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Original Article

# The *In-Vitro* Antiviral Activity of *Carissa Edulis, Tulbaghia Acutiloba*, and *Tetradenia Riparia* on Severe Acute Respiratory Syndrome Coronavirus-2

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**ABSTRACT**

The current treatment landscape for COVID-19 is largely supportive or experimental in nature, highlighting the need for alternative approaches. Ethanolic extracts of three South African medicinal plants (*Carissa edulis, Tulbaghia acutiloba*, and *Tetradenia riparia)* were tested for their antiviral activity against SARS-CoV-2. Viral loads were quantified using real-time PCR, and their anti-inflammatory effects were also assessed using ELISA. Both pre-treatment and post-treatment of H1299-hACE2-E3 cells with *C. edulis* extract significantly reduced viral loads by two- to four-fold decreases as compared to untreated controls, which suggests potential antiviral activity. *T. acutiloba* and *T. riparia* also exhibited moderate antiviral suppression, especially when the extracts were pre-treated prior to infection at early time points (24 hours). All three plants showed anti-inflammatory activity. The findings from this research demonstrate *C. edulis*' potent antiviral activity and positions it as a promising candidate for further research in antiviral therapy development.

**Keywords:** *Carissa edulis, Tulbaghia acutiloba, Tetradenia riparia,* Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), Antiviral activity.

## INTRODUCTION

SARS-CoV-2 continues to cause disease throughout the year and seasonal spikes have been identified in COVID-19 cases, this has been consistent with typical winter months where seasonal respiratory viruses usually increase in circulation.[30, 27, 28] Extensive research has been conducted on SARS-CoV-2, but there are still unknowns regarding the potential long-term health effects of COVID-19 infection and the genetic changes of the virus over time.[1, 2, 6, 19] Omicron and its descendent lineages are responsible for a large proportion of the currently circulating variant landscape, and it is believed that it would constitute the genetic background from which new SARS-CoV-2 variants will likely emerge in the future.[43] Continued viral evolution remains a concern and ongoing sequencing has provided monitoring of mutations and guidance of public health strategies.[29]

Vaccination remains the most effective protection against infection and severe disease.[43, 44] Regular booster vaccination is still a key part of the ongoing management of COVID-19, especially among older and more clinically vulnerable populations.[22] Treatment strategies and guidelines vary between different countries as numerous agents are tested and either removed from or added to protocols.[33, 13, 18, 37, 17, 14, 34, 38, 15, 45, 36, 16, 4, 26, 10, 12, 9, 39, 25, 7, 5, 20, 21]

With the onset of the COVID-19 pandemic, scientists looked to explore traditional plant remedies as potential sources of antiviral compounds.[23] Traditional herbal medicines represent a rich source of potential therapeutic compounds and chemical diversity in the search for new COVID-19 treatments. Plant-based therapies may have fewer adverse effects than synthetic drugs and could bolster natural immune function while also improving accessibility, especially in poorer countries.[48] *Tetradenia riparia, Tulbaghia acutiloba*, and *Carissa edulis* have been widely and traditionally used in folk medicine to treat respiratory ailments, as well as their reported pharmacological activities, including antiviral and anti-inflammatory properties, which make them potential candidates for investigating their antiviral activity against SARS-CoV-2.[3, 32, 24]

## MATERIALS AND METHODS

### Materials

#### *Cells*

The H1299-hACE2-E3 cells, Cercopithecus aethiops kidney epithelial cell line (Vero E6) overexpressing human ACE2 receptor were kindly donated by Prof. Alex Sigal, African Health Research Institute, University of KwaZulu-Natal, South Africa. In cellular cytotoxicity, infectivity, and anti-inflammatory experiments, the cell line was propagated in RPMI supplemented with 10% FBS, 1% NEAA, Na-pyruvate, HEPES, and 2% L-Glutamine, which were all acquired from Gibco, USA.

#### *SARS-CoV-2*

The South African SARS-CoV-2 Omicron BA 2. 12. 1 variant was originally isolated from a confirmed COVID-19 PCR-positive patient sample. The virus was grown and propagated in H1299-hACE2-E3 cells at the Pathogen Research Laboratory at the University of the Free State, South Africa. Briefly, samples were prepared by a 1:1 dilution with

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RPMI 1640 maintenance media (Gibco, USA), supplemented with 2% FBS, 2%L-glutamine (L-glut), 1% NEAA and 1% antibiotics, 1% C3H3NaO3, and 1% HEPES, and then filter-sterilised using a 0.2 µM nylon filter (GVS North America, USA). A 75 µl aliquot of the sample was inoculated on a confluent monolayer of cells in a T25 vented cell culture flask.

Cells were incubated at 37°C for 30 minutes to allow virus adsorption. Post-incubation, a 5 ml aliquot of maintenance media was added to the flask, and the cells were incubated at 37°C in a carbon dioxide ($CO_2$) incubator with 5% $CO_2$. Cells were monitored daily for cytopathic effects (CPEs). At the first sign of CPEs, an aliquot of cell culture media was tested using the rapid SARS-CoV-2 antigen test card (Boson Biotech, China). Positive cell cultures were incubated until 90% CPEs were observed, at which time the cells were freeze-thawed and clarified by centrifugation at 1 200 xg for five minutes. Confirmation of the isolates was performed using next-generation sequencing. The 50% tissue culture infectious dose (TCID50) was quantified in replicates of six by monitoring CPEs and the virus titer was measured using the Reed and Muench (1938) method expressed as infectious units per millilitre (IU/ml)[35]. For infectivity studies, the multiplicity of infection (MOI), referred to as the number of infectious viral particles used to infect a single cell, needed to be quantified in order to obtain the value, the TCID50 was converted to plaque-forming units (PFU) as described by Wulff, Tzatzaris and Young (2012), and the MOI was then calculated using the following formula:

$$\text{MOI} = \frac{PFU\ of\ SARS-CoV-2}{Number\ of\ cells\ seeded\ per\ well}$$

Formula 1: Multiplicity of infection (MOI) calculation

Prior to the infectivity experiments, a working virus suspension was prepared at a low MOI of 0.01. This suspension was prepared in a 5 ml falcon tube in RPMI media; specifically, 100 µl of stock SARS-CoV-2 was diluted with 5 ml media in a 1:50 ratio to produce a 0.01 MOI. The experiments directly involving infection with replication competent virus were performed at the Biosafety Level-3 facility at the Pathogen Research Laboratory, under the supervision of Prof. Felicity Burt with protocols approved by the Environmental and Biosafety Research Ethics Committee and the NdoH.

### *Preparation of plant extracts*

Mature whole plants of *C. edulis*, *T. acutiloba*, and *T. riparia* purchased from various plant nurseries in South Africa. Fresh *T. acutiloba* plants were obtained from the SA Bulb Company in August 2022 (Hartbeespoort, South Africa; 25°47'03.9"S 27°47'37.7"E). Whole *C. edulis* plants were sourced in September 2022 from the Free State National Botanical Garden (Bloemfontein, South Africa; 29°03'12.7"S 26°12'46.8"E). In the same month, *T. riparia* was collected from the Kruger National Park (Phalaborwa, South Africa; 23°59'17.9"S 31°33'17.1"E). Plant specimens were authenticated by each of the nurseries and all plant material was delivered in single batches of varying weights between August and September 2022. The plant material was inspected for any possible contamination and was washed to remove soil, and the leaves, stems, and roots were then separated and cut into smaller pieces. The plant parts were then oven-dried at 60 °C for five days and subsequently ground into a fine powder using a blender. The powdered samples were stored at 4 °C pending extraction.

Powdered plant samples were weighed out to 10 g and prepared for extraction. The samples were extracted by maceration separately using 70% $C_2H_6O$ as the solvent. In a 1 000 ml sterile conical flask, 10 g of powdered plant sample was dissolved in 400 ml of solvent and the mixture was placed on an orbital shaker to be agitated over 72 hours at 165 revolutions per minute. The solvent supernatant was then filtered through using Whatman No. 1 filter paper and then allowed to dry in a 40 °C incubator. The extract samples were reconstituted in 1.5 ml microcentrifuge tubes in the same 70% ethanol used in extraction and filtered using a 0.22 µm syringe filter. A stock concentration of 1 000 mg/ml was initially prepared and stored in a -20 °C freezer. For the infectivity, cytoxicity, and ELISA anti-inflammatory experiments, serial dilutions of stock solutions of extracts were prepared using culture medium initially at a broad full log-scale dilution series, with concentrations of 1 000 µg/ml, 100 µg/ml, and 10 µg/ml (1:10, 1:100, and 1:1000).

## *Cell viability and cytotoxicity activity on* H1299-hACE2-E3 *cells*

After passaging, 100 µl of the H1299-hACE2-E3 cells were seeded at a density of 2 x $10^5$ cells/ml in a 96-well plate (Thermofischer Scientific, USA) and incubated for 24 hours at 37 °C in a humidified 5% $CO_2$ incubator. After incubating for 24 hours, the supernatant was aspirated from the cell monolayer and discarded and 100 µl of plant extracts at varying concentrations (25 to 1 000 µg/ml) were added in triplicate. Seventy percent $C_2H_6O$ was used as a vehicle control and cells-only controls were also included in triplicate. After incubating the cell monolayers with extracts for 48 hours, the cytotoxicity activity was measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA) colorimetric method according to the manufacturer's instructions. For this assay, the culture supernatant was first carefully aspirated, and 20 µl of CellTiter 96® AQueous One Solution Reagent obtained from the kit was added into each well of the 96-well assay plate containing the samples and controls. The plate was then re-incubated for four hours, and absorbance was recorded at 490 nm using a multi-well spectrophotometer (Thermofischer Scientific, USA). Background absorbance was corrected by a triplicate set of control wells (without cells) containing the same volumes of culture medium and CellTiter 96® AQueous One Solution Reagent as in the experimental wells. To obtain the corrected absorbance, the average 490 nm absorbance from the no-cell control wells was subtracted from all other absorbance values. To obtain the percentage inhibition, the following calculation was used:

$$\%\ \text{Cell inhibition} = \frac{Mean\ abs\ of\ extract}{Mean\ abs\ untreated\ cells\ control}\ x\ 100$$

Formula 2: Percentage cellular inhibition calculation

Non-linear regression analysis was used to calculate IC50 values, indicating the concentration of the extracts that reduces the viability of the cells by 50% compared to untreated cells.

## Antiviral screening of infectious SARS-CoV-2 virus

The antiviral screening of infectious SARS-CoV-2 virus comprised time of extract-addition assay, viral RNA extraction, quantitative real-time PCR, and the generation of a viral loads standard curve:

### *Time of extract-addition assay*

The antiviral effects of the extracts were evaluated at different times of viral infection, as described by Chiang et al. (2002)[11]. Prior to the experiment, H1299-hACE2-E3 cells were seeded at 2 × $10^5$ cells per well in 96-well plates and incubated overnight at 37°C in a humidified $CO_2$ incubator. The following day, the seeded H1299-hACE2-E3 cells were used in two independent experiments. $IC_{50}$ concentrations of the three plant extracts were prepared in RPMI media prior to experimentation. An hour before the analysis, the virus stock was removed from the -80 °C freezer and thawed on ice. A working virus suspension was

prepared in a 5 ml falcon tube diluted in RPMI media by diluting 100 µl of stock SARS-CoV-2 in 5 ml media in a 1:50 ratio in order to produce a 0.01 MOI. $IC_{50}$ concentrations of the extracts were added before and after infection in two independent experiments.

In the first experiment, the H1299-hACE2-E3 cells were treated with extracts prior to virus infection to evaluate whether or not the extracts can block early viral attachment/entry steps before replication begins by interacting directly with viral particles or block cell receptors to inhibit binding/entry. This analysis sought to determine if the extracts have prophylactic effects. Briefly, H1299-hACE2-E3 cells cultured on 96-well plates were treated with 100 µl of extracts, and then incubated at 37°C for an hour. After incubation, the supernatant was removed and the cells were subsequently inoculated with 100 µl of SARS-CoV-2 MOI of 0.01 and re-incubated at 37 °C for 48 hours.

In the second experiment, the H1299-hACE2-E3 cells were first infected with 100 µl of the virus suspension at an MOI of 0.01 and incubated at 37 °C for an hour then treated with the plant extracts. This experiment was to evaluate the potential therapeutic effects of the extracts against SARS-CoV-2 after infection had been established, in order to determine whether the extracts could interfere with the virus's replication cycle or mitigate its effects within already-infected cells.

### *Viral ribonucleid acid (RNA) extraction*

Viral RNA was extracted from the culture supernatants of the samples collected directly from the 96-well plates of the antiviral assay at 24-, 48-, and 72-hours post-infection, according to the manufacturer's instructions using the Quick-RNA™ Viral Kit (Zymo Research, USA). Briefly, 200 µl of SARS-CoV-2 supernatant culture was added to 200 µl of DNA/RNA Shield™ Buffer (2X concentrate) in a 1:1 ratio. Viral RNA buffer was then prepared according to the kit's protocol by adding 250 µl of beta-mercaptoethanol ($C_2H_6OS$) to 50 ml of viral RNA buffer. Viral wash buffer was prepared by adding 24 ml of 100% ethanol to the 6 ml viral wash buffer concentrate.

To purify the RNA samples, 800 µl of viral RNA buffer was added to each 400 µl sample in (2:1) ratio and mixed well. The mixture was transferred into a Zymo-Spin™ IC Column in a collection tube and centrifuged for two minutes at 16 000 g. Thereafter, the column was then transferred into a new 1.5 ml microcentrifuge collection tube. A volume of 500 µl of viral wash buffer was added to the column, centrifuged for 30 seconds, and the flow-through discarded. This was repeated twice as per the protocol. To the column, 500 µl of $C_2H_6O$ 100% was added to the sample and centrifuged for one minute to ensure the complete removal of the wash buffer. The column was then carefully transferred into a nuclease-free tube. To elute RNA, 15 µl of DNase/RNase-free water (Thermofischer Scientific, USA) was added directly to the column matrix and centrifuged for 30 seconds. The 15 µl eluted sample was collected in a 1.5 ml nuclease-free tube and RNA was stored at -80 °C until used.

### *Quantitative real-time polymerase chain reaction (PCR)*

Undiluted RNA samples were analyzed using the CFX96 Real-Time Thermal Cycler (Bio-Rad, USA) with the Allplex™ 2019-nCoV assay (Seegene, USA). RT-PCR reactions were prepared as shown in Table 1, and PCR products were detected in real time using a fluorescent dye-based method. Briefly, the reagent master mix was prepared prior to use in a labelled, sterile 1.5 ml microcentrifuge tube. In a 96-well plate, 18 µl of master mix was aliquoted into each well and 8 µl of RNA sample was added thereafter. In parallel, positive and negative controls were included: 8 µl of a known internal positive control and 8 µl of DNase/RNase-free water as a negative control were added to the reagent master mix in separate wells. The total volume after the addition of samples and controls was 26 µl. The plate was covered with a permanent clear heat seal, and spun down at 2 500 revolutions per minute for five seconds and liquid containing all PCR components was verified to be at the bottom of each PCR tube. The plate was then analysed on a CFX96 Touch Real-Time PCR machine according to the PCR-run conditions shown in Table 2. Fluorescent probes targeting specific SARS-CoV-2 genes, particularly the N gene for this study, were used to quantify viral loads based on relative fluorescence and Ct values. Samples were confirmed positive if one or more gene targets (N, RdRp, E) were detected within 45 cycles. Ct values were plotted against standard RNA concentrations to generate a standard curve, allowing for viral load quantification.

**Table 1: Reagent mixtures for the amplification of cDNA in SARS-CoV-2 culture supernatant using the Allplex™ 2019-nCoV assay (Seegene, USA).**

| Reagents | Volume |
|---|---|
| 2019-nCoV MuDT* Oligo Mix | 5 µl |
| DNase/RNase-free water | 5 µl |
| 5X Real-time one-step buffer | 5 µl |
| Real-time one-step enzyme | 2 µl |
| Internal control | 1 µl |
| **Total reaction** | **18 µl** |

**Table 2: RT-PCR cycling conditions for SARS-CoV-2 reverse transcription and cDNA amplification.**

| Programmes | | | | |
|---|---|---|---|---|
| **Programme name** | **Cycles** | | **Analysis mode** | |
| Initial denaturation | 1 | | None | |
| Amplification | 45 | | Quantification | |
| Annealing | 1 | | None | |
| **Temperature targets** | | | | |
| Step | PCR cycle | Target [°C] | Hold [hh:min:ss] | Average ramp rate [°C/sec.] |
| Step 1 | Reverse transcription | 50 | 00:20:00 | 3.3 |
| Step 2 | Denaturation | 95 | 00:15:00 | 3.3 |
| Step 3 | Amplification | 94 | 00:00:15 | 3.3 |
| Step 4 | Annealing | 58 | 00:00:30 | 3.3 |
| Step 5 | Repeat amplification and annealing steps 44 more times. | | | |

### *Anti-inflammatory activity of expressed cytokines*

In addition to direct antiviral effects, the anti-inflammatory activity of the extracts was evaluated by testing their ability to inhibit SARS-CoV-2-induced inflammatory responses in infected H1299-hACE2-E3 cells.

Excessive production of pro-inflammatory cytokines like IL-6, TNF-α, and IL-8 mediated by viral infection contributes significantly to COVID-19 pathogenesis. Extracts that can modulate this hyperinflammatory state without compromising antiviral efficacy can provide additional therapeutic benefits.

Supernatant was collected at 24-, 48-, and 72-hours post-infection from infected cells and centrifuged at 1 000 g to remove particulates and used immediately. The cytokine levels of IL-6, TNF-α, and IL-8 were measured and quantified using commercially available ELISA kits supplied by ABclonal (USA) according to the manufacturer's instructions. Briefly, prior to the experiment, the reagents were brought to room temperature before use and prepared.

Wash buffer was prepared by diluting it 1:20 with double distilled or deionised water before use, where 20 ml of wash buffer concentrate was added to 380 ml of deionised or distilled water to prepare 400 ml of wash buffer. The standard was serially diluted with standard diluent to produce seven different concentrations in order to prepare the standard curve.

ELISA wells were first washed with 350 µl per well of wash buffer, each well was aspirated after holding for 40 seconds, and the process was

repeated twice for a total of three washes. Thereafter, 100 µl of sample diluent was added in a blank well and 100 µl of different concentrations of standard and sample in the other wells in duplicate. The plate was covered with the adhesive strip provided and incubated for two hours at 37 °C in a humidified 5% $CO_2$ incubator.

Working Biotin Conjugate Antibody solution was prepared 15 minutes before use by diluting 1:100 of Concentrated Biotin Conjugate Antibody (100x) with Biotin Conjugate Antibody Diluent before use, 20 µl of Concentrated Biotin Conjugate Antibody (100x) was added to 1 980 µl of Biotin Conjugate Antibody Diluent to prepare a 2 000 µl working solution of Biotin Conjugate Antibody Buffer. After incubation, the plates were aspirated and washed three times. To each well was then added 100 µl of Working Biotin Conjugate Antibody, and then covered once more with new adhesive sealer and incubated for an hour.

The H1299-hACE2-E3 cells were infected with SARS-CoV-2 as described earlier, either pre-treated or treated after infecting with non-cytotoxic concentrations of the extracts.

Streptavidin-HRP Concentrated (100x) Working Solution was prepared 15 minutes before use. The solution was prepared by diluting 1:100 of concentrated Streptavidin-HRP (100x) with Streptavidin-HRP Diluent and 20 µl of Concentrated Streptavidin-HRP (100x) to 1 980 µl Streptavidin-HRP Diluent to prepare 2 000 µl working solution of Streptavidin-HRP Buffer. After incubation, the plates were aspirated and washed once more, and 100 µl of working Streptavidin-HRP was added to each well. The plate was covered with new adhesive sealer and re-incubated for 50 minutes at 37 °C. During the incubation, the microplate reader was warmed up for 30 minutes before measuring. After incubation, the aspiration and wash steps were repeated once more and 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was then added to the wells and incubated for 20 minutes at 37 °C and protected from light.

Stop solution of 50 µl was added to each well and the optical density of each well was determined within five minutes, using a compact microplate reader (Byonoy, Germany) set to 450 nm. Wavelength correction was made by reading the plate at 620 nm, whereby the reading at 620 nm was subtracted from the reading at 450 nm.

A standard curve was generated to quantify the unknown cytokines by employing already pre-quantified standards with known concentrations as reference. The concentrations (x-axis) were plotted against the optical density absorbance values (y-axis) and a linear correlation was determined. The expressed cytokine levels of the samples and controls were analysed at different time points. The cytokine standards were serially diluted in seven 10-fold increments in duplicate.

The absorbance values obtained from the serial dilutions of the cytokine standards were plotted against the corresponding concentrations (pg/ml) to generate the standard curves. The equation generated from the linear regression model was then used to determine the unknown cytokine concentrations corresponding to the absorbance values of the unknown samples.

# RESULTS AND DISCUSSIONS

## Evaluation of cytotoxicity

The cytotoxicity of *T. riparia*, *T. acutiloba*, and *C. edulis* extracts on H1299-hACE2-E3 cells was evaluated using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA).

Dose-response curves were generated by plotting the percentage of cell death against the extract concentration (µg/ml) in a semi-logarithmic scale for each extract (see Figure 1). Non-linear regression analysis was performed to fit the curves and to determine the $IC_{50}$ values, which represent the concentration required to inhibit cell viability by 50%.

Lower IC50 values indicate higher cytotoxicity, which means that the extract requires a lower concentration to inhibit cell viability by 50%. Based on the obtained IC50 values, *C. edulis* exhibited the lowest cytotoxicity on H1299-hACE2-E3 cells, with a concentration of 419.76 µg/ml, followed by *T. acutiloba* at 364.75 µg/ml, and *T. riparia* at 244.03 µg/ml, which showed the highest cytotoxicity.

## Evaluation of antiviral activity

The antiviral effects of *T. riparia*, *T. acutiloba*, and *C. edulis* extracts were evaluated against SARS-CoV-2 using an *in vitro* assay. Two independent experiments were conducted to assess the impact of the extracts on different stages of viral infection.

In the first experiment, the H1299-hACE2-E3 cells were treated with $IC_{50}$ concentrations of the extracts an hour prior to virus infection to evaluate their potential prophylactic effects.

In the second experiment, the H1299-hACE2-E3 cells were first infected with SARS-CoV-2 and then treated with the extracts post-infection to be able to evaluate if the extracts can help reduce or hinder the progression of the infection after it is already established. The results were represented as nucleocapsid gene estimated viral load copies/ml, as shown in Tables 3 and 4.

In the pre-treatment experiment, all samples, including controls, showed increases in viral loads over time, consistent with active viral replication. At 24 hours, *C. edulis* pre-treatment displayed a marked 81.23% reduction in viral load relative to the virus-infected control, although this reduction did not achieve statistical significance ($p > 0.05$). This trend suggests a potential inhibitory effect early on in viral replication post-infection, but further investigation with additional replicates would be necessary to confirm significance. By 48 and 72 hours, *C. edulis* maintained a lower viral load compared to controls, achieving a statistically significant reduction at 48 hours with a p-value < 0.05 (49.83% reduction) and a lesser reduction of 11.16% by 72 hours. *T. acutiloba* and *T. riparia* also showed some reduction in viral load during pre-treatment, with *T. acutiloba* achieving a 45.16% reduction and *T. riparia* a 43.33% reduction at 24 hours. However, these reductions were not statistically significant ($p > 0.05$). By 72

**Table 3: SARS-CoV-2 viral loads after pre-treatment with extracts.**

| Plant extract | Time post-infection | N gene $C_t$ value Mean ± SD | Mean N gene +/- estimated viral load ± SD ($Log_{10}$ copies/mL) | % Reduction vs control virus-infected cells only |
|---|---|---|---|---|
| *C. edulis* | 24 hours | 38.06 ± 3.02 | <1 ± 0.60 | 81.23% |
| | 48 hours | 28.48 ± 2.43 | 3.01 ± 0.35 | 49.83% |
| | 72 hours | 18.60 ± 4.10 | 5.33 ± 1.20 | 11.16% |
| *T. acutiloba* | 24 hours | 30.09 ± 3.45 | 2.62 ± 0.52 | 45.16% |
| | 48 hours | 21.14 ± 3.15 | 4.73 ± 0.83 | 21.16% |
| | 72 hours | 10.34 ± 4.20 | >6 ± 1.80 | - |
| *T. riparia* | 24 hours | 29.63 ± 3.20 | 2.73 ± 0.55 | 43.33% |
| | 48 hours | 24.76 ± 2.95 | 3.88 ± 0.91 | 35.33% |
| | 72 hours | 18.52 ± 3.85 | 5.35 ± 1.32 | 10.83% |
| Vehicle control | 24 hours | 29.96 ± 3.75 | 2.65 ± 0.64 | 55.83% |
| | 48 hours | 24.58 ± 3.10 | 3.92 ± 0.87 | 34.66% |
| | 72 hours | 13.09 ± 4.40 | >6 ± 1.76 | - |
| Cells only | 24 hours | | | |
| | 48 hours | | | |
| | 72 hours | | | |
| Virus-infected cells only | 24 hours | 18.60 ± 4.15 | 5.33 ± 1.18 | |
| | 48 hours | 9.79 ± 3.85 | >6 ± 1.62 | |
| | 72 hours | 8.81 ± 4.30 | >6 ± 1.90 | |

**Table 4: SARS-CoV-2 viral loads after post-treatment with plant extracts.**

| Plant extract | Time post-infection | Mean N gene $C_t$ values ± SD | Mean N gene +/- estimated viral load ± SD ($Log_{10}$ copies/mL) | % Reduction vs control virus-infected cells only |
|---|---|---|---|---|
| *C. edulis* | 24 hours | 29.58 ± 3.31 | 2.74 ± 0.85 | 44.75% |
| | 48 hours | 11.33 ± 2.62 | >6 ± 0.56 | - |
| | 72 hours | 10.33 ± 2.54 | >6 ± 0.75 | - |
| *T. acutiloba* | 24 hours | 24.78 ± 1.76 | 3.87 ± 1.10 | 21.97% |
| | 48 hours | 10.33 ± 2.6 | >6 ± 0.95 | - |
| | 72 hours | 9.55 ± 2.49 | >6 ± 1.13 | - |
| *T. riparia* | 24 hours | 19.71 ± 1.48 | 5.07 ± 1.31 | - |
| | 48 hours | 11.78 ± 2.13 | >6 ± 0.42 | - |
| | 72 hours | 10.21 ± 2.26 | >6 ± 0.35 | - |
| Vehicle control | 24 hours | 20.17 ± 3.51 | 4.96 ± 0.78 | - |
| | 48 hours | 10.45 ± 4.92 | >6 ± 0.16 | - |
| | 72 hours | 9.97 ± 1.87 | >6 ± 0.52 | - |
| Cells only | 24 hours | | | |
| | 48 hours | | | |
| | 72 hours | | | |
| Virus-infected cells only | 24 hours | 17.85 ± 2.97 | 4.96 ± 1.41 | |
| | 48 hours | 13.11 ± 1.62 | >6 ± 0.25 | |
| | 72 hours | 8.03 ± 1.13 | >6 ± 0.38 | |

**Table 5: Cytokine concentration in cell culture supernatant after pre-treatment with plant extracts.**

| Plant extract | Time post-infection | Mean cytokine concentrations (pg/ml) ± SD | | |
|---|---|---|---|---|
| | | IL-6 | IL-8 | TNF- α |
| *C. edulis* | 24 hours | 88.71 ± 1.26 | 200.28 ± 0.69 | 550.46 ± 1.22 |
| | 48 hours | 85.48 ± 1.13 | 119.90 ± 0.25 | 381.20 ± 1.71 |
| | 72 hours | 70.36 ± 0.74 | 121.44 ± 1.41 | 87.34 ± 1.58 |
| *T. acutiloba* | 24 hours | 72.52 ± 1.35 | 25.64 ± 2.12 | 716.59 ± 1.62 |
| | 48 hours | 43.78 ± 2.56 | <1.56 ± 1.65 | 514.41 ± 3.12 |
| | 72 hours | 53.36 ± 2.31 | <1.56 ± 3.91 | 220.55 ± 4.09 |
| *T. riparia* | 24 hours | 72.39 ± 1.74 | 266.35 ± 2.14 | 784.76 ± 1.42 |
| | 48 hours | 56.33 ± 3.32 | 185.97 ± 0.83 | 566.92 ± 2.50 |
| | 72 hours | 123.39 ± 1.61 | 103.38 ± 1.43 | 50.51 ± 3.17 |
| Vehicle control | 24 hours | 37.17 ± 3.41 | 250.94 ± 3.74 | 897.61 ± 1.64 |
| | 48 hours | 48.77 ± 2.10 | 172.76 ± 0.16 | 983.02 ± 0.81 |
| | 72 hours | 61.86 ± 1.52 | 90.17 ± 1.48 | 876.45 ± 0.47 |
| Untreated cells | 24 hours | 69.01 ± 0.66 | 264.15 ± 1.32 | 997.91 ± 0.85 |
| | 48 hours | 161.58 ± 2.14 | 185.97 ± 1.25 | 961.08 ± 3.06 |
| | 72 hours | 165.89 ± 1.93 | 103.38 ± 1.41 | 910.14 ± 1.31 |
| No cells | 24 hours | | | |
| | 48 hours | | | |
| | 72 hours | | | |
| Prednisolone | 24 hours | 11.94 ± 3.15 | <1.56 ± 2.56 | <1.56 ± 1.34 |
| | 48 hours | 13.02 ± 2.23 | <1.56 ± 0.82 | <1.56 ± 1.25 |
| | 72 hours | 26.24 ± 2.18 | <1.56 ± 1.37 | <1.56 ± 1.73 |

hours, both extracts' effects diminished substantially, with viral loads approximating control levels, underscoring the limited prophylactic efficacy relative to *C. edulis*.

In the post-treatment experiment, *C. edulis* again showed the most pronounced reduction in viral load, with statistically significant decreases observed at both 48 hours ($p < 0.05$) and 72 hours ($p < 0.01$). At 24 hours, *C. edulis* exhibited a 44.75% reduction in viral load, suggesting an early antiviral response, although this reduction was not statistically significant. In contrast, *T. acutiloba* and *T. riparia* exhibited lower reductions of 21.97% and 17.45%, respectively, at 24 hours, without statistical significance at any time point ($p > 0.05$). These observations suggest that *C. edulis* exerts a stronger antiviral effect when used post-infection, possibly due to its ability to interfere with viral replication processes rather than viral entry.

The percent reduction in viral loads varied across the extracts and was most notable for *C. edulis*, with reductions of 81.23% and 49.83% at 24 and 48 hours in pre-treatment and 44.75% post-treatment at 24 hours. This indicates a robust antiviral effect that supports further exploration of its active compounds.

Fold Change (FC) values were used as a standardized metric for comparing viral loads between treated and untreated cells, with an FC threshold of 2 established to signify antiviral activity beyond random fluctuations[49]. *C. edulis* consistently met this threshold across pre- and post-treatment conditions, indicating consistent and possibly active antiviral properties. Both *T. acutiloba* and *T. riparia* displayed antiviral effects, though these were less consistent and pronounced than those observed with *C. edulis* (Figure 3).

Across all conditions, higher viral suppression was observed in pre-treatment scenarios, warranting further investigation into the extracts' potential as prophylactic agents. Vehicle and untreated controls exhibited increasing viral loads over time, reflecting natural SARS-CoV-2 replication, which reinforces the observed viral reductions in treated groups as effects of the extracts. Overall, these findings highlight the potential antiviral activity of *C. edulis*, with additional, though less pronounced, effects seen with *T. acutiloba* and *T. riparia*. Further studies are recommended to elucidate mechanisms and active compounds responsible for these antiviral effects.

## Evaluation of anti-inflammatory activity

To assess the anti-inflammatory potential of *C. edulis*, *T. acutiloba*, and *T. riparia*, cytokine concentrations for IL-6, IL-8, and TNF-α were measured in cell culture supernatants following both pre- and post-treatment protocols (see Tables 6 and 7). The study included vehicle

**Table 6: Cytokine concentration in cell culture supernatant after post-treatment with plant extracts.**

| Plant extract | Time post-infection | Mean cytokine concentrations (pg/ml) ± SD | | |
|---|---|---|---|---|
| | | IL-6 | IL-8 | TNF- α |
| *C. edulis* | 24 hours | 64.70 ± 3.14 | 134.22 ± 2.54 | 501.09 ± 1.40 |
| | 48 hours | 69.69 ± 1.78 | 84.66 ± 4.52 | 395.30 ± 1.34 |
| | 72 hours | 53.63 ± 2.52 | 2.08 ± 1.23 | 258.17 ± 0.27 |
| *T. acutiloba* | 24 hours | 167.38 ± 2.30 | 180.46± 0.63 | 807.49 ± 3.88 |
| | 48 hours | 132.97 ± 2.51 | 124.30 ± 0.31 | 287.16 ± 1.43 |
| | 72 hours | 81.02 ± 1.42 | 39.52 ± 0.47 | 228.39 ± 0.23 |
| *T. riparia* | 24 hours | 179.52 ± 4.67 | 200.28 ± 3.34 | 894.47 ± 2.71 |
| | 48 hours | 142.42 ± 1.35 | 139.72 ± 1.57 | 742.45 ± 0.50 |
| | 72 hours | 91.82 ± 0.41 | 57.14 ± 1.64 | 338.10 ± 0.45 |
| Vehicle control | 24 hours | 20.71 ± 0.59 | 248.29 ± 1.51 | 784.76 ± 0.23 |
| | 48 hours | 40.81 ± 1.61 | 174.52 ± 1.23 | 846.67 ± 1.67 |
| | 72 hours | 79.67 ± 4.48 | 91.93 ± 1.55 | 715.02 ± 4.84 |
| Untreated cells | 24 hours | 88.85 ± 2.53 | 261.51 ± 2.81 | 392.95 ± 3.21 |
| | 48 hours | 137.96 ± 3.07 | 187.73 ± 0.76 | 801.22 ± 2.44 |
| | 72 hours | 184.78 ± 1.54 | 105.14 ± 1.91 | 693.86 ± 3.60 |
| No cells | 24 hours | | | |
| | 48 hours | | | |
| | 72 hours | | | |
| Prednisolone | 24 hours | 13.96 ± 0.83 | <1.56 ± 0.75 | <1.56 ± 0.33 |
| | 48 hours | 2.09 ± 1.66 | <1.56 ± 0.42 | <1.56 ± 0.81 |
| | 72 hours | 10.86 ± 0.51 | <1.56 ± 0.44 | <1.56 ± 0.27 |

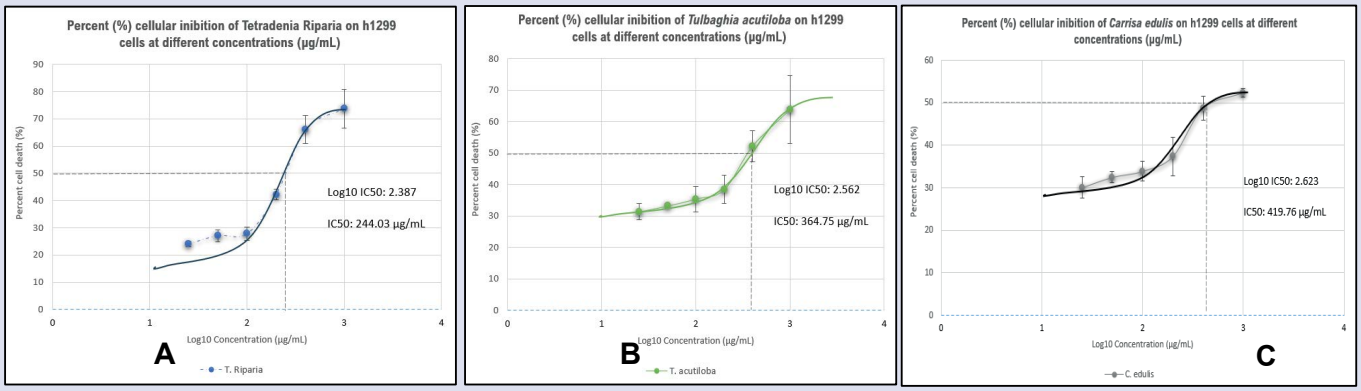



**Figure 1: Dose-response curves of *T. riparia*, *T. acutiloba*, and *C. edulis* extracts on H1299-hACE2-E3 cells.** Data are presented as the mean of the triplicate values and the ± standard deviation is represented by the error bars.

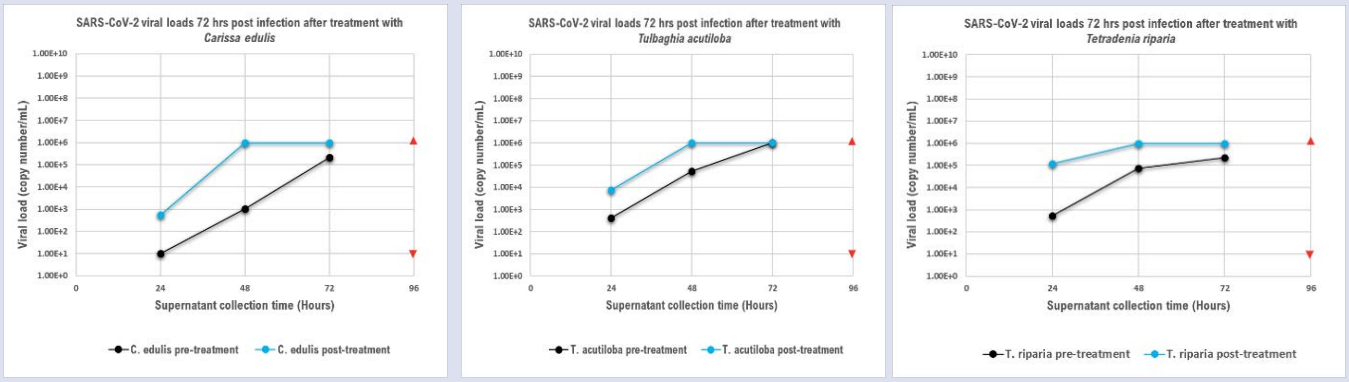



**Figure 2:** A-C: SARS-CoV-2 viral loads 72 hours post-infection after treatment with $IC_{50}$ concentrations of plant extracts.

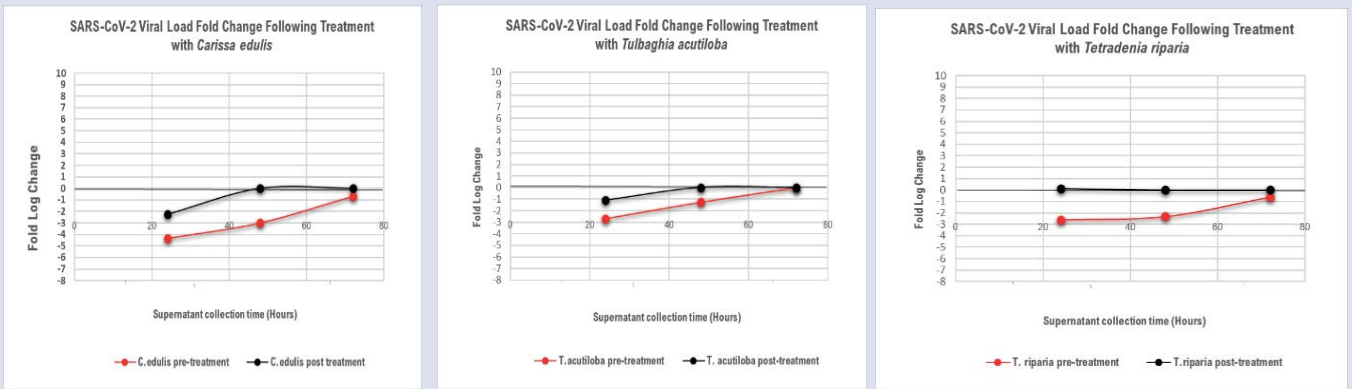
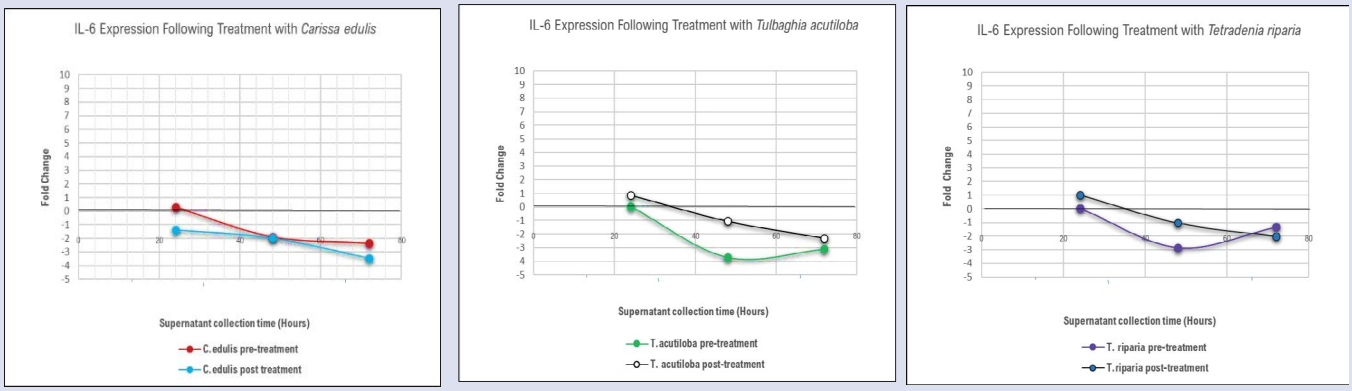
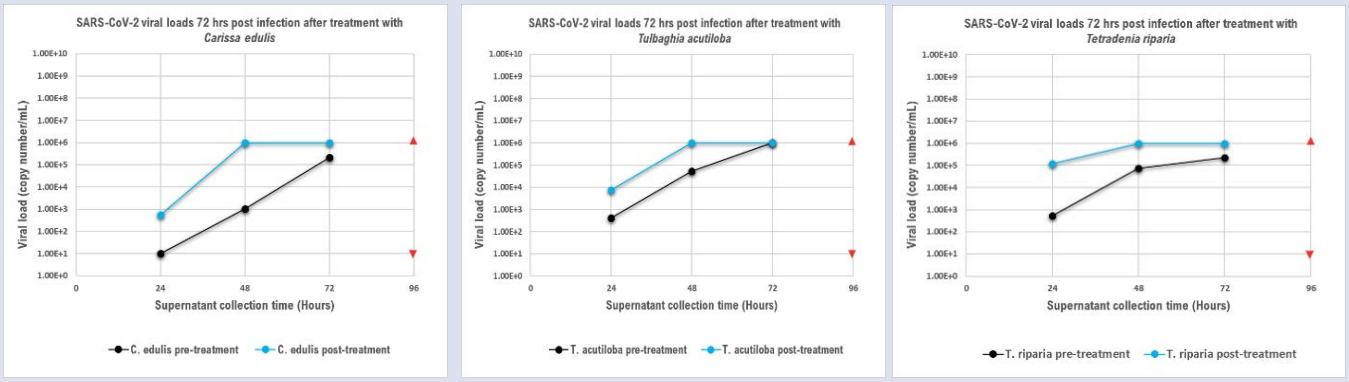



**Figure 3:** A-C: SARS-CoV-2 viral load fold change (FC) following treatment with $IC_{50}$ concentrations of plant extracts relative to the untreated control.



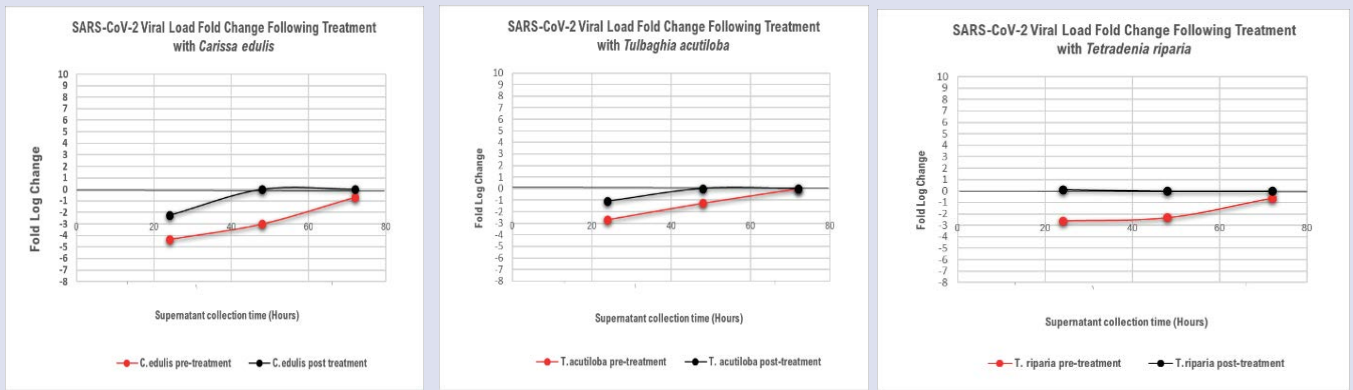

A B C

**Figure 4:** FC in concentration levels of IL-6 following treatment with the extracts.




A B C

**Figure 5:** FC in concentration levels of IL-8 following treatment with the extracts.




A B C

**Figure 6:** FC in concentration levels of TNF-α following treatment with the extracts.

and untreated controls, a no-cell control, and prednisolone as a positive control. Prednisolone significantly reduced cytokine levels, affirming its known anti-inflammatory effects and establishing a benchmark.

Across the three extracts, pre-treatment with *C. edulis* yielded notable reductions in cytokine levels, particularly at 48 hours, where IL-6 and TNF-α decreased significantly compared to the untreated control ($p < 0.05$). At 72 hours, both IL-6 and TNF-α levels showed a reduction >2-fold relative to the untreated control, indicating potential anti-inflammatory activity. In contrast, pre-treatment with *T. acutiloba* and *T. riparia* showed varying efficacy, with cytokine reductions that were generally non-significant ($p > 0.05$) except in isolated instances. For example, *T. riparia* reduced IL-6 levels by >50% at 24 hours but did not maintain significance over time.

In the post-treatment setup, *C. edulis* consistently demonstrated the strongest effects, achieving significant TNF-α reductions at 72 hours ($p < 0.01$), which may reflect an active anti-inflammatory response in reducing late-stage cytokine production. *T. acutiloba* and *T. riparia* showed some degree of TNF-α reduction, though less pronounced than *C. edulis*, aligning with findings from other cytokines. These results suggest that *C. edulis* may interfere more effectively with cytokine-mediated inflammatory pathways after infection onset, whereas the other extracts may have limited or delayed efficacy.

Overall, FC analysis further supported these findings (see Figures 4 to 6), with >2-fold reductions noted primarily in *C. edulis* post-treatment groups for both IL-6 and TNF-α[8, 31]. This aligns with the interpretation that *C. edulis* possesses measurable anti-inflammatory properties, while *T. acutiloba* and *T. riparia* display mild to moderate effects that warrant further investigation.

In conclusion, the study suggests that *C. edulis*, particularly in a post-infection scenario, may exert the most substantial anti-inflammatory effects on SARS-CoV-2 infected cells. This effect may contribute to mitigating cytokine-mediated inflammation in viral infections, although further studies are needed to confirm these findings and determine the active components responsible.

## CONCLUSIONS

his study evaluated the antiviral and anti-inflammatory properties of *T. riparia*, *T. acutiloba*, and *C. edulis* extracts against SARS-CoV-2 in vitro, with particular focus on their potential as both prophylactic and post-infection treatments.

In terms of cytotoxicity, all three extracts displayed tolerable effects on H1299-hACE2-E3 cells, with *C. edulis* showing the lowest cytotoxicity (IC50 = 419.76 µg/ml). This made *C. edulis* a particularly promising candidate for further testing in infection scenarios where high concentrations are needed to achieve therapeutic efficacy.

The antiviral activity assessments revealed that *C. edulis* consistently exhibited the most substantial antiviral effects, particularly in post-infection settings where it significantly reduced viral loads at 48 hours ($p < 0.05$) and 72 hours ($p < 0.01$). This suggests that *C. edulis* may be more effective in targeting viral replication rather than viral entry, indicating a mechanism that likely interferes with intracellular viral processes. In contrast, *T. acutiloba* and *T. riparia* displayed limited antiviral effects with reductions that were not statistically significant in most cases, although both extracts showed some initial efficacy in pre-treatment scenarios.

Anti-inflammatory effects were evaluated by measuring reductions in IL-6, IL-8, and TNF-α cytokine levels. *C. edulis* once again demonstrated the strongest activity, achieving >2-fold reductions in IL-6 and TNF-α levels during post-treatment. These findings were statistically significant, particularly for TNF-α at 72 hours ($p < 0.01$), highlighting *C. edulis*'s potential in attenuating cytokine-mediated inflammation. *T. acutiloba* and *T. riparia* also displayed mild to moderate reductions in cytokine levels, although the effects were less consistent and did not achieve the same level of significance as *C. edulis*.

In conclusion, *C. edulis* emerged as the most promising extract, showing both antiviral and anti-inflammatory properties that could support its further exploration as a treatment option for SARS-CoV-2 infections. Future studies should aim to isolate the active compounds within *C. edulis* to better understand its mechanisms of action and to evaluate its potential in in vivo models. While *T. acutiloba* and *T. riparia* demonstrated mild effects, additional investigation may clarify their roles and optimize their use as adjunctive therapies.

## STATISTICAL ANALYSIS

Data are presented as means ± standard deviation (SD) from three biological replicates. Unpaired t-tests were used for pairwise comparisons of viral loads and cytokine concentrations between treated and untreated groups at each time point. Statistical significance was set at $p < 0.05$, with specific p-values reported where applicable. The IC50 values were determined using GraphPad Prism software version 9.0, based on dose-response curves, to identify the extract concentrations necessary for 50% inhibition of cell viability.

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## ETHICS

The study was approved by the University of the Free State Environment and Biosafety Ethics Committee (UFS-ESD2022/0177/22).

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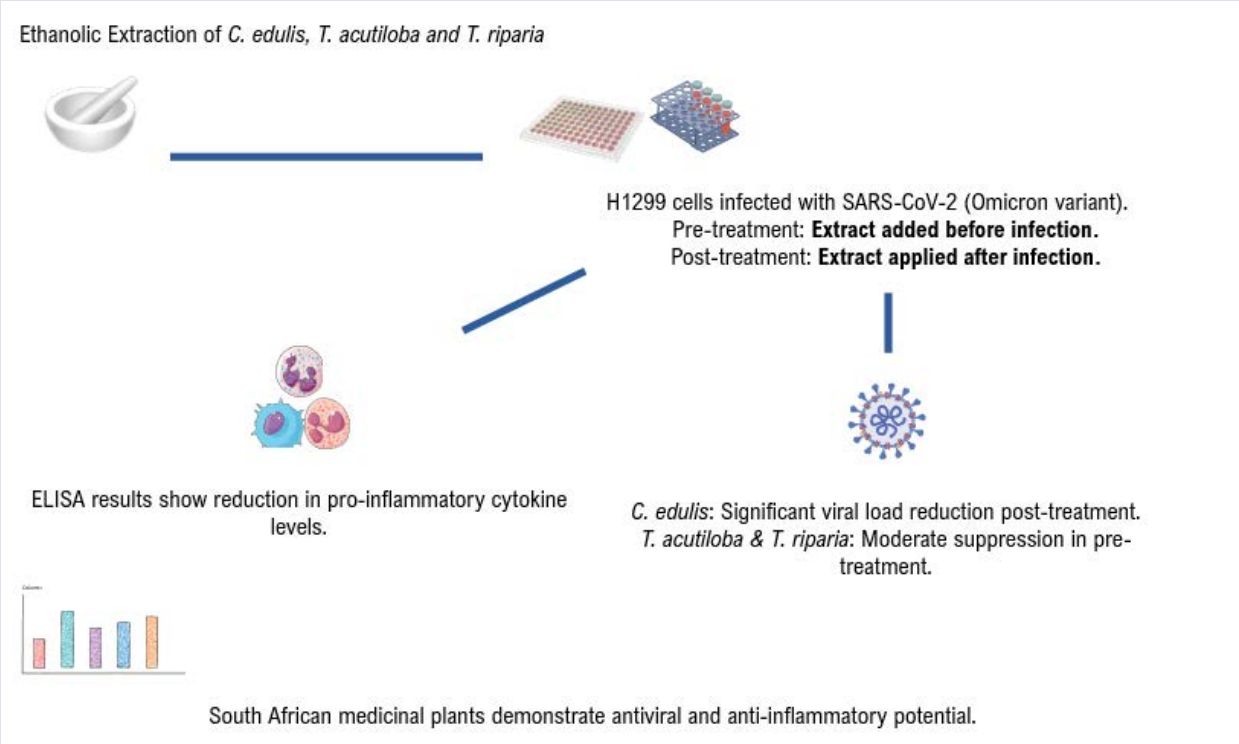
## GRAPHICAL ABSTRACT

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