The effects of curcumin derivatives on SARS-CoV-2 spike S_1 protein induced oxidative stress on macrophage cells

by

Maureen Ramadimetsa Nkwana

Research dissertation

Submitted in fulfilment of the requirements for the degree of

Master of Science

in

Biochemistry

in the

Faculty of Science and Agriculture

(School of Molecular and Life Sciences)

at the

University of Limpopo

Supervisor: Dr RT Makola

Co-supervisor: Prof. TM Matsebatlela

2024

CONFERENCE PRESENTATION

1. 13th Annual Faculty of Science and Agriculture research day.
   Venue, Date: Bolivia Lodge, Limpopo, 20-22 September 2023.
   Title: The effects of curcumin derivatives on SARS-CoV-2 spike S_1 protein induced oxidative stress on macrophage cells.
   Authors: Nkwana M.R., Makola R.T. and Matsebatlela T.M.
   Prize: Second place in the category: Masters in Biological Sciences.

2. The 28TH South African Society for Biochemistry and Molecular Biology Congress **Venue, Date:** The Ranch Hotel, Polokwane, 7-10 July 2024
   Title: The effect of curcumin derivatives on SARS-CoV-2 spike S1 protein induced oxidative stress on macrophage cells.
   Authors: Nkwana, M.R., Maluleke M.M., Matsebatlela, T.M., Makola, R.T.

Table of Contents

DECLARATION

I declare that the study titled: **The effects of curcumin derivatives on SARS-CoV-2 spike S_1 protein induced oxidative stress on macrophage cells** is my work. This work is being submitted for the degree of Master of Science in Biochemistry at the University of Limpopo. This work has not been submitted to any other University and I further declare that all sources used are duly acknowledged.

Surname and initials: **Nkwana MR** *Date:* **10/10/2024**

signature

DEDICATION

I dedicate this work to all the people who always believed in me and positively contributed to my success.

ACKNOWLEDGEMENTS

I would like to extend my sincere and heartfelt gratitude to the following people who have contributed to the success of this project:

- Supervisor, **Dr. R.T Makola**, for the dedication, time, and support to help me with my project regardless of his tight schedule. Thank you very much.
- Co-supervisor, **Prof. T.M Matsebatlela**, for his assistance and guidance.
- I would like to acknowledge **Dr Marole Maluleke** for providing us with curcumin compounds.
- My mom [**Elizabeth Nkwana**] and siblings, for all the efforts you made for me to remain encouraged and interested especially during the times I thought of giving up.
- My bestfriend, **Tshedimosho Kgoadi**, for being my pillar of strength.
- **Boitumelo Radingoana**, thank you for being supportive and listening to all my frustrations during the whole process of completing my project. You are the best.
- The **Biochemistry, Microbiology and Biotechnology (BMBT) department** of the University of Limpopo for the opportunity to pursue this study.

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACE 2	Angiotensin-converting enzyme 2
BCA	Bicinchoninic acid
COVID-19	Coronavirus Disease of 2019
DAF-2 DA	Diaminofluorescein-2 diacetate
DMEM	Dulbecco's modified eagle media
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HRP	Horseradish peroxidase
IAV	Influenza A viruses
IFN	Interferon
IκB-α	Nuclear factor-kappa-B inhibitor alpha
IKK	Inhibitor of nuclear factor-κB (IκB) kinase
IL-6	Interleukin-6
iNOS	inducible nitric oxide synthase
IRF	Interferon regulatory factors
LPS	Lipopolysaccharide
MDA-5	Anti-Melanoma Differentiation-Associated gene 5

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NRF2	Nuclear factor erythroid 2-related factor 2
PAMPs	Pathogen-associated molecular pattern molecules
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline Tween 20
PI	Propidium Iodide
PRRs	Pattern Recognition Receptors
PSN	Penicillin, streptomycin, and neomycin
PVDF	Polyvinylidene fluoride
RBD	Receptor-binding domain
RIG-I	Retinoic acid-inducible gene-I
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive oxygen species
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
WHO	World Health Organization

ABSTRACT

The corona virus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has emerged as a global health crisis that claimed almost 7 million lives and a total of more than 700 million cases to date with new variants still being reported in various countries. Currently, there is no specific treatment for COVID-19 patients and the sporadic emergence of new variants make vaccine development a delayed response towards management of future outbreaks. Infection of host cells via interaction with SARS-CoV-2 spike proteins is characterised by hyper-inflammation of the innate immune response leading to overproduction of reactive oxygen species (ROS) by macrophage. The increased ROS production ultimately overwhelms the antioxidant pool and promote elevated oxidative stress levels, converting mild symptoms to severe COVID-19 outcomes. Curcumin has shown potent antioxidant and anti-inflammatory properties in various pathological conditions. Thus, this study investigated the antioxidant potential of curcumin derivatives in ameliorating SARS-CoV-2 spike protein-induced oxidative stress using macrophage cells as the innate immune model system. The cytotoxicity effect of curcumin derivatives was assessed by using two viability assays, namely the colorimetric MTT and propidium iodide (PI) fluorescence assays on Raw 264.7 macrophage cells. The curcumin derivatives were shown to exert no cytotoxic effect on Raw 264.7 cells at low doses (1.25-0.625 mM). The Annexin-V/ PI assay was employed to evaluate the mode of cell death induced by these curcumin derivatives. The results obtained showed that the derivatives at 5 mM induce apoptosis as a mode cell death. The production of IL-6 pro-inflammatory cytokines was examined using ELISA from supernatant after stimulation with 100 ng/ml SARS-CoV-2 spike S_1 and treated with 1 mM curcumin derivatives. Treatment of SARS-CoV-2 spike S_1-stimulated Raw 264.7 macrophages with curcumin resulted in lower production of IL-6 compared to untreated SARS-CoV-2 spike S_1 stimulated control cells. The oxidative stress muse kit and DAF-2 DA were used to determine the levels of reactive oxygen species (ROS) and nitric oxide (NO) produced by Raw 264.7 cells post SARS-CoV-2 spike S_1 stimulation and curcumin

derivative treatment. Treatment with 1 mM curcumin derivatives reduced the production of ROS and NO. The Western blot assay was used to measure the expression levels of Nrf2, IκB-α and NF-κB inflammatory proteins. Western blot protein expression assay demonstrated that these curcumin derivatives upregulate the expression of Nrf2 and IκB-α while downregulating that of NF-κB. Collectively, this study suggests that curcumin derivatives possess remarkable anti-inflammatory and antioxidant properties, making them a potential therapeutic treatment option to explore for the management of the SARS-Cov-2 induced oxidative stress and hyper-inflammation associated with COVID-19 infection.

Keywords: Macrophages, inflammation, SARS-CoV-2, curcumin derivatives and NF-κB

CHAPTER 1

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), originated in Wuhan, China, in December 2019 (Samrat *et al*., 2020). The Huanan Seafood Wholesale Mark*et* was initially linked to COVID-19 cases (Zhai *et al*., 2020). The rapid global spread and significant fatalities prompted the World Health Organisation to declare it a pandemic on March 11, 2020, (Tagde *et al*., 2021).

COVID-19 has continued to have a profound effect on global health, with over 770 million confirmed cases and more than 6 million deaths worldwide (Sibanda and Haryanto, 2024). Although daily case and death reports have become less frequent, weekly updates reveal that infection rates fluctuate due to factors such as the emergence of new variants, varying levels of vaccination coverage, and shifts in public health measures (Lima *et al*., 2024). Current primary treatments for COVID-19 include vaccines, antiviral drugs like Paxlovid, and supportive care (Zong *et al*., 2024). However, with the virus evolving and new variants emerging, there is growing interest in exploring alternative or supplementary treatments, especially for patients with specific vulnerabilities or those who do not respond well to standard therapies (Moura *et al*., 2023). This outbreak necessitates the development of effective prophylactics against SARS-CoV-2 (Weisblum *et al*., 2020).

The SARS-CoV-2 virus is an enveloped virus with a single-stranded positive-sense RNA genome (Zhou *et al*., 2020).

The spike (S) glycoprotein is crucial in the virus's entry into host cells, as it binds to the angiotensin-converting enzyme 2 (ACE2) receptor present on various cell types, including type 2 alveolar cells and gobl*et* cells in the airways (Tai *et al*., 2020). The S glycoprotein consists of two subunits, S_1 and S_2, within each monomer of the trimeric structure (Kozlov *et al*., 2021). The S_1 subunit determines the virus's cellular tropism, housing essential receptor binding motif in the receptor binding domain

(Sodeifian *et al*., 2022). On the other hand, the S_2 subunit facilitates the fusion of the virus with the cell membranes (Felsenstein *et al*., 2020). This binding process is activated by transmembrane protease serine 2 (TMPRSS2) and the host cell protease, furin (Ciotti *et al*., 2020).

Viral entry stimulates the immune system especially the innate immune system, that initiates inflammatory response to combat and eradicate the viral infection (Ahmad and Haque, 2022). This initial defence comprises immune cells like macrophages (Yazdanpanah *et al*., 2020). These immune cells release signalling molecules such as chemokines and cytokines, these inflammatory mediators attract additional immune cells to the inflamed site (García, 2020). Severe instances of COVID-19 are frequently linked to an overwhelmed immune reaction, that include observed cytokine storm (Brown *et al*., 2023). This represents an unregulated discharge of pro-inflammatory cytokines, capable of inducing tissue damage (Mortaz *et al*., 2020).

An effective defence against invading pathogens relies on the prompt activation of innate immunity, a nonspecific response that can control infections through the release of antiviral and pro-inflammatory molecules (Ahmad and Haque, 2022). This initial response also plays a crucial role in supporting the subsequent adaptive immune response, which is essential for clearing infections and preventing reinfection by the same pathogen (Ricci *et al*., 2021). In the case of SARS-CoV-2, local immune cells in the lung promptly recognise the virus, triggering a localised immune response that recruits additional innate immune cells from the bloodstream (Gusev *et al*., 2022). However, immune reactions to SARS-CoV-2 can occasionally take an unpredictable turn when too much of the inflammatory mediators are produced. Resulting in tissue damage, leading to a rapid progression from mild to severe disease marked by multiorgan failure and fatal outcomes (Wang *et al*., 2020).

Both soluble factors and cellular mediators contribute to these contrasting outcomes, though the specific mechanisms that tip the balance between a protective and harmful immune response are not fully understood (Ricci *et al*., 2021). Growing body of evidence indicates that the virus trigger production of vast inflammatory mediators that include detrimental reactive oxygen species leading to oxidative stress, a

condition associated with various health problems (Yazdanpanah *et al*., 2020). Oxidative stress happens when there is an imbalance between the generation of oxidants and the capacity of the cellular antioxidant defence to counteract the oxidants (García-Caparrós *et al*., 2021). This imbalance can lead to cellular damage, contributing to the disruption of immune responses and worsening the progression of the infection (He *et al*., 2020).

Macrophages are crucial components of the immune system; they are first line of defence and particularly central to developments of oxidative stress (Yazdanpanah *et al*., 2020). Under-controlled inflammation and oxidative stress disrupt macrophage usual functions and playing a role in the overall development of coronavirus disease (Yazdanpanah *et al*., 2020). COVID-19 encompasses a range of clinical conditions, spanning from mild flu-like symptoms to severe pneumonia (Adhikari *et al*., 2021). Severe cases of COVID-19 are believed to be linked to inflammation (Zhou *et al*., 2024), as indicated by elevated levels of proinflammatory cytokines like interleukin-1 b*et*a (IL-1B), interleukin-8 (IL-8), and tumor necrosis factor alpha (TNF-α) in the plasma of severely affected patients (Ong *et al*., 2020).

Thus, the primary focus is on developing antiviral drugs targeting SARS-CoV-2, there are calls to investigate host-directed approaches aimed at mitigating the inflammatory response associated with severe COVID-19 (Benarba and Pandiella, 2020). However, this study makes use of curcumin derivatives to treat COVID-19 in an attempt to manage the inflammatory sequel. Curcumin, a polyphenolic compound extracted from Curcuma longa rhizomes, has been extensively researched due to its antioxidative, anti-inflammatory, and antiviral properties (Worachartcheewan *et al*., 2011).

Recent studies have focused on enhancing curcumin's bioavailability and effectiveness through derivative development (Sahu *et al*., 2016). These derivatives display diverse pharmacological activities and show potential in addressing the oxidative stress and inflammatory responses associated with SARS-CoV-2 infection (Oglah *et al*., 2020). Investigating the relationship between curcumin derivatives and oxidative stress in the context of SARS-CoV-2 infection could lead to targeted

interventions, boosting the immune response and reducing COVID-19 severity and a need for hospitalisation. Therefore, this study aims to examine impact of curcumin derivatives on oxidative stress induced by the SARS-CoV-2 spike S_1 protein in macrophage cells.

CHAPTER 2

LITERATURE REVIEW

2.1. Background

The immune system is a sophisticated network made up of lymphoid organs, cells, and cytokines (Parkin and Cohen, 2001), which plays a crucial role in defending the body against infectious agents (Jerne, 1973; Lewis *et al*., 2019). The immune system is subdivided in to innate and adaptive immune system that are effectively coordinated to protect the host (Glencross *et al*., 2020;Lewis *et al*., 2019). Natural killer cells, along with phagocytic cells like neutrophils, monocytes, and macrophages, and cells that release inflammatory mediators such as basophils, mast cells, and eosinophils, are essential in directly combating invading pathogens (Eter *et al*., 2000; Lewis *et al*., 2019). Additionally, molecular components like acute-phase proteins and cytokines contribute significantly to the activation of the adaptive immune system. (Eter *et al*., 2000; Lewis *et al*., 2019).

The adaptive immune system is highly specialised and takes time to develop. It responds to specific pathogens that the body has previously encountered and creates a targeted response to eliminate them (Sun *et al*., 2020). Lymphocytes, such as Thymus and Bursa cells, are part of the system and are responsible for coordinating immune responses and producing antibodies (Sun *et al*., 2020). Adaptive immune response is characterised by the proliferation of Thymus (T) and Bursa (B) cells that are antigen-specific, which happens when the cell surface receptors attach to antigen (Eter *et al*., 2000). Thymus cells kill intracellular pathogens and assist B cells in producing antibodies (Eter *et al*., 2000). While on the other hand the host's first line of defence against invasive infections is the innate immune system (Eter *et al*., 2000).

Pathogen-associated molecular patterns (PAMPs), such as glycoproteins and nucleic acids, are detected by the innate immune system (Mogensen, 2009). It offers broad, instantaneous defence against infections, using the pattern recognition receptors (PRR) (Mogensen, 2009). The system involves different cells, such as

neutrophils, macrophages, and natural killer cells that identify and eliminate infections, as well as physical barriers like the skin and mucous membranes (Abaricia *et al*., 2021). The recruitment of immune cells to the inflamed or infection site is an essential part of this response (Kozlov *et al*., 2021). Cytokines and chemokines are signalling molecules that orchestrate this recruitment (Turner *et al*., 2014). During innate immunity, the body's defense mechanisms are triggered by the release of cytokines, which also triggers local cellular responses to infection or injury (Kozlov *et al*., 2021).

Key pro-inflammatory cytokines released during the early response to infection include interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor alpha (Zhang and An, 2007). Many infections are eliminated from the body by the aid of these cytokines, which also promote local inflammation and cell recruitment (Turner *et al*., 2014). Since autoimmune or inflammatory diseases are usually associated with the dysregulated production of these inflammatory cytokines, these cytokines represent important therapeutic targets (Marshall *et al*., 2018).

2.2. Inflammation

Inflammation is the defence mechanism to stimulated by injury and infection caused by pathogens, chemical irritants, or damaged cells (Antonelli and Kushner, 2017), as well as genetic changes in the intestinal tract caused by reactive oxygen and nitrogen species (ROS and RNS), inflammatory chemokines and cytokines (Gasparrini *et al*., 2018). This regenerative process is divided into acute and chronic inflammation (Liu *et al*., 2023). Blood flow and vascular permeability increase during the acute phase as inflammatory mediators and white blood cells accumulate (Liu *et al*., 2023). Similarly, chronic inflammation frequently develops without obvious signs and symptoms and is typically overlooked until the condition becomes clinically apparent (Liu *et al*., 2023).

Infection with a pathogen causes an acute inflammatory response, in which innate immune system cells travel to the inflamed site (Eter *et al*., 2000). Inflammation plays the most important part of Innate immunity, in addition to facilitating the killing of the engulfed pathogen (Zhang and Cao, 2021). However, when the immune system's

activation is prolonged and under-controlled, chronic inflammation that leads to oxidative stress occurs (Lewis *et al*., 2019).

Uncontrolled reactive oxygen and nitrogen species degrade lipids and other biomolecules in cells during inflammatory processes (Gasparrini *et al*., 2018). This eventually leading to DNA damage and mutations, which emanate from cellular oxidative state and reduce antioxidant defence systems (Gasparrini *et al*., 2018). Damage to biological systems can occur when there is an imbalance between oxidant and antioxidant production (also known as oxidative stress) (Turner *et al*., 2014). Oxidative stress has been linked to pathogenesis of several illnesses, thus, knowing the chemistry of processes involving reactive species formed from oxygen and nitrogen might assist in enhancing treatment methods for antioxidant defence (Forman and Zhang, 2021).

2.3. Macrophages as central cell in viral infection

Originating from monocytes generated in the bone marrow, macrophages are an essential subset of white blood cells in the immune system (Zhang *et al*., 2021). These blood-circulating monocytes infiltrate tissues and undergo a differentiation process to become macrophages (Zhang *et al*., 2021). Macrophages can adopt different activation states, most notably M1 and M2 (Lendeckel *et al*., 2022). M1 macrophages participate in pro-inflammatory responses, while M2 macrophages are associated with tissue repair, the reduction of inflammation, and immunoregulation (Lendeckel *et al*., 2022). The balance between these states significantly influences immune responses and tissue health (Lavin *et al*., 2015). As versatile and essential components of the immune system, macrophages are involved in pathogen defence, inflammation regulation, and maintaining tissue homeostasis (Rankin and Artis, 2018). Dysregulated macrophage function can contribute to various diseases, including chronic inflammatory conditions and autoimmune disorders (Lavin *et al*., 2015).

Viral infections and macrophages have a complex and diverse connection, involving roles in virus detection, antigen presentation, and inflammation (Newton *et al*., 2016). When macrophages detect viral components, they become activated and

release various cytokines and chemokines (Sládková and Kostolanský, 2006). These signaling molecules enhance the antiviral status of surrounding cells and draw additional immune cells to the infection site, aiding in the coordination of the larger immune response (Ramos and Fernandez-Sesma, 2015). While macrophages are crucial for controlling viral infections, their activity can sometimes lead to excessive inflammation and tissue damage (Catanzaro *et al*., 2020). During severe infections, the overproduction of pro-inflammatory cytokines, known as a cytokine storm, can occur (Catanzaro *et al*., 2020). This excessive inflammatory response can cause significant tissue damage and contribute to the pathology of diseases such as influenza and COVID-19 (Flerlage *et al*., 2021).

2.4. Cytokines and Chemokines

Cytokines and chemokines are multifunctional, secreted proteins that influence growth, differentiation, and activation, playing a pivotal role in regulating immune responses (Uchi *et al*., 2000). Cytokines are small, non-structural proteins with low molecular weights exerting complex regulatory effects on inflammation and immunity (Ray, 2016). They serve as intercellular messengers within the immune system (Commins *et al*., 2010). Cytokines coordinate the activities of various cell types across different body compartments to produce a unified immune response (Steinke and Borish, 2006). Cytokines include a wide range of interleukins, interferons, tumor necrosis factors, and growth factors (Ray, 2016). They play a major role in many diverse functions including immune cell differentiation, inflammation and viral pathogenesis (Steinke and Borish, 2006).

Chemokines are a group of small proteins secreted by numerous cell types (Juan and Colobran, 2009). Their name, which comes from "chemoattractant cytokines," reflects their role in directing the movement of nearby responsive cells (Ozga *et al*., 2021). Some chemokines are pro-inflammatory, meaning they can be produced during an immune response to attract immune cells to an infection site (Laing and Secombes, 2004). Others are homeostatic, playing a role in regulating cell migration during normal processes of tissue maintenance and development (Ozga *et al*., 2021).

2.5. Corona Virus disease 2019 (COVID-19)

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is identified as the causative agent of COVID-19 (Fu *et al*., 2021). This novel beta-coronavirus belongs to order Nidovirales, suborder Cornidovirineae, and family Coronaviridae (Fu *et al*., 2021). This viral infection is thought to have originated in late 2019 in Hubei Province, China, with a possible association with the Huanan Seafood Market (Hafeez *et al*., 2020). Coronaviruses are characterised by their large, enveloped structure and possess a single-stranded, non-segmented, positive-sense RNA genome, making it the largest among RNA viruses (Felsenstein *et al*., 2020). While the zoonotic source of SARS-CoV-2 is not definitively confirmed, sequence-based analysis points to bats as the primary reservoir (Hafeez *et al*., 2020).

The geographical significance of Wuhan as a major transportation hub facilitated the rapid spread of the virus, particularly during the Spring Festival travel rush, allowing millions to leave the city and disseminate the virus (Tsang *et al*., 2021). The global impact of the pandemic caused by SARS-CoV-2 is significant, with over 100 million confirmed COVID-19 cases and more than 2 million deaths reported worldwide (Pohl *et al*., 2021). The virus primarily spreads through respiratory droplets produced during coughing and sneezing. However, transmission can also occur through surface contamination when these droplets land on objects surfaces, subsequently being touched by others who then touch their eyes, nose, or mouth (Hafeez *et al*., 2020). Despite the lungs being the main target of coronavirus infection, the widespread distribution of ACE2 receptors in various organs raises concerns about potential damage to the cardiovascular, gastrointestinal, and central nervous systems (Ciotti *et al*., 2020).

SARS-CoV-2 is a single-stranded RNA virus enveloped in a membrane, with a genome consisting of 29,891 nucleotides encoding 9,860 amino acids (Tsang *et al*., 2021). The virus membrane includes four structural components: spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (Felsenstein *et al*., 2020). The receptor binding domain (RBD) in the S1 subunit of the S protein plays a crucial role in direct host entry (Tai *et al*., 2020). Additionally, the M protein

contributes to virion shaping and nucleocapsid binding, the N protein coats the viral RNA genome, and the E protein is involved in viral pathogenesis (Pohl *et al*., 2021).

The primary target for interfering with the virus entry process is the SARS-CoV-2 spike protein, particularly the receptor-binding domain (RBD) region (Tai *et al*., 2020). The spike glycoprotein forms homotrimers and undergoes cleavage into S_1 and S_2 subunits (Fu *et al*., 2021). The S_1 subunit comprises the RBD and the N terminal domain (NTD), which facilitate the recognition and binding to human receptors such as human angiotensin-converting enzyme 2 (hACE2) and other potential co-receptors, mediating the virus entry process (Fu *et al*., 2021).

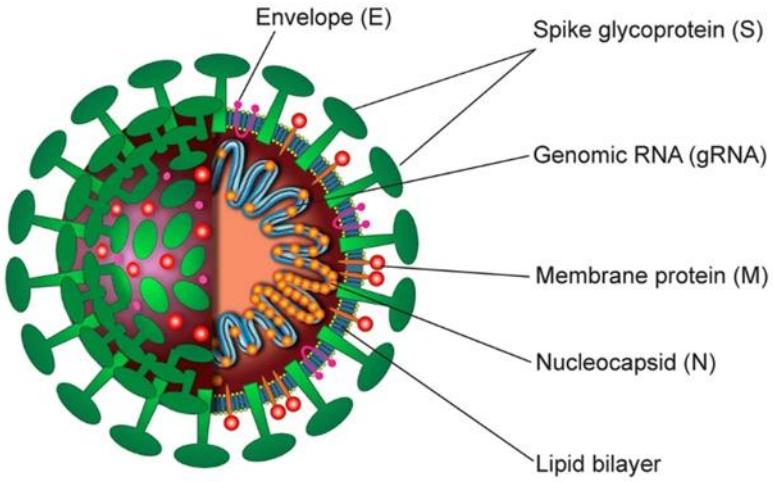


Figure 1: Depiction of the SARS-CoV-2 structure. the lipid bilayer influences viral entry by mediating fusion; the spike protein (S) facilitates binding to the trans-membrane ACE2 host receptor; the envelope (E) and membrane (M) proteins form the viral envelope and determine its shape; and the nucleocapsid (N) protein is bound to the virus's genomic RNA (gRNA) to form the nucleocapsid (Beig Parikhani *et al*., 2021).

Viral entry into host cells is facilitated by the proteolysis of the S protein at two specific locations, a process mediated by host proteases (Dessie and Malik, 2021). This proteolytic activity induces irreversible conformational changes in the S protein (Felsenstein *et al*., 2020). Certain antibodies in humans against SARS-CoV-2 mimic receptor engagement, providing a model for the conformational changes in the S

protein during antigen-antibody interaction (Dessie and Malik, 2021). The virus enters cells by binding to the host's cellular receptor/membrane-bound enzyme, angiotensin-converting enzyme 2 (ACE2), utilizing its spike (S) glycoprotein displayed on the virion's surface (Jackson *et al*., 2021).

ACE2 is highly expressed in surfactant-producing type 2 alveolar cells, as well as in ciliated and goblet cells in the airways, suggesting these cells serve as entry points for the virus in humans (Ricci *et al*., 2021). While ACE2 is also expressed in monocytes and macrophages, the levels are lower and not ubiquitous, providing an alternative entry mechanism for SARS-CoV-2 (Felsenstein *et al*., 2020). The infection process initiates when the virus enters the host cell, and the uncoated virus particle's spike protein attaches to its complementary host cell receptor (Hafeez *et al*., 2020). Subsequently, a host cell proteolytic enzyme cleaves and activates the receptor-bound spike macromolecule (Ricci *et al*., 2021). Depending on the available host cell proteolytic enzyme, cleavage and activation facilitate cell entry through either endocytosis or direct fusion of the viral envelope with the host membrane (Hafeez *et al*., 2020).

The symptoms induced by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) include fever, cough, shortness of breath, and diarrhoea (Adhikari *et al*., 2021). Elevated levels of inflammatory cytokines, such as interleukin-6 (IL-6), granulocyte macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor alpha (TNF-α), are associated with the severity of COVID-19 (Hirano, 2021). SARS-CoV-2 affects the immune system both directly and indirectly by triggering a cytokine storm and hyper-inflammation, resulting in severe damage to multiple organs and, ultimately, death (Ahmad and Haque, 2022). The innate immune response is initiated by lung epithelial cells, alveolar macrophages, and neutrophils, followed by adaptive immunological responses involving T and B lymphocytes to complete the immune response (Yazdanpanah *et al*., 2020).

The effective response and elimination of viral infections depend significantly on the expression of type I interferon (T1IFN) (Sodeifian *et al*., 2022). T1IFN expression, along with downstream signals, plays a crucial role in modulating cellular responses

and transforming cells into an anti-viral state, thereby facilitating infection control and the clearance of pathogens (Felsenstein *et al*., 2020). In the initial stages, immune cells detect viral infections by recognizing pattern-associated molecular patterns (PAMPs) derived from the virus, such as viral RNA (Sodeifian *et al*., 2022). These PAMPs bind to and activate pattern recognition receptors (PRRs) on or within immune cells, leading to the activation of immune cells (Yazdanpanah *et al*., 2020).

The RNA viruses like SARS-CoV-2, detection involves endosomal RNA PRRs, including Toll-like receptors (TLR-3 and 7), as well as cytoplasmic RNA sensors like retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) (Li *et al*., 2022). Typically, activation of TLR3/7 leads to the nuclear translocation of transcription factors NF-κB and IRF3, while RIG-I/MDA5 activation results in the activation of IRF3 (Li *et al*., 2022). Consequently, there is an increase in the expression of T1IFN (via IRF3) and other innate pro-inflammatory cytokines (IL-1, IL-6, TNF-α via NFκB) (Felsenstein *et al*., 2020). The activation and priming of both innate and adaptive immune responses are expected to contribute to pathogen clearance and recovery (Brown *et al*., 2023). However, in a subset of infected individuals, SARS-CoV-2 can evade immune system recognition by suppressing the immunological mechanisms that plays a role in eliminating the pathogens (Felsenstein *et al*., 2020).

2.6. Nuclear factor kappa-B (NF-κB) and Interferon Regulatory Factors (IRFs) inflammatory transcription factor

The NF-κB and IRF transcription factor families are crucial in inflammation, proliferation, differentiation mostly in system that allow cells to adapt and survive. In the antiviral response NF-κB act as key effector for the innate immune and inflammatory response (Iwanaszko and Kimmel, 2015). The NF-κB family consists of structurally related five members divided into two groups: RelA (p65), RelB, and c-Rel form the first group, while NF-κB1 (p50) and NF-κB2 (p52) make up the second group (Ghosh and Wang, 2021). The precursors p105 and p100 undergo proteolytic processing to become the mature p50 and p52, respectively (Yilmaz *et al*., 2014).

NF-κB subunits can form various homo- or heterodimers, such as p65-p50 or p50-p50, with each dimer exhibiting unique functions (Yilmaz *et al*., 2014).

These activated dimers typically move from the cytoplasm to the nucleus, where they bind to promoter regions of target genes that contain specific consensus sequences (Kusiak and Brady, 2022). Although generally cytosolic in their inactive state due to sequestration, NF-κB subunits can sometimes be found in mitochondrial fractions or pre-existing in the nucleus (Kusiak and Brady, 2022). NF-κB dimers are primarily recognised as pro-inflammatory transcription factors, commonly associated with the expression of pro-inflammatory cytokines, chemokines, and adhesion molecules (Iwanaszko and Kimmel, 2015).

The Interferon Regulatory Factors (IRFs) are a family of transcription factors essential for various immune responses, including immune cell development and responses to pathogens (Feng *et al*., 2021). Among them, IRF3, IRF5, and IRF7 are crucial to produce type I interferons, which are activated downstream of pathogen recognition receptors that detect viral RNA and DNA (Jefferies, 2019). Another member, IRF9, is key in regulating interferon-driven gene expression (Jefferies, 2019). Additionally, IRF4, IRF8, and IRF5 play significant roles in the development and phenotype of myeloid cells, thereby influencing inflammatory responses (Feng *et al*., 2021). Understanding how the levels and activities of these IRFs are regulated is vital, as disturbances can lead to dysregulated immune responses and potentially result in autoimmune diseases (Feng *et al*., 2021).

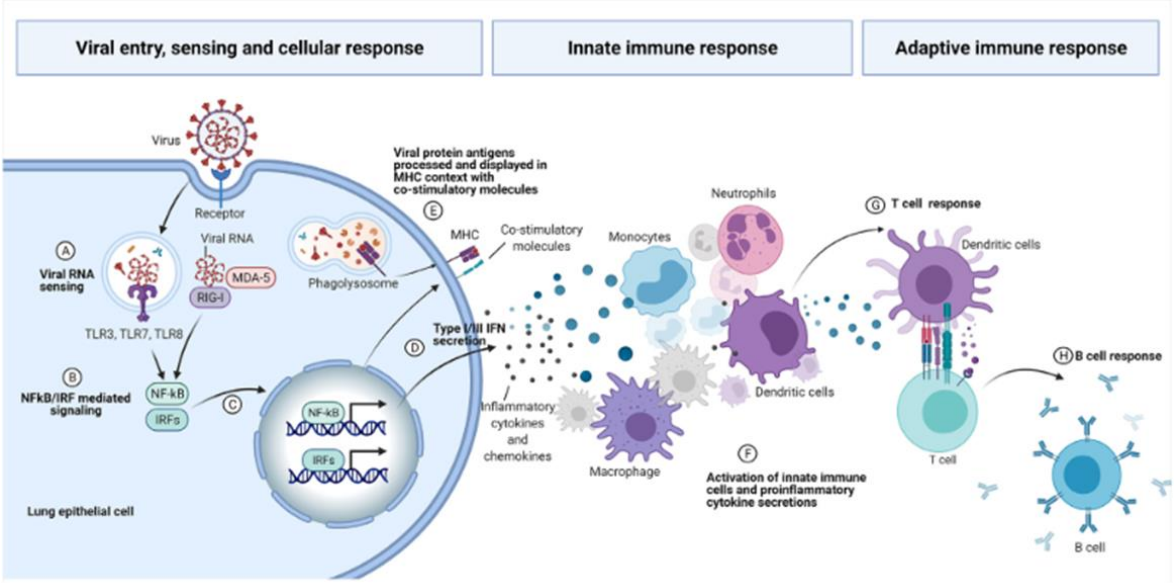


Figure 2: Schematic view of the viral entry, sensing, and host immune response. The viral entry process involves the RNA virus binding to cellular receptors and subsequent internalization. Pattern Recognition Receptors (PRRs) play a crucial role in sensing the viral RNA (A). This trigger cellular signalling mediated by NF-kB and IRFs transcription factors (B), ultimately leading to the nucleolus activation (C). This activation results in the expression of specific genes, including Type I and Type III interferons (IFNs) and proinflammatory cytokines (D). Simultaneously, viral protein antigens are processed and presented in the MHC context with co-stimulatory molecules (E). The pro-inflammatory cytokines and chemokines generated contribute to the cellular innate response, leading to a notable infiltration of monocytes and neutrophils (F). Activated dendritic cells then present these antigens to adaptive T cells (G), which, in turn, secrete cytokines to stimulate B cells for the production and secretion of immunoglobulins (H) (Akamatsu *et al.*, 2021).

2.7. Pathological effects and potential treatment of Covid-19

Severe cases of COVID-19 have been associated with a life-threatening condition known as cytokine storm, characterized by uncontrolled cytokine expression (Brown *et al.*, 2023). Within the airways, key players of the innate immune system, such as epithelial cells, macrophages, and dendritic cells, detect viral particles, initiating inflammatory cascades (Kozlov *et al.*, 2021). Given SARS-CoV-2's affinity for the airway epithelium, virus-infected cells induce the expression of cytokines, chemokines, and cell adhesion molecules (Gusev *et al.*, 2022). These molecules act as signals, attracting more immune cells that contribute to tissue damage and

escalate the inflammatory response (Gusev *et al*., 2022). Consequently, cytokines emerge as crucial players in SARS-CoV-2 infection (Costela-Ruiz *et al*., 2020).

Simultaneously, the activation of pattern recognition receptors (PRRs) and the production of pro-inflammatory cytokines play a significant role in the development of oxidative stress (Kozlov *et al*., 2021). Toll-like receptors (TLRs) signalling, for instance, has been implicated in the priming, activation, and translocation of NADPH Oxidases (NOX) towards the plasma membrane through NOX subunit phosphorylation (Li *et al*., 2019). Thus, TLR signalling serves as a potent mechanism that promotes the assembly of NADPH oxidase, thereby accelerating the production of reactive oxygen species (ROS) (Li *et al*., 2019). Additionally, the activation of NF-κB signalling increases the expression of the gp91 subunit, an integral component of the NADPH oxidase complex responsible for generating ROS (Moghadam *et al*., 2021). The rise in ROS concentration, coupled with a decrease in antioxidant defence, contributes to the development of oxidative stress (Kozlov *et al*., 2021).

There is a growing body of evidence suggesting the potential involvement of oxidative stress in the underlying mechanisms of SARS-CoV-2 infection (Suhail *et al*., 2020). Currently, the search for both existing and novel medications to alleviate symptoms and reduce mortality is a high priority, with a particular focus on considering antioxidant drugs (Kozlov *et al*., 2021). Certain pathways related to oxidative stress signalling may directly influence the onset of a cytokine storm during the infection (Brown *et al*., 2023). Therefore, it is crucial to investigate the impact of inflammatory mediators that encompasses protein polypeptides and reactive molecules such as ROS and NOS on the severity of coronavirus infection. Implementing appropriate antioxidant therapy is likely to play a significant role in reducing the rate of hyperinflammation.

Despite ongoing research on various therapeutic molecules, there is a lack of developed vaccines or specific treatments for COVID-19 (Benarba and Pandiella, 2020). Since the outbreak of the pandemic, there has been a growing interest in exploring the potential of different medicinal plants, either independently or in conjunction with conventional medications, for treating infected individuals (Tagde

et al., 2021). Medicinal plants and natural products continue to be viewed as promising alternatives for the prevention or treatment of various diseases (Khan and Ahmad, 2019). Evaluating the potential of combining natural products or herbal mixtures with established anti-COVID-19 drugs presents a promising avenue for preventive and therapeutic exploration (Tagde *et al*., 2021).

Plants in general are abundant in antioxidants that are necessary for their survival in the environment (García-Caparrós *et al*., 2021). Plant-based traditional medicines are still widely used because they are inexpensive, effective with minimal side effects when used as common conditions regiments (Anand *et al*., 2019). Growing body of evidence pinpoint the use of medicinal plants as the replacement of the current pharmaceutical treatments (Dhyani *et al*., 2022).

2.8. Curcumin as the possible treatment

Turmeric, derived from the rhizome of a perennial plant belonging to the Zingiberaceae family and primarily cultivated in India and Southeast Asia, is a well-known Indian spice (Hay *et al*., 2019). While its medicinal properties have been acknowledged for millennia, the specific mechanisms of action and active components have only recently undergone analysis (Khan and Ahmad, 2019). Across Asian countries, turmeric has been traditionally utilised as a medicinal herb due to its noted antioxidant and anti-inflammatory properties (Permatananda *et al*., 2021). However, its recognition and usage have transcended regional boundaries, finding application worldwide for its potential health benefits (Permatananda *et al*., 2021). As one of the most extensively researched plants, turmeric stands out as a primary source of curcumin (Kozlov *et al*., 2021) .

Figure 3: *Curcuma longa* plant and turmeric rhizomes. The rhizome, from which the turmeric is derived, is tuberous, with a rough and segmented skin. The rhizomes mature beneath the foliage in the ground. They are yellowish brown with a dull orange interior. The main rhizome is pointed or tapered at the distal end and measures 2.5–7.0 cm (1–3 inches) in length and 2.5 cm (1 inch) in diameter, with smaller tubers branching off. (Abdel-Hafez *et al*., 2021).

Curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), is a bright yellow phytochemical (Malhotra *et al*., 2019). It has been linked to several biological and pharmacological activities, including antioxidant, anti-viral and anti-inflammatory (Tavaf *et al*., 2020; Worachartcheewan *et al*., 2011). Despite the therapeutic potential of curcumin, its clinical applications are hindered by factors such as low bioavailability, which stems from poor water solubility, limited oral absorbability, and a rapid metabolic rate (Karthikeyan *et al*., 2020).

Thus, laboratory curcumin derivatives with various structural changes have been produced to improve its pharmacological profile (Khor *et al*., 2019; Sahu, 2016). several unique ways to improve the pharmacokinetic characteristics of curcumin have been employed to synthesise new curcumin derivatives with established pharmacological designs (Tavaf *et al*., 2020), moreover curcumin derivatives have been synthesised to improve the anti-inflammatory, anti-viral and antioxidant activities (Worachartcheewan *et al*., 2011). Many structural modifications involving

the carbonyl moiety and active methylene group have recently been made, and it has been discovered that some of the active methylene and carbonyl substituted curcumin derivatives/analogues have greater antioxidant activity than curcumin (Lal *et al*., 2016; Sahu *et al*., 2016)

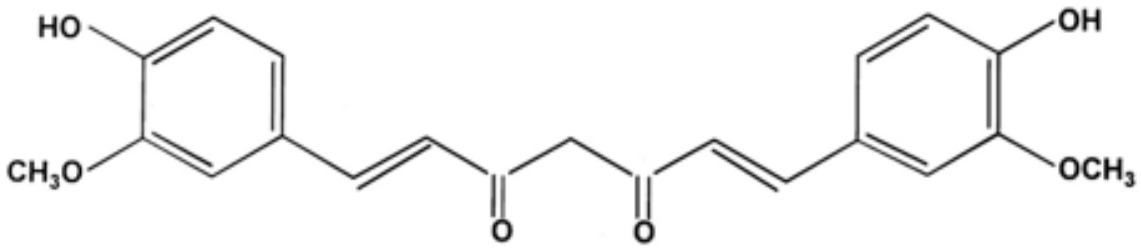


Figure 4: The chemical structure of curcumin. Curcumin is a symmetric molecule, also known as diferuloyl methane. The IUPAC name of curcumin is (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, with a chemical formula $C_{21}H_{20}O_6$, and molecular weight of 368.38 (Abdel-Hafez *et al*., 2021).

According to various research papers evaluated, curcumin derivatives seem to be one of the best drugs that can be used to help the immune system to maintain balance between antioxidants and oxidants in cases when the cells fails since they have shown to be effective antioxidants (Lee *et al*., 2013). However, there is still paucity in data regarding the effect of curcumin derivatives in relation to the oxidative stress induced by SARS-CoV-2 spike S_1 protein on macrophage cells. Hence, this study aims to investigate the effects of curcumin derivatives on SARS-CoV-2 spike S_1 protein induced oxidative stress on Raw 264.7 macrophage cells.

AIM

The aim of this study is to evaluate the effects of curcumin derivatives compounds on SARS-CoV-2 spike S_1 protein induced oxidative stress on Raw 264.7 macrophage cells.

OBJECTIVES

i. Effects of curcumin derivatives on viability of Raw 264.7 macrophage cells after stimulation with SARS-Cov-2 spike S_1 proteins.

ii. Effects of curcumin derivatives on reactive oxygen species production post Raw 264.7 macrophage cells stimulation with SARS-Cov-2 spike S1 proteins.

iii. Effects of curcumin derivatives on Nitric oxide production post Raw 264.7 macrophage cells stimulation with SARS-Cov-2 spike S_1 proteins.

iv. Effects of curcumin derivatives on the level of immune-protein expression in Raw 264.7 macrophage cells following exposure to SARS-Cov-2 spike S_1 proteins.

v. Apoptotic effects of curcumin derivatives on Raw 264.7 macrophage cells after exposure to SARS-Cov-2 spike S_1 proteins.

CHAPTER 3

EXPERIMENTAL PROCEDURE

3.1 Materials

The Raw 264.7 macrophage cells were collected from cellonex. JHB. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (South Logan, UT, USA). Antibiotic mixture containing penicillin and streptomycin (Pen-Strep), phosphate buffered saline (PBS); Pierce® Bicinchoninic Acid (BCA) protein assay kit; Polyvinylidene fluoride (PVDF) membranes and MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] were obtained from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA, USA) while Dimethyl Sulfoxide (DMSO), Trypan-blue dye, curcumin, Lipopolysaccharide (LPS), Propidium Iodide (PI), RIPA lysis buffer and DAF-2 DA were purchased from Sigma-Aldrich® (St Louis, MO, USA). Curcumin derivatives were collected from University of Johannesburg in South Africa and SARS-CoV-2 spike s_1 proteins were purchased from Sino Biological (Beijing, China). Muse Annexin-V & Dead Cell Kit and Oxidative stress kit were purchased from Luminex (USA). Primary antibodies were purchased from Santa Cruz biotechnology (USA). The curcumin derivatives were synthesised by Dr Marole Maluleke University of Johannesburg.

3.2 Cell culture

Raw 264.7 cells were propagated in a cell culture flask at 37 °C in a humidified environment (incubator) of 95 % air and 5 % CO_2. They were maintained in Dulbecco's Modification of Eagle Medium (DMEM), supplemented with; 10 % FBS, 2 mM L-glutamine and 1 x PSN. The density of the cells was determined by 1:1 volume of cells to 1 x trypan blue dye onto an Invitrogen countess 3 automated cell counter.

3.3 Cytotoxicity assays

3.3.1 Examination of cytotoxic levels of curcumin derivatives using MTT

This assay relies on the mitochondrial enzyme succinate dehydrogenase to reduce the water-soluble yellow MTT into a water-insoluble purple formazan, which is then

solubilised by DMSO (Boyraz *et al*., 2021) . In this study, Raw 264.7 cells were seeded at 1 × 10^5 cells/well in a 96-well culture plate and allowed to attach, overnight. Cells were then treated with different concentrations of curcumin derivatives (5; 2.5; 1.25; and 0.625 mM) or curcumin (50 µM) for 24 hours. After 24 hours, the solutions from the wells were discarded then 80 µL of 2 mg/ml MTT was added to each well and the plate was incubated for 2 hours. Following incubation, supernatants from each well were discarded and 100 µL DMSO was added to dissolve formazan crystals and the absorbance readings were taken at 560 nm using Multiskan Sky microplate spectrophotometer (Thermo Fisher Scientific, USA). Percentage viability was calculated using this formula:

$$\text{Cell viability (\%)} = \frac{Average\ OD\ (experimental\ group)}{Average\ OD\ (untreated\ group)} \text{ x } 100$$

3.3.2 Examination of curcumin derivatives impact on membrane integrity using propidium Iodide (PI)

This assay is based on the principle that when cells die, they lose their membrane integrity. propidium iodide (PI) was used for nuclear staining of dead cells resulting in red fluorescing dead cells. Raw 264.7 cells were seeded at 1×10^5 cells/well in a 96-well culture plate and allowed to attach overnight. Cells were then treated with different concentrations of curcumin derivatives (5; 2.5; 1.25; and 0.625 mM) or curcumin (50 µM) for 24 hours. Thereafter, the cell culture medium was discarded followed by the addition of 50 µg/ml of PI to each well and 30 minutes incubation in the dark. Fluorescence intensity reading was carried out using Multiskan Sky microplate spectrophotometer at 525 nm (Thermo Fisher Scientific, USA).

3.4 Examination of apoptosis using Annexin-V/PI apoptosis assay

The mode of cell death was evaluated using the Muse Annexin V & Dead Cell Kit (Luminex, USA). The assay depends on the attachment of fluorescently labeled Annexin V to phosphatidylserine (PS), which moves to the outer surface of the cell membrane when apoptosis begins(Chinnasamy *et al*., 2020) . Raw 264.7 cells were seeded at 3×10^5 cells/well in a 24 well culture plate and incubated overnight. Cells were then treated with different concentrations of curcumin derivatives (5; 2.5; 1.5;

and 1 mM) or curcumin (50 µM) for 24 hours. After 24 hours of treatment, cells were harvested followed by centrifugation at 300 x g for 5 minutes at 25°C. Thereafter, the supernatant was discarded, and the pellet was washed once with 1 x PBS by centrifugation. The cell pellet was further resuspended in 50 µL of 1 x PBS and stained with 50 µL of annexin V-PI reagent followed by 20 minutes incubation in the dark at room temperature. The mode of cell death was analysed using Guava Muse Cell Analyser (Luminex, USA).

3.5 Determination of IL-6 production using ELISA

To quantify the production of Interleukin 6 (IL-6), Raw 264.7 cells were seeded at 3 x 10^5 cells/well in a 24 well plate and allowed to attach overnight. The cells were then stimulated with 10 µg/ml of LPS, and 100 ng/ml of SARS-CoV-2 spike S_1 and treated with 1 mM of curcumin derivatives (TP011, TP012 and TP014) for 24 hours. After 24 hours of treatment, the supernatant was collected and frozen -20°C for later use. Enzyme-linked immunosorbent assay (ELISA) was carried out according to the manufacturer's protocol (Elabscience, Wuhan). The standard was serially diluted 2-fold and 100 µL was added in duplicates in a 96-well plate followed by addition of samples for 90 minutes at 37°C. The added samples were discarded and immediately 100 µL of biotinylated detection antibody, diluted 1:99 in a diluent, was added to each well. This was then followed by aspiration of excess detection antibody and plate was washed 3 times with wash buffer. The Horseradish peroxidase (HRP) solution was then diluted 1: 99 in a diluent, thereafter 100 µL of this diluted HRP added to each well and incubated for 30 min at 37°C. This solution was aspirated, and wells were washed 5 x with wash buffer. After blotting of wells, 90 µL of substrate reagent was added to each well and the plates were incubated for 15 minutes at 37°C. Thereafter, 50 µL of stop solution was added followed by measurement of colour colour at 450 nm using Multiskan Sky microplate spectrophotometer (Thermo Fisher Scientific, USA).

3.6 Determination of the production of Nitric oxide (NO)

DAF-2 Diacetate (DAF-2 Da) is a cell-permeable nitric oxide (NO) probe that is initially non-fluorescent (Kataria *et al*., 2020). Inside the cell, it is deacetylated by

intracellular esterase, converting it to DAF-2, which reacts with NO to produce a fluorescent product called trazolofluorescein (DAF-2T) (Kataria *et al*., 2020). Raw 264.7 cells were seeded at $3x10^5$ cells/well in a 24 well plate and incubated overnight. The cells were then treated with 1 mM of curcumin derivatives (TP011, TP012 and TP014); 10 µg/ml of LPS or 100 ng/ml of SARS-CoV-2 spike S_1 for 24 hours. After 24 hours of treatment, cells were harvested followed by centrifugation at 300 x g for 5 minutes at 25°C. Thereafter, the supernatant was discarded, and the pellet was washed once with 1 x PBS by centrifugation. After washing, the supernatant was discarded followed by the addition of 1 x PBS (200 µL) and DAF-2 Da (200 µL) in a 1:1 proportion. The solution was further added with 20 µL of PI (20 µg/ml) and then incubated for 30 min in the dark at room temperature. The production of NO was analysed using Guava Muse Cell Analyser (Luminex, USA).

3.7 Determination of the production of reactive oxygen species (ROS)

Muse® Oxidative Stress Reagent is a cell permeable reagent based on dihydroethidium (DHE) (Nam *et al*., 2019). DHE upon reaction with superoxide anions undergoes oxidation to form the DNA-binding fluorophore ethidium bromide or a structurally similar product which intercalates with DNA resulting in red fluorescence (Nam *et al*., 2019). Raw 264.7 cells were seeded at $3x10^5$ cells/well in a 24 well plate and allowed to attach overnight. This was followed by treatment with 1 mM of curcumin derivatives (TP011, TP012 and TP014); 10 µg/ml of LPS or 100 ng/ml of SARS-CoV-2 spike S_1 for 24 hours. After 24 hours of treatment, cells were harvested followed by centrifugation at 300 x g for 5 minutes at 25°C. Thereafter, the supernatant was discarded, and the pellet was washed once with 1 x PBS by centrifugation. The ROS production was measured using an oxidative stress kit (Luminex, USA). Muse oxidative stress reagent was diluted 1:100 with 1 x assay buffer to make intermediate solution which was further diluted 1:80 with 1 x assay buffer to make muse oxidative stress working solution. The cell pellet was added with 10 µL of 1 x assay buffer followed by the addition of 190 µL of muse oxidative stress working solution and 30 minutes incubation at 37°C. After

incubation, the ROS production was analysed using Guava Muse Cell Analyser (Luminex, USA).

3.8 SDS-PAGE and western blotting

The effect of curcumin derivatives (TP011, TP012 and TP014) on NF-κB, IκB-α and Nrf2 expression levels was examined using western blotting. Raw 264.7 cells were cultured in T 25 culture flasks and treated for 24 hours with 1 mM of curcumin derivatives or 100 ng/ml SARS-CoV-2 spike S_1. The attached cells were harvested and centrifuged at 300 x g for 5 minutes. The cell pellet was resuspended in a RIPA lysis buffer supplemented with protease inhibitor, incubated for 30 minutes on ice, and centrifuged at 12 000 rpm for 20 minutes at 4°C. The total cellular protein content was quantified using a BCA protein quantification kit (Thermo Scientific, Rockford, USA), according to the manufacturer's protocol. Proteins were separated on a 12 % sodium dodecyl sulphate-polyacrylamide gel at 100 volts for 2 hours. Proteins were transferred to PVDF membranes for 90 minutes. Membranes were blocked with 5 % fat-free milk for 1 hour and incubated in the presence of primary antibodies in phosphate-buffered saline-tween-20 (PBST) at 4°C, overnight. The primary antibodies (1:1000) used were mouse anti-NF-κB, IκB-α and Nrf2. The membranes were washed three times at 5-minute intervals using 1× PBST and incubated in the presence of HRP-conjugated mouse-(anti-mouse) secondary antibody (1:20000) in 1× PBST-milk for 1 hour at room temperature, with constant shaking. Chemiluminescence the membranes were washed again as described above and 1:1 mixture of Enhanced chemiluminescence (ECL) substrates was pipetted on each membrane and incubated for 2 minutes. The membranes were then developed and captured using a chemiluminescence imaging system. Mean band densities were measured using the ImageJ software and quantitative analysis done by normalising to GAPDH. Data was expressed as relative protein expression. The formulas below were used to measure band signals.

$$\text{Lane normalisation factor} = \frac{\text{observed signal of GAPDH for each lane}}{observed\ signal\ of\ GAPDH\ for\ untreated\ control}$$

Normalised experimental signal (%) = $\frac{\text{observed experimental signal}}{\text{Lane normalisation factor}}$ x 100

3.9 Statistical analysis

Statistical differences between treatments and the controls were calculated using the Graphpad prism version 8.4.2 software by one-way of variance (ANOVA) followed by Dunnett's comparison tests. The data were expressed as mean ± SEM of two or three independent experiments done in duplicate and the p-values ≤ 0.05 were considered significant.

CHAPTER 4

RESULTS

4.1 Effects of curcumin derivatives on Raw 264.7 cells viability using MTT assay

Curcumin derivatives were evaluated for their cytotoxicity effect on Raw 264.7 cells using the MTT viability assay. Raw 264.7 cells were treated for 24 hours with various concentrations (5-0.625 mM) of curcumin derivatives (TP011, TP012 and TP014) and 50 μM curcumin as a positive control. The change in percentage cell viability was in a dose-dependent manner, such that, as the concentration of the derivatives decreased the cell viability increased **(figures 4.1.1B, 4.1.2B, 4.1.3B)**. As shown in **figure 4.1.1B**, a significant ($p \leq 0.05$) reduction in the viability of Raw 264.7 cells was observed in cells treated with 5 mM and 2.5 mM of the TP011 curcumin derivative when compared to untreated control, however, those treated with 1.25 mM and 0.625 mM showed no significant reduction in viability. In **figure 4.1.2B**, Raw 264.7 cells treated with 5 mM, 2.5 mM and 1.25 mM concentrations of TP012 curcumin derivative resulted in a significant reduction in viability as compared to the untreated control, whereas the concentration of 0.625 mM resulting in non-significant decrease. **Figure 4.1.3B** showed no significant reduction in viability for cells treated with TP014 curcumin derivative of 2.5 mM to 0.625mM as compared to the untreated control and for the concentration of 5mM a significant reduction was observed. These curcumin derivatives at low doses (1.25 mM-0.625 mM), showed no cytotoxic effect on Raw 264.7 macrophage cells. Exposure to 50 μM curcumin resulted in a significant reduction in Raw 264.7 cells. Moreover, Raw 264.7 cells treated with 1.25 mM and 0.625 mM of curcumin derivatives (TP011, TP012 and TP014) and untreated cells retained their spindle shape morphology **(figures 4.1.1A, 4.1.2A, 4.1.3A**). However, cells treated with 5mM and 2.5 mM of curcumin derivatives and 50 μM curcumin resulted in morphological change from spindle to spherical shape.

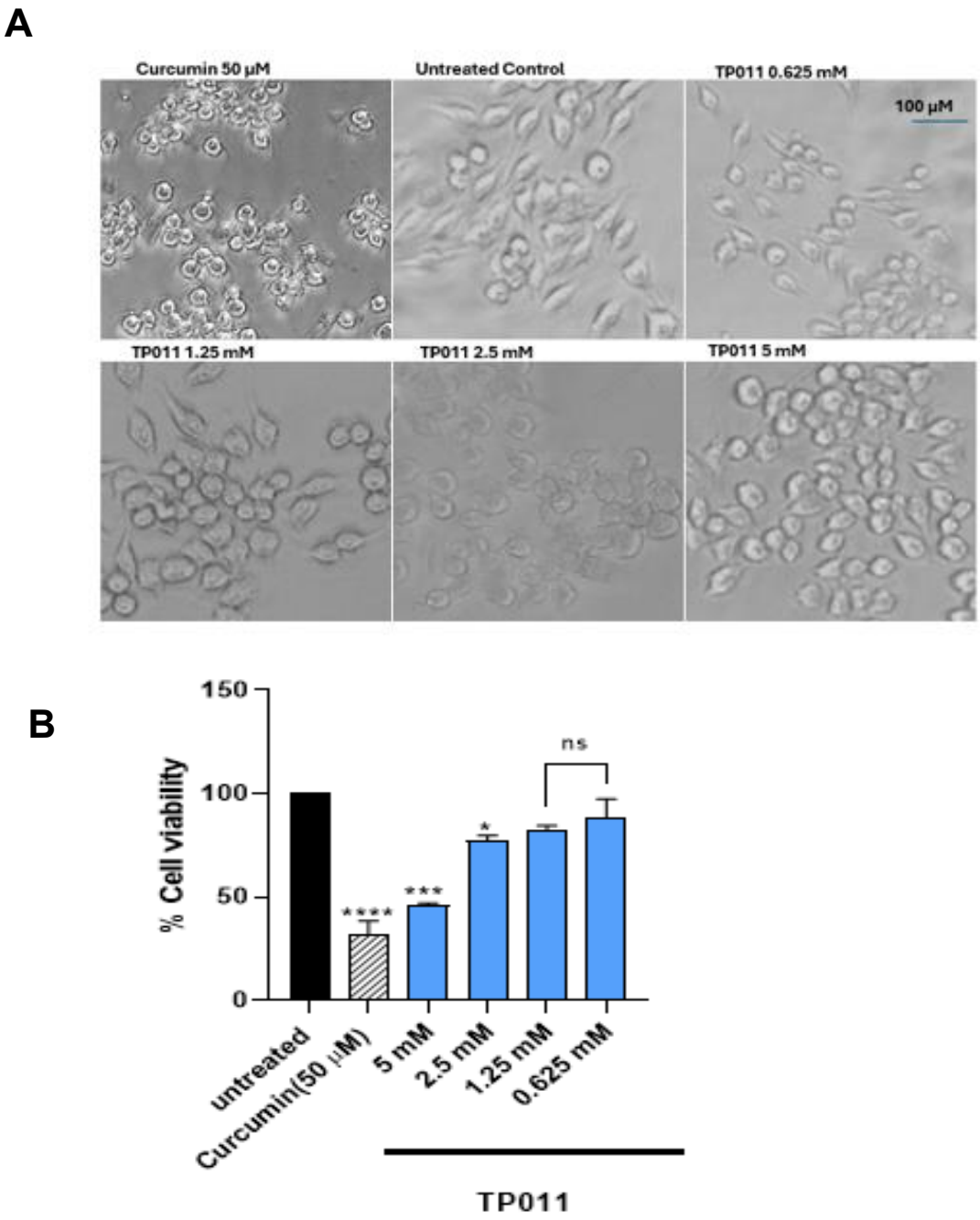
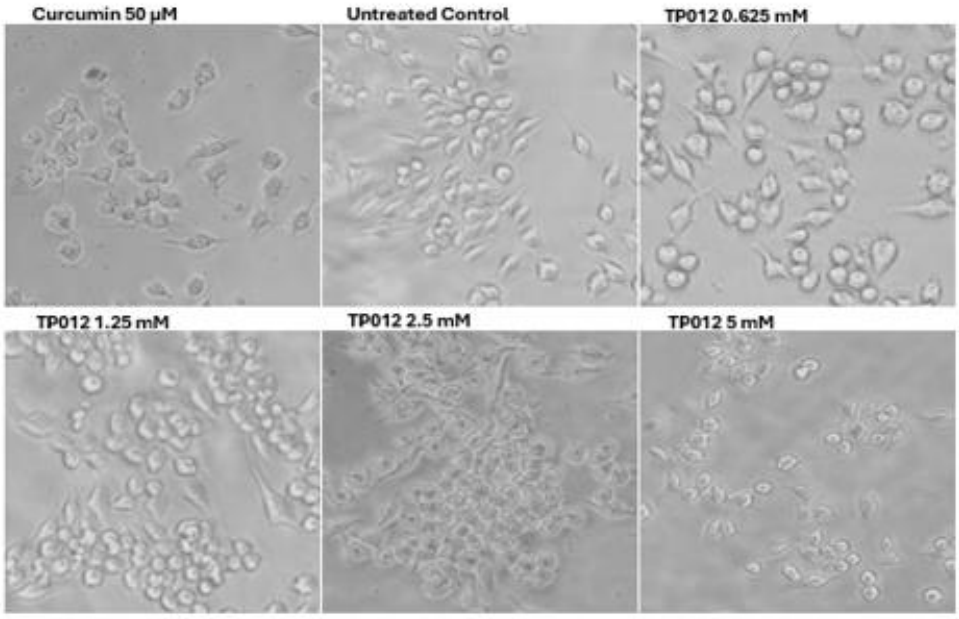


Figure 4.1.1: Effect of TP011 curcumin derivative on Raw 264.7 macrophage cell viability. The cells were seeded at $1x10^5$ cells/well in a 96-well culture plate and treated with varying concentrations (5 – 0.625 mM) of TP011 and 50 µM of curcumin as a positive control for 24 hours. Cells imaging was executed with the Olympus CKX53 Inverted microscope, (Olympus, Japan) **(A)**; thereafter MTT was added, followed by absorbance measurement using GloMax-Multi microplate reader at 560 nm (Promega, USA) **(B)**. Values are expressed as mean ± SEM. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to the untreated control. Imaging Scale: 100 µM.

A



B

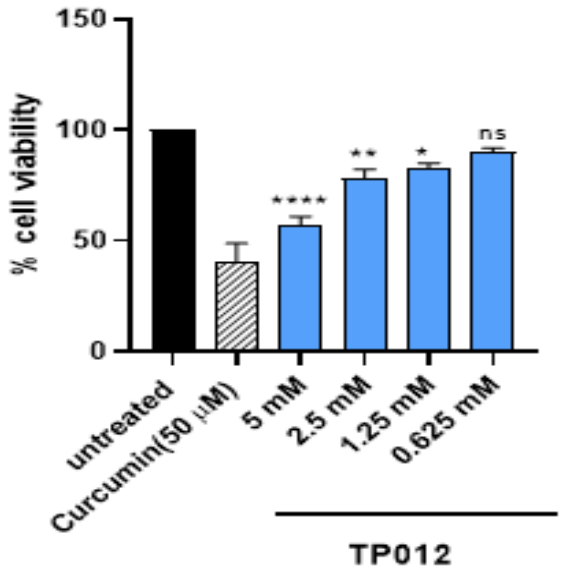


Figure 4.1.2: Effect of TP012 curcumin derivative on Raw 264.7 macrophage cell viability. The cells were seeded at $1x10^5$ cells/well in a 96-well culture plate and treated with varying concentrations (5 – 0.625 mM) of TP012 or 50 µM of curcumin as a positive control for 24 hours. Cells imaging was executed with the Olympus CKX53 Inverted microscope, (Olympus, Japan) **(A)**; thereafter MTT was added, followed by absorbance measurement using GloMax-Multi microplate reader at 560 nm (Promega, USA) **(B)**. Values are expressed as mean ± SEM. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to the untreated control. Imaging scale: 100 µM.

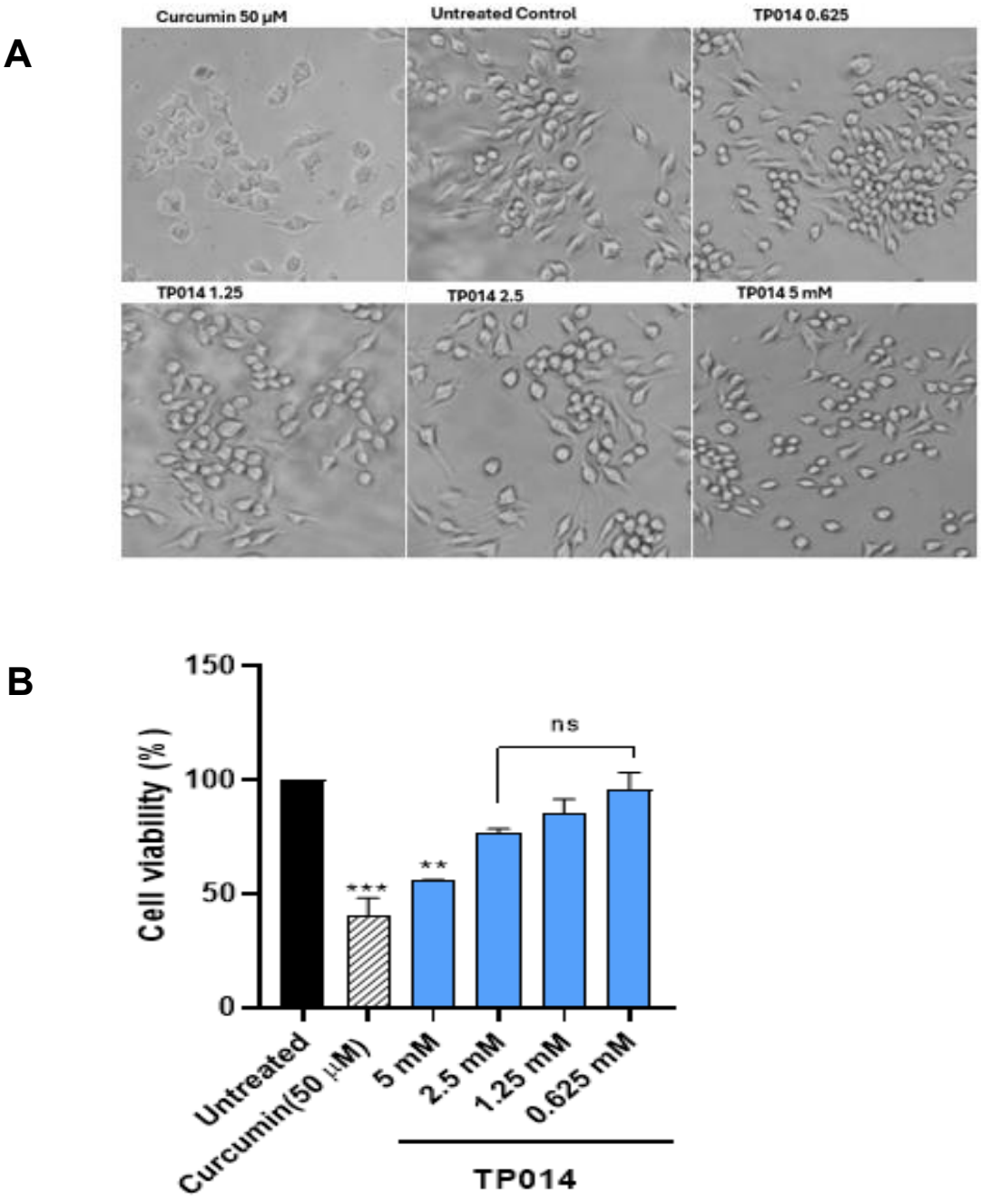


Figure 4.1.3: Effect of TP014 curcumin derivative on raw 264.7 macrophage cell viability. The cells were seeded at $1x10^5$ cells/well in a 96-well culture plate and treated with varying concentrations (5 – 0.625 mM) of TP014 or 50 µM of curcumin as a positive control for 24 hours. Cells imaging was executed with the Olympus CKX53 Inverted microscope, (Olympus, Japan) **(A)**; thereafter MTT was added, followed by absorbance measurement using GloMax-Multi microplate reader at 560 nm (Promega, USA) **(B)**. Values are expressed as mean ± SEM. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to the untreated control. Imaging scale: 100 µM.

4.2 Effects of curcumin derivatives on cell membrane integrity of Raw 264.7 cells using Propidium Iodide

A fluorescence dye (Propidium Iodide) was used to further examine the effect of curcumin derivatives (TP011, TP012 and TP014) on membrane integrity as one of the cell viability phenomena. Cells were treated with various concentrations (5-0.625 mM) of curcumin derivatives and 50 µM of curcumin as a positive control. It was observed that the change in the population density of cells that have retained their membrane integrity was in a concentration dependent manner, such that, as the concentration of the curcumin derivatives decreased the viability increased **(figure 4.2)**. A significant ($p \leq 0.05$) reduction in viability for Raw 264.7 cells treated with 5 mM-0.625 mM of curcumin derivatives was observed when compared with untreated control cells except for 0.625 mM TP012 treated cells which showed non-significant reduction. The curcumin control cells showed a significant reduction in viability indicating the high population of cells with compromised membrane integrity. Compared to curcumin control, the curcumin derivatives were shown to have less negative effect on the membrane integrity of Raw 264.7 cells.

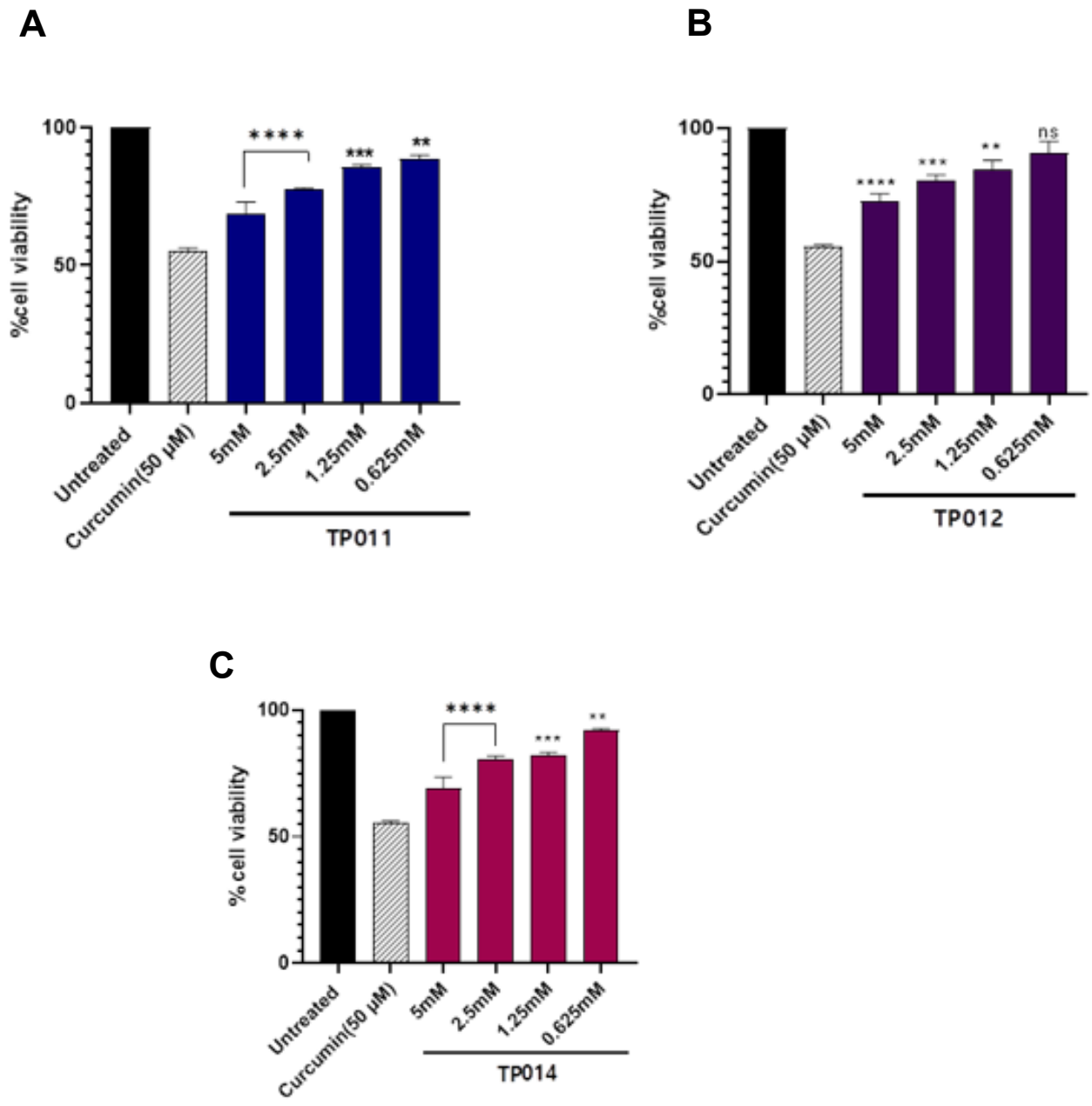


Figure 4.2: Effect of curcumin derivatives on Raw 264.7 cells membrane integrity using PI. To examine the effects of curcumin derivatives on membrane integrity of Raw 264.7 macrophage cells in a fluorescence-based assay, cells were seeded at $1x10^5$ cells/well in a 96-well culture plate, treated with curcumin derivatives and 50 µM curcumin as positive control for 24 hours. Thereafter the cells were stained with 50 µg/ml PI for 30 minutes in the dark. Fluorescence intensity was measured using Multiskan Sky microplate spectrophotometer at 525 nm (Thermo Fisher Scientific, USA). Each data point represents the mean ± SEM of three independent experiments, performed in duplicates. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to the untreated control.

4.3 Apoptotic effects of curcumin derivatives on Raw 264.7 cells.

The mode of cell death induced by the curcumin derivatives (TP011, TP012 and TP014) on Raw 264.7 cells was evaluated using muse Annexin-V and dead cell assay kit following the manufacturer's protocol. The chromatograms show the analysis of live cells and cells at the early stages of apoptosis, those at the late stages of apoptosis and necrotic cells **(figures 4.3.1A, 4.3.2A, 4.3.3A)**. As shown in **figures 4.3.1B, 4.3.2B, 4.3.3B**, the percentage of apoptotic cells is higher than that of necrotic cells. The number of cells undergoing apoptosis decreased in a concentration-dependent manner, after treatment with different concentrations of curcumin derivatives. Moreover, a significant ($p \leq 0.05$) decrease in Raw 264.7 total apoptotic cells was observed following treatment with 2.5 mM-0.625 mM of curcumin derivatives **(figures 4.3.1B, 4.3.2B, 4.3.3B)**. On the other hand, treatment with 5 mM revealed a non-significant decrease in total apoptotic cells. High percentages of apoptotic cells were observed in curcumin control as compared to untreated control.

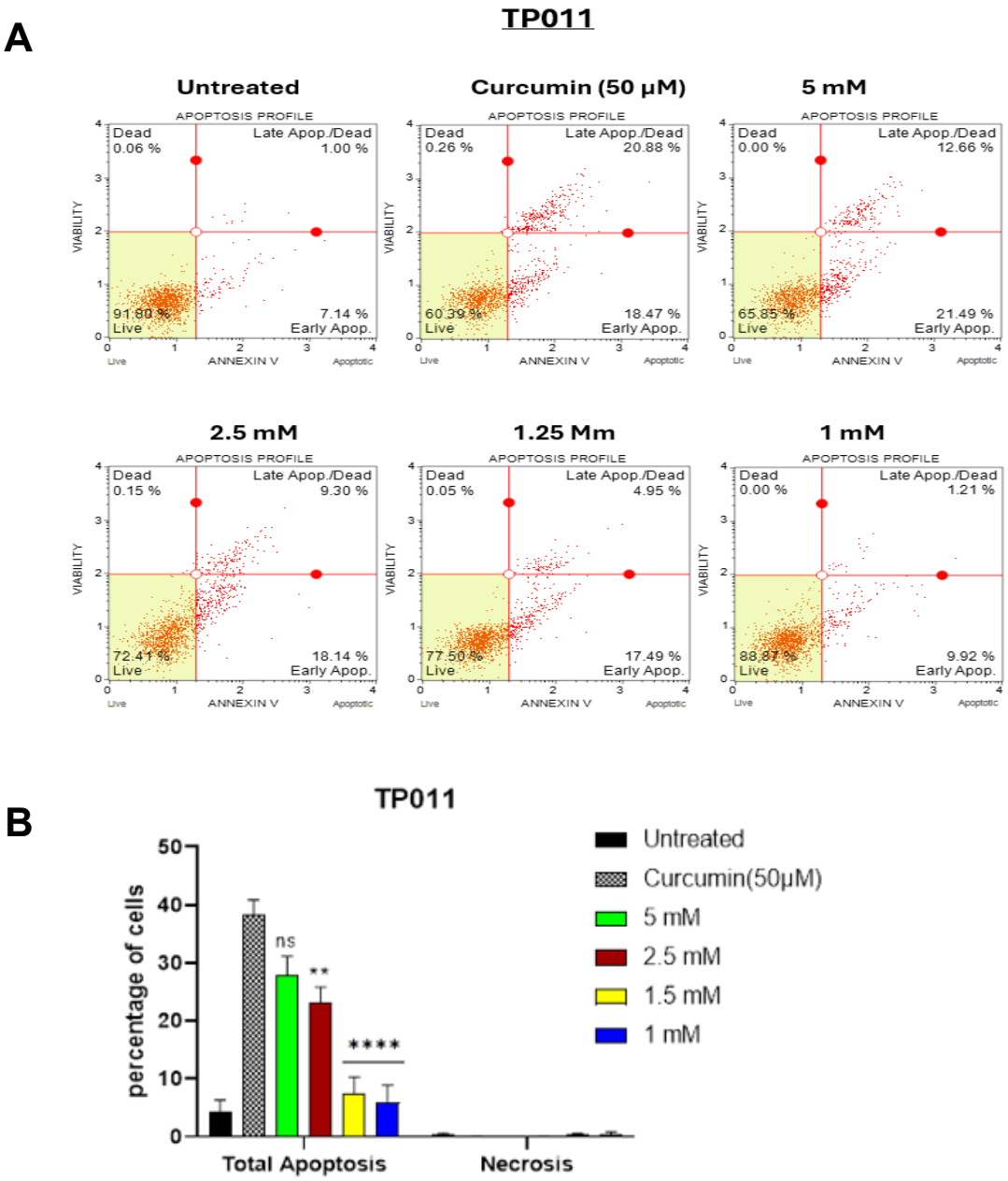


Figure 4.3.1: Examination of the mode of cell death induced by TP011 curcumin derivative on Raw 264.7 cells. Cells were seeded at $1x10^5$ cells/well in 24 well plate and a treated with curcumin derivatives and 50 µM curcumin as positive control for 24 hours. The number of cells undergoing cell death were quantified using Annexin-V and dead cell assay kit and analysed using Muse analyser. Data is expressed as mean ± SEM of three independent experiments, performed in duplicates. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to the untreated control.

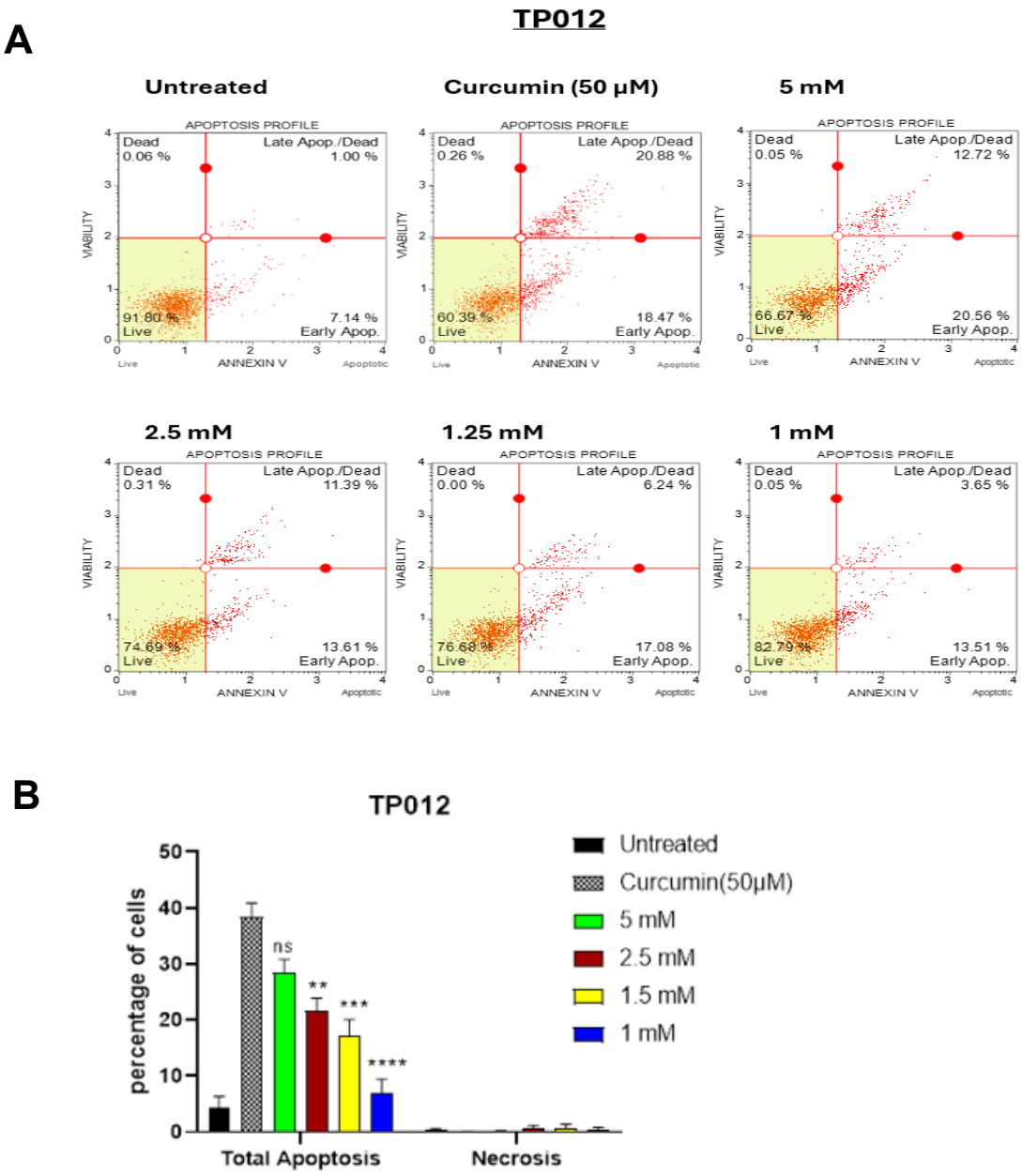


Figure 4.3.2: Examination of the mode of cell death induced by TP012 curcumin derivative on Raw 264.7 cells. Cells were seeded at $1x10^5$ cells/well in 24-well plate and treated with curcumin derivatives and 50 µM curcumin as positive control for 24 hours. The number of cells undergoing cell death were quantified using Annexin-V and dead cell assay kit and analysed using Muse analyser. Data is expressed as mean ± SEM of three independent experiments, performed in duplicates. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to the untreated control.

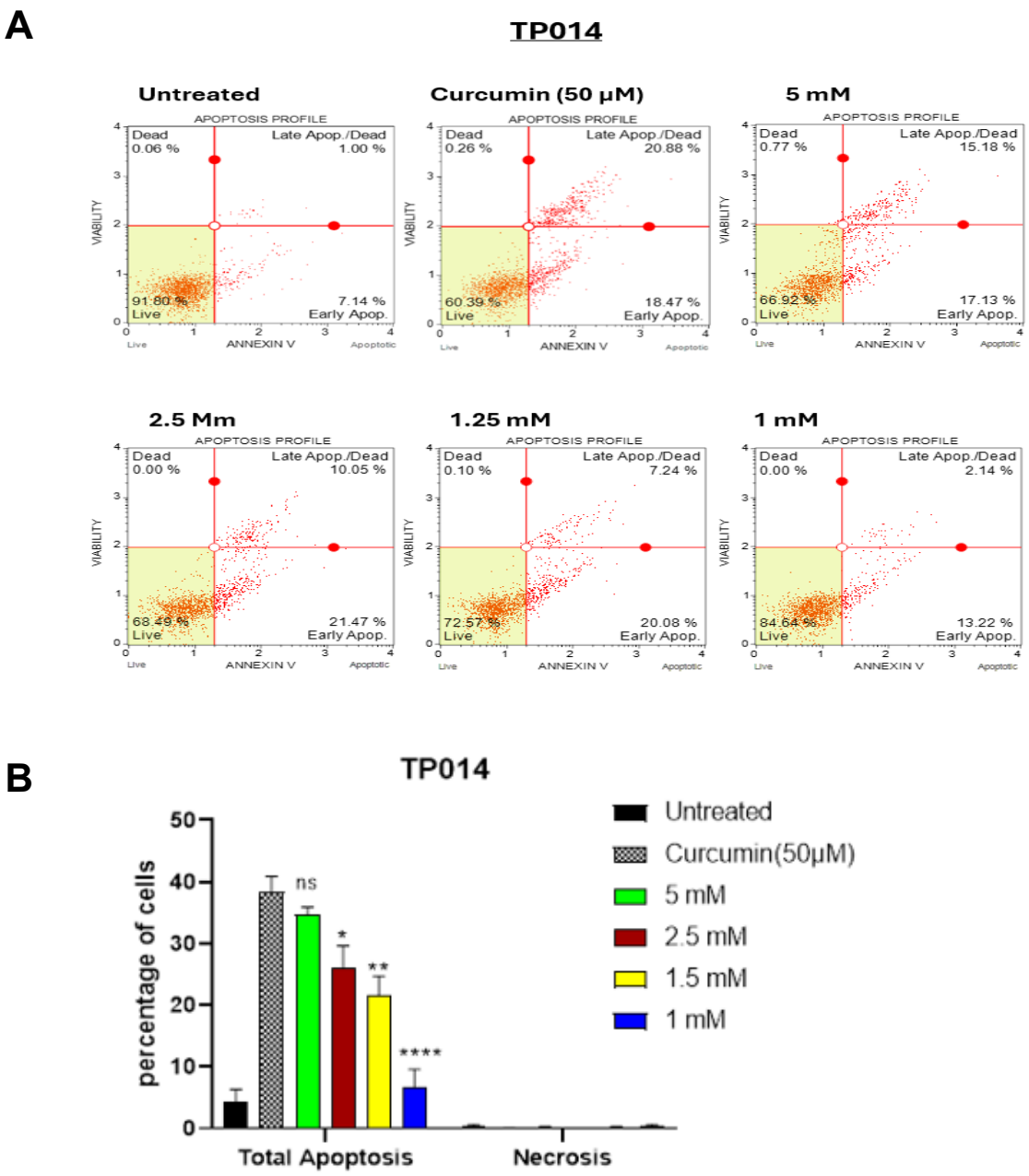


Figure 4.3.3: Examination of the mode of cell death induced by TP014 curcumin derivative on Raw 264.7 cells. Cells were seeded at 1x10^5 cells/well in 24-well plate and treated with curcumin derivatives and 50 µM curcumin as positive control for 24 hours. The number of cells undergoing cell death were quantified using Annexin-V and dead cell assay kit and analysed using Muse analyser. Data is expressed as mean ± SEM of three independent experiments, performed in duplicates. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to the untreated control.

4.4 Effects of curcumin derivatives on the expression of Interleukin 6 in Raw 264.7 cells

Interleukin (IL)-6 is one of the most fundamental pro-inflammatory cytokines that promotes inflammation (Nara and Watanabe, 2021). To evaluate the effect of curcumin derivatives (TP011, TP012 and TP014) on the expression levels of pro-inflammatory cytokines using IL-6 as a model, Raw 264.7 cells were stimulated with spike SARS-CoV-2 spike S_1 (100 ng/ml) glycol-proteins, LPS-stimulated, and treated with curcumin derivatives (1 mM) for 24 hours. The expression levels of IL-6 significantly ($p \leq 0.05$) decreased after treatment with curcumin derivatives in SARS-CoV-2 spike S_1 stimulated Raw 264.7 cells compared to untreated SARS-CoV-2 spike S_1 stimulated control cells **(figure 4.4A)**. The significant reduction in the expression levels of IL-6 pro-inflammatory cytokine in Raw 264.7 cells was also displayed by the LPS-stimulated cells treated with curcumin derivatives as opposed to untreated LPS-stimulated control cells **(figure 4.4B)**. The inhibitory effect of curcumin derivatives on the expression levels of IL-6 pro-inflammatory cytokine was more on LPS-stimulated Raw 264.7 cells than SARS-CoV-2 spike S_1 stimulated cells.

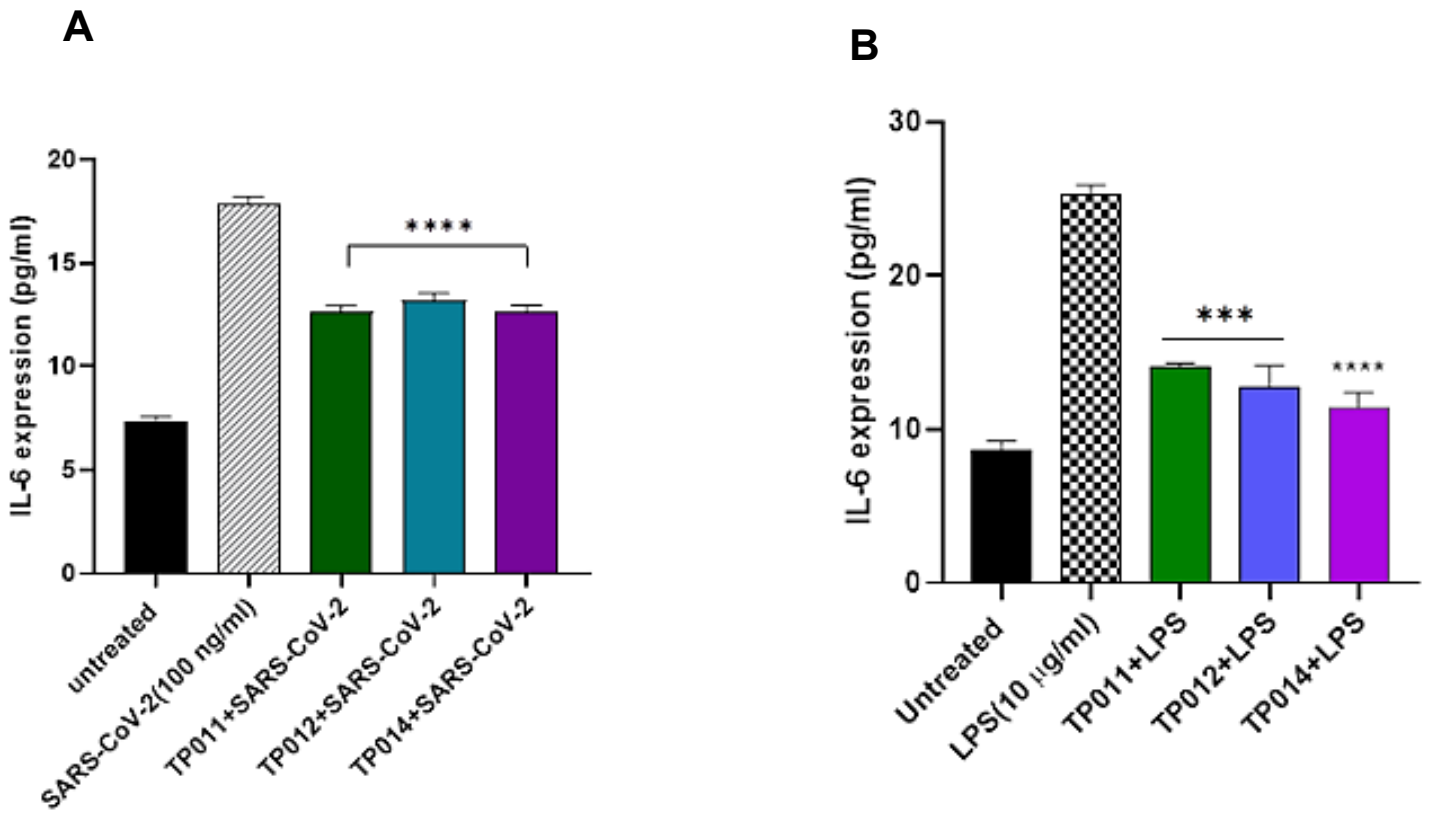
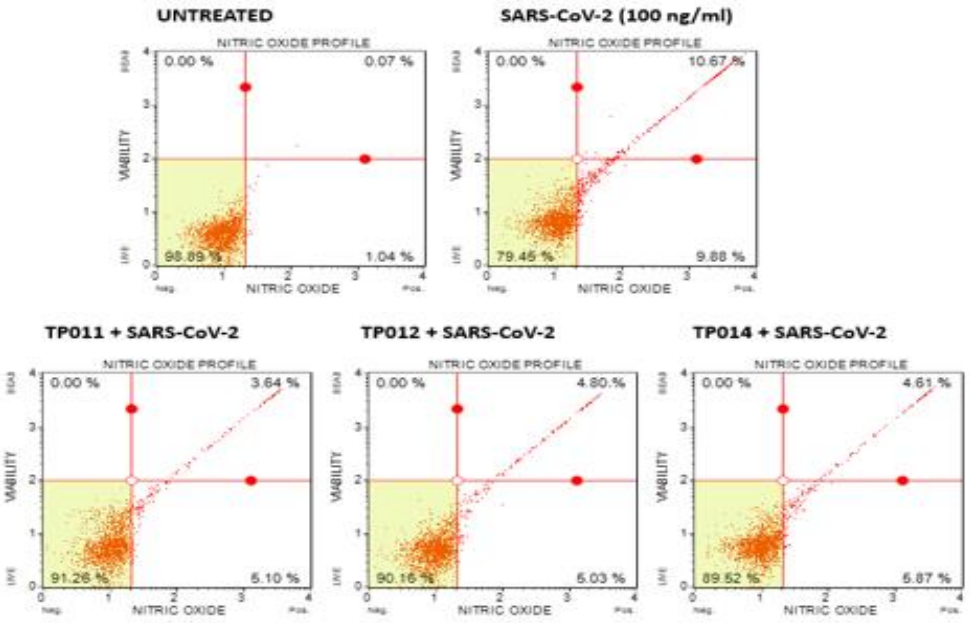


Figure 4.4: Effect of curcumin derivatives on the expression of Interleukin 6 in Raw 264.7 cells. To examine the effects of curcumin derivatives on the expression of Interleukin 6, the Raw cells were seeded at $3x10^5$ cells/well in 24-well plate, thereafter, treated with 1 mM of curcumin derivatives (TP011, TP012 and TP014); 10 µg/ml of LPS or 100 ng/ml of SARS-CoV-2 spike S_1 for 24 hours. After 24 hours of treatment, the supernatant was collected and frozen -20°C for later use. Mouse ELISA kit (Elabscience, Wuhan) was used for examination of the level of IL-6 expressed. The level of protein expressed was then quantified using Multiskan Sky microplate spectrophotometer at 525 nm (Thermo Fisher Scientific, USA). Each data point represents the mean ± SEM of three independent experiments, performed in duplicates. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to the SARS-CoV-2 spike S_1 **(A)** and LPS **(B)** control.

4.5 Effects of curcumin derivatives on Nitric Oxide production in Raw 264.7 cells

To investigate the effect of curcumin derivatives (TP011, TP012 and TP014) on nitric oxide production, 4.5-diamino-fluorescein diacetate (DAF-2 DA) and propidium Iodide were used. Raw 264.7 cells were stimulated with SARS-CoV-2 spike S_1 (100 ng/ml), 10 µg/ml LPS and treated with curcumin derivatives (1 mM) for 24 hours. Nitric oxide muse representative plots are divided in four cell populations-live with no nitric oxide activity, live with nitric oxide activity, dead with nitric oxide activity and dead with no nitric oxide activity **(figure 4.5.1A and 4.5.2A).** The quantification of the nitric oxide production levels after stimulation (SARS-CoV-2 spike S_1 and LPS) and treatment with curcumin derivatives was done using the percentages of viable cell population with nitric oxide activity. Treatment with curcumin derivatives significantly ($p \leq 0.05$) inhibited NO production in SARS-CoV-2 spike S_1 stimulated Raw 264.7 cells compared to untreated SARS-CoV-2 spike S_1 stimulated control cells **(figure 4.5.1B)**. The same outcomes were observed in LPS-stimulated Raw 264.7 cells treated with curcumin derivatives **(figure 4.5.2B)**. Curcumin derivatives shown to inhibit the production of nitric oxide.

A



B

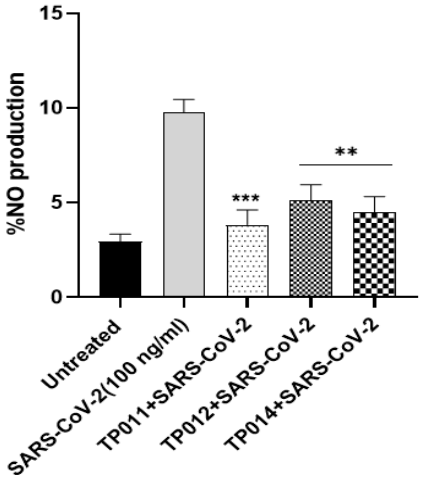
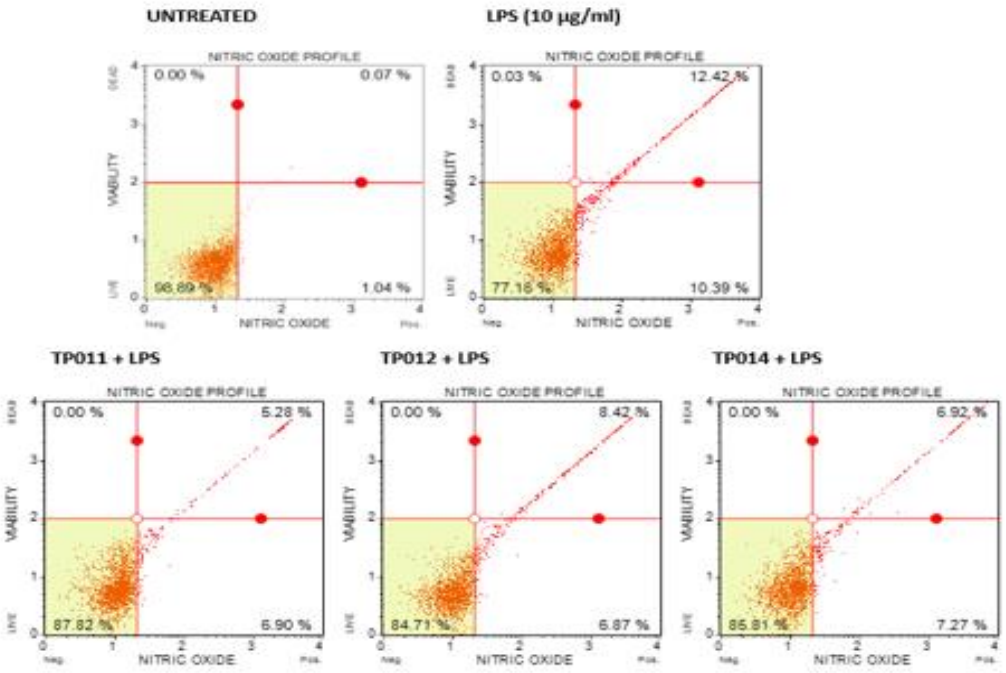


Figure 4.5.1: Effect of curcumin derivatives on Nitric Oxide production on Raw 264.7 cells. To examine the effects of curcumin derivatives on NO production, cells were seeded at $3x10^5$ cells/well in 24-well plate and stimulated with SARS-CoV-2 spike S_1, thereafter, treated with curcumin derivatives for 24 hours. The nitric oxide produced was quantified using DAF-2 DA combined with propidium iodide and analysed using Muse Flow cytometry. Data is expressed as mean ± SEM of three independent experiments, performed in duplicates. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to SARS-CoV-2 spike S_1 control.

A



B

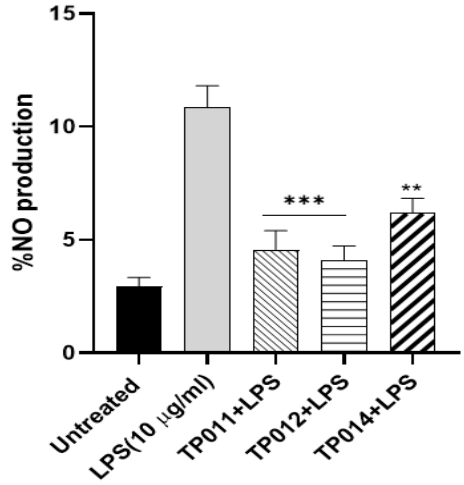
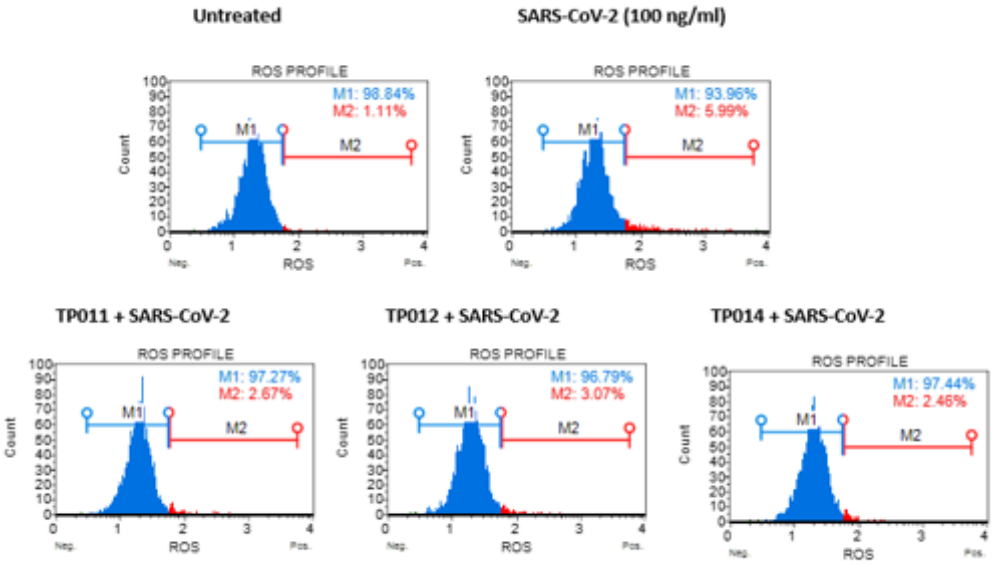


Figure 4.5.2: Effect of curcumin derivatives on Nitric Oxide production on Raw 264.7 cells. To examine the effects of curcumin derivatives on NO production, cells were seeded at $3x10^5$ cells/w in 24-well plate and stimulated with LPS, thereafter, treated with curcumin derivatives for 24 hours. The nitric oxide produced was quantified using DAF-2 DA combined with propidium iodide and analysed using Muse Flow cytometry. Data is expressed as mean ± SEM of three independent experiments, performed in duplicates. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to LPS control.

4.6 Effects of curcumin derivatives on Reactive Oxygen Species production in stimulated Raw 264.7 cells

The determination of the effect of curcumin derivatives (TP011, TP012 and TP014) on reactive oxygen species (ROS) in SARS-CoV-2 spiked Raw 264.7 cells was done using Muse® Oxidative stress Assay Kit. Cells were stimulated with SARS-CoV-2 spike S_1 (100 ng/ml), LPS-stimulated, and treated with curcumin derivatives (1 mM) for 24 hours. The representative histograms show the distribution of cells into two populations of cells with reactive oxygen species (ROS) and those without ROS. M1 refers to the ROS-negative population of cells and M2 indicates ROS-positive cells **(figure 4.6.1A and 4.6.2A)**. As evident by the percentages of M1 and M2, stimulation with 100 ng/ml SARS-CoV-2 spike S_1 and 10 µg/ml LPS increased the intracellular ROS production compared to untreated control and stimulated cells treated with curcumin derivatives **(figures 4.6.1B and 4.6.2B).** Treatment of Raw 264.7 cells with curcumin derivatives resulted in a significant ($p \leq 0.05$) reduction in ROS production levels as opposed to SARS-CoV-2 spike S_1 control **(figure 4.6.1B)**. Similarly, treatment with 1 mM of curcumin derivatives significantly reduced ROS production levels as opposed to LPS control **(figure 4.6.2B)**. The lower fluorescence intensity observed in Raw 264.7 cells after treatment with curcumin derivatives suggests that these derivatives inhibit production of ROS.

A



B

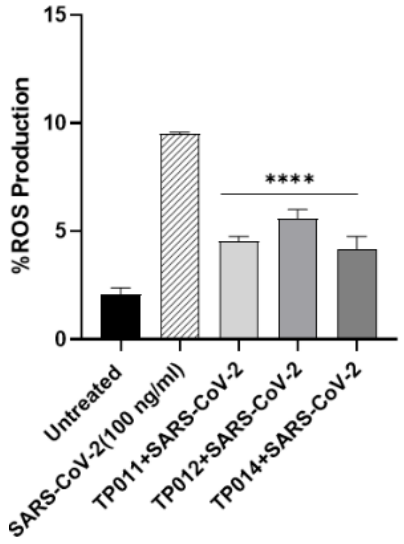
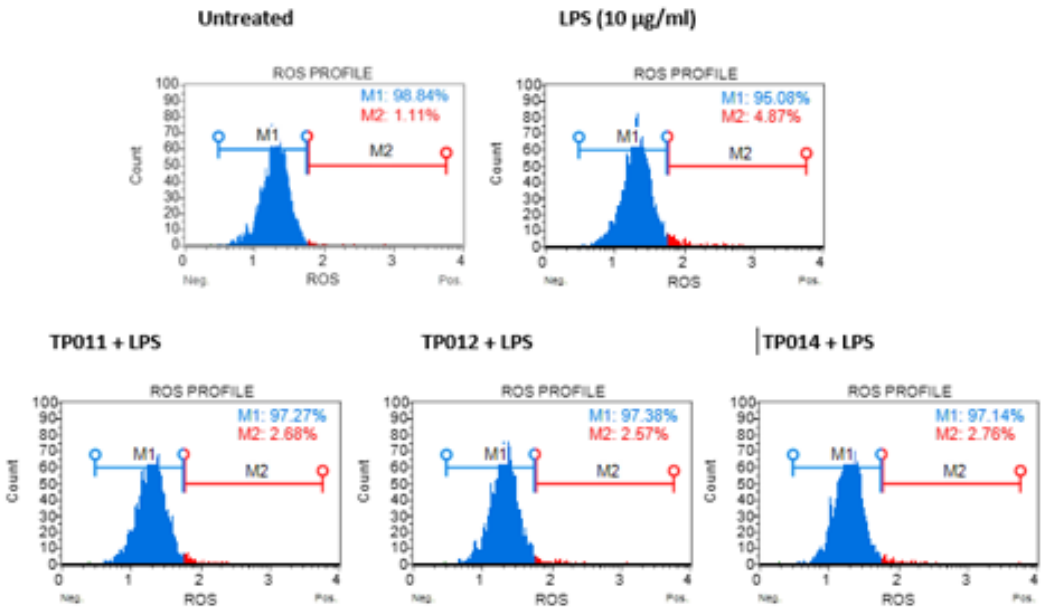


Figure 4.6.1: Effect of curcumin derivatives on Reactive Oxygen Species production on Raw 264.7 cells. To examine the effects of curcumin derivatives on ROS production, cells were seeded at $3x10^5$ cells/well in 24-well plate and stimulated with SARS-CoV-2 spike S_1, thereafter, treated with curcumin derivatives for 24 hours. Reactive oxygen species produced were quantified using Muse oxidative stress kit and analysed using Muse Flow cytometry. Data is expressed as mean ± SEM of three independent experiments, performed in duplicates. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to SARS-CoV-2 spike S_1 control.

A



B

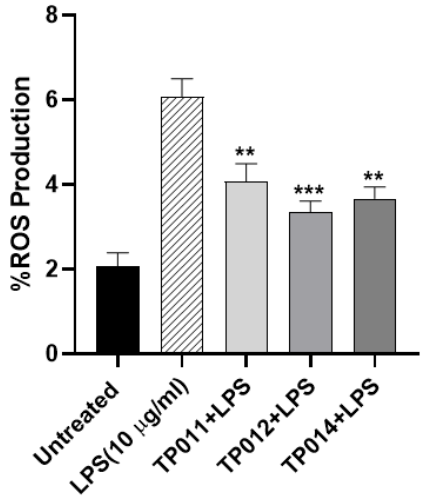
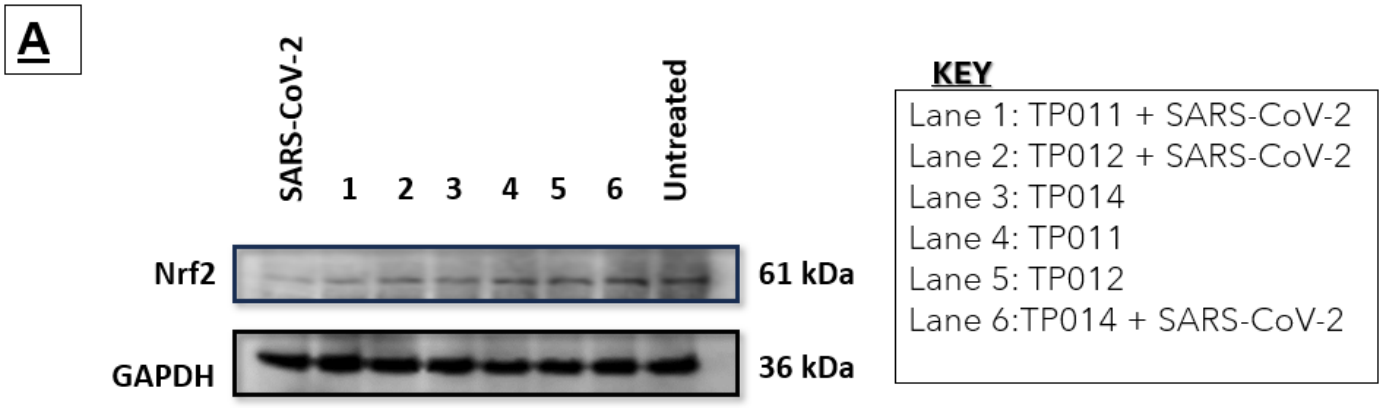


Figure 4.6.2: Effect of curcumin derivatives on Reactive Oxygen Species production on Raw 264.7 cells. To examine the effects of curcumin derivatives on ROS production, cells were seeded at $3x10^5$ cells/well 24-well plate and stimulated with LPS, thereafter, treated with curcumin derivatives for 24 hours. Reactive oxygen species produced were quantified using Muse oxidative stress kit and analysed using Muse Flow cytometry. Data is expressed as mean ± SEM of three independent experiments, performed in duplicates. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to LPS control.

4.7 The effect of curcumin derivatives on the expression of Nrf-2 protein on stimulated Raw 264.7 cells

The nuclear factor erythroid 2-related factor (Nrf2) is recognised as the regulator of cellular resistance to oxidants (Ngo and Duennwald, 2022). Therefore, the effect of curcumin derivatives on the expression of Nrf2 was assessed using western blotting. As shown in **figure 4.7A**, treatment of Raw 264.7 cells for 24 hours with 1 mM of curcumin derivatives (TP011, TP012 and TP014) resulted in the upregulation of Nrf2 protein as compared to the SARS-CoV-2 spike S_1 control. Furthermore**, figure 4.7B, C, D** shows that the upregulation was significant ($p \leq 0.05$).



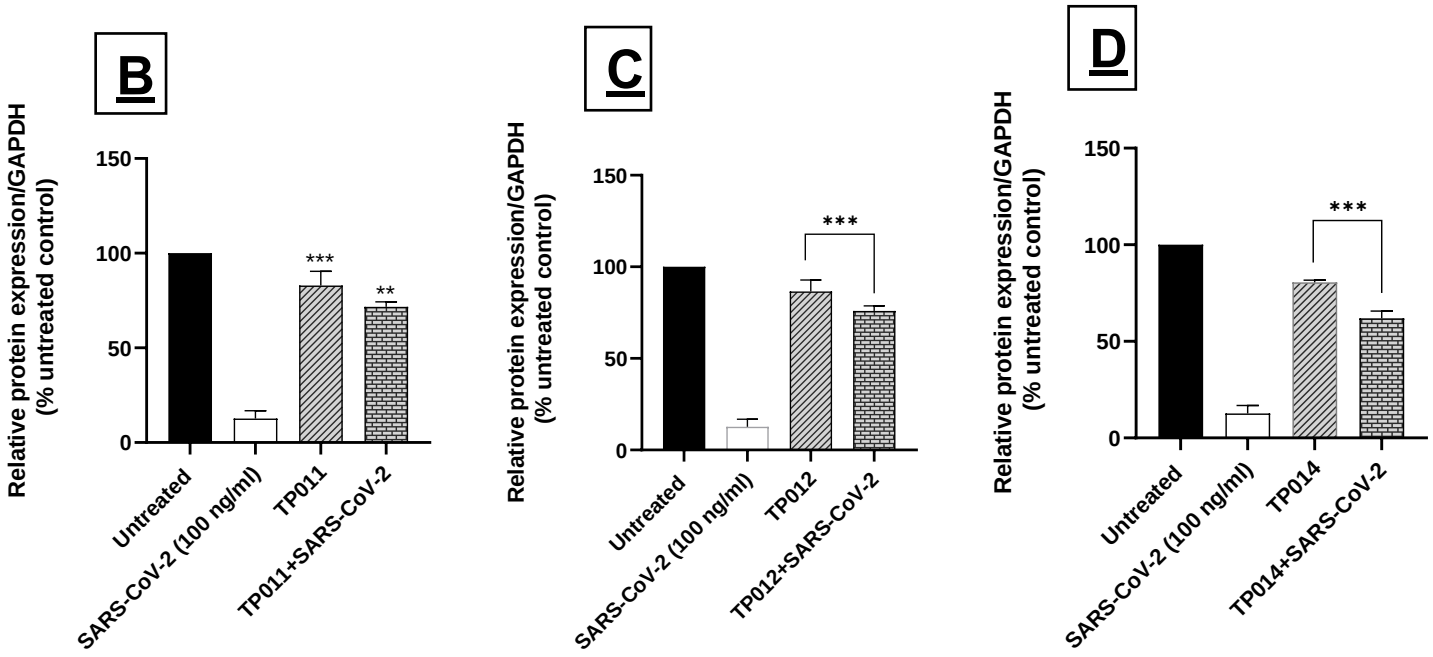
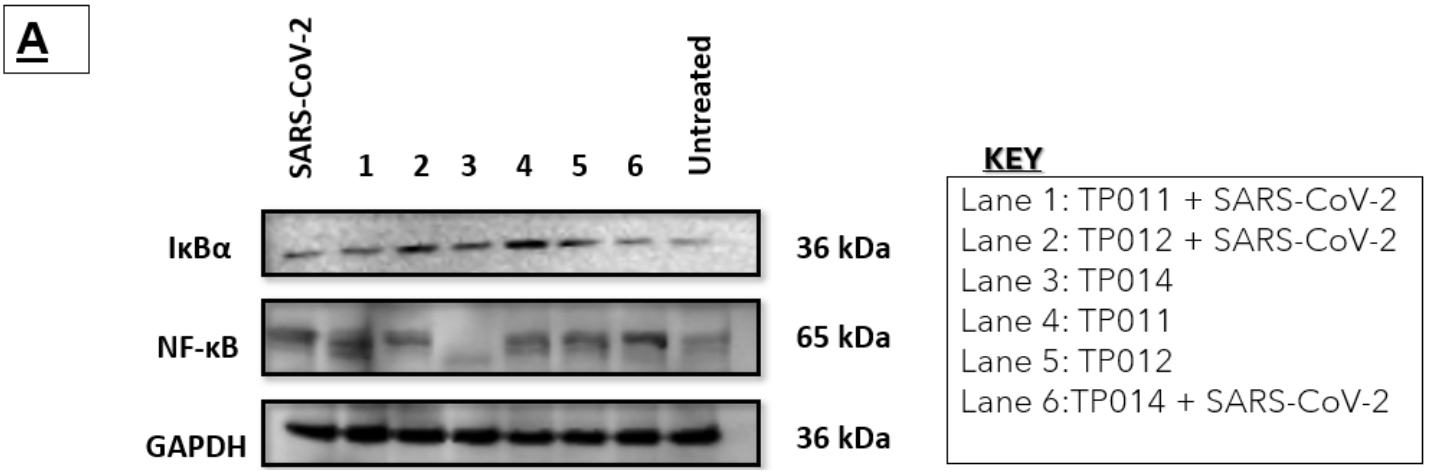


Figure 4.7: The effect of curcumin derivatives on the expression of Nrf2 protein in Raw 264.7 cells. Cells were stimulated with 100 ng/ml of SARS-CoV-2 spike S_1 and treated with 1 mM of curcumin derivatives. The expression of Nrf2 protein was assessed using western blotting. ImageJ software was used to analyse the density of the bands, which were then quantified by normalising to GAPDH. **Lane 1, 2, 3, 4, 5 and 6**, represent TP011+SARS-CoV-2, TP012+SARS-CoV-2, TP014, TP011, TP012 and TP014+SARS-CoV-2, respectively **(A)**. Each data point represents mean ± SEM of two independent experiments. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to the SARS-CoV-2 spike S_1 control.

4.8 The effect of curcumin derivatives on the expression of NF-κB and IκBα proteins in Raw 264.7 cells

Nuclear factor-kappa B (NF-κB) comprises a group of inducible transcription factors that regulate numerous genes involved in various immune and inflammatory responses (Liu *et al*., 2017). NF-κB activation depends on the phosphorylation-triggered degradation of inhibitors of nuclear factor kappa B (IκBs), which keep NF-κB dimers inactive in the cytosol of unstimulated cells (Liu *et al*., 2017). Therefore, the effect of curcumin derivatives (TP011, TP012 and TP014) on the expression of both NF-κB and IκB-α proteins was assessed using western blotting. As shown in **figure 4.8 A**, treatment of Raw 264.7 cells for 24 hours with 1 mM of curcumin derivatives resulted with the downregulation of NF-κB and upregulation of IκBα as compared to the SARS-CoV-2 spike S_1 control. Furthermore, **figures 4.8 B, C, D** show that the downregulation and upregulation of these proteins was significant ($p \leq 0.05$).



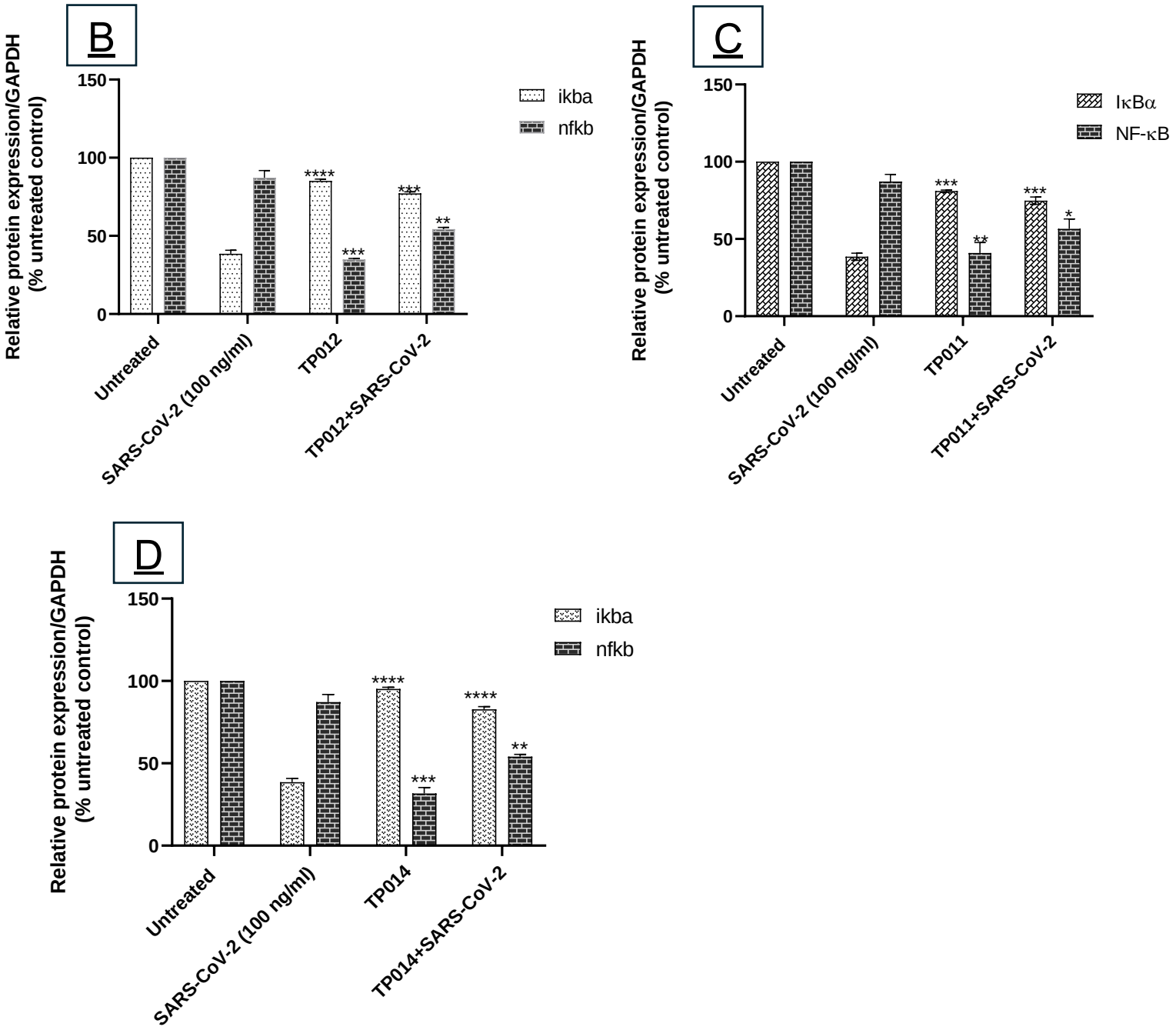


Figure 4.8: The effect of curcumin derivatives on the expression of IκBα and NF-κB proteins in Raw 264.7 cells. Cells were stimulated with 100 ng/ml of SARS-CoV-2 spike S_1 and treated with 1 mM of curcumin derivatives. The expression of NF-κB and IκBα proteins was assessed using western blotting. ImageJ software was used to analyse the density of the bands, which were then quantified by normalising to GAPDH. **Lane 1, 2, 3, 4,**

5 and 6, represent TP011+SARS-CoV-2, TP012+SARS-CoV-2, TP014, TP011, TP012 and TP014+SARS-CoV-2, respectively **(A)**. Each data point represents mean ± SEM of two independent experiments. The inclusion of asterisks indicates significant differences ($p \leq 0.05$) to the SARS-CoV-2 spike S_1 control.

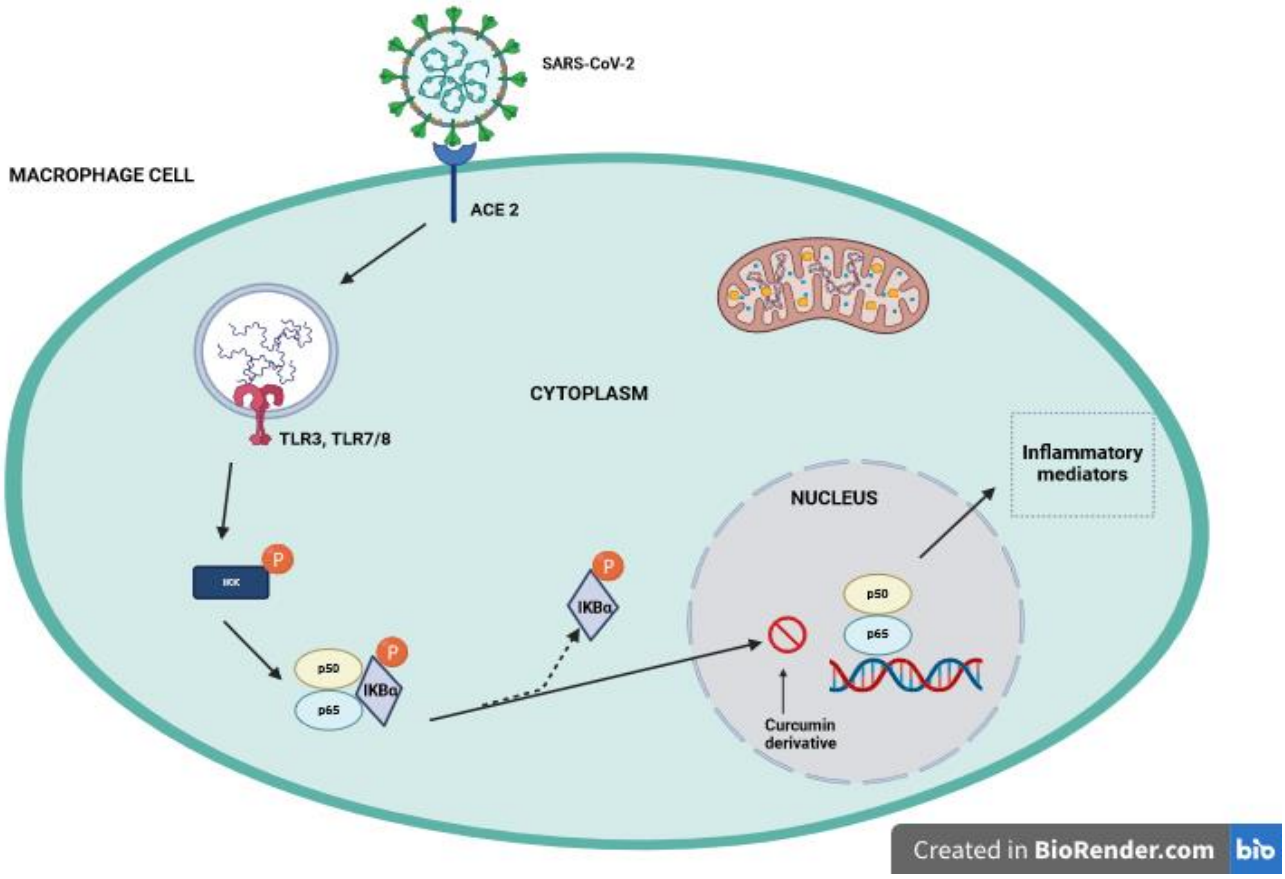


Figure 4.9: Effect of curcumin derivatives on the NF-κB signalling pathway after SARS-CoV-2 infection. Viral envelope glycoproteins are cleaved or activated when SARS-CoV-2 binds to its receptors, angiotensin-converting enzyme 2 (ACE2). This process permits SARS-CoV-2 to enter the host cell. The Toll-like receptors (TLRs), including TLR3, TLR4, and TLR7/8, are then activated by viral single-stranded RNA virus. These receptors trigger the activation of IKK, which phosphorylates the cytoplasmic inhibitor factor IκB-α. This phosphorylation tag IκB-α for ubiquitination consequently causes its degradation. NF-κB p50 and p65 are released from IκB-α and translocate from the cytoplasm into the nucleus to induce transcription of various genes coding for pro-inflammatory proteins such as cytokines and chemokines. This figure indicates that curcumin derivatives (TP011, TP012, and TP014) may repair dysregulated inflammation following SARS-CoV-2 infection through NF-κB suppression due the upregulated IκB-α. This figure was developed using the BioRender online software tool.

CHAPTER 5

DISCUSSION

COVID-19 is a novel and highly contagious infection caused by the SARS-CoV-2 virus, its emergence threatened global public health (Yazdanpanah *et al*., 2020). This viral infection has led to the death of more than 2 million patients worldwide (Pohl *et al*., 2021) (Fihel *et al*., 2024). The early immune response to SARS-CoV-2 infection is initiated by innate immune cells like macrophages, neutrophils, and dendritic cells (Khan *et al*., 2021). These cells produce inflammatory mediators that suppress virus replication, promote tissue healing, and trigger the adaptive immune system (Khan *et al*., 2021). The inflammatory response during infection is very critical, because proper control and maintenance of the anti- and pro-inflammatory molecules are crucial for elimination of the viral infection (Oliveira *et al*., 2020).

Studies have linked SARS-CoV-2 infection with cytokine storm and hyper-inflammation which leads to severe multi-organ damage, including the development of lung injuries and even death as a result of aberrant oxidative stress levels (García, 2020). Most of the patients die, not solely because of the infection itself, but due to the multiple organ failure as a result of over-production of inflammatory molecules such as cytokines, reactive oxygen, and nitrogen species (Perlman and Dandekar, 2005). The pursuit of an effective treatment targeting the oxidative stress and hyper-inflammation caused by this viral infection remains a primary focus of current study (Yazdanpanah *et al*., 2020). Curcumin has garnered significant attention from medical researchers due to its various biological effects, including antioxidant and anti-inflammatory activities (Oglah *et al*., 2020). However, its poor bioavailability and susceptibility to intestinal metabolism are major limitations, largely due to its unfavorable pharmaceutical profile (Shahcheraghi *et al*., 2022).

Currently, the use of curcumin derivatives represents a well-established strategy to improve metabolic stability (Shahcheraghi *et al*., 2022). On this account, this study investigated the effect of curcumin derivatives on SARS-CoV-2 spike S_1 protein-induced oxidative stress in Raw 264.7 macrophage cells. The cytotoxicity effect of

curcumin derivatives (TP011, TP012 and TP014) was assessed using the colorimetric (MTT) and fluorometric (PI) assays. The results from both assays revealed that these curcumin derivatives are not toxic at low doses (1.25 mM-0.625 mM) **(figures 4.1.1B, 4.12B, 4.1.3B)** and **(figure 4.2 A, B, C)**, respectively. The cell images were used to determine if the MTT results corresponds with the morphology of the treated cells **(figures 4.1.1A, 4.1.2A, 4.1.3A)**. This is the first time the cytotoxic effects of these curcumin derivatives have been evaluated. However, numerous other curcumin derivatives have previously been reported to show no toxic effects on healthy cells (Zhang *et al*., 2014).

According to Vanden Berghe and colleagues, (2013), reduction in cell viability is directly proportional to the induction of cell death, through modes such as apoptosis and necrosis (Vanden Berghe *et al*., 2013). For that reason, it was paramount to determine the mode of cell death that resulted from the decrease in cell viability. In this study, the mode of cell death induced by high doses of these curcumin derivatives on Raw 264.7 macrophage cells was examined using the Annexin-V/ PI assay. The findings showed that the cells treated with high doses of curcumin derivatives stained positive for Annexin-V FITC and only a small percentage stained positive for both annexin-V and propidium iodide (PI) **(figures 4.3.1, 4.3.2, 4.3.3)**. This staining pattern is indicative of apoptosis as mode of cell death in high doses of curcumin derivatives, and this imply that curcumin derivatives are safe to use at low doses, as low doses did not show any apoptotic signs in macrophage cells.

Macrophages serve as a critical component of the first line of defense, bridging the innate and adaptive immune responses (Hosseini *et al*., 2020). Viruses like SARS-CoV-2 target macrophages to invade the innate immune system and get access to other tissues (Amor *et al*., 2020). The diminished inflammatory responses resulting from SARS-CoV-2 infection are thought to contribute to the virus's pathogenesis and its fatal outcomes (Hosseini *et al*., 2020). Given the crucial role of pro-inflammatory cytokines in the inflammatory response, research has focused on strategies to either inhibit their functions or enhance the effects of anti-inflammatory cytokines (Xu and Liu, 2017).

Inflammation studies revealed that upon the SARS-CoV-2 spike S_1 stimulation, Raw 264.7 macrophage cells produced higher levels of Interleukin 6 (IL-6) pro-inflammatory cytokine, compared to that with no viral infection **(figure 4.4A)**. This was also observed when Lipoposaccharides (LPS) was used as a stimulant **(figure 4.4B)**. Curcumin derivatives were shown to reduce the production of IL-6 in both SARS-CoV-2 spike S_1 and LPS stimulated cells **(figure 4.4)**. The inhibitory effect of IL-6 by these curcumin derivatives coincides with observations by Xu and Liu, (2017), which showed that curcumin indeed downregulate the pro-inflammatory cytokine (IL-6) production of Influeza A viruses (IAV) infected human macrophages. The transcription of inducible nitric oxide synthase (iNOS) and genes related to oxidative stress have been implicated in the development of various diseases and disorders (Ruhee *et al*., 2019).

Oxidative stress leads to the oxidation of proteins, lipids, and nucleic acids, ultimately causing cellular dysfunction and cell death, thereby contributing to the onset of diseases and disorders (Hajam *et al*., 2022). This study suggests that the anti-inflammatory properties of curcumin derivatives in SARS-CoV-2 spike S_1 stimulated Raw 264.7 macrophage cells could be attributed to the observed inhibition of nitric oxide (NO) and reactive oxygen species (ROS) production. SARS-CoV-2 spike S_1 stimulated cells treated with curcumin derivatives showed lowered production of reactive nitrogen and oxygen species. The lowered production of these reactive molecules has been depicted on the qualitative assay results in **figure 4.5.1A and figure 4.6.1A**, respectively. The quantitative findings **(figures 4.5.1B and 4.6.1B)** agree with the qualitative results. To further examine the NO and ROS inhibitory properties of curcumin derivatives, Raw 264.7 cells were stimulated with LPS and treated with curcumin derivatives. Results suggest that the treatment of Raw 264.7 cells with curcumin derivatives downregulates LPS-stimulated NO and ROS production **(figures 4.5.2B and 4.6.2B)**. This work aligns with anti-inflammatory properties of curcumin outlined in a review article by Jurenka and Research, 2009 Moreover, these findings suggest that curcumin derivatives could interfere with some molecules along the inflammation signaling pathways.

Nuclear factor erythroid 2-related factor 2 (Nrf2) plays a role in the anti-inflammatory process by coordinating the recruitment of inflammatory cells and controlling gene expression via the antioxidant response (ARE) pathway (Saha *et al*., 2020). In this study, the results showed lowered expression of Nrf2 in SARS-CoV-2 spike S_1 stimulated Raw 264.7 cells as compared to infected cells treated with curcumin derivatives **(figure 4.7)**. The selected curcumin derivative lowered the expression of the Nrf2 as reported in the review by Shahcheraghi and colleagues, (2022), which outlined Curcumin to be natural activator of Nrf2 pathway.

Moreover, NF-κB signalling pathway is crucially activated in macrophages when they encounter pathogens (Xu and Liu, 2017). Its persistent activation have been associated with the development of severe COVID-1 complications (Olajide *et al*., 2021). It was found that SARS-CoV-2 infection leads to the decrease in IκB-α and enhanced levels of NF-κB/p65 **(figure 4.8)**, which means inflammation and production of inflammatory mediators. However, administration of curcumin derivatives reversed this phenomenon. When cells are exposed to SARS-CoV-2, an inhibitor of NF-κB, IκB-α, can undergo phosphorylation and degradation, allowing NF-κB p65 subunit to be activated. RelA/ p65, a subunit of the NF-κB heterodimer, plays a role in facilitating the nuclear translocation and activation of NF-κB (Olajide *et al*., 2021).

When cells are stimulated with SARS-CoV-2, IκB-α was phosphorylated and degraded facilitating the activation of NF-κB. Subunits of the NF-κB heterodimer, p50 and p65, can move from the cytoplasm to the nucleus, where they enhance the expression of genes related to inflammation (Xu and Liu, 2017). This study suggests that curcumin derivatives target NF-κB for inhibition of the pro-inflammatory mediators produced by macrophage cells. The effect of TP011, TP012 and TP014 curcumin derivatives on SARS-CoV-2 spike protein S_1 induced NF-κB and IκB-α expression is consistent with the study by Xu and Liu, (2017); which revealed that upon the treatment of IAV-infected human macrophages with curcumin, NF-κB signalling pathway was inhibited by promoting the expression of nuclear factor of

kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκB-α), and inhibiting the translocation of p65 from cytoplasm to nucleus.

This study is the first to show that TP011, TP012 and TP014 curcumin derivatives inhibit the production of IL-6 pro-inflammatory cytokine, nitrogen, and reactive oxygen species in stimulated Raw 264.7 macrophage cells. The result from this work also suggests that the anti-inflammatory mode of action of these derivatives involves proteins such as Nrf2, NF-κB/p65 and IκB-α. Although these results are based on the outcome of *in vitro* experiments on Raw 264.7 cells, the ability these derivatives to exert these anti-inflammatory effects without inducing cell death, makes curcumin derivatives promising candidates in clinical applications for the treatment of hyper-inflammation and oxidative stress associated with SARS-CoV-2 infection.

REFERENCES

Abaricia, J.O., Farzad, N., Heath, T.J., Simmons, J., Morandini, L., and Olivares-Navarrete, R. (2021). Control of innate immune response by biomaterial surface topography, energy, and stiffness. *Acta biomaterialia* 33, 58-73.

Abdel-Hafez, S.M., Hathout, R.M., and Sammour, O.A. (2021). Attempts to enhance the anti-cancer activity of curcumin as a magical oncological agent using transdermal delivery. *Advances in traditional medicine*, 2-10.

Adhikari, B., Marasini, B.P., Rayamajhee, B., and Bhattarai, B.R., Lamichhane, G., Khadayat, K., Adhikari, A., Khanal, S., Parajuli, N. (2021). Potential roles of medicinal plants for the treatment of viral diseases focusing on covid-19: a review. *Phytotherapy research* 35, 1298-1312.

Khan, M.S.A. and Ahmad, I. (2019). Herbal Medicine: Current trends and prospects. In: New Look to Phytomedicine: Advancements in herbal products as novel drug leads. *Elsevier*, 3–13.

Ahmad, R. and Haque, M. (2022). Surviving the storm: cytokine biosignature in SARS-CoV-2 severity prediction. *Vaccines* 10, 1-38.

Akamatsu, M.A., de Castro, J.T., Takano, C.Y., and Ho, P.L. (2021). Off balance: interferons in covid-19 lung infections. *EBiomedicine* 73, 2-6.

Amor, S., Fernández Blanco, L., and Baker, D. (2020). Innate immunity during SARS-CoV-2: evasion strategies and activation trigger hypoxia and vascular damage. *Clinical experimental immunology* 202, 193–209.

Anand, U., Jacobo-Herrera, N., Altemimi, A., and Lakhssassi, N. (2019). A comprehensive review on medicinal plants as antimicrobial therapeutics: potential avenues of biocompatible drug discovery. *Metabolites* 9, 2-6.

Antonelli, M. and Kushner, I. (2017). It's time to redefine inflammation. *FASEB Journal* 31, 1787–1791.

Beig Parikhani, A., Bazaz, M., Bamehr, H., Fereshteh, S., Amiri, S., Salehi-Vaziri, M., Arashkia, A., and Azadmanesh, K. (2021). The inclusive review on SARS-CoV-2 biology, epidemiology, diagnosis, and potential management options. *Current microbiology* 78, 1099-1114.

Benarba, B., and Pandiella, A., (2020). Medicinal plants as sources of active molecules against covid-19. *Frontiers pharmacolology* 11, 1-16.

Boyraz, M.Ü., Shekhany, B., and Süzergöz, F. (2021). Cellular imaging analysis of MTT assay based on tetrazolium reduction. *Harran universitesi tıp Fakültesi Dergisi* 18, 95–99.

Brown, B., Ojha, V., Fricke, I., Al-Sheboul, S.A., Imarogbe, C., Gravier, T., Green, M., Peterson, L., Koutsaroff, I.P., Demir, A., Andrieu, J., Leow, C.Y., and Leow, C.H. (2023). Innate and adaptive immunity during SARS-CoV-2 infection: biomolecular cellular markers and mechanisms. *Vaccines* 11, 11-60.

Catanzaro, M., Fagiani, F., Racchi, M., Corsini, E., Govoni, S., and Lanni, C. (2020). Immune response in covid-19: addressing a pharmacological challenge by targeting pathways triggered by SARS-CoV-2. *Signal transduction targeted therapy* 5, 2-8.

Chinnasamy, S., Zameer, F., and Muthuchelian, K. (2020). Molecular and biological mechanisms of apoptosis and its detection techniques. *Journal of oncological sciences* 6, 49–64.

Ciotti, M., Ciccozzi, M., Terrinoni, A., Jiang, W.C., Wang, C. Bin, and Bernardini, S. (2020). The covid-19 pandemic. *Critical reviews in clinical laboratory sciences* 57, 365-388.

Commins, S.P., Borish, L., and Steinke, J.W. (2010). Immunologic messenger molecules: cytokines, interferons, and chemokines. *Journal of allergy and clinical immunology* 125, 53-68.

Costela-Ruiz, V.J., Illescas-Montes, R., Puerta-Puerta, J.M., Ruiz, C., and Melguizo-Rodríguez, L. (2020). SARS-CoV-2 infection: the role of cytokines in covid-19 disease. *Cytokine growth factor reviews* 54, 62-75.

Dessie, G. and Malik, T. (2021). Role of serine proteases and host cell receptors involved in proteolytic activation, entry of SARS-CoV-2 and its current therapeutic options. *Infection drug resistance* 14, 1883–1892.

Dhyani, P., Quispe, C., Sharma, E., Bahukhandi, A., Sati, P., Attri, D.C., Szopa, A., Sharifi-Rad, J., Docea, A.O., Mardare, I., Calina, D., and Cho, W.C. (2022). Anticancer potential of alkaloids: a key emphasis to colchicine, vinblastine, vincristine, vindesine, vinorelbine and vincamine. *Cancer cell international* 22, 2-20.

Eter, P., Elves, J.D., and Oitt, V.M.R. (2000). The immune system first of two parts. *Advances in immunology* 343, 37-48.

Felsenstein, S., Herbert, J.A., McNamara, P.S., and Hedrich, C.M. (2020). COVID-19: immunology and treatment options. *Clinical immunology* 215, 2-13.

Feng, H., Zhang, Y.B., Gui, J.F., Lemon, S.M., and Yamane, D. (2021). Interferon regulatory factor 1 (IRF1) and anti-pathogen innate immune responses. *PLoS pathogens* 17, 1-22.

Fihel, A., Janicka, A., Buschner, A., Ustinavičiene, R., and Trakiene, A. (2024). Unrecognised covid-19 deaths in central Europe: the importance of cause-of-death certification for the COVID-19 burden assessment. *PLoS One* 19, 1-14.

Flerlage, T., Boyd, D.F., Meliopoulos, V., Thomas, P.G., and Schultz-Cherry, S. (2021). Influenza virus and SARS-CoV-2: pathogenesis and host responses in the respiratory tract. *Nature reviews microbiology* 19, 425-441.

Forman, H.J. and Zhang, H. (2021). Targeting oxidative stress in disease: promise and limitations of antioxidant therapy. *Nature reviews drug discovery* 20, 689-709.

Fu, D., Zhang, G., Wang, Yuhui, Zhang, Z., Hu, H., Shen, S., Wu, J., Li, B., Li, X., Fang, Y., Liu, J., Wang, Qiao, Zhou, Y., Wang, W., Li, Y., Lu, Z., Wang, X., Nie, C., Tian, Y., Chen, D., Wang, Yuan, Zhou, X., Wang, Qisheng, Yu, F., Zhang, C., Deng, C., Zhou, L., Guan, G., Shao, N., Lou, Z., Deng, F., Zhang, H., Chen, X., Wang, M., Liu, L., Rao, Z., and Guo, Y. (2021). Structural basis for SARS-CoV-2 neutralizing antibodies with novel binding epitopes. *PLoS biology* 19, 1-22.

García, L.F. (2020). Immune response, inflammation, and the clinical spectrum of COVID-19. *Frontiers in immunology* 11, 1-13.

García-Caparrós, P., De Filippis, L., Gul, A., Hasanuzzaman, M., Ozturk, M., Altay, V., and Lao, M.T. (2021). Oxidative stress and antioxidant metabolism under Adverse environmental conditions: a review. *Botanical review* 87, 421–466.

Gasparrini, M., Afrin, S., Forbes-Hernández, T.Y., Cianciosi, D., Reboredo-Rodriguez, P., Amici, A., Battino, M., and Giampieri, F. (2018). Protective effects of Manuka honey on lps-treated raw 264.7 macrophages. Part 2: Control of oxidative stress induced damage, increase of antioxidant enzyme activities and attenuation of inflammation. *Food and chemical toxicology* 120, 578–587.

Ghosh, G. and Wang, V.Y.F. (2021). Origin of the functional distinctiveness of NF-κB/p52. *Frontiers in cell developmental biology* 9, 1-13.

Glencross, D.A., Ho, T.R., Camiña, N., Hawrylowicz, C.M., and Pfeffer, P.E. (2020). Air pollution and its effects on the immune system. *Free radical biology medicine* 151, 56-68.

Gusev, E., Sarapultsev, A., Solomatina, L., and Chereshnev, V. (2022). SARS-CoV-2-specific immune response and the pathogenesis of COVID-19. *International journal of molecular sciences* 23, 1-34.

Hafeez, A., Ahmad, S., Siddqui, S.A., Ahmad, M., and Mishra, S. (2020). A review of covid-19 (coronavirus disease-2019) diagnosis, treatments and prevention. *Eurasian journal of medicine and oncology* 4, 116-125.

Hajam, Y.A., Rani, R., Ganie, S.Y., Sheikh, T.A., Javaid, D., Qadri, S.S., Pramodh, S., Alsulimani, A., Alkhanani, M.F., Harakeh, S., Hussain, A., Haque, S., and Reshi, M.S. (2022). Oxidative stress in human pathology and aging: molecular mechanisms and perspectives. *Cells* 11, 1-27.

Hay, E., Lucariello, A., Contieri, M., Esposito, T., De Luca, A., Guerra, G., and Perna, A. (2019). Therapeutic effects of turmeric in several diseases: an overview. *Chemico- biological interactions* 310, 1-6.

He, F., Ru, X. and Wen, T. (2020). NRF2, a transcription factor for stress response and beyond. *International journal of molecular sciences* 21, 1-23.

Hirano, T. (2021). IL-6 in inflammation, autoimmunity and cancer. *International immunology* 33, 127-148.

Hosseini, A., Hashemi, V., Shomali, N., Asghari, F., Gharibi, T., Akbari, M., Gholizadeh, S., and Jafari, A. (2020). Innate and adaptive immune responses against coronavirus. *Biomedicine and pharmacotherapy* 132, 1-7.

Iwanaszko, M. and Kimmel, M. (2015). NF-KB and IRF pathways: Cross-regulation on target genes promoter level. *BMC genomics* 16, 1-8.

Jackson, C.B., Zhang, L., Farzan, M., and Choe, H. (2021). Functional importance of the D614G mutation in the SARS-CoV-2 spike protein. *Biochemical biophysical research communications* 538, 108–115.

Jefferies, C.A. (2019). Regulating IRFs in IFN driven disease. *Frontiers immunology* 10, 1-15.

Jerne, N.K. (1973). The immune system. *Scientific American* 229, 52–63.

Juan, M. and Colobran, R. (2009). Chemokines and chemokine receptors. *Encyclopedia of life sciences*, 1-11.

Jurenka, J. and Research, T. (2009). Anti-inflammatory properties of curcumin, a major constituent of Curcuma longa. *Journal of clinical therapeutic* 14, 141-153.

Karthikeyan, A., Senthil, N., and Min, T. (2020). Nanocurcumin: a promising candidate for therapeutic applications. *Frontiers in pharmacology* 11, 1-24.

Kataria, S., Jain, M., Tripathi, D.K., and Singh, V.P. (2020). Involvement of nitrate reductase-dependent nitric oxide production in magnetopriming-induced salt tolerance in soybean. *Physiologia plantarum* 168, 422–436.

Khan, S., Shafiei, M.S., Longoria, C., Schoggins, J.W., Savani, R.C., and Zaki, H. (2021). SARS-CoV-2 spike protein induces inflammation via TLR2-dependent activation of the NF-κB pathway. *Elife* 10, 1-26.

Khor, P.Y., Mohd Aluwi, M.F.F., Rullah, K., and Lam, K.W. (2019). Insights on the synthesis of asymmetric curcumin derivatives and their biological activities. *European journal of medicinal chemistry* 183, 1-21.

Kozlov, E.M., Ivanova, E., Grechko, A. V., and Wu, W.-K., Starodubova, A. V., Orekhov, A.N. (2021). Involvement of oxidative stress and the innate immune system in SARS-CoV-2 infection. *Diseases* 9, 17.

Kusiak, A. and Brady, G. (2022). Bifurcation of signalling in human innate immune pathways to NF-kB and IRF family activation. *Biochemical pharmacology* 205, 1-14.

Laing, K.J. and Secombes, C.J. (2004). Chemokines. *Developmental and comparative immunology* 28, 443–460.

Lal, J., Gupta, S.K., Thavaselvam, D., and Agarwal, D.D. (2016). Synthesis and pharmacological activity evaluation of curcumin derivatives. *Chinese chemical letters* 27, 1067–1072.

Lavin, Y., Mortha, A., Rahman, A., and Merad, M. (2015). Regulation of macrophage development and function in peripheral tissues. *Nature reviews immunology* 15, 731-744.

Lee, W.-H., Loo, C.-Y., Bebawy, M., Luk, F., Mason, R.S., and Rohanizadeh, R. (2013). Curcumin and its Derivatives: their application in neuropharmacology and neuroscience in the 21st century. *Current neuropharmacology* 11, 338-378.

Lendeckel, U., Venz, S., and Wolke, C. (2022). Macrophages: shapes and functions. *ChemTexts* 8, 1-12.

Lewis, E.D., Meydani, S.N., and Wu, D. (2019). Regulatory role of vitamin E in the immune system and inflammation. *IUBMB Life* 71, 487–494.

Li, W., Wang, H. and Zheng, S.J. (2022). Roles of RNA sensors in host innate response to influenza virus and coronavirus infections. *International journal of molecular sciences* 23, 1-26.

Li, Y., Deng, S.L., Lian, Z.X., and Yu, K. (2019). Roles of toll-like receptors in nitroxidative stress in mammals. *Cells* 8, 1-16.

Lima, H.S., Tupinambás, U., and Guimarães, F.G. (2024). Estimating time-varying epidemiological parameters and underreporting of covid-19 cases in Brazil using a mathematical model with fuzzy transitions between epidemic periods. *PLoS One* 19, 1-35.

Liu, T., Zhang, L., Joo, D., and Sun, S.C. (2017). NF-κB signaling in inflammation. *Signal Transduction targeted therapy* 2, 1-9.

Liu, X., Yin, L., Shen, S., and Hou, Y. (2023). Inflammation and cancer: paradoxical roles in tumorigenesis and implications in immunotherapies. *Genes and diseases* 10, 151-164.

Malhotra, M., Rai, A., and Malhotra, V. (2019). Curcumin in the management of oral potentially malignant disorders. *World journal of pharmaceutical research World journal of pharmaceutical research SJIF impact factor* 8, 1-21.

Marshall, J.S., Warrington, R., Watson, W., and Kim, H.L. (2018). An introduction to immunology and immunopathology. *Allergy, asthma and clinical immunology* 14, 5-14.

Mogensen, T.H. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical microbiology reviews* 22, 240-273.

Moghadam, Z.M., Henneke, P., and Kolter, J. (2021). From flies to men: ros and the nadph oxidase in phagocytes. *Frontiers in cell developmental biology* 9, 1-16.

Mortaz, E., Tabarsi, P., Varahram, M., Folkerts, G., and Adcock, I.M. (2020). The immune response and immunopathology of covid-19. *Frontiers in immunology* 11, 1-9.

Moura, A., Lopes, L., Matos, L.C., Machado, J., and Criado, M.B. (2023). Converging paths: a comprehensive review of the synergistic approach between complementary medicines and western medicine in addressing covid-19 in 2020. *BioMed* 3, 282–308.

Nam, G.H., Jo, K.J., Park, Y.S., Kawk, H.W., Kim, S.Y., and Kim, Y.M. (2019). In vitroand in vivoinduction of p53-dependent apoptosis by extract of euryale ferox salisbin A549 human caucasian lung carcinoma cancer cells is mediated through akt signaling pathway. *Frontiers in oncology* 9, 1-13.

Nara, H. and Watanabe, R. (2021). Anti-inflammatory effect of muscle-derived interleukin-6 and its involvement in lipid metabolism. *International journal of molecular sciences* 22, 1-15.

Newton, A.H., Cardani, A., and Braciale, T.J. (2016). The host immune response in respiratory virus infection: balancing virus clearance and immunopathology. *Seminars immunopathology* 38, 471-482.

Ngo, V. and Duennwald, M.L. (2022). Nrf2 and oxidative stress: a general overview of mechanisms and implications in human disease. *Antioxidants* 11, 1-27.

Oglah, M.K., Mustafa, Y.F., Bashir, M.K., and Jasim, M.H. (2020). Curcumin and its derivatives: a review of their biological activities. *Systematic reviews in pharmacy* 11, 472–481.

Olajide, O.A., Iwuanyanwu, V.U., Lepiarz-Raba, I., Al-Hindawi, A.A., Aderogba, M.A., Sharp, H.L., and Nash, R.J. (2021). Garcinia kola and garcinoic acid suppress SARS-CoV-2 spike glycoprotein s_1-induced hyper-inflammation in human pbmcs through inhibition of NF-κB activation. *Phytotherapy research* 35, 6963–6973.

Oliveira, D.S., Medeiros, N.I., and Gomes, J.A.S. (2020). Immune response in covid-19: What do we currently know? *Microbial pathogenesis* 148, 1-7.

Ong, E.Z., Chan, Y.F.Z., Leong, W.Y., Lee, N.M.Y., Kalimuddin, S., Haja Mohideen, S.M., Chan, K.S., Tan, A.T., Bertoletti, A., Ooi, E.E., and Low, J.G.H. (2020). A dynamic immune response shapes covid-19 progression. *Cell host microbe* 27, 879-882.

Ozga, A.J., Chow, M.T., and Luster, A.D. (2021). Chemokines and the immune response to cancer. *Immunity* 54, 859-874.

Parkin, J. and Cohen, B. (2001). An overview of the immune system. *Lancet* 357, 1777-1789.

Perlman, S. and Dandekar, A.A. (2005). Immunopathogenesis of coronavirus infections: Implications for SARS. *Nature reviews in immunology* 5, 917-927.

Permatananda, P.A.N.K., Aryastuti, A.A.S.A., Cahyawati, P.N., Udiyani, D.P.C., Wijaya, D., Pandit, I.G.S., and Wirajaya, A.A.N.M. (2021). Phytochemical and antioxidant capacity test on turmeric extract (Curcuma longa) traditionally processed in Bali. *Journal of physics: conference series* 1869, 1-6.

Pohl, M.O., Busnadiego, I., Kufner, V., Glas, I., Karakus, U., Schmutz, S., Zaheri, M., Abela, I., Trkola, A., Huber, M., Stertz, S., and Hale, B.G. (2021). SARS-CoV-2 variants reveal features critical for replication in primary human cells. *PLoS biology* 19, 1-22.

Ramos, I. and Fernandez-Sesma, A. (2015). Modulating the innate immune response to influenza A virus: potential therapeutic use of anti-inflammatory drugs. *Frontiers in immunology* 6, 1-19.

Rankin, L.C. and Artis, D. (2018). Beyond host defense: emerging functions of the immune system in regulating complex tissue physiology. *Cell* 173, 554-567.

Ray, A. (2016). Cytokines and their role in health and disease: a brief overview. *MOJ immunology* 4, 2-9.

Ricci, D., Etna, M.P., Rizzo, F., Sandini, S., Severa, M., and Coccia, E.M. (2021). Innate immune response to SARS-COV-2 infection: From cells to soluble mediators. *International journal of molecular sciences* 22, 1-20.

Ruhee, R.T., Ma, S., and Suzuki, K. (2019). Sulforaphane protects cells against lipopolysaccharide-stimulated inflammation in murine macrophages. *Antioxidants* 8, 1-11.

Saha, S., Buttari, B., Panieri, E., Profumo, E., and Saso, L. (2020). An overview of Nrf2 signaling pathway and its role in inflammation. *Molecules* 25, 1-31.

Sahu, P.K. (2016). Design, structure activity relationship, cytotoxicity and evaluation of antioxidant activity of curcumin derivatives/analogues. *European journal of medicinal chemistry* 121, 510–516.

Sahu, Pramod K., Sahu, Praveen K., Sahu, P.L., and Agarwal, D.D. (2016). Structure activity relationship, cytotoxicity and evaluation of antioxidant activity of curcumin derivatives. *Bioorganic and medicinal chemistry letters* 26, 1342–1347.

Samrat, S.K., Tharappel, A.M., Li, Z., and Li, H. (2020). Prospect of SARS-CoV-2 spike protein: potential role in vaccine and therapeutic development. *Virus research* 288, 1-17.

Shahcheraghi, S.H., Salemi, F., Peirovi, N., Ayatollahi, J., Alam, W., Khan, H., and Saso, L. (2022). Nrf2 regulation by curcumin: molecular aspects for therapeutic prospects. *Molecules* 27, 1-20.

Sibanda, B. and Haryanto, B. (2024). Assessing the impact of covid-19 vaccination programs on the reduction of covid-19 cases: a systematic literature review. *Annals of global health* 90, 1-11.

Sládková, T. and Kostolanský, F. (2006). The role of cytokines in the immune response to influenza a virus infection, *Acta virologica* 27, 151-162.

Sodeifian, F., Nikfarjam, M., Kian, N., Mohamed, K., and Rezaei, N. (2022). The role of type I interferon in the treatment of covid-19. *Journal of medical virology* 94, 63-81.

Steinke, J.W. and Borish, L. (2006). Cytokines and chemokines. *Journal of allergy and clinical immunology* 117, 460-475.

Suhail, S., Zajac, J., Fossum, C., Lowater, H., McCracken, C., Severson, N., Laatsch, B., Narkiewicz-Jodko, A., Johnson, B., Liebau, J., Bhattacharyya, S., and Hati, S. (2020). Role of oxidative stress on SARS-CoV (SARS) and SARS-CoV-2 (covid-19) infection: a review. *Protein Journal* 39, 644-656.

Sun, L., Wang, X., Saredy, J., Yuan, Z., Yang, X., and Wang, H. (2020). Innate-adaptive immunity interplay and redox regulation in immune response. *Redox biology* 37, 1-13.

Tagde, P., Tagde, S., Tagde, P., Bhattacharya, T., Monzur, S.M., Rahman, M.H., Otrisal, P., Behl, T., Hassan, S.S.U., Abdel-Daim, M.M., Aleya, L., and Bungau, S. (2021). Nutraceuticals and herbs in reducing the risk and improving the treatment of covid-19 by targeting SARS-COV-2. *Biomedicines* 9, 1-27.

Tai, W., He, L., Zhang, X., Pu, J., Voronin, D., Jiang, S., Zhou, Y., and Du, L. (2020). Characterization of the receptor-binding domain (RBD) of 2019 novel coronavirus: implication for development of rbd protein as a viral attachment inhibitor and vaccine. *Cellular and molecular immunology* 17, 613–620.

Tavaf, Z., Dangolani, S.K., Yousefi, R., Panahi, F., Shahsavani, M.B., and Khalafi-Nezhad, A. (2020). Synthesis of new curcumin derivatives as influential antidiabetic α-glucosidase and α-amylase inhibitors with antioxidant activity. *Carbohydrate research* 494, 1-10.

Tsang, H.F., Chan, L.W.C., Cho, W.C.S., Yu, A.C.S., Yim, A.K.Y., Chan, A.K.C., Ng, L.P.W., Wong, Y.K.E., Pei, X.M., Li, M.J.W., and Wong, S.C.C. (2021). An update on Covid-19 pandemic: the epidemiology, pathogenesis, prevention and treatment strategies. *Expert review of anti-infective therapy* 19, 877-888.

Turner, M.D., Nedjai, B., Hurst, T., and Pennington, D.J. (2014). Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. *Biochimica et biophysica acta molecular cell research* 1843, 2563-2583.

Uchi, H., Terao, H., Koga, T., and Furue, M. (2000). Cytokines and chemokines in the epidermis. *Journal of dermatological science* 24, 19-38.

Vanden Berghe, T., Grootjans, S., Goossens, V., Dondelinger, Y., Krysko, D. V., Takahashi, N., and Vandenabeele, P. (2013). Determination of apoptotic and necrotic cell death *in vitro* and *in vivo*. *Methods* 61, 117–129.

Wang, M.Y., Zhao, R., Gao, L.J., Gao, X.F., Wang, D.P., and Cao, J.M. (2020). SARS-CoV-2: structure, biology, and structure-based therapeutics development. *Frontiers in cellular and infection microbiology* 10, 1-17.

Weisblum, Y., Schmidt, F., Zhang, F., DaSilva, J., Poston, D., Lorenzi, J.C.C., Muecksch, F., Rutkowska, M., Hoffmann, H.H., Michailidis, E., Gaebler, C., Agudelo, M., Cho, A., Wang, Z., Gazumyan, A., Cipolla, M., Luchsinger, L., Hillyer, C.D., Caskey, M., Robbiani, D.F., Rice, C.M., Nussenzweig, M.C., Hatziioannou, T., and Bieniasz, P.D. (2020). Escape from neutralizing antibodies 1 by SARS-CoV-2 spike protein variants. *Elife* 9, 1-31.

Worachartcheewan, A., Nantasenamat, C., Isarankura-Na-Ayudhya, C., Prachayasittikul, S., and Prachayasittikul, V. (2011). Predicting the free radical scavenging activity of curcumin derivatives. *Chemometrics and intelligent laboratory systems* 109, 207–216.

Xu, Y. and Liu, L. (2017). Curcumin alleviates macrophage activation and lung inflammation induced by influenza virus infection through inhibiting the NF-κB signaling pathway. *Influenza other respiratory viruses* 11, 457–463.

Yazdanpanah, F., Hamblin, M.R., and Rezaei, N. (2020). The immune system and covid-19: friend or foe? *Life sciences* 256, 1-5.

Yilmaz, Z.B., Kofahl, B., Beaudette, P., Baum, K., Ipenberg, I., Weih, F., Wolf, J., Dittmar, G., and Scheidereit, C. (2014). Quantitative dissection and modeling of the NF-κB p100-p105 module reveals interdependent precursor proteolysis. *Cell reports* 9, 1756–1769.

Zhai, P., Ding, Y., Wu, X., Long, J., Zhong, Y., and Li, Y. (2020). The epidemiology, diagnosis and treatment of covid-19. *International journal of antimicrobial agents* 55, 1-13.

Zhang, C., Yang, M., and Ericsson, A.C. (2021). Function of macrophages in disease: current understanding on molecular mechanisms. *Frontiers in immunology* 12, 1-12.

Zhang, J.M. and An, J. (2007). Cytokines, inflammation, and pain. *International anesthesiology clinics* 45, 27-37.

Zhang, Q. and Cao, X. (2021). Epigenetic remodeling in innate immunity and inflammation. *Annual review of immunology* 39, 279-311.

Zhang, Y., Jiang, X., Peng, K., Chen, C., Fu, L., Wang, Z., Feng, J., Liu, Z., Zhang, H., Liang, G., and Pan, Z. (2014). Discovery and evaluation of novel anti-inflammatory derivatives of natural bioactive curcumin. *Drug design, development and therapy* 8, 2161–2171.

Zhou, Q., Zhang, L., Dong, Y., Wang, Y., Zhang, B., Zhou, S., Huang, Q., Wu, T., and Chen, G. (2024). The role of SARS-CoV-2-mediated NF-κB activation in covid-19 patients. *Hypertension research* 47, 375-384.

Zhou, Y., Hou, Y., Shen, J., Mehra, R., Kallianpur, A., Culver, D.A., Gack, M.U., Farha, S., Zein, J., Comhair, S., Fiocchi, C., Stappenbeck, T., Chan, T., Eng, C., Jung, J.U., Jehi, L., Erzurum, S., and Cheng, F. (2020). A network medicine approach to investigation and population-based validation of disease manifestations and drug repurposing for covid-19. *PLoS biology* 18, 1-43.

Zong, K., Xu, L., Luo, Chun, Luo, Chen, Liu, B., Chen, J., Wu, H., Liu, Z., Zhuang, R., and Guo, S. (2024). Paxlovid reduces the 28-day mortality of patients with covid-19: a retrospective cohort study. *BMC infectious diseases* 24, 1-7.