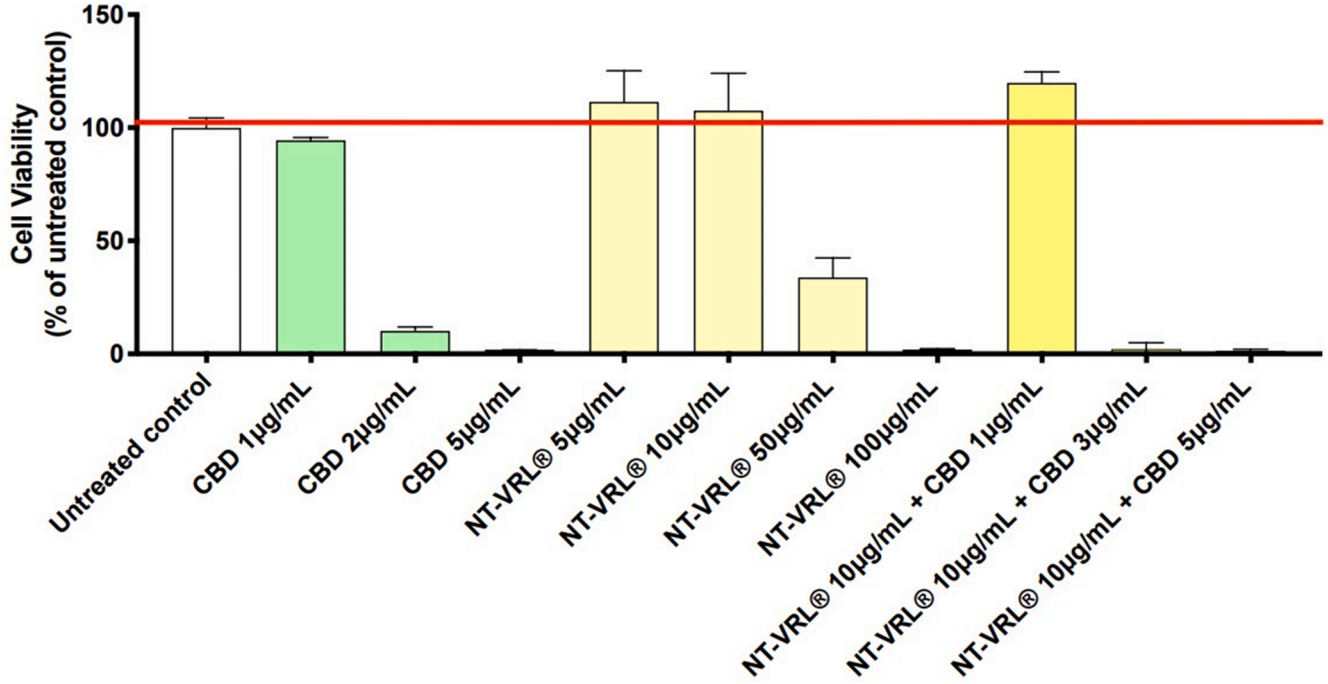

Fig. 2. Cytotoxicity of treatments to MRC-5 cells. MRC-5 cells were treated with different concentrations of the compounds for 72 h. Cell viability was then determined using an XTT assay that measures metabolically active live cells. Results represent mean percent viability ± SD ($n = 4$). CBD—cannabidiol.

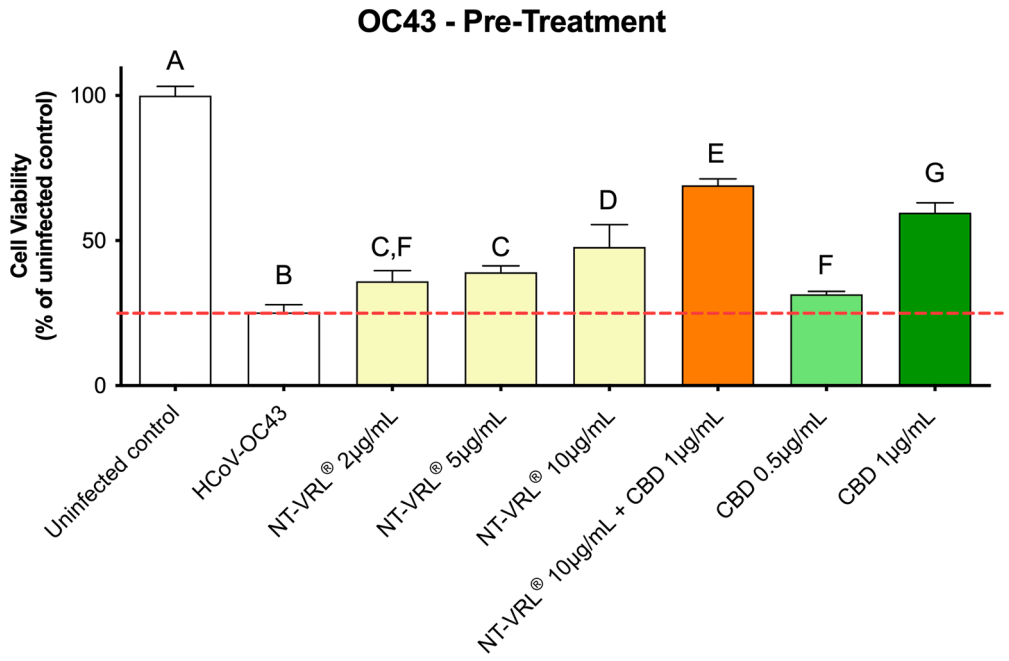

Fig. 3. Cell viability in each treatment group. The bars represent means + standard deviation (SD). Bars that are not labeled with a common letter are significantly different from one another ($p < 0.05$). $n = 4$.

3.1.3. Efficacy of compounds against HCoV-OC43: post-adsorption

When the compounds were added to the cells after virus adsorption, the viability of the HCoV-OC43-infected cells was reduced to 33 % of the control, as shown in Fig. 4. Under these conditions, 10 µg/mL NT-VRL® enhanced cell viability to around 65 % ($p < 0.05$) and the addition of 1 µg/mL CBD did not amplify the effect of NT-VRL®. One µg/mL CBD applied on its own had an effect like that of 10 µg/mL NT-VRL®.

3.1.4. Effect of NT-VRL® against HCoV-OC43

Morphological change associated with viral infection was observed on the cell surface at 72 h post-infection (Fig. 5B). Treatment of cells with NT-VRL® and CBD (Fig. 5C) before viral infection prevented that morphological change and the treated MRC-5 cells appeared similar to healthy cells (Fig. 5A).

3.1.5. Efficacy of compounds against influenza A (H1N1): cell pre-treatment

MRC-5 cells were pre-treated with the compounds prior to inoculation with influenza A (H1N1) virus. As shown in Fig. 6, the viability of cells that were infected with influenza A (H1N1) virus, but otherwise

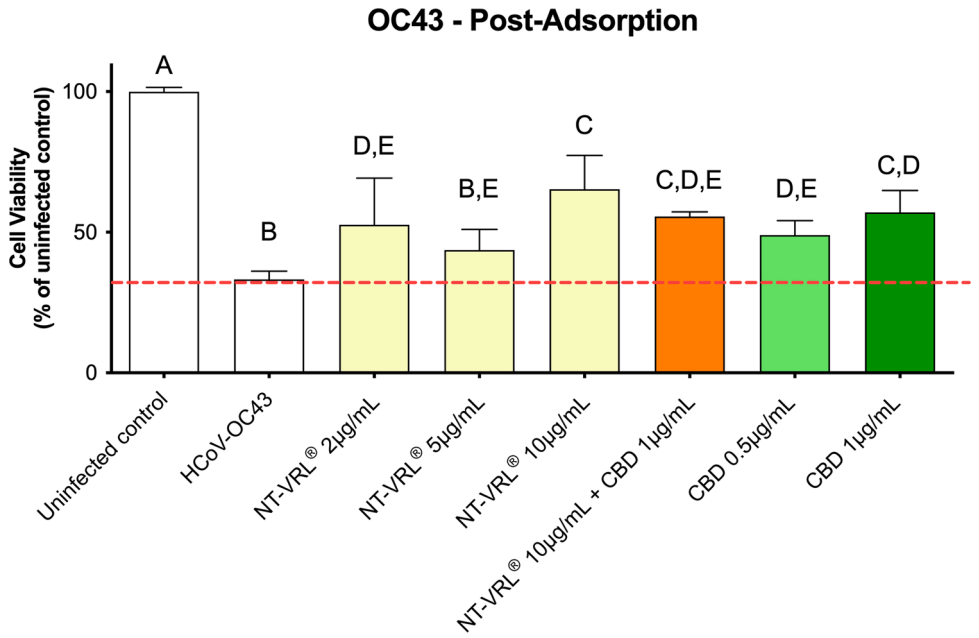

Fig. 4. Cell viability in each treatment group. The bars represent means + SD. Bars that are not labeled with a common letter are significantly different from one another ($p < 0.05$). $n = 4$.

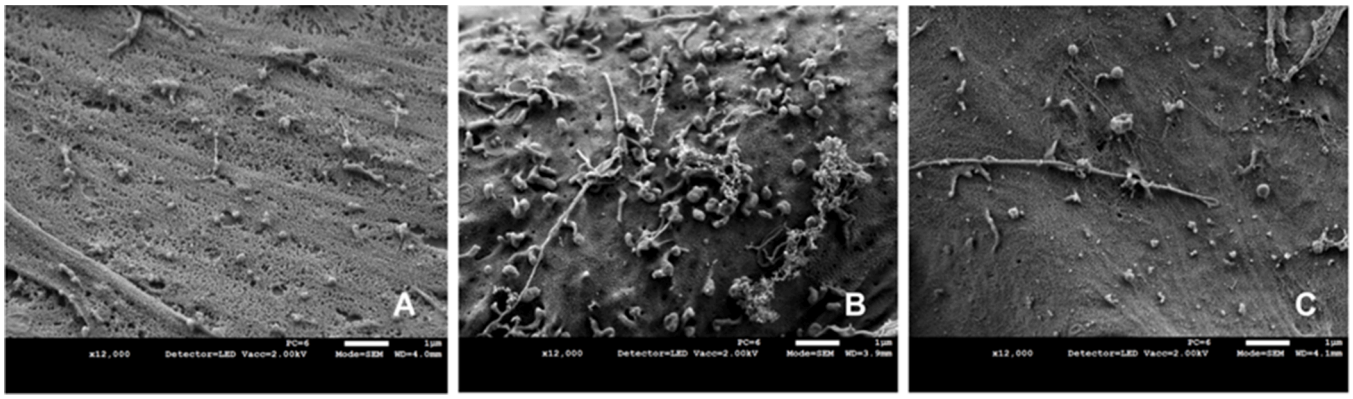

Fig. 5. Protective effects of pre-treatment of MRC-5 cells with NT-VRL®ℊ and CBD against the morphological effect of HCoV-OC43 infection. **(A)** Healthy MRC-5 cells. **(B)** MRC-5 cells that were pre-treated with assay medium, photographed 72 h after inoculation with HCoV-OC43. **(C)** MRC-5 cells that were pre-treated with NT-VRL®ℊ and CBD, photographed at 72 h after exposure to HCoV-OC43.

untreated, was reduced to ~36 % of the viability of the uninfected control cells. Pre-incubation of the cells with all the compounds prior to virus inoculation increased the level of cell viability. NT-VRL®ℊ exhibited a dose-response efficacy against influenza A (H1N1) virus. The combination of 10 µg/mL NT-VRL®ℊ with 1 µg/mL CBD was the most effective treatment associated with the highest level of cell viability ($p < 0.05$).

3.1.6. *Effect of NT-VRL®ℊ against influenza (H1N1) virus*

As shown in Fig. 7, this pattern was also observed in terms of the cytopathic effect seen microscopically after 72 h. Healthy MRC5 cells adhered efficiently to the surface and were evenly spread out, as can be seen in Fig. 7A. Lengthening and clumping of the MRC-5 cells was observed 72 h after viral infection (Fig. 7B). Pre-treatment of cells with NT-VRL®ℊ alone (Fig. 7C) before viral infection preserved cell viability and halted the cytopathic effect.

3.1.7. *Efficacy of compounds against influenza A (H1N1): post-adsorption*

When the compounds were added to the cells after virus adsorption, the viability of the influenza A (H1N1) virus-infected cells was reduced to 39 % of the control, as shown in Fig. 8. Under these conditions, NT-VRL®ℊ enhanced cell viability in a dose-dependent manner and the 10 µg/mL NT-VRL®ℊ treatment was associated with the highest level of cell viability (around 57 %; $p < 0.05$). The addition of 1 µg/mL CBD did not amplify the effect of NT-VRL®ℊ and even caused a decrease in cell viability, as compared to infected cells ($p < 0.05$).

3.1.8. *Effect of NT-VRL®ℊ against HCoV-229E*

In a previous study by Chatow et al. [7], NT-VRL®ℊ exhibited an antiviral effect when it was pre-incubated with host cells prior to virus infection. The addition of CBD potentiated that antiviral effect and did so more effectively than the positive controls pyrazofurin and glycyrrhizin. This pattern was also observed in terms of the cytopathic effect observed under a microscope after 72 h. Healthy MRC5 cells adhered to the surface, as can be seen in Fig. 9A. There was a drastic decrease in cell confluence presented by cell fragments left on the slide (Fig. 9B). Treatment of cells with NT-VRL®ℊ and CBD (Fig. 9C) before viral infection preserved cell viability and halted the cytopathic effect.

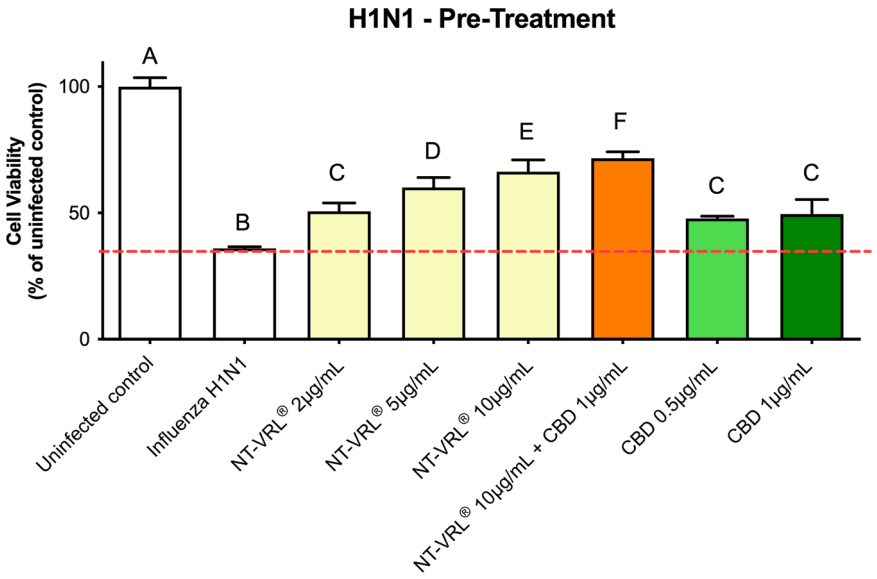

Fig. 6. Cell viability in each treatment group. The bars represent means + SD. Bars that are not labeled with a common letter are significantly different from one another ($p < 0.05$). $n = 4$.

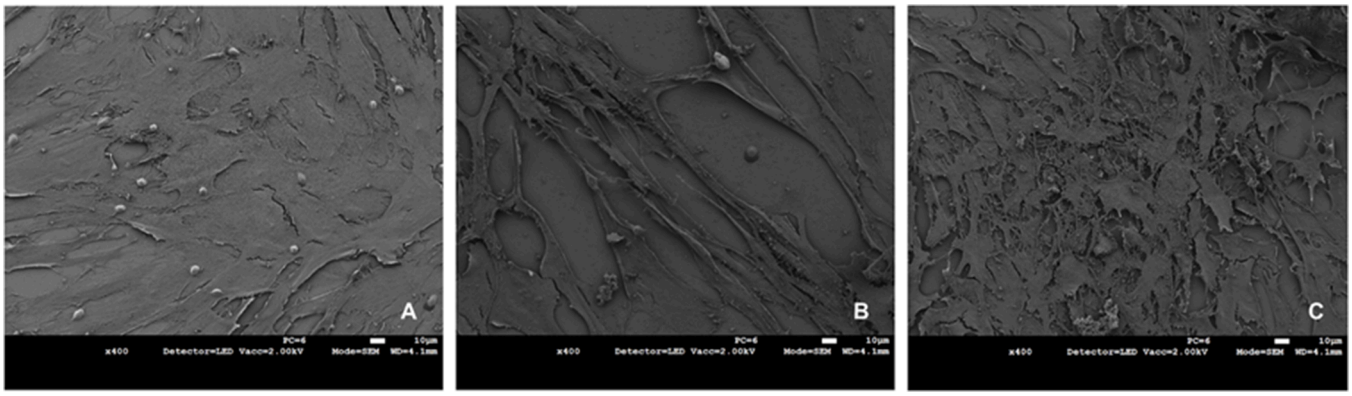

Fig. 7. Effects of NT-VRL®ℊ and CBD applied pre-infection on the cytopathic effect of influenza A (H1N1) virus in MRC-5 cells. **(A)** Healthy MRC-5 cells. **(B)** MRC-5 cells at 72 h after infection with influenza A (H1N1) virus. **(C)** MRC-5 cells that were pre-treated with NT-VRL®ℊ, photographed 72 h after exposure to influenza A (H1N1) virus.

3.2. Anti-inflammatory effect

3.2.1. Cytotoxicity of compounds

PBMC cells were screened against a range of concentrations of NT-VRL®ℊ (2–20 µg/mL), dexamethasone (40 µg/mL), and CBD (2 µg/mL; data not shown). The chosen concentrations for the efficacy test did not affect cell viability and were considered non-toxic. The non-toxic concentrations were: ≤1 µg/mL CBD and ≤10 µg/mL NT-VRL®ℊ.

3.2.2. Inhibition of pro-inflammatory cytokines

The results for all four cytokines (IL-1β, TNF-α, IL-6, and IL-8) revealed that the NT-VRL®ℊ formulation had a dose-dependent inhibitory effect on the secretion of pro-inflammatory cytokines (Fig. 10), which did not compromise cell viability. The anti-inflammatory effect of NT-VRL®ℊ could be seen at concentrations of 2–20 µg/mL. The inhibitory effect of 20 µg/mL NT-VRL®ℊ was slightly greater than the effect of 2 µg/mL CBD for most cytokines. The NT-VRL®ℊ formulation exhibited a stronger inhibitory effect than 1 µg/mL dexamethasone. Finally, the addition of CBD (2 µg/mL) together with NT-VRL®ℊ provided the greatest inhibition of cytokine secretion.

4. Discussion

Viral infections remain a major worldwide cause of morbidity and mortality. Each year, there are 3 million cases of influenza and 300,000–500,000 deaths from that disease [28]. The development of novel, effective antiviral treatments with low toxicity and few side effects is a matter of great interest. The rapid mutations that occur within both coronaviruses and influenza viruses indicate that there is a great need for the development of novel and potent antiviral drugs that are not genome-specific, and which have multiple antiviral properties. Furthermore, the efficacy of vaccinations wanes over time and vaccines are unavailable for certain populations. Thus, a natural and safe antiviral solution may be useful as an additional protection especially for population groups that may not be eligible for vaccination, such as children, pregnant women, the immune-suppressed, and people with other limiting medical conditions.

Drug repurposing involves testing a drug for a medical application that is different from its original indications [29]. This approach led to the approval of remdesivir as the first specific drug for the treatment of hospitalized COVID-19 patients [30]. Natural compounds are one of the most valuable sources for drug discovery and can be “repurposed” for the treatment of different conditions. For example, terpenes and CBD are

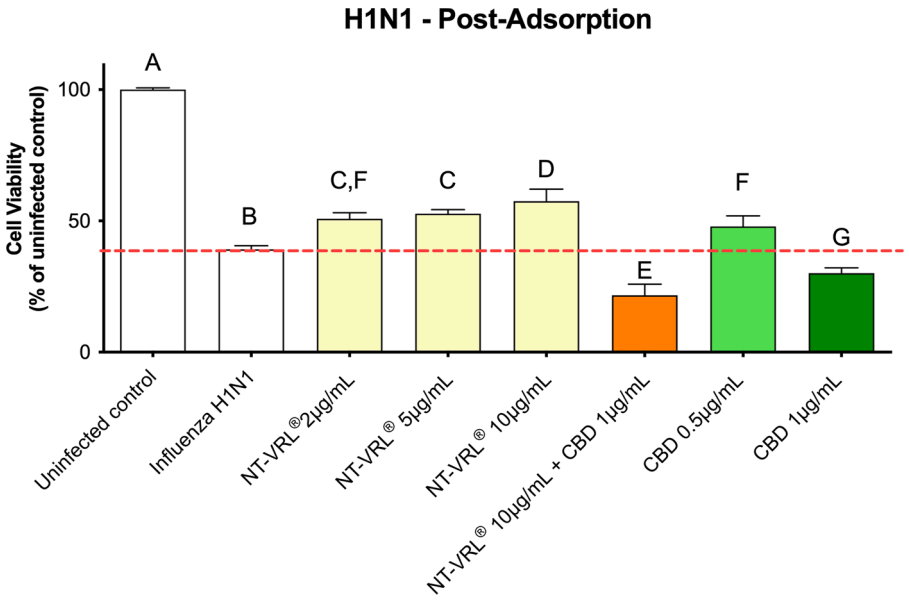

Fig. 8. Cell viability in each treatment group. The bars represent means + SD. Bars that are not labeled with a common letter are significantly different from one another ($p < 0.05$). $n = 4$.

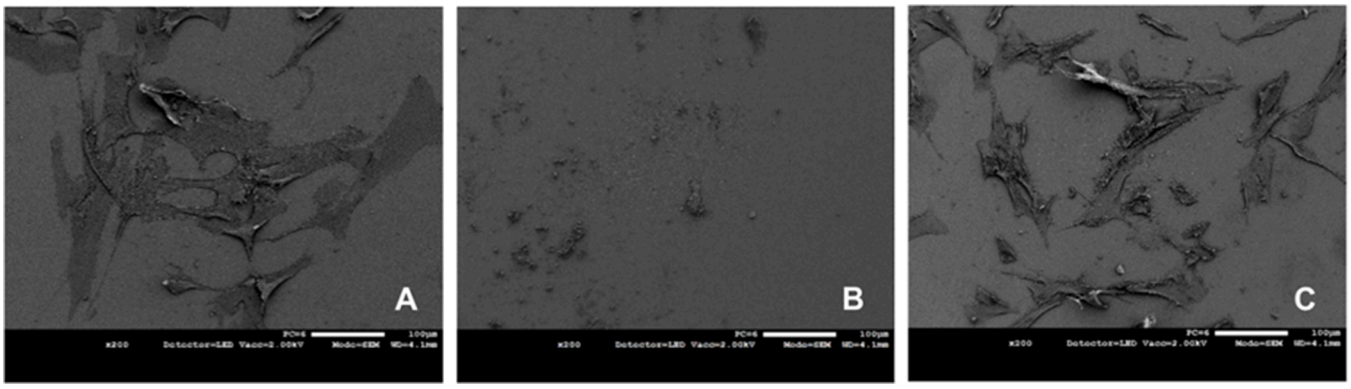

Fig. 9. Effects of NT-VRL®2 and CBD applied pre-infection on the cytopathic effect of HCoV-229E on MRC-5 cells. **(A)** Healthy MRC-5 cells. **(B)** MRC-5 cells at 72 h after infection with HCoV-229E. **(C)** MRC-5 cells that were pre-treated with NT-VRL®2, photographed 72 h after exposure to HCoV-229E.

used in both folk and Western medicine as treatments for various disorders such as cancer [31], bile stones [32], and anxiety [33] and other psychiatric disorders [34]. Terpenes are known to have efficient bioavailability via inhalation [35], a property that can be utilized for therapeutic applications related to the respiratory system. In addition, terpenes have known antiviral potential and low toxicity, making them good candidates as antiviral and anti-inflammatory agents with minimal side effects [36]. In this work, we suggest the consideration of terpenes as potential antiviral and anti-inflammatory agents for human use.

In a previous paper, we reported the antiviral efficacy of a proprietary terpene formulation (NT-VRL®2) against HCoV-229E [7]. In the current study, we sought to determine whether this formulation is effective against other viruses and whether it decreases the secretion of inflammatory cytokines. In this study, we demonstrate the in vitro efficacy of NT-VRL®2, with and without CBD, against the HCoV-OC43 and influenza A (H1N1) viruses. HCoV-OC43 is the most frequently infective virus of the HCoV family in infants [37]. As opposed to SARS-CoV-1,2 and MERS-CoV which present significant mortality rate, both HCoV-OC43 and HCoV-229E are endemic in human populations with clinical representation resembles that of many other agents of the common cold [38,39]. They have been the subject of many research endeavors long before the outbreak of COVID-19 [40–42]. Further, following the outbreak [43–45], both HCoV-OC43 and HCoV-229E could be studied at the biosafety level 2, thus research using them as a model system may help discover effective drugs or SARS-CoV-2 pathogenesis [39]. Influenza viruses and coronaviruses are both groups of enveloped viruses, but they have very different surface proteins and viral RNA. The observed antiviral effect against both viruses suggests that NT-VRL®2 may have a non-specific antiviral effect.

The activity of NT-VRL®2 against the two viruses was determined by the addition of the compounds to lung cells before or after those cells were inoculated with the viruses. The time-of-addition assays can help in determining the point(s) at which the formulation inhibits viral replication. NT-VRL®2 and CBD had different effects on the two viruses. NT-VRL®2 was more effective against HCoV-OC43 when it was applied post-adsorption, as compared to a pre-treatment of host cells. In contrast, NT-VRL®2 + CBD was more effective in the host-cell pre-treatment experiment. The effects of NT-VRL®2 against influenza A (H1N1) were similar in both the post-adsorption and pre-treatment tests. However, the addition of CBD enhanced cell survival in the pre-treatment test, but drastically decreased cell survival in the post-adsorption test. That decrease in cell survival is still under investigation.

When working with phytochemicals, studies suggest that combination treatments based on synergism between plant metabolites have the

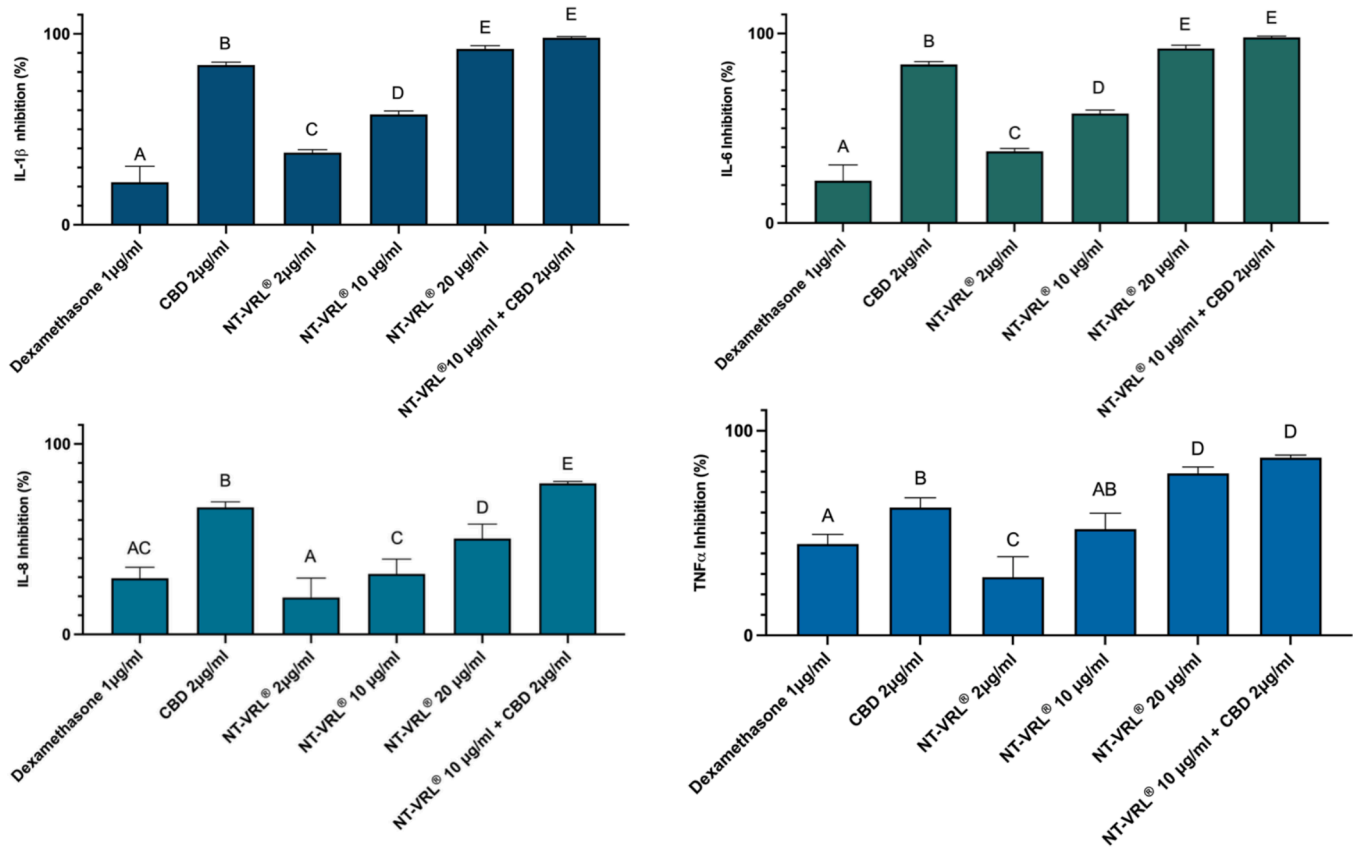

Fig. 10. Inhibition of secretion of each pro-inflammatory. The bars represent means + SD. Bars that are not labeled with a common letter are significantly different from one another ($p < 0.05$). $n = 3$.

potential to provide stronger effects than any single compound [46]. One example of this phenomenon is Rowatinex®, a formulation of terpenes that is used to improve the clearance of urinary stones [47]. Here, we suggest the use of a carefully planned terpene formulation as an effective in vitro antiviral and anti-inflammatory treatment. The in vitro efficacy of NT-VRL®, a 30-terpene formulation, could indicate a pharmacodynamic synergy between the compounds in that formulation ("entourage effect"). Plant extracts have been shown to have higher in vitro or in vivo activity than isolated ingredients at equivalent doses. NT-VRL® comprises several compounds that may operate on several targets to improve the overall therapeutic impact, without compromising cell viability or safety.

Since respiratory failure caused by acute respiratory distress syndrome is the primary cause of COVID-19 mortality [48], it is very important to utilize anti-inflammatory treatment to prevent the extreme inflammatory response. That initial inflammatory response is usually associated with an induced cytokine storm syndrome [48]. The cytokine storm is characterized by elevated levels of several cytokines such as interleukin (IL)-6, IL-8, and tumor necrosis factor alpha (TNFα; [49]). We demonstrated the effectiveness of NT-VRL® against the inflammatory response as measured by cytokines. Thus, the dual efficacy of cellular protection from the viruses and the reduction of cytokines increases the potential value of the compound. Recently published research demonstrated the virucide effectiveness of cannabidiol and terpene-based formulations [50] but neglected the anti-inflammatory point of view.

If proven effective in animal and human studies, NT-VRL® may be a valuable treatment for viral illnesses throughout the world. The results of these in vitro studies demonstrating protection of human lung cells by NT-VRL®, with or without CBD, prior to or after inoculation with either an influenza virus or a coronavirus suggest that this formulation may be helpful for preventing or alleviating the illnesses caused by those viruses.

5. Conclusion

NT-VRL® exhibited an antiviral and anti-inflammatory effect both pre-treatment and post-adsorption. Electron microscopy images revealed the antiviral properties of NT-VRL®, showing the protective effects of the formulation on the morphology of the cells. The combination of NT-VRL® with CBD provided an amplified antiviral effect. These results suggest that NT-VRL®, with or without CBD, may be useful as a preventative measure against coronaviruses and influenza viruses. Animal studies are ongoing to determine the antiviral efficacy of NT-VRL® and the efficacy of this formulation against additional viruses will also be examined.

Author contributions

L.C., N.E, A.N., S.R., I.N., R.B. contributed to conceptualization and design of the study. E.Z. performed the electronic microscopy imaging. L.C., A.N, N.E. and R.B. oversaw the analysis and interpretation of results. A.N. performed the statistical analysis. L.C., A.N., R.B. wrote the manuscript. N.E. was in charge of funding acquisition. All authors contributed to manuscript revision, read and the final version.

Declaration of competing interest

Lior Chatow, Nadav Eyal, Silvia Ramirez and Adi Nudel are employees of Eybna Technologies, a company that manufactures terpene-based formulations. Iris Nesher and Richard Boxer are advisors of Eybna Technologies.

Data availability

No data was used for the research described in the article.

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References

[1] A. Astani, J. Reichling, P. Schnitzler, Comparative study on the antiviral activity of selected monoterpenes derived from essential oils, Phytotherapy Res. 24 (5) (2010) 673–679.
[2] A. Astani, P. Schnitzler, Antiviral activity of monoterpenes beta-pinene and limonene against herpes simplex virus in vitro, Iran. J. Microbiol. 6 (3) (2014) 149–155.
[3] Z. Yang, N. Wu, Y. Zu, Y. Fu, Comparative anti-infectious bronchitis virus (IBV) activity of (-)-pinene: effect on nucleocapsid (N) protein, Molecules 16 (2) (2011) 1044–1054.
[4] A. Zamora, J.H. Edmonds, M.J. Reynolds, A.A. Khromykh, S.J. Ralph, The in vitro and in vivo antiviral properties of combined monoterpene alcohols against West Nile virus infection, Virology 495 (2016) 18–32.
[5] C. Bicchi, P. Rubiolo, M. Ballero, C. Sanna, M. Matteodo, F. Esposito, et al., HIV-1-inhibiting activity of the essential oil of *Ridolfia segetum* and *Oenanthe crocata*, Planta Med. 75 (12) (2009) 1331–1335.
[6] A.C. Guimarães, L.M. Meireles, M.F. Lemos, M.C. Cunegundes Guimarães, D. C. Endringer, M. Fronza, R. Scherer, Antibacterial activity of terpenes and terpenoids present in essential oils, Molecules 24 (13) (2019) 2471.
[7] L. Chatow, A. Nudel, I. Nesher, D. Hayo Hemo, P. Rozenberg, H. Voropaev, et al., In vitro evaluation of the activity of terpenes and cannabidiol against human coronavirus E229, Life 11 (4) (2021) 290.
[8] S. Belouzard, J.K. Millet, B.N. Licitra, G.R. Whittaker, Mechanisms of coronavirus cell entry mediated by the viral spike protein, Viruses 4 (2012) 1011–1033.
[9] J. Signer, H.R. Jonsdottir, W.C. Albrich, M. Strasser, R. Züst, Ryter, et al., In vitro virucidal activity of Echinaforce®, an *Echinacea purpurea* preparation, against coronaviruses, including common cold coronavirus 229E and SARS-CoV-2, Virol. J. 17 (2020) 136.
[10] World Health Organization. Weekly epidemiological update on COVID-19 - 18 May 2022. Available from: https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19—18-may-2022.
[11] J. Cinatl, B. Morgenstern, G. Bauer, P. Chandra, H. Rabenau, H.W. Doerr, Glycyrrhizin, an active component of liquorice roots, and replication of SARS-associated coronavirus, Lancet 361 (9374) (2003) 2045–2046.
[12] L. Haiying, H. Na, X. Xiaoyuan, The Curative Effects of Glycyrrhizin On Patients With SARS, Presented at the Annual Meeting of The Society of Infectious and Parasitic Diseases, Chinese Medical Association, Wuhan, China, 2003. Oct. 18–22.
[13] M.R. Loizzo, A.M. Saab, R. Tundis, G.A. Statti, F. Menichini, I. Lampronti, et al., Phytochemical analysis and in vitro antiviral activities of the essential oils of seven Lebanon species, Chem. Biodivers. 5 (3) (2008) 461–470.
[14] D. Giordano, A. Facchiano, V. Carbone, Food plant secondary metabolites antiviral activity and their possible roles in SARS-CoV-2 treatment: an overview, Molecules 28 (6) (2023) 2470.
[15] T.A. Bouback, A.M. Aljohani, A. Albeshri, H. Al-Talhi, Y. Moatasim, M. GabAllah, M.A. Ali, Antiviral activity of *Humulus lupulus* (HOP) aqueous extract against MERS-CoV and SARS-CoV-2: in-vitro and in-silico study, Biotechnol. Biotechnol. Equip. 37 (1) (2023) 167–179.
[16] H. Ulbegi Polat, M. Serhatli, A. Taş Ekiz, I. Abaci, Ö. Aksoy, G. Çakirca, C. Peru, Investigation of the Antiviral Effects of *Saussurea lappa* Root Extract against SARS-CoV-2 Virus, Mol. Nutr. Food Res. 67 (14) (2023) 2200804.
[17] Q. Ma, R. Li, W. Pan, W. Huang, B. Liu, Yuqi Xie, Phillyrin (KD-1) exerts anti-viral and anti-inflammatory activities against novel coronavirus (SARS-CoV-2) and human coronavirus 229E (HCoV-229E) by suppressing the nuclear factor kappa B (NF-κB) signaling pathway, Phytomedicine 78 (2020) 153296.
[18] K. Parang, N.S. El-Sayed, A.J. Kazeminy, R.K. Tiwari, Comparative antiviral activity of remdesivir and anti-HIV nucleoside analogs against human coronavirus 229E (HCoV-229E), Molecules 25 (10) (2020) 2343.
[19] N.M. Bouvier, P. Palese, The biology of influenza viruses, Vaccine 26 (Suppl. 4) (2008) D49–D53.
[20] W.P. Glezen, R.B. Couch, Influenza viruses. Viral Infections of Humans, Springer, Boston, MA, 1997, pp. 473–505.
[21] Y. Matsuzaki, N. Katsushima, Y. Nagai, M. Shoji, T. Itagaki, M. Sakamoto, et al., Clinical features of influenza C virus infection in children, J. Infect. Dis. 193 (2006) 1229–1235.
[22] A.C. Kalil, P.G. Thomas, Influenza virus-related critical illness: pathophysiology and epidemiology, Crit. Care 23 (1) (2019) 258.
[23] J.S. Min, D.E. Kim, Y.H. Jin, S. Kwon, Kurarinone inhibits HCoV-OC43 infection by impairing the virus-induced autophagic flux in MRC-5 human lung cells, J. Clin. Med. 9 (7) (2020) 2230.
[24] P.W. Cheng, L.T. Ng, L.C. Chiang, C.C. Lin, Antiviral effects of saikosaponins on human coronavirus 229E in vitro, Clin. Experim. Pharmacol. Physiol. 33 (7) (2006) 612–616.
[25] O.A. Husain, J.A. Millett, J.M. Grainger, Use of polylysine-coated slides in preparation of cell samples for diagnostic cytology with special reference to urine sample, J. Clin. Pathol. 33 (3) (1980) 309.
[26] S. Arimilli, B.E. Damratoski, B. Bombick, M.F. Borgerding, G.L. Prasad, Evaluation of cytotoxicity of different tobacco product preparations, Regul. Toxicol. Pharmacol. 64 (3) (2012) 350–360.
[27] L.G. Arlian, M.S. Morgan, J.S. Neal, Extracts of scabies mites (Sarcoptidae: sarcoptes scabiei) modulate cytokine expression by human peripheral blood mononuclear cells and dendritic cells, J. Med. Entomol. 41 (1) (2004) 69–73.
[28] R. Gasparini, D. Amicizia, P.L. Lai, D Panatto, Clinical and socioeconomic impact of seasonal and pandemic influenza in adults and the elderly, Hum. Vaccin. Immunother. 8 (1) (2012) 21–28.
[29] M.S. Hossen, M.A. Barek, N. Jahan, M.S. Islam, A review on current repurposing drugs for the treatment of COVID-19: reality and challenges, SN Compreh. Clin. Med. 2 (2020) 1777–1789.
[30] J.H. Beigel, K.M. Tomashek, L.E. Dodd, A.K. Mehta, B.S. Zingman, A.C. Kalil, et al., Remdesivir for the treatment of Covid-19 - final report, N. Engl. J. Med. 383 (19) (2020) 1813–1826.
[31] A.M. Tomko, E.G. Whynot, L.D. Ellis, D.J. Dupré, Anti-cancer potential of cannabinoids, terpenes, and flavonoids present in cannabis, Cancers (Basel) 12 (2020) 1985.
[32] J. Han, J.H. Moon, H.C. Koo, J.H. Kang, J.H. Choi, S. Jeong, et al., Effect of biliary stenting combined with ursodeoxycholic acid and terpene treatment on retained common bile duct stones in elderly patients, Am. J. Gastroenterol. 104 (10) (2009) 2418–2421.
[33] E.W. Leen-Feldner, T.M. Bynion, R. Gournay, M.O. Bonn-Miller, M.T. Feldner, Practical considerations for testing the effects of cannabidiol on human anxiety, J. Anxiety Disord. 82 (2021) 102429.
[34] J. Sarris, J. Sinclair, D. Karamacoska, M. Davidson, J. Firth, Medicinal cannabis for psychiatric disorders: a clinically-focused systematic review, BMC Psychiatry 20 (2020) 24.
[35] A. Falk-Filipsson, A. Löf, M. Hagberg, E. Wigaeus Hjelm, Z Wang, D-Limonene exposure to humans by inhalation: uptake, distribution, elimination, and effects on the pulmonary function, J. Toxicol. Environ. Health 38 (1) (1993) 77–88.
[36] D. Biswas, S. Nandy, A. Mukherjee, D.K. Pandey, A. Dey, *Moringa oleifera* Lam and derived phytochemicals as promising antiviral agents: a review, S. Afr. J. Botany 129 (2020) 272–282.
[37] R. Dijkman, M.F. Jebbink, E. Gaunt, J.W. Rossen, K.E. Templeton, T.W. Kuijpers, L. Van der Hoek, The dominance of human coronavirus OC43 and NL63 infections in infants, J. Clin. Virol. 53 (2) (2012) 135–139.
[38] V.M. Corman, D. Muth, D. Niemeyer, C. Drosten, Hosts and sources of endemic human coronaviruses, Adv. Virus Res. 100 (2018) 163–188.
[39] M.M. Kesheh, P. Hosseini, S. Soltani, M. Zandi, An overview on the seven pathogenic human coronaviruses, Rev. Med. Virol. 32 (2) (2022) e2282.
[40] H.K. Lee, B.H. Lee, S.H. Seok, M.W. Baek, H.Y. Lee, D.J. Kim, Y.R. Na, K.J. Noh, S. H. Park, D.N. Kumar, H. Kariwa, Production of specific antibodies against SARS-coronavirus nucleocapsid protein without cross reactivity with human coronaviruses 229E and OC43, J. Vet. Sci. 11 (2) (2010) 165–167.
[41] A. Vabret, F. Mouthon, T. Mourez, S. Gouarin, J. Petitjean, F. Freymuth, Direct diagnosis of human respiratory coronaviruses 229E and OC43 by the polymerase chain reaction, J. Virol. Methods 97 (1–2) (2001) 59–66.
[42] P.C. Woo, S.K. Lau, B.H. Wong, K.H. Chan, W.T. Hui, G.S. Kwan, J.M. Peiris, R. B. Couch, K.Y. Yuen, False-positive results in a recombinant severe acute respiratory syndrome-associated coronavirus (SARS-CoV) nucleocapsid enzyme-linked immunosorbent assay due to HCoV-OC43 and HCoV-229E rectified by Western blotting with recombinant SARS-CoV spike polypeptide, J. Clin. Microbiol. 42 (12) (2004) 5885–5888.
[43] V. Demers-Mathieu, C. DaPra, G.B. Mathijssen, E. Medo, Previous viral symptoms and individual mothers influenced the leveled duration of human milk antibodies cross-reactive to S1 and S2 subunits from SARS-CoV-2, HCoV-229E, and HCoV-OC43, J. Perinatol. 41 (5) (2021) 952–960.
[44] D. Forni, R. Cagliani, F. Arrigoni, M. Benvenuti, A. Mozzi, U. Pozzoli, M. Clerici, L. De Gioia, M. Sironi, Adaptation of the endemic coronaviruses HCoV-OC43 and HCoV-229E to the human host, Virus Evol. 7 (2) (2021) veab061.
[45] R. Kubota-Koketsu, Y. Terada, M. Yunoki, T. Sasaki, E.E. Nakayama, W. Kamitani, T. Shioda, Neutralizing and binding activities against SARS-CoV-1/2, MERS-CoV, and human coronaviruses 229E and OC43 by normal human intravenous immunoglobulin derived from healthy donors in Japan, Transfusion 61 (2) (2021) 356–360.
[46] L. Rui Hai, Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals, Am. J. Clin. Nutr. 78 (3) (2003) 517S–520S.
[47] I. Romics, G. Siller, R. Kohnen, S. Mavrogenis, J. Varga, E. Holman, A special terpene combination (Rowatinex®) improves stone clearance after extracorporeal shockwave lithotripsy in urolithiasis patients: results of a placebo-controlled randomised controlled trial, Urol. Int. 86 (2011) 102–109.

[48] P. Mehta, D.F. McAuley, M. Brown, E. Sanchez, R.S. Tattersall, J.J. Manson, COVID-19: consider cytokine storm syndromes and immunosuppression, Lancet 395 (2020) 1033.
[49] P. Ruscitti, O. Berardicurti, A. Iagnocco, R. Giacomelli, Cytokine storm syndrome in severe COVID-19, Autoimmun. Rev. 19 (2020) 102562.
[50] S. Santos, P. Barata, A. Charmier, I. Lehmann, S. Rodrigues, M.M. Melosini, P. J. Pais, A.P. Sousa, C. Teixeira, I. Santos, A.C. Rocha, Cannabidiol and terpene formulation reducing SARS-CoV-2 infectivity tackling a therapeutic strategy, Front. Immunol. 13 (2022).