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Original Article

Effect of PHELA, an African traditional drug, on levels of selected inflammatory cytokines in mammalian cells infected with SARS-CoV-2

MM Litabe[1], M Matsabisa[2], FJ Burt[1,3]*

ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes a severe illness, often characterized by an excessive release of proinflammatory cytokines, leading to a cytokine storm associated with disease severity. In this study the effect of PHELA, an accepted herbal combination of four exotic African medicinal plants, namely; Clerodendrum glabrum E. Mey. Lamiaceae, Gladiolus dalenii van Geel, Rotheca myricoides (Hochst.) Steane & Mabb, and Senna occidentalis (L.) Link, was investigated to determine the effect on viral replication of SARS-CoV-2 Omicron infected mammalian cells and the effect on cytokine release. PHELA has previously been proposed for use as an immune booster. In this study mammalian cells were treated with plant extracts before or after infection with SARS-CoV-2. Viral RNA was measured at intervals and selected cytokine levels (IL-1β, IL-2Rα, IL-6, TNF-α, IFN-γ) were monitored. No significant difference in viral RNA was observed between infected cells treated with plant extracts and untreated infected cells. However, PHELA-treated cells showed a delay in viral RNA increase. Infected cells treated with PHELA exhibited significantly lower levels of IL-1β, IL-2Rα, and TNF-α compared to untreated infected cells at 48- and 72-hours post-infection. Post-treatment was more effective in reducing proinflammatory cytokine production than pre-treatment, highlighting the potential of PHELA and its constituents in modulating cytokine responses during SARS-CoV-2 infection.

Keywords: PHELA, African traditional medicine, SARS-CoV-2, immune-modulating, proinflammatory

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INTRODUCTION

Coronavirus disease 19 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in December 2019 in Wuhan, China, with subsequent rapid global spread. The development of vaccines and natural immunity contributed towards reducing disease transmission and severity. In addition to vaccine development, various approaches to treatment and prevention have been described including monoclonal antibodies that target the receptor binding domain for virus neutralisation, or that mediate the destruction of infected cells via the opsonization pathway; small molecules and anti-viral peptide inhibitors; protease inhibitors; and antiviral agents such as Remdesivir (GS-5734)[1]. Host targets have also been investigated for interrupting viral replication.

Infection with SARS-CoV-2 can result in dysregulated release of proinflammatory cytokines, resulting in a cytokine storm. In particular, IL-6 has been identified as one of the main proinflammatory cytokines and used as a disease severity biomarker[2]. High levels of IL-6 have been associated with disease severity, organ failure, and death[3]. Although vaccines have been developed against SARS-CoV-2, effective and safe antivirals are still needed to treat and manage the disease. Currently, some attempts at treatment have focused on repurposing drugs, including HIV antivirals such as HIV-1 protease inhibitors lopinavir[4], the hepatitis C virus protease inhibitor danoprevir[2], the influenza antiviral favipiravir[5], and the RNA polymerase inhibitor, remdesivir[6,7]. The strategy of repurposing provides a potentially rapid trajectory toward an approved treatment. Traditional medicines have been proposed as affordable treatments and alternatives for patients suffering from COVID-19. At the beginning of the COVID-19 pandemic, traditional medicines from plant extracts were used in different countries as a form of treatment[8,9,10]. Previous studies have shown the effectiveness of traditional medicinal plants against various viral infections[11,12]. Traditional medicines are proposed to strengthen healing and regeneration processes by modulating the host's immune responses[13]. Traditional medicines can potentially interfere with various steps of the viral replication cycle and induce anti-inflammatory responses that may play a role in reducing severity of illness[14]. PHELA is prepared from four South African traditional medicinal plant extracts, combining *Gladiolus dalenii Van Geel*, *Senna occidentalis (L). Link*, *Rotheca myriciodes (Hochst.) Steane & Mabb*, and *Clerodendrum glabrum E. Mey var. glabrum* at specific fixed ratios. Using a rodent animal model, PHELA was shown to have immunostimulatory effects and restored cyclosporine-induced immunosuppression, indicating activation of IL-2[15]. In this study, PHELA and its four components, a traditional medicinal plant, were tested *in vitro* to determine its immunomodulatory effects against SARS-CoV-2 infected cells. PHELA is a traditional herbal medicinal plant under development as an immune booster in South Africa. The study aimed to determine whether these plant extracts have the potential to reduce viral RNA load in infected

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mammalian cells and/or modulate selected cytokine levels reported to be associated with the cytokine storm that occurs in some COVID-19 patients.

METHODS AND MATERIALS

Ethics consideration

The study was approved by the University of the Free State Health Sciences Research Ethics Committee (UFS-HSD2020/2001/2601-0005) and the University of the Free State Environment and Biosafety Research Ethics Committee (UFS-ESD2020/0181/22).

Cells, Plant Materials, and Virus

H1299-hACE2-E3 cells, a human lung cell line overexpressing the ACE-2 receptor (kindly donated by Professor Alex Sigal, African Health Research Institute, University of KwaZulu-Natal), which is highly susceptible to SARS-CoV-2 infection, was infected with SARS-CoV-2 Omicron and used to determine the cytotoxicity and antiviral activity or immune modulatory activity of the different plant extracts. Cells were grown in RPMI media supplemented with 10% FBS, penicillin (100 IU/ml), and streptomycin (100 mg/ ml) in a 5% CO_2 atmosphere at 37 °C. Growth media for the cytotoxicity assays contained 2% FBS and no additional supplements.

The plant extracts, including PHELA, *Gladiolus dalenii* (GD), *Senna occidentalis* (SO), *Rotheca myriciodes* (RM), and *Clerodendrum glabrum* (CG), were kindly provided by Professor Motlalepula G. Matsabisa from the African Medicines Innovations and Technologies Development (AMITD) Platform, Department of Pharmacology, University of the Free State, Bloemfontein, South Africa. PHELA is manufactured by the AMITD. The plants used in the experiments are exotic African medicinal plants. *Gladiolus dalenii, Rotheca myricoides,* and *Clerodendrum glabrum* were purchased from local plant nurseries. *Senna occidentalis* powder extracts were purchased from Dhanvantari Botanicals & Nutraceuticals in India. Voucher specimens of the plants have been deposited at the Geo Potts Herbarium at the University of the Free State. Each plant is assigned a specific voucher specimen number for reference: *Gladiolus dalenii* (BLFU MGM 001), *Rotheca myricoides* (BLFU MGM 0013), *Clerodendrum glabrum* (BLFU MGM 004), and *Senna occidentalis* (BLFU MGM 0012). Extracts were obtained from four plants using hydro-alcoholic extraction through maceration, dried into a homogenous powder, and then sterilized by gamma irradiation. The powder is tested before and after irradiation for bacterial and fungal contamination. To produce PHELA, the plant extracts were combined at specific ratios and supplied as a powder for further experimentation, and all the procedures were performed in accordance with the institutional and national guidelines and regulation. Stock solutions of the plant extracts were prepared in deionized water at a final concentration of 2 mg/ml and were filter sterilized and stored in aliquots at -80 before use. SARS-CoV-2 Omicron virus was isolated from a human sample by inoculating h1299-hACE2-E3 cells and viral stocks were stored at -80 °C prior to use. The tissue culture infectious dose 50 ($TCID_{50}$) was determined by performing tenfold serial dilutions of virus stock on h1299-hACE2-E3 cells in flat-bottomed 96-well plates, from 10^{1} to 10^{-7}, in replicates of six and calculations done using the Reed–Muench method[30,31]. All infectious studies were performed in a biosafety level 3 laboratory.

Cell cytotoxicity assay

The cytotoxicity of the plant extracts was determined using a CellTiter Aqueous One Solution Cell Proliferation Assay (MTS) [3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]-based viability assay (Promega, Wisconsin, USA) to determine a suitable dose for treating h1299-hACE2-E3 cells in which the plant extracts were not toxic. To test for cell viability, monolayers of $1x10^5$ H1299-hACE2-E3 cells were seeded into a 96-well plate and treated with plant extracts diluted twofold from 1600 µg/ml to 100µg/ml, then 80µg/ml to 10µg/ml and incubated at 37°C. Seventy-two hours post-treatment, the cell culture supernatant was removed, the cells were treated with cell proliferation solution, and the cell viability was measured according to the manufacturer's instructions. Briefly, 20µl of MTS reagent was added directly to the wells, and cell plates were incubated at 37°C for 2 hours. Absorbance was measured at 490nm on a SpectraMax Plus384 reader (Molecular Devices; Sunnyvale, CA). The average of the duplicate readings of each sample was obtained. Corrected absorbance was determined by subtracting the absorbance value obtained from culture media only from that obtained in cells treated with plant extracts, and the percentage of viable cells was calculated using the equation below. Dose-response curves were constructed, and the concentration causing death in 50% of the cells (CC_{50}) was determined.

% cell viability= ((sample-media)/(control-media)) x 100

Assessment of the effect of the plant on viral load

Pretreatment of virus with immune modulator

H1299-hACE-E3 cells were first treated with 80µg/mL of each plant extract, including Phela, at 37°C for 30 minutes in a humidified 5% CO_2 incubator. Post-incubation, 100µL of $1x10^5$ h1299-h1299-ACE2-E3 cells were infected with 50µL 100 $TCID_{50}$ treated SARS-CoV-2 virus in a final volume of 200µL/well and incubated in a humidified 5% CO_2 incubator at 37°C. A total of 200µL/well of cell supernatant was collected from each well at 12-, 24-, 48-, and 72-hours post-infection to determine the viral load and levels of selected cytokines using ELISA, separate wells were used for each time point. The viral load was determined at each time point from one sample. Each cytokine level was tested at each time point using two biological replicates. Virus-infected cells, not treated with plant extracts, and untreated and uninfected were used as controls. To normalize the data obtained, all cell supernatants were stored at -20°C before analysis. Each sample had a single freeze-thaw, using the same control, and all were tested on the same day.

Treatment of cells with plant extracts post-infection with SARS-CoV-2

A total of 100 µl of $1x10^5$ h1299-hACE2-E3 cells seeded in a 96-well plate were infected with 50 µl 100 TCID50 of SARS-CoV-2 Omicron virus and incubated for 30 minutes at 37°C to allow the adsorption of the virus. Post-infection cells were treated with 50µl of 80µg/ml of each individual plant extract or the combination, PHELA, and incubated in a humidified 5% CO_2 incubator at 37°C at a final volume of 200µl/well. The viral load was determined at each time point. Each cytokine level was tested at each time point using two biological replicates. A total volume of 200µl of cell culture supernatant was collected from each well at 12-, 24-, 48-, and 72-hours post-infection to determine viral load. Virus-infected cells, not treated with plant extracts, and untreated and uninfected were used as controls. All cell supernatants were stored at -20°C after collection and each sample subsequently had a single freeze-thaw, and were tested on the same day.

Detection of SARS-CoV-2

SARS-CoV-2 RNA in samples was evaluated using a qualitative commercial assay commonly used during the pandemic for diagnostic purposes. RNA was extracted from the cell culture supernatant of infected cells using the Zymo Quick RNA Viral extraction kit (Zymo Research, USA) according to the manufacturer's instructions. RNA was eluted from the spin columns by adding 15µl of RNase-free water

to the column matrix and centrifuging for 30 seconds. Eluted RNA was stored at -80°C before use.

The real-time qPCR was performed using the Allplex 2019-nCoV assay (Seegene, Seoul, South Korea). The assay is designed to detect the RNA-dependent RNA polymerase (RdRP) and the nucleocapsid (N) genes specific to SARS-CoV-2 and the envelope (E) gene for all Sarbecovirus, including SARS-CoV-2. Briefly, the master mix was prepared by adding 5µl of 2019-nCoV MOM, 5µl of buffer 5×, 5µl of RNase-free water, 1µl of internal control (IC), and 2µl of enzymes for each reaction. An 8µl aliquot of RNA sample, 8µl of positive control, or 8µl of RNase-free water for negative control was added to the master mix, resulting in a final volume of 26µl. Plates were then spun down at 2500rpm for 5 seconds and analyzed on a CFX96 Touch Real-Time PCR from BioRad using the following conditions: reverse transcription reaction one cycle: 50°C/20 minutes– 95°C/15minutes and the PCR reaction of 45 cycles: 94°C/15seconds– 58°C/30seconds. Gene amplifications were analyzed using FAM (E gene), HEX (IC), Cal Red 610 (RdRP), and Quasar 670 (N gene) fluorophores. Results were analyzed using a 2019-nCoV viewer from Seegene Inc. according to the manufacturer's instructions.

Quantification of cytokine expression by ELISA

Cytokine levels in 100µL aliquots of cell-free supernatant collected at different times post-infection were determined using Elabscience ELISA kits (Elabscience, Texas, USA). Briefly, a standard curve was generated for each cytokine using twofold serial dilutions of reference standards (supplied in each kit) tested in duplicate. The mean optical density (OD) value at 450nm for each dilution was used to generate a standard curve for each cytokine. To quantify each cytokine, 100µL of known standards (in duplicate) and samples (in duplicate) were incubated for 90 minutes at 37°C. Post-incubation, the samples (and standards) were removed from each well, and plates were incubated with 100µL of biotinylated detection antibody. Post-incubation, plates were washed three times with 350µL of wash buffer and incubated with 100µL horseradish peroxidase (HRP) conjugated streptavidin solution, and the plates were incubated at 37°C for 30 minutes. Post-incubation, the plate was washed five times with 350µL of wash buffer. A 90µL aliquot of substrate reagent was added to each well and incubated for 15 minutes at 37°C, and the reaction stopped with a 50µL aliquot of stop solution. The OD values at 450nm were determined after 15 minutes using a SpectraMax Plus384 reader.

Statistical analysis

Statistical analysis and graphical presentation of results were performed using GraphPad Prism version 9.2.0. Differences in viral RNA and cytokine levels were determined using the ANOVA test, and a p-value of less than 0.05 was considered statistically significant.

RESULTS

Cytotoxicity assay

H1299-hACE2-E3 cells were chosen as they are permissive to SARS-CoV-2 due to the expression of ACE-2 receptors. The cells were incubated with dilutions of PHELA and the four individual components of PHELA, including *Gladiolus dalenii* (GD), *Rotheca myricoides* (RM), *Clerodendrum glabrum* (CG), and *Senna occidentalis* (SO) to determine suitable concentrations that could be used to treat the cells without causing cytotoxic effects. The cells were incubated with plant extracts prepared from stock solutions of 2mg/ml and diluted twofold from 1600µg/ml to 100µg/ml, then 80µg/ml to 10µg/ml in cell culture media. Dilutions between 400µg/ml and 1600µg/ml of plant extracts were found to be toxic to the cells, resulting in cell death. MTS assays showed that PHELA exhibited dose-dependent cytotoxicity on h1299-hACE2-E3 cells with 50% cytotoxic concentration (CC_{50}) values of 400µg/ml ± 200µg/ml, while three of the components of PHELA, including CG, RM, and SO, exhibited a CC_{50} of 200 ± 100µg/ml respectively (data not shown). The exception was GD, whose CC_{50} was undetermined. Treatment of cells with GD resulted in more than 50% cell death, even at the lowest concentration, and thus was excluded from the infection studies. A final concentration of 80µg/ml (>90% cell viability) was selected as the concentration for downstream investigations (Figure 1). Due to its noticeable cytotoxicity in mammalian cells, GD was excluded from downstream analyses. The extent of its toxicity impacted cell viability, making it challenging to accurately assess the effects of SARS-CoV-2 infection in the presence of the extract. Including GD in further experiments could have confounded the results, as cells would be forced to simultaneously manage both the stress of viral infection and the cytotoxic effects of the compound. This dual burden would not provide a true representation of infection dynamics or immune responses, in measuring viral replication and cytokine production. Despite its exclusion from follow-up experiments, GD was initially tested because it is an integral component of the PHELA formulation. In the PHELA formulation and in the traditional preparation, the synergistic activities of the plants appear to mask the possible toxicity of the single GD plant as this could be observed in Figure 1 below.

Infection of h1299-hACE2-E3 with SARS-CoV-2 Omicron variant

Growth curves for virus infected cells pre- and post-treated with plant extracts were determined by incubating cells with plant extracts for 1 hour before inoculation with 100 $TCID_{50}$ of SARS-CoV-2, and by infecting cells with 100 $TCID_{50}$ prior to treatment with plant extracts. Viral RNA was detected using RT-qPCR. Briefly RNA was extracted from cell supernatants at 12-, 24-, 48-, and 72-hours post-infection. Detection of RNA based on cycle threshold (Ct) values from treated cells were compared to those obtained from infected, untreated cells. Results were represented as Ct values, where a low Ct value represented a high viral load and a high Ct value represented a low viral load. The

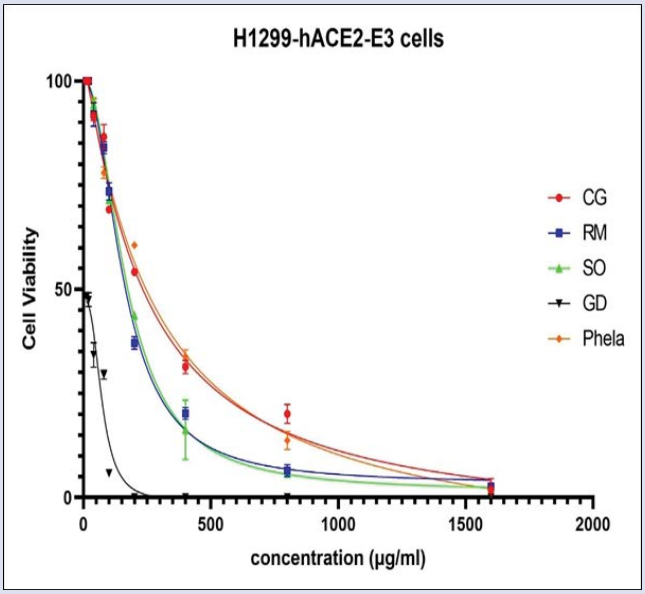


Figure 1. Cell viability dose-response curve. H1299-hACE2-E3 cells were treated with plant extracts from 1600, 800, 400, 200, 100, 80, 40, 20, to 10µg/ml for 72 hours, and cell viability was evaluated using the MTS assay. Results were represented as mean ± SD of two independent experiments and are expressed as relative values compared to the untreated cells.

assay targets three genes, including envelope (E), RNA-dependent RNA polymerase (RdRP), and the nucleocapsid (N) gene. All samples were positive for at least two of three viral targets. Figure 2 A-C represents the RNA detected at different time points for the E, RdRP, and N genes.

There was no statistical significance in Ct values obtained from cells treated with plant extracts compared to the virus-infected control however post-treatment of cells with PHELA, although not statistically significant, resulted in a higher Ct value 12 hours post infection, suggesting some initial inhibition of viral replication. Cells post-treated with PHELA had a higher Ct value, (31.5, 34.2, and 32.4 for E, RdRP, and E gene, respectively), at 12h post-infection compared to Ct values ranging from 12 to 19. In addition, the lower levels of viral RNA were detected at 72 hours post-infection for cells pretreated with PHELA, which was not noted in other samples.

Cytokine levels post-infection with SARS-CoV-2 infection

The study was performed to assess the ability of PHELA and its components to modulate immune responses by analysing selected cytokine levels specifically known to be associated with cytokine storms. The levels of IL-1β, IL2Rα, IL-6, TNF-α, and IFN-γ were determined in cells treated with plant extracts pre- and post-infection and compared to untreated cells and uninfected cells at 12-, 24-, 48-, and 72-hours post-infection. Higher levels of extracellular TNF-α, IL-1β, and IL-2Rα were detected in SARS-CoV-2 Omicron-infected cells in samples collected at 12 hours compared with mock-infected or treated cells (Figures 3-5). At 48- and 72-hours post-infection, levels of TNF-α were significantly higher in virus-infected untreated cells compared with pre- and post-treated infected (Figure 3). Levels of TNF-α were significantly lower in post-treated cells compared to pre-treated cells at 72 hours post-infection, except for cells treated with CG. TNF-α was detected in samples collected 24- and 48-hours post-infection for RM and CG pretreated cells, respectively (Figure 6A). In contrast, TNF-α was detected with significantly higher levels at 48 (p=0.0001) and 72 (p=0.0001) hours for all post-treated cells (Figure 6B). Lower levels of TNF-α were detected in the supernatant of cells collected 48 hours and 72 hours post-infection from cells pretreated with CG and RM compared with cells pretreated with SO and PHELA (Figure 6A). In

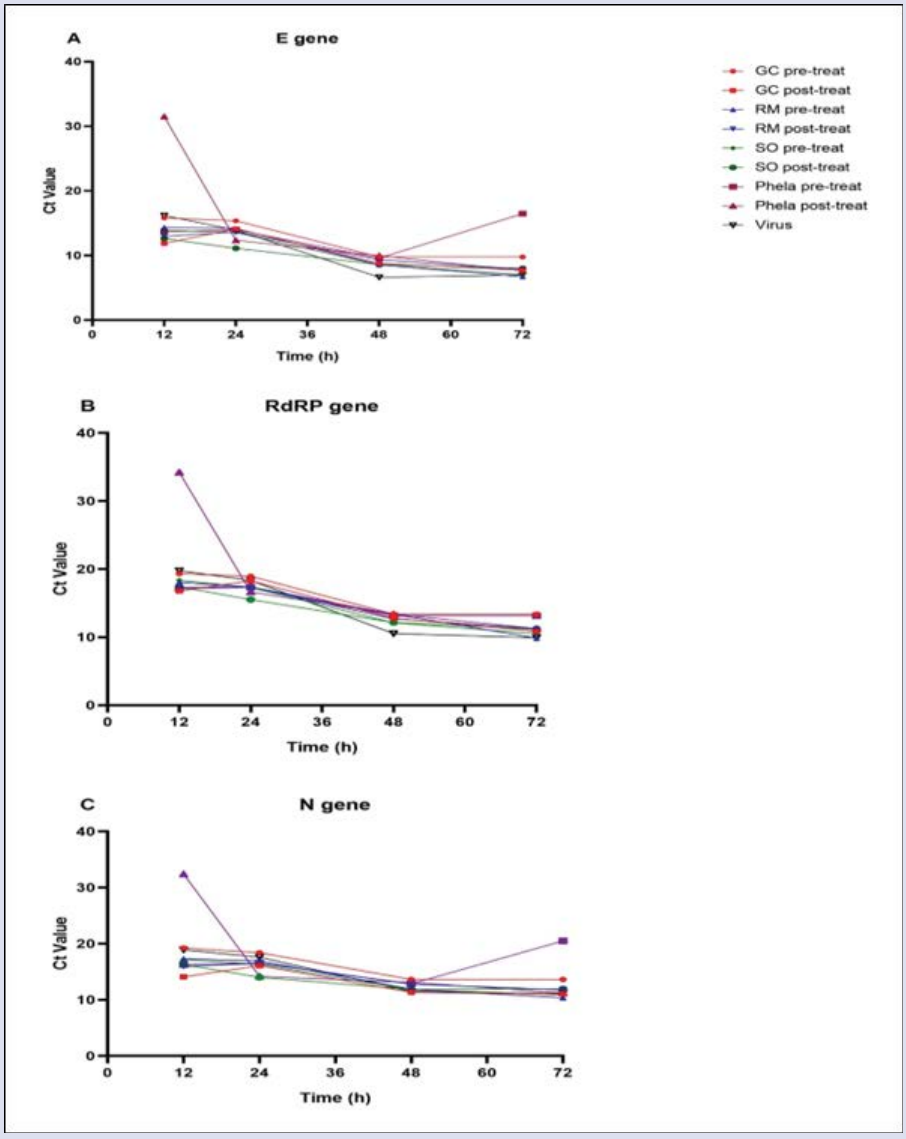
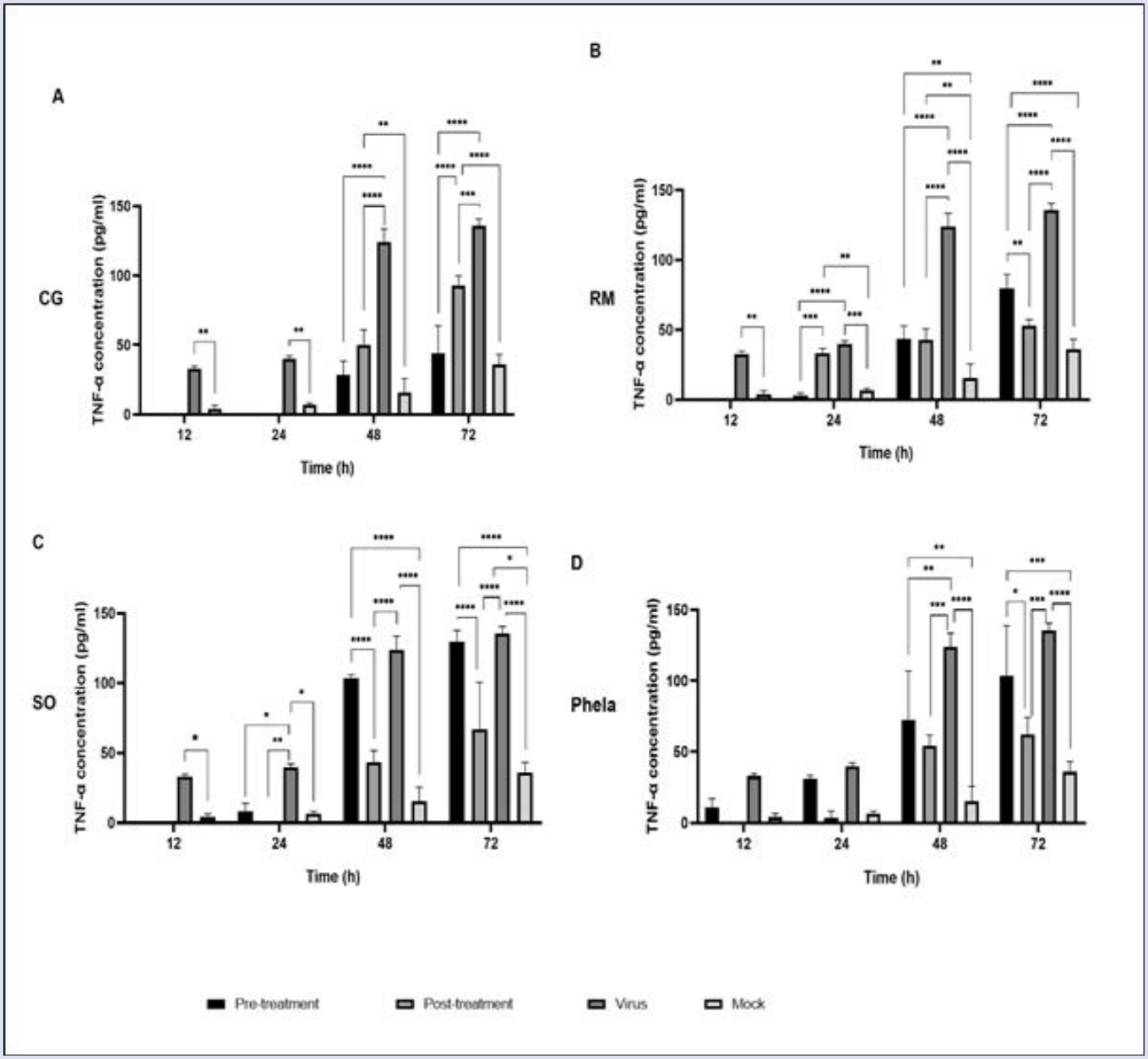


Figure 2. SARS-CoV-2 growth curves pretreatment and post-treatment with plant extracts. H1299-hACE2-E3 cells were incubated with 80µg/mL of *CG*, *RM*, *SO*, and Phela for 30 minutes before infection, or h1299- hACE2-E3 cells were infected with SARS-CoV-2 Omicron v*arian*t before treatment with 80µg/mL of *CG*, *RM*, *SO*, and Phela. RNA was extracted at 12, 24, 48, and 72 hours, and viral loads were evaluated by qRT-PCR.



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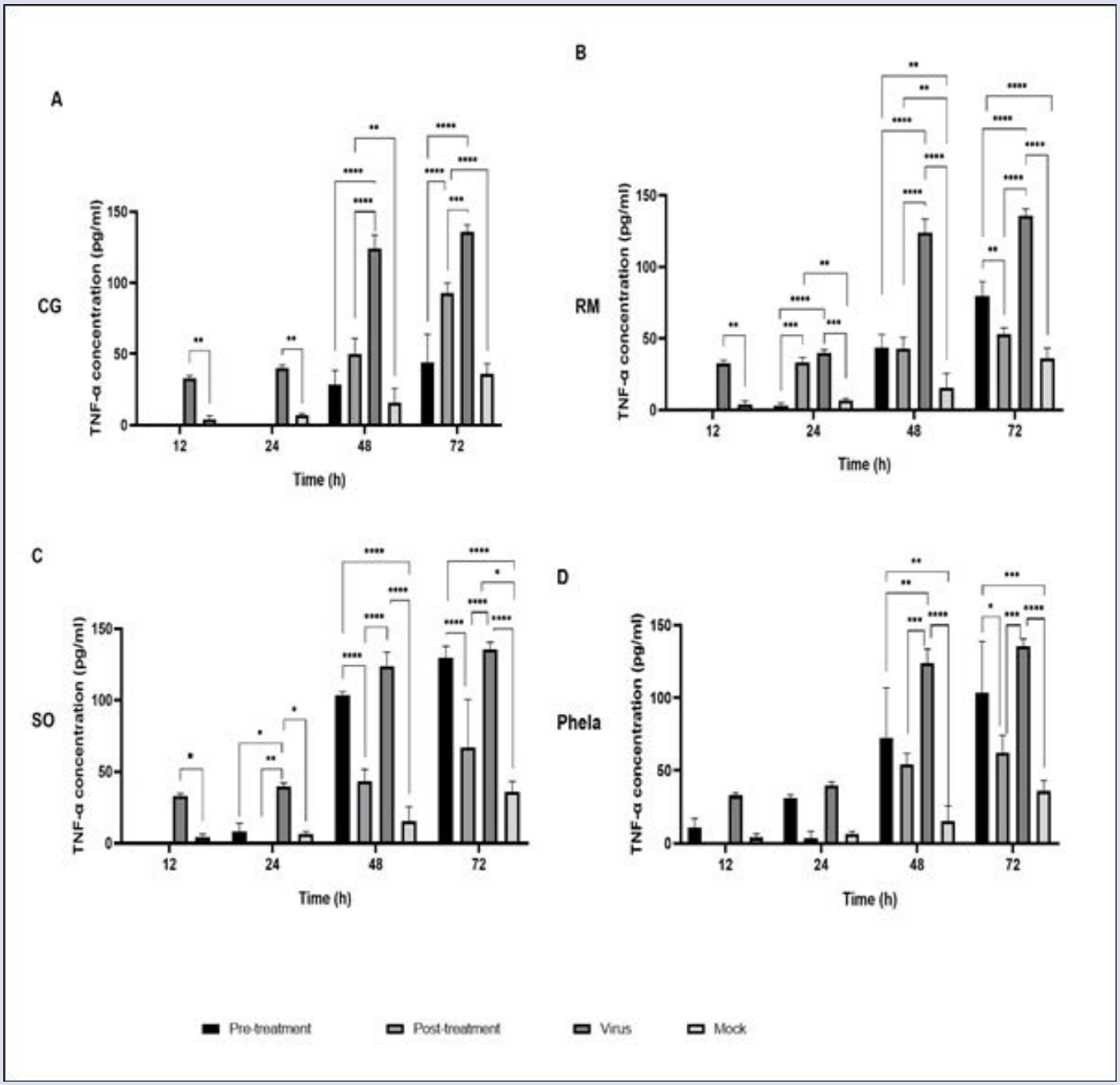

Figure 3. Cytokine levels in SARS-CoV-2 Omicron infected h1299-hACE-2-E3 cells. Cells were infected with SARS-CoV-2 pre- and post-treatment with plant extracts. Cell supernatant was collected at 12, 24, 48, and 72 hours post-infection, and extracellular release of TNF-α quantified by ELISA. A. TNF-α levels in cells treated with CG. B. TNF-α levels in cells treated with RM. C. TNF-α levels in cells treated with SO. D. TNF-α cytokine levels in cells treated with PHELA. ****p<0.0001, *** p<0.001, **p< 0.01, *p<0.05.

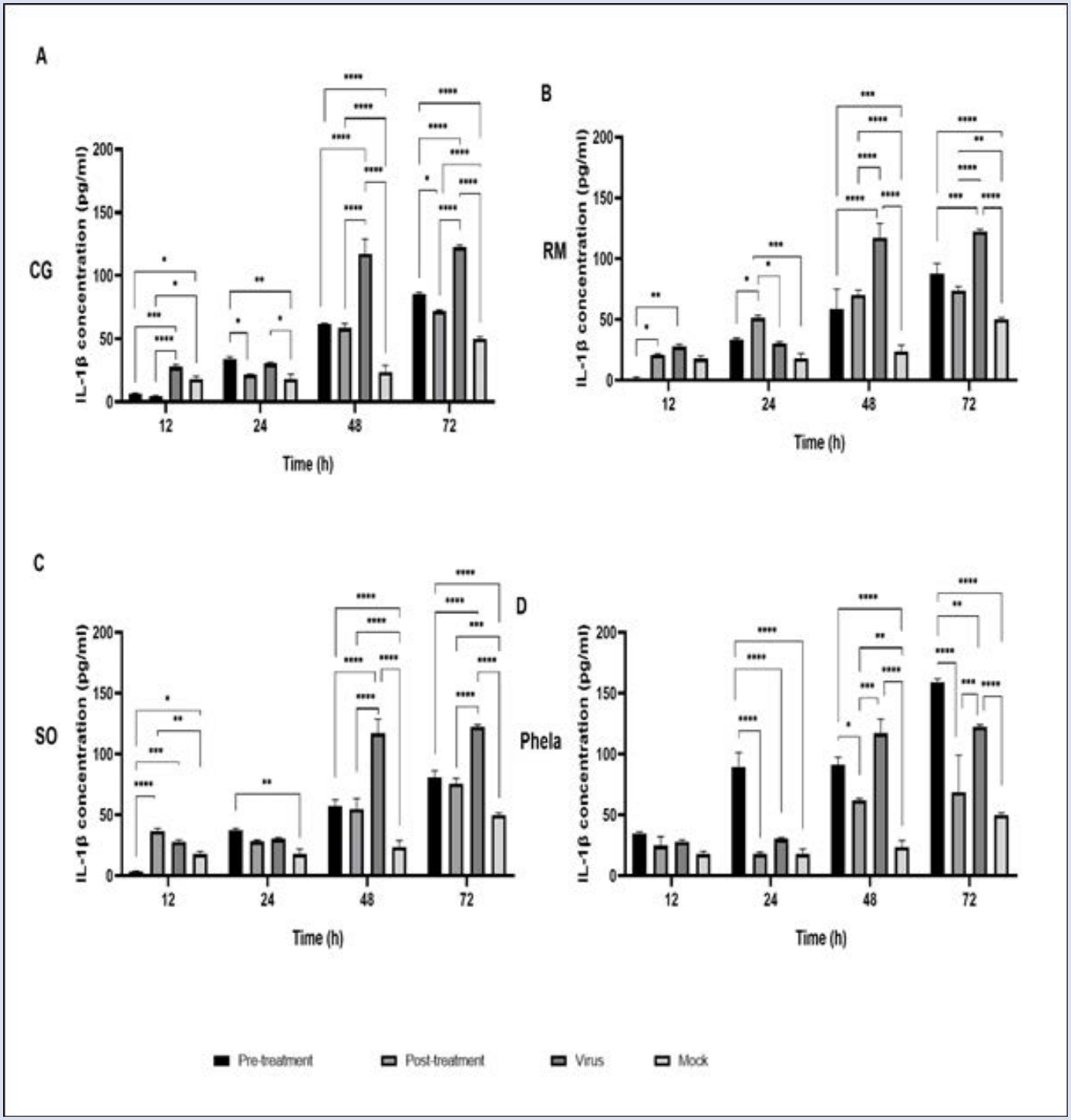

Figure 4. Cytokine levels in SARS-CoV-2 Omicron infected h1299-hACE-2-E3 cells. Cells were infected with SARS-CoV-2 pre- and post-treatment with plant extracts. Cell supernatant was collected at 12-, 24-, 48-, and 72-hours post-infection, and extracellular cytokine release of IL-1β quantified by ELISA. A. IL-1β levels in cells treated with CG. B. IL-1β levels in cells treated with RM. C. IL-1β levels in cells treated with SO. D. IL-1β levels in cells treated with PHELA. ****p<0.0001, *** p<0.001, **p< 0.01, *p<0.05.

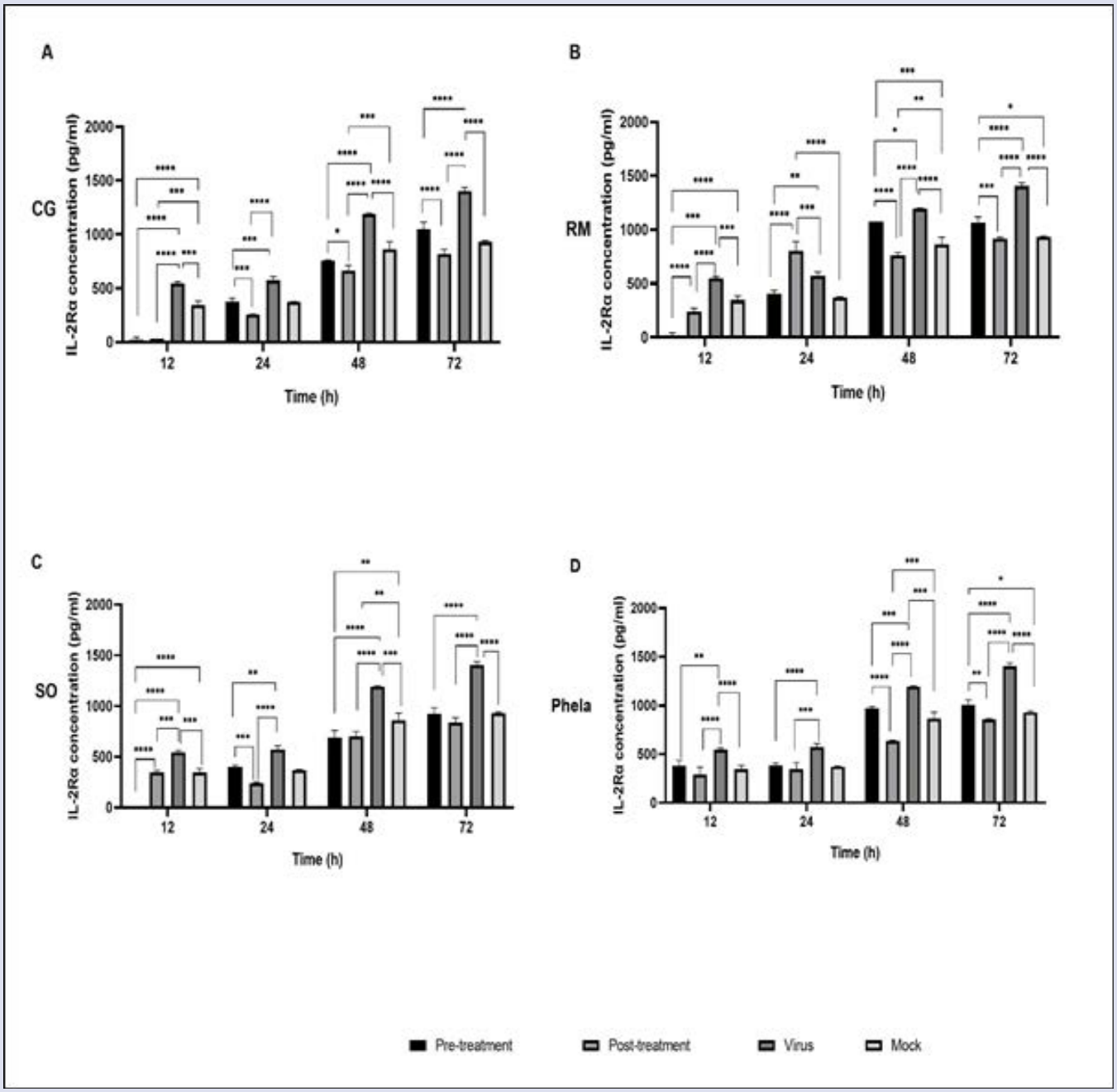

Figure 5. Cytokine levels in SARS-CoV-2 Omicron infected h1299-hACE-2-E3 cells. Cells were infected with h1299 pre-and post-treatment with plant extracts. Cell supernatant was collected at 12-, 24-, 48-, and 72-hours post-infection, and extracellular cytokine release of IL-2Rα were quantified by ELISA. A. IL-2Rα levels in cells treated with CG. B. IL-2Rα levels in cells treated with RM. C. IL-2Rα levels in cells treated with SO. D. IL-2Rα levels in cells treated with PHELA. ****p<0.0001, *** p<0.001, **p< 0.01, *p<0.05.

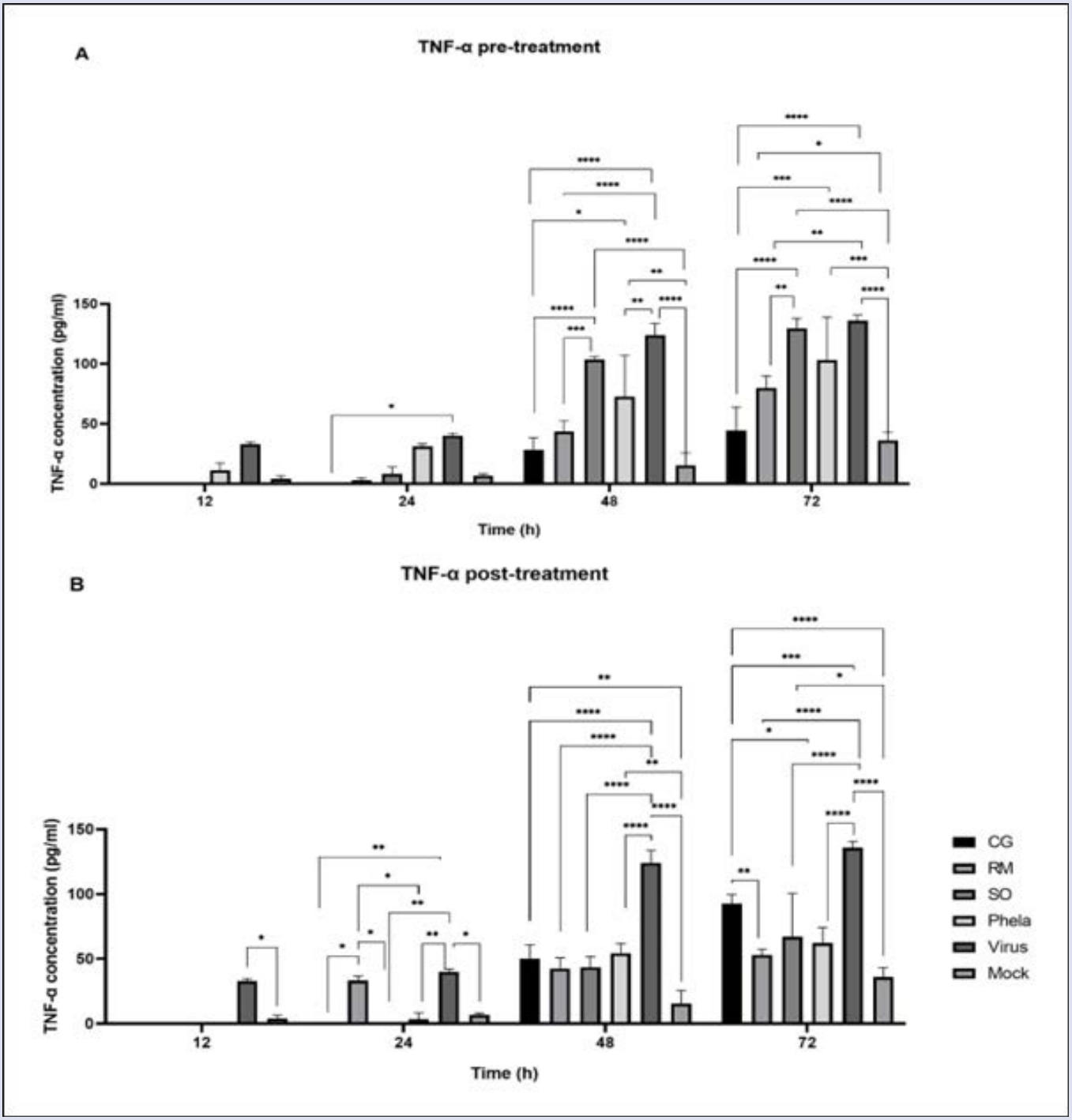

Figure 6. TNF-α levels in cells pretreated and post-treated with plant extracts and infected with SARS-CoV-2 Omicron. A: Cells pretreated with plant extracts before infection. B: Cells treated post-infection. Cell supernatant was collected 12-, 24-, 48-, and 72-hours post-infection, and TNF-α levels quantified by ELISA.

****p<0.0001, *** p<0.001, **p< 0.01, *p<0.05.

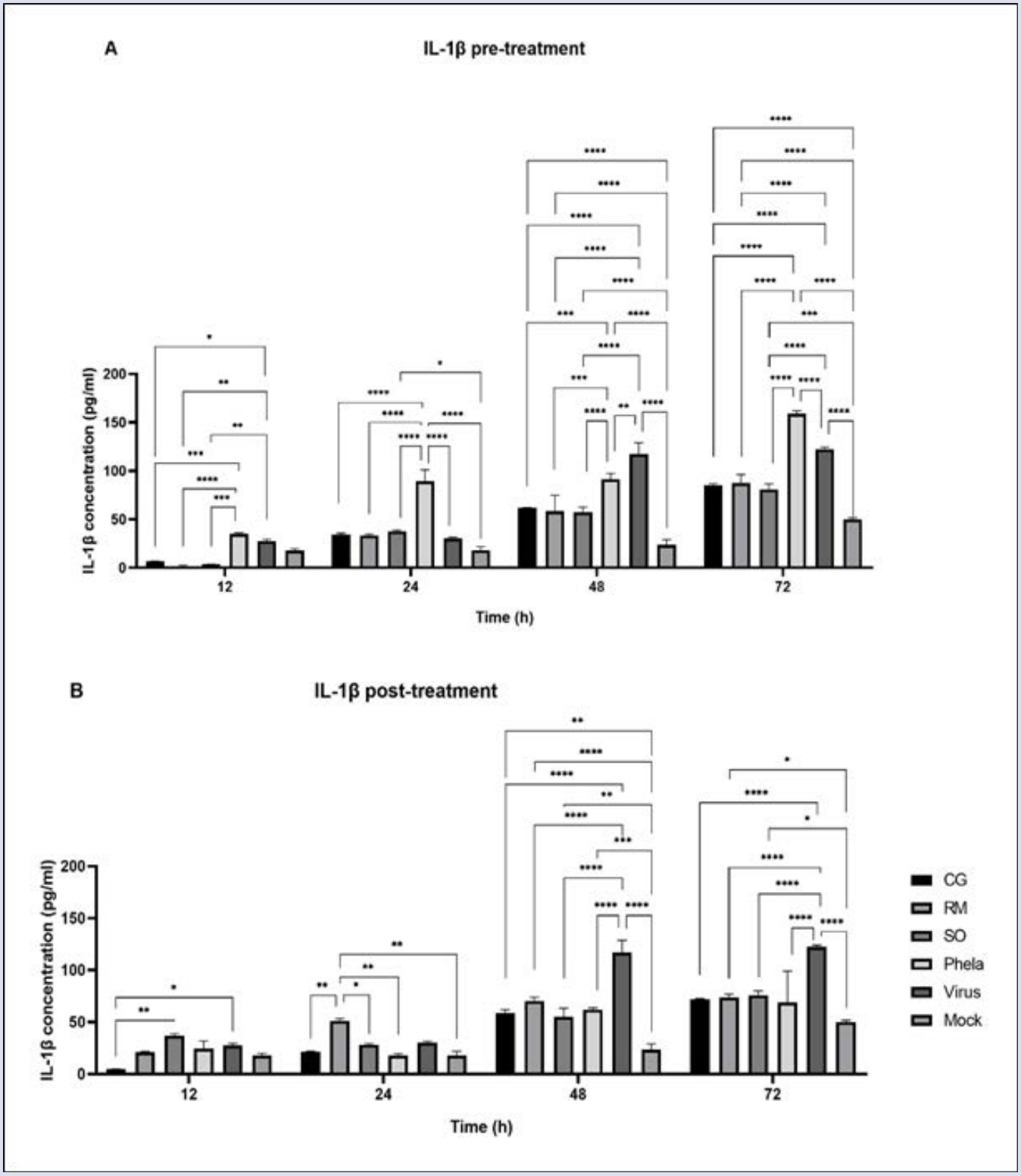

Figure 7. IL-1β levels in cells pretreated and post-treated with plant extracts and infected with SARS-CoV-2 Omicron. Cell supernatant was collected 12-, 24-, 48-, and 72-hours post-infection, and IL-1β levels quantified by ELISA. A: Cells pretreated with plant extracts before infection. B: Cells treated post-infection. ****p<0.0001, *** p<0.001, **p< 0.01, *p<0.05.

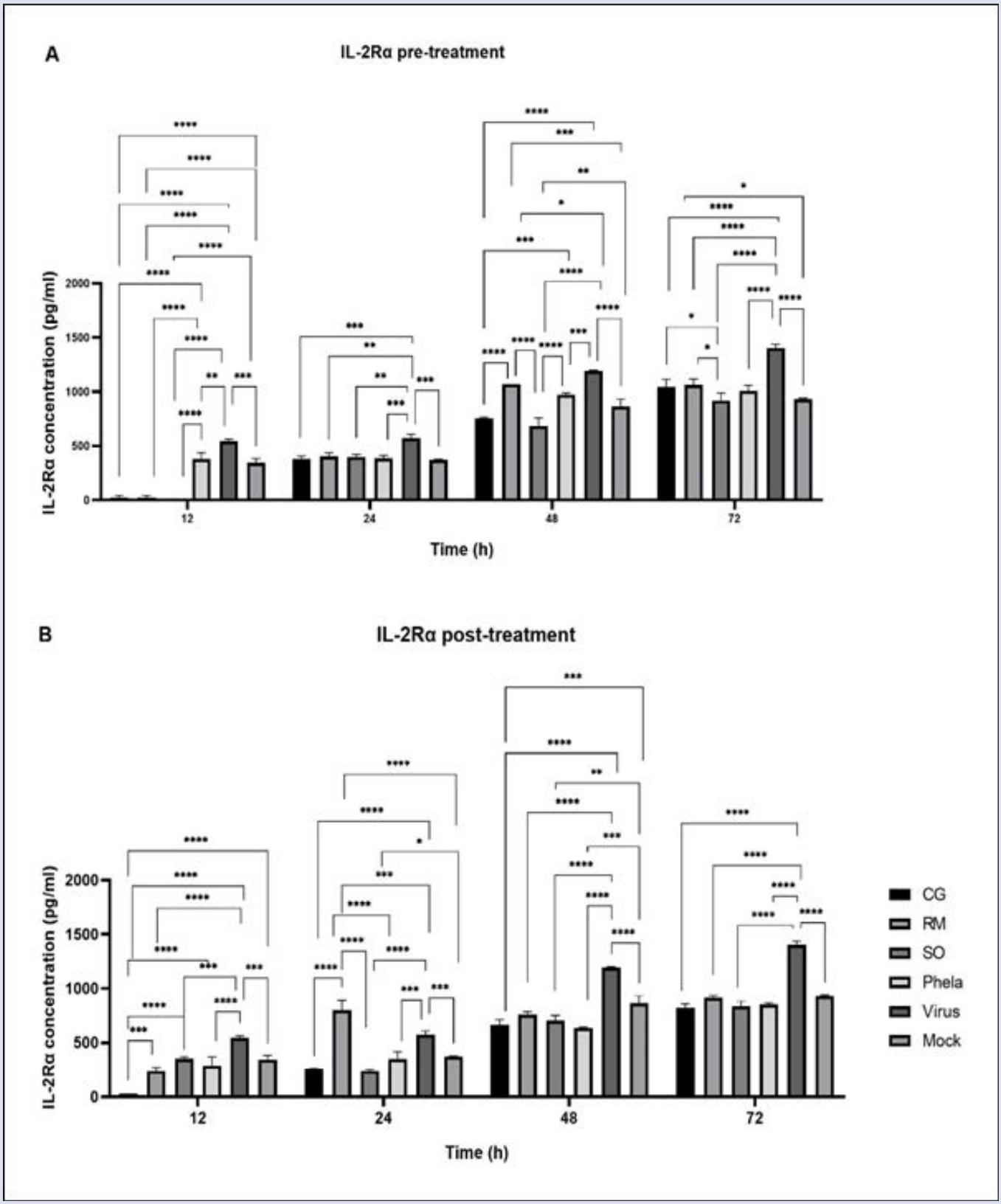

Figure 8. IL-2Rα levels in cells pretreated and post-treated with plant extracts and infected with SARS-CoV-2 Omicron. Cell supernatant was collected 12-, 24-, 48-, and 72-hours post-infection, and IL-2Rα levels quantified by ELISA. A: Cells pretreated with plant extracts before infection. B: Cells treated post-infection. ****p<0.0001, *** p<0.001, **p< 0.01, *p<0.05.

contrast, TNF-α levels were similar at 48 hours post-infection in all post-treated cells (Figure 6B), and significantly higher in CG-treated cells at 72 hours post-infection.

Similarly, IL-1β levels were significantly higher 48- and 72-hours post-infection in virus-infected control cells compared to cells treated with plant extracts, except for PHELA at 72 hours post-infection, where the pretreatment group was higher compared to the control (Figure 4). Similar trends were noted between pretreatment and post-treatment, except for when treated with PHELA, which had significantly higher levels at 24 (p=0.0001) and 72 (p=0.0001) hours post-infection compared with virus-infected control cells (Figure 7A). In the post-treatment group, levels of IL-1β were comparable between cells treated with the different plant extracts and significantly lower compared to the virus-infected untreated cells at 48 (p=0.0001) and 72 (p=0.0001) hours post-treatment (Figure 7B).

IL-2Rα was detected in all cell cultures including mock infected, regardless of treatment however, virus-infected control cells still had significantly higher cytokine levels than those treated with plant extracts (Figure 5). Cytokine levels in cells pretreated with plant extracts compared to virus-infected control differed 48 hours post-infection (range of p=0.01 and 0.0001) (Figure 8A). At 72 hours post-infection, all pretreated cells had significantly lower levels of IL-2Rα compared to the virus-infected control cells (p=0.0001) (Figure 8A). Significantly higher levels of IL-2Rα were evident in virus-infected control cells compared to cells post-treated with plant extracts at 48 (p=0.0001) and 72 (p=0001) hours post-infection (Figure 8B). Similarly to TNF-α and IL-1β, there were differences in levels of IL-2Rα in pretreated cells, whereas levels were comparable in post-treated cells.

IFN-γ was not detectable in supernatant from cells infected with virus and treated with plant extracts or from uninfected control cells. However, increased levels of IFN-γ were detected at 48- and 72-hours post-infection in virus-infected, untreated cells (Figure 9).

IL-6 was not detectable during infection with SARS-CoV-2, although high levels of viral RNA were detected in infected cells and the proinflammatory cytokine has previously been associated with high viral loads and severity of disease.

The results suggested that the timing of treatment (pre- or post-treatment) appeared to influence the cytokine upregulation or downregulation, with lower cytokine levels detected in cells that were post-treated with extracts, except in the case of CG, where pretreatment had more influence. Overall PHELA and its components modulated the release of proinflammatory cytokines like TNF-α, IL-1β, and IL-2Rα, which are implicated in the cytokine storms observed in severe COVID-19 cases.

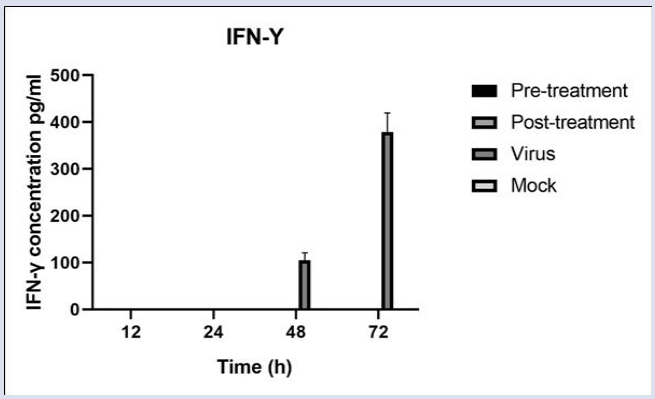

Figure 9. IFN-γ levels in cells pretreated and post-treated with plant extracts and infected with SARS-CoV-2 Omicron. Cell supernatant was collected 12-, 24-, 48-, and 72-hours post-infection, and extracellular release of IFN-γ quantified by ELISA.

DISCUSSION

The current vaccines approved for use have been shown to reduce disease severity and lower fatality rates. However, novel therapeutics are still required for the treatment and management of disease. Treatments would have implications for breakthrough infections, unvaccinated populations, and for emergence of variants that evade the immune response induced by the current vaccines. Traditional medicinal plants have been used previously to treat various respiratory infections, as they are believed to have promising antiviral capacities[12,16,17]. Different countries have tested the potential of indigenous plants for immune-modulating capabilities against SARS-CoV-2[8, 9,10], and compounds in plant extracts with the potential to have antiviral activity against SARS-CoV-2 have been identified using *in silico* molecular docking[18].

This study investigated the effects of PHELA and its components on cytokine expression and viral load using SARS-CoV-2-infected mammalian cell lines overexpressing the ACE-2 receptor. Viral RNA was detected using a qualitative commercially available RT-PCR[19], the gold standard for diagnosis and which has been shown to correlate with quantitative viral loads and hence was used to represent viral load. PHELA is under development as an immune booster in South Africa, with recent studies showing its effectiveness in the treatment of gram-positive *Staphylococcus aureus*[20] and possible use for the treatment of Alzheimer's disease[21]. An *in vitro* investigation of PHELA showed over 90% inhibition of SARS-CoV-2 and SARS-CoV infections at 0.005 mg/ml to 0.03 mg/ml[22] using pseudovirus infected cells. Additionally, PHELA demonstrated close to 100% inhibition of MERS-CoV infection at concentrations ranging from 0.1 mg/ml to 0.6 mg/ml[22]. Based on Ct values representing viral loads, treatment of cells with plant extracts did not appear to inhibit viral replication. There was no statistical significance in Ct values between cells treated with plant extracts and the virus-infected control cells, suggesting that the plant extracts had limited inhibition of SARS-CoV-2 replication, although this may have been dose dependent. It was necessary to use a concentration that was not toxic for the cell line, but it may be necessary to consider other cell lines where the higher concentrations of extract can be used to determine an outcome. Although not significant, post-treatment with PHELA resulted in higher Ct values compared to virus-infected control cells and cells treated with other plant extracts.

There were clear differences in levels of cytokines due to treatment with plant extracts. Multiple studies have reported on the dysregulated production of inflammatory cytokines in patients with severe COVID-19 disease[23,24,25]. Release of these cytokines has been shown to be an essential indicator of the severity of disease for SARS-CoV-2, associated mainly with organ failure rather than viral load. Previous studies have also associated the release of IL-6, IL-1β, and TNF-α with the severity of the disease[26,27]. In this study, key inflammatory cytokines, including IL-1β, IL-2Rα, IL-6, TNF-α, and IFN-γ, that have been associated with cytokine storm were included in our analysis. Some cytokines, including TNF-α, IL-1β, and IL-2Rα, showed significantly higher release in virus-infected cells than those treated with plant extracts. Post-treatment of cells with immune modulators resulted in a significant reduction of IL-1β, IL-2Rα, and TNF-α at 48 and 72 hours post-infection of PHELA and all its components, suggesting a potential role of these plant extracts in modulating the immune response by reducing TNF-α release during SARS-CoV-2 infection. This cytokine-modulating effect is particularly relevant, given that severe COVID-19 cases are often driven not just by viral replication but by an exaggerated host immune response or better referred at the cytokine storm. Rather than directly targeting the virus, PHELA may help to restore immune balance by reducing inflammatory response that contributes to disease

severity. In our previous study, PHELA was found to reconstitute the immune system acting through both cellular and humoral immunity. This could be especially important in supportive treatment for patients with advanced or complicated COVID-19, where controlling inflammation is often just as critical as suppressing viral replication. IFN-γ was not detected in cells treated with PHELA, all its components, and the uninfected cells. In contrast, high levels of IFN-γ were detected in h1299-hACE2-E3 virus-infected untreated cells at 48- and 72-hours post-infection. Results also show a better response post-treatment than pretreatment, suggesting a role in managing cytokine storms associated with severe symptoms of COVID-19 disease. Given that severe COVID-19 cases are often associated with a cytokine storm, with an excessive release of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α. Understanding how PHELA modulates these responses is critical. These findings may indicate that PHELA has immunomodulatory properties capable of reducing inflammation, suggesting potential therapeutic value of PHELA and relevance in managing pro-inflammatory responses in SARS-CoV-2 infection.

The results show the complexity of innate immune response to viral infections and the potential of PHELA and components of PHELA in modulating innate immune responses. Understanding the underlying mechanisms through which these compounds interact with the immune system could provide valuable insights into possible treatments for SARS-CoV-2 and related viruses to manage the dysregulated immune responses in severe cases. Previous studies have associated IL-6 with severity of disease[28], with high viral loads being associated with increased levels of IL-6. However, in this study, IL-6 was not detectable in cells treated with plant extracts or the controls, even though infected cells demonstrated high viral loads[29]. This could possibly be due to the ELISA methods not being sensitive enough to detect low levels of these cytokines. Another reason for the IL-6 being undetectable could be the early sampling post-infection, the timing of sample collection in relation to the progression of the infection may influence the detectability of the proinflammatory cytokine. Majority of individuals in which high levels of IL-6 is detected are usually in the severe stage of the disease, with disease severity usually occurring five days post-infection, in our case, sampling was only performed for up to 72 hours in infected cells.

In summary, PHELA and three out of its four component plants reduced pro-inflammatory cytokine responses but not viral load *in vitro*. Treating cells with plant extracts did not result in reduced viral loads post-infection with SARS-CoV-2 Omicron virus, suggesting that they may have a limited role in reducing viral load or being antiviral. The inability to directly suppress viral replication suggests that the primary value of PHELA may lie in its immunomodulatory properties, particularly in dampening the excessive inflammatory responses associated with severe COVID-19, such as the cytokine storm. This could make PHELA useful as a therapeutic drug aimed at managing immune-mediated damage, rather than controlling viral spread. Whether the observed effects could be relevant for use in humans needs to be further investigated. These results warrant further studies on the effect of traditional medicinal plants on modulating cytokine release during viral infections and support further exploration of PHELA in *in vivo* models and eventual clinical testing as a supportive therapy for severe COVID-19, especially in cases where inflammation contributes significantly to disease severity.

The limitation of the study is that the small design and sample number warrant repeating the experiments with more samples and additional parameters, and using gene expression studies or microarrays to further characterize the effect on the release of cytokines. IL-6 was not detected in any of the infected or control cell samples. This absence may be attributed to limitations in the ELISA assay, as its accuracy can be influenced by factors such as antibody specificity and detection sensitivity. It is possible that the assay's detection threshold was not low enough to capture the presence of IL-6 at biologically relevant, yet low, concentrations, thereby limiting the ability to measure this cytokine accurately. Additionally, the results obtained in *in vitro* studies are not readily transferrable to a clinical setting to test in humans. However, they do provide the potential that these extracts may have against SARS-CoV-2. Cytokine storms are not unique to SARS-CoV-2 and can occur in response to various viral infections and other inflammatory conditions. If PHELA or its components prove effective in modulating cytokine responses, they could have broader applications in diseases characterized by excessive immune activation. These findings provide valuable insights into understanding the immunological aspects of viruses and pave the way for further exploration of plant-based therapies for managing COVID-19 and related inflammatory responses.

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