

**The *in-vitro* antiviral activity of *Carissa edulis*, *Tulbaghia acutiloba*,
and *Tetradenia riparia* on severe acute respiratory syndrome
coronavirus-2**

By

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DECLARATION OF INDEPENDENT WORK

I, **Carlos da Silva**, identity number ##### and student number #####, hereby declare that this research project submitted to the Central University of Technology, Free State, for the Master of Health Sciences in Biomedical Technology degree, is my own independent work; that it complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules, and regulations of the Central University of Technology, Free State; and that it has not been submitted before to any institution by me or any other person in fulfilment or partial fulfilment of the requirements for the attainment of any qualification.

Signature

Date

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LIST OF ABBREVIATIONS

+ssRNA	Single-stranded RNA
2019-nCoV	Novel coronavirus 2019
ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme 2
Ang	Angiotensin
Ang II	Angiotensin II
ATP	Adenosine triphosphate
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA

c-FLIP	IL-1-converting enzyme-inhibitory protein
COVID-19	Coronavirus disease of 2019
CPE	Cytopathic effects
Ct	Cycle threshold
dec-RVKR-cmk	Decanoyl-RVKR-chloromethylketone
DMSO	Dimethyl sulfoxide
DMV	Double membrane vesicle
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
dsRNA	Double-stranded RNA
ELISA	Enzyme-linked immunosorbent assay
ERGIC	Endoplasmic-reticulum–Golgi intermediate compartment
FBS	Foetal bovine serum
FC	Fold change
FDA	Food and Drug Administration
gRNA	Genomic RNA
hACE2	Human angiotensin-converting enzyme 2
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
IBV	Infectious bronchitis virus
IC ₅₀	Half-maximal inhibitory concentration
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon
IFN- γ	Interferon-gamma
IL	Interleukin
IRF3	Interferon regulatory factor 3
JAK	Janus kinase
Keap1-Nrf2	Kelch-like epichlorohydrin-associated protein 1- nuclear factor E2-related factor 2
MCP-1	Monocyte chemoattractant protein-1
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
MHV	Mouse hepatitis virus
MOI	Multiplicity of infection
Mpro	Main protease
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAAT	Nucleic acid amplification test
NADPH	Nicotinamide adenine dinucleotide phosphate
NDoH	National Department of Health
NEAA	Non-essential amino acids
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor-κB
NICD	National Institute for Communicable Diseases
NSP	Non-structural protein
NTD	N-terminal domain
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque-forming units
pH	Potential of hydrogen
PLpro	Papain-like protease
qPCR	Quantitative PCR
RBD	Receptor-binding domain
RdRp	RNA-dependent RNA polymerase
REACT	Rapid Evidence Appraisal for COVID-19 Therapies
REMAP-CAP	Randomized Embedded Multifactorial Adaptive Platform for Community-Acquired Pneumonia
RNA	Ribonucleid acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RTC	Replication–transcription complex
RT-PCR	Reverse transcription quantitative PCR
SAHPRA	South African Health Products Regulatory Authority
SARS	Severe acute respiratory syndrome
SARS-CoV	Severe acute respiratory syndrome coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SARSr-CoV	Severe acute respiratory syndrome-related coronavirus
SC ₅₀	Radical scavenging ability required to scavenge 50% of free radicals
sg-mRNA	Subgenomic mRNA
sgRNA	Subgenomic RNA
STAT	Signal transducer and activator of transcription

TCID ₅₀	50% tissue culture infectious dose
TMB	3,3',5,5'-Tetramethylbenzidine
TMPRSS2	Transmembrane protease serine 2
TNF- α	Tumour necrosis factor-alpha
TP	Triphosphate
USA	United States of America
WHO	World Health Organization

LIST OF UNITS

°C	Degree(s) Celsius
μ l	Microlitre(s)
μ M	Micrometre(s)
Cm	Centimetre(s)
cm ²	Square centimetre(s)
G	Gravity
G	Gram(s)
g BE/g	Berberine equivalents per gram
g BE/mg	Berberine equivalents per milligram
g GAE/g	Gallic acid equivalents per gram
IU/ml	Infectious units per millilitre
Kb	Kilobase
M	Metre(s)
Mg	Milligram(s)
mg/ml	Milligram(s) per millilitre
ml	Millilitre(s)
Mm	Millimetre(s)
mM	Millimolar(s)
Nm	Nanometre(s)
pg/ml	Picogram(s) per millilitre
xg	Relative centrifugal force / times gravity
μ g/ml	Microgram(s) per millilitre

LIST OF CHEMICAL SYMBOLS

$C_{20}H_{18}ClNO_4$	Berberine hydrochloride
$C_2H_3NaO_2$	Sodium acetate
$C_2H_3O^{2-}$	Acetate
C_2H_6O	Ethanol
C_2H_6OS	Beta-mercaptoethanol
$C_3H_3NaO_3$	Sodium pyruvate
C_6H_{14}	Hexane
$C_6H_3N_3O_7$	Picric acid
$C_6H_8O_6$	L-Ascorbic acid
$C_7H_6O_5$	Gallic acid
Ca	Calcium
CH_3OH	Methanol
$CHCl_3$	Chloroform
$CHNaO_3$	Sodium bicarbonate
CO_2	Carbon dioxide
Fe	Iron
Fe^{3+}	Iron ion
$FeCl_3$	Ferric chloride
H_2O_4S	Sulphuric acid
HCl	Hydrochloric acid
HNaO	Sodium hydroxide
N_2	Nitrogen
NH_3	Ammonia
O_2	Oxygen
OH	Hydroxyl group
$OH^{\cdot}$	Hydroxyl radical

ABSTRACT

Introduction: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative virus of the coronavirus disease of 2019 (COVID-19). Despite the availability of some antiviral drugs, the treatment options for COVID-19 remain limited, which highlights the need for alternative approaches. Traditional medicines represent an underexplored source of potential antiviral compounds. This study assessed the antiviral activity of three South African medicinal plants that are traditionally used to treat lower respiratory tract infections such as SARS-CoV-2.

Methods: Different concentrations of ethanolic extracts of *Carissa edulis*, *Tulbaghia acutiloba*, and *Tetradenia riparia* were prepared and screened for their cytotoxicity on human non-small cell lung carcinoma cell line (H1299 cells) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The extracts with non-cytotoxic effects were further investigated for their antiviral activity against SARS-CoV-2-infected H1299 cells using a time-of-addition assay. Real-time polymerase chain reaction quantified viral loads post-treatment and the anti-inflammatory activity was determined by measuring inflammatory cytokine levels via enzyme-linked immunosorbent assay (ELISA). The anti-oxidant capacity of the extracts was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Phytochemical screening of the plant extracts was performed using the coulometric assay.

Results: Both pre-treatment and post-treatment of H1299 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). The anti-inflammatory effects were assessed by measuring pro-inflammatory cytokine (interleukin [IL]-6, IL-8, and tumour necrosis factor-alpha [TNF- α]) levels. All three plants extracts demonstrated greater than two-fold decreases in IL-6 and TNF- α production over time compared to controls, which is indicative of potential anti-inflammatory activity. The anti-oxidant capacity was determined using the DPPH radical scavenging assay, with *T. riparia* exhibiting the

strongest activity with an inhibitory concentration of 50% (IC₅₀) of 144 µg/ml, followed by *T. acutiloba* (IC₅₀ 158 µg/ml) and *C. edulis* (IC₅₀ 234 µg/ml). Phytochemical analysis revealed the presence of flavonoids, saponins, tannins, and alkaloids, with *T. riparia* containing the highest phenolic content (12.57 g GAE/g) and alkaloid levels (7.86 mg BE/g).

Discussion: The study investigated the antiviral and anti-inflammatory potential of three South African medicinal plants and revealed significant insight into traditional medicine's role in contemporary therapeutic approaches. 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. Similarly, *T. acutiloba* and *T. riparia* demonstrate moderate antiviral suppression, which is particularly notable when the extracts were applied as a pre-treatment before infection. The effectiveness observed at early time points (24 hours) suggests that the timing of treatment application plays a crucial role in maximising antiviral effects, which highlights the potential for these plants to contribute to early intervention strategies.

The study also delved into the anti-inflammatory properties of the plant extracts by measuring the levels of pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α. The results showed greater than two-fold decreases in IL-6 and TNF-α production over time in comparison to controls, which indicates significant anti-inflammatory activity. This outcome is particularly relevant in the context of SARS-CoV-2 infections, where the cytokine storm is a critical factor in disease severity, which suggests that these plant extracts could offer dual benefits in both antiviral and anti-inflammatory capacities. *T. riparia* displayed the highest anti-oxidant capacity, followed by *T. acutiloba* and *C. edulis*. This anti-oxidant activity is crucial as oxidative stress plays a significant role in the pathogenesis of viral infections, including those caused by SARS-CoV-2. The phytochemical analysis conducted as part of the study revealed the presence of flavonoids, saponins, tannins, and alkaloids, with *T. riparia* exhibiting the highest levels of phenolic content and alkaloid levels. These compounds are known for their therapeutic effects, including antiviral, anti-inflammatory, and anti-oxidant properties, which could explain the observed biological activities of the extracts.

Conclusion: This study not only reaffirms the traditional use of *C. edulis*, *T. acutiloba*, and *T. riparia* in treating respiratory infections but also highlights their potential as sources of bioactive compounds for the development of novel antiviral and anti-inflammatory therapies. The significant reductions in viral load, coupled with anti-inflammatory and anti-oxidant effects, highlights the need for further investigation into these plants' mechanisms of action and their potential integration into treatment protocols for viral infections, including COVID-19. Future research should aim to isolate and characterise the specific active compounds that are responsible for these effects, thereby paving the way for the development of new pharmacological agents derived from traditional medicinal plants.

CHAPTER 1:

INTRODUCTION

1.1 BACKGROUND

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a coronavirus strain that was first discovered in Wuhan, China, in 2019 (Gorbalenya et al., 2020; Wang, Horby, et al., 2020; World Health Organization [WHO], 2019, 2020a, 2020b, 2023b;). It is the causative virus of the respiratory illness responsible for the coronavirus disease of 2019 (COVID-19) and is a member of the species *Betacoronavirus pandemicum* of the genus *Betacoronavirus*, and genetically related to the virus responsible for the 2002 SARS outbreak (Chen, 2020). Genomic analysis reveals close similarities to bat coronaviruses, which suggests a natural spillover event likely in 2019 (Andersen et al., 2020; MacLean et al., 2021; Wu, Zhao, Yu, et al., 2020).

CoVs are a large family of enveloped, positive-sense ribonucleic acid (RNA) viruses that belong to the family *Coronaviridae* that infects a diverse range of hosts (International Committee on Taxonomy of Viruses [ICTV], 2022; Woo et al., 2023). The family is divided into four main genera, with bats considered natural reservoirs for alpha- and betacoronaviruses, and avian species for delta- and gammacoronaviruses (Banerjee et al., 2019; Lin et al., 2017; Woo, Lau, Li, et al., 2006; Tang et al., 2006; Woo et al., 2012). Among the seven CoVs known to infect humans, four cause mild respiratory infections associated with common cold-like symptoms and circulate seasonally (Cui et al., 2019; Gaunt et al., 2010; Van der Hoek et al., 2004; Vlasova et al., 2022; Woo, Lau, Yip, et al., 2006). The remaining three, including SARS-CoV-2, illustrate the pathogenic and zoonotic potential of coronaviruses and the risks of their emergence (Peiris, Yuen, et al., 2003; Wu, Zhao, Yu, et al., 2020; Zaki et al., 2012).

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 (Agrawal et al., 2022; Annweiler et al., 2020; Bobrovitz et

al., 2023; Harris, 2023;). Current drug treatments are primarily supportive and to manage associated complications from severe disease. Key approved drug treatments for hospitalised cases include the immune modulators dexamethasone and baricitinib (National Department of Health [NDoH], 2021). While there are some approved antiviral treatments for COVID-19, regulatory authorisation has varied between countries. Challenges of cost, unclear long-term benefits, drug interactions, and limited availability have affected their widespread adoption (Reubenson et al., 2023; South African Health Products Regulatory Authority [SAHPRA], 2023).

Traditional herbal medicines represent a rich source of potential therapeutic compounds and chemical diversity in the search for new COVID-19 treatments. Initial studies on the antiviral properties of medicinal plants against SARS-CoV-2 may reveal promising candidates worthy of further investigation (Chikowe et al., 2021; Runfeng et al., 2020; Ryu et al., 2010). Some current commercially available respiratory drugs have historical origins from plant sources (Albrecht, Dicipinigaitis & Guenin, 2017; Patra et al., 2020). Further research on the antiviral and anti-inflammatory properties of additional herbal medicines is warranted, as fully utilising the pharmacological potential of plants could yield novel natural product-derived COVID-19 treatments. *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 (Aremu & Van Staden, 2013; Panda et al., 2022; Kaunda & Zhang, 2017).

To date, several medicinal plants have been tested for their antiviral and anti-inflammatory activity against SARS-CoV-2, with some showing promising results. For instance, plants like *Allium sativum* (Keyaerts et al., 2007) and *Glycyrrhiza glabra* (Nourazarian et al., 2016) are known to target the CoV replication. Another plant, *Clerodendrum inerme*, deactivates viral ribosomes. This can be used as a drug that targets the translation of the CoV-2 protein (Olivieri et al., 2021). Similarly, *Cusia Strobilanthes* blocked the synthesis of viral RNA genomes and induced PLpro activity that targets CoVs (Tsai & Wilson, 2020).

Most importantly, various medicinal plants exhibit inhibitory effects against ACE-2, including *Boerhaavia diffusa*, *Coriandrum sativum*, *Coscinium fenestratum*, *Cynara scolymus*, *Punica granatum*, and *Cassia occidentalis*. Among them, *Punica granatum* shows a competitive mode of action, while others are non-specific inhibitors (Khan & Kumar, 2019; Hasan et al., 2020; Hussain et al., 2020). *Andrographis paniculata* was found to inhibit the increase in NOD-like receptor protein 3, interleukin-1 and β caspase-1, which are heavily involved in the pathogenesis of SARS-CoV and possibly CoV-2 (Liu, Chen, Lii et al., 2020; Liu, Zhou, Li, Garner et al., 2020; Liu & Wang, 2020; Liu, Gayle, Wilder-Smith et al., 2020;).

However, the knowledge base on the antiviral and anti-inflammatory properties of medicinal plants specific to SARS-CoV-2 remains limited. The selected plants for this study, *Tetradenia riparia*, *Tulbaghia acutiloba*, and *Carissa edulis*, have been traditionally used in folk medicine for treating respiratory ailments and possess reported antiviral and anti-inflammatory activities. By investigating these plants, this study aims to contribute new information to the existing knowledge base, potentially identifying novel natural product-derived treatments for COVID-19. Specifically, research on these plants could reveal unique mechanisms of action, such as targeting different stages of the viral life cycle or modulating the host immune response. The findings could also expand the repertoire of medicinal plants with demonstrated activity against SARS-CoV-2, offering additional options for the development of accessible and affordable treatments.

1.2 RESEARCH AIM AND OBJECTIVES

The main objective of this study was to explore the *in vitro* antiviral potential of three plant extracts, namely *T. riparia*, *T. acutiloba*, and *C. edulis*, against SARS-CoV-2.

The secondary objectives of the study were:

- To perform preliminary phytochemical screening of the extracts to identify major chemical constituents.

- To determine the cytotoxicity profiles of the plant extracts using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on H1299 cells.
- To assess the antiviral activity of non-toxic extract concentrations against SARS-CoV-2-infected H1299 cells using the time-of-addition assay and quantifying viral replication in the presence of plant extracts using real-time polymerase chain reaction (PCR).
- To assess the inhibitory effects of extracts on pro-inflammatory cytokines (tumour necrosis factor-alpha [TNF- α], interleukin [IL]-8, and IL-6) secretion in H1299 cells infected with SARS-CoV-2 using enzyme-linked immunosorbent assay (ELISA).
- To determine the anti-oxidant capacity of the plant extracts using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.

1.3 DISSERTATION STRUCTURE

The first chapter contains the introduction to the study, as well as its aim, motivation, and research objectives. Chapter 2 presents the literature review, with the materials and methods presented in Chapter 3, as well as the link between the different methodologies examined in this study and the information needed to understand and interpret the results. Chapter 4 presents the findings, while Chapter 5 discusses the results, limitations, and conclusions of the study and provides recommendations and suggestions for further research.

CHAPTER 2:

LITERATURE REVIEW

2.1 DISCOVERY AND EARLY RESEARCH

Coronaviruses were first isolated in 1931 as the causative agent of infectious respiratory disorders in poultry. This first coronavirus was termed infectious bronchitis virus (IBV) due to the disease pathology it induced (Beaudette & Hudson, 1937; Schalk & Hawn, 1931). Between the 1940s and 1950s, coronaviruses were also discovered to infect several other animal species and leading to conditions such as a contagious gastroenteritis in young pigs and severe hepatitis and neurologic diseases in mice (Doyle & Hutchings, 1946; Gledhill & Andrewes, 1951). In humans, the first human coronavirus, designated B814, was isolated in 1961 by the then British Common Cold Research Unit led by virologist David Tyrell (Kendall, Bynoe & Tyrrell, 1962). The second strain, 229E, was isolated in 1962 by infectious disease scientists Dorothy Hamre and John J. Procknow at the University of Chicago, while the third distinct and structurally identical human coronavirus strain, OC43, was identified in 1967 (Hamre & Procknow, 1966; McIntosh, Becker & Chanock, 1967).

Through epidemiological associations with the winter months, these human viruses are also likely responsible for a fraction of common colds, particularly those that circulate during the cold seasons. They are associated with upper respiratory infections with occasional cases of pneumonia and asthma exacerbations in infants and young adults, as well as chronic bronchitis in older adults and the elderly (McIntosh et al., 1970). The identification of a third distinct but structurally identical human virus suggests that these viruses were an under-recognised cause of common colds and lower respiratory illness at the time.

By mid-1968, it was recognised that IBV, B814, 229E, OC43, and the mouse hepatitis virus (MHV) were sufficiently structurally and biologically similar to consider them a distinct group with shared properties (Almeida & Tyrell, 1967; Berry et al., 1964; Ferreira

& Manaker, 1965; McIntosh, Dees, et al., 1967; McIntosh, Becker & Chanock, 1967). The most notable common feature was a cluster of widely spaced, crown-like spikes that projected from the virion surface, which gave the viral particle the appearance of the solar corona (Berry et al., 1964). This prompted the name that was formally adopted thereafter: *Coronavirus* (Estola, 1970).

2.2 CLASSIFICATION

First established in 1966 as the International Committee on Nomenclature of Viruses (Fenner, 1971), but later renamed to the International Committee on Taxonomy of Viruses (Fenner, 1974), *Coronavirus* was accepted as a genus name in the committee's first report in 1971 (ICTV, 1971). The genus included the renamed avian IBV (formerly IBV), as well as human respiratory virus, MHV, and transmissible gastroenteritis virus of swine. IBV was recognised as representative of the coronavirus genus and established as the type species in this earliest classification. Coronaviruses identified during this period were separated and classified based on their host species, which included the human coronaviruses 229E and OC43, which were collectively grouped as human respiratory virus. The first discovered human coronavirus strain, B814, was excluded from initial classification taxonomy and taxonomy reports thereafter as the virus could not be propagated in culture and all available virus was used up during experiments in 1968 (Myint, 1994).

The emergence and classification of coronaviruses have been a dynamic process since then with the establishment of the family *Coronaviridae* in 1975 and the subsequent discovery of more coronaviruses that cause a variety of diseases from respiratory, gastrointestinal, neurological, to immune-mediated ailments in both humans and animals (Fenner, 1976; Spaan, Cavanagh & Horzinek, 1988; Cavanagh et al., 1990). With improvements in techniques of virus detection and viral metagenomics, the sequencing of the avian IBV genome in 1987 was a significant milestone, as it allowed for a better understanding of coronavirus genetics and paved the way for the discovery of additional RNA viruses with similar genomic features (Bournsnel et al., 1987).

The order Nidovirales was first proposed in 1996 to reclassify recently discovered genetically related viruses and the order was initially split into two viral families, *Coronaviridae* and *Arteriviridae*, which contained newly discovered equine, murine, simian, and swine coronaviruses (Pringle, 1996). A third viral family was proposed in 2002, *Roniviridae*, after the discovery of the presence of coronaviruses in shellfish (Mayo, 2002). The emergence of three highly pathogenic human coronaviruses between the 2000s and 2010s demonstrated the zoonotic potential of coronaviruses to spill over from animals and cause worldwide epidemics in humans. In 2002, a novel coronavirus was identified as the cause of an outbreak of severe acute respiratory syndrome (SARS) viruses in Southern China (Peiris, Lai, et al., 2003; Marra et al., 2003; Drosten et al., 2003; Rota et al., 2003; Centers for Disease Control and Prevention [CDC], 2003). The virus name “severe acute respiratory syndrome coronavirus (SARS-CoV)” was introduced and approved by the ICTV, and the species was classified under the genus *Coronavirus* (ICTV, 2004). With the influx of research efforts after SARS, by 2008, two additional human coronaviruses were discovered, HCoV-NL63 (Van der Hoek et al., 2004; Fouchier et al., 2004) and HCoV-HKU1 (Woo et al., 2005), with the addition of avian coronaviruses (Cavanagh et al., 2002; Jonassen et al., 2005).

The ICTV proposed a major reclassification in 2009 owing to the increasing number and diversity of new coronaviruses being discovered (ICTV, 2009). Sequencing showed members of the genus to consistently form three distinct clusters/groups in phylogenetic trees (see Figure 2.1). These three clusters were unofficially referred to as phylogroups 1, 2, and 3 in publications and literature and were organised into the genera *Alphacoronavirus*, *Betacoronavirus*, and *Gammacoronavirus*, with *Deltacoronavirus* included in 2011 to represent a fourth cluster of recently discovered virus species (Cavanagh & Britton, 2008; Woo, Lau, Lam, et al., 2009; De Groot et al., 2011).

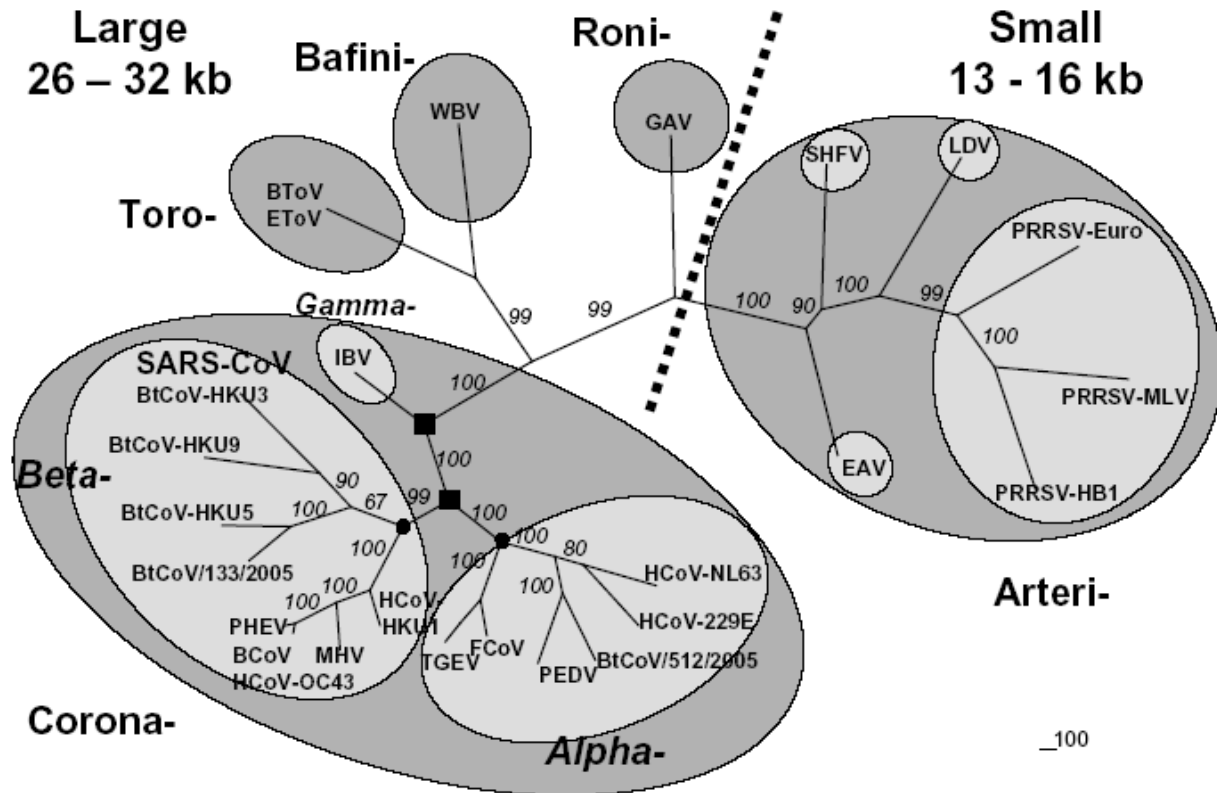


Figure 2.1: Nidovirales phylogeny as of 2009

Source: Adapted from Gorbalenya (2008)¹

Figure 2.1 illustrates the unrooted maximum parsimonious tree representing the evolutionary relationships between the different virus groups. This unrooted tree was based on comparing nucleotide sequences of the RNA-dependent RNA polymerase (RdRp)-helicase region from the species of the five groups of the nidovirus order in 2009, which included the *Coronavirinae*, *Torovirus* spp., *Roniviridae*, *Bafinivirus* spp., and *Arteriviridae* and the newly formed genera of *Alpha-*, *Beta-*, and *Gammacoronavirus*.

The classification was based on genetic relatedness, and new species were created by a merger within the genus when existing species shared high sequence identity (Gorbalenya, 2008; ICTV, 2009). This reclassification also led to the creation of new virus species, such as SARSr-CoV, which replaced and extended the existing *Betacoronavirus* species SARS-CoV by including the virus directly linked to the 2003 outbreak, but also

¹ Image reproduced from the ICTV's 9th report in 2009; see Appendix C for permission to use this material.

more distantly related viruses, such as SARS-Rh-BatCoV HKU3, SARSr-Rh-BatCoV, and SARSr-Rh-BatCoV 273 that were isolated from bats (Guan et al., 2003; Li, Shi, et al., 2005; Lau et al., 2005; Tang et al., 2006).

The first coronavirus, IBV, was united with existing *Coronavirus* species of birds (turkey coronavirus, duck coronavirus, goose coronavirus, pheasant coronavirus, and pigeon coronavirus) to form the new *Avian coronavirus* species in the genus *Gammacoronavirus*. Human coronavirus OC43 was united with previously established *Betacoronavirus* species of humans, cattle, swine, and horses (human enteric coronavirus, bovine coronavirus, porcine hemagglutinating encephalomyelitis virus, and equine coronavirus) to form a new species *Betacoronavirus 1* in the genus *Betacoronavirus*. Since its discovery, MHV was key in model studies to evaluate coronavirus replication and pathogenesis. It was renamed murine hepatitis virus in 1976, and united with puffinosis coronavirus and rat coronavirus to form the new species *Murine coronavirus* in the genus *Betacoronavirus* (Fenner, 1976; Lai et al., 1982; Frana et al., 1985; Williams et al., 1991; Bender et al., 2010).

In 2012, 10 years after the 2002 SARS outbreak, the Ministry of Health in Jordan reported a similar outbreak of respiratory illness in Zarqa that was also caused by a novel coronavirus strain (Zaki et al., 2012). The novel strain was named Middle East Respiratory Syndrome Coronavirus (MERS-CoV), on the basis that initial investigations linked viral cases between countries in the Middle East (De Groot et al., 2013). The ICTV named the species Middle East respiratory syndrome coronavirus (MERS-CoV) and in 2015 modified the name to incorporate “-related”, following the example that was set for SARS-CoV and related viruses in 2009. The virus was classified as *Betacoronavirus cameli* (ICTV, 2015).

At this stage, coronaviruses were well known by the scientific community. In the years following the 2012 MERSr-CoV outbreak, ongoing surveillance of animal reservoirs such as bats and camels was aimed at identifying new coronaviruses crossing over to humans (Woo, Lau, Wernery, et al., 2014; Woo, Lau, Teng, Tsang, Joseph, Wong, Tang, Sivakumar, Xie, et al., 2014; Woo, Lau, Teng, Tsang, Joseph, Wong, Tang, Sivakumar, Bai, et al., 2014). It was generally thought that SARSr-CoV, which is mainly found in bats,

could cause a future disease outbreak (Hu et al., 2017; Fan et al., 2019). In 2018, the ICTV proposed to expand the virus classification system from five ranks (species, genus, subfamily, family, and order) to a new system with 15 ranks to better accommodate all levels of virus diversity (species, genus, family, order, class, phylum, and kingdom) (Gorbalenya, 2018). As part of this change, a new high-level realm rank was created to unite all RNA viruses that use the same enzyme (RdRp) to copy their genetic material (Gorbalenya, 2018). This would include many genetically diverse RNA viruses that infect different hosts, which also included the Nidovirales order. Evidence suggested that these RNA viruses evolved from a common ancestor; they were thus grouped under the proposed Riboviria realm rank (Gorbalenya, 2018). Creating this realm was the first step to use the new expanded rank system and to establish a link between the most distantly related viruses. The goal was to classify viruses at every level of diversity, from narrow species up to the very broad, using a consistent ranking system.

In late 2019, another outbreak of unidentified pneumonia cases in Wuhan, Hubei province, central China, emerged (Wuhan Municipal Health Commission, 2020). A novel coronavirus was detected and was temporarily named the novel coronavirus 2019 (2019-nCoV), the seventh known human coronavirus to infect humans (see Figure 2.2) (WHO, 2019; Zhou et al., 2020). Based on recommendations by the Coronavirus Study Group of the ICTV, based on sequencing identity, phylogeny, taxonomy, and established practice, the group formally recognised the virus as a sister to the 2002-2004 SARS-CoV of the species SARSr-CoV and was therefore designated as SARS-CoV-2 (Gorbalenya et al., 2020).

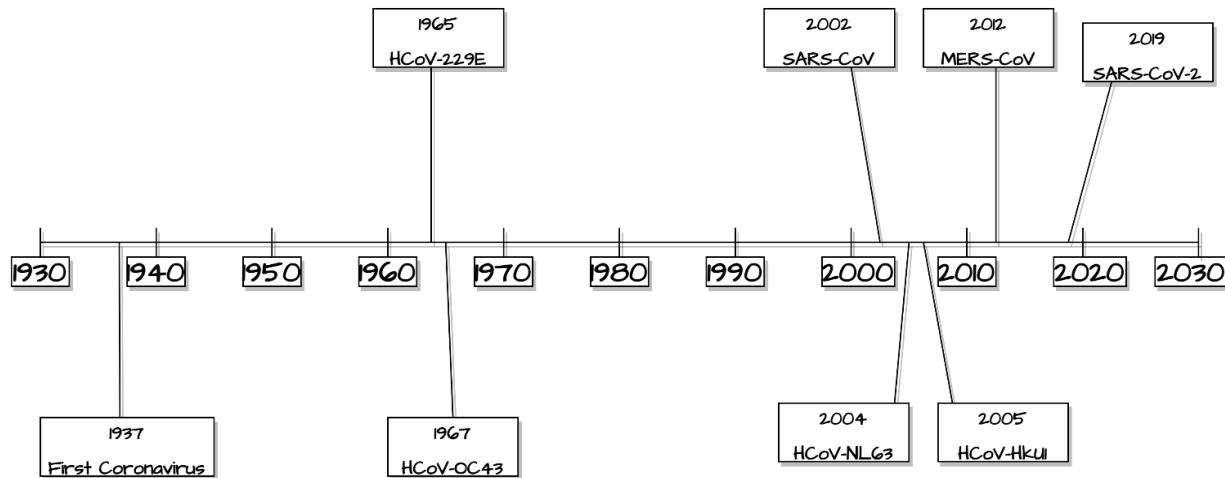


Figure 2.2: Timeline of the emergence of human coronaviruses

Source: Researcher (2023)

As of 2023, there are eight recognised viral suborders belonging to the Nidovirales order: Abnidoirineae, Arnidoirineae, Cornidoirineae, Mesnidoirineae, Monidoirineae, Nanidoirineae, Ronidoirineae, and Tornidoirineae, and they encompass 14 viral families, 25 subfamilies, 39 genera, 65 subgenera, and a total of 109 viral species (ICTV et al., 2022). Within Nidovirales, the *Coronaviridae* family is the largest and most diverse, comprising coronaviruses that infect many animal species, including important human respiratory pathogens (see Figure 2.3). It is further subdivided into three subfamilies, *Letovirinae*, *Pitovirinae*, and *Orthocoronavirinae*, with the orthocoronaviruses being the most genetically varied and encompassing key human strains. *Orthocoronavirinae* further divides into four genera that infect different host ranges. *Orthocoronavirinae* consists of four genera, namely *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*, that infect different host ranges (Li, 2016).

Figure 2.3 shows the latest classification of the *Coronaviridae* family determined by the ICTV (2022).

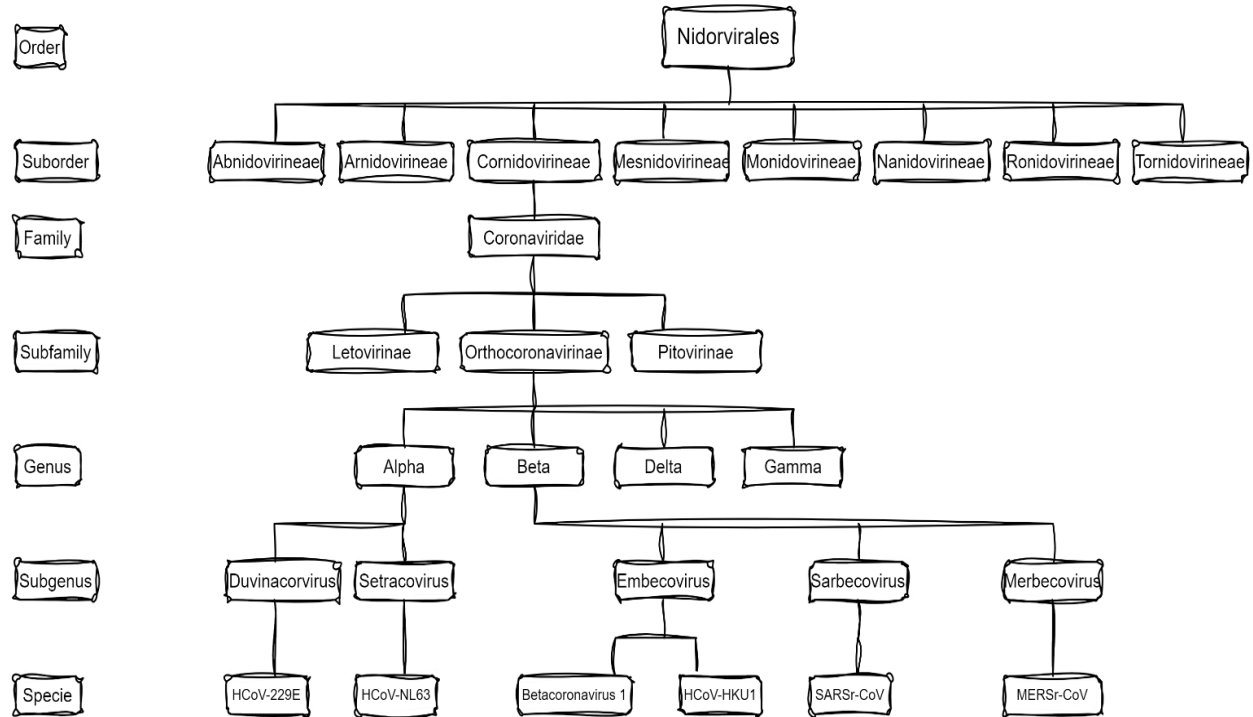


Figure 2.3: Summary of the latest classification of members of the family *Coronaviridae*

Source: Researcher (2023)²

2.3 SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 (SARS-COV-2) BIOLOGY

2.3.1 Virus structure

Coronavirus virions appear pleiomorphic and spherically shaped under electron microscopy (Barcena et al., 2009). *Orthocoronavirinae* are between 80 and 160 nm in diameter, with their most distinctive feature being large spike-like surface projections that are approximately 20 nm long in electron micrographs. These projections are made up of spike proteins that form groups of three. Some betacoronaviruses also display smaller projections, specifically those that belong to the subgenus *Embecovirus*, which are around 5 to 7 nm long and made of homodimeric hemagglutinin-esterase glycoprotein (Hurdiss et al., 2020). When viewed with cryo-electron tomography, coronavirions appear uniformly spherical and around 85 nm in diameter. Their outer envelope is unusually thick

² The figure was created with app.diagrams.net. A more detailed list of members of the Nidovirales order can be obtained from the ICTV's official website (<https://talk.ictvonline.org/taxonomy/>).

at around 7.8 nm compared to the typical biological membranes of cells and other viruses, which have an average thickness of 4 nm. The nucleocapsid consists of RNA and protein contents in a coiled-up spiral shape that appears tightly folded and separated from the envelope by a gap of approximately 4 nm, as shown in Figure 2.4.

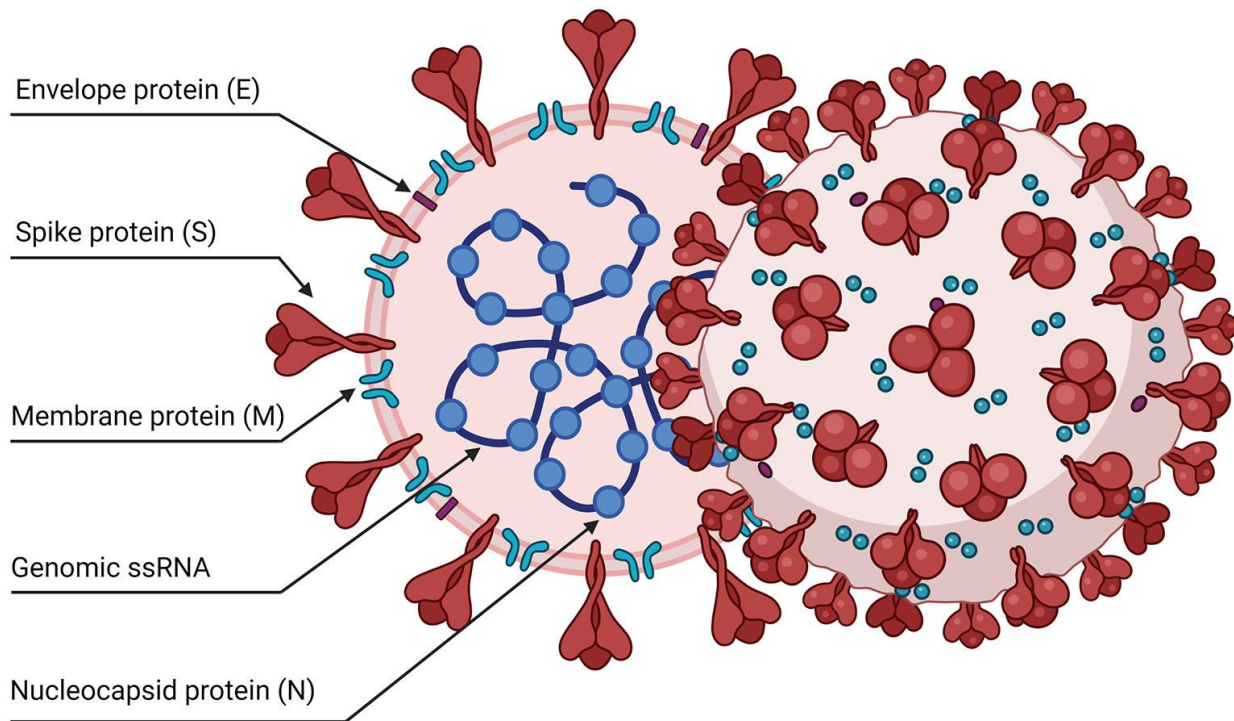


Figure 2.4: Coronavirus virion structure

Source: Adapted from Pizzato et al. (2022)³

The virions are embedded with spike, envelope, and membrane surface proteins. The virion contains a positive-sense, single-stranded RNA genome (+ssRNA) enclosed by a lipid envelope nucleocapsid protein.

2.3.2 Viral genome

The SARS-CoV-2 genome is made up of single-stranded positive-sense RNA that is approximately 30 000 bases in length (30 kb). The genes are arranged in a set order along the genome from 5' to 3' (Naqvi et al., 2020). The genome encodes multiple open reading frames (ORFs). The 5' end contains an untranslated leader sequence that is key

³ See Appendix C for permission to use this material.

in replication. These frameshifted polyproteins (ORF1a and ORF1b) occupy two-thirds of the genome, encoding a total of 16 non-structural proteins (NSPs) (Bredenbeek et al., 1990). The 3' end contains genes which encode the main structural proteins spike, envelope, membrane, and nucleocapsid. The 3' end also contains an untranslated region and a polyadenine (poly A) tail of 27 bases that stabilise the viral genome, as shown in Figure 2.5.

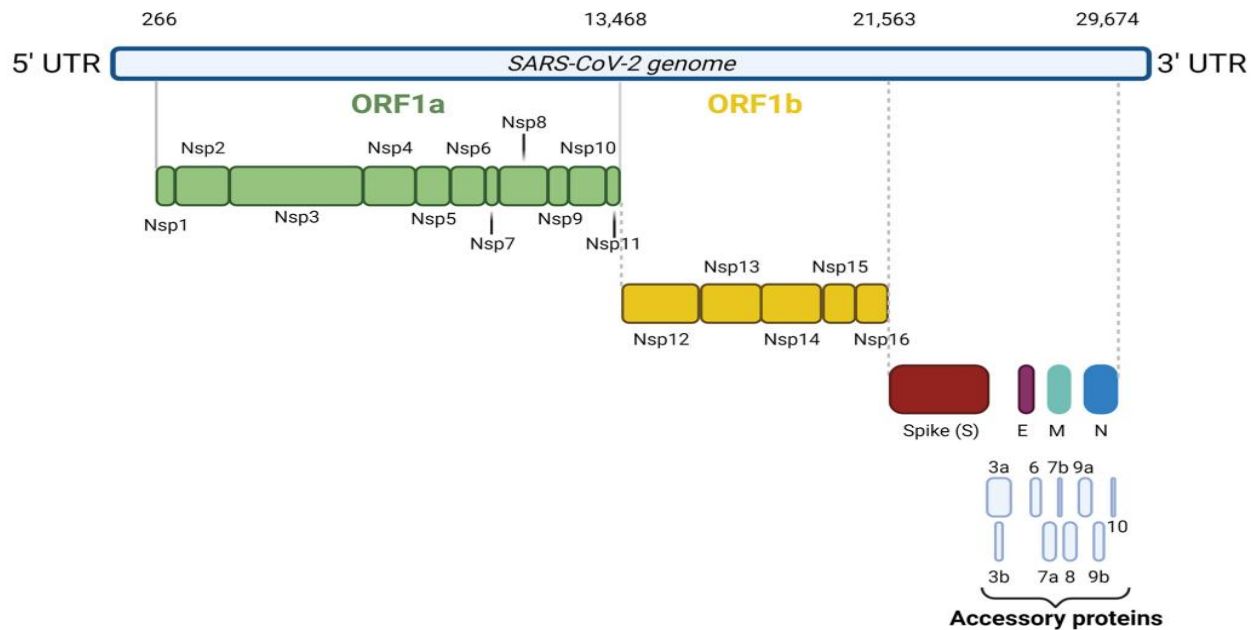


Figure 2.5: Genomic arrangement of SARS-CoV-2

Source: Adapted from Pizzato et al. (2022)⁴

Figure 2.5 illustrates the 30 kb genomic organisation of SARS-CoV-2, which shows the arrangement of various non-structural, structural, and accessory genes flanked by 5' and 3' untranslated regions.

When translated, the replicase genes (ORF1a and ORF1b) produce the polyproteins pp1a and following programmed-1 ribosomal frameshifting, the C-terminally extended product, pp1ab (Bredenbeek et al., 1990). Following proteolytic processing by viral protease enzymes (Ziebuhr, Snijder & Gorbalenya, 2000; Ziebuhr, 2005), a total of 16

⁴ See Appendix C for permission to use this material.

NSPs are produced that are involved in one or more steps in viral replication (see Table 2.1).

These proteins include RdRp/NSP12, which is a critical composition of coronavirus replication/transcription. In addition to NSP12, other NSPs work together to form a large protein replication–transcription complex (RTC) that serves as the site for viral replication and transcription taking place within the infected host cells (Hillen, 2021). It is within the RTC that the viral RNA genome is replicated to produce more copies and is also transcribed to produce new viral messenger RNA (mRNA). ORFs that are distributed towards the 3' end of the viral genome encode the structural proteins spike, envelope, membrane, and nucleocapsid, as well as accessory proteins that play supporting roles in the viral lifecycle (Sola et al., 2015). Upon transcription of this region, a set of subgenomic mRNAs (sg-mRNAs) are produced in addition to the full-length transcript. These sg-mRNAs are partial transcripts that overlap with the 3' end of the genome. Translation of the sg-mRNAs then produces the four key structural proteins, as well as assorted accessory proteins encoded by the smaller ORFs located in this region.

The four main structural proteins each plays an important role in maintaining the viral integrity (Shang, Ye, et al., 2020). The spike protein is composed of three identical monomeric spike polypeptides that form a homotrimeric structure, each monomer has two non-covalently associated parts, the S1 subunit consists of the N-terminal domain (NTD), and the receptor-binding domain (RBD), which binds to the receptor of the host cell, while the S2 subunit anchors the spike protein to the membrane and contains the fusion peptides that form coiled structures that mediate viral and cellular membrane fusion during entry (Duan et al., 2020). Many of the variants of SARS-CoV-2 are characterised by mutations of the spike protein that can influence their function (Harvey et al., 2021), which can make the virus more infectious. Many of these mutations are unique to each variant; however, for example, D614G is found on each of the variants of concern (Zhang, Cai, et al., 2021).

The envelope protein is a small hydrophobic integral membrane protein with viroporin activity that is crucial for completing the virus' infection cycle and plays roles in assembly, envelope formation, and budding (Ruch & Machamer, 2011). The nucleocapsid protein

binds the genomic RNA to form the nucleoprotein complex and primarily serves to package the viral RNA, although it may also be involved in processes such as host cell response and assembly/budding (Huang, Yu & Petros, 2004). The membrane protein is multifunctional; it maintains the integrity of the viral envelope while also participating in virion formation and assembly by binding to and stabilising the nucleocapsid-RNA complex, which is essential for producing new virus particles (Alsaadi & Jones, 2019).

Table 2.1: Summary of SARS-CoV-2 non-structural proteins (NSPs) and their proposed functions

NSP	Function	Reference
NSP1	Interferon (IFN) antagonist and degradation of host mRNAs.	Huang et al. (2011); Tidu et al. (2021)
NSP2	Interacts with host prohibitin.	Slavin et al. (2021)
NSP3	Papain-like protease activity.	Osipiuk et al. (2021)
NSP4	Participates in the formation of sites for viral RNA synthesis and double membrane vesicles (DMVs).	Gadlage et al. (2010)
NSP5	Main protease.	Lee, Worrall, et al. (2020); Fung et al. (2021)
NSP6	Participates in the formation of sites for viral RNA synthesis and DMVs.	Cottam, Whelband & Wileman (2014); Xia et al. (2020)
NSP7	Involved in the primase complex.	Te Velthuis et al. (2012)
NSP8	Involved in the primase complex.	Te Velthuis et al. (2012)
NSP9	RNA replicase activity.	Yan et al. (2021)
NSP10	Cofactor for NSPs 14 and 16.	Decroly et al. (2011)
NSP11	Unknown function.	-
NSP12	RdRp suppresses nuclear translocation of phosphorylated interferon regulatory factor 3 (IRF3).	Te Velthuis et al. (2010); Wang et al. (2021)
NSP13	Helicase/triphosphatase activity; inhibits signal transducer and activator of transcription (STAT) 1 and STAT2 phosphorylation.	Jia et al. (2019); Guo, Gao, et al. (2021)
NSP14	Exoribonuclease activity.	Minskaia et al. (2006)
NSP15	RNA endonuclease and inhibits autophagy and blocks IRF3 nuclear translocation.	Bhardwaj et al. (2006); Yuen et al. (2020)
NSP16	Inhibits retinoic acid-inducible gene I and anti-melanoma differentiation-associated gene 5.	Shi et al. (2019); Vithani et al. (2021)

2.4 LIFECYCLE

2.4.1 Viral attachment

The SARS-CoV-2 spike glycoprotein is key for viral entry into host cells (Fehr & Perlman, 2015). SARS-CoV-2 preferentially targets ciliated and alveolar type 2 cells in airway and

alveolar regions co-expressing the human angiotensin-converting enzyme 2 (hACE2) and co-receptor transmembrane protease serine 2 (TMPRSS2) (Shang, Wan, et al., 2020). For SARS-CoV-2 to enter host cells, the receptor-binding domain must recognise and bind to the hACE2 receptor located on the host. After the spike protein makes contact, it is cleaved by host protease enzymes furin and TMPRSS2 at a cleavage site bordering the S1 and S2 subunits (Glowacka et al., 2011). Cleavage at this site separates the two subunits, which exposes a second cleavage site within the S2 domain, which is termed the S2' site. Cleavage at this S2' site by host cellular proteases activates the spike protein, which liberates the S2 fusion peptide and initiates viral-host membrane fusion. This membrane fusion occurs through an irreversible conformational change in the spike protein, which allows the full particle to be endocytosed and the viral contents to enter the host cell cytosol and initiate infection.

Although SARS-CoV-2 prefers activation by TMPRSS2, cleavage of the S2' site can also be mediated by cathepsins, especially cathepsin L (Bayati et al., 2021). If the target cells express low or insufficient TMPRSS2 or if a virus–angiotensin-converting enzyme 2 (ACE2) complex does not encounter TMPRSS2, the whole ACE2-bound virus particle is internalised via clathrin-mediated endocytosis, where the vesicle matures into a late endolysosomes. Within this compartment, host cathepsin proteases can cleave the viral spike at an alternative S2' site, which allows the virus to still fuse to the cell membrane and enter the cellular cytoplasm, although less efficiently than through the optimal TMPRSS2 cleavage pathway (Bayati et al., 2021).

2.4.2 Viral translation and replication

As with all other positive-stranded RNA viruses of eukaryotes, the replication of SARS-CoV-2 occurs primarily in the cytoplasm of the host cell (Den Boon, Diaz & Ahlquist, 2010). Upon entry, viral uncoating releases viral genomic RNA (gRNA) from the nucleocapsid, which acts as a template for both transcription and replication. It first recruits host ribosomes to initiate translation and then serves as a functional mRNA that carries the necessary protein-coding information for protein synthesis (Wu, Zhao, Yu, et al., 2020) (see Figure 2.6). When bound to these ribosomes, translation of the open

reading frames ORF1a and ORF1b produces two large polyproteins, pp1a and pp1ab, of which the production of pp1ab depends on the occurrence of a programmed ribosomal frameshift (Finkel et al., 2021). Following their translation, the two polyproteins undergo an autoproteolytic cleavage, performed by virus-encoded papain-like protease (PLpro) in NSP3 or main protease (Mpro) in NSP5 (Ziebuhr et al., 2000). This results in the yield of 16 mature NSPs that either mediate the shutdown of translation of host mRNAs by hijacking host ribosomes to preferentially translate viral RNAs instead of host mRNAs (Lokugamage et al., 2012), or to form protein complexes that engage in viral RNA synthesis referred to as RTCs (Te Velhuis et al., 2012). The RTCs are involved in two processes of the coronavirus replicative cycle, namely full-length viral genome replication and transcription of subgenomic mRNAs (Angelini et al., 2013). During the replication process, short regions of double-stranded RNA (dsRNA) are also produced as intermediate and by-products (Weber et al., 2006).

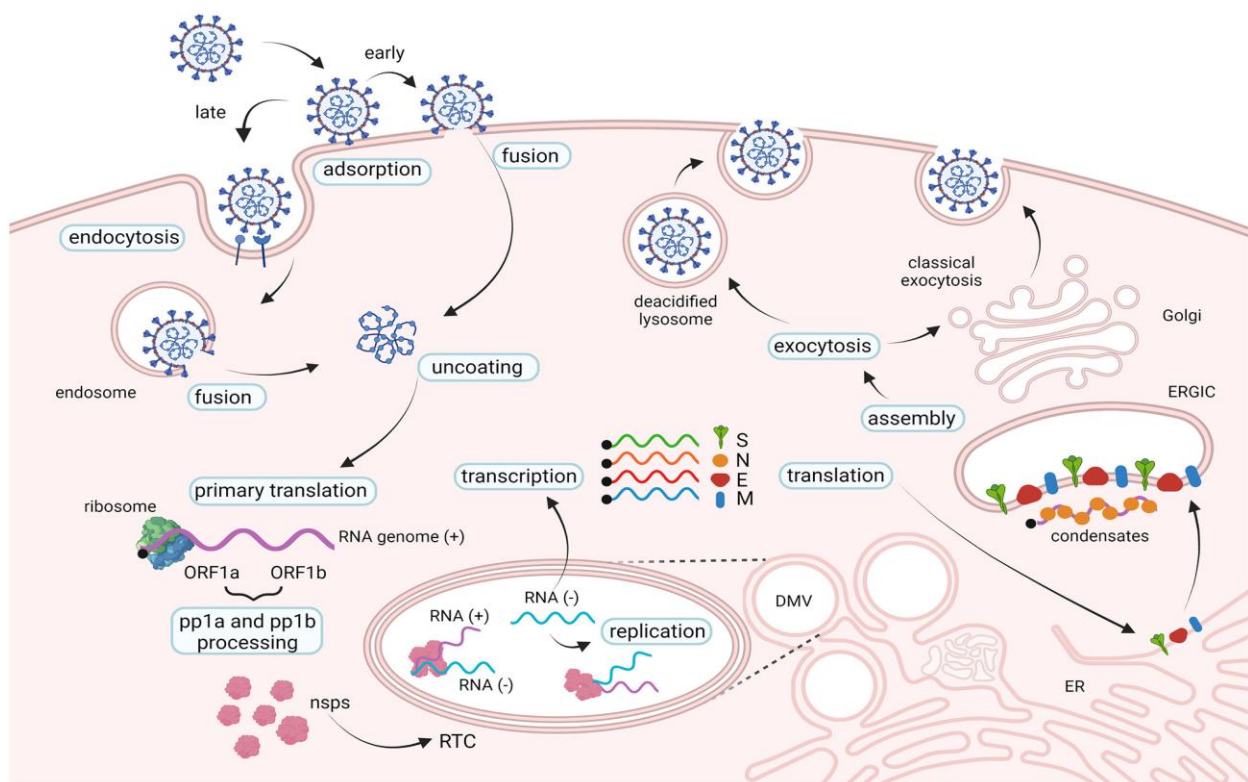


Figure 2.6: The lifecycle of SARS-CoV-2

Source: Adapted from Pizzato et al. (2022)⁵

⁵ See Appendix C for permission to use this material.

As illustrated in Figure 2.6, entry into host cells by SARS-CoV-2 can either be through endocytosis or directly by fusion on the host cell surface. Following primary translation, the viral gRNA replicates inside protective DMVs. Transcription and translation result in the formation and resulting assembly of structural proteins that bud out from the cell in secretory vesicles through the Golgi pathway or by hijacking lysosomes, and are released via exocytosis.

The cleavage products NSP12, NSP13, NSP14, and NSP16 are the primary drivers of replication and transcription (Slanina et al., 2021; Yan, Yang, et al., 2021; Gorbalenya et al., 2006; Seybert et al., 2000; Ivanov et al., 2004; Snijder et al., 2003; Bouvet et al., 2010; Decroly et al., 2008). The NSP12 subunit, which contains the active RdRp domain, requires the assistance of NSP7 and NSP8 to form the fully active RdRp enzyme complex, which is termed holoenzyme RdRp. The RdRp complex initiates RNA synthesis using gRNA as a template. This results in the production of two RNA products, namely a full-length antigenome (positive-sense RNA), which is complementary to the gRNA; and negative-sense subgenomic RNAs (sgRNAs) that are derived from the region of the gRNA located beyond ORF1ab, which encodes the replicase proteins (Cui et al., 2015). The synthesised antigenome then serves as the template for synthesising new gRNAs, while sgRNAs direct the production of a nested set of sg-mRNAs that are used to produce viral structural and accessory proteins that are enhanced by NSP7 and NSP8 (Cui et al., 2015).

In addition to NSP12, other NSPs are part of the larger RTC that play supporting roles. NSP1 is among the first viral proteins to be expressed and serves in suppressing the synthesis of host proteins, while allowing viral mRNA to escape this suppression and be translated, which modulates the host's innate immune responses against the virus (Mosquera-Sulbaran et al., 2021; Mu et al., 2020). NSP3, NSP4, and NSP6 play a crucial role in remodelling of host cell membranes, such as those of the endoplasmic reticulum, endolysosomes, or mitochondria, by forming double-layered structures known as replication organelles (Osipiuk et al., 2021; Gadlage et al., 2010; Cottam et al., 2014; Xia et al., 2020). These organelles provide a sheltered environment that is separated from the cell for viral RNA synthesis to take place. Typically, the formation of these replication

organelles occurs before the virus enters the exponential or log phase of rapidly amplifying its genome (Malone et al., 2022). The RTCs produce new gRNAs and a set of sg-mRNAs that include ORFs that encode the structural spike, membrane, envelope, and nucleocapsid proteins, as well as a number of accessory proteins (Wolff et al., 2020). The majority of the roles that these accessory proteins are thought to play relate to host immune evasion (Redondo et al., 2021), such as the regulation of cytokine synthesis by ORF9c or the inhibition of Type I IFN activity by ORF3b, ORF6, ORF7a, ORF8, or ORF9b. These auxiliary proteins also influence other important cellular processes, such as ORF3a's involvement in autophagy or apoptosis (Zhang, Sun, et al., 2021) and ORF9b's function in inflammasome activation. Newly made gRNAs can be translated to yield additional NSPs, serve as a template for further RNA synthesis, or be packaged into new virions.

SARS-CoV-2 assembly starts with the coating of gRNAs with nucleocapsid proteins, generating nucleocapsid structures that bud into the endoplasmic-reticulum–Golgi intermediate compartment (ERGIC) (Haan et al., 2005; Siu et al., 2008), thereby acquiring a lipid bilayer that contains the viral spike, membrane, and envelope proteins. The membrane protein, once synthesised, localises to the ERGIC, where it provides a platform for recruiting other viral structural proteins like spike and envelope proteins (De Haan & Rottier, 2005). The membrane protein provides a critical scaffolding function and orchestrates virion formation and development of shape and structure through direct physical interactions, which are known as heterotypic interactions with the other structural proteins. These heterotypic interactions facilitated by the membrane protein scaffold are essential for proper shaping and formation of the virus particle during assembly by guaranteeing the accurate incorporation of structural proteins like spike and envelope proteins into the developing virion structure at the ERGIC (Lu et al., 2021). After molecular assembly, mature virions are transported to smooth-walled vesicles through the secretory pathway to the plasma membrane via exocytosis (Stertz et al., 2007). The virions then exit the cell and spread to other parts of the body through the secretory pathway. Recent studies also suggest that SARS-CoV-2 and other betacoronaviruses may exploit a lysosomal exocytic pathway that results in the non-lytic release of its virions (Ghosh et al., 2020), which indicate the formation of fusion of lysosomes that contain multiple virus

particles to the cell membrane, which possibly supports lysosomal-mediated exocytosis (Mendonça et al., 2021).

2.5 DISEASE PATHOLOGY

2.5.1 Viral entry and replication

Orthocoronaviruses, such as SARS-CoV-2, are primarily transmitted through fomites, respiratory droplets, or faecal-oral transmission (Zhang, Li, et al., 2020; Guo, Tao, et al., 2021). They typically target the epithelial cells that line the gastrointestinal and respiratory tract, often causing asymptomatic or mild infections (Sims et al., 2005; Van Kampen et al., 2021). However, in severe COVID-19, manifestations are associated with aberrant immune responses and resultant tissue damage (Merad & Martin, 2020). The disease pathogenesis is broad and not fully characterised as yet; however, it does involve dysfunctional IFN signalling, cytokine storms, recruitment of ineffective immune cells, and uncontrolled complement activation, resulting in neutrophil extracellular trap (NET) formation and thrombosis (Lee, Park, et al., 2020; Prompetchara, Ketloy & Palaga, 2020; Yu et al., 2022; Twaddell et al., 2019; Porto & Stein, 2016). Although the respiratory route is the dominant infection route, the highest ACE2 and TMPRSS2 expression is in the small intestine, testes, kidney, heart, colon, and thyroid glands (Wang, Wang, Luo, et al., 2020; Ye et al., 2004). Consistent with this multi-organ expression, cardiac, renal, and gastrointestinal viral infiltration has been observed in autopsy cases (Lindner et al., 2020; Safari et al., 2020; Ahmed et al., 2020; Bryce et al., 2021). Additionally, SARS-CoV-2 may also exhibit neurotropism, possibly related to the finding of ACE2 expression in the brain, which is a feature that is also observed in the 2002 SARS-CoV (Puelles et al., 2020; Chen et al., 2021; Xu et al., 2005; Gu et al., 2005). ACE2 is also expressed in some immune cells, such as alveolar macrophages and T-lymphocytes in lung tissue, providing a possible immune evasion mechanism and a route of viral dissemination to other organs or tissues in the body (Muus et al., 2021). Overall, this wide tissue distribution of ACE2 likely explains the multi-system manifestations seen in severe disease.

The severity of COVID-19 is influenced by various factors, including genetics and pre-existing health conditions (Meftahi et al., 2020; Rakotoarivelo et al., 2018; Xiao et al.,

2014; Randeria et al., 2019; Tanase et al., 2019). The innate immune system's IFN production is an important early defence mechanism that inhibits viral replication (Acharya, Liu & Gack, 2020; Dansako et al., 2013; Nguyen et al., 2017; Feinberg & Hunter, 2003; Van Boxel-Dezaire, Rani & Stark, 2006). However, SARS-CoV-2 employs various strategies to antagonise this IFN signalling (Channappanavar & Perman, 2017; Kindler, Thiel & Weber, 2016; Schoggins et al., 2011; Yuen et al., 2020). Proteins like viral nucleocapsid protein and the accessory ORF9b, NSP13, and ORF7a proteins disrupt important components of the IFN signalling pathway (Sui et al., 2022; Liu, Qin, et al., 2021; Kouwaki et al., 2021). Moreover, genetic defects or auto-antibodies that affect IFN generation are also evident risk factors for a wide range of viral infections that now include COVID-19 (Bastard et al., 2020).

In response to infection, overwhelmed host cells will activate apoptosis (Thompson, 1995). However, SARS-CoV-2 can manipulate this mechanism by both inhibiting and inducing it, depending on the stage of infection. The virus suppresses initial IFN signalling and suppresses pro-apoptotic regulators such as FADD-like-IL-1 β -converting enzyme-inhibitory protein (c-FLIP) and nuclear factor- κ B (NF- κ B) to promote replication and propagation before detection by the immune system (Lavrik & Krammer, 2012; Nilsson-Payant et al., 2021; Kucharczak et al., 2003; Gordon, Tchesnokov & Woolner, 2020). After new viral copies are made, the infected cell can continue to produce more virions that infect nearby cells or form syncytiums with other cells through fusion proteins (Hu et al., 2020; González-Reyes et al., 2001; Porotto et al., 2019). Syncytium formation aids in the virus evading host defences and has frequently been observed in autopsies of lung tissue samples (Zhang, Zheng, et al., 2021; Lin et al., 2021; Li et al., 2022; Chaudhary et al., 2023; Braga et al., 2021; Zhang, Sun, et al., 2021; Merolli et al., 2022). Recent studies have also demonstrated that the virus exploits tunnelling nanotubes between adjacent or even remote cells to facilitate spread in the absence of ACE2 receptors on neighbouring cells, to enable the virus to evade neutralising antibodies (Merolli et al., 2022; Pepe et al., 2021; Sattentau, 2008; Zeng et al., 2022; Jackson et al., 2021).

In the early stages of infection, a coordinated response involving various cell types, including immune cells and potentially even tissue cells, contributes to the overall pro-

inflammatory cytokine production (Hasan, Elgazzar & Mostafa, 2020; Wang, Nie, et al., 2020; Hue et al., 2020). Complement proteins in circulation are also activated at sites of infection, which triggers potent inflammatory responses (Janeway et al., 2001). Tissue resident immune cells are the first responders to infection and engulf foreign pathogens by producing additional inflammatory mediators and chemoattractant proteins, and enhancing phagocytosis (Mjosberg & Spits, 2016). This attraction of immune cells and the resulting inflammation activate the adaptive immune system (Garcia, 2020; Perlman & Dandekar, 2005; Chen & Subbarao, 2007; Amodio et al., 2013). The coordinated involvement of both the innate and adaptive immune systems amplifies inflammation, clears infected cells and viral particles, and establishes immunological memory to prevent future reinfections. Patients with diminished immune functionality have difficulty mounting an effective immune response, especially against new and novel viruses such as SARS-CoV-2 (Rydyznski Moderbacher et al., 2020).

2.5.2 Excessive immune response

A complex interplay of various factors ultimately leads to the excessive inflammatory response observed in some cases of severe COVID-19. The mechanisms that the virus uses in initial evasion allow the virus to spread efficiently. Pyroptosis of these infected cells results in the simultaneous release of damage-associated molecular patterns, a burst of newly replicated viral particles, and several inflammatory molecules all at once. Overall, this significant contributing factor and the overwhelming exposure to these elements can trigger extensive hyperactivation of pathogen recognition receptors and resultant hyperinflammation (Junqueira et al., 2022; Sefik et al., 2022; Zhong et al., 2013; Jessie & Dobrovolny, 2021). The most significant pathogen recognition receptor that is activated is the nucleotide-binding oligomerisation domain-like receptor protein 3 inflammasome, which results in the formation of activated Gasdermin D pores in the cell membrane that leads to the secretion of inflammatory cytokines into the extracellular environment and the influx of extracellular fluid and the subsequent cellular rupture. Released virions can go on to infect more cells and propagate, and the eventual pyroptotic cycle results in a damaging positive feedback loop. This repetitive rupturing of infected cells continuously overwhelms tissues with inflammation-inducing signals and new viral

particles. This sustained hyperstimulation is known as a cytokine storm (Frühbeck et al., 2021; Sun et al., 2022; Kim, Kim & Song, 2021; Wu, Zhao, Yu, et al., 2022).

The overactivation of the complement system contributes to the observed cytokine storm (Magro et al., 2020). NETs are net-like structures composed of deoxyribonucleic acid (DNA)-histone complexes and proteins released by activated neutrophils (Brinkmann et al., 2004). Extensive activation and recruitment of neutrophils results in the excessive production of these NETs, which significantly contribute to additional inflammation and tissue damage (Brinkmann et al., 2004). Studies have found elevated levels of NET formations in both peripheral blood and lung tissues of COVID-19 patients (Janiuk, Jabłońska & Garley, 2021; Cavalcante-Silva et al., 2021; Domínguez-Díaz et al., 2021; Joshi et al., 2022; Ondracek & Lang, 2021; Ng et al., 2021; Masso-Silva et al., 2022). The exact mechanisms underlying thrombosis in COVID-19 is still not fully understood (Rodrigues et al., 2020); however, based on the role of NETs in other diseases, it has been proposed that this formation might be directly related to thrombotic events seen in COVID-19 patients (Zuo et al., 2020; Barnes et al., 2020). The activation of NETs promotes blood clotting by stimulating platelet and red blood cell adhesion through the concentration of coagulation proteins and factors (Brill et al., 2012; Fuchs et al., 2010; Von Bruhl et al., 2012). Thrombus-resident neutrophils play an important role in propagating these clots by binding to and activating factor XII, during which the neutrophils release webs of NETs that enhance clotting and promote further thrombus growth (Von Bruhl et al., 2012). Disseminated intravascular coagulation involving systemic blood clotting throughout the vessels can obstruct blood flow to vital organs, and in severe disease this pathologic clotting is a common feature and occurs via overactivation of the coagulation cascade (Nguyen, Cruz & Carcillo, 2015; Fraissé et al., 2020; McKay, 1965).

2.5.3 Oxidative stress

SARS-CoV-2 entry into host cells and subsequent viral replication are thought to lead to oxidative phosphorylation dysfunction, which leads to increased mitochondrial reactive oxygen species (ROS) production (Delgado-Roche & Mesta, 2020). This in turn results in

the disruption of cellular calcium (Ca) homeostasis by viral proteins such as the spike protein, which further elevates ROS levels within infected cells (Gatti et al., 2020; Su, Yu & Zhou, 2021; Ren et al., 2020). The presence and severity of oxidative imbalance in COVID-19 depend on the stage of the disease (Lage et al., 2022). Oxidative imbalance is present in both mild and severe COVID-19, but the severity increases with disease progression (Delgado-Roche & Mesta, 2020). This imbalance contributes to the inflammatory response seen in COVID-19, particularly in severe cases. Studies have shown that angiotensin II (Ang II) can bind to receptors that activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which is an enzyme complex that produces ROS inside cells (Dikalov & Nazarewicz, 2013). As Ang II levels are most commonly increased in severe disease, a positive feedback loop may occur between mitochondrial and NADPH oxidase-derived ROS that promotes cell and tissue degeneration as a consequence of ROS accumulation (Dikalov, 2011; Daiber, 2010). As a result, markers of oxidative damage to lipids, proteins, and DNA are elevated in COVID-19 patients, which correlates with disease severity (Basaran et al., 2023). Furthermore, inflammasomes assemble in response to SARS-CoV-2 and ROS, firing up inflammatory cascades but also generating more damaging oxidants (Lage et al., 2022). This oxidative stress is what promotes pyroptosis, which can potentially explain diffuse lung damage in severe COVID-19 (Wang et al., 2019). Notably, individuals with underlying conditions linked to chronic oxidative stress, such as diabetes, have poorer COVID-19 outcomes (Kountouri et al., 2021).

2.5.4 Pathological consequences

Several studies have reported elevated serum levels of various cytokines, including IL-2, IL-6, IL-8, IL-10, TNF- α , interferon-gamma (IFN- γ), and monocyte chemoattractant protein-1 (MCP-1) in severe cases of COVID-19 (see Table 2.2) (Ronit et al., 2021; McElvaney et al., 2020; Lucas et al., 2020; Azkur et al., 2020). Among these, higher levels of IL-6 have been associated with increased mortality risk. Elevated IL-6 levels are a key feature of COVID-19 cytokine storms and are known to increase vascular permeability by inducing the overproduction of vascular endothelial growth factor (Nakahara et al., 2003). This increased permeability in the lungs leads to the influx of fluid from the surrounding

blood capillaries to flow into the alveoli, which impairs gaseous exchange and causes symptoms such as hypoxaemia and dyspnea (Matthay, Ware & Zimmerman, 2012; Bachofen & Weibel, 1982). If not promptly treated, the condition can progress to acute respiratory distress syndrome, which is a major cause of respiratory failure and mortality in 90% of COVID-19 cases (Nuckton et al., 2002; Tzotzos et al., 2020).

In addition to IL-6, other potential indicators of severe and fatal COVID-19 cases include lymphopenia, elevated levels of ferritin, D-dimer, aspartate aminotransferase, lactate dehydrogenase, C-reactive protein, neutrophils, procalcitonin, and creatinine (Wang, Wang, Chen, et al., 2020; Amgalan & Othman, 2020; Zhang, Zheng, et al., 2020; Lippi & Plebani, 2020; Tang, Wang, et al., 2020). Ferritin, IL-6, and C-reactive protein are considered to be possible immunological biomarkers for the early detection of systemic inflammatory response syndromes in COVID-19 (Melo et al., 2021).

Table 2.2: Pro-inflammatory cytokines associated with COVID-19 severe disease

Cytokine	Main source	Proposed function	References
IL-2	CD4+ T helper cells	Proliferation and activation of T and B cells.	Keene and Forman (1982); Kamimura and Bevan (2007); Bamford et al. (1994)
IL-6	Monocytes, macrophages, and endothelial cells	Acute phase response, and involved in the transition to chronic inflammation that leads to the recruitment of more immune cells, especially neutrophils. Also shown to prolong neutrophil survival through regulatory effects on neutrophil apoptosis.	Mauer et al. (2014); Fielding et al. (2008); Asensi et al. (2004)
IL-8	Multiple cell types, including monocytes, neutrophils, epithelial, fibroblast, and endothelial cells	Chemoattractant recruiting T cells, as well as non-specific inflammatory cells into sites of inflammation, also activating neutrophils.	Matsushima and Oppenheim (1989); Schröder (1992)
IL-10	CD4+ and CD8+ T helper cells	Inhibit cytokine production, promote B cell proliferation antibody production and mast cell growth.	Moore et al. (2001); Kessler et al. (2017); Boulland et al. (1998); Go et al. (1990)
IFN- γ	Natural killer cells and activated T cells	Promote macrophage activation, mediate antiviral immunity, enhance antigen	Billiau (1996); Boehm et al. (1997)

Cytokine	Main source	Proposed function	References
		presentation, orchestrate activation of the innate immune system, coordinate lymphocyte–endothelium interaction, regulate T cell balance, and control cellular proliferation and apoptosis.	
TNF- α	Macrophages, CD4+ T helper cells and natural killer cells	Key regulator of acute and chronic inflammation and, in certain circumstances, cause cell death by apoptosis and necroptosis.	Horiuchi et al. (2010); Brenner, Blaser and Mak (2015); Van Loo and Bertrand (2022)
MCP-1	Monocytes and macrophages	Chemoattractant.	Deshmane et al. (2009); Yoshimura, Robinson, et al. (1989); Yoshimura, Yukhi, et al. (1989)

2.6 LABORATORY DIAGNOSIS

Infection is confirmed by the detection of SARS-CoV-2 RNA using nucleic acid amplification tests (NAATs), with reverse transcription quantitative PCR (RT-PCR) recommended as the most sensitive NAAT method (CDC, 2023). The RT-PCR process begins with RNA extraction from respiratory specimens, followed by reverse transcription where the purified total RNA (viral RNA and host RNA) is converted to complementary DNA (cDNA) by an enzyme reverse transcriptase (Artika, Wiyatno & Ma'roef, 2020). This cDNA is then used in quantitative PCR (qPCR) to exponentially amplify the target viral gene of interest, which will be quantified at each cycle to indicate viral load.

SARS-CoV-2 NAAT is most commonly performed on upper respiratory tract samples, with the South African National Institute for Communicable Diseases (NICD, 2021) recommending that swabs be obtained from the nasopharynx, oropharynx, nasal mid-turbinate, or anterior nares deep in the nasal/oral cavities. Nasal or nasopharyngeal washes or aspirates are also appropriate sample types.

RT-PCR tests usually target highly conserved and abundantly expressed SARS-CoV-2 genes such as the structural proteins (Alsuliman et al., 2020). Common gene targets include spike, nucleocapsid, RdRp, and ORF1ab proteins, with multiple gene targets helping to reduce false negatives from primer mutations.

Serological assays play a limited role in diagnosis; however, rapid antigen detection assays have been shown to have application in rapid diagnosis.

2.7 EPIDEMIOLOGY AND TRANSMISSION

The natural reservoir of SARS-CoV-2 is still unknown but the virus is believed to have originated in bats and then transmitted to an intermediate host before humans were infected (Wu, Zhao, Yu, et al., 2020; Yip et al., 2009; Li, Guan, et al., 2020). Researchers have identified several coronaviruses in bats that are genetically similar to SARS-CoV-2 (Rahalkar & Bahulikar, 2020; Murakami et al., 2020; Delaune et al., 2021; Zhou et al., 2021; Wacharapluesadee, 2021). Notably, the bat coronavirus RaTG13, first isolated in 2013, shares approximately 96% of its genome with SARS-CoV-2. This high level of genetic similarity suggests that RaTG13 could potentially be a direct ancestral virus that SARS-CoV-2 evolved from over time (Zhang, Wu & Zhang, 2020). Mutation rates deduced from observations in humans estimate that its divergence was likely between 25 and 65 years ago (Zhang, Wu & Zhang, 2020). Differences in spike protein genetic sequences and SARS-CoV-2's increased infectivity versus RaTG13 suggest that RaTG13 infected another species, which then transmitted SARS-CoV-2 to humans (Wrapp et al., 2020; Zhang, Wu & Zhang, 2020).

The typical incubation period of COVID-19 infection ranges from four to five days after exposure to the onset of symptoms, which can extend up to 14 days (Guan et al., 2020). The basic reproduction number (R_0) is estimated between two and three, which means each infected person could statistically be expected to spread the virus to two to three other people on average if no containment measures are implemented (Read et al., 2020; Li, Guan, et al., 2020). There is no general difference in levels of viral shedding between people who present with symptoms and those that are asymptomatic or pre-symptomatic (Zuin et al., 2021). A systematic review found that approximately 40% of COVID-19 confirmed positive cases in the population are individuals who did not exhibit any symptoms. This high rate of asymptomatic infection possibly helped to fuel the spread of SARS-CoV-2 as infected individuals passed on the virus without realising, they were infected earlier on in the pandemic (Moghadas et al., 2020; Chen, Fan, et al., 2020; Huang

et al., 2020; Li, Ji, et al., 2020; Qiu et al., 2020; Tong et al., 2020; Wong et al., 2020; Zhang et al., 2023; Ma et al., 2021). According to data from the WHO COVID-19 dashboards, as of January 2024, the majority of COVID-19 cases occurred in Europe, the Americas, and the South-East Asian regions (see Figure 2.7), which encompass mainly heavily populated areas and international travel destinations. The overall global case fatality rate of SARS-CoV-2 is estimated to be around 1% to 2% (Wang et al., 2022). SARS-CoV-2 continues to cause disease throughout the year and seasonal spikes have been identified in COVID-19 cases, which is consistent with typical winter months where seasonal respiratory viruses usually increase in circulation (Lofgren et al., 2007; Nichols et al., 2021; Liu, Huang, et al., 2021; Merow & Urban, 2020).

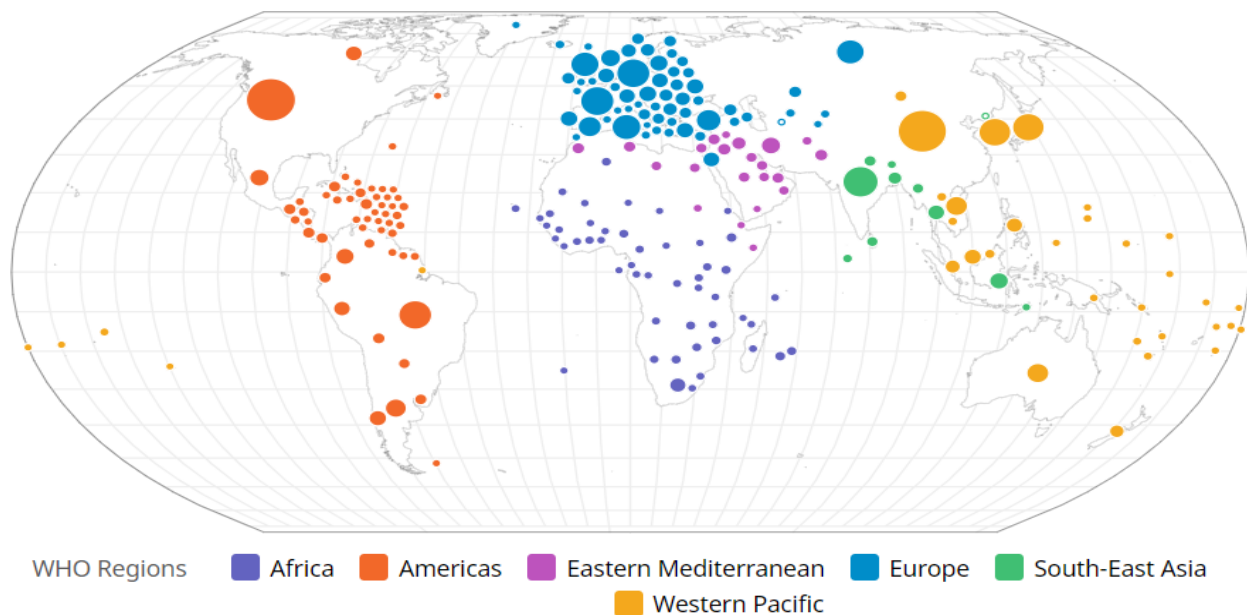


Figure 2.7: Global distribution of total accumulative SARS-CoV-2 cases as of January 2024

Source: Adapted from WHO (2024)⁶

2.8 SARS-CoV-2 VARIANTS

The original SARS-CoV-2 viral strain that was first detected in Wuhan, China, in late 2019 is referred to as the wild type, and subsequent viral variants formed through natural mutations when errors occur in the RNA replication process as the virus replicates and circulates (Wu, Zhao, Yu, et al., 2020; Sanjuán et al., 2010; Tang et al., 2020). It has been

⁶ See Appendix C for permission to use this material.

suggested that prolonged viral survival in long-COVID patients may be a major influence of viral evolution, which possibly provides more opportunities for the virus to mutate (Chaguzza et al., 2023). The formation of these variants has aided in the increased transmissibility or ability of the virus to evade immunity and gain selective advantages (Aleem et al., 2021; Mohaptra et al., 2022). These mutations have primarily clustered within the spike protein of SARS-CoV-2 and have arisen within the RBD of the S1 subunit (Rambaut, Holmes, et al., 2020). The Alpha variant (B.1.1.7) contains N501Y mutation; Beta (B.1.351) with N501Y, K417N, and E484K mutations; Gamma (P.1) with N501Y, K417T, and E484K; Delta (B.1.617.2) with T478K and L452R; while the highly transmissible Omicron variant (B.1.1.529) displays over 15 mutations in the RBD region alone, which include S371L, G339D, S375F, S373P, K417N, N440K, S477N, G446S, E484A, T478K, Q493K, Q498R, G496S, N501Y, and Y505H (Cherian et al., 2021; Davies et al., 2021; Tegally et al., 2022; Volz et al., 2021).

These specific mutations within the spike protein RBD appear to facilitate improved binding to host cells and drive increased infectivity, which contribute to the rising global dominance of emerging SARS-CoV-2 variants over time (Taylor et al., 2022). As of November 2023, 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 (WHO, 2023a, 2023d). Continued evolution of mutations in the virus remains a concern for pandemic control efforts. Ongoing sequencing has provided monitoring of mutations and guidance of public health strategies (NICD, 2023).

2.9 PREVENTION AND TREATMENT

Vaccination remains the most effective protection against infection and severe disease (Wu, Joyal-Desmarais, et al., 2022; WHO, 2023c). As of January 2024, a total of 64 vaccines have been approved for use by at least one national regulatory authority, and of these, six vaccines were part of the vaccine rollout in South Africa (United Nations Children's Fund, 2024). Updated 2023-2024 vaccine formulas are expected to provide better protection and to more closely target variants of concern such as Omicron and its

subvariants (Food and Drug Administration [FDA], 2024a). Regular booster vaccination is still a key part of the ongoing management of COVID-19, especially among older and more clinically vulnerable populations (Hogan et al., 2023).

The current treatment landscape is largely supportive or experimental, with many drugs currently in ongoing clinical trial stages. Treatment strategies and guidelines vary between different countries as numerous agents are tested and either removed from or added to protocols. The global research environment pertaining to randomised controlled trials and their use in the context of the COVID-19 pandemic has been extensive, with a significant number of randomised controlled trials being conducted to evaluate various treatments. Early on in the pandemic this included the repurposing of existing drugs used for other illnesses, which included antimalarials, antiretrovirals, antiparasitics, and antidepressants (Sanders et al., 2020; Felsenstein et al., 2020; Grein et al., 2020; Wang, Horby, et al., 2020; Gordon, Jang, Bouhaddou, et al., 2020; Frediansyah et al., 2021; Simonis et al., 2021; Wang, Zhang, Du, et al., 2020; Geleris et al., 2020; WHO Solidarity Trial Consortium, 2021; Uzun & Toptas, 2020; Gendrot et al., 2020; Bae et al., 2020; Lin et al., 2020; Chan et al., 2003; Chu, 2004; Cao et al., 2020; Warren et al., 2016; Lo et al., 2017; Bray et al., 2020; Biber et al., 2021; Hashimoto, Suzuki & Hashimoto, 2022a, 2022b).

Remdesivir, an inhibitor of the SARS-CoV-2 RdRp gene, has previously shown broad-spectrum activity against members of several viral families, including filoviruses such as Ebola, the coronaviruses SARS-CoV and MERS-CoV, and more recently SARS-CoV-2 (Sheahan et al., 2020; Wang, Zhang, Du, et al., 2020; De Wit et al., 2020). It was granted regulatory approval as a treatment option for COVID-19 in a number of countries, including South Africa (SAHPRA registration number 55/20.2.8/0458); however, like the protease inhibitor nirmatrelvir-ritonavir (SAHPRA registration number 57/20.2.8/0360), both drugs have shown potential in reducing mortality and hospitalisation, but further studies are still warranted, especially in specific subpopulations. Access to the treatments has also been limited, and the price of the product remains high. It is for these reasons that remdesivir and nirmatrelvir-ritonavir have not been widely adopted for public sector use in South Africa (SAHPRA, 2024; National Institutes of Health, 2024; FDA, 2024b;

National Health Service, 2024; Hammond et al., 2022; Pfizer, 2022; Cheema et al., 2023; Reubenson et al., 2023; Beigel et al., 2020; Goldman et al., 2020; The COVID-NMA Initiative, 2023; Spinner et al., 2020; WHO Solidarity Trial Consortium et al., 2021; Gottlieb et al., 2022; Ader et al., 2022; Mahajan, Singh & Gifty, 2021).

Type I IFN has also been suggested as a treatment option; however, there has not been any clear evidence of its efficacy (Davoudi-Monfared et al., 2020; WHO Solidarity Trial Consortium et al., 2021; Rahmani et al., 2020; Monk et al., 2021). Antibody treatments, including convalescent plasma and monoclonal antibodies, have been used with good effect for other infections, but definitive evidence of clinical benefit for COVID-19 has not yet been shown (Roback & Guarner, 2020; Chen, Xiong, et al., 2020; Bartoli et al., 2021; Han et al., 2021; ACTIV-3/TICO LY-CoV555 Study Group, 2020).

Several treatments have also been studied and tested for their potential to reduce the hyperinflammatory response seen in severe COVID-19 cases. These include colchicine, tocilizumab, and anakinra (Lopes et al., 2021; Reyes et al., 2021; Cumhur Cure, Kucuk & Cure, 2020; Toniati et al., 2020; De Rossi et al., 2020; Biran et al., 2020; Dimopoulos et al., 2020; Filocamo et al., 2020; Aouba et al., 2020). As yet, only the corticosteroids dexamethasone and its alternatives prednisolone and betamethasone have been recommended (Ledford, 2020; RECOVERY Collaborative Group et al., 2020; NDoH, 2021; WHO Rapid Evidence Appraisal for COVID-19 Therapies [REACT] Working Group et al., 2020). Baricitinib, a Janus kinase (JAK) 1 and 2 inhibitor with anticytokine effects, can be given in addition to the above corticosteroids, but it requires dose modification as adverse events have been seen in patients with renal disease (NDoH, 2021).

The binding of SARS-CoV-2 to ACE2 leads to decreased availability of Ang 1-7, which causes excessive Ang II levels, which can result in lung and heart injury (Vaduganathan et al., 2020; Albin et al., 2020). ACE inhibitors and angiotensin receptor blockers are believed to mitigate these effects by reducing Ang II levels or blocking its receptor (Albashir, 2021; Annweiler et al., 2020). Recombinant human ACE2 is being studied for its potential to allow the conversion of Ang II while blocking viral binding to cellular ACE2. Initial results have shown decreased Ang II, IL-6, and viral loads in early clinical cases (Vaduganathan et al., 2020; Pang, Cui & Zhu, 2020; Abd El-Aziz, Al-Sabi & Stockand,

2020). For severe COVID-19 patients with respiratory symptoms and low oxygen (O_2) levels, supplemental O_2 is the mainstay supportive treatment (Caputo, Strayer & Levitan, 2020; Xu et al., 2020). Thromboprophylaxis or therapeutic anticoagulation guided by D-dimer levels is recommended due to the association between inflammation and coagulation in seriously ill COVID-19 patients, with subsequent studies showing improved survival with the use of low molecular weight heparin (Kollias et al., 2020; Tang, Bai, et al., 2020; Shen, Qiu, et al., 2022; NDoH, 2021).

2.10 DRUG DISCOVERY

This section discusses potential drug targets, ancient remedies and modern medicine, chemical classes of plant metabolites with antiviral activity, and the study's plant selection and rationale.

2.10.1 Potential drug targets

The COVID-19 pandemic highlighted the urgent need for effective antiviral therapies. Drug discovery efforts focused on various targets in the SARS-CoV-2 lifecycle, including viral entry, protease activity, polymerase inhibition, and immune modulation.

2.10.1.1 Viral entry inhibition

Entry inhibitors typically target the early stages of infection, such as receptor binding, the proteolytic activation of the spike protein, or viral-cell membrane fusion. Convalescent plasma containing neutralising antibodies and synthetic monoclonal antibodies targeting the SARS-CoV-2 spike protein are potential therapeutic approaches (Peng, Rhind & Beckett, 2021; Renn et al., 2020; Jiang et al., 2020). Several potential drug candidates, including small molecules/peptides such as cepharantine, VE607, SSAA09E2, emodin, HTCC, and HM-HTCC have been screened and designed to directly target either the ACE2 receptor-binding site on the spike protein RBD, or the binding site on the ACE2 receptor (Xiu et al., 2020; Khare et al., 2020; Artese et al., 2020). Engineered recombinant human soluble ACE2 is another potential treatment strategy, possibly with the added fusion of the fragment crystallisable region of the structural domain segment of human immunoglobulin G (Strohl, 2015; Yamin et al., 2021; Linsky et al., 2020). This form of

ACE2 does not pose any catalytic activity as compared to the membrane-bound ACE2, but can competitively bind to the virus and outcompete transmembrane ACE2, which will prevent its entry into host cells (Linksy et al., 2020).

The three types of host proteases involved during viral entry, namely furins, cathepsins, and surface serine TMPRSS2 proteases, are also evident drug targets. Furin inhibitors such as peptidomimetic decanoyl-RVKR-chloromethylketone (dec-RVKR-cmk) and MI-1851 have shown their ability to inhibit SARS-CoV-2 infection in *in vitro* experiments, while small molecule inhibitors of TMPRSS2 such as camostat and nafamostat mesylate are already FDA-approved treatments (Hoffmann, Kleine-Weber, et al., 2020; Hoffmann, Kleine-Weber, Schroeder, et al., 2020; Hoffmann, Schroeder, et al., 2020; Bestle et al., 2020). Cathepsin has been reported to be inhibited by the broad-spectrum antibiotic teicoplanin in pseudovirions by directly binding to the enzyme active site (Zhang, Ma, et al., 2020). Indirect inhibition of protease activity has also been reported, specifically through pH modulation (Yang & Shen, 2020). Endosome acidification inhibitors, such as the quinine derivatives hydroxychloroquine and chloroquine and the antibiotic azithromycin work by raising the pH within endosomes, thereby preventing cathepsin L activation and viral entry (Bereczki et al., 2022; Ceccarelli et al., 2021; Andreani et al., 2020). However, especially in the case of the antimalarials, there is concern over their side effects, where some clinical trials have not supported benefits of their use (Das et al., 2021).

2.10.1.2 Viral protease inhibition

Protease inhibitors are drugs that target the two integral proteases of SARS-CoV-2, the virus-encoded PLpro (NSP3) and the Mpro (NSP5), which are essential for cleaving viral polyproteins for replication. There are three main categories of PLpro and Mpro inhibitors based on their mechanisms of action: covalent inhibitors that form a bond with the catalytic cysteine, non-covalent inhibitors that block substrate entry to the catalytic site, and non-covalent inhibitors that target allosteric binding pockets. Class (i) PLpro inhibitors such as VIR250 are small molecule peptidomimetics structures that are designed to mimic the viral substrate to inhibit protease activity by binding covalently via a thioether

linkage to the catalytic cysteine residue (Rut et al., 2020). Nirmatrelvir, the most successful Mpro inhibitor, is a class (i) covalent inhibitor that forms a reversible covalent bond with the cysteine protease Cys145 in Mpro's active site to inhibit viral replication and reduce viral loads (Zhao et al., 2022). PLpro class (ii) non-covalent inhibitors such as GRL0617 and F0213 work by physically blocking the substrate entry channel, which indirectly inhibits protease activity rather than direct active site targeting (Shen, Ratia, et al., 2022). Penicillin and ebselen derivatives are major Mpro class (ii) inhibitors but lack specificity in their target (Malla et al., 2022; Gao et al., 2021). Class (iii) inhibitors like HE9, which targets PLpro, bind to allosteric pockets/sites distant to the main catalytic site and cause a conformational change that makes the active site less receptive to binding to substrates (Li et al., 2023). Ensitrelvir is a non-covalent class (iii) inhibitor that targets Mpro that occupies the substrate binding pockets (S1', S1, and S2) that was approved in Japan for emergency use (Mukae et al., 2023; Unoh et al., 2022). Class (iv) Mpro inhibitors are allosteric; for example, NB1A2 targets the Mpro dimer interface to inhibit formation; however, they require further optimisation (Li et al., 2023). Developing potent molecules has been a challenge for PLpro and Mpro inhibitors. Inhibitors that target PLpro run the risk of also inadvertently inhibiting human endogenous enzymes, which can potentially cause toxicity.

2.10.1.3 Polymerase inhibition

For most RNA viruses, an RdRp (NSP12) is essential for replication and transcribing genomic material, which makes it a critical component of the viral lifecycle. The SARS-CoV-2 active site is especially conserved and accessible and provides an effective target for polymerase inhibitors (Li et al., 2023). RdRp inhibitors can be classified into nucleos(t)ide analogues (such as remdesivir, molnupiravir, favipiravir, and bemnifosbuvir) and non-nucleos(t)ide analogues (such as suramin) with different mechanisms of action (Li et al., 2023). Remdesivir targets the RdRp active site after conversion through phosphorylation to its triphosphate form remdesivir-TP (Slanina et al., 2021; Kokic et al., 2021). Remdesivir-TP then competes with adenosine triphosphate (ATP), the substrate used by RdRp during replication, incorporating into new RNA copies and blocking further extension of the RNA strand to effectively terminate viral replication and transcription

(Gordon, Tchesnokov, Feng, et al., 2020). Remdesivir has shown clinical benefit in some COVID-19 patients and has proven effective when administered intravenously; however, it is not well absorbed when taken orally in pill/capsule form (Xie & Wang, 2021). Molnupiravir mutates viral RNA through its active metabolite to induce lethal errors. It reduces complications in COVID-19 patients but safety concerns remain regarding mutagenesis (Gordon et al., 2021; Kabinger et al., 2021). Favipiravir, another mutagenic nucleoside analogue, inhibits SARS-CoV-2 RNA synthesis but shows no benefit in COVID-19 patients, which is likely due to insufficient plasma drug levels (Li et al., 2023; Kaptein et al., 2020; Nguyen et al., 2017). Other candidates like bemnifosbuvir and suramin inhibit RdRp and/or N-terminal nidovirus RdRp-associated nucleotidyltransferase domain *in vitro* but require further evaluation and optimisation, such as addressing safety or delivery issues (Shannon et al., 2022). Overall, targeting SARS-CoV-2's replication machinery provides opportunities for antiviral drug development.

2.10.1.4 Other potential viral targets

SARS-CoV-2 helicase (NSP13) unwinds viral RNA and has ATPase activity. RNA-binding and ATPase sites show potential for inhibitors such as FPA-124, but specificity needs improving due to selectivity shared with human helicases (Zeng et al., 2021; Yan, Ge, et al., 2021; Chen, Malone, et al., 2020; Newman et al., 2021). Exoribonuclease (NSP14), along with cofactor NSP10, proofreads viral RNA and removes mismatched nucleotides and errors in the newly made viral RNA chain (Liu, Shi, et al., 2021; Yan et al., 2021). NSP14 also methylates viral RNA caps and aids in the immune system's virus evasion (Baddock et al., 2022). Promising drug candidates have been identified for each function; however, they have undesirable side effects because they also interact with similar proteins found in human cells (Li et al., 2023). Endoribonuclease (NSP15) cleaves specific regions of the viral RNA that contain uridine. Specific locations on the protein are potentially effective and safe desirable targets as no close human cellular equivalent currently exists (Pillon et al., 2021; Kim et al., 2020). Current drug treatments, however, have showed weak inhibition (Kim, Wower, et al., 2021). 2'-O-methyltransferase (NSP16) and its activator NSP10 also methylate viral RNA caps by further modifying them (Nencka

et al., 2022; Viswanathan et al., 2021). Several drugs have been shown to inhibit NSP16 in cell culture; however, they have multiple limitations, including poor membrane permeability and toxicity (Bobileva et al., 2021). The nucleocapsid protein has several important functions; however, developing drugs that target it has been reported to be challenging due to lack of stable drug-binding pockets. Its abundance and complex structure also make it hard to target without affecting healthy cells (Cubuk et al., 2021). Current inhibitors have shown weak potency and are less effective than those that target other parts of the virus. Other non-structural and accessory proteins have also been explored but have also faced challenges (Yan et al., 2022; Liu et al., 2014; Kulkarni et al., 2021).

2.10.1.5 Immune and inflammatory modulation

Systemic corticosteroids, especially glucocorticoids such as dexamethasone, are widely used to reduce inflammation and suppress the immune system and have been shown to be effective in reducing mortality in severe COVID-19 (Hardy, Raza & Cooper, 2020; Sinha et al., 2022). Dexamethasone binds to the glucocorticoid receptor within cells and triggers its translocation to the nucleus (Croxtall, Choudhury & Flower, 2000). This glucocorticoid-dexamethasone complex interacts with host DNA and influences the transcription of various genes, including the suppression of genes that encode pro-inflammatory cytokines, chemokines, and adhesion molecules (Chikanza, 2002; Newman, Flower & Croxtall, 1994). Corticosteroids can have severe side effects, especially in high doses and with prolonged use. Suppressing the immune system can be harmful in early COVID-19 stages; time and dosage are therefore crucial (Liu et al., 2013; Matthay & Thompson, 2020).

JAK inhibitors block the activity of JAK enzymes in immune response signalling, which is especially crucial in the JAK-STAT signalling pathway (Philips et al., 2022). By hindering the phosphorylation of STAT proteins, the transcription of various genes involved in immune responses and inflammation is blocked (Philips et al., 2022). JAK inhibitors such as baricitinib and tofacitinib have reported benefit in treating hospitalised patients, and

are usually given in combined therapy along with dexamethasone (Stebbing et al., 2021; RECOVERY Collaborative Group, 2022; Marconi et al., 2021; Guimarães et al., 2021).

IL antagonists have varied in effectiveness, where promising agents are those that target cytokines that are more pronounced in severe disease, such as IL-6 (tocilizumab, siltuximab) and TNF- α (infliximab) (Leisman et al., 2020; RECOVERY Collaborative Group, 2021; Randomized Embedded Multifactorial Adaptive Platform for Community-Acquired Pneumonia [REMAP-CAP] Investigators, 2021; Del Valle et al., 2020). Other agents have offered less benefits, such as IL-1 β (canakinumab) (Caricchio et al., 2021; Resende et al., 2022). Other treatments that have been investigated include non-steroidal anti-inflammatories, the anticancer drug topotecan, and the complement anti-C5a antibody vilobelimab (Ho et al., 2021; Carvelli et al., 2020).

2.10.1.6 Targeting oxidative imbalances as a complementary approach

Although still theoretical, with limited and inconclusive evidence, the potential role of anti-oxidants to mitigate oxidative stress and concurrently modulate the inflammatory response in COVID-19 requires further investigation. The endogenous anti-oxidant system plays an important role in maintaining redox balance and protecting against oxidative stress (Niture, Khatri & Jaiswal, 2014). Enzymatic and some non-enzymatic anti-oxidants are synthesised within endogenous cells such as the major anti-oxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, and thioredoxin reductase, and the non-enzymatic molecules uric acid, bilirubin, and coenzyme Q10 (Jones et al., 2003; Tomasetti et al., 1999; Neužil & Stocker, 1993; Ames et al., 1981; Goszcz et al., 2015; Salvayre, Negre-Salvayre & Camaré, 2016; Kalyanaraman, 2013; Hanschmann et al., 2013; Rahman et al., 2005). Non-enzymatic anti-oxidants can also be obtained through dietary and supplementary sources such as vitamin C, vitamin E, curcumin, beta-caroten, and lycopene through ingesting various fruits and vegetables (Moussa, Judeh & Ahmed, 2020).

Severe COVID-19 is characterised by the excessive production of ROS, which contributes significantly to cellular damage and disease progression (Borges et al., 2021). Drugs targeting specific ROS or their production pathways are crucial supplementary

strategies to prevent and treat disease progression. Promising targets include the inhibition of NADPH oxidases, which are elevated in severe disease (Sorce et al., 2017). The kelch-like epichlorohydrin-associated protein 1- nuclear factor E2-related factor 2 (Keap1-Nrf2) pathway regulates the expression of anti-oxidant and detoxification enzymes. Activating Nrf2 with small molecules could boost the body's natural anti-oxidant defences and combat oxidative stress such as the synthetic the Nrf2 activator PB125[®] (McCord et al., 2020). SARS-CoV-2 has been reported to disrupt iron (Fe) homeostasis, which leads to free Fe levels that readily react with O₂ to form highly reactive hydroxyl radicals (OH[•]) (Kronstein-Wiedemann et al., 2022; Liu & Li, 2020; Wenzhong & Hualan, 2021). The enzyme, heme Oxygenase-1 (HO-1), degrades heme and inducing its expression could reduce both heme-derived ROS and inflammation (Naito, Takagi & Yoshikawa, 2004; Takagi et al., 2018; Chora et al., 2007; Protzer et al., 2007). Developing effective anti-oxidant drugs with immunomodulatory effects for severe COVID-19 remains an active area of research. Overall, supporting the anti-oxidant defence mechanisms is a promising supportive approach for the treatment of SARS-CoV-2 infections.

2.10.2 From ancient remedies to modern medicine

Traditional medicinal plants have been utilised for thousands of years by indigenous cultures around the world as natural sources of remedies, as plant extracts were commonly used to treat illnesses when conventional medicine was less advanced (Yuan et al., 2016). Medicinal plants have evolved various chemical compounds for protection against microbial pathogens, viruses, and other stressors in the environment, and contain potent antiviral, anti-inflammatory, and immune-modulating alkaloids, terpenoids, and flavonoids (Vaou et al., 2021). With the onset of the COVID-19 pandemic, scientists looked to explore traditional plant remedies as potential sources of antiviral compounds that are effective against SARS-CoV-2, and built on previous research that had already identified some plant-derived drugs that work against other coronaviruses or respiratory viruses with analogous mechanisms (Jamal, 2022). High-throughput screening of medicinal plant extracts enables rapid identification of potential lead compounds for drug development (Latha, Hrishikesh, Shibani, et al., 2022). 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 (Zebeaman et al., 2023). Ongoing studies hope to discover multi-target botanical treatments through investigations of medicinal plants from different regions.

2.10.3 Chemical classes of plant metabolites with antiviral activity

Key chemical classes of plant metabolites that demonstrate antiviral and anti-inflammatory activity include polyphenols such as flavonoids, phenolic acids, and stilbenes, which are widely found in fruits, vegetables, and herbs, with many studies showing that they inhibit viral entry and replication and modulate inflammation (Jennings & Parks, 2020; Liu et al., 2023; Ullah et al., 2020). Terpenoids, the isoprenoid essential oil compounds in plants, contain potent antiviral and anti-inflammatory monoterpenes such as carvacrol and thymol (Masyita et al., 2022). Alkaloids, the nitrogen-containing compounds concentrated in specialised plant tissues, contain berberine from goldenseal, which has strong antiviral efficacy against respiratory viruses (Li & Wang, 2014). Glycosides derived from terpenoids, flavonoids, and alkaloids, such as the immunomodulating saponins from ginseng, have anti-influenza and antipneumovirus properties (De Rotte et al., 2014). Vitamins C, D, and E act as anti-oxidants to reduce the oxidative stress caused by viral infections and excessive inflammation. Immunostimulatory polysaccharides such as the beta-glucans in medicinal mushrooms boost cellular immunity against viruses (Kim et al., 2018). Anti-inflammatory omega-3 fatty acids from plants, including flaxseed and chia, inhibit viral replication of coronaviruses while suppressing damaging cytokine release (Liu et al., 2018). These phytochemicals demonstrate therapeutic potential individually or in combination for treating viral illnesses such as COVID-19 by targeting multiple stages of the viral lifecycle and modulating the cytokine storm reaction (Sruthi et al., 2023).

2.10.4 Plant selection and rationale

This section discusses the rationale behind the selection of *C. edulis*, *T. acutiloba*, and *T. riparia* for this study.

2.10.4.1 *Carissa edulis*

C. edulis is a multi-stemmed and multi-branched prickly evergreen shrub or small tree that can grow up to 6 m tall. It produces a lush canopy with near complete coverage of sunlight (Kafache et al., 2022). *C. edulis* belongs to the plant family Apocynaceae, which is also known as the dogbane family (Kafache et al., 2022). The plants that belong to this family are typically shrubs with thorns or spines that are located at the junction where the leaves meet the stem. When cut or damaged, the plant releases a milky latex substance (Kafache et al., 2022). The fruits are fleshy, oval, and range between 6 and 11 mm in diameter, with red to purple blackberries (Orwa et al., 2009). The fruits undergo a colour transformation; starting from red and gradually turning a deeper shade of purple until they reach a near-black colour when fully ripe. The fruits are nutritionally rich, have several health benefits, and can be used in various processed foods. Since they are readily available in the wild, people in rural areas often harvest and eat them as a source of essential nutrients (Djoueche, Azebaze & Dongmo, 2011; Ibrahim et al., 2010; Bagchi et al., 2004).

C. edulis is commonly found in warm, drier areas and thrives in deciduous forests, coastal thickets, bushes, and scrubs (Opande, 2022). The plant is widely distributed across various tropical regions in Africa and Asia, including the Arabian subcontinent (Burkill, 1985; Sofowora, 1980; Hutchinson & Dalziel, 1963). In traditional medicine, the plant has been used to treat various infections, including those affecting the reproductive, respiratory, and digestive systems (Burkill, 1985; Irvine, 1961; Sofowora, 1980; Omino & Kokwaro, 1993; Oliver, 1994; Dalziel, 1937; Irvine, 1961; Watt & Breyer-Brandwijk, 1962; Bentley, Bracket & Chapya, 1984; Gelfand et al., 1985; Bazeeb, 1991; Addis, Abebe & Urga, 2001; Giday, Asfaw, et al., 2003; Nedi, Mekonnen & Urqa, 2004; Jeruto et al., 2008). They have also shown a variety of pharmacological activities with several classes of chemical constituents being isolated, such as sesquiterpenes, cardiac glycosides, phenolic compounds, flavonoids, lignans, sterols, tannins, proanthocyanidins, chlorogenic acid derivatives, and 2'-hydroxyacetophenone (Pakrashi et al., 1968; Raina, Bhatnagar & Atal, 1971; Achenbach, Waibal & Addae-Mensah, 1983, 1985; Bentley et al., 1984; Mathuram et al., 1998; Rao et al., 2005; Festus et al., 2009).



Figure 2.8: *C. edulis* (Forssk.) Vahl.

Source: L. Balugani (1737-1770)⁷

2.10.4.2 *Tulbaghia acutiloba*

T. acutiloba is a fragrant flowering plant with characteristics that are typical of many monocots (Pooley, 1998; Fabian & Germishuizen, 1997). It is a perennial plant with non-woody stems that die annually and stores its nutrients underground in bulb-like structures, although for some South African species within this genus, these structures might be more accurately classified as corms or rhizomes (Pooley, 1998). *T. acutiloba* is a stemless plant with underground rhizomes that serve as storage organs. The plant typically grows between 15 to 60 cm tall and has long, narrow leaves that emerge from the base. The flowers have a distinct feature, namely a central structure called a “corona” that resembles a raised crown, or in some cases a fleshy ring. The number of flowers per plant can vary significantly; ranging from three to 40 (Fabian & Germishuizen, 1997; Pooley, 1998; Manning, 2009; Vlok & Schutte-Vlok, 2010). The pollen grains are described as having furrows and a net-like pattern reticulate. The plant relies on insects for pollination (Kubitzki, 1998). The seeds are black, flat, and elongated (Kubitzki, 1998).

⁷ See Appendix C for permission to use this material.

T. acutiloba's common names include wild garlic, of which the strong smell is a characteristic shared with garlic (Aremu & Van Staden, 2013). Indigenous to specific regions of South Africa, locals call the plant *ishaladi lezinyoka* in isiZulu, *sefothafotha* in Sesotho, and *lisela* in siSwati (Aremu & Van Staden, 2013). Traditionally, the plant's leaves, bulb, and roots are boiled and used in herbal remedies to treat various ailments, including dysentery, diarrhoea, high blood pressure, asthma, colds, and flu, and are also used as a lotion to wash wounds to prevent infections and to promote healing. There is, however, limited scientific research on the plant (Watt & Breyer-Brandwijk, 1962; Ncube et al., 2011).



Figure 2.9: *T. acutiloba*

Source: University of Oxford (2024)⁸

2.10.4.3 *Tetradenia riparia*

T. riparia is a woody plant with multiple stems and a bushy structure. It is well-branched and the stems are not rigid (Codd, 1983; Philipson & Steyn, 2008). *T. riparia* typically grows 1 to 3 m tall and possesses a pleasant smell (Bazihizina et al., 2016). The stems start with four ridges and a hairy texture, but become smooth and round as the plant matures (Martins et al., 2008). The bark of these shrubs is usually pale brown (Sabec

⁸ See Appendix C for permission to use this material.

et al., 2020). The leaves are attached to the stem by a stalk and have a shape that can broadly be described as egg-shaped to round (Martins et al., 2008). Additionally, the leaves have small hairs with glands that can secrete substances (Codd, 1983; Phillipson & Steyn, 2008). The single-layered outer epidermis of the plant's leaves share the presence of hair-like structures called trichomes with variations in cell size on opposite leaf surfaces (Martins et al., 2008). Flowering only occurs in areas with subtropical, temperate, and frost-free climates (Blythe et al., 2020). The flowering season is usually in winter (Zardeto-Sabec et al., 2020). Typically preferring locations at lower elevations and more humid environments, the plant can also thrive in diverse climates (Phillipson & Steyn, 2008; Njau & Ndakidemi, 2017; Godoy et al., 1999; Gazim et al., 2010; Zelnik et al., 1978; Powo, 2022). *T. riparia* is a popular medicinal plant in South Africa and is used to treat numerous health issues (Van Puyvelde et al., 1986; Van Puyvelde et al., 1987; Weaver et al., 1994; Campbell et al., 1997; Van Puyvelde & De Kimpe, 1998; De Melo et al., 2015; Lorenzi & Matos, 2008; Zelnik et al., 1978; Godoy et al., 1999; Gazim et al., 2010). As a commonly used medicinal plant, *T. riparia* has shown potent antimicrobial and antiviral activity (Panda et al., 2022).

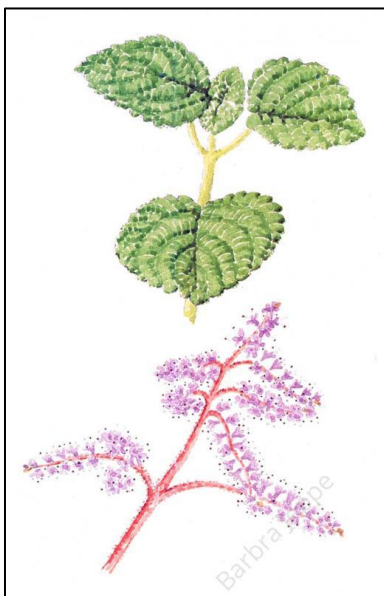


Figure 2.10: *T. riparia*

Source: Casabio (2023)⁹

⁹ See Appendix C for permission to use this material.

CHAPTER 3:

MATERIALS AND METHODS

3.1 MATERIALS

The materials used in this study comprised disposable materials, commercial kits, the H1299/hACE-2/E3 cell line, and a sample of SARS-CoV-2.

3.1.1 Disposable materials

Tables 3.1 and 3.2 list the disposable materials and solutions, chemicals, drugs, buffers, and media used in this study and their suppliers.

Table 3.1: Disposable materials used in this study and their suppliers

Disposables	Suppliers
Scrub suits or Tyvek coveralls	Firebreak SA (Pty) Ltd, South Africa
Disposable gowns (folio-dress)	HARTMANN South Africa, South Africa
Tyvek protective sleeves	Pienaar Bros North Pty Ltd, South Africa
Rubber boots	Hinterland, South Africa
Disposable shoe covers (overshoes)	Supra Healthcare (Pty) Ltd, South Africa
Nitrile examination gloves	Labex, South Africa
Red waste container bucket	Compass Medical Waste Services, South Africa
T25 and T75 cm ² cell culture flask	Thermofischer Scientific, United States of America (USA)
1.25 ml microcentrifuge tubes	Eppendorf, Germany
96-well plate	Thermofischer Scientific, USA

Table 3.2: Solutions, chemicals, drugs, buffers, and media used in this study and their suppliers

Buffers, medium, and solutions	Supplier
Sulphuric acid (H ₂ O ₄ S)	Merck, USA
Hydrochloric acid (HCl)	Merck, USA
Ferric chloride (FeCl ₃)	Merck, USA
Picric acid (C ₆ H ₃ N ₃ O ₇)	Sigma Aldrich, USA
Folin-ciocalteu reagent	Sigma Aldrich, USA
Sodium bicarbonate (CHNaO ₃)	Clicks, South Africa
Gallic acid (C ₇ H ₆ O ₅)	Thermofischer, USA
Bromocresol Green	Sigma Aldrich, USA
Chloroform (CHCl ₃)	Sigma Aldrich, USA

Buffers, medium, and solutions	Supplier
Berberine hydrochloride (C ₂₀ H ₁₈ ClNO ₄)	Sigma Aldrich, USA
2x phosphate buffered saline (PBS)	Gibco, USA
Roswell Park Memorial Institute medium (RPMI 1640)	Gibco, USA
Dimethyl sulfoxide	Thermofischer, USA
Trypsin/Ethylenediaminetetraacetic acid (EDTA)	Gibco, USA
Methanol (CH ₃ OH) and ethanol (C ₂ H ₆ O)	Merck, USA
Trypan blue	Sigma Aldrich, USA
Foetal bovine serum (FBS)	Gibco, USA
Non-essential amino acids (NEAA)	Gibco, USA
Sodium pyruvate (C ₃ H ₃ NaO ₃)	Gibco, USA
N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffer	Gibco, USA
L-Glutamine	Gibco, USA
DPPH	Thermofischer, USA
Triton X-100	Sigma Aldrich, USA
DNase/RNase-free water	Thermofischer, USA
Prednisolone (40 mg)	MediREX Pharmacy, South Africa
Sodium acetate (C ₂ H ₃ NaO ₂)	Thermofischer, USA
Sodium hydroxide (HNaO)	Thermofischer, USA
Rely+ on ® Virkon ® Tablets	LanXess, Germany
L-ascorbic acid (C ₆ H ₈ O ₆)	Sigma Aldrich, USA

3.1.2 Commercial kits

Table 3.3 lists the commercial kits used in this study and their suppliers.

Table 3.3: List of commercial kits used in this study and their suppliers

Enzymes and kits	Suppliers
Quick-RNA™ Viral Kit	Zymo Research, USA
Allplex™ 2019-nCoV assay	Seegene, USA
Human IL-6 ELISA Kit	ABclonal, USA
Human TNF-α ELISA Kit	ABclonal, USA
Human IL-8 ELISA Kit	ABclonal, USA
CellTiter 96® Aqueous One Solution Cell Proliferation Assay	Promega, USA

3.1.3 H1299/hACE-2/E3 cell line

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. The cell line was originally modified to express H2AZ histone variant with nuclear-labelled Yellow Fluorescent Protein (Sigal et al., 2007) and further constructed to overexpress human ACE2 (Cele,

2021). Briefly, this was done by generating lentivirus containing the human ACE2 gene. The lentivirus was generated by co-transfecting HEK-293T cells with the pHAGE2-EF1aInt-ACE2-WT plasmid along with several lentiviral helper plasmids. Supernatant containing the lentivirus was collected, filtered, and used to infect the H1299-H2AZ cells in the presence of polybrene. The transduced cells were then subcloned and screened by infection with SARS-CoV-2. The clones with the highest expression of ACE2 were expanded and then denoted as H1299-E3. 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, as described by the datasheet that was attached along with the cells.

3.1.4 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/ACE-2 cells at the Pathogen Research Laboratory at the University of the Free State. Briefly, samples were prepared by a 1:1 dilution with RPMI 1640 maintenance media (Gibco, USA), supplemented with 2% FBS, 2%L-glutamine (L-glut), 1% NEAA and 1% antibiotics, 1% $C_3H_3NaO_3$, and 1% HEPES, and then filter-sterilised using a 0.2 μ M nylon filter (GVS North America, USA). A 75 μ l 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 (CO₂) incubator with 5% CO₂. 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) (Reed & Muench, 1938). 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:

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

Formula 3.1: Multiplicity of infection (MOI) calculation

Prior to the infectivity experiments, a working virus suspension was prepared at a low MOI of 0.01. This MOI simulates *in vivo* exposure and infection kinetics by allowing single infections to establish without overwhelming the host cells. 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 (see Appendices A and B).

3.2 METHODS

This section discusses the study's methods in relation to plant collection and authentication, extraction of plant samples, preparation of extract dilutions, preliminary qualitative and quantitative phytochemical screening, cultivation and maintenance of the human cell line, cell viability and cytotoxicity activity on H1299 cells, antiviral screening of infectious SARS-CoV-2 virus, anti-inflammatory activity, and anti-oxidant activity.

3.2.1 Plant collection and authentication

Mature whole plants of *C. edulis*, *T. acutiloba*, and *T. riparia* were 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.

Table 3.4: Source and characteristics of plant samples used in the experiments

Plant name	Family name	Plant material used in extraction	Place of collection
<i>T. acutiloba</i>	Alliaceae	Rhizomes	Hartbeespoort, Gauteng
<i>C. edulis</i>	Apocynaceae	Leaves	Bloemfontein, Free State
<i>T. riparia</i>	Lamiaceae	Root	Mpumalanga, Limpopo

3.2.2 Extraction of plant samples

Powdered plant samples were weighed out to 10 g and prepared for extraction. The samples were extracted by maceration separately using 70% C₂H₆O 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. Formula 3.2 shows how the percentage yield was calculated, while Table 3.5 shows the yield of the crude extract.

$$\text{Percentage Yield (\%)} = \frac{\text{Mass of crude extract obtained (g)}}{\text{Mass of plant sample used (g)}} \times 100$$

Formula 3.2: Percentage yield calculation

Table 3.5: Yield of crude extract

Plant name	Yield (mg)	Percentage yield (%)
<i>T. acutiloba</i>	289.6 mg	2.89%
<i>C. edulis</i>	150.3 mg	1.50%
<i>T. riparia</i>	325.8 mg	3.25%

3.2.3 Preparation of extract dilutions

The extract samples were reconstituted in 1.5 ml microcentrifuge tubes in the same 70% C₂H₆O 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, cytotoxicity, and ELISA anti-inflammatory experiments, serial dilutions of stock solutions of extracts were prepared using culture medium initially at a broad full logarithmic scale dilution series, with concentrations of 1 000 µg/ml, 100 µg/ml, and 10 µg/ml (1:10, 1:100, and 1:1000). For experiments to determine the phytochemical content and anti-oxidant activity of the extracts, the samples were diluted with concentrations of 1 000 mg/ml, 100 mg/ml, and 10 mg/ml.

3.2.4 Preliminary qualitative and quantitative phytochemical screening

3.2.4.1 Preliminary qualitative phytochemical screening

The preliminary qualitative phytochemical screening involved flavonoids, saponins, tannins, and alkaloids.

(a) Flavonoids

Approximately 200 mg of plant material was diluted in 3 ml of ammonia (NH₃), followed by the addition of 1 ml of concentrated H₂O₄S (Merck, USA). Adding H₂O₄S acidifies the basic solution and creates an acidic environment for the reaction. Flavonoids are a diverse group of plant chemicals with a common structure and under acidic conditions, the structure undergoes a specific change that causes the molecule to be conjugated. This conjugation allows the detection of its presence, where a yellow colouration in each extract shows the presence of flavonoids (Harborne, 1973).

(b) *Saponins*

To detect the presence of saponins, 5 ml of distilled water was added to 500 mg of powdered plant samples and vortexed for 10 minutes. Saponins are a class of amphiphilic molecules that are both polar and non-polar. When saponins are mixed with water, the polar part interacts with water, while the non-polar part tries to avoid contact with water. This creates surface tension and allows the saponins to trap air bubbles, which forms a stable foam. The presence of saponins is indicated by the formation of a foam column. Confirmation is done by adding HCl afterwards. Saponin-based foam is relatively stable. However, adding a strong acid like HCl disrupts the saponin molecules and reduces the surface tension and the formation of a foam column that does not disappear with the addition of HCl (Merck, USA) was evaluated as positive for saponin (Sati & Kumar, 2015).

(c) *Tannins*

The presence of tannins is indicated by a colour change in a solution of FeCl_3 . Tannins are a complex group of plant polyphenols known for their taste and ability to bind to proteins. When FeCl_3 reacts with tannins, it forms a complex between the iron ions (Fe^{3+}) and the hydroxyl groups ($-\text{OH}$) present in the tannin structure. A brownish green or blue-black colouration in the solution is considered a positive test for tannins. Three to five drops of FeCl_3 solution (Merck, USA) were added to 1 000 mg/ml of plant aqueous extracts to observe the brownish green or blue-black colouration that is indicative of the presence of tannins (Trease & Evans, 2009).

(d) *Alkaloids*

Two ml of hexane (C_6H_{14}) was added to 100 mg of powdered plant samples in a test tube. C_6H_{14} is a non-polar solvent, which means it has no electrical charge separation and interacts poorly with water. Adding C_6H_{14} to the powdered plant sample aimed to remove non-polar fats, oils, waxes, and other lipophilic (compounds present in the plant) material. These non-polar compounds can interfere with the detection of alkaloids in later steps. The mixture was vortexed to facilitate interaction between the solvent and the plant material, which was then filtered to separate the C_6H_{14} extract (containing the removed non-polar compounds) from the remaining plant material in the test tube. This was

followed by the addition of 3 ml of 2% HCl (Merck, USA) to the filtrate. HCl is a polar solvent (water-based) that can dissolve ionic compounds such as salts of alkaloids. Most alkaloids are weak bases and react readily with acids to form water-soluble salts. This step aimed to extract the alkaloids from the plant material into the acidic water solution. The solution was then heated briefly and filtered again. As an additional step, the solution was heated briefly to enhance the extraction of alkaloids from the plant cells. Heat can increase the solubility of some alkaloids in the acidic water solution. The mixture was filtered once more to separate the acidic water extract (that hopefully contained the alkaloids) from the remaining plant material residue. Two to three drops of $C_6H_3N_3O_7$ (Sigma Aldrich, USA) were then added to the filtrate. $C_6H_3N_3O_7$ is a reagent that can form precipitates with various compounds, including some alkaloids. The formation of a yellow precipitate is indicative of the presence of alkaloids, according to the method described by Harborne (1973).

3.2.4.2 Preliminary quantitative phytochemical screening

The preliminary quantitative phytochemical screening involved total phenolic content and total alkaloid content.

(a) Total phenolic content

The content of the total phenolic compounds of the crude ethanolic extracts were determined spectrophotometrically using the Folin-Ciocalteu assay as described by Attard (2013), with minor modifications. An aliquot of 30 μ l of extracts with varying concentrations ranging from 25 to 1 000 μ g/ml were mixed with 150 μ l of freshly prepared Folin reagent (Sigma Aldrich, USA) and allowed to stand at 25 °C for five minutes, and thereafter 120 μ l of a concentration of 9.5 mg/ml of $CHNaO_3$ solution (Clicks, South Africa) was added to the mixture. The mixture was allowed to incubate at 25 °C for 90 minutes away from light, and absorbance was measured at 630 nm using a microplate spectrophotometer (Thermofischer Scientific, USA) and done in triplicate. $C_7H_6O_5$ (25 to 1 000 μ g/ml) (Thermofischer Scientific, USA) was used to prepare the standard curve (see Figure 3.1), and the level of phenolics was expressed in terms of gallic acid equivalents per gram of dried plant material (g GAE/g).

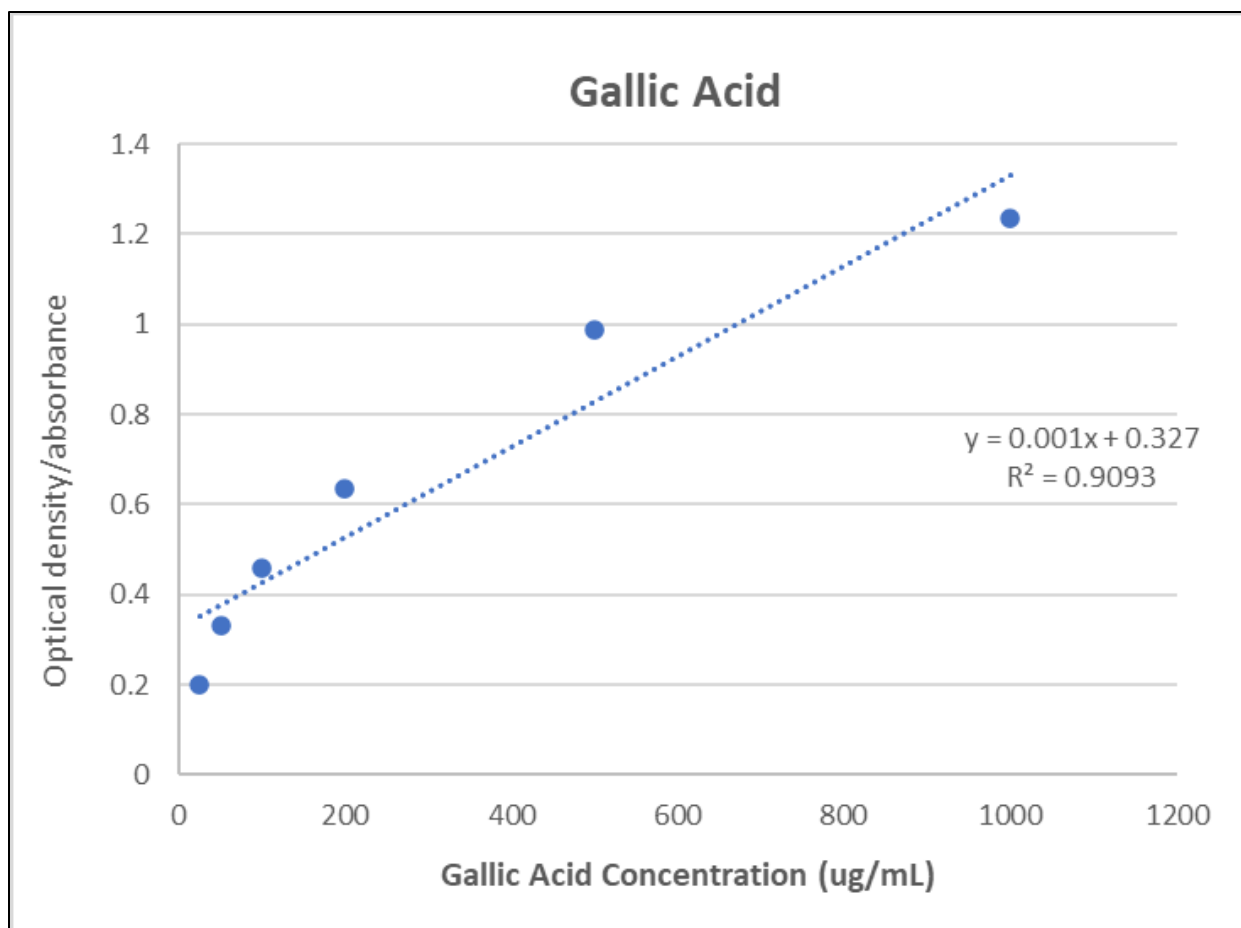


Figure 3.1: Gallic acid ($C_7H_6O_5$) standard curve for the determination of total phenolic content

(b) Total alkaloid content

The total alkaloid content of the plant extracts was determined as described by Li et al. (2015). An aliquot of 3 ml of extracts with varying concentrations ranging from 25 to 1 000 $\mu\text{g/ml}$ were vortex-mixed with 3 ml of 0.03% Bromocresol green (Sigma Aldrich, USA). After 30 minutes of incubation, 5 ml of CHCl_3 (Sigma Aldrich, USA) was added and vortexed once more for two minutes. $\text{C}_{20}\text{H}_{18}\text{ClNO}_4$ (Sigma Aldrich, USA) was used as a standard and was prepared by dissolving the powdered extract in PBS (Gibco, USA) with concentrations ranging from 25 to 1 000 $\mu\text{g/ml}$. All samples were prepared in triplicate and absorbances were read at 418 nm and the alkaloid content was expressed as grams of Berberine equivalents per gram of dried plant material (g BE/g).

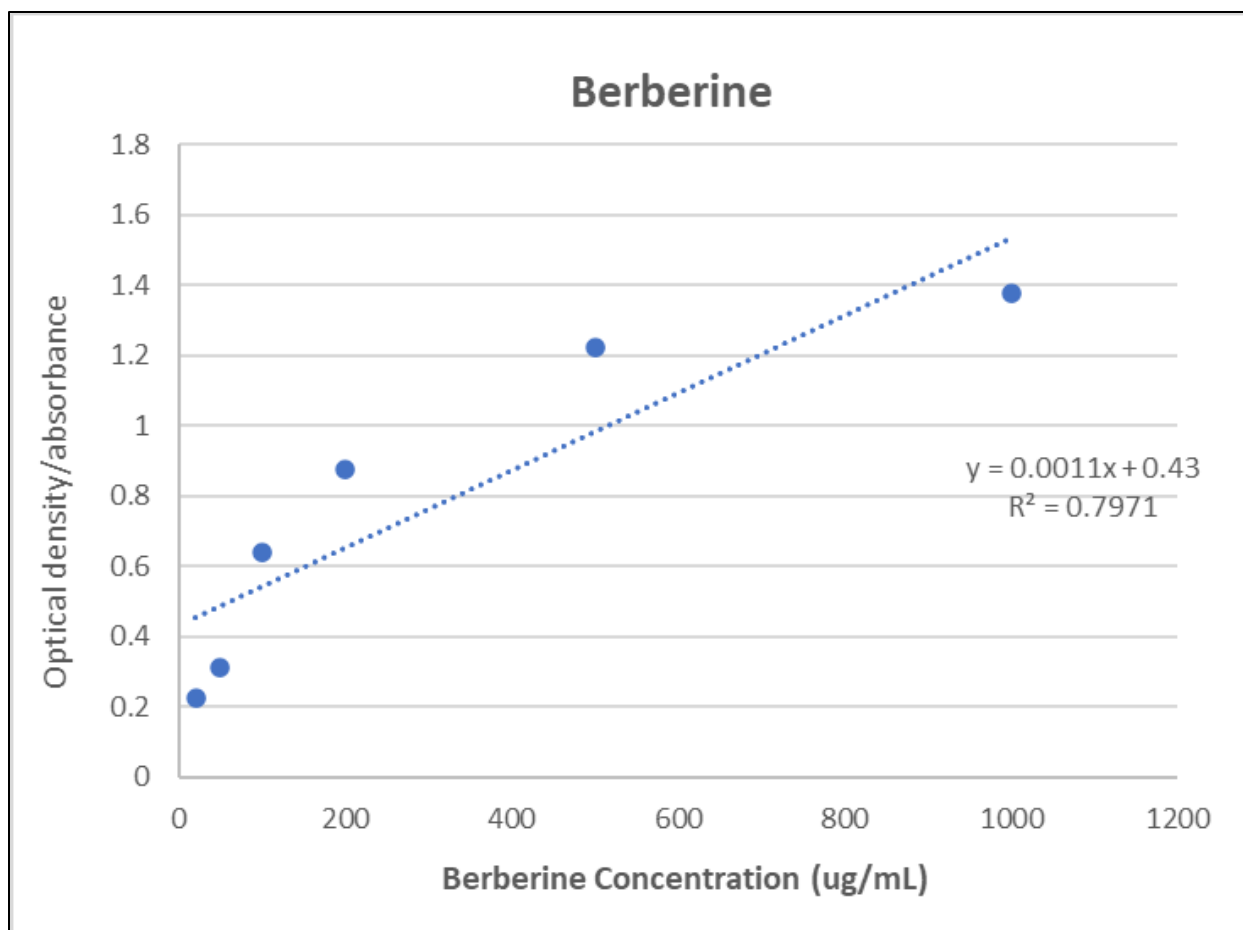


Figure 3.2: Berberine standard curve for the determination of total alkaloid content

3.2.5 Cultivation and maintenance of the human cell line

This process involved thawing and passaging of cells, cell counting, and cell freezing.

3.2.5.1 Thawing cells

To start a fresh culture from stock, 10 ml of growth media was pre-warmed to 37 °C using a water bath. Cryotubes were retrieved from the liquid nitrogen tank, 1 ml of warm media was added immediately, and transferred back into the 15 ml falcon tube. To wash away the cytotoxic dimethyl sulfoxide (DMSO) (Thermofischer Scientific, USA), the cells were centrifuged at 800 g for five minutes at room temperature, resuspended in fresh medium, and seeded into a T75 cm² cell culture flask (Thermofischer Scientific, USA).

3.2.5.2 Passaging cells

The cells were passaged every two to three days, preferably when the cells reached 80% to 90% confluency with a split ratio of 1:7 to 1:10 (Liao, Zhao, Qu et al., 2015; Zhao, Xue, Liu, et al., 2016; Chen, Lei, Zhang et al., 2023). When splitting, media and Trypsin/EDTA (Gibco, USA) were first warmed to 37 °C in the water bath. To passage cells, old medium was aspirated from the flask and adherent cells were washed twice with sterile 10 ml PBS. Afterwards, 2 ml of Trypsin/EDTA solution was added to detach the cells from the flask. The cells were incubated for three minutes at 37 °C and then taken out to observe if detachment was successful under microscopy. Approximately 10 ml of fresh medium was added to inactivate the trypsin and the cells were resuspended and cell clumps were broken down using a 2 ml pipette by gently pipetting up and down. An aliquot of the suspension was diluted in ratios between 1:7 and 1:10 with fresh medium in a new culture flask.

3.2.5.3 Cell counting

Cell counting was performed when seeding the cells for all experiments that required cell culture. The number of cells in a suspension was counted using the Countess II FL cell counter (Thermofischer Scientific, USA). Briefly, an aliquot of 10 µl of cell suspension prepared as described above was mixed with 10 µl of trypan blue to count viable cells. Non-viable cells were stained blue, while viable cells were not. The mixture was used to fill one side of the counting chamber and the viable cells were counted. To obtain the desired concentration of cells required for seeding, the following formula was used:

$$V = \frac{(\text{Desired cell density} \times \text{Desired Volume})}{\text{Current cell density (Hemocytometer result or viable cells)}}$$

Formula 3.3: Cell culture seeding volume calculation

3.2.5.4 Freezing cells

To prepare culture stocks, adherent cells were detached with Trypsin/EDTA and resuspended in media and centrifuged at 800 g for five minutes at 4 °C to pellet the cells.

The cell pellet was resuspended in ice-cold freezing medium and DMSO in a ratio of 1:9 (volume per volume), and the volume of freezing medium added was approximately five times the volume of the pellet. Aliquots of approximately 1.5×10^6 cells per cryotube were prepared and frozen at -80°C . In short, the cells were harvested and counted the cells to ensure they are in the optimal condition for freezing, and were then centrifuged to form a pellet and the supernatant was removed. The cells were then resuspended in a cryoprotectant medium, 10% DMSO in FBS to protect the cells during freezing. The cell suspension was aliquoted into sterile cryotubes, ensuring that each tube contains the desired number of cells. Cryotubes were then cooled in a controlled-rate freezing container, designed to provide a consistent cooling rate. The cryotubes were then transferred to a -20°C freezer for approximately 1 hour to gradually cool the cells to 4°C . This slow cooling rate helped to minimize thermal shock and allowed the cryoprotectant to penetrate the cells. For long storage, cells were transferred into liquid nitrogen after in a -80°C freezer.

3.2.6 Cell viability and cytotoxicity activity on H1299 cells

After passaging, 100 μl of the H1299 cells were seeded at a density of 2×10^5 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 cell monolayer was viewed under microscopy, and upon 80% to 90% confluence, the supernatant was aspirated and discarded and 100 μl of plant extracts at varying concentrations (25 to 1 000 $\mu\text{g/ml}$) were added in triplicate. Seventy percent $\text{C}_2\text{H}_6\text{O}$ was used as a vehicle control and cells-only controls were also included in triplicate. The cells were also treated with 1% Triton X-100 as a positive control (Acito et al., 2022).

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:

$$\text{Percent (\%) cell inhibition} = \frac{\text{Mean absorbance of extract}}{\text{Mean absorbance of untreated cells control}} \times 100$$

Formula 3.4: Percentage cellular inhibition calculation

Applying non-linear regression analysis, a standard curve was generated using Excel (Microsoft Office Professional Plus 2019; Microsoft, USA), the percentage cellular inhibition (Y-axis) was plotted versus the concentration of the extracts (X-axis) converted into a semi-logarithmic scale in µg/ml. From the curve-fitting analysis, the concentration of the extracts that reduces the viability of the cells by an IC₅₀ compared to the control (untreated cells) was calculated. This indicates the toxicity of the extracts to the cells, and represents the concentration at which half of the cell population is no longer viable.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD	STD		Ex1 1000	Ex1 1000	Ex1 1000	Ex2 200	Ex2 200	Ex2 200	Ex3 50	Ex1 50	Ex3 50
B	STD	STD		Ex1 500	Ex1 500	Ex1 500	Ex2 100	Ex2 100	Ex2 100	Ex3 25	Ex3 25	Ex3 25
C	STD	STD		Ex1 200	Ex1 200	Ex1 200	Ex2 50	Ex2 50	Ex2 50			
D	STD	STD		Ex1 100	Ex1 100	Ex1 100	Ex2 25	Ex2 25	Ex2 25			
E	STD	STD		Ex1 50	Ex1 50	Ex1 50	Ex3 1000	Ex3 1000	Ex3 1000			
F	STD	STD		Ex1 25	Ex1 25	Ex1 25	Ex3 500	Ex3 500	Ex3 500	Vehicle	Vehicle	Vehicle
G	STD	STD		Ex2 1000	Ex2 1000	Ex2 1000	Ex3 200	Ex3 200	Ex3 200	Untreated	Untreated	Untreated
H				Ex2 500	Ex2 500	Ex2 500	Ex3 100	Ex3 100	Ex3 100	"No cells"	"No cells"	"No cells"

Figure 3.3: Plate layout for cytotoxicity activity experiment

STD: 1% Triton X-100. Vehicle Control: C₂H₆O 70%. Negative Control: Untreated Cells. Ex1, Ex2 and Ex3: extract 1 (*T. riparia*), extract 2 (*T. acutiloba*), extract 3 (*C. edulis*)

3.2.7 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.

3.2.7.1 *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). Prior to the experiment, the H1299/ACE-2 cells were seeded at 2×10^5 cells per well in two 96-well plates and incubated overnight at 37 °C in a humidified CO₂ incubator. The following day, upon reaching 80% to 90% confluence, the seeded H1299 cells were used in two independent experiments. IC₅₀ 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.

IC₅₀ concentrations of the extracts were added before and after infection in two independent experiments. In the first experiment, the H1299/ACE-2 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 potential as prophylactics to prevent infection. Briefly, H1299/ACE-2 cells cultured on 96-well plates were treated with 100 µl of extracts, and then incubated at 37 °C for one 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/ACE-2 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. After incubation, the infected cells were treated with the extracts and re-incubated for 48 hours. Treating

the infected cells with extracts post-infection was to evaluate if the extracts would be able to suppress ongoing infection by potentially inhibiting intracellular viral replication/assembly.

Cell culture supernatant was collected at three time points post-infection for RNA extraction, at 24, 48, and 72 hours. The collection at these time intervals after initial infection was to evaluate the overall effects the extracts have on the inhibition of the SARS-CoV-2 replication cycle. The collected RNA supernatant would represent both intracellular RNA within infected cells, as well as extracellular/released virions that were shed into the media during the course of infection. The relative comparison of viral loads was thereafter quantified by RT-qPCR.

3.2.7.2 *Viral ribonucleic 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 (C₂H₆OS) 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 C₂H₆O 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.

3.2.7.3 Quantitative real-time polymerase chain reaction (PCR)

RNA-extracted samples were used undiluted and were subjected to quantitative RT-PCR using the CFX96 Real-Time Thermal Cycler (Biorad, USA). The Allplex™ 2019-nCoV assay (Seegene, USA) was used according to the manufacturer's protocol to perform RT-PCR. The kit reverse-transcribes RNA into cDNA and amplifies the cDNA with a thermal cycling reaction in one tube through by a real-time one-step enzyme. Reactions for RT-PCR were prepared, as shown in Table 3.6. PCR products are detected in real time using a fluorescent dye-based method.

Table 3.6: 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

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 3.7.

Table 3.7: 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.			

Fluorescent probes with attached fluorophores targeted specific sequences in the SARS-CoV-2 viral genome, as shown in Table 3.8. When incorporating itself in the amplified double-stranded DNA, fluorescence was emitted, read, and detected at 58 °C. As more double-stranded DNA molecules were generated through each PCR cycle, the fluorescence signal increased. The fluorescence observed is proportional to the amount of DNA present in the tubes. The instrument reports the amount of detected fluorescence as relative fluorescence units and cycle threshold (Ct) values.

Table 3.8: Fluorophores targeting specific sequences in the SARS-CoV-2 viral genome

Fluorophore	Fluorescence	Analyte
Fluorescein amidites	Blue	E gene
Hexachlorofluorescein	Green	Internal Control
Cal Red 610	Pink	RdRP gene
Quasar 670	Red	N gene

The Allplex 2019-nCoV assay from Seegene is a commercially available and validated one-step real-time RT-PCR kit for the qualitative detection of SARS-CoV-2. The manufacturer has already established the limit of detection through serial dilutions of quantified RNA standards; thus providing confidence in the analytical sensitivity of the assay. Precision was assessed through replicate testing of positive and negative controls

run with each batch of samples to demonstrate the reproducibility of results. Accuracy was ensured by the detection of an internal control in each reaction to verify that nucleic acid extraction and PCR amplification proceeded without inhibition.

Samples were confirmed as SARS-CoV-2 positive if at least one of the three gene targets (envelope, RdRp and nucleocapsid genes) were detected within the 45 cycles. The results were compiled and analysed using the 2019-nCoV Viewer from Seegene Inc. according to the manufacturer's instructions.

3.2.7.4 Generation of a viral loads standard curve

A standard curve was generated using Excel (Microsoft Office Professional Plus 2019; Microsoft, USA) using an already quantified SARS-CoV-2 RNA standard with documented C_t values and known copies/ml serving as reference for the determination of viral RNA copies/ml of the unknown samples. Serial 10-fold dilutions of the RNA standard were prepared, ranging from an estimated 10^6 copies/ml to 10^1 copies/ml. The C_t values were plotted against the log concentration of the DNA, and a linear correlation was determined, as shown in Figure 3.4. The SARS-CoV-2 N gene encodes the nucleocapsid protein, which is the most abundant viral protein produced during coronavirus replication. The nucleocapsid gene target was analysed for each of the samples and the controls at different time points.

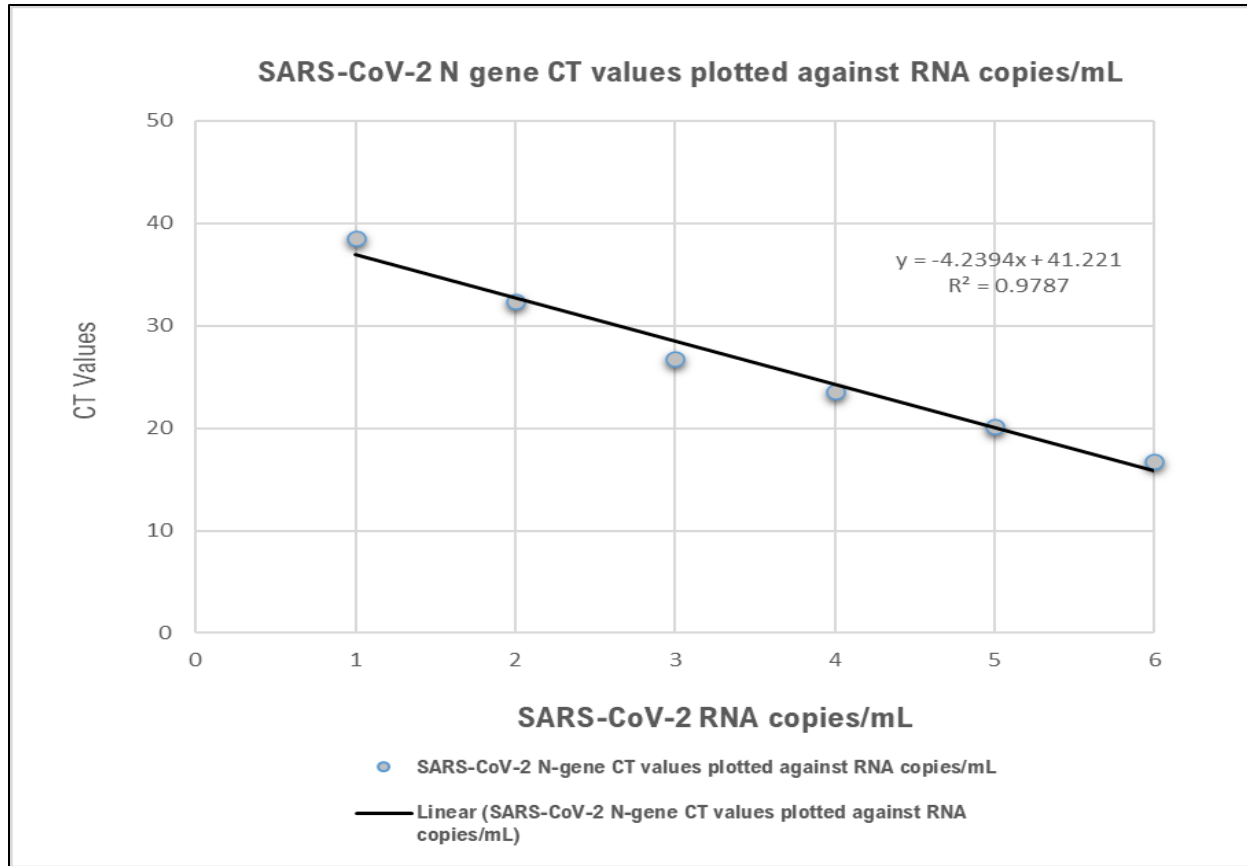


Figure 3.4: Standard curve for the quantification of SARS-CoV-2 RNA copies/ml

The C_t values obtained from the serial dilutions of the SARS-CoV-2 standard were plotted against the corresponding concentrations (RNA copies/ml) to generate the standard curve. The equation generated from the linear regression model was then used to determine the unknown viral loads corresponding to the C_t values of the unknown samples. The calculated parameters of the linear regression model include the R^2 value, which can be observed on the graph, and the linear equation was represented as $y = (\text{slope})x + (\text{y-intercept})$, where y is the C_t value and x is the concentration in RNA copies/ml. The equation can then be rearranged to calculate the unknowns as shown in Formula 3.5:

$$\text{Copy number} = \frac{\text{Observed } C_t - (41.221)}{-4.2394}$$

Formula 3.5: Calculation to determine unknown RNA copies/ml of samples

3.2.8 Anti-inflammatory activity

This section discusses the anti-inflammatory activity of expressed cytokines and the generation of ELISA standard curves.

3.2.8.1 *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 cells, as described by Dolci et al. (2022). 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. The H1299/ACE-2 cells were infected with SARS-CoV-2 as described earlier, either pre-treated or treated after infecting with non-cytotoxic concentrations of the extracts. Vehicle control and cells-only controls were also included in triplicate. Prednisolone 40 mg generic (MediRex Pharmacy, South Africa) was used as a positive control. It is an immunomodulator used for the treatment of a variety of inflammatory and immune-mediated disorders, especially in severe COVID-19. The plate layout is shown in Figure 3.5.

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 μ l 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 μ l of sample diluent was added in a blank well and 100 μ l 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% CO₂ 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 μ l of Concentrated Biotin Conjugate Antibody (100x) was added to 1 980 μ l of Biotin Conjugate Antibody Diluent to prepare a 2 000 μ l working solution of Biotin Conjugate Antibody Buffer. After incubation, the plates were aspirated and washed three times. To each well was then added 100 μ l of Working Biotin Conjugate Antibody, and then covered once more with new adhesive sealer and incubated for an hour.

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 μ l of Concentrated Streptavidin-HRP (100x) to 1 980 μ l Streptavidin-HRP Diluent to prepare 2 000 μ l working solution of Streptavidin-HRP Buffer. After incubation, the plates were aspirated and washed once more, and 100 μ l 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 μ l 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 μ l 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.

				Pre-Treatment				Post-Treatment				
	1	2	3	4	5	6	7	8	9	10	11	12
A	STD	STD		Ex1 (IC ₅₀) 24hrs	Ex1 (IC ₅₀) 48hrs	Ex1 (IC ₅₀) 72hrs		Ex1 (IC ₅₀) 24hrs	Ex1 (IC ₅₀) 48hrs	Ex1 (IC ₅₀) 72hrs		
B	STD	STD		Ex2 (IC ₅₀) 24hrs	Ex2 (IC ₅₀) 48hrs	Ex2 (IC ₅₀) 72hrs		Ex2 (IC ₅₀) 24hrs	Ex2 (IC ₅₀) 48hrs	Ex2 (IC ₅₀) 72hrs		
C	STD	STD		Ex3 (IC ₅₀) 24hrs	Ex3 (IC ₅₀) 48hrs	Ex3 (IC ₅₀) 72hrs		Ex3 (IC ₅₀) 24hrs	Ex3 (IC ₅₀) 48hrs	Ex3 (IC ₅₀) 72hrs		
D	STD	STD										
E	STD	STD		Vehicle 24hrs	Vehicle 48hrs	Vehicle 72hrs		Vehicle 24hrs	Vehicle 48hrs	Vehicle 72hrs		
F	STD	STD		Untreated 24hrs	Untreated 48hrs	Untreated 72hrs		Untreated 24hrs	Untreated 48hrs	Untreated 72hrs		
G	STD	STD		Media only 24hrs	Media only 48hrs	Media only 72hrs		Media only 24hrs	Media only 48hrs	Media only 72hrs		
H				PRED 24hrs	PRED 48hrs	PRED 72hrs		PRED 24hrs	PRED 48hrs	PRED 72hrs		

Figure 3.5: Plate layout for anti-inflammatory activity experiment

STD – Standard, extract 1 (Ex1) – *C. edulis*, extract 2 (Ex2) – *T. riparia*, extract 3 (Ex3) – *T. acutiloba*. Controls: vehicle control – 70% C₂H₆O, cells-only control, media only control, prednisolone.

3.2.8.2 Generation of enzyme-linked immunosorbent assay (ELISA) standard curves

A standard curve was generated using Excel (Microsoft Office Professional Plus 2019; Microsoft, USA) to quantify the unknown cytokines in the ELISA by employing already pre-quantified standards with known concentrations as reference. The cytokine standards had concentrations of 1000 pg/ml (TNF- α), 250 pg/ml (IL-8), and 100 pg/ml (IL-6), and were serially diluted in seven 10-fold increments in duplicate. The concentrations (x-axis) were plotted against the optical density absorbance values (y-axis) and a linear correlation was determined as shown in Figure 3.6. The expressed cytokine levels of the samples and controls were analysed at different time points.

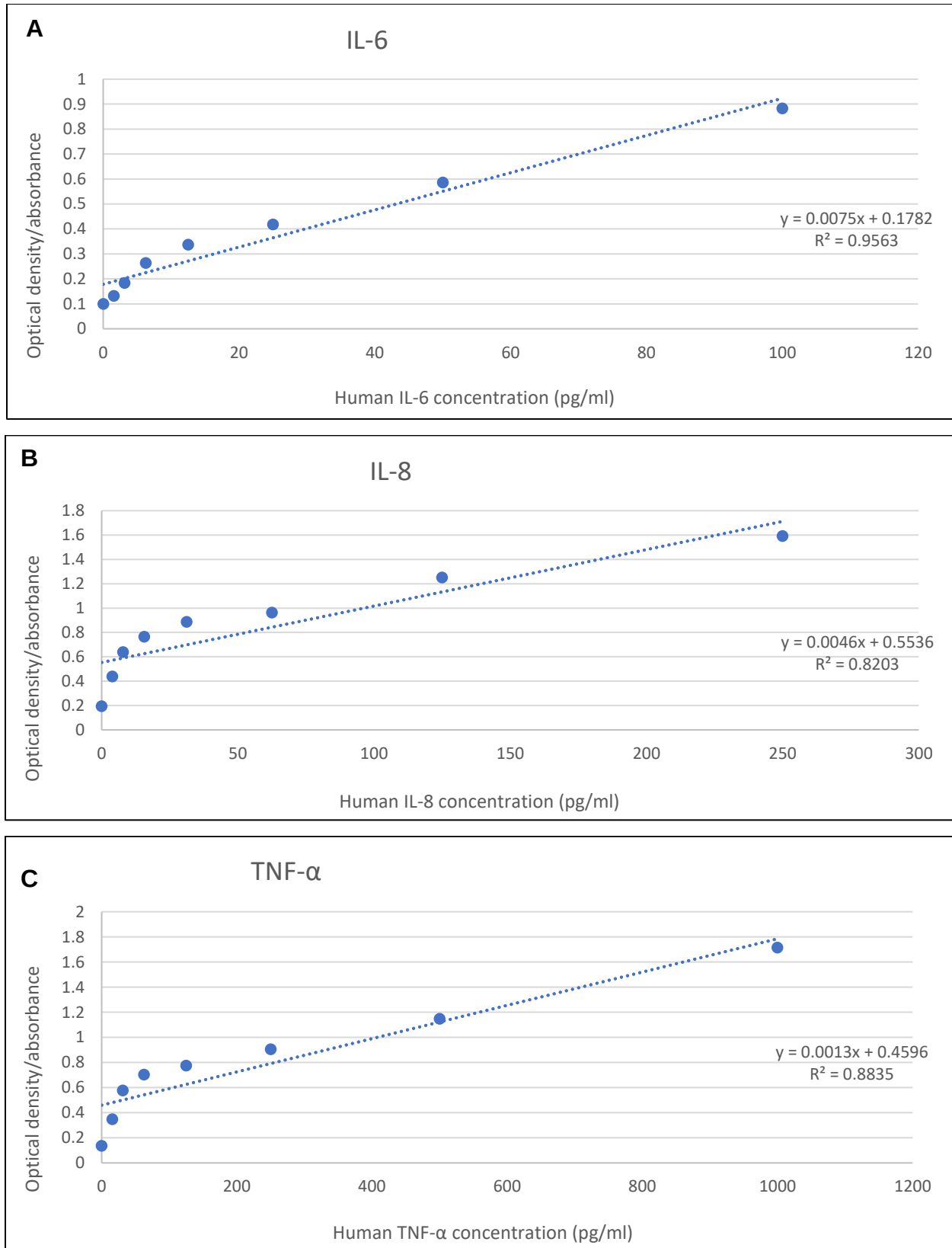


Figure 3.6: Standard curves for ELISA quantification of TNF- α , IL-8, and IL-6

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. The calculated parameters of the linear regression model include the R^2 values that can be observed on the graphs, and the linear equations are represented as $y = (\text{slope})x + (\text{y-intercept})$, where y is the absorbance value and x is the concentration in pg/ml. The equation can then be rearranged to calculate the unknown cytokine concentrations as shown in Formula 3.6:

$$\text{Cytokine concentration} = \frac{\text{Absorbance} - (\text{y-intercept})}{\text{slope}}$$

Formula 3.6: Unknown cytokine concentrations calculation

3.2.9 Anti-oxidant activity

To measure the anti-oxidant activity of the extracts, their free radical scavenging ability was determined using the DPPH assay method as described by Bertalaníč et al. (2012), with minor modifications. DPPH is a stable organic free radical, and anti-oxidants are known for their ability to scavenge free radicals and neutralise their activity. Organic free radicals are derived from an organic compound and, unlike most stable molecules, which have electrons paired together, an organic free radical possesses one unpaired electron in its outermost electron shell. This unpaired electron makes the molecule highly reactive because it seeks to form a pair with another electron to achieve stability. When DPPH interacts with an anti-oxidant, the anti-oxidant donates an electron to DPPH, which neutralises its free radical state. This process causes DPPH to lose its characteristic purple colour and become colourless or pale yellow. When measured at a wavelength of usually between 515 and 520 nm (Bandoniene et al., 2002; Pavlov et al., 2002; Gazi et al., 2004), this colour change translates to a decrease in absorbance. The greater the decrease in absorbance after the reaction with the extract, the more effective the extract is at scavenging free radicals, which signifies higher anti-oxidant potential.

For this experiment, a solution of 200 μM DPPH (Thermofischer Scientific, USA) was prepared in pure CH_3OH (Merck, USA). To prepare the acetate ($\text{C}_2\text{H}_3\text{O}_2^-$) buffer, a concentration of 50 mM $\text{C}_2\text{H}_3\text{NaO}_2$ (Thermofischer Scientific, USA) at pH 5.25 was made by titrating the acid with HNaO (Thermofischer Scientific, USA). A sample solution of 100 μl of extracts and $\text{C}_6\text{H}_8\text{O}_6$ standard solution at concentrations of 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 200 $\mu\text{g/ml}$, 500 $\mu\text{g/ml}$, and 1 000 $\mu\text{g/ml}$ were pipetted to the premixed assay solution of 1 000 μl of DPPH and 900 μl of $\text{C}_2\text{H}_3\text{O}_2^-$ buffer in 2 ml microcentrifuge tubes. The solutions were incubated in the dark at 25 $^\circ\text{C}$ for 60 minutes before 2 ml was pipetted into a cuvette and absorbance was measured at 520 nm (Beckmann Coulter, USA). DPPH 200 μM with 50 mM $\text{C}_2\text{H}_3\text{O}_2^-$ buffer was used as a blank and the absorbances of the samples were subtracted from the blanks. The percentage DPPH inhibition was calculated (see Formula 3.7) for each of the extracts and the standard, and a graph was plotted thereafter with the concentrations (x-axis) against the DPPH percentage inhibition values (y-axis).

$$\text{Percent (\%)} \text{ DPPH inhibition} = \frac{(\text{Mean abs Blank} - \text{Mean abs sample})}{\text{Mean abs of blank}} \times 100$$

Formula 3.7: Percentage DPPH inhibition calculation

CHAPTER 4:

RESULTS

4.1 QUALITATIVE AND QUANTITATIVE PHYTOCHEMICAL SCREENING

The phytochemical screening of *C. edulis*, *T. acutiloba*, and *T. riparia* extracts revealed the presence of the secondary metabolites flavonoids, saponins, tannins, and alkaloids. Several flavonoids and alkaloids possess anti-inflammatory properties, and one of the mechanisms involves inhibiting the production of inflammatory cytokines (Al-Khayri et al., 2022; Souto et al., 2011). The presence of both may explain the potential anti-inflammatory properties that the extracts have shown to possess.

Flavonoids, saponins, tannins, and alkaloids have shown antiviral activities against various viruses. Alkaloids have previously shown strong antiviral activity against SARS-CoV-2 (Majnooni et al., 2021) – the presence of which in *C. edulis* may explain its antiviral effects. It is important to note that the effectiveness of these compounds *in vivo* might differ from the observed laboratory studies. Further research is required to explore the potential of these secondary metabolites as antiviral agents.

Table 4.1: Phytochemical content of the plant extracts *C. edulis*, *T. acutiloba*, and *T. riparia*

Plant extract	Flavonoids	Saponins	Tannins	Alkaloids
<i>C. edulis</i>	+++	++	+	++
<i>T. acutiloba</i>	+++	++	+	+
<i>T. riparia</i>	+++	+	+	-

+++ = high presence, ++ = moderate presence, + = low presence, - = not detected

The phenolic and alkaloid contents of the plant extracts are shown in Table 4.2. The total phenolic content was the highest in *T. riparia* extracts, with intermediate and the lowest content in *T. acutiloba* and *C. edulis* extracts respectively. Compared to the other two plants, *T. riparia* emerges as a promising source for both total phenolics and total alkaloids. This makes it a good candidate for further research on its potential health benefits. Further studies need to be conducted to identify and quantify the specific types of phenolics and alkaloids present in order to provide a more complete picture.

Additionally, understanding the biological effects of these compounds would be crucial for any definitive conclusions.

Table 4.2: Phenolic and alkaloid composition of *C. edulis*, *T. acutiloba*, and *T. riparia* extracts

Totals	Treatment		
	<i>C. edulis</i>	<i>T. acutiloba</i>	<i>T. riparia</i>
Total phenols (g GAE/g)	4.13 ± 0.25	5.24 ± 0.62	12.57 ± 0.42
Total alkaloids (mg BE/g)	5.41 ± 0.24	6.61 ± 0.33	7.86 ± 0.18

Data are presented as the mean of the triplicate values ± standard deviation.

4.2 CELL VIABILITY AND CYTOTOXICITY ASSAY

The cytotoxicity of *T. riparia*, *T. acutiloba*, and *C. edulis* extracts on H1299 lung cancer cells was evaluated using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA). The positive control (1% Triton X-100) exhibited significant cell death with over 90% inhibition (>90% inhibition), confirming the assay's effectiveness in detecting cytotoxicity (see Figure 4.1). The vehicle control (C₂H₆O 70%) showed minimal cell death (<10% inhibition), which indicates that the solvent had no significant impact on cell viability. 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 4.2). Non-linear regression analysis was performed to fit the curves and to determine the IC₅₀ values, which represent the concentration required to inhibit cell viability by 50%.

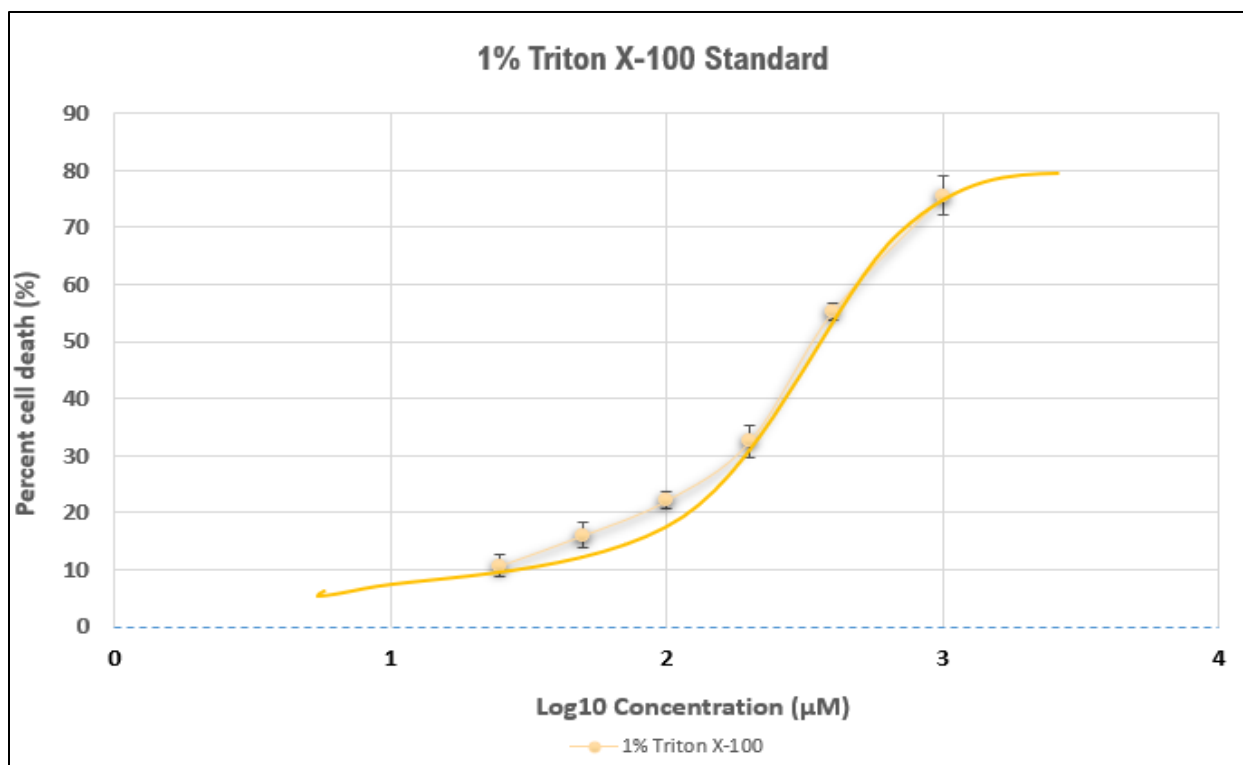
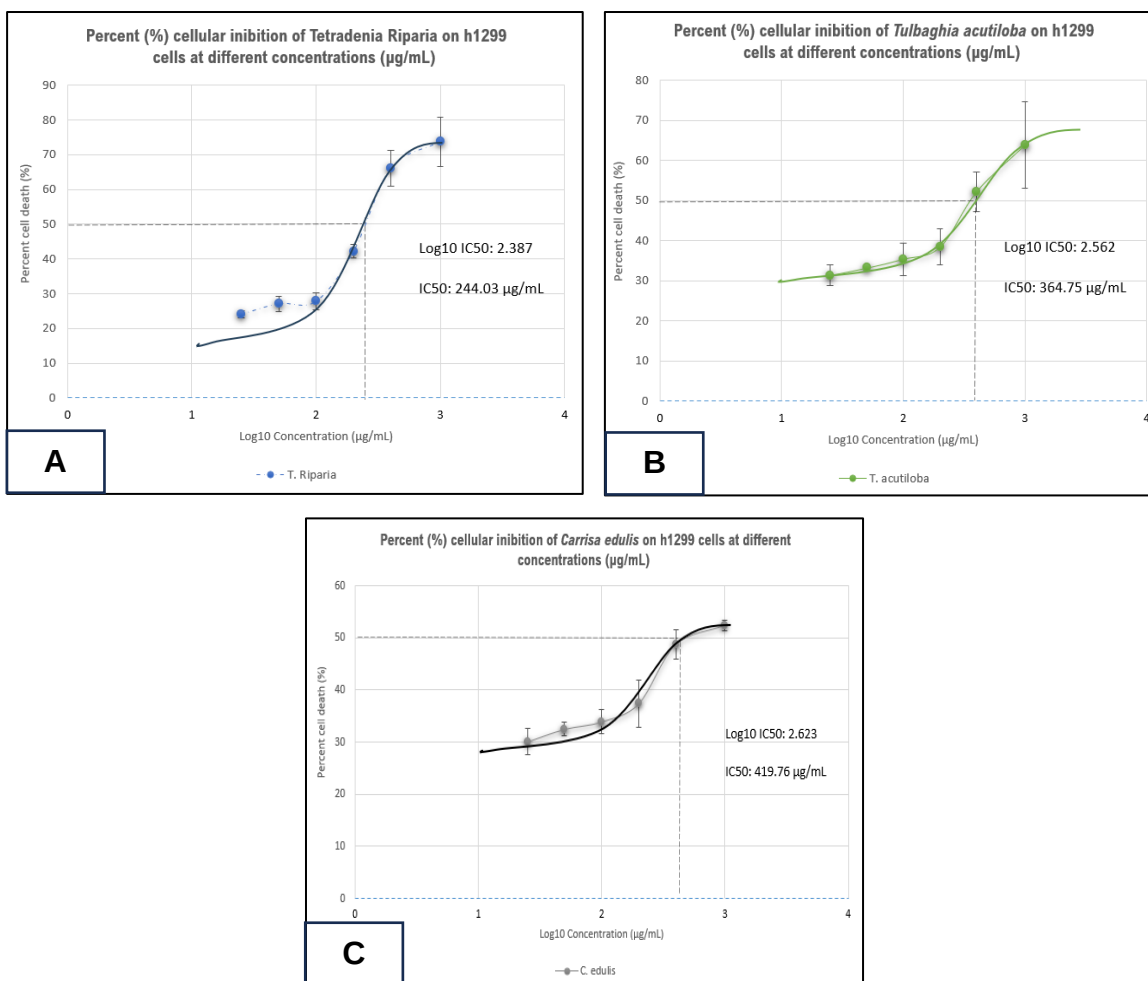


Figure 4.1: 1% Triton X-100 standard

The positive control (1% Triton X-100) exhibited significant cell death with over 90% inhibition (>90% inhibition), which confirmed the assay's effectiveness in detecting cytotoxicity.

Lower IC_{50} values indicate higher cytotoxicity, which means that the extract requires a lower concentration to inhibit cell viability by 50%. Based on the obtained IC_{50} values, *C. edulis* exhibited the lowest cytotoxicity on H1299-ACE-2 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.



Data are presented as the mean of the triplicate values and the $\pm$ standard deviation is represented by the error bars.

Figure 4.2: Dose-response curves of *T. riparia*, *T. acutiloba*, and *C. edulis* extracts on H1299 cells

4.3 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/ACE-2 cells were treated with IC₅₀ concentrations of the extracts an hour prior to virus infection to evaluate their potential prophylactic effects. In the second experiment, the H1299/ACE-2 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

are represented as nucleocapsid gene estimated viral load copies/ml, as shown in Tables E1 and E2 (see Appendix E).

All pre-treated and post-treated samples, including the controls, showed increasing viral loads over time, which indicated ongoing viral replication (see Figure 4.3). When pre-treated, at 24 hours, the plant extracts showed no significant difference compared to the control. At later time points, *C. edulis* and *T. riparia* displayed slightly lower viral loads than the control and *T. acutiloba*, which suggests a potential trend of reduced viral replication, but the difference was not substantial. Post-treatment with *C. edulis* displayed a significant reduction in viral load compared to the control at later time points, which suggests a possible antiviral effect. *T. acutiloba* and *T. riparia* also showed some reduction, but the effect was less pronounced than with *C. edulis*. Importantly, all groups, except the control, still had high viral loads at the final time point, which highlights the need for further investigation.

Fold change (FC) was used to provide a standardised method to compare viral loads between the treated and untreated cells. FC in the viral loads of SARS-CoV-2-infected cells treated with extracts was evaluated relative to the baseline viral load of infected cells without any plant extract treatment at different times post-infection (see Figure 4.4). An FC threshold of 2 was established, and any change in viral loads by greater than 2 ($FC > 2$) was considered as being indicative of antiviral activity by the extracts rather than random fluctuations in the experiment, as described by Litabe (2020). This means that a decrease in viral load at least twice the level observed in the control groups likely resulted from extract activity and not experimental variations. Greater than two-fold decreases in viral loads were seen in all the plant extracts, especially when the extracts were pre-treated before infection with the virus. This effect was not sustained at later time points at 48 and 72 hours as observed with the FCs. The most effective extract in decreasing viral load after post-treatment was *C. edulis*, which was shown to be more effective in suppressing viral loads over the three-day period. Overall, the data suggest that *C. edulis* might have a promising effect, while the effects of *T. acutiloba* and *T. riparia* were less pronounced. However, further research is needed to confirm these observations and to understand the mechanisms of action.

Across all conditions, viral suppression was observed when the extracts were pre-treated prior to infection, which warrants further investigation. The vehicle and untreated controls, without plant extract treatment, showed an increase in viral load over time, which confirms that the observed increase in viral load reflects the natural course of viral replication in the absence of any interventions. Overall, these findings highlight the potential antiviral activity observed in the tested plant extracts against SARS-CoV-2 in this experiment. Further investigation with different concentrations, extended time points, and additional assays to investigate the mechanisms by which the extracts might have interacted with the virus are crucial to draw definitive conclusions about their potential antiviral properties.

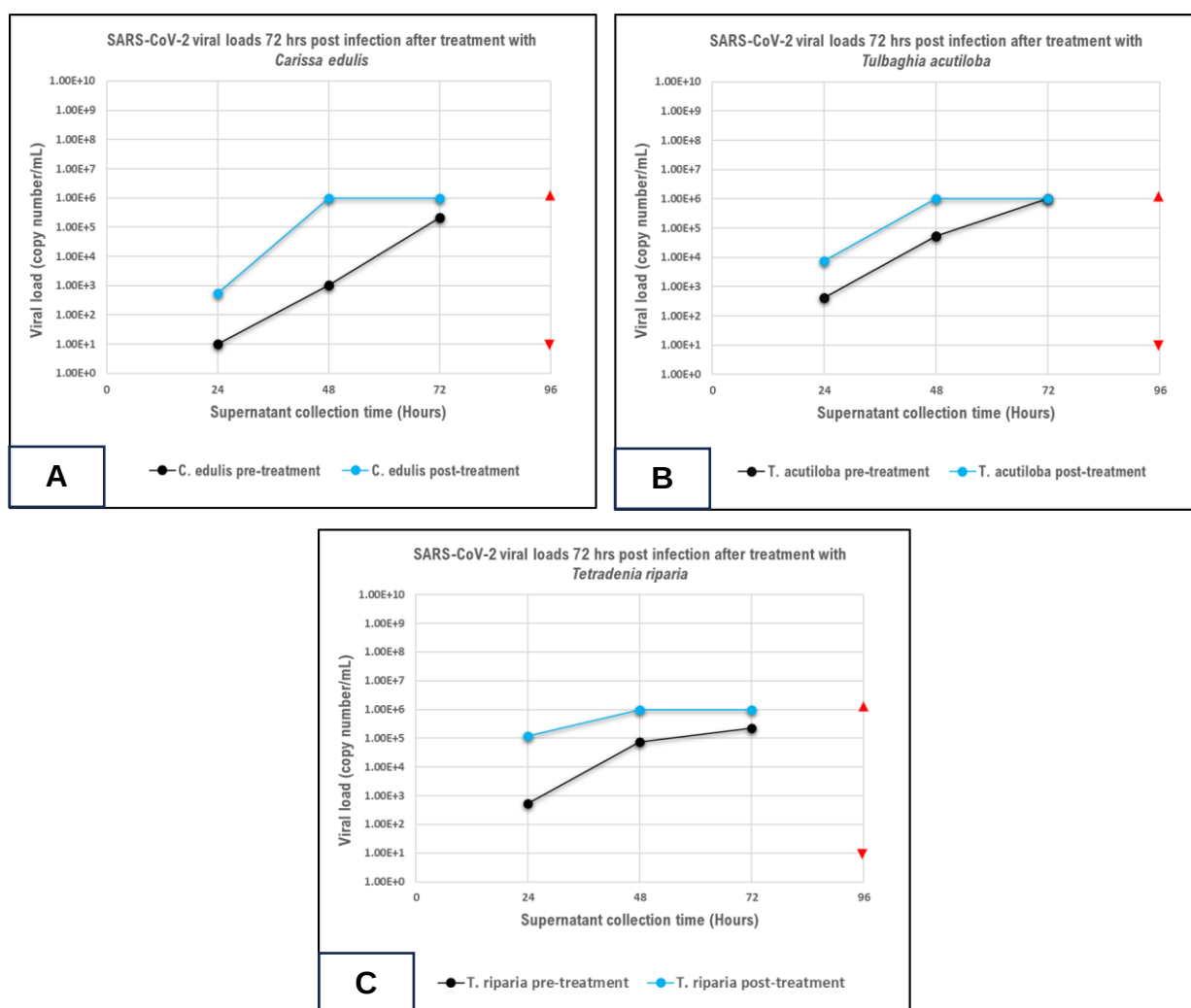


Figure 4.3: A-C: SARS-CoV-2 viral loads 72 hours post-infection after treatment with IC₅₀ concentrations of plant extracts

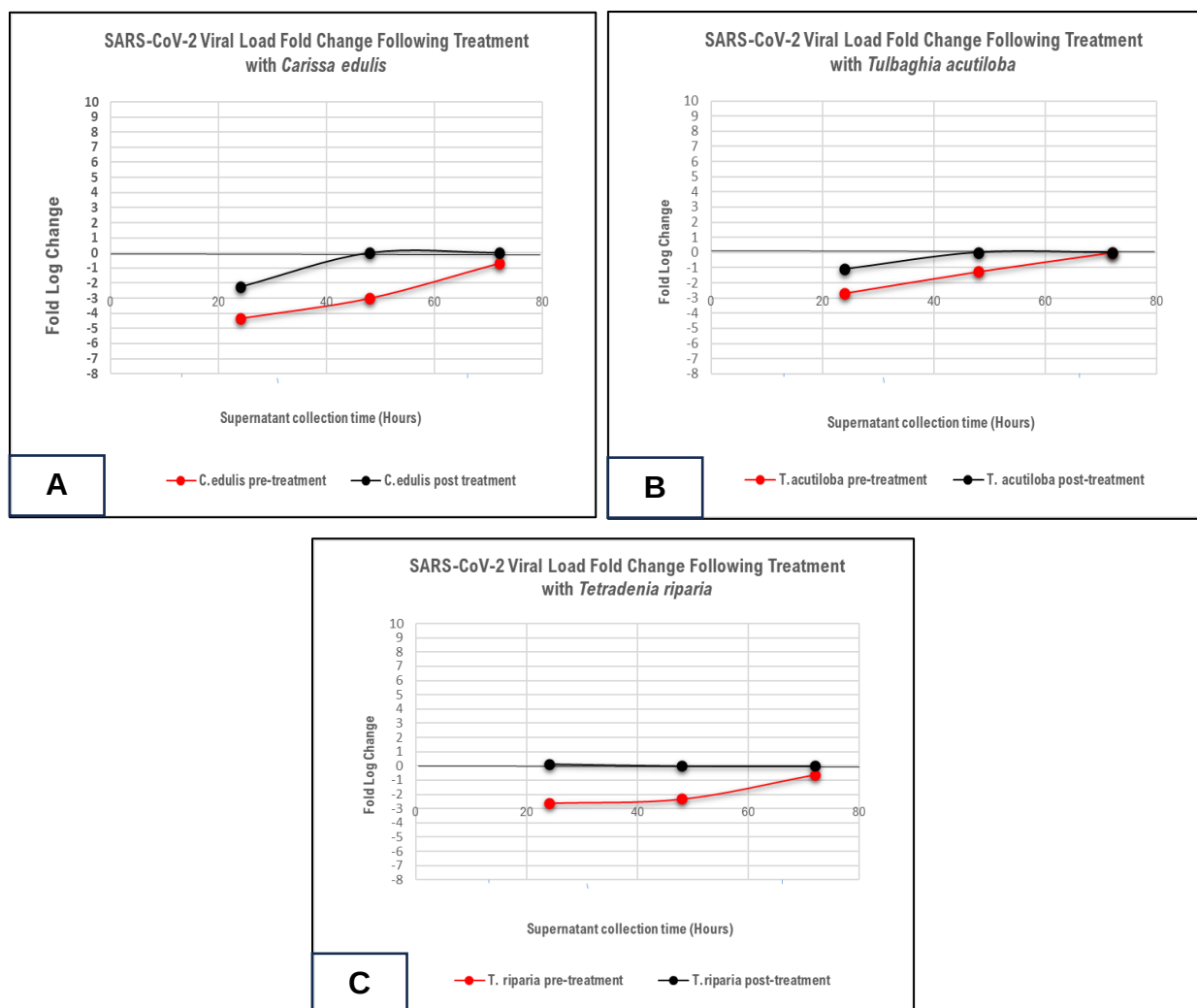


Figure 4.4: A-C: SARS-CoV-2 viral load fold change (FC) following treatment with IC₅₀ concentrations of plant extracts relative to the untreated control

4.4 ANTI-INFLAMMATORY ACTIVITY

The concentrations of the three cytokines (IL-6, IL-8, and TNF- α) were measured in cell culture supernatant after treatment with the plant extracts. This included pre-treatment with plant extracts (*C. edulis*, *T. acutiloba*, and *T. riparia*), post-treatment with plant extracts, vehicle control, untreated cells, no-cells control, and positive control (prednisolone). The measurements were taken at three different time points post-infection (24, 48, and 72 hours) and cytokine concentrations were calculated in pg/ml (see Tables F1 and F2 in Appendix F).

The untreated cells group showed the highest levels of all three cytokines across all time points, which indicated an ongoing inflammatory response. Prednisolone significantly reduced the levels of all three cytokines compared to untreated cells, which confirmed its anti-inflammatory effect. At the 48- and 72-hour time points, the cytokine concentrations were not detectable and were observed to be out of the detectable range, for both IL-8 (between 3.9 and 250 pg/ml) and TNF- α (between 15.6 and 1 000 pg/ml), as specified by the manufacturer. The no-cells control values were near zero for all cytokines, which confirmed the absence of cells, potential contaminants, and non-specific signals.

Cytokine secretion can vary naturally, even in healthy cells. Non-specific cytokine secretion was a factor that was taken into consideration as it can occur due to several non-experimental reasons as well. FC was used to compare the cytokine levels between the treated and untreated cells. FC in cytokine concentrations of SARS-CoV-2-infected cells treated with extracts were evaluated relative to the baseline secretion levels of the infected cells without any plant extract treatment at different times post-infection (see Figures 4.5 to 4.7). A threshold of 2 was therefore established, and any change in cytokine levels by greater than 2 ($FC > 2$) was considered as being indicative of anti-inflammatory activity by the extracts. Greater than two-fold decreases in cytokine expression were seen in all the plant extracts, especially in IL-6 and TNF- α at later time points post-infection.

Overall, the three extracts showed general decreases in cytokine levels over time compared to the untreated cells control. This largely suggests possible mild anti-inflammatory effects, and potential resolution of the inflammatory response or degradation of the cytokines over time. As a single experiment, further studies are needed to confirm these findings and to explore the mechanisms of action of the plant extracts. Overall, the results suggest that *C. edulis*, *T. acutiloba*, and *T. riparia* might possess some anti-inflammatory properties, but further research is needed to fully understand their potential therapeutic effects.

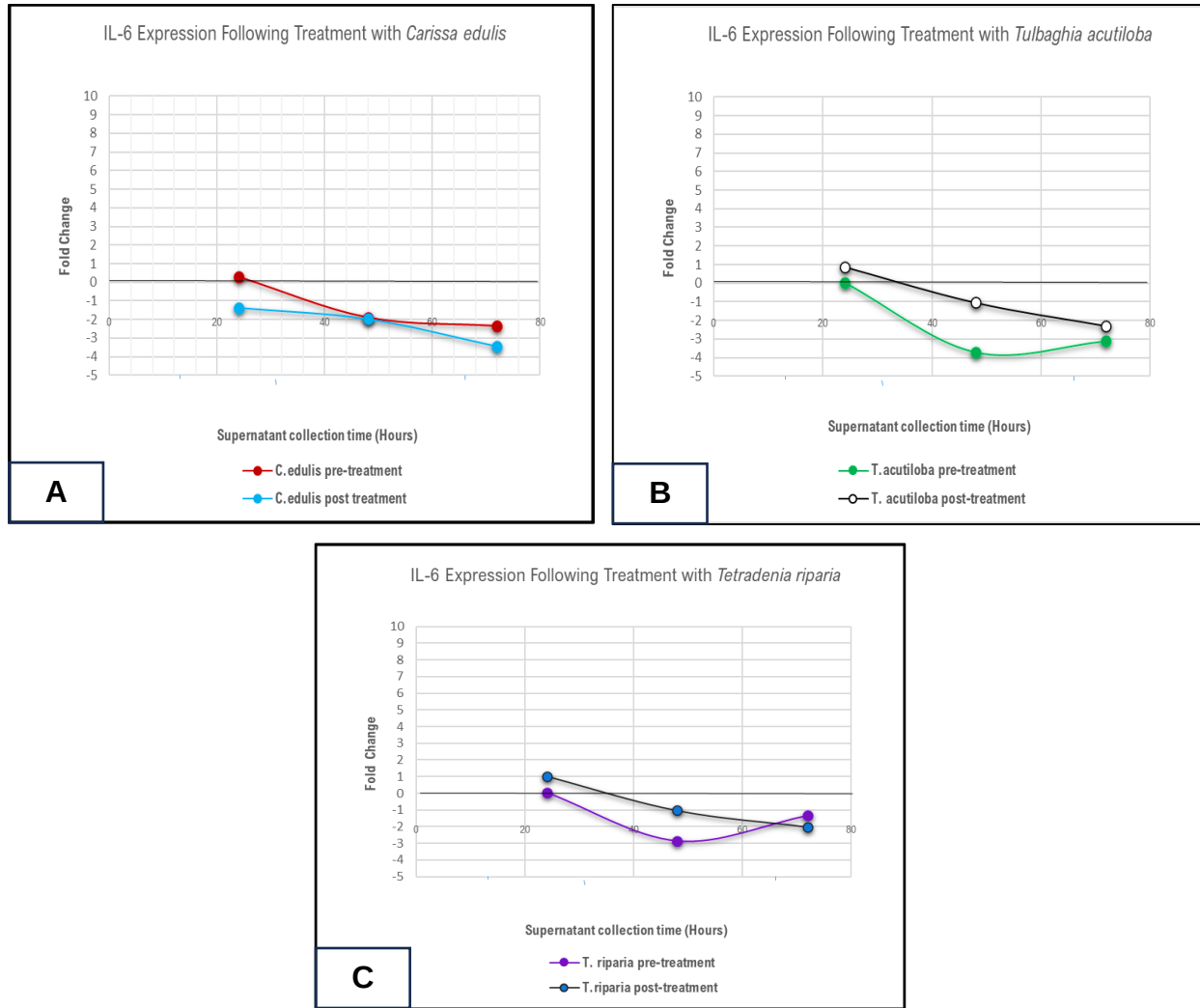
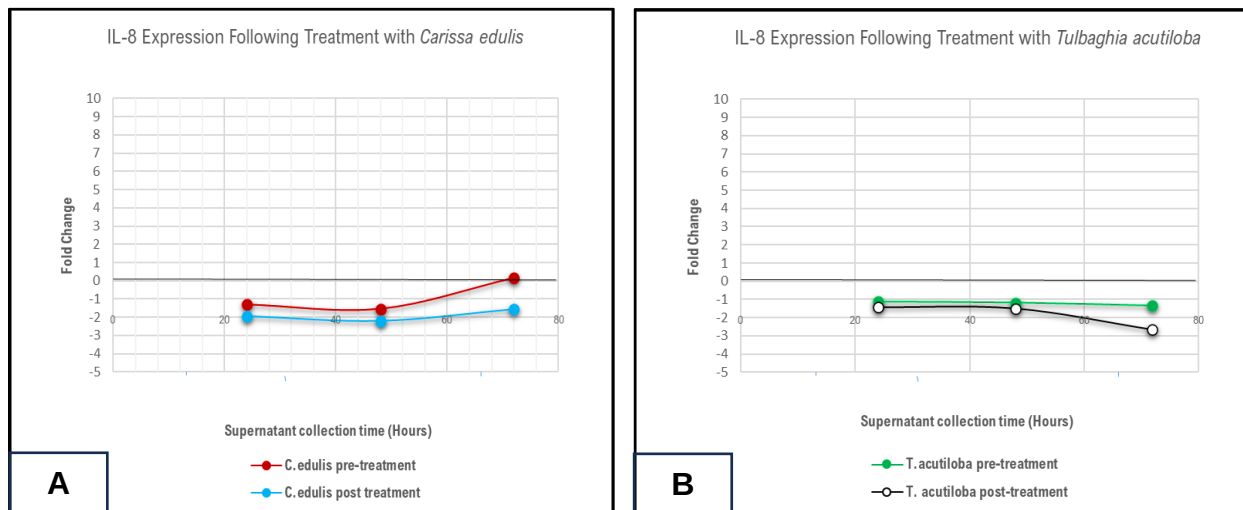


Figure 4.5: FC in concentration levels of IL-6 following treatment with the extracts



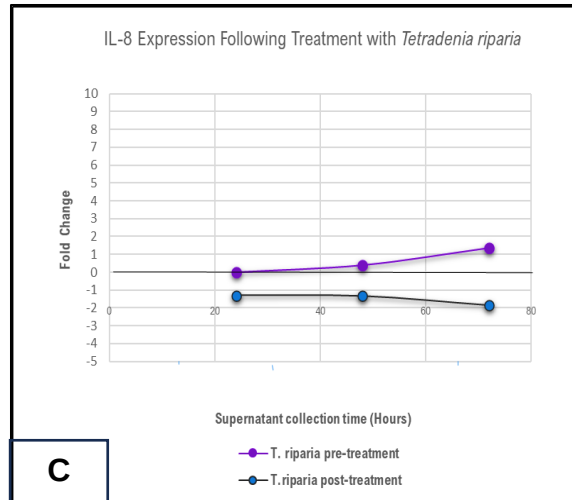


Figure 4.6: FC in concentration levels of IL-8 following treatment with the extracts

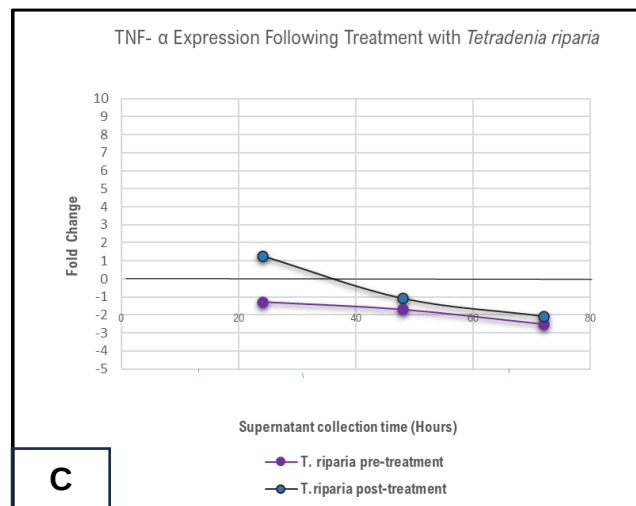
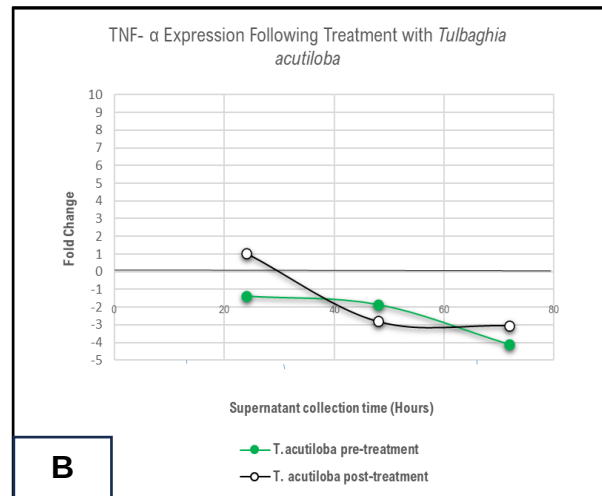
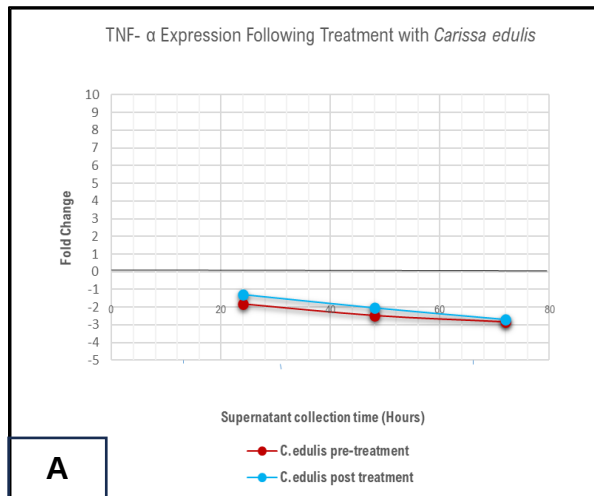


Figure 4.7: FC in concentration levels of TNF- α following treatment with the extracts

4.5 ANTI-OXIDANT ACTIVITY

T. riparia extracts exhibited the strongest radical scavenging activity, followed by *C. edulis* and *T. acutiloba* in decreasing order. Comparatively, the three plant extracts had lower SC₅₀ values as compared to the ascorbic acid standard. These findings could be attributed to the presence of phenolic compounds such as flavonoids and tannins. This is supported by the negative correlation between DPPH IC₅₀ values with the phenolic content of the extracts, which suggests a low effective concentration (i.e., high potency) in extracts with high contents of phenolics, as shown in Table 4.2. Dobrinás et al. (2021) reported that the anti-oxidant activity is highly correlated with the total polyphenol content of extracts. Polyphenols are a large group of natural compounds with chemical structures that are rich in -OH attached to aromatic rings. The -OH in polyphenols can act as electron donors by donating an electron to free radicals and stabilising them. The structure of many polyphenols allows them to delocalise the unpaired electron they receive from the free radical. This further reduces the damaging potential of the free radical. Even though the DPPH assay does not perfectly mimic the *in vivo* environment, it is still considered a valuable tool because the ability of an extract to donate electrons to DPPH is believed to be a good indicator of its general ability to counteract free radicals and act as an anti-oxidant. These results therefore demonstrate that the three extracts have anti-oxidant properties that perform better than the ascorbic acid standard and require lower concentrations (234 µg/ml for *C. edulis*, 158 µg/ml for *T. acutiloba*, and 144 µg/ml for *T. riparia*) to demonstrate 50% anti-oxidant activity as compared to 288 µg/ml for C₆H₈O₆.

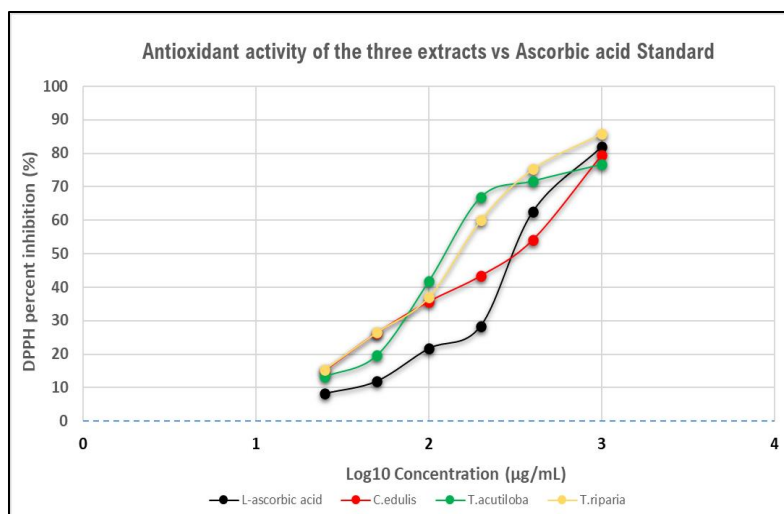
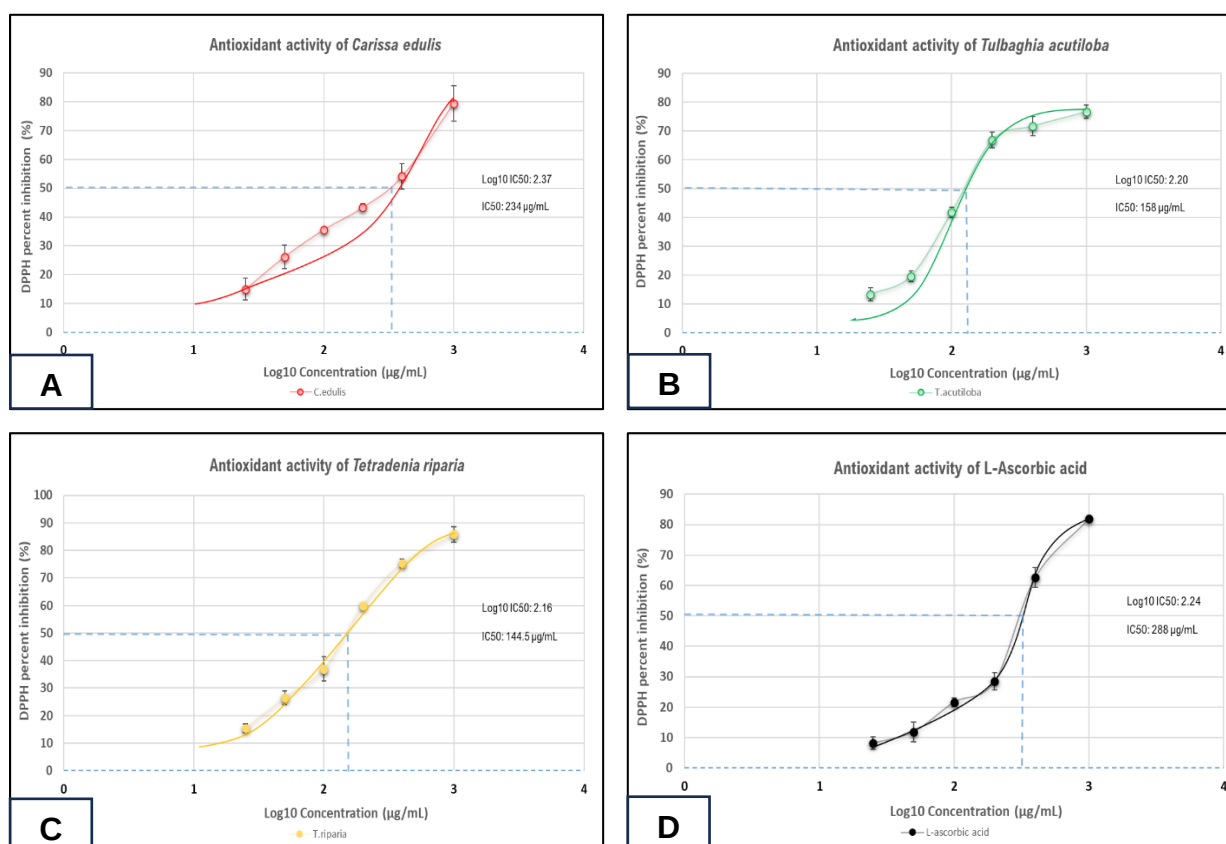


Figure 4.8: Comparison of the anti-oxidant activity of the three plant extracts *C. edulis*, *T. acutiloba*, and *T. riparia* versus ascorbic acid



Data are presented as mean of the triplicate values and the $\pm$ standard deviation is represented by error bars.

Figure 4.9: Dose-response curves of *T. riparia*, *T. acutiloba*, and *C. edulis* extracts on DPPH inhibition

CHAPTER 5:

DISCUSSION AND CONCLUSIONS

5.1 INTRODUCTION

Vaccines remain the primary approach to preventing severe illness, hospitalisation, and death from COVID-19. Multiple effective vaccines are available, and booster shots are recommended to maintain protection. No single treatment is a cure-all for COVID-19 and the best course of treatment depends on the severity of the illness, individual patient factors, and the specific circulating viral strains. Early diagnosis and intervention are crucial for maximising treatment effectiveness. The main objective of this study was to explore the *in vitro* antiviral potential of three plant extracts, namely *T. riparia*, *T. acutiloba*, and *C. edulis*, against SARS-CoV-2. The investigation aimed to assess the impact of these extracts on different stages of the viral infection cycle (prophylactic and post-infection treatment). Key observations from the study included that all treated and control groups displayed increasing viral loads over time, which indicated ongoing viral replication.

5.2 FINDINGS

Pre-treatment with the extracts showed a potential trend towards reduced viral replication compared to the control, particularly for *C. edulis* and *T. riparia* at later time points. Post-treatment with *C. edulis* displayed a significant reduction in viral load compared to the control at later time points, which suggests a possible antiviral effect for *T. acutiloba*, and *T. riparia* also showing some reduction, but the effect was less pronounced. These findings suggest a potential antiviral effect, particularly for *C. edulis*. However, several points warrant further investigation, including the limited sustained effect of the extracts, as the initial reduction in viral load observed with pre-treatment and post-treatment with *C. edulis* needs further exploration to understand if it can be sustained or enhanced. Mechanism-of-action studies are also needed to elucidate the mechanisms by which

these extracts might be interfering with the virus and evaluating the effects of different extract concentrations might identify more potent antiviral dosages.

Monitoring viral loads beyond 72 hours can also provide insight into the long-term effects of the extracts, as well as complementary assays investigating the impact on viral attachment, entry, or replication. Overall, this study highlights the potential of *C. edulis*, *T. acutiloba*, and *T. riparia* extracts as candidates for further investigation and development as antiviral agents against SARS-CoV-2.

5.3 SUMMARY AND SUGGESTIONS FOR FURTHER RESEARCH

In addition to direct antiviral effects, the anti-inflammatory activity of the extracts was also evaluated by testing their ability to inhibit SARS-CoV-2-induced inflammatory responses. The study investigated the effects of pre-treatment and post-treatment with the extracts on the production of pro-inflammatory cytokines (IL-6, IL-8, and TNF- α) by infected cells. Untreated SARS-CoV-2-infected cells displayed the highest levels of all three cytokines (IL-6, IL-8, and TNF- α) across all time points, which indicated an ongoing inflammatory response. Prednisolone, a known anti-inflammatory drug used as an alternative treatment for COVID-19 (Ledford, 2020; RECOVERY Collaborative Group et al., 2020; NDoH, 2021; WHO REACT Working Group et al., 2020), which served as a positive control, significantly reduced the levels of all three cytokines. Cytokine concentrations, particularly IL-8 and TNF- α , became undetectable at later time points (48 and 72 hours) due to limitations of the assay's detection range. Compared to untreated controls, all three extracts generally showed a decrease in cytokine levels, particularly IL-6 and TNF- α , at later time points. These findings suggest that the plant extracts might have some anti-inflammatory potential; however, several points warrant further investigation, such as that the observed reductions in cytokine levels were not substantial, which suggests a possible mild anti-inflammatory effect. Mechanism-of-action studies are also needed to elucidate the mechanisms by which these extracts might be modulating the inflammatory response. Further studies with a larger sample size and a wider range of extract concentrations are required to confirm these observations and identify potentially more potent anti-inflammatory dosages.

The anti-oxidant potential and phytochemical composition of the extracts were also evaluated. All three extracts exhibited free radical scavenging activity, with *T. riparia* demonstrating the strongest effect, followed by *C. edulis* and *T. acutiloba*. The extracts displayed lower IC₅₀ values compared to the ascorbic acid standard, which indicates potentially higher potency. This anti-oxidant activity could be attributed to the presence of phenolic compounds (flavonoids and tannins) detected in the extracts (Pietta, 2000). The negative correlation between DPPH IC₅₀ values and total phenolic content supports this link, which suggests that extracts that are richer in phenolics have a lower effective concentration (higher potency) (Kumar, Sandhir & Ojha, 2014). The anti-oxidant activity of the extracts, particularly *T. riparia*, is consistent with previous studies on similar plant species (Fernandez et al., 2017), as is for *C. edulis* and *T. acutiloba* (Muruthi et al., 2023; Isaiah et al., 2020). Interestingly, the anti-inflammatory effects observed in the study aligns with previous reports of flavonoids inhibiting inflammatory cytokine production (Borghi et al., 2018; Verri et al., 2012).

Additionally, the anti-oxidant activity might correlate with the observed anti-inflammatory effects since free radicals contribute to inflammation (Halliwell & Gutteridge, 2007; Droge, 2002; Young & Woodside, 2001; Valko et al., 2007). Qualitative phytochemical analysis of the plant extracts revealed the presence of flavonoids, saponins, tannins, and alkaloids in all three, similar to previous reports (Isaiah et al., 2020; Opande, 2022; Panda et al., 2022). Alkaloids, particularly those present in *C. edulis*, might contribute to the antiviral effects observed earlier, which is in alignment with research that suggested antiviral activity of some alkaloids against SARS-CoV-2 (Choy et al., 2020; Hassan, 2020). The specific types and quantities of identified phytochemicals (flavonoids and alkaloids) need further investigation for a more complete picture. Understanding the biological effects of these individual compounds is crucial for drawing definitive conclusions about their potential health benefits. Overall, this study demonstrated the anti-oxidant potential and the presence of various bioactive compounds in *T. riparia*, *C. edulis*, and *T. acutiloba* extracts. *T. riparia* emerged as particularly promising due to its high total phenolic and alkaloid content.

5.4 CONCLUSIONS

This study investigated the phytochemical composition and biological activities of extracts from *C. edulis*, *T. acutiloba*, and *T. riparia*, with a focus on their antiviral, anti-inflammatory, and anti-oxidant properties. The results of this comprehensive study offer several important insights and conclusions.

The phytochemical screening revealed that all three plant extracts contain significant amounts of secondary metabolites such as flavonoids, saponins, tannins, and alkaloids. These compounds are known for their diverse biological activities, including anti-inflammatory, antiviral, and anti-oxidant effects. Notably, *T. riparia* showed the highest total phenolic and alkaloid content, which makes it a promising candidate for further investigation.

The antiviral assays against SARS-CoV-2 demonstrated that the extracts have the potential to reduce viral replication. Pre-treatment with the extracts suggested a trend towards reduced viral loads, particularly for *C. edulis* and *T. riparia*. Post-infection treatment with *C. edulis* notably reduced viral loads, which indicates a potential antiviral effect. However, the antiviral activity was not sustained at later time points, which suggests the need for further research to optimise the timing and dosage of these treatments.

The study also assessed the anti-inflammatory effects of the extracts by measuring the cytokine levels (IL-6, IL-8, and TNF- α) in infected cells. The extracts generally reduced cytokine levels compared to untreated controls, particularly at later time points, which indicates mild anti-inflammatory properties. These findings align with the known anti-inflammatory effects of flavonoids and alkaloids present in the extracts.

The anti-oxidant potential of the extracts was evaluated using the DPPH assay, which showed that *T. riparia* had the strongest radical scavenging activity, followed by *C. edulis* and *T. acutiloba*. The high anti-oxidant activity is likely due to the presence of phenolic compounds, which are known to neutralise free radicals and reduce oxidative stress.

The cytotoxicity assays on H1299 lung cancer cells revealed that *T. riparia* exhibited the highest cytotoxicity, followed by *T. acutiloba* and *C. edulis*. While this suggests potential

therapeutic uses, it also underscores the importance of determining safe and effective dosages for potential treatments.

5.5 STUDY LIMITATIONS AND RECOMMENDATIONS

While the findings of this study are promising, several limitations should be acknowledged. The antiviral, anti-inflammatory, and cytotoxic activities of the extracts were evaluated *in vitro* using cell cultures. *In vitro* results may not fully replicate the complex interactions that occur *in vivo*. Further studies using animal models and human clinical trials are thus necessary to confirm the therapeutic potential of these extracts.

The antiviral activity was tested against a single strain of SARS-CoV-2. Given the genetic variability and emergence of new variants, it is essential to evaluate the efficacy of these extracts against a broader range of viral strains to ensure comprehensive antiviral activity. The study used fixed concentrations of the plant extracts due to a shortage of laboratory materials, including PCR kits.

Further research is needed to identify optimal dosages that maximise therapeutic benefits while minimising potential cytotoxic effects. The cytokine concentrations for IL-8 and TNF- α fell outside the detectable range of the assay at later time points. This limitation may have affected the accuracy of the measured anti-inflammatory effects. Utilising more sensitive assays could provide a clearer picture of the extracts' impact on cytokine production.

The extracts contain multiple bioactive compounds, and their individual contributions to the observed effects were not isolated or characterised in this study. Future research should focus on identifying and testing these individual compounds to understand their specific roles and interactions.

The study did not explore the potential synergistic effects of combining the plant extracts with other antiviral or anti-inflammatory agents. Investigating such combinations could reveal enhanced therapeutic efficacy.

The study did not fully address the statistical significance of the results. Future studies could include comprehensive statistical analyses to validate the findings and ensure their reliability.

Given these findings, several areas warrant further investigation. Detailed mechanism-of-action studies are needed to elucidate the specific mechanisms by which these extracts exert their antiviral and anti-inflammatory effects. Longer-term extended time points and dosage optimisation studies and optimisation of extract concentrations could provide insight into how to sustain and enhance their therapeutic effects. Testing the extracts against a wider range of SARS-CoV-2 strains and other viruses could validate and expand their potential use as antiviral agents.

Identifying and quantifying the specific bioactive compounds within the extracts will help to understand their individual contributions to the observed effects and could lead to the development of more targeted therapies. This study contributed valuable preliminary data on the phytochemical composition and biological activities of *C. edulis*, *T. acutiloba*, and *T. riparia* extracts. While the findings are promising, particularly for *C. edulis* in antiviral applications, comprehensive future studies are essential to confirm these results and explore their potential therapeutic benefits in greater detail.

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APPENDICES

Appendix A: Environmental and Biosafety Research Ethics Committee Approval Letter



Environment & Biosafety Research Ethics Committee

23-Jul-2022

Dear Carlos da Silva

Project Title: **The in-vitro antiviral activity of Carissa edulis, Tulbaghia acutiloba and Tetradenia riparia on Severe acute respiratory syndrome coronavirus 2.**

Department: CUT - Central University of Technology

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Environment & Biosafety Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2022/0177/22**

Please note the following:

1. This ethical clearance is valid for two years from the issuance of this letter.
2. If the research takes longer than two years to complete, please submit a **Continuation Report to the Ethics Committee before ethical clearance expires.**
3. If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an **Amendment.**
4. When the research is concluded, please submit a **Final Report to the Ethics Committee.**

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely



Prof. RR (Robert) Bragg
Chairperson: Environment & Biosafety Research Ethics Committee
University of the Free State

Directorate: Research Development
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Johannes Brill Building, Room 106D, First Floor
205 Nelson Mandela Drive | Park West, Bloemfontein 9301 | South Africa



Appendix B:

National Department of Health Permit to Perform Research on SARS-CoV-2



DIRECTOR GENERAL
HEALTH
REPUBLIC OF SOUTH AFRICA

PRETORIA
Private Bag X828, PRETORIA, 0001, 19th Floor, Civitas Building, Cnr Andries and Struben Street, PRETORIA, 0002 Tel (012) 395 8402 Fax (012) 395 8422
CAPE TOWN
P.O. Box 3875, CAPE TOWN, 8000, 103 Parliament Towers, Room 615, 120 Plain Street, CAPE TOWN, 8000 Tel (021) 461 2040 Fax (021) 461 6864

Dr Felicity Burt
Division of Virology
School of Pathology
Faculty of Health Sciences
University of the Free State
Bloemfontein
FREE STATE

Dear Dr Felicity Burt

PROVISIONAL PERMIT FOR YOUR LABORATORY TO PERFORM RESEARCH ON SARS CoV-2 (Issued in terms of Regulations R.178 of March 2012)

Your laboratory has been given provisional permit to conduct research on SARS CoV-2.

Please note that this permit is valid until the National Department of Health is able to conduct a physical inspection of your laboratory as stipulated in our R178 Regulations Relating to the Registration of Microbiological Laboratories and the Acquisition, Importation, Handling, Maintenance and Supply of Human Pathogens.

You are entrusted to comply with all the evidence/documents submitted with your application and believe you will take full responsibility should there be any accidental release of the virus during your research activities.

Failure to comply with the above conditions will result in your permit being revoked.

Kind regards



DR SSS BUTHELESI
DIRECTOR-GENERAL OF HEALTH

DATE: 24-02-2021

Appendix C: Documentation Indicating That Material Is Available in the Public Domain and/or Permission to Use It

Figure 2.1: Nidovirales phylogeny. Unrooted maximum parsimonious tree-inferred from multiple nucleotide alignments of the RdRp-Hel region- depicting the evolutionary relationships between the five groups of nidoviruses (Coronavirinae, Toroviruses, Roniviridae, Bafiniviruses, and Arteriviridae) and the formed genera of Alpha-, Beta-, and Gammacoronavirus along with Toro 2009 the three clusters that formed.

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File: [2008.085-122V.v4.Coronaviridae](#)

Source:

https://ictv.global/taxonomy/taxondetails?taxnode_id=20090624&taxon_name=Coronavirinae

Author(s): International Committee on Taxonomy of Viruses: ICTV

Date: 20th November 2023

Figure 2.4: Coronavirus virion structure. The virions are embedded with spike (S), envelope (E) and membrane (M) surface proteins. The virion contains a positive-sense, single-stranded RNA genome (+ssRNA) enclosed by a lipid envelope nucleocapsid (N) protein. Figure is adapted from Pizzato et al. (2022)

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Author(s): Pizzato, M., Baraldi, C., Sopetto, G. B., Finozzi, D., Gentile, C., Gentile, M. D., Marconi, R., Paladino, D., Raoss, A., Riedmiller, I., Rehman, H. U., Santini, A., Succetti, V. & Volpini, L.

Date: 12th of January 2022

Figure 2.5: Genomic arrangement of SARS-CoV-2. The 30 kb genomic organisation of SARS-CoV-2, showing the arrangement of various non-structural, structural and accessory genes flanked by 5' and 3' untranslated regions. Figure was adapted from Pizzato et al. (2022).

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Date: 12th of January 2022

Figure 2.6: The lifecycle of SARS-CoV-2. Entry into host cells by SARS-CoV-2 can either be through endocytosis or directly by fusion at the host cell surface. Following primary translation, the viral genomic RNA replicates inside protective double-membrane vesicles. Transcription and translation results in the formation and resulting assembly of structural proteins that bud out from the cell in secretory vesicles through the Golgi pathway or by hijacking lysosomes, and is released via exocytosis. Figure is adapted from Pizzato et al. (2022).

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Author(s): Pizzato, M., Baraldi, C., Sopetto, G. B., Finozzi, D., Gentile, C., Gentile, M. D., Marconi, R., Paladino, D., Raoss, A., Riedmiller, I., Rehman, H. U., Santini, A., Succetti, V. & Volpini, L.

Date: 12th of January 2022

Figure 2.7: Global distribution of total accumulative SARS-CoV-2 cases as of January 2024. Figure is adapted from WHO (2024).

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License	The World Health Organization ("WHO") encourages public access and use of the data that it collects and publishes on its web site data.who.int. The data are organized in datasets and made available in machine-readable format ("Datasets"). The Datasets have been compiled from data provided by WHO's Member States under the WHO policy on the use and sharing of data collected by WHO in Member States outside the context of public health emergencies.

Source: <https://data.who.int/dashboards/covid19/cases>

Author(s): World Health Organization.

Date: January 2024

Figure 2.8: *Carissa edulis* (Forssk.) Vahl.

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Source: <https://collections.britishart.yale.edu/catalog/tms:58110>

Author(s): Balugani, L.

Date: November 2023

Figure 2.9: *T. acutiloba*

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Source: <https://herbaria.plants.ox.ac.uk/bol/plants400/Profiles/st/Tulbaghia>

Author(s): The University of Oxford.

Date: November 2023

Figure 2.10: *T. riparia*



Anyone can contribute

Casabio is free to use for anyone: students, teachers, researchers, conservationists, any everyone else. Photographs of plants and animals can be uploaded in bulk or individually.



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Anyone in the community can vote to help identify observations, and tag the interaction between species in the photos. Members with higher reliability scores have more weight when giving their opinion.



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Source: <https://casabio.org/taxa/tetradenia-riparia>

Author(s): Casabio

Date: November 2023

Appendix D:

Cytotoxicity MTT Assay Plate Raw Data

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.111	0.085	0.021	0.231	0.215	0.212	0.295	0.365	0.31	0.415	0.461	0.457
B	0.161	0.132	0.061	0.226	0.265	0.251	0.325	0.398	0.329	0.474	0.472	0.488
C	0.192	0.212	0.005	0.238	0.28	0.243	0.432	0.477	0.522	0.088	0.056	0.014
D	0.278	0.315	0.077	0.372	0.377	0.406	0.474	0.663	0.612	0.024	0.018	0.082
E	0.495	0.514	0.069	0.549	0.637	0.622	0.272	0.298	0.253	0.049	0.025	0.003
F	0.711	0.667	0.049	0.674	0.607	0.737	0.291	0.31	0.288	0.315	0.397	0.389
G	0.808	0.774	0.031	0.286	0.31	0.263	0.286	0.328	0.314	0.883	0.957	0.898
H	0.032	0.065	0.019	0.308	0.298	0.303	0.352	0.376	0.295	0.084	0.064	0.061

Appendix E:

SARS-CoV-2 Viral Loads Obtained Pre- and Post-Treatment with Plant Extracts

Table E1: SARS-CoV-2 viral loads after pre-treatment with extracts

Plant extract	Time post-infection	N gene C _t value Mean ± SD	N gene estimated viral load ± SD (Log ₁₀ copies/mL)
<i>C. edulis</i>	24 hours	38.06 ± 3.02	<1 ± 0.60
	48 hours	28.48 ± 2.43	3.01 ± 0.35
	72 hours	18.60 ± 4.10	5.33 ± 1.20
<i>T. acutiloba</i>	24 hours	30.09 ± 3.45	2.62 ± 0.52
	48 hours	21.14 ± 3.15	4.73 ± 0.83
	72 hours	10.34 ± 4.20	>6 ± 1.80
<i>T. riparia</i>	24 hours	29.63 ± 3.20	2.73 ± 0.55
	48 hours	24.76 ± 2.95	3.88 ± 0.91
	72 hours	18.52 ± 3.85	5.35 ± 1.32
Vehicle control	24 hours	29.96 ± 3.75	2.65 ± 0.64
	48 hours	24.58 ± 3.10	3.92 ± 0.87
	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 ± 1.62

Table E2: SARS-CoV-2 viral loads after post-treatment with plant extracts

Plant extract	Time post-infection	N gene C _t value Mean $\pm$ SD	N gene estimated viral load $\pm$ SD (Log ₁₀ copies/mL)
<i>C. edulis</i>	24 hours	29.58 $\pm$ 3.31	2.74 $\pm$ 0.85
	48 hours	11.33 $\pm$ 2.62	>6 $\pm$ 0.56
	72 hours	10.33 $\pm$ 2.54	>6 $\pm$ 0.75
<i>T. acutiloba</i>	24 hours	24.78 $\pm$ 1.76	3.87 $\pm$ 1.10
	48 hours	10.33 $\pm$ 2.6	>6 $\pm$ 0.95
	72 hours	9.55 $\pm$ 2.49	>6 $\pm$ 1.13
<i>T. riparia</i>	24 hours	19.71 $\pm$ 1.48	5.07 $\pm$ 1.31
	48 hours	11.78 $\pm$ 2.13	>6 $\pm$ 0.42
	72 hours	10.21 $\pm$ 2.26	>6 $\pm$ 0.35
Vehicle control	24 hours	20.17 $\pm$ 3.51	4.96 $\pm$ 0.78
	48 hours	10.45 $\pm$ 4.92	>6 $\pm$ 0.16
	72 hours	9.97 $\pm$ 1.87	>6 $\pm$ 0.52
Cells only	24 hours		
	48 hours		
	72 hours		
Virus-infected cells only	24 hours	17.85 $\pm$ 2.97	4.96 $\pm$ 1.41
	48 hours	13.11 $\pm$ 1.62	>6 $\pm$ 0.25
	72 hours	8.03 $\pm$ 1.13	>6 $\pm$ 0.38

Appendix F:

Anti-Inflammatory Activity ELISA Plate Raw Data and Cytokine Concentrations

IL-6

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.874	0.892	0.069	0.848	0.824	0.712	0.81	0.67	0.707	0.588	0.047	0.072
B	0.571	0.601	0.074	0.728	0.515	0.586	0.105	0.45	0.846	1.262	0.079	0.032
C	0.435	0.401	0.079	0.727	0.608	1.105	0.096	0.413	1.017	1.028	0.088	0.079
D	0.351	0.323	0.093	0.064	0.059	0.052	0.109	0.074	0.081	0.102	0.081	0.027
E	0.267	0.261	0.081	0.466	0.552	0.649	0.091	0.344	0.493	0.781	0.115	0.035
F	0.197	0.171	0.088	0.702	1.388	1.42	0.023	0.849	1.213	1.56	0.028	0.002
G	0.153	0.111	0.102	0.381	0.339	0.787	0.042	0.315	0.425	0.471	0.02	0.004
H	0.105	0.096	0.109	0.279	0.287	0.385	0.047	0.294	0.206	0.271	0.01	0.016

IL-8

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.684	1.498	0.152	1.521	1.156	1.163	0.178	1.221	0.996	0.621	0.155	0.19
B	1.255	1.249	0.097	1.671	1.326	0.961	0.153	1.431	1.176	0.791	0.127	0.176
C	0.917	1.01	0.174	1.821	1.456	1.081	0.062	1.521	1.246	0.871	0.108	0.105
D	0.869	0.903	0.113	0.195	0.168	0.186	0.199	0.174	0.168	0.166	0.15	0.098
E	0.747	0.784	0.185	1.751	1.396	1.021	0.189	1.739	1.404	1.029	0.139	0.091
F	0.655	0.621	0.068	1.811	1.456	1.081	0.093	1.799	1.464	1.089	0.103	0.17
G	0.491	0.386	0.119	0.751	0.396	0.321	0.074	0.739	0.404	0.229	0.114	0.133
H	0.196	0.193	0.077	0.341	0.166	0.171	0.194	0.439	0.274	0.331	0.151	0.121

TNF- α

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.746	1.683	0.218	1.222	1.006	0.631	0.097	1.159	1.024	0.849	0.214	0.1
B	1.002	1.294	0.152	1.434	1.176	0.801	0.189	1.55	0.886	0.811	0.147	0.122
C	0.817	0.994	0.088	1.521	1.243	0.584	0.148	1.661	1.467	0.951	0.121	0.175
D	0.779	0.772	0.197	0.201	0.108	0.163	0.121	0.199	0.218	0.092	0.105	0.151
E	0.684	0.725	0.107	1.665	1.774	1.638	0.146	1.521	1.6	1.432	0.131	0.198
F	0.511	0.641	0.076	1.793	1.746	1.681	0.122	1.021	1.542	1.405	0.14	0.097
G	0.339	0.356	0.219	0.394	0.404	0.519	0.206	0.651	0.251	0.394	0.186	0.211
H	0.174	0.101	0.212	0.319	0.166	0.174	0.171	0.181	0.23	0.163	0.223	0.221

Table F1: Cytokine concentration in cell culture supernatant after pre-treatment with plant extracts

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

Table F2: Cytokine concentration in cell culture supernatant after post-treatment with plant extracts

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

Appendix G:

Research Publication

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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. Ethanol 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.^{1, 2, 3, 4} 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.^{5, 6, 7, 8, 9, 10} 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.¹¹ Continued viral evolution remains a concern and ongoing sequencing has provided monitoring of mutations and guidance of public health strategies.¹²

Vaccination remains the most effective protection against infection and severe disease.^{13, 14} Regular booster vaccination is still a key part of the ongoing management of COVID-19, especially among older and more clinically vulnerable populations.¹⁵ Treatment strategies and guidelines vary between different countries as numerous agents are tested and either removed from or added to protocols.^{16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100}

With the onset of the COVID-19 pandemic, scientists looked to explore traditional plant remedies as potential sources of antiviral compounds.²⁹ 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.³⁰ *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.^{31, 32, 33}

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, 2mM-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 (CO₂) incubator with 5% CO₂. 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)²⁶. 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:

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

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. acutifolia*, and *T. riparia* purchased from various plant nurseries in South Africa. Fresh *T. acutifolia* 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% C₂H₅O 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, cytotoxicity, 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 2×10^5 cells/ml in a 96-well plate (ThermoFischer Scientific, USA) and incubated for 24 hours at 37 °C in a humidified 5% CO₂ 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 C₂H₅O 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:

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

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)¹⁶. 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 CO₂ incubator. The following day, the seeded H1299-hACE2-E3 cells were used in two independent experiments. IC₅₀ 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. IC₅₀ 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 ribonucleic 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 (C₂H₅OS) 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 C₂H₅O 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 Alplex™ 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

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

Reagents	Volume
2019-nCoV MaDT™ Oligo Mix	5 µl
DNase/RNase-free water	5 µl
SX 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:mm: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.			

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.

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% CO₂ 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. acutifolia*, 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 IC₅₀ 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. acutifolia* 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. acutifolia*, 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 IC₅₀ 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. acutifolia* and *T. riparia* also showed some reduction in viral load during pre-treatment, with *T. acutifolia* 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
<i>C. edulis</i>	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%
<i>T. acutifolia</i>	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	-
<i>T. riparia</i>	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			
	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 values $\pm$ SD	Mean N gene $\pm$ estimated viral load $\pm$ SD (Log ₁₀ copies/mL)	% Reduction vs control virus-infected cells only
<i>C. edulis</i>	24 hours	29.58 $\pm$ 3.31	2.74 $\pm$ 0.85	44.75%
	48 hours	11.33 $\pm$ 2.62	>6 $\pm$ 0.56	-
	72 hours	10.33 $\pm$ 2.54	>6 $\pm$ 0.75	-
<i>T. acutifolia</i>	24 hours	24.78 $\pm$ 1.76	3.87 $\pm$ 1.10	21.97%
	48 hours	10.33 $\pm$ 2.6	>6 $\pm$ 0.95	-
	72 hours	9.55 $\pm$ 2.49	>6 $\pm$ 1.13	-
<i>T. riparia</i>	24 hours	19.71 $\pm$ 1.48	5.07 $\pm$ 1.31	-
	48 hours	11.78 $\pm$ 2.13	>6 $\pm$ 0.42	-
	72 hours	10.21 $\pm$ 2.26	>6 $\pm$ 0.35	-
Vehicle control	24 hours	20.17 $\pm$ 3.51	4.96 $\pm$ 0.78	-
	48 hours	10.45 $\pm$ 4.92	>6 $\pm$ 0.16	-
	72 hours	9.97 $\pm$ 1.87	>6 $\pm$ 0.52	-
Cells only	24 hours			
	48 hours			
	72 hours			
Virus-infected cells only	24 hours	17.85 $\pm$ 2.97	4.96 $\pm$ 1.41	
	48 hours	13.11 $\pm$ 1.62	>6 $\pm$ 0.25	
	72 hours	8.03 $\pm$ 1.13	>6 $\pm$ 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) $\pm$ SD		
		IL-6	IL-8	TNF- α
<i>C. edulis</i>	24 hours	88.71 $\pm$ 1.26	200.28 $\pm$ 0.69	550.46 $\pm$ 1.22
	48 hours	85.48 $\pm$ 1.13	119.90 $\pm$ 0.25	381.20 $\pm$ 1.71
	72 hours	70.36 $\pm$ 0.74	121.44 $\pm$ 1.41	87.34 $\pm$ 1.58
<i>T. acutifolia</i>	24 hours	72.52 $\pm$ 1.35	25.64 $\pm$ 2.12	716.59 $\pm$ 1.62
	48 hours	43.78 $\pm$ 2.56	<1.56 $\pm$ 1.65	514.41 $\pm$ 3.12
	72 hours	53.36 $\pm$ 2.31	<1.56 $\pm$ 3.91	220.55 $\pm$ 4.09
<i>T. riparia</i>	24 hours	72.39 $\pm$ 1.74	266.35 $\pm$ 2.14	784.76 $\pm$ 1.42
	48 hours	56.33 $\pm$ 3.32	185.97 $\pm$ 0.83	566.92 $\pm$ 2.50
	72 hours	123.39 $\pm$ 1.61	103.38 $\pm$ 1.43	50.51 $\pm$ 3.17
Vehicle control	24 hours	37.17 $\pm$ 3.41	250.94 $\pm$ 3.74	897.61 $\pm$ 1.64
	48 hours	48.77 $\pm$ 2.10	172.76 $\pm$ 0.16	983.02 $\pm$ 0.81
	72 hours	61.86 $\pm$ 1.52	90.17 $\pm$ 1.48	876.45 $\pm$ 0.47
Untreated cells	24 hours	69.01 $\pm$ 0.66	264.15 $\pm$ 1.32	997.91 $\pm$ 0.85
	48 hours	161.58 $\pm$ 2.14	185.97 $\pm$ 1.25	961.08 $\pm$ 3.06
	72 hours	165.89 $\pm$ 1.93	103.38 $\pm$ 1.41	910.14 $\pm$ 1.31
No cells	24 hours			
	48 hours			
	72 hours			
Prednisolone	24 hours	11.94 $\pm$ 3.15	<1.56 $\pm$ 2.56	<1.56 $\pm$ 1.34
	48 hours	13.02 $\pm$ 2.23	<1.56 $\pm$ 0.82	<1.56 $\pm$ 1.25
	72 hours	26.24 $\pm$ 2.18	<1.56 $\pm$ 1.37	<1.56 $\pm$ 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. acutifolia* 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 fluctuations⁴⁶. *C. edulis* consistently met this threshold across pre- and post-treatment conditions, indicating consistent and possibly active antiviral properties. Both *T. acutifolia* 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. acutifolia* 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. acutifolia*, 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) $\pm$ SD		
		IL-6	IL-8	TNF- α
<i>C. edulis</i>	24 hours	64.70 $\pm$ 3.14	134.22 $\pm$ 2.54	501.09 $\pm$ 1.40
	48 hours	69.69 $\pm$ 1.78	84.66 $\pm$ 4.52	395.30 $\pm$ 1.34
	72 hours	53.63 $\pm$ 2.52	2.08 $\pm$ 1.23	258.17 $\pm$ 0.27
<i>T. acutifolia</i>	24 hours	167.38 $\pm$ 2.30	180.46 $\pm$ 0.63	807.49 $\pm$ 3.88
	48 hours	132.97 $\pm$ 2.51	124.30 $\pm$ 0.31	287.16 $\pm$ 1.43
	72 hours	81.02 $\pm$ 1.42	39.52 $\pm$ 0.47	228.39 $\pm$ 0.23
<i>T. riparia</i>	24 hours	179.52 $\pm$ 4.67	200.28 $\pm$ 3.34	894.47 $\pm$ 2.71
	48 hours	142.42 $\pm$ 1.35	139.72 $\pm$ 1.57	742.45 $\pm$ 0.50
	72 hours	91.82 $\pm$ 0.41	57.14 $\pm$ 1.64	338.10 $\pm$ 0.45
Vehicle control	24 hours	20.71 $\pm$ 0.59	248.29 $\pm$ 1.51	784.76 $\pm$ 0.23
	48 hours	40.81 $\pm$ 1.61	174.52 $\pm$ 1.23	846.67 $\pm$ 1.67
	72 hours	79.67 $\pm$ 4.48	91.93 $\pm$ 1.55	715.02 $\pm$ 4.84
Untreated cells	24 hours	88.85 $\pm$ 2.53	261.51 $\pm$ 2.81	392.95 $\pm$ 3.21
	48 hours	137.96 $\pm$ 3.07	187.73 $\pm$ 0.76	801.22 $\pm$ 2.44
	72 hours	184.78 $\pm$ 1.54	105.14 $\pm$ 1.91	693.86 $\pm$ 3.60
No cells	24 hours			
	48 hours			
	72 hours			
Prednisolone	24 hours	13.96 $\pm$ 0.83	<1.56 $\pm$ 0.75	<1.56 $\pm$ 0.33
	48 hours	2.09 $\pm$ 1.66	<1.56 $\pm$ 0.42	<1.56 $\pm$ 0.81
	72 hours	10.86 $\pm$ 0.51	<1.56 $\pm$ 0.44	<1.56 $\pm$ 0.27

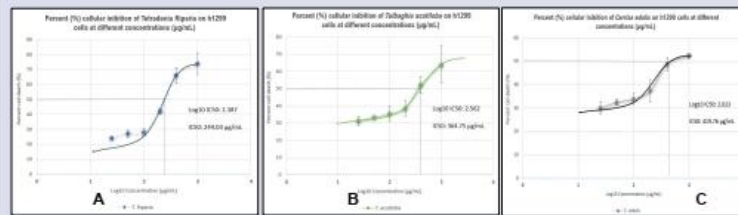


Figure 1: Dose-response curves of *T. riparia*, *T. acutifolia*, and *C. edulis* extracts on H1299-hACE2-E3 cells. Data are presented as the mean of the triplicate values and the $\pm$ standard deviation is represented by the error bars.

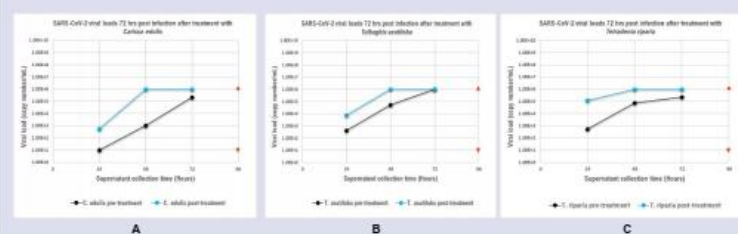


Figure 2: A-C: SARS-CoV-2 viral loads 72 hours post-infection after treatment with IC_{50} concentrations of plant extracts.

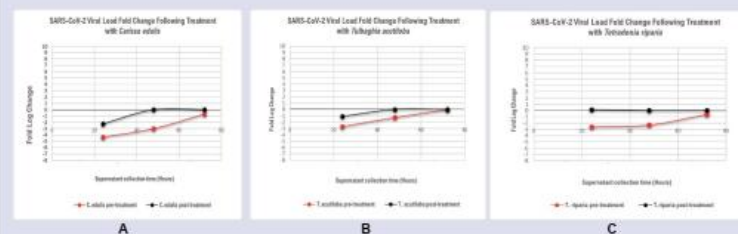


Figure 3: A-C: SARS-CoV-2 viral load fold change (FC) following treatment with IC_{50} concentrations of plant extracts relative to the untreated control.

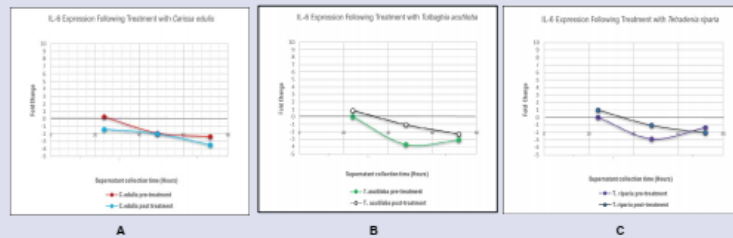


Figure 4: FC in concentration levels of IL-6 following treatment with the extracts.

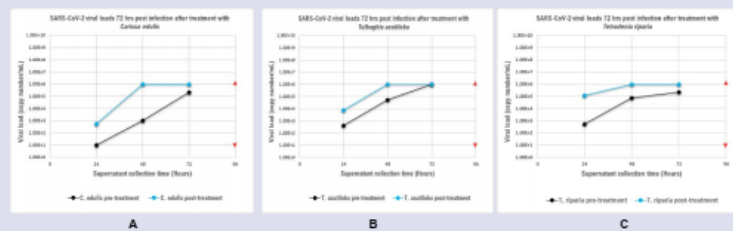


Figure 5: FC in concentration levels of IL-8 following treatment with the extracts.

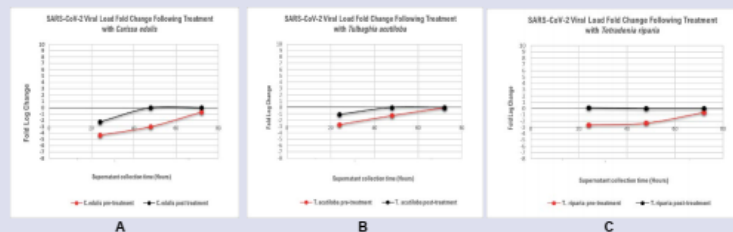


Figure 6: FC in concentration levels of TNF-α following treatment with the extracts.

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. acutifolia* 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. acutifolia* 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 . This aligns with the interpretation that *C. edulis* possesses measurable anti-inflammatory properties, while *T. acutifolia* 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. acutifolia*, 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 μ g/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. acutifolia* 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. acutifolia* 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. acutifolia* 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 $\pm$ 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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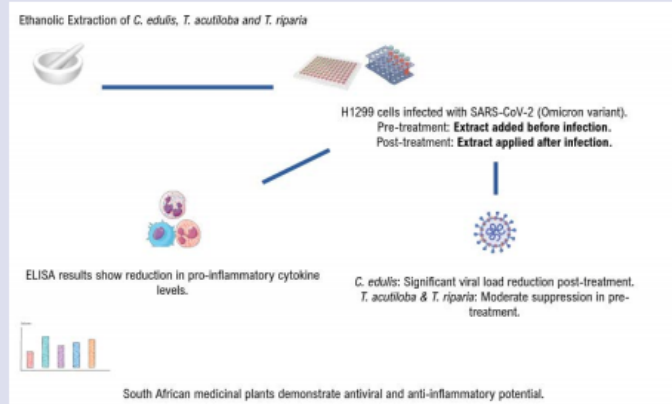
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GRAPHICAL ABSTRACT



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