UNIVERSITY OF HELSINKI

Evaluating the antioxidant and anti-inflammatory effects of *Amomum villosum* Lour.

Tiina Pohjankoski

Master's Thesis

Master's Programme in Translational Medicine

Health Industry and Innovations

Faculty of Medicine

Supervisors:

Dr Belal Chami

Professor Päivi Tammela

4/2024

University of Helsinki & University of Sydney

Abstract

Amomum villosum Lour. (Zingiberaceae) has been a fundamental component of traditional Chinese medicine for centuries, primarily employed in managing gastrointestinal disorders. The commonly utilized component of the plant is the dried and mature fruit known as *Amomi fructus*, which has been shown to contain multiple active chemical components including volatile oils, polysaccharides and polyphenols.

Despite extensive research into the medicinal properties of *A. villosum*, which encompasses anti-inflammatory, antioxidant, antimicrobial, antitumoral, hypolipidemic and hypoglycaemic effects, much of the focus has been on local inflammatory disease models such as inflammatory bowel disease. However, investigations into its potential efficacy in systemic diseases like rheumatoid arthritis are notably lacking. Rheumatoid arthritis is a chronic autoimmune disease characterized by inflammation of the synovial joints, leading to joint damage and disability.

The effects of novel water and methanol extracts of *A. villosum* was studied on murine RAW 264.7 macrophages and human dermal fibroblasts (HDF). RAW 264.7 cells are widely used as a model for studying inflammation and immune responses, while HDF cells exhibit relevance to RA through their similarities to synovial fibroblasts and involvement in inflammatory processes, angiogenesis and tissue remodelling. High-performance liquid chromatography analysis revealed the presence of vanillic acid in our extracts, a potent antioxidant with potential therapeutic applications in various inflammatory conditions. Vanillic acid served as a baseline for subsequent *in vitro* experiments with our extracts.

Results from the PrestoBlue cell viability assay demonstrated that water and methanol extracts from *A. villosum* enhanced HDF cell proliferation. Furthermore, both extracts exhibited antioxidant effects against hydrogen peroxide stimulation in both cell lines. Additionally, the water extract from *A. villosum,* inhibited the secretion of interleukin-6 in lipopolysaccharide-stimulated RAW 264.7 cells. Vanillic acid provided similar results. These findings suggest that the anti-inflammatory properties of *A. villosum* may offer a novel therapeutic approach for chronic inflammatory diseases.

Keywords: *Amomum villosum* Lour., chronic inflammation, oxidative stress, rheumatoid arthritis, vanillic acid, anti-inflammatory, antioxidant

Abbreviations

ACPAs	Anti-citrullinated protein antibodies
COX-	Cyclooxygenase-
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DMARDs	Disease-modifying antirheumatic drugs
FLSs	Fibroblast-like synoviocytes
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H_2O_2	Hydrogen peroxide
HDF	Human dermal fibroblasts
HLA	Human leukocyte antigen
HO-1	Heme oxygenase-1
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease
IFNγ	Interferon-γ
IL-	Interleukin-
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MLSs	Macrophage-like synoviocytes (MLSs)
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor-κB
NMR	Nuclear magnetic resonance

Nrf2	Nuclear factor-erythroid 2 p45-related factor 2
NSAID	Nonsteroidal anti-inflammatory drug
PAMPs	Pathogen-associated molecular patterns
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
RF	Fc region of immunoglobulin G, Rheumatoid Factor
RFU	Relative fluorescence units
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TCM	Traditional Chinese medicine
Tfh	Follicular helper cells
Th	T helper lymphocytes
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
Tph	Peripheral helper cells
Treg	Regulatory T lymphocyte
VA	Vanillic acid

TABLE OF CONTENTS

1. INTRODUCTION

Inflammation is a complex process characterized by diverse interactions among various components. The recruitment of leukocytes to the site of inflammation is a crucial event in the pathogenesis of inflammatory disorders. Upon infection, resident macrophages and lymphocytes can be activated by bacteria, their by-products or other antigens (Schett *et al.*, 2018). It has been shown that the activation of macrophages plays a critical role in initiating the inflammatory cascade, leading to the production of potent pro-inflammatory cytokines such as interleukin (IL)-6 (Kadomoto *et al.*, 2022; Na *et al.*, 2019).

IL-6, in conjunction with nuclear factor-κB (NF-κB), orchestrates inflammation and facilitates the recruitment of other immune cells like neutrophils and T-lymphocytes (Kadomoto *et al.*, 2022; Na *et al.*, 2019). While IL-6 and NF-κB act as beneficial components of the host defence system, their overexpression can contribute to the development of pathological conditions. For instance, elevated levels of these inflammatory cytokines have been implicated in various chronic inflammatory disorders including rheumatoid arthritis (RA), asthma, colitis and Crohn's disease (Na *et al.*, 2019; Schett *et al.*, 2018). Consequently, there is considerable interest in identifying agents capable of modulating the production or actions of inflammatory cytokines to mitigate the progression of inflammatory diseases.

RA is a chronic, systemic autoimmune disorder primarily affecting the synovial joints, characterized by inflammation, joint damage and systemic perturbations (Kronzer & Davis, 2021). RA may be classified into two categories: seropositive, characterized by the presence of autoantibodies to the Fc region of immunoglobulin G (rheumatoid factor, RF) and to citrullinated cyclic peptide (ACPAs), or seronegative, where no autoantibodies are detected. Patients harbouring RF or ACPAs typically manifest a more aggressive and severe disease, which frequently results in a poorer disease prognosis, particularly among individuals who are smokers (Dedmon, 2020). RA typically presents with an initial phase of peripheral joint inflammation localized in the synovial membrane, or synovium, which lines the interior of the joint capsule. This inflammation gives rise to morning stiffness, which usually eases throughout the day, and inflammatory pain. RA is further characterized by joint swelling and prolonged stiffness, which, if left uncontrolled, can lead to the erosion of both bone and cartilage, ultimately resulting in joint deformities (Toumi, 2022). It is one of the most prevalent forms of inflammatory arthritis and contributes significantly to disability

burden. The pathogenesis of RA involves a complex interplay of genetic, environmental and immunological factors (Kronzer & Davis, 2021).

The treatment of RA is commonly aiming for sustained disease remission since there is currently no cure for the disease. The current recommendations for treatment are conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), biologic DMARDs (bDMARDs), targeted synthetic DMARDs (tsDMARDs) and corticosteroids (Fraenkel *et al*., 2021). In addition, multiple novel therapeutic approaches, including anti-TNF agents, interleukin inhibitors and B-cell targeted therapies, have been studied with limited success (Huang *et al*., 2021). Although significant progress in therapeutic approaches has substantially improved the quality of life for individuals afflicted by RA, the challenges the disorder offers are still far from being completely solved.

Over the past decade, there has been a significant increase in scholarly focus on traditional medicine. This surge in academic interest has led to a greater acknowledgement of various medicinal plants that have received limited attention in scientific research, despite their potential therapeutic attributes. Consequently, a substantial knowledge gap persists with concerning the specific therapeutic effects and underlying mechanisms of action associated with these relatively unexamined botanical resources.

Amomum villosum Lour. is classified within the Zingiberaceae genus. This botanical species holds a significant history in traditional Chinese medicine (TCM) (Zhou, 1993). *A. villosum* has been studied to have an immunomodulatory effect on macrophages, anticancer activity, hepatoprotective activities and a protective effect against oxidative stress (Zhang *et al*., 2024; Liu *et al.*, 2022; Zhou *et al*., 2021 Zhang *et al*., 2013). Natural herbs belonging to the Zingiberaceae genus have also been shown to have an effective result in the treatment of RA (Toumi, 2022).

The focus of this thesis is to evaluate the anti-inflammatory effect of *A. villosum.* Given the relevance of inflammatory cytokines, particularly IL-6, in the pathogenesis of RA, our study aims to elucidate the potential therapeutic benefits of *A. villosum* extracts on inflammatory processes implicated in this chronic autoimmune disorder.

2. REVIEW OF THE LITERATURE

2.1 INFLAMMATION

2.1.1 ACUTE AND CHRONIC INFLAMMATION

Inflammation is a complex physiological response initiated by harmful stimuli, such as pathogens, tissue damage or environmental triggers. Its primary objective is to eliminate pathogens and dead cells from the affected site and to initiate the healing process. Inflammation involves a coordinated cascade of cellular and molecular events, including vasodilation, increased vascular permeability, recruitment of immune cells (such as neutrophils, macrophages and T-lymphocytes) from circulation to injured tissues, release of inflammatory mediators (such as cytokines, chemokines and prostaglandins) and activation of immune responses (Na *et al.*, 2019; Schett *et al.*, 2018).

Acute inflammation is typically a protective and self-limiting process, ultimately contributing to restoring homeostasis. The resolution phase of inflammation is therefore essential and tightly controlled (Na *et al.*, 2019), whereas failure to resolve inflammation can lead to chronic inflammatory diseases such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD) or asthma (Schett *et al.*, 2018). These chronic inflammatory diseases can contribute to the pathogenesis of various conditions by causing permanent tissue damage, including cardiovascular diseases and cancer (Na *et al.*, 2019; Schett *et al.*, 2018).

2.1.2 OXIDATIVE STRESS IN CHRONIC INFLAMMATORY DISEASES

Oxidative stress is characterized by an imbalance between the production of reactive molecules, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), and the body's capacity to eliminate these reactive substances or repair the consequent damage (Lim *et al.*, 2020). In pathological conditions, immune cells such as macrophages produce reactive molecules like superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot HO$) and nitric oxide ($\cdot NO$). Excessive production of ROS and RNS can activate inflammatory pathways and trigger the release of pro-inflammatory cytokines, leading to sustained inflammation (Djordjevic *et al.*, 2023). Additionally, these reactive molecules can directly damage cellular components such as DNA, proteins and lipids, further exacerbating inflammation and tissue injury (Lim *et al.*, 2020). Oxidative

stress can therefore contribute to chronic inflammatory diseases such as RA by promoting inflammation and tissue damage (Djordjevic *et al.*, 2023).

In RA, oxidative stress plays an important role in perpetuating joint inflammation and damage. ROS activation of intracellular signalling molecules causes increased cell proliferation in the synovial membrane and damage to cartilaginous matrix components. For instance, H_2O_2 has been observed to inhibit chondrocyte proteoglycan synthesis (Schalkwijk *et al*., 1986). Specifically, the activation of the transcription factor NF-κB, a principal inflammatory mediator, by ROS is implicated in the induction of various proinflammatory cytokines (Kaur *et al*., 2022).

The primary activated immune cells infiltrating the joints such as T- and B-lymphocytes as well as macrophages produce pro-inflammatory cytokines like tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6). These cytokines stimulate osteoclast activation and matrix metalloproteinase (MMP) production, initiating processes that lead to joint cartilage damage and bone erosion.

Neutrophils and macrophages present in the synovial fluid further contribute to synovial inflammation, cartilage degradation and bone erosion by facilitating MMP activity, complement system activation and ROS generation (Djordjevic *et al.*, 2023).

2.1.3 MACROPHAGES

Recent advancements have elucidated specific molecular and cellular mechanisms involved in the resolution of inflammation, with macrophages playing a vital role in preventing excessive immune responses. Upon tissue damage, phagocytosis of apoptotic cells triggers a functional switch in macrophages towards an anti-inflammatory transcriptional program, similar to the alternative macrophage activation pattern, leading to the production of cytokines, growth factors and lipid mediators essential for the restoration of homeostasis (Na *et al.*, 2019).

Macrophages are crucial immune cells that play diverse roles in both innate and adaptive immune responses. Derived from circulating monocytes, they are found in nearly all tissues throughout the body, where they serve as protectors of the immune system. Macrophages exhibit remarkable plasticity, adopting distinct phenotypes including M1 macrophages and various subclasses of M2 macrophages (M2a, M2b, M2c and M2d). This dynamic capability, referred to as "polarization," facilitates rapid adjustments in macrophage behaviour to effectively address the diverse stimuli encountered within the local microenvironmental. Their primary functions include

phagocytosis of pathogens and cellular debris, antigen presentation to T cells, secretion of cytokines and chemokines and tissue remodelling. Additionally, macrophages contribute to host defence against infection, tissue repair and maintenance of tissue homeostasis (Kadomoto *et al.*, 2022; Mosser *et al.*, 2021; Na *et al.*, 2019).

Macrophage activation and polarization are processes crucial for immune function and tissue homeostasis. Macrophages possess a diverse range of receptors, including Toll-like receptors (TLRs), which recognise pathogen- and damage-associated molecular patterns (PAMPs and DAMPs). In response to PAMPs, such as bacterial lipopolysaccharide (LPS), or other stimuli like interferon-γ (IFN-γ) or TNF-α, macrophages undergo alterations in their gene expression through TLR4 activation and upregulation of nuclear factor-κB (NF-κB). This leads to the differentiation of M1 macrophages, often referred to as pro-inflammatory macrophages. Subsequently, these cells begin to produce elevated levels of pro-inflammatory cytokines, including IL-6, IL-1β, IL-12, IL-23, TNF-α and CCL2, which promote immunity against foreign pathogens and tumour cells, as well as recruit other leukocytes to the site of inflammation (Figure 1) (Kadomoto *et al.*, 2022; Na *et al.*, 2019).

In contrast, cytokines such as IL-4, IL-10 and IL-13, glucocorticoids and immune-complexes, drive the polarization of macrophages toward the M2 phenotype. M2 macrophages are characterized by their capacity for anti-inflammatory responses and phagocytosis. However, there is a downside, as tumour-associated macrophages often exhibit an M2 phenotype, which promotes tumour progression (Figure 1) (Kadomoto *et al.*, 2022; Na *et al.*, 2019).

A key aspect of macrophage polarization is the alteration of cell surface marker expression. M1 macrophages exhibit elevated expression of cell surface markers CD80 and CD86, as well as intracellular markers such as inducible nitric oxide synthase (iNOS), along with secretion of pro-inflammatory cytokines. Conversely, M2 macrophages show increased expression of intracellular arginase-1 (Arg-1), extracellular scavenger receptors such as CD163 and CD204, mannose receptor CD206 and chemokine receptors CXCR1 and CXCR2. M2 macrophages release the anti-inflammatory cytokine IL-10 and chemokines such as CCL1, CCL17, CCL18 and CCL22, as well as growth factors like VEGF and CSF-1. These molecules are essential for tissue repair, angiogenesis and metabolism (Figure 1) (Kadomoto *et al.*, 2022; Na *et al.*, 2019).

Since macrophages play a vital role in the immune response, dysregulated macrophage activation is implicated in various diseases, including infections, autoimmune disorders, cancer and metabolic disorders (Kadomoto *et al.*, 2022; Mosser *et al.*, 2021; Na *et al.*, 2019).

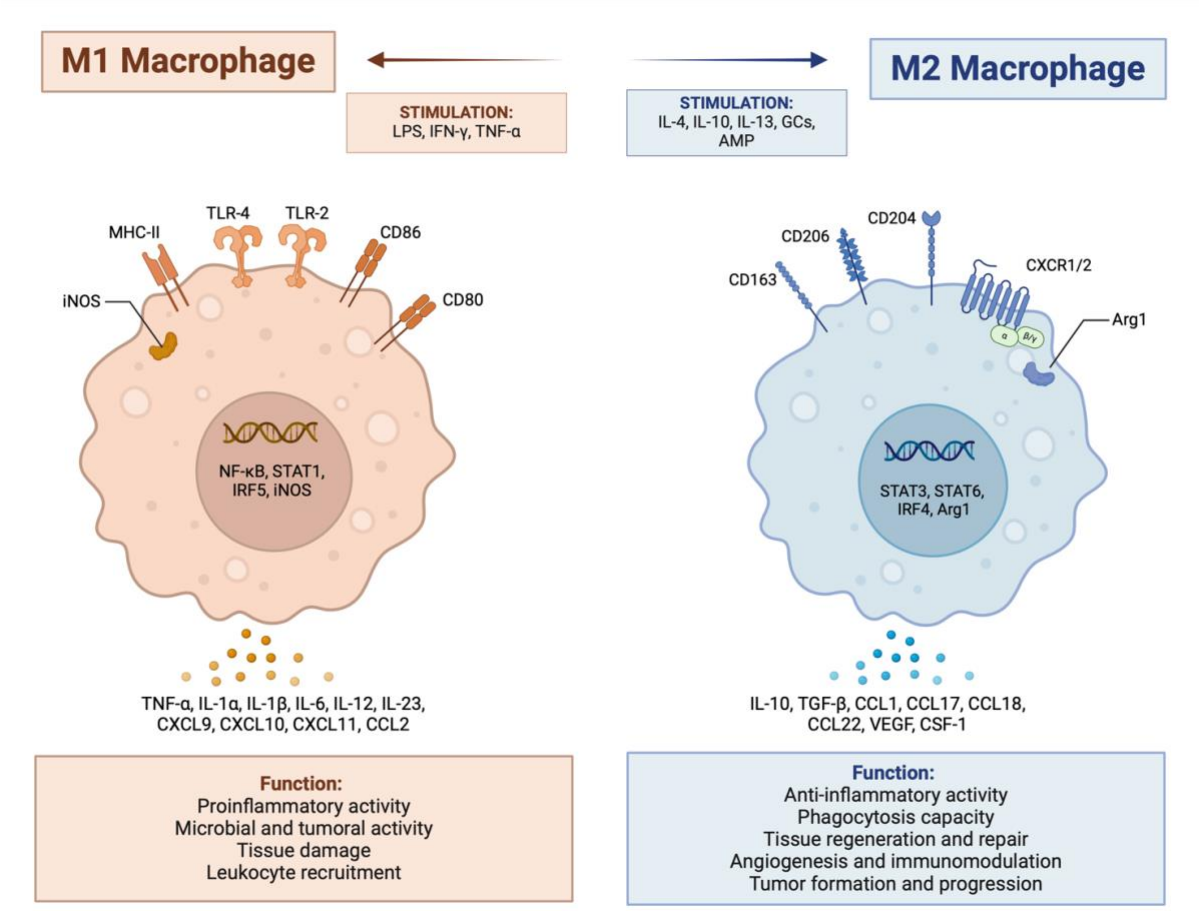

Figure 1. Macrophage polarization: M1 and M2 subtypes. LPS, lipopolysaccharide; IFN, interferon; TNF, tumour necrosis factor; IL, interleukin; GCs, glucocorticoid; AMP, adenosine monophosphate; NOS, nitric oxide synthase; MHC major histocompatibility complex; TLR, Toll-like receptor; CD, cluster of differentiation; CXCR, CXC chemokine receptor; ARG, Arginase; NF-κB, nuclear factor-kappa B; STAT, signal transducer and activator of transcription; IRF, interferon regulatory factor; ARG, arginase; CXCL, CXC chemokine ligand; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; CSF-1, colony-stimulating factor-1; CCL, CC chemokine ligand (modified with BioRender from Kadomoto *et al.*, 2022).

2.2 RHEUMATOID ARTHRITIS

2.2.1 AETIOLOGY OF RHEUMATOID ARTHRITIS

RA is one of the most common chronic autoimmune diseases (Kronzer & Davis, 2021). It reduces life expectancy with 3-10 years and is also associated by a 2-3-fold increase in cardiovascular diseases (Dedmon, 2020; Toledano *et al.*, 2012). RA also demonstrates a pronounced gender bias, with a significantly higher prevalence in females compared to males. This gender disparity is underscored

by a lifetime risk of 3.6% in women, whereas in men, it is notably lower at 1.7% (Crowson *et al*., 2011).

In 2019, according to the World Health Organization, 18 million people worldwide had RA (WHO, 2023). The aetiology of RA started to unravel itself in the 1950s, with Anthony Caplan's publication. In this paper, Caplan established a significant association between coal miners and the incidence of RA, thereby introducing coal exposure as the initial risk factor for the development of the disease (Caplan, 1953). Over the years, several risk factors have been identified, and the aetiology of RA has been acknowledged to involve a complex interplay of various genetic and environmental factors.

Twin studies have shown that the heritability of RA has been estimated to be around 60%, with a substantial genetic component (Okada *et al.,* 2019). This high heritability primarily applies to patients with seropositive RA. RA is recognised as a multi genetic disorder, encompassing multiple different mutations in the human leukocyte antigen (HLA) class II family, found within the human major histocompatibility complex (MHC) on chromosome 6. Alleles that are mostly affected by *HLA-DRB1* alleles include *HLA-DRB1*01*, *HLA-DRB1*04* and *HLA-DRB1*10* (Dedmon, 2020; Okada *et al*., 2019; Weyand *et al*., 1992). Additional genetic risk factors contributing to susceptibility to RA, such as *PTPN22*, *CTLA4*, *PADI4*, *STAT4* and *IL2RA* have successfully been identified by numerous genome-wide association studies and meta-analyses (Dedmon, 2020; Okada *et al.,* 2014; The Wellcome Trust Case Control Consortium, 2007). Polymorphism of the *PTPN22* gene together with the *HLA-DRB1* alleles account for approximately 50% of the familial clustering of RA (The Wellcome Trust Case Control Consortium, 2007). However, in genetically predisposed individuals, environmental triggers, such as smoking and certain infections, can initiate or worsen the disease process.

Environmental risk factors for RA are silica exposure, smoking, infectious agents, vitamin D deficiency, obesity, changes in the microbiome, western diet and periodontal disease (Kronzer & Davis, 2021; Smolen *et al*., 2018). These stressors, for example, cigarette smoke, appear to influence cells at mucosal sites, leading to a process called citrullination, where the amino acid arginine is converted to citrulline in various proteins. This includes proteins found within cells (like histones) and those in the extracellular matrix (such as fibronectin, collagen, fibrinogen, enolase and vimentin). This conversion is facilitated by the activation of peptidyl arginine deiminases (Figure 3a) (Smolen *et al*., 2018). Smoking has been shown to have the greatest effect on these contributing to a 20-fold increase in getting RA in anti-circular citrullinated protein antibody-positive patients (Klareskog *et al*., 2020; Smolen *et al*., 2018). Environmental risk factors suggest that autoimmune inflammation

initiates in the joints due to exposure to various antigens external to the joint areas but located elsewhere in the patient's body. These remote locations encompass the lungs, oropharynx and gastrointestinal tract (Klareskog *et al*., 2020). Exposure to external antigens plays an essential role in initiating the autoimmune inflammatory response observed in the pathogenesis of RA.

2.2.2 PATHOGENESIS OF RHEUMATOID ARTHRITIS

RA is a diverse disease, showing differences in how it presents clinically, and the underlying mechanisms involved. This diversity is observed not only among individuals who share the same formal diagnosis but also across various stages of the disease. The precise pathogenesis of RA is still mostly unknown, despite the recognized autoimmune nature of the disease.

RA is driven by an immune response that leads to chronic inflammation of the synovium. The immune system, particularly adaptive immunity, has a central function. However, in recent decades the role of the innate immune system in the initiation and progression of RA has increased (Jang *et al*., 2022). There are therefore multiple cellular players that affect RA.

2.2.2.1 SYNOVIUM

In early and established RA, chronic inflammation and immune cell infiltration in the synovium lead to hyperplasia of the lining layer, the formation of new blood vessels from the synovium and the development of a thickened area known as pannus, which will later on mediate damage and formation of erosion (Figure 2) (Smolen *et al*., 2018). Pannus may consist of macrophages, fibroblast-like synoviocytes (FLSs), dendritic cells or plasma cells and mast cells (Jang *et al*., 2022). Under normal conditions, the synovium typically possesses a sparse cellular composition, characterized by a delicate intimal lining (Figure 2) (Smolen *et al*., 2018).

Another important pathogenic change in the synovium of RA is the expansion of the intima primarily caused by the proliferation and activation of macrophage-like synoviocytes (MLSs) and FLSs. These synoviocytes are major sources of various cytokines and proteases, including integrins, selectins and members of the immunoglobulin superfamily. MLSs secrete several pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. FLSs produce IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF), in addition to small-molecule mediators like prostaglandins and leukotrienes. They also produce large quantities of MMPs, including MMP-1 and MMP-3, which

contribute to cartilage degradation (Figure 3c) (Jang *et al*., 2022; Lofti *et al*., 2019). Moreover, FLSs contribute to the activation of immune responses by interacting with immune cells and facilitating the formation of ectopic lymphoid structures in synovial tissues (Jang *et al*., 2022; Smolen *et al*., 2018). MLSs and FLSs reside in close proximity within the synovial intimal lining, where the cytokines they produce can activate either themselves or neighbouring cells (Smolen *et al*., 2018).

Infiltrating cells to the synovial sublining are mostly composed of CD4+ T cells, B cells and macrophages, which can organise themselves into lymphoid aggregates with germinal centres where mature B cells, proliferate, differentiate and produce antibodies. B cells, plasmablasts and plasma cells in the synovium are mainly responsible for producing rheumatoid factor (RF) or anti-citrullinated protein antibodies (ACPAs) contributing to immune dysregulation (Jang *et al*., 2022). The presence of ACPAs alone is unlikely to induce the inflammation of the synovium; an additional hit, such as immune complex formation, activation of complement, or damage to the microvasculature, is likely necessary to trigger clinical synovitis. This condition is distinguished by heightened vascular permeability and the infiltration of inflammatory cells into the synovium (Figure 3c, d) (Smolen *et al*., 2018).

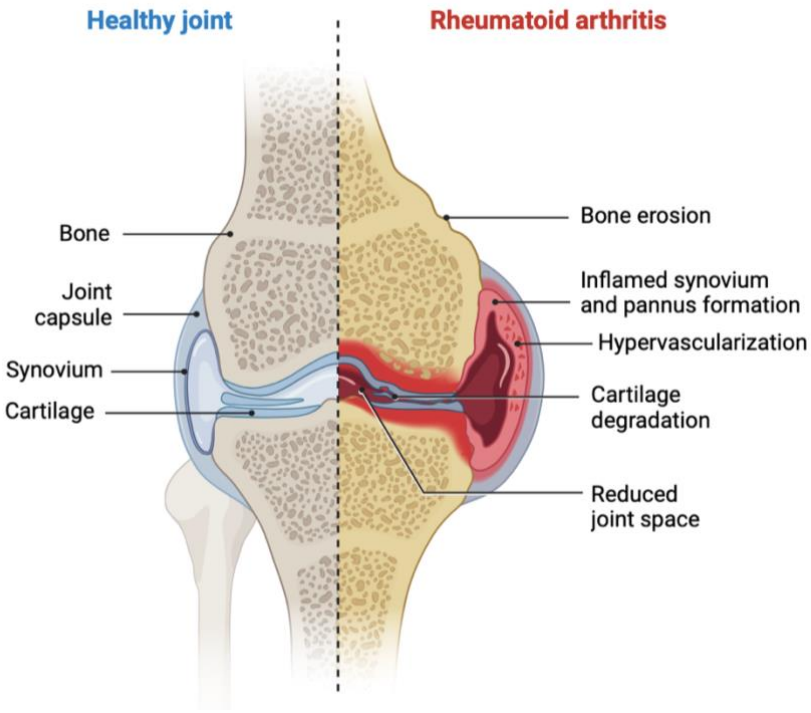

Figure 2. Healthy joint vs. rheumatoid arthritis (modified with BioRender from Smolen *et al*., 2018).

2.2.2.2 B CELL-MEDIATED IMMUNE RESPONSE IN RHEUMATOID ARTHRITIS

The main functions of B cells, autoantibody production, cytokine secretion and antigen presentation, contribute to the pathogenesis of RA. Autoantibodies to immunoglobulin G (IgG; known as, RF) and ACPAs relate to the disease process by targeting self-antigens, leading to immune complex formation and complement activation (Figure 3b) (Jang *et al*., 2022; Smolen *et al*., 2018). These autoantibodies are considered hallmarks of RA and aid in diagnosis and disease stratification.

Given the central role of inflammation in RA pathogenesis, ACPAs have been shown to activate immune cells and increase the production of pro-inflammatory cytokines. Although autoantibodies constitute a significant hallmark of RA (seropositive RA), there exists a subset of patients who do not exhibit these autoantibodies (seronegative RA) (Jang *et al*., 2022; Smolen *et al*., 2018).

Activated B cells present in the peripheral blood of RA patients demonstrate the ability to produce numerous cytokines, including CCL3, TNF-α, IFN-γ, IL-6, IL-1β, IL-17 and IL-18. These cytokines function by activating other cell types, including T lymphocytes, macrophages and osteoclasts. Particularly, TNF-α, in conjunction with IL-1β, has been observed to enhance the expression of receptor activator of NF-κB ligand (RANKL) by B cells, thereby facilitating osteoclastogenesis (Jang *et al*., 2022).

Another crucial function of mature B cells involves presenting antigens to T cells via MHC II receptors. The HLA region in MCH comprises both class I and class II genes. Specifically, class II genes (*HLA-DR*, *-DQ* and *-DP*) are responsible for encoding the alpha and beta chains of the corresponding MHC class II molecule (Dedmon, 2020). Particularly the alleles within the *HLA-DRB1* gene, which are associated with a heightened susceptibility to RA, encode specific amino acid sequences in the *HLA-DRB1* chain. These sequences form a shared epitope that predisposes individuals to autoimmune responses against self-antigens (Kronzer & Davis, 2021; Smolen *et al*., 2018). B cells exhibit the capability to present antigens primarily to CD4+ T helper cells (Jang *et al*., 2022).

2.2.2.3 T CELL-MEDIATED IMMUNE RESPONSE IN RHEUMATOID ARTHRITIS

Chronically activated T cells are considered to be the trigger for RA. The main subset of T lymphocytes associated with RA is CD4+ helper T cells (Th), which makes IL-6 an important mediator of bone degradation by regulating T lymphocyte production and inflammation (Smolen *et al*., 2018). CD4+ helper T cells are further categorized into follicular helper cells (Tfh) and peripheral helper cells (Tph). Numerous studies indicate an elevation in the frequency of circulating Tph and Tfh cells in the blood of RA patients compared to healthy individuals (Jang *et al*., 2022).

Pathogenic Th17 cells are known to secrete pro-inflammatory cytokines like IL-21, which significantly contribute to synovial inflammation by activating, proliferating, differentiating and enhancing the affinity maturation and antibody production of B cells. Th17 cells, in particular, are also implicated in driving inflammation through the production of IL-17, IL-22 and GM-CSF. IL-17 triggers the release of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 in the cartilage, synovial cells, macrophages and bone cells. Additionally, it promotes the production of several chemokines, including CXCL1, CXCL2, CXCL8, CCL2, CCL7 and CCL20, which recruit neutrophils, macrophages and lymphocytes to the synovium. TNF-α and IL-1 are instigated within the synovial membrane, giving rise to localized swelling and pain. These factors also prompt the generation of ROS and the activation of osteoclasts, thereby precipitating the destructive processes affecting bone and cartilage (Toumi, 2022). IL-22, like IL-17, promotes inflammation in the synovium by stimulating the proliferation of FLSs and inducing the production of chemokines (Jang *et al*., 2022). Notably, GM-CSF enhances the activation and movement of myeloid cells to areas of inflammation, thereby supporting the survival of effector granulocytes and macrophages involved in immune responses. Increased frequencies of GM-CSF-producing B and T cells in individuals with RA have been proposed to indicate its role in the production of autoantibodies and the progression of the disease (Figure 3c) (Lofti *et al*., 2019).

The inflammation site includes also regulatory T lymphocytes (Treg), which normally suppress the pro-inflammatory immune response (Jang *et al*., 2022; Schett *et al.*, 2018). In RA IL-1β and IL-6 downregulate forkhead box P3 (Foxp3) expression, which is important in regulating the survival and function of Treg cells. This leads to a decrease in the suppressive activity of Treg cells (Jang *et al*., 2022). Despite the abundance of T cells in the synovium, the functional role of T cells remains unclear, yet they are considered crucial in the pathogenesis of the disease.

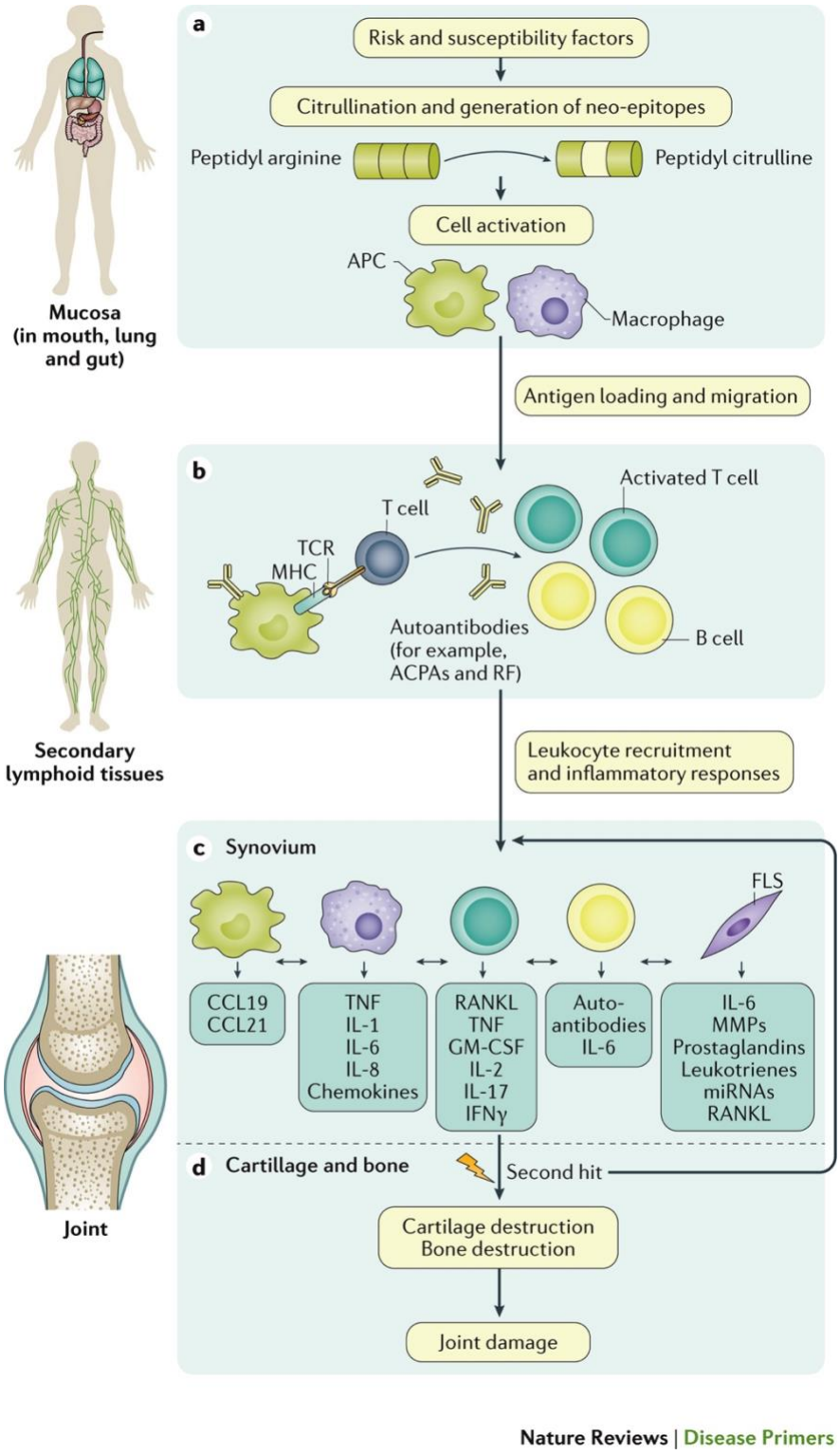

Figure 3. Mechanisms involved in intitiation and progression of rheumatoid arthritis. **a**. Post-translational modifications like citrullination in the mucosa can generate neo-epitopes that can be recognized by the adaptive immune system. **b.** Antigen presenting cells (APCs) can initiate an adaptive immune response and the production of autoantibodies by presenting these modified peptides in lymphoid tissues. **c**. Locally activated stromal cells (such as fibroblast-like synoviocytes (FLS)), APCs and macrophages produce various inflammatory factors. While the immune system's autoimmune response triggers synovial inflammation, an additional trigger, like immune complex formation and complement activation, may be necessary to induce or intensify cytokine production and synovial vascular leakage. **d**. Paracrine and autocrine actions of cytokines,

coupled with sustained adaptive immune responses, can perpetuate the disease process, , eventually resulting in damage to cartilage and bone. APCAs, anti-citrullinated protein antibodies; CCL19, CC-chemokine ligand 19; CCL21, CC-chemokine ligand 21; GM-CSF, granulocyte–macrophage colony-stimulating factor; MHC, major histocompatibility complex; miRNA, microRNA; MMP, matrix metalloproteinase; RANKL, receptor activator of nuclear factor-κB ligand; RF, rheumatoid factor; TCR, T cell receptor; TNF, tumour necrosis factor (Smolen *et al*., 2018, License number: 5756880298805, License date:Mar 27, 2024).

2.2.2.4 INNATE IMMUNITY-MEDIATED IMMUNE RESPONSE IN RHEUMATOID ARTHRITIS

Innate immune cells such as macrophages, monocytes and dendritic cells play a pivotal role in the onset and advancement of RA, activating the adaptive immune system, which becomes indispensable in the disease's later phases. Among these cells, macrophages are notably abundant in the synovium of RA patients. Research has shown that complexes formed by citrullinated fibrinogen and ACPA (known as CitFibr-ACPA) found in the synovial membrane of RA patients can activate macrophages by simultaneously engaging TLR-4 and Fc-γ receptor (FcγR). This dual activation leads to a synergistic increase in the production of TNF-α. These findings imply that citrullination may enhance the effectiveness of natural immune ligands and offer insights into how anti-citrulline autoimmunity could contribute to the development and progression of inflammation in RA (Jang *et al*., 2022; Smolen *et al*., 2018).

Macrophages produce pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α, chemoattractant factors (CCL2 and IL-8) and metalloproteinases (MMP-3 and MMP-12). Macrophages also act as antigen-presenting cells therefore activating T lymphocytes. Classically activated macrophages, known as the M1 phenotype, promote joint erosion by predominantly releasing pro-inflammatory cytokines like TNF-α and IL-1β. Conversely, anti-inflammatory cytokines such as IL-10 and TGF-β stimulate the activation of macrophages into an alternatively activated state, referred to as the M2 phenotype, which helps regulate inflammation and contributes to processes like angiogenesis, tissue remodelling and repair. Recent research indicates an imbalance in macrophage subsets within the synovial fluid of RA patients, with a higher M1/M2 ratio observed compared to patients with osteoarthritis (Jang *et al*., 2022; Zhou *et al*., 2021). In addition, functional genomics has shown that the disruption of synovial macrophages and fibroblasts has been linked with disease activity and remission (Kronzer & Davis, 2021). Furthermore, GM-CSF prompts multipotent mesenteric mesothelial cells to differentiate into macrophages via the ERK1/2 and NF-κB signalling pathway (Lofti *et al*., 2019).

In RA dendritic cells (DCs) are the most prevalent antigen-presenting cells in the synovium. They express both MHC class I and II molecules, along with co-stimulatory molecules necessary for T cell activation. DCs have the capacity to either foster tolerance or trigger autoimmunity, influenced by the diverse signals they encounter in the joint. DCs can foster immune tolerance through various means, such as the production and preservation of T regs, as well as inducing a state of unresponsiveness in T cells. Conversely, DCs' role in presenting antigens may facilitate the activation and differentiation of self-reactive T cells, potentially leading to immune responses against self-components (Jang *et al*., 2022).

In summary, these events finally lead to the destruction of cartilage and bone, as well as joint damage (Figure 3d) (Smolen *et al*., 2018). These processes are driven by the release of MMPs and ROS that degrade joint structures, in addition to the inflammation insult that happens with RA (Djordjevic *et al.*, 2023).

2.2.3 CHALLENGES IN THE TREATMENT OF RHEUMATOID ARTHRITIS

RA is not only limited to joint involvement since it can also have systemic effects. Patients may experience fatigue, malaise and anaemia due to chronic inflammation. Extra-articular manifestations can affect various organs, such as the skin (rheumatoid nodules), lungs (pleuritis) and blood vessels (rheumatoid vasculitis) (Cojocaru *et al*., 2010). In addition, inflammation causes fluid accumulation in the joints, which leads to swelling and more experience of pain in RA patients. Finally, the persistence of inflammation and the absence of sufficient repair mechanisms lead to joint deformities and functional limitations, significantly adding to the burden of disability (Kronzer & Davis, 2021).

In the first place, nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylate, naproxen, ibuprofen and etodolac are used to relieve pain and swelling and reduce inflammation, without an effect on disease progression (Huang *et al*., 2021). Corticosteroids, for example, glucocorticoids (non-specific anti-inflammatory drugs), modify gene expression by binding to glucocorticoid receptors to display anti-inflammatory and immunosuppressive effects (Huang *et al.,* 2021).

The overall treatment of RA is commonly aiming for sustained disease remission since there is currently no cure for the disease. Nevertheless, early diagnostics remains an important factor in the course of the disease (Toumi, 2022; Fraenkel *et al*., 2021). The correlation between RF levels and clinical disease activity remains a subject of debate. RF levels may fluctuate and change during the

early stages of the disease, yet these fluctuations do not appear to influence clinical outcomes. Despite this, RF remains a valuable diagnostic marker for RA and is routinely utilized in clinical practice. On the other hand, ACPAs also serve as an important diagnostic parameter for identifying early RA and initiating timely treatment (Jang *et al*., 2022).

The current recommendations for treatment, alongside NSAIDs and corticosteroids, are conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), biological DMARDs (bDMARDs) and targeted synthetic DMARDs (tsDMARDs), which are a set of immunosuppressive and immunomodulatory drugs (Fraenkel *et al*., 2021). Conventional synthetic DMARDs, like hydroxychloroquine, sulfasalazine, methotrexate leflunomide and gold salts, are used as a first-line treatment after diagnosis of RA. They can affect multiple cell signalling pathways, but their mechanism of action is not yet fully understood. It is advisable to alternate between multiple drugs of DMARDs, rather than relying solely on one, as prolonged use of a single DMARDs may lead to a decrease in its effectiveness over time (Toumi, 2022; Fraenkel *et al*., 2021).

Biological DMARDs are monoclonal antibodies targeting specific molecules, including TNF inhibitors (etanercept, adalimumab, infliximab, golimumab, certolizumab pegol), T cell costimulatory inhibitors (abatacept), IL-6 receptor inhibitors (tocilizumab, sarilumab) and anti-CD20 antibodies (rituximab) (Toumi, 2022; Fraenkel *et al*., 2021). The effect of bDMARDs is to downregulate the chronic inflammatory reaction in RA by modulating the immune response and inhibiting pro-inflammatory cytokines through these specific targets (Fraenkel *et al*., 2021).

Targeted synthetical DMARDs, such as Janus Kinase (JAK) inhibitors (tofacitinib, baricitinib, upadacitinib), are the first oral option compared to other DMARDs. JAK facilitates signalling via the IL-6 receptor as well as numerous other transmembrane receptors, including cytokine receptors, G protein-coupled receptors and receptor tyrosine kinases (Fraenkel *et al*., 2021; Huang *et al*., 2021).

The treatment of RA has evolved significantly in recent decades. Targeted synthetic and biologic disease-modifying antirheumatic drugs (DMARDs) have revolutionized management after NSAIDs (Fraenkel *et al*., 2021; Huang *et al*., 2021). Biologics, including anti-TNF agents, interleukin inhibitors and B cell-targeted therapies, have improved treatment outcomes in RA. Developed treatments alleviate certain symptoms and hinder further bone and cartilage damage. Nevertheless, there are still challenges associated with marketed biologics and drugs, which include instances of non-responsiveness and partial responses, along with the occurrence of adverse effects (Table 1) (Toumi, 2022; Fraenkel *et al*., 2021; Huang *et al*., 2021). In addition to the adverse effects listed in Table 1, the use of DMARDs has been associated with various serious adverse events. These include

opportunistic infections (such as tuberculosis and fungal infections), serious bacterial infections, irreversible organ damage (such as to the liver, kidneys, bone marrow and lungs), ocular toxicity and morbidity from conditions like diabetes, cardiovascular disease and hepatic disease. Other serious outcomes may include cataracts, osteoporosis, fractures, cancer, foetal toxicity and even death. Furthermore, reversible adverse effects have been observed, including hair loss, weight gain, mucosal and dermatologic reactions, gastrointestinal side effects like nausea and diarrhoea and changes in mental health such as depression, brain fog, dizziness, insomnia and headaches (Fraenkel *et al.,* 2021).

Table 1.

Challenges with current treatment methods in rheumatoid arthritis

Medication	**Side effects**	**References**
Conventional synthetic disease-modifying antirheumatic drugs (csDMARDs)	Stomatitis, exanthema, pneumonitis, nodulosis, retinopathy, neuropathy/myopathy, haemolytic anaemia (g6pd deficiency), irreversible dermatologic changes, CNS/neuromuscular adverse effects, hypoglycaemia, fertility, peripheral neuropathy and hypertension	(Fraenkel *et al*., 2021)
Biologic DMARDs (bDMARDs)	Demyelinating disease, CHF exacerbation, COPD exacerbation, hypogammaglobulinemia, progressive multifocal leukoencephalopathy, severe mucocutaneous reactions, severe infusion reactions, hyperlipidemia, GI perforation and hypertension	(Fraenkel *et al*., 2021)
Targeted synthetic DMARDs (tsDMARDs)	GI perforation, hyperlipidemia, thrombosis and hypertension	(Fraenkel *et al*., 2021)
Corticosteroids	Nausea, abdominal pain, ulceration, osteoporosis and diabetes	(Huang *et al*., 2021)
NSAIDS	Bleeding and gastrointestinal ulceration	(Huang *et al*., 2021)

As the challenges in treating RA continue, the request for alternative therapeutic options has intensified, leading researchers to explore the potential of natural remedies. Among the diverse array of plant species employed in RA treatment, Acacia species have emerged as promising contenders. Renowned for their ability to inhibit cyclooxygenase-1 and -2 (COX-1 & COX-2) enzymes, Acacia species offer a natural approach to alleviating RA symptoms. *In vitro* studies have further corroborated their efficacy in suppressing these enzymes, underscoring their potential as therapeutic

agents. Additionally, extracts from the Zingiberaceae family have demonstrated remarkable anti-inflammatory properties, particularly in inhibiting cytokine production in FLSs from RA patients. This growing body of research signifies a new era in RA treatment, one that utilizes the therapeutic potential of natural remedies to combat this debilitating disease (Toumi, 2022).

2.3 THE AMOMUM GENUS

Traditional Chinese medicine (TCM) is regarded as one of the oldest forms of medicine (Tang *et al*., 2008). Most adverse effects linked to TCM are due to contaminations and inappropriate use rather than risks posed by the herbal components themselves. By ensuring quality control and adherence to clear guidelines, the occurrence of adverse reactions can be significantly minimized (Tang *et al*., 2008). TCM could therefore offer a safer approach to patient treatment, particularly for those concurrently using multiple medications. Furthermore, the expanding population, coupled with the increasing healthcare demands it presents, underscores the imperative that further scientific research is needed to comprehensively investigate the full potential offered by TCM.

The genus *Amomum* belongs to the Zingiberaceae family and consists of around 150 species which mainly grow in the tropical regions of Asia and Oceania. In traditional medicine, there are 16 species considered significant. These species offer various parts such as fruits, seeds, stems, roots, rhizomes and leaves for medicinal use (Cai *et al*., 2021). Studies on the pharmacological properties of the Amomum genus have shown its potential effectiveness in treating inflammation, gastrointestinal diseases, liver diseases, cancer, malaria and other conditions (Table 2) (Kim *et al*., 2016; Zhang *et al*., 2015; Kumar *et al*., 2014; Heilmann *et al*., 2001).

Table 2.

Medicinal plants of the genus *Amomum*.

Species	Medicinal parts	Medicinal uses	Distribution	References
Amomum villosum Lour.	Fruits, stems and leaves	Gastrointestinal disorders, inflammation, antioxidant, hypolipidemic, anti-tumoral,	China, Vietnam, Cambodia, Laos and Thailand	(Zhang et al., 2024; Cai et al., 2021; Yue *et al*., 2021)

		antimicrobial and hypoglycaemic		
Amomum xanthioides	Seeds and fruits	Atopic dermatitis, allergic reactions, inflammation	Korea and Seoul	(Choi *et al*., 2017)
Amomum tsao-ko	Fruits	Inflammation, digestive disorders and cancer	Guangxi, Guangdong and Yunnan	(Zhang *et al*., 2015; Kim *et al*., 2016).
Amomum subulatum Roxb.	Seeds and fruits	Stomach, hepatic and pulmonary disorders	Bhutan, Nepal and India	(Kumar *et al*., 2014).
Amomum aculeatum Roxb.	Rhizomes, roots and leaves	Fever and malaria	Malaysia, Indonesia, and Papua New Guinea.	(Heilmann *et al*., 2001)
Amomum maximum Roxb.	Roots and rhizomes	Gastrointestinal disorders and hypoglycaemic	South China and Southeast Asia	(Luo *et al*., 2014; Lu *et al*., 2022)
Amomum compactum	Seeds	Asthma, inflammation	Korea	(Lee *et al*., 2010)
Amomum koenigii J.F.Gmel	Rhizomes and fruits	Antimicrobial and ACE inhibition	Vietnam	(Darsih *et al*., 2023)
Amomum kravanh	Fruits	Gastrointestinal disorders, antioxidant, antimicrobial and DNA damage protection	Cambodia, Thailand and Vietnam	(Zhang *et al*., 2020; Li *et al*., 2023)
Amomum muricarpum	Rhizomes	Antioxidant	China, Philippines and Vietnam	(Huong, *et al*., 2015)
Amomum masticatorium	Rhizomes	Treating ulcer, inflammation, hypolipidemic, antioxidant and antibacterial	Korea	(Sinitha & Thoppil, 2017)
Amomum cardamomum L.	Seeds	Hepatic injury and dyspepsia	India	(Lim *et al*., 2016)

Amomum nilgiricum	Leaves	Bacterial and fungal diseases, antioxidant, antidiabetic	India	(Konappa *et al*., 2020)
Amomum krervanh Pierre.	Fruits	Malaria; Antibacterial	Thailand	(Kamchonwongpaisan *et al*., 1995)
Amomum uliginosum J. Koenig	Rhizomes	Gastrointestinal diseases	Thailand and Malaysia	(Wannaporn *et al*., 2015)

2.3.1 AMOMUM VILLOSUM LOUR.

Amomum villosum Lour. is native to several Southeast Asian countries, including China, Vietnam, Cambodia, Laos and Thailand (Cai *et al*., 2021). In China, its principal cultivation is primarily observed in the southern regions of the country, particularly within the provinces of Yunnan, Guangdong and Jiangxi, as well as in Hainan and Fujian. The commonly utilized component of the plant is the dried and mature fruit known as *Amomi fructus* (Figure 4), which is typically ground and prepared for consumption in the form of herbal tea or as a spice in food (Zhou, 1993).

Early studies into *A*. *villosum* was characterized by examining its chemical composition, pharmacological effects and potential applications in traditional medicine. The studies conducted in the early 1990s encompassed various investigations, including the analysis of chemical constituents within root rhizomes and stems, as well as the comparison of pharmacological distinctions between volatile oil and water extracts. Notably, a majority of the early research papers published during this period were authored in the Chinese language, making them inaccessible to individuals unfamiliar with the language. The research on the constituents of the plant and its therapeutic effects has increased since 2018.

Figure 4. Dried fruit of *Amomum villosum* Lour., *Amomi fructus* (picture by Tiina Pohjankoski).

Amomum villosum Lour. has been shown to contain multiple active chemical components, including over 120 volatile oils like terpenoids, sesquiterpenes, and approximately 30-40 non-volatile components, such as phenolic compounds, polysaccharides and saponins (Table 3) (Zhang *et al*., 2024; Zeng *et al*., 2022; Cai *et al*., 2021). The most abundant components of volatile oils have been shown to include camphor, bornyl acetate, caryophyllene, β-bisabolene, (E)-nerolidol and cubenol (Zeng *et al*., 2022; Chen *et al*., 2020). Phenolic compounds of *A*. *villosum* consist mostly of polyphenols like phenolic acids, flavonoids and flavonols (Zhang *et al*., 2024). While naming polysaccharides can be challenging, specific neutral and acidic polysaccharides such as AVLP-2, AVPG-1 and AVPG-2 have been isolated and identified. The polysaccharides from *A*. *villosum* are primarily composed of different monosaccharides, such as glucose, arabinose, galactose and mannose, which contribute to the inflammatory effect of *A. villosum* (Table 3) (Kim *et al*., 2022; Liu *et al*., 2022; Zhou *et al*., 2021; Zhang *et al*., 2013).

Table 3.

Chemical constituents of *Amomum villlosum* Lour.

Polyphenols	**Volatile oils**	**Carbohydrates**
Phenolic acids:	**Terpenoids:**	**Monosaccharides:**
Vanillic acid	Camphor	Glucose
Gallic acid	Bornyl acetate	Arabinose
Ferulic acid	Caryophyllene	Galactose
Caffeic acid	Cubenol	Mannose

Syringic acid	Copaene	
p-coumaric acid	α-Santalene	
4-hydroxybenzoic acid	γ-Elemene	
Protocatechuic acid	δ-Cadinene	
Flavonoids:	**Sesquiterpenes:**	**Polysaccharides:**
Quercetin	β-bisabolene	AVLP-2
Quercitrin	δ-Cadinol	AVPG-1
Isoqercitrin	Torreyol	AVPG-2
Flavonols:		
(+/-) Catechin		
Epicatechin		
Isorhamnetin		
Rutin		

The medicinal properties of *A*. *villosum*'s active components have been employed in TCM for over a millennium, primarily to address gastrointestinal disorders. Studies conducted using in-vitro methods, animal models and compound analysis have demonstrated that *A. villosum* contains a wide range of medicinal properties, including anti-inflammatory, antioxidant, antimicrobial, anti-ulcer, hypolipidemic, anti-tumoral and hypoglycaemic effects (Zhang *et al*., 2024; Gao *et al*., 2023; Kim *et al*., 2022; Cai *et al*., 2021; Yue *et al*., 2021). In addition, it has demonstrated promise in the treatment of hepatoma, functional dyspepsia, antibiotic-associated diarrhoea, digestive tract disease, inflammatory bowel disease and gouty arthritis (Dong *et al*., 2023; Cai *et al*., 2021; Chen *et al*., 2018). Furthermore, numerous studies have demonstrated that the consumption of natural antioxidants is associated with a reduction in oxidative stress (Zhang *et al*., 2024; Cai *et al*., 2021; Lim *et al*., 2020; Zhang *et al*., 2013). Extracts from *A. villosum* have successfully enhanced antioxidant capacity and regulated inflammatory factors making it a potential anti-inflammatory medicine (Liu *et al.*, 2022).

2.3.2 ANTI-INFLAMMATORY EFFECT OF AMOMUM VILLOSUM LOUR.

2.3.2.1 POLYPHENOLS

The anti-inflammatory properties of *A.* villosum stem from its rich array of chemically active compounds. A major phenolic component of *A. villosum* is found to be vanillic acid, 4-hydroxy-3-methoxybenzoic acid (Figure 5) (Zhang *et al*., 2024). Vanillic acid is a phenolic acid and an oxidized form of vanillin. It is regarded as an antioxidant with a broad impact on the immune defence (Kim *et al.*, 2011).

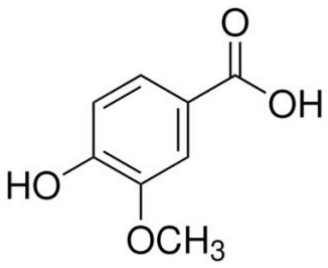

Figure 5. The molecular form of vanillic acid.

Several studies have demonstrated the efficacy of vanillic acid in modulating immune responses. For instance, vanillic acid strongly enhanced the proliferation of human peripheral blood mononuclear cells and the secretion of IFNγ (Chiang *et al.*, 2003). Additionally, vanillic acid has been shown to have antimicrobial, anti-DNA oxidation and anti-tumoral properties (Aziz *et al.*, 1998; Guimarães *et al.*, 2007; Kampa *et al.*, 2004). Furthermore, vanillic acid demonstrated a notable decrease in clinical symptoms and inflammatory mediator levels in an experimental model of colitis (Kim *et al.*, 2010) and a hepatoprotective effect by suppressing immune-mediated liver inflammation (Itoh *et al.*, 2009).

Vanillic acid has shown to reduce inflammation through the regulation of NF-κB. The LPS-induced anti-inflammatory effects of vanillic acid are inhibition of TNF-α, IL-6, IFN-γ and prostaglandin E2 production (Kim *et al.*, 2011; Itoh *et al.*, 2009). Vanillic acid also decreases the expression of COX-2, as well as NO and caspase-1 levels (Kim *et al.*, 2011).

Quercetin, another polyphenol in *A. villosum,* has been observed to inhibit the NF-*κ*B signalling pathway, resulting in decreased levels of proinflammatory cytokines, NO and prostaglandins in peritoneal macrophages and RAW 264.7 cells treated with LPS. This effect was mediated through the activation of the nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)/ heme

oxygenase-1 (HO-1) pathway (Lim *et al*., 2020). HO-1 is predominantly expressed in M2 macrophages, leading to a transition from an inflammatory to an anti-inflammatory state. This shift promotes the resolution of inflammatory processes (Lim *et al*., 2020).

Phenolic compounds in addition to vanillic acid and quercetin, include quercitrin, catechin, epicatechin and protocatechuic acid have too demonstrated strong antioxidant capacity through their ability to scavenge free radicals (Zhang *et al*., 2024).

2.3.2.2 VOLATILE OILS

Bornyl acetate, a natural compound found in the essential oil of *A. villosum,* has been studied for its pharmacological effects, including its anti-inflammatory and analgesic properties. A study found that the volatile oil extracted from *A.* villosum effectively regulated the intestinal microflora, improved chronic low-grade inflammation by increasing the expressions of ZO-1 and occludin proteins, as well as inhibited the TLR4/NF-κB signalling pathway (Lu *et al*., 2018).

Bornyl acetate has also been found to reduce the expression of proinflammatory cytokines both *in vitro* and *in vivo*. A study showed bornyl acetate decreased the total cell count, as well as the numbers of neutrophils and macrophages in bronchoalveolar lavage fluid. It mitigated histological lung alterations, lowered the wet-to-dry weight ratio in bronchoalveolar lavage fluid, and inhibited the activation of NF-κB inhibitor alpha, extracellular regulated protein kinases, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (Chen *et al*., 2014). Additionally, Gao *et al.,* 2023 proved that an extracted component, a norditerpene, from *A. villosum,* demonstrated significant antioxidant activity via the Nrf2/ HO-1 pathway.

The water extract and volatile oils derived from *A.* villosum exhibit a multifaceted impact on immune and inflammatory processes in rats with IBD. These effects include the regulation of inflammatory cytokines, modulation of CD4+CD25+FOXP3+ T cells (Treg) and support for intestinal microecological balance, all contributing to the observed anti-inflammatory effects (Chen *et al.*, 2018).

2.3.2.3 POLYSACCHARIDES

Polysaccharides constitute a significant portion of *A. villosum*'s chemical composition. These macromolecules typically consist of more than ten monosaccharide units connected by various

glycosidic bonds. Their biological activities are affected by factors such as molecular weight, monosaccharide composition, glycosidic linkages and sulphate content (Luo *et al.*, 2022; Liu *et al.*, 2021; Zhang *et al.*, 2013).

Ulcerative colitis, a prominent form of IBD, primarily affects the colon and rectum. It is characterized by chronic inflammation and dysregulation of intestinal macrophages (Na *et al.*, 2019). Administration of AVLP (presumed to be *A. villosum* polysaccharides) yielded notable beneficial outcomes in a mouse model of colitis. These findings propose that incorporating AVLP into the diet could serve as a promising nutritional approach for addressing inflammatory bowel diseases. The observed improvements in body weight, colon metrics, inflammatory indicators and intestinal barrier integrity, along with the significant alterations in gut microbiota composition, underscore the potential therapeutic advantages of AVLP in the context of colitis (Luo *et al.*, 2022).

Zhang *et al.*, 2013 extracted and purified polysaccharides from *A. villosum,* which exhibited inhibitory activity against the growth of HepG2 cancer cells. Additionally, it demonstrated potent free radical scavenging activities *in vitro*. In a mouse model of CCl4-induced liver injury, the polysaccharides significantly prevented the formation of malondialdehyde and boosted the activities of antioxidant enzymes.

Another study on *A. villosum* extracted polysaccharides, AVLP-2, effectively ameliorated oxidative stress in the gastric mucosa by enhancing the activity of superoxide dismutase and elevating glutathione levels, while concurrently suppressing the excessive production of malondialdehyde in tissues. Additionally, AVLP-2 treatment resulted in the downregulation of myeloperoxidase, IL-1β, IL-10 and NF-κB p65, whereas TNF-α levels were upregulated. These effects collectively reduced alcohol-induced inflammation (Liu *et al.*, 2021).

The extracts from *A. villosum* have also shown an interesting immunomodulatory effect on RAW 264.7 macrophages. AVPG-2, an acidic polysaccharide from *A. villosum,* induced the generation of NO, the secretion of IL-6 and TNF-α, and enhanced the phagocytic abilities of RAW 264.7 cells. AVPG-2 could also turn the polarization of macrophages to the M1 direction (Zhou *et al*., 2021, thus addressing the imbalance between pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages associated with RA (Jang *et al*., 2022).

3. AIM OF THE STUDY

Previous studies support the strong anti-inflammatory and antioxidant properties of *Amomum villosum* Lour. (Zhang *et al*., 2024; Gao *et al*., 2023). Furthermore, vanillic acid, a major phenolic component of *A. villosum*, has exhibited various anti-inflammatory and antioxidant properties, making it a beneficial compound against oxidative stress (Kim *et al.*, 2011). Oxidative stress can contribute to chronic inflammatory diseases such as rheumatoid arthritis by promoting inflammation and tissue damage (Djordjevic *et al.*, 2023).

While *A. villosum* has been predominantly studied using RAW 264.7 macrophage cell models, its effect on human dermal fibroblasts (HDF), crucial in wound healing, tissue repair, collagen production and regulating the inflammatory response (Li *et al*., 2022), remains unexplored. Additionally, various extraction methods employed on *A. villosum* have yielded distinct active compounds, thereby exhibiting divergent anti-inflammatory effects (Zhang *et al*., 2024; Lim *et al*., 2020; Chen *et al.*, 2018; Zhang *et al.*, 2013).

It is hypothesized that treatment with extracts of *A. villosum* could demonstrate antioxidant and anti-inflammatory effects by modulating the oxidative stress response and inflammatory pathways in RAW 264.7 and HDF. Specifically, it is expected that the extracts will inhibit pro-inflammatory cytokine secretion and promote cell viability and proliferation in both cell types.

The scientific aim of this thesis is to assess novel methods for studying water and methanol extracts from *Amomum villosum* Lour. More specifically, the aims were to:

- Evaluate their antioxidant and cytotoxic effects using cell models including RAW 264.7 macrophages and HDF.
- Establish a non-toxic dose using cell line cytotoxicity models to guide subsequent *in vitro* studies.
- Examine the effect of the extracted components against oxidative damage and inflammation.

This effort aims to gain deeper knowledge of *A. villosum* extracts with different extraction methods, potentially opening new therapeutic strategies to tackle chronic inflammatory diseases.

4. MATERIALS AND METHODS

4.1 REAGENTS

Dulbecco's modified Eagle's medium (DMEM) and phosphate buffered saline (PBS), fetal bovine serum (FBS, F9423), L-glutamine (G7513), vanillic acid (H36001), trolox (648471), dexamethasone (D1756), hydrogen peroxide solution (H1009) and trypsin-EDTA (59430C) were purchased from Sigma-Aldrich Co. LLC (St. Louis, Missouri, USA). Penicillin-streptomycin was purchased from Invitrogen Corporation (Carlsbad, California, USA). PrestoBlue (A13261) was purchased from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, US). Lipopolysaccharide (LPS) derived from *Escherichia coli* (Sigma-Aldrich, L2880) was dissolved in PBS to form a 1 mg/mL stock solution.

4.2 AMOMUM VILLOSUM LOUR.

The dried fruits of *A. villosum* Lour. were purchased from a local eastern Chinese medicine store.

Methanol extraction

Amomum villosum powder (10 mg) was sonicated in 20 mL of petroleum ether to disrupt cell structures and release bioactive compounds. Sonicate for 2 min with maximum frequency. The mixture was then incubated at +4°C overnight to remove non-polar compounds. To prevent polyphenol degradation, the bottle was shielded from light with aluminium foil. After defatting, the powder was allowed to evaporate in a hood. The remaining powder was sonicated at 10°C for 5 min, and the supernatant was separated. This process was repeated with 20 mL of methanol 3-4 times to collect approximately 100 mL of polyphenol solution. The solution was then concentrated using a rotary evaporator until 80% of the solvent evaporated. The concentrated solution was transferred to a glass bottle wrapped in foil and left in a vacuum chamber overnight for further evaporation until a dry gel formed (Figure 6).

Water extraction

50 mL of deionized water was added to 10 mg of dry powder in a short bottle. The mixture was sonicated in a 0°C water bath for 10 min. After sonication, the solution was transferred to a 50 mL falcon tube to separate the supernatant from the solution. The supernatant was transferred to a clean

short bottle, and the powder in the falcon tube was returned to the bottle containing the herbs. This process was repeated 3-4 times with an additional 50 mL of deionized water each time to collect approximately 200 mL of crude water extract solution. The solution was evaporated in a centrifugal evaporator at 35°C for 5 h until the solvent volume was halved. Subsequently, the solution was transferred to a 250 mL aluminium cup and frozen at -30°C overnight. The frozen solution was then placed in a freeze-dryer for 3 days to obtain freeze-dried powder (Figure 6).

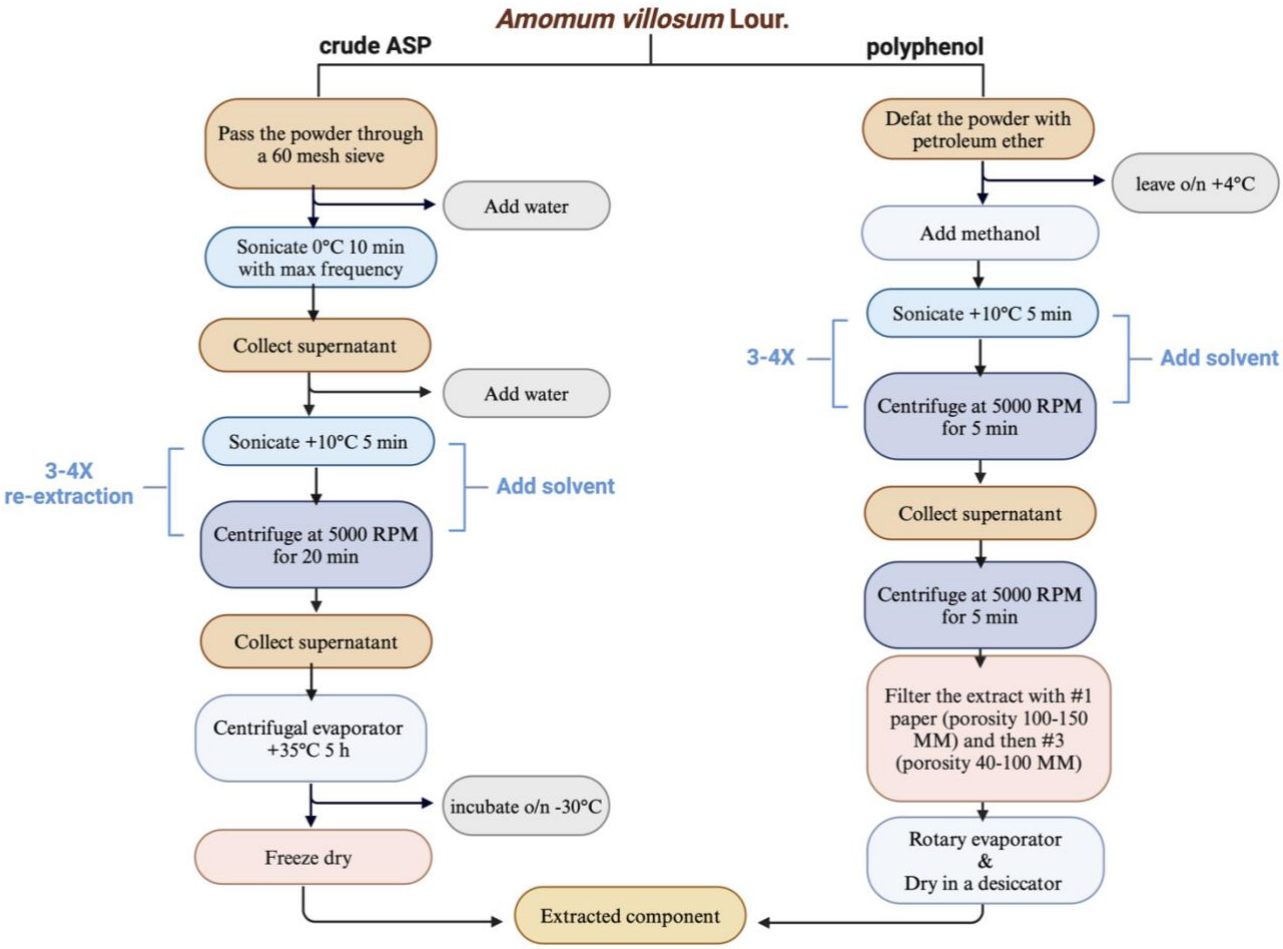

Figure 6. Extraction method of *Amomum villosum* Lour. (created with BioRender). ASP, *Amomum villosum* Lour. aqueous extract.

Nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC) were used to determine the constituents of water and methanol extract previously.

Vanillic acid served as a standard reference to guide our subsequent *in vitro* studies. The molarity of our extracts was determined as follows: The relative concentration of vanillic acid in our water and methanol extract was found to be 14.5% and 20.7%, respectively, as determined by HPLC

(Section 5.1). This corresponds to a dilution factor of 1:6.8 for the water extract and 1:4.8 for the methanol extract.

4.3 CELL LINES AND CELL CULTURE

The RAW 264.7 cell line (ATCC, TIB-71) and Human dermal fibroblasts (HDF) (ATCC, ATCPCS201012) were purchased from ATCC (Manassas, Virginia, USA).

Both RAW 264.7 macrophages cells and HDF were cultured in DMEM high-glucose medium, which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

4.4 CELL PROLIFERATION ASSAY

HDF and RAW 264.7 macrophages were seeded on a 96-well plate (Greiner bio-one cellstar, using a seeding density of 10,000 cells/well (HDF) and 8,000 cells/well (RAW 264.7)). The cells were then cultured for 4 h. To obtain a baseline for proliferation, PrestoBlue was added directly to the media, after 4 h incubation, to a final concentration of 1:10. We utilized a TECAN SPARK plate reader (TECAN, Männedorf, Switzerland) equipped with fluorescence capabilities and HT-FRET+GSM technology for our experiments. The plate was read at excitation/emission wavelengths of 540 nm and 610 nm. After this, the cells were treated with different concentrations of vanillic acid (12.5, 50, 100 and 200 µM). The plate was cultured for a further 24 h. For three days cell proliferation was assessed daily using PrestoBlue. The measured fluorescent signal was expressed as relative fluorescent units (RFU). For each measurement, the baseline signal of the measurement solution was established using an equivalent volume of medium without cells under the same experimental conditions. This baseline signal was then deducted from the signal recorded in the presence of cells to derive the signal specifically caused by the presence of the cells.

The effect of the extracted components on HDF cell proliferation was evaluated using the same method outlined previously. HDF cells were subjected to 12.5 µM (equivalent to vanillic acid) of water and methanol extract in place of vanillic acid.

4.5 H_2O_2-INDUCED OXIDATIVE STRESS

The chosen hydrogen peroxide (H_2O_2) concentration for our subsequent studies was determined with a titration experiment on RAW 264.7 macrophages. RAW 264.7 cells were seeded on a 96-well plate (Greiner bio-one cellstar, using a seeding density of 0,25 x 10^5 cells/well). The cells were then cultured for 24 h, treated with different concentrations of H_2O_2 (250, 500, 750 µM and 1mM) and cultured for a further 6 h. Cell viability was assessed using PrestoBlue which was added directly to the media to a final concentration of 1:10. The plate was read with our TECAN SPARK plate reader at excitation/emission wavelengths of 540 nm and 610 nm. The measured fluorescent signal was expressed as RFU. For each measurement, the baseline signal of the measurement solution was established using an equivalent volume of medium without cells under the same experimental conditions. This baseline signal was then deducted from the signal recorded in the presence of cells to derive the signal specifically caused by the presence of the cells.

The effect of vanillic acid on H_2O_2-induced oxidative stress on RAW 264.7 macrophages was assessed using the PrestoBlue assay described above. RAW 264.7 cells were seeded on a 96-well plate (Greiner bio-one cellstar, using a seeding density of 0,25 x 10^5 cells/well). The cells were then cultured for 24 h. Cells were pre-treated with varying concentrations of vanillic acid (12.5, 50, 100 and 200 µM) or 750 µM of H_2O_2 alone. A concentration of 200 µM of trolox served as a positive control. Following a 10-min incubation period, 750 µM of H_2O_2 was added to both the vanillic acid-treated group and the positive control group. The cells were then cultured for an additional 6 h. Cell viability was assessed using the TECAN SPARK plate reader.

The same experiment was conducted on HDF using the method described above. HDF cells were seeded on a 24-well plate (Falcon, using a seeding density of 2,5 x 10^5 cells/mL). Following treatment, HDF cells were cultured for 16 h, a duration longer than that used for RAW 264.7 cells (6 h). Cell viability was assessed using the Trypan Blue assay, which relies on the principle that live cells have an intact cell membrane that excludes the negatively charged dye. Specifically, an aliquot of collected cells (10 µl) was mixed with an equal volume of Trypan Blue (10 µl), and subsequently, the numbers of blue (dead) and white (live) cells were counted. The cell counting will be determined by optical density.

The impact of the extracted components from *A. villosum* on cell viability under H_2O_2-induced oxidative stress was assessed using the PrestoBlue assay. RAW 264.7 and HDF cells were exposed

to various concentrations of water and methanol extract (3.135, 6.35, 12.5, 25 and 50 µM equivalent to vanillic acid) instead of vanillic acid.

4.6 LPS-INDUCED CELL CYTOTOXICITY

The chosen LPS concentration for our subsequent studies was determined with a titration experiment by using the PrestoBlue assay to evaluate RAW 264.7 cell viability with LPS stimulation. RAW 264.7 macrophages were seeded on a 96-well plate (Greiner bio-one cellstar, using a seeding density of 0,25 x 10^5 cells/well). The cells were then cultured for 24 h, treated with different concentrations of LPS (0.1, 0.5 and 1.0 µg/mL) and cultured for a further 48 h, after which cell viability was determined using the TECAN SPARK plate reader.

The effect of vanillic acid on LPS-induced cytotoxicity was assessed using the PrestoBlue assay. RAW 264.7 macrophages were seeded on a 96-well plate (Greiner bio-one cellstar, using a seeding density of 0,25 x 10^5 cells/well). Cells were pre-treated with various concentrations of vanillic acid (12.5, 50, 100 and 200 µM) or 1.0 µg/mL of LPS alone. After a 1 h incubation period, 1.0 µg/mL of LPS was added, and the cells were cultured for an additional 48 h. Cell viability was subsequently assessed using the TECAN SPARK plate reader. A concentration of 1 µM of dexamethasone served as a positive control in the experiment.

The impact of the extracted components from *A. villosum* on LPS-induced cytotoxicity in RAW 264.7 cells was assessed using the PrestoBlue assay, employing the identical method delineated previously. Instead of vanillic acid, RAW 264.7 cells were subjected to different concentrations of water and methanol extract (3.135, 6.35, 12.5, 25 and 50 µM equivalent to vanillic acid).

4.7 IMMUNOCYTOCHEMISTRY

The production of IL-6 and NF-κB in RAW 264.7 macrophages after exposure to LPS, along with the impact of vanillic acid and water extract from *A. villosum,* was evaluated using immunocytochemistry. RAW 264.7 cells were cultured in cell culture flasks and subsequently seeded onto coverslips in a 12-well plate (Corning, Costar) at a seeding density of 0.25 x 10^5 cells/well. After 24 h incubation, the cells were treated with various concentrations of vanillic acid (12.5, 50, 100 and 200 µM) or 50 ng/mL of LPS alone, along with a positive control group treated with 1 µM of dexamethasone. After a 1 h incubation, 50 ng/mL of LPS was added to both the vanillic acid-

treated group and the positive control group. The cells were then cultured for a further 48 h. The cells were fixed by adding 4% freshly prepared paraformaldehyde (PFA) and incubating for 20 min at room temperature. Subsequently, the coverslips were removed and subjected to staining with either recombinant anti-IL-6 polyclonal primary antibody (bs-0781R, Biossusa, Woburn, Massachusetts, United States) or anti-phospho-NF-κB p65 ($pSer^{276}$) antibody (SAB4504488, Sigma-Aldrich) in a ratio of 1 µL of primary antibody to 50 µL of antibody diluent (1% BSA, 0.5% triton-X 100, 0.01M PBS) for 2 h, followed by another 30 min incubation period with Alexa Fluor 594-conjugated anti-rabbit secondary antibody (A11012, Thermo Fisher Scientific) in a ratio of 1 µL of secondary antibody in 200 µL of antibody diluent. Tris-buffered saline with Tween 20 (TBST) was used in between and after staining and fixing. Following this, the coverslip in each sample was mounted on the microscopic slide using Glycerol Mounting Medium with DAPI and DABCO (Abcam, ab188804), and was imaged using the ZEISS Axioscope fluorescence microscope (Carl Zeiss AG, Germany.). The quantification of the IL-6 and NF-κB intensity density was measured and normalised to cell count using the software Fiji ImageJ and bar charts were constructed using GraphPad Prism.

The same method was then repeated by treating the cells with different concentrations of water extract (3.135, 6.35, 12.5, 25 and 50 µM equivalent to vanillic acid).

4.8 STATISTICAL ANALYSIS

All experiments were conducted in at least three independent experiments and each treatment group was replicated three times. All results were shown as mean value ± standard deviation that was determined for quantitative analyses. Statistical tests as indicated were performed using Prism 7 (Graph Pad) software (V9) while one-way ANOVA for multiple comparisons was with the Tukey post hoc test. In addition, an unpaired parametric two-tailed t-test was performed for the proliferation assay. Statistical significance was accepted at $p<0.05$.

5. RESULTS

5.1 *AMOMUM VILLOSUM* LOUR. EXTRACTS CONTAIN TERPENOIDS, VANILLIC ACID AND OTHER POLYPHENOLS

Amomum villosum Lour. has been shown to contain multiple active chemical components including volatile oils like terpenoids, phenolic compounds, polysaccharides and saponins (Zhang *et al*., 2024; Zeng *et al*., 2022). The essential phenolic components include polyphenols such as vanillic acid and quercetin (Zhang *et al*., 2024). Four compounds were identified in our extracts using nuclear magnetic resonance (NMR) retention time data. These include bornyl acetate (Figures 7B & 8B), vanillic acid (Figures 9B & 10B), quercetin (Figures 9C & 10C) and tannic acid (Figures 9D & 10D).

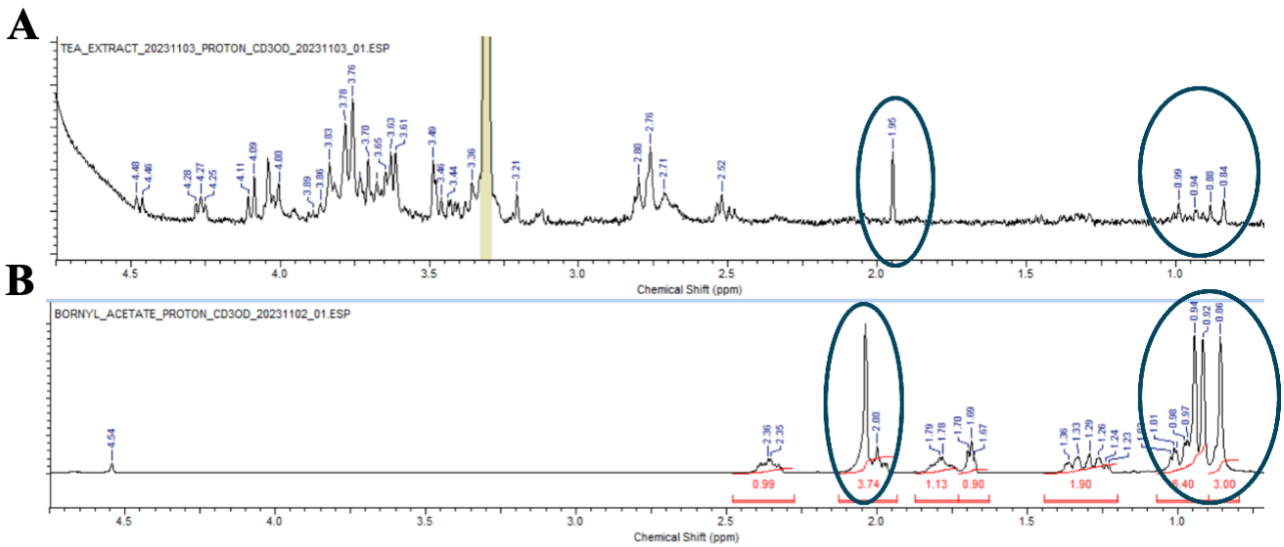

Figure 7. A. Characterization of water extract from *A. villosum* with nuclear magnetic resonance NMR spectrum. **B.** NMR for bornyl acetate standard. Chemical shifts 'x' are reported in parts per million (ppm), and the blue circles indicate the chemical shift.

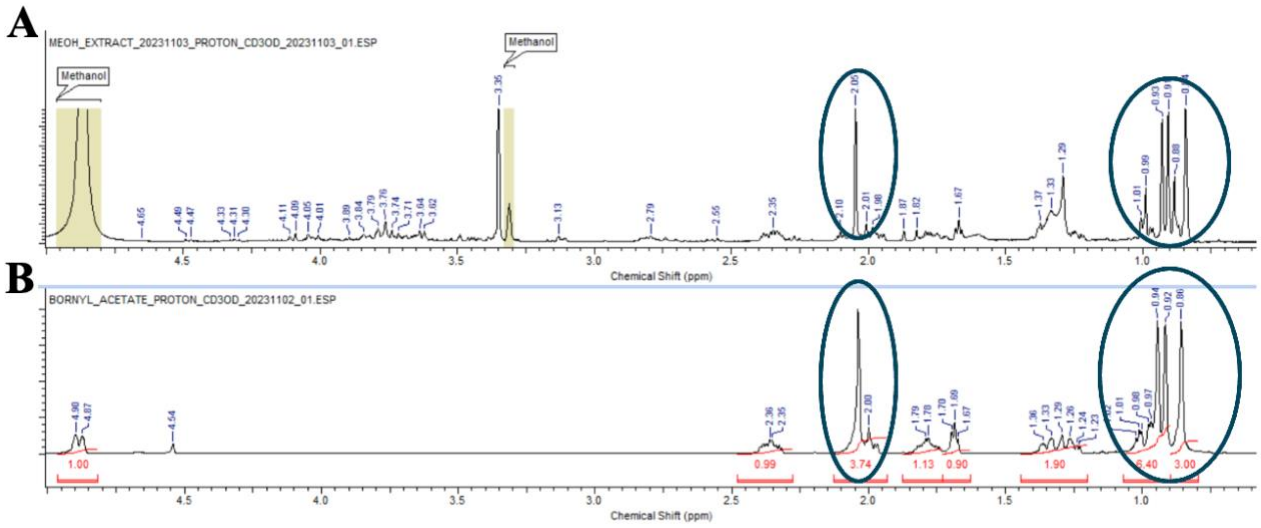

Figure 8. A. Characterization of methanol extract from *A. villosum* with nuclear magnetic resonance (NMR) spectrum. **B.** NMR for bornyl acetate standard. Chemical shifts 'x' are reported in parts per million (ppm), and the blue circles indicate the chemical shift.

We identified vanillic acid by observing the elution of the compound at approximately 15 min (Figures 11 & 12). Upon spiking the sample, the chromatogram reveals an increase in the peak corresponding to vanillic acid. Subsequent analysis revealed that the water extract contains approximately 14.5% vanillic acid compared to the 20.7% methanol extract. This calculation was derived by dividing the peak area of vanillic acid by the total peak area. Further verification for other components was not conducted. The evaluation of our extracts was done in collaboration with Kaiser Hamid, Colin Duke and Wilson Tran from Sydney Pharmacy School, Faculty of Medicine and Health.

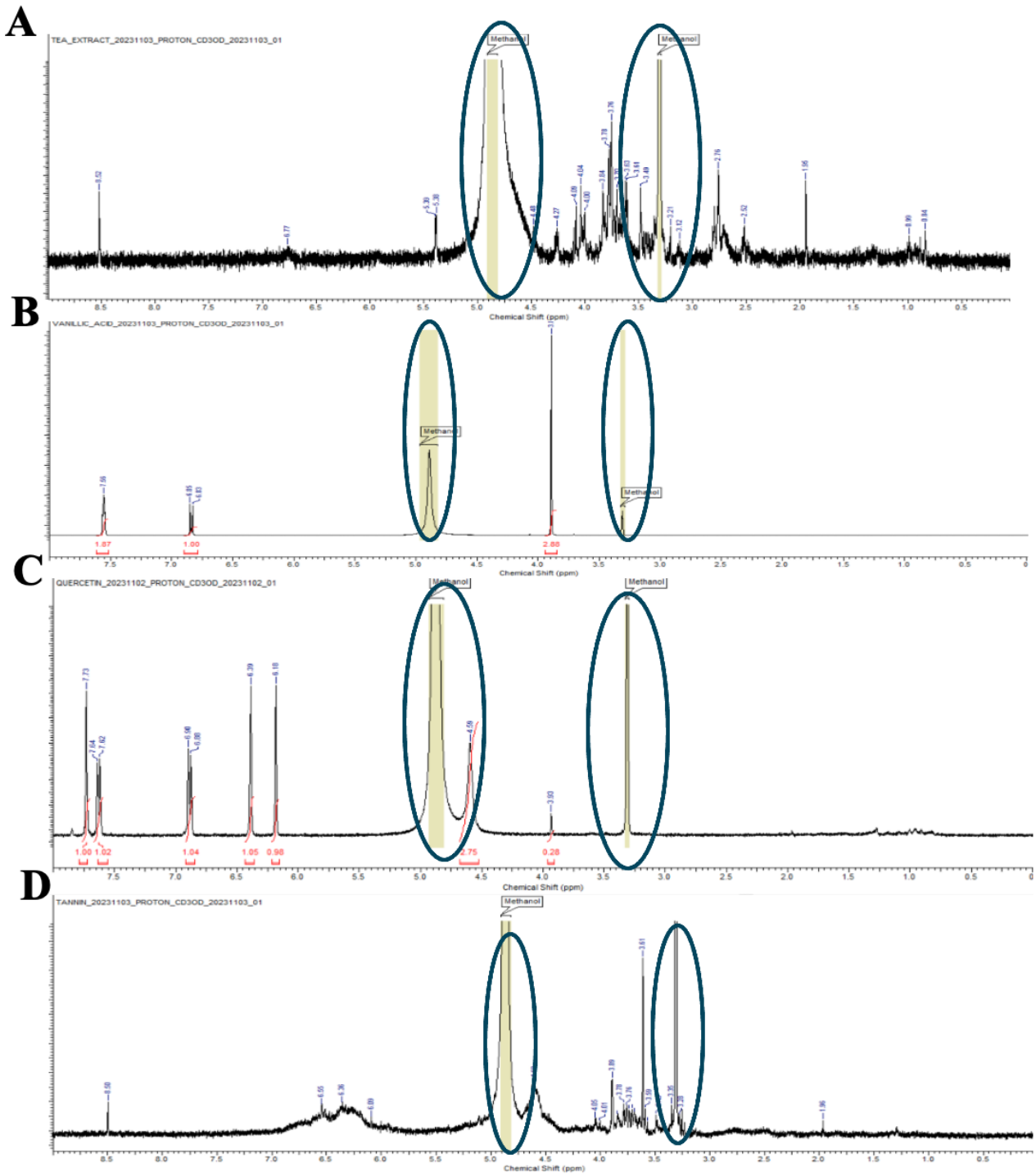

Figure 9. A. Characterization of water extract from *A. villosum* with nuclear magnetic resonance (NMR) spectrum. **B.** NMR for vanillic acid standard. **C.** NMR for quercetin standard. **D.** NMR for tannic acid standard. Chemical shifts 'x' are reported in parts per million (ppm), and the blue circles indicate the chemical shift.

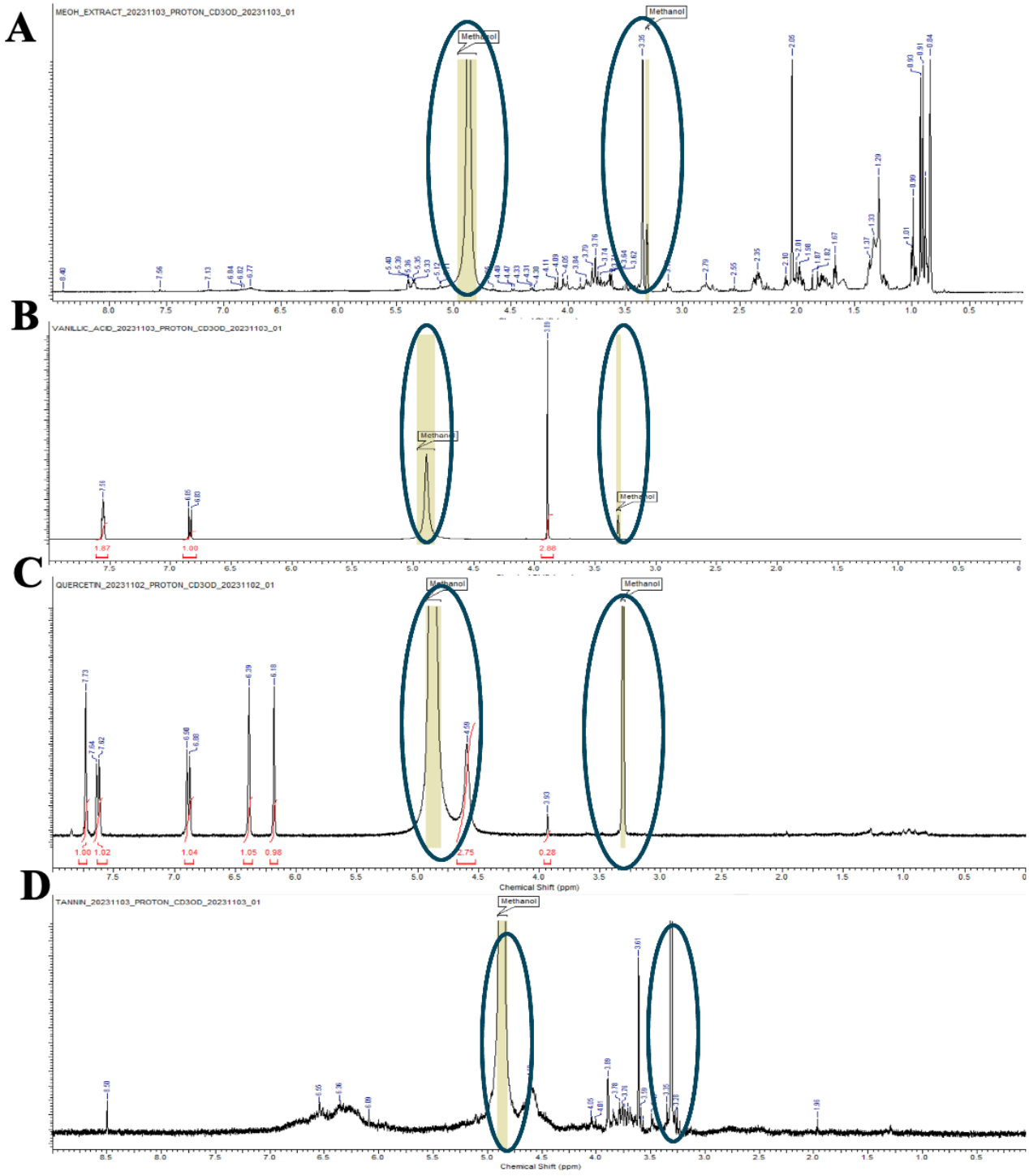

Figure 10. A. Characterization of methanol extract from *A. villosum* with nuclear magnetic resonance (NMR) spectrum. **B.** NMR for vanillic acid standard. **C.** NMR for quercetin standard. **D.** NMR for tannic acid standard. Chemical shifts 'x' are reported in parts per million (ppm), and the blue circles indicate the chemical shift.

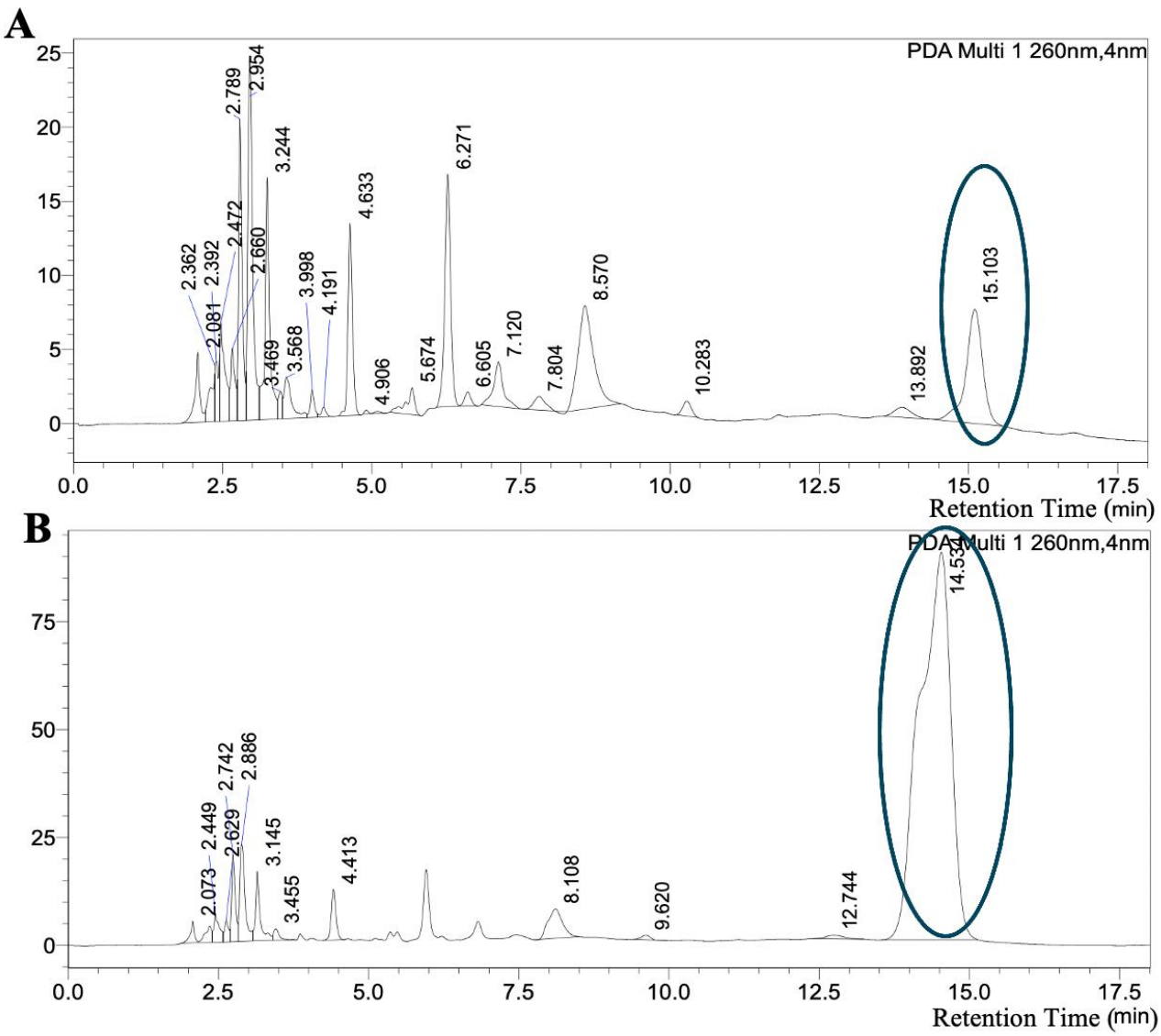

Figure 11. A. HPLC chromatogram of water extract from *A. villosum*. **B.** HPLC chromatogram of water extract from *A. villosum* with a spike for vanillic acid. Retention time (min) 'x' represents water extract A. and vanillic acid B. Blue circles correspond to retention times A. 15.103 and B. 14.534, respectively.

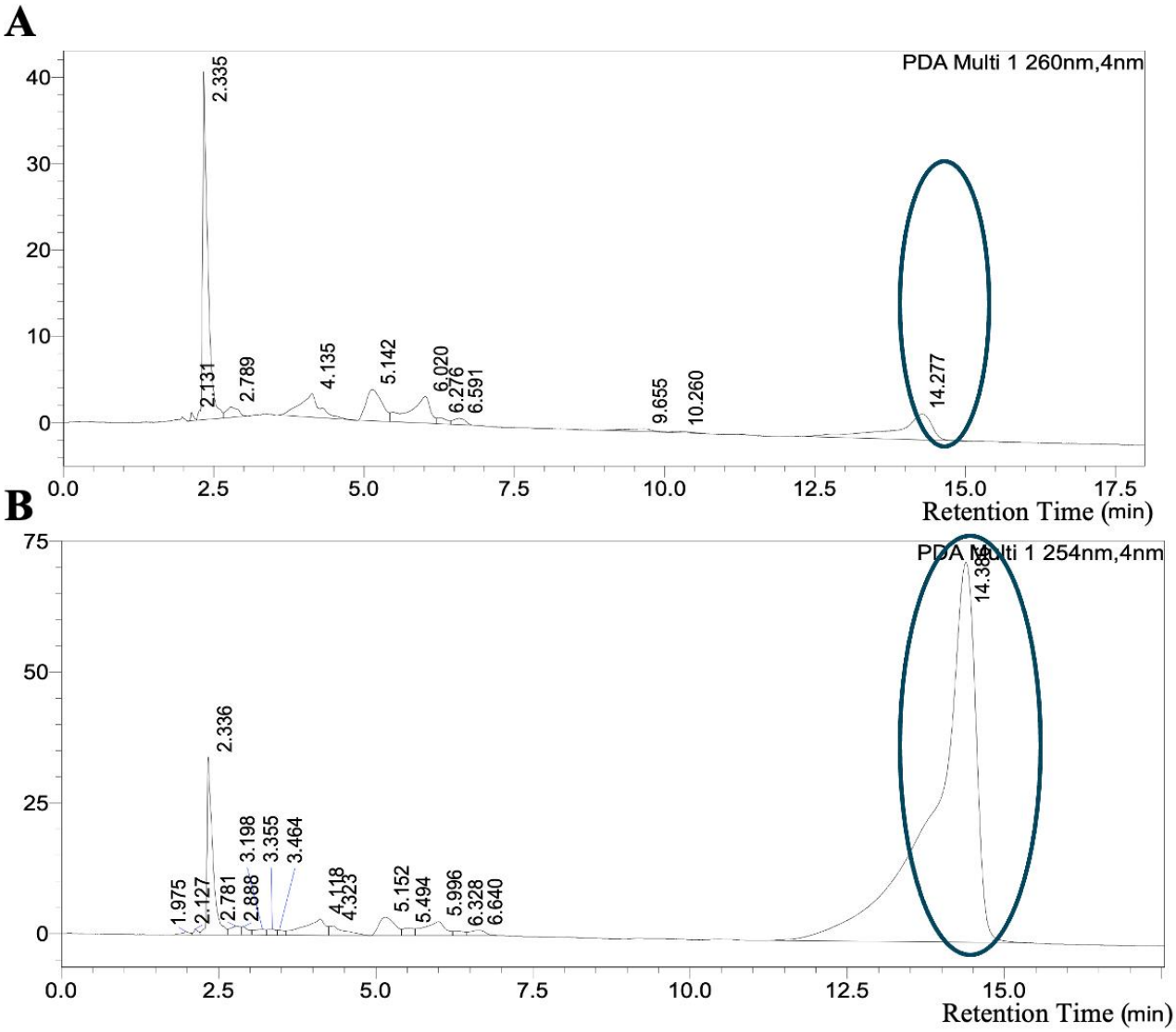

Figure 12. A. HPLC chromatogram of methanol extract of *A. villosum*. **B.** HPLC chromatogram of methanol extract of *A. villosum* with spike for vanillic acid. Retention time (min) 'x' represents methanol extract A. and vanillic acid B. Blue circles correspond to retention times A. 14.277 and B. 14.386, respectively.

5.2 VANILLIC ACID AND EXTRACTS OF *AMOMUM VILLOSUM* LOUR. ENHANCE HDF PROLIFERATION

To investigate the effect of vanillic acid on the proliferation of HDF and RAW 264.7 macrophages, PrestoBlue assay was performed using these cells treated with different concentrations of vanillic acid (12.5, 50, 100 and 200 µM) for 0, 24, 48 and 72 h, to develop our method and obtain a baseline for the extracts. HDF showed exponential cell proliferation from 0 to 72 h in all groups. However, the proliferation rate of HDF significantly increased between 0 to 24 h and 48 to 72 h post-treatment with vanillic acid (Figure 13A, C & D). Additionally, the results showed that at 12.5, 50 and 200 µM of vanillic acid promoted the proliferation of HDF significantly at 72 h compared to the control group

p<0.01 (12.5 and 50 µM) and p<0.001 (200 µM) (Figure 13D). No toxicity of vanillic acid at (12.5-200 µM, 0 – 72 h) on HDF or RAW 264.7 macrophages was observed (Figure 13A & 15A). 12.5 µM of vanillic acid was chosen to guide subsequent *in vitro* experiments.

Furthermore, HDF were treated with 12.5 µM (equivalent to vanillic acid) of water and methanol extracts for 0, 24, 48 and 72 h. The proliferation rate of HDF increased from 0 to 24 h, attenuated between 24 to 48 h, and then increased significantly between 48 to 72 h post-treatment. It was observed that treatment with both 12.5 µM of water and methanol extracts for 72 h significantly induced HDF proliferation compared to the control group, with the methanol extract exhibiting a stronger effect (p<0.01) (Figure 14A). No toxicity of water and methanol extracts at 12.5 µM was observed at 0 to 72 h (Figure 14A).

The effects of vanillic acid on RAW 264.7 macrophage cell proliferation are shown in Figure 15. No significant effect (p<0.05) on RAW 264.7 proliferation was noticed at 0 to 72 h compared to the control group. The results of three repeated and four individual experiments were similar (Figure 15B, C & D)

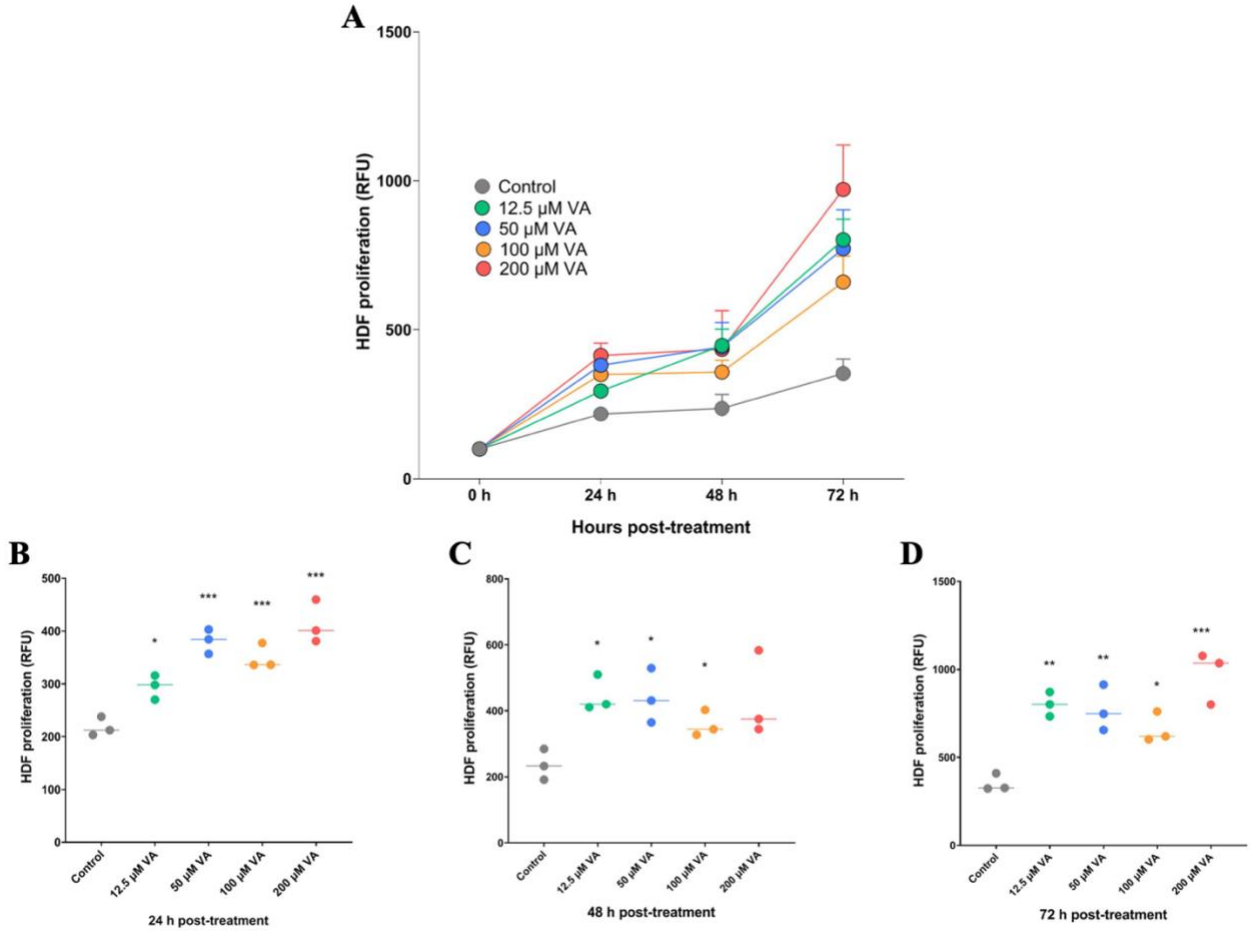

Figure 13. The effect of vanillic acid treatment on HDF cell proliferation. **A.** Cell proliferation rate of HDF cells treated with concentration gradient (12.5–200 µM) of vanillic acid (VA) for 0, 24, 48 and 72 h. **B.** 24 h

post-treatment. **C.** 42 h post-treatment. **D.** 72 h post-treatment. RFU: relative fluorescence units. Data are presented as mean ± standard deviation of three repeated and independent experiments. * Different from the Control group. *p<0.05, **p<0.01 and ***p<0.001.

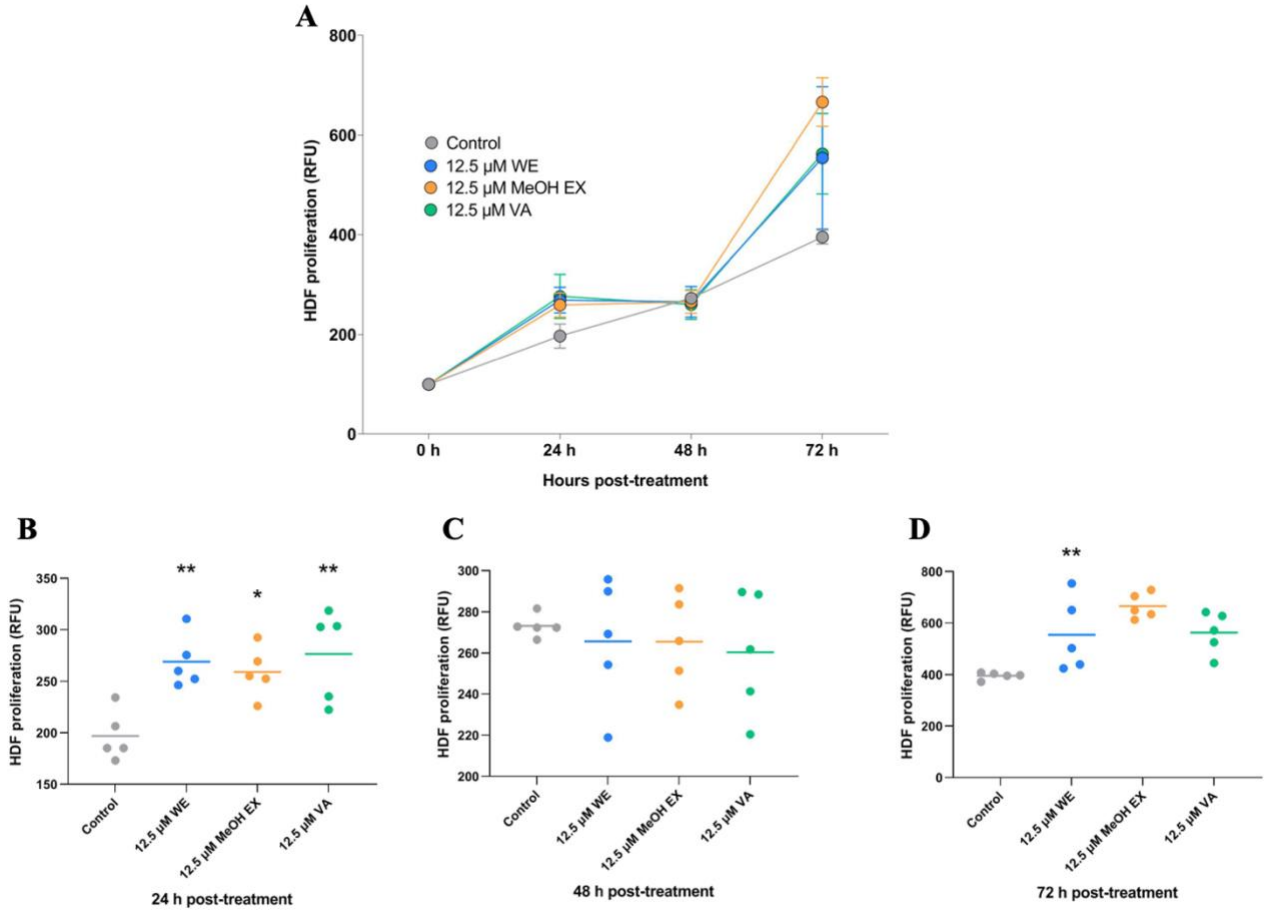

Figure 14. The effect of treating HDF with *A. villosum* extracts on cell proliferation. **A.** Cell proliferation rate of HDF cells treated with 12.5 µM (equivalent to vanillic acid (VA)) of water extract (WE) and methanol extract (MeOH EX) for 0, 24, 48 and 72 h. **B.** 24 h post-treatment. **C.** 42 h post-treatment. **D.** 72 h post-treatment. RFU: relative fluorescence units. Data are presented as mean ± standard deviation of three repeated and five independent experiments. * Different from the Control group. *p<0.05, **p<0.01 and ***p<0.001.

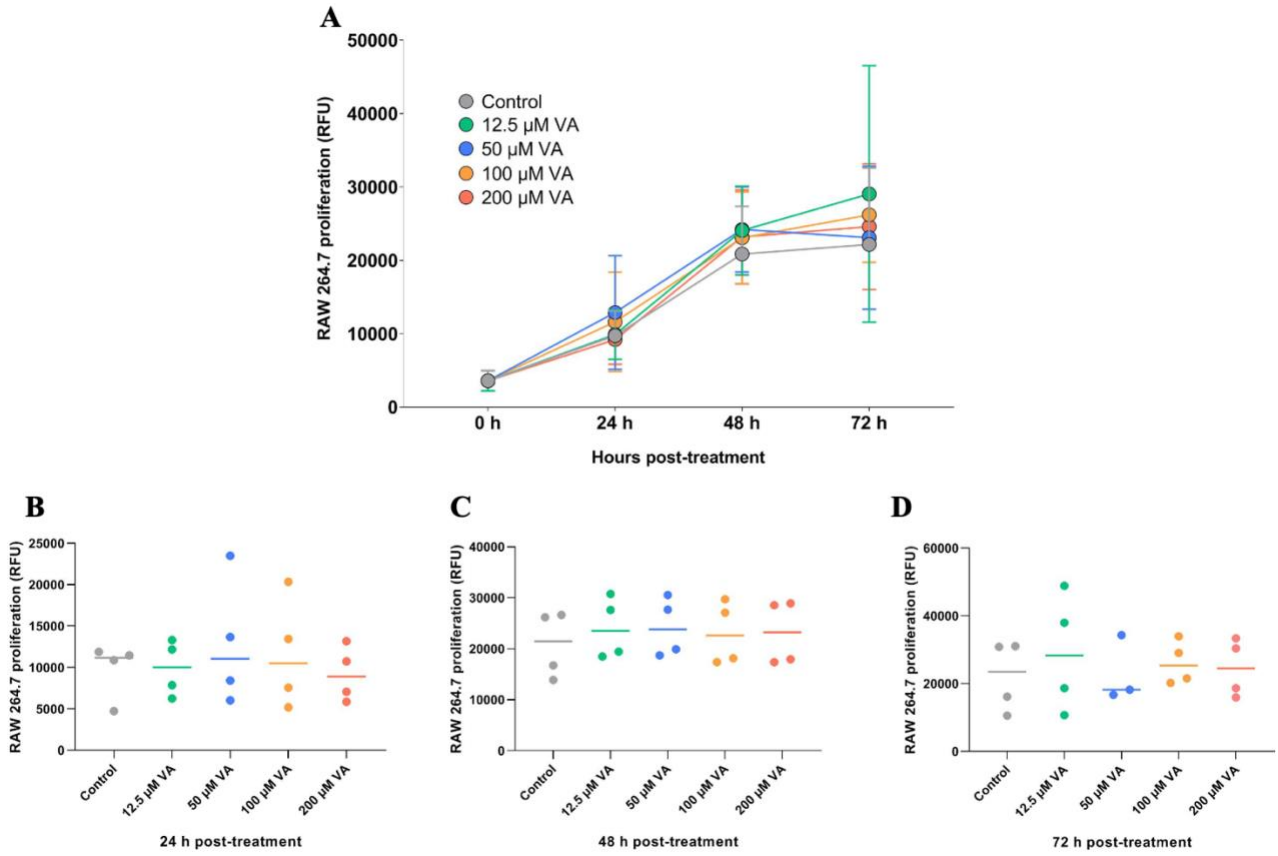

Figure 15. The effect of vanillic acid treatment on RAW 264.7 cell proliferation. **A.** Cell proliferation rate of RAW 264.7 macrophages treated with concentration gradient (12.5–200 µM) of vanillic acid (VA) for 0, 24, 48 and 72 h. **B.** 24 h post-treatment. **C.** 42 h post-treatment. **D.** 72 h post-treatment. RFU: relative fluorescence units. Data are presented as mean ± standard deviation of three repeated and four independent experiments. * Different from the Control group. *p<0.05, **p<0.01 and ***p<0.001.

5.3 WATER AND METHANOL EXTRACTS FROM *AMOMUM VILLOSUM* LOUR. PROTECT CELLS FROM H_2O_2-INDUCED OXIDATIVE STRESS

The determination of a maximum tolerated concentration of H_2O_2-induced oxidative stress at (250, 500, 750 µM and 1mM) for 6 h was evaluated by measuring the cell viability of RAW 264.7 macrophages using PrestoBlue assay (Figure 16A). PrestoBlue assesses cell viability by detecting the reducing environment of live cells, causing the reagent to turn red and become highly fluorescent. This colour change can be detected using fluorescence or absorbance measurements. H_2O_2 decreased cell viability in a dose-dependent manner. The results showed that the cell viability of cells subjected to H_2O_2 at 750 µM was diminished by 26.8%, compared with the control group. This concentration was therefore chosen for subsequent *in vitro* experiments. Our previously conducted experiments have shown that the maximum tolerated concentration for HDF cells is similar to that of RAW 264.7

cells, with 1mM resulting in approximately 35% (± 10% SD) decrease in cell viability (data not shown).

Amomum villosum Lour. as a medicinal plant can display various biological activities, including anti-inflammatory and antioxidant activity (Zhang *et al*., 2024). After determining a suitable concentration of H_2O_2, we developed our method and investigated the protective impacts of vanillic acid, as well as our water and methanol extracts on the viability of RAW 264.7 and HDF cells. This was achieved by inducing oxidative stress with H_2O_2 stimulation to establish a baseline with vanillic acid for subsequent treatment with our water and methanol extracts. Cells were pre-treated with various concentrations of vanillic acid (12.5, 50, 100 and 200 µM) for 10 min after which H_2O_2 was added and incubated for either 6 h (RAW 264.7) or 16 h (HDF) (Figure 16B & 17A). No significant results (p<0.05) were observed with RAW 264.7 cells, although vanillic acid demonstrated some protection against H_2O_2-induced oxidative stress (Figure 16B). Conversely, treatment with 12.5 µM of vanillic acid exhibited the highest level of protection on HDF against H_2O_2-induced oxidative stress (p<0.05) (Figure 17B). 12.5 µM of vanillic acid was therefore chosen for the subsequent *in vitro* experiments. Trolox, a synthetic derivative of vitamin E commonly used as an antioxidant in scientific research, was used as a positive control giving a significant p-value of 0.01 (Figure 17B). The concentration of trolox was determined based on previous research that utilized similar concentrations as an antioxidative agent (Roh *et al.,* 2013).

To evaluate the protective effect of *A. villosum* extracts, we examined the cell viability rate that was pre-protected with water and methanol extracts (3.125, 6.25, 12.5, 25 and 50 µM equivalent to vanillic acid) for 10 min before H_2O_2 stimulation. The cells were further incubated for either 16 h (HDF) or 6 h (RAW 264.7 macrophages) (Figure 16C & 17B). As you can see from Figure 16C, 3.125 µM of water extract (p<0.05), 6.25 µM of methanol extract (p<0.01) and 12.5 µM of vanillic acid (p<0.001), protected RAW 264.7 cells from H_2O_2-induced oxidative stress compared to the group treated with H_2O_2 only. For that of HDF, the water extract at concentrations ranging from 3.125 to 25 µM showed significant protection. Similarly, the methanol extract at concentrations ranging from 3.125 to 12.5 µM demonstrated protective effects in HDF cells (Figure 17C). These results were consistent across three repeated and/or six individual experiments.

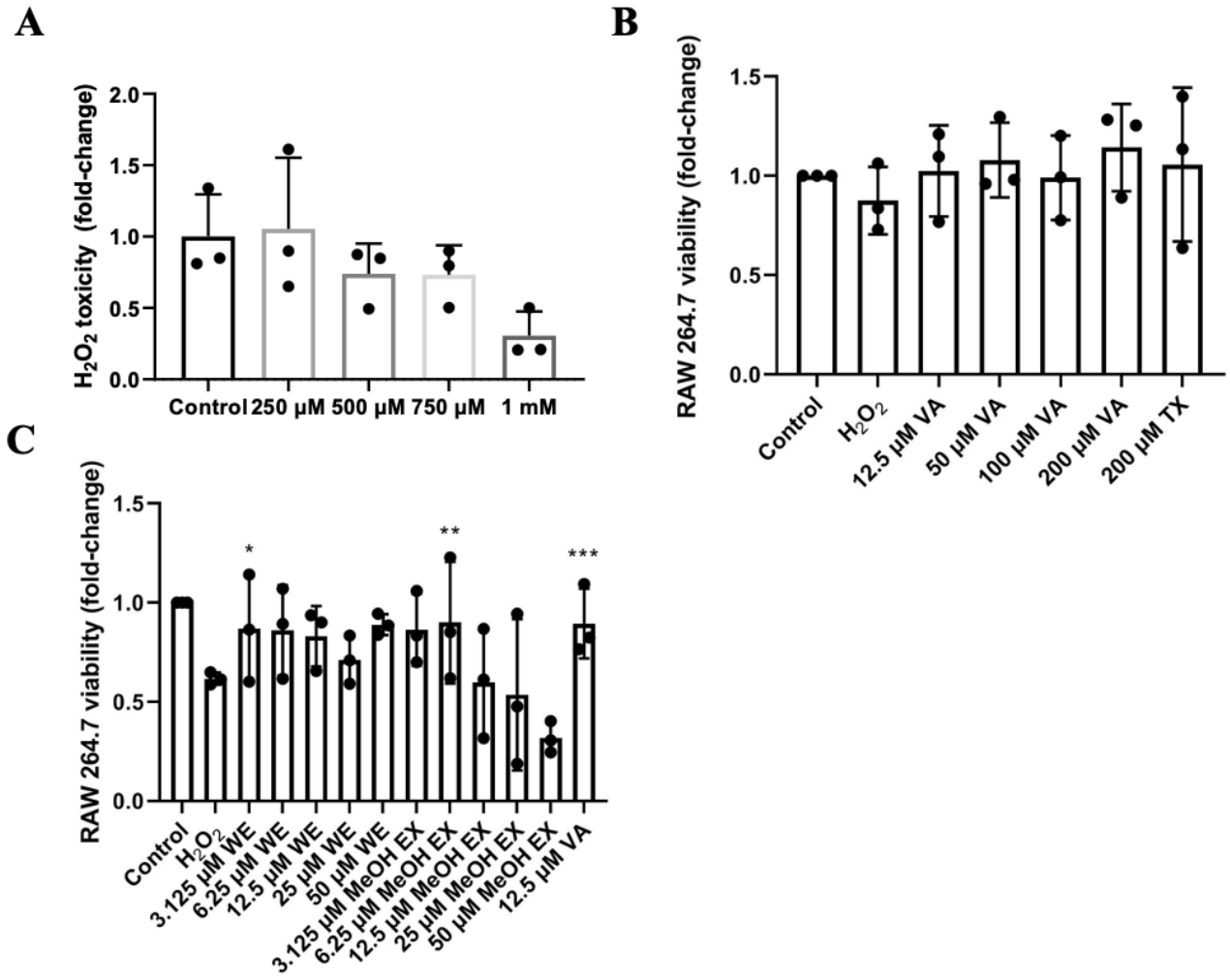

Figure 16. Antioxidant activity detection of compounds extracted from *A. villosum*. **A.** Cell viability rate of RAW 264.7 cells treated with concentration gradient (250 µM–1.0 mM) of hydrogen peroxide (H_2O_2) for 6 h. **B.** Cell viability rate of RAW 264.7 cells treated with concentration gradient (12.5–200 µM) of vanillic acid (VA) for 10 min after which H_2O_2 was added. Cell viability measured after 6 h. TX: trolox was used as a positive control. **C.** Inhibitory effect (3.125–50 µM equivalent to VA) of water extract (WE) and methanol extract (MeOH EX) in H_2O_2-induced RAW 264.7 cells. VA was used as a positive control. Data are presented as mean ± standard deaviation of three repeated and independent experiments. * Different from the H_2O_2 group. *p<0.05, **p<0.01, ***p<0.001.

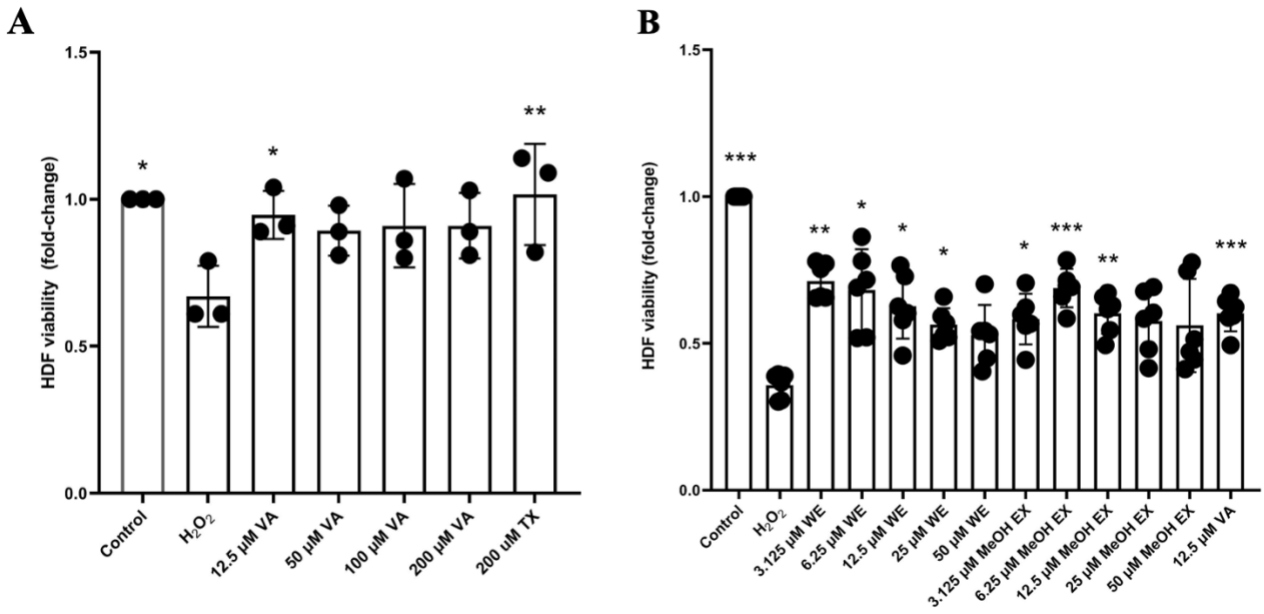

Figure 17. Antioxidant activity detection of compounds extracted from *A. villosum*. **A.** Cell viability rate of HDF after 16 h, cells treated with concentration gradient (12.5–200 µM) of vanillic acid (VA) for 10 min after which hydrogen peroxide (H_2O_2) was added. TX: trolox was used as a positive control. **B.** Inhibitory effect (3.125–50 µM equivalent to VA) of water extract (WE) and methanol extract (MeOH EX) in H_2O_2-induced HDF cells. VA was used as a positive control. Data are presented as mean ± standard deviation of three repeated and/or six independent experiments. * Different from the H_2O_2 group. *p<0.05, **p<0.01 and ***p<0.001.

5.4 THE ANTI-APOPTOTIC EFFECT OF VANILLIC ACID AND *AMOMUM VILLOSUM* LOUR. EXTRACTS ON LPS-INDUCED CELL TOXICITY

First, we evaluated the maximum tolerated concentration of LPS (0.1–1.0 µg/mL) by assessing the viability of RAW 264.7 macrophages using the PrestoBlue assay (Figure 18A). LPS exhibited a dose-dependent reduction in cell viability, with 1.0 µg/mL resulting in a 42.8% decrease compared to untreated cells (Figure 18A). Consequently, the concentration of 1.0 µg/mL was selected for subsequent *in vitro* experiments.

Following the determination of the optimal LPS concentration, we examined the anti-apoptotic effects of vanillic acid, as well as our water and methanol extracts, on RAW 264.7 cell viability. This was achieved by inducing cytotoxicity with LPS stimulation to establish a baseline with vanillic acid for subsequent treatment with our water and methanol extracts. Cells were pre-treated with various concentrations of vanillic acid (12.5, 50, 100 and 200 µM) for 1 h, followed by the addition of LPS and further incubation for 48 h (Figure 18B). According to the results shown in Figure 18B, LPS-induced cytotoxicity was most effectively reduced by 100 and 200 µM of vanillic acid. Dexamethasone (1 µM), a synthetic corticosteroid commonly used as an anti-inflammatory

agent, served as a positive control and exhibited similar effects to those observed with 200 µM of vanillic acid (Figure 18B). To determine the concentration of dexamethasone we referred to previous research by Chuang *et al.,* 2017 where similar concentrations of dexamethasone were employed as an anti-inflammatory agent.

Subsequently, we investigated the protective effect of *A. villosum* extracts on LPS-induced RAW 264.7 macrophages. Cell viability was assessed after pre-treatment with varying concentrations of water and methanol extracts (3.125, 6.25, 12.5, 25 and 50 µM equivalent to vanillic acid) for 1 h prior to LPS stimulation, followed by 48 h of incubation (Figure 18C). As illustrated in Figure 18C, no significant protective effects were observed. However, 25 and 50 µM of water extract demonstrated cytotoxic effects on RAW 264.7 macrophages as shown in Figure 18C.

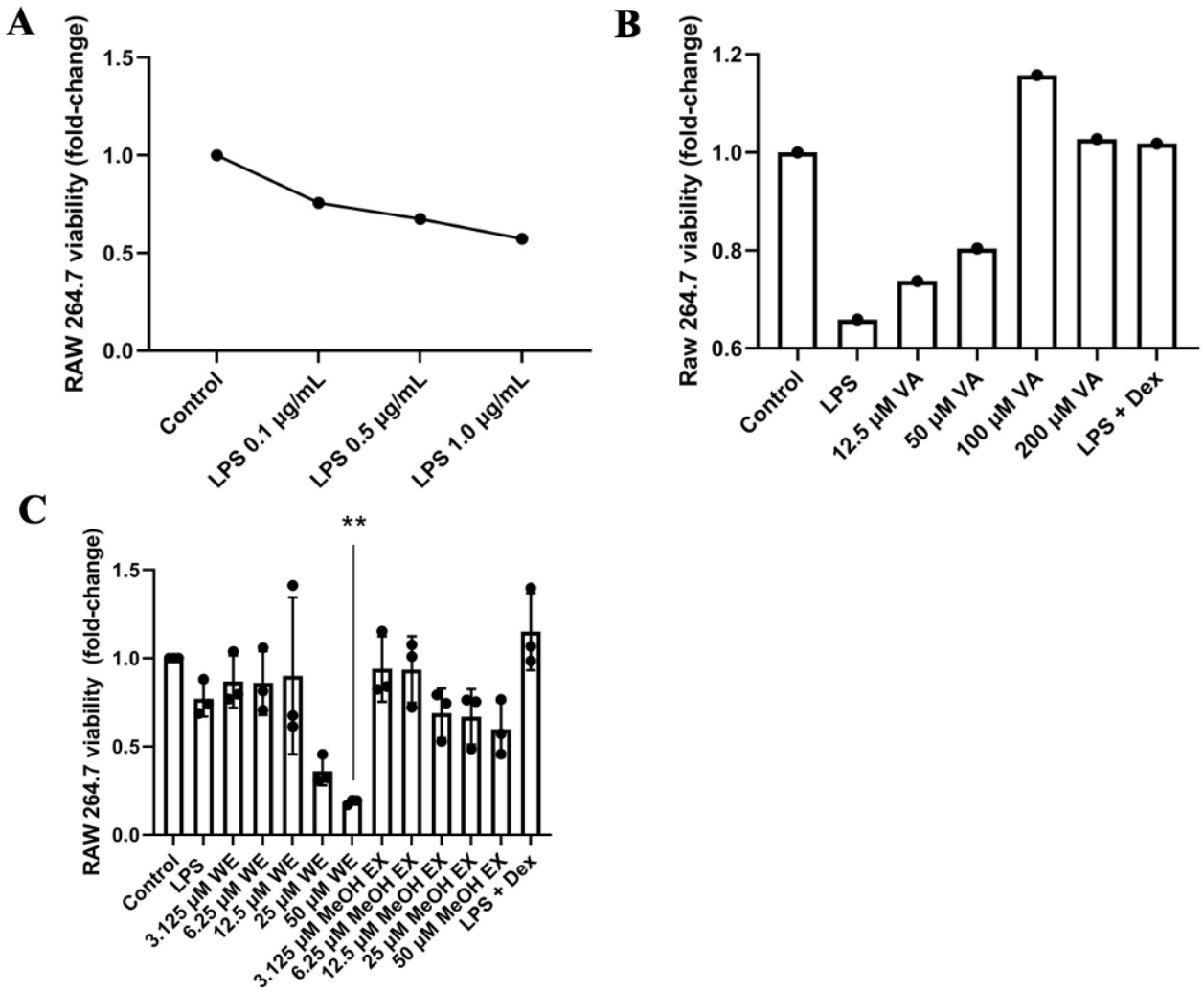

Figure 18. Anti-inflammatory activity detection of compounds extracted from *A. villosum*. **A.** Cell viability rate of RAW 264.7 cells treated with concentration gradient (0.1–1.0 µg/mL) of Lipopolysaccharide (LPS) for 48 h. **B.** Cell viability rate of RAW 264.7 cells treated with concentration gradient (12.5–200 µM) of vanillic acid (VA) for 1h before LPS stimulation, with further 48 h incubation in the presence of LPS (1.0 µg/mL) (n=1). Dex: dexamethasone was used as a positive control. **C.** Inhibitory effect (3.125–50 µM equivalent to

VA) of water extract (WE) and methanol extract (ME) in LPS-induced RAW 264.7 cells. Data are presented as mean ± standard deviation of three repeated and independent experiments. * Different from the LPS group. **p<0.01.

5.5 THE ANTI-INFLAMMATORY ACTIVITY OF VANILLIC ACID AND *AMOMUM VILLOSUM* LOUR. EXTRACTS ON LPS-STIMULATED RAW 264.7 MACROPHAGES

IL-6 and NF-κB are key mediators of inflammation. Furthermore, these two are involved in the pathogenesis of RA (Smolen *et al.,* 2018). Therefore, we aimed to examine the effect of our water extract treatment on LPS-induced inflammatory cytokines on IL-6 and NF-κB. Firstly, vanillic acid was used for method development and to guide subsequent *in vitro* studies. Each data point is presented as one field of view, we therefore formed additional independent experiments to confirm our findings.

The effect of vanillic acid on LPS-induced RAW 264.7 macrophage cytokine production is seen in Figure 19A. Cells were pre-treated with various concentrations of vanillic acid (12.5, 50, 100 and 200 μM) for 1 h, followed by the addition of 50 ng/mL of LPS and further incubation for 48 h. After this cells were fixed with PFA on coverslips and subjected to staining with either anti-IL-6 or anti-phospho-NF-κB p65 antibody. Treatment of RAW 264.7 cells with LPS alone resulted in significant increases in cytokine production compared to unstimulated cells (Figure 19A). In contrast, the significantly high levels of IL-6 and NF-κB in RAW 264.7 macrophages exposed to LPS were decreased in all vanillic acid pre-treatment groups alongside a positive control group treated with 1 μM dexamethasone (Figure 19A & 20). Moreover, 100 μM of vanillic acid demonstrated the highest effect when compared to the cells treated with LPS only (Figure 19A & 20).

The results illustrated in Figure 19B depict the effects of the water extract on the LPS-induced RAW 264.7 macrophage inflammatory factor IL-6. Water extract treatment at concentrations of 3.125, 6.25 and 50 μM (equivalent to vanillic acid) exhibited increased IL-6 production in RAW 264.7 macrophages compared to the cells treated with LPS only. However, at a concentration of 25 μM, the water extract attenuated IL-6 production significantly (p<0.05) (Figure 19B & 21).

Researchers have confirmed that the NF-κB signalling pathway participates in LPS-induced inflammatory injury (Kim *et al.*, 2011). As shown in Figure 19C and 22, LPS stimulation increased NF-κB production, which was significantly inhibited by vanillic acid or dexamethasone pre-treatment (50 μM (vanillic acid) & 1 μM dexamethasone, p<0.01), when compared to the cells treated with LPS only. Immunofluorescence staining can be seen on Figures 20, 21 & 22.

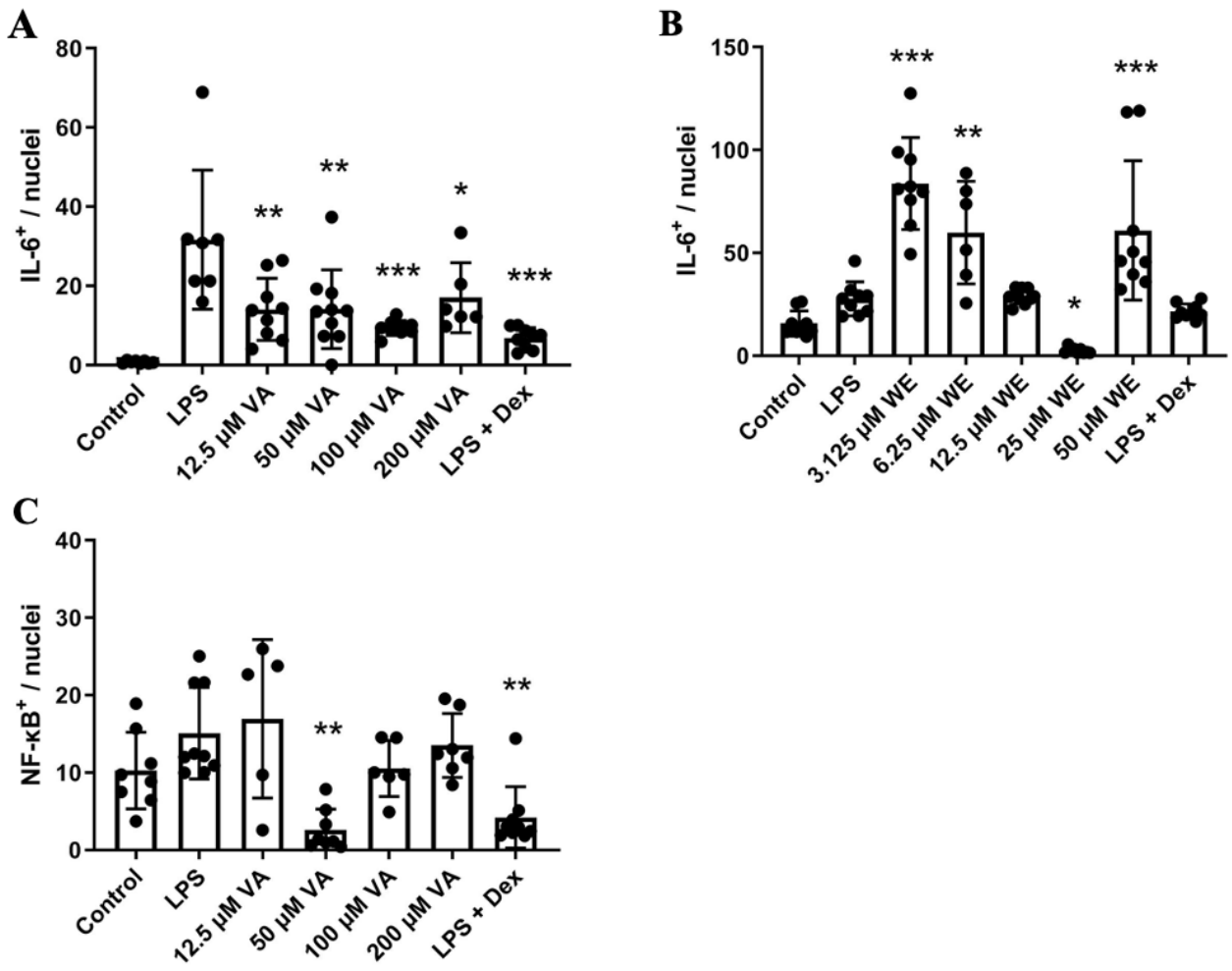

Figure 19. Effect of vanillic acid on LPS-induced cytokine production in RAW 264.7 macrophages **A.** IL-6 production of cells treated with LPS alone (1.0 µg/mL) or LPS plus different concentrations (12.5–200 µM) of vanillic acid (VA). Pre-treatment of VA was done 1h before LPS stimulation. The cells were then incubated for further 48 h in the presence of LPS. Dex: dexamethasone was used as positive control. **B.** Inhibitory effect (3.125–50 µM equivalent to VA) of water extract (WE) on IL-6 production of LPS-induced RAW 264.7 cells. **C.** NF-κB production of RAW 264.7 cells treated with h LPS alone or LPS plus different concentrations (12.5–200 µM) of VA. Data are presented as mean ± standard deviation. Each data point is one field of view (magnification x20). * Different from the LPS group. *p<0.05, **p<0.01 and ***p<0.001.

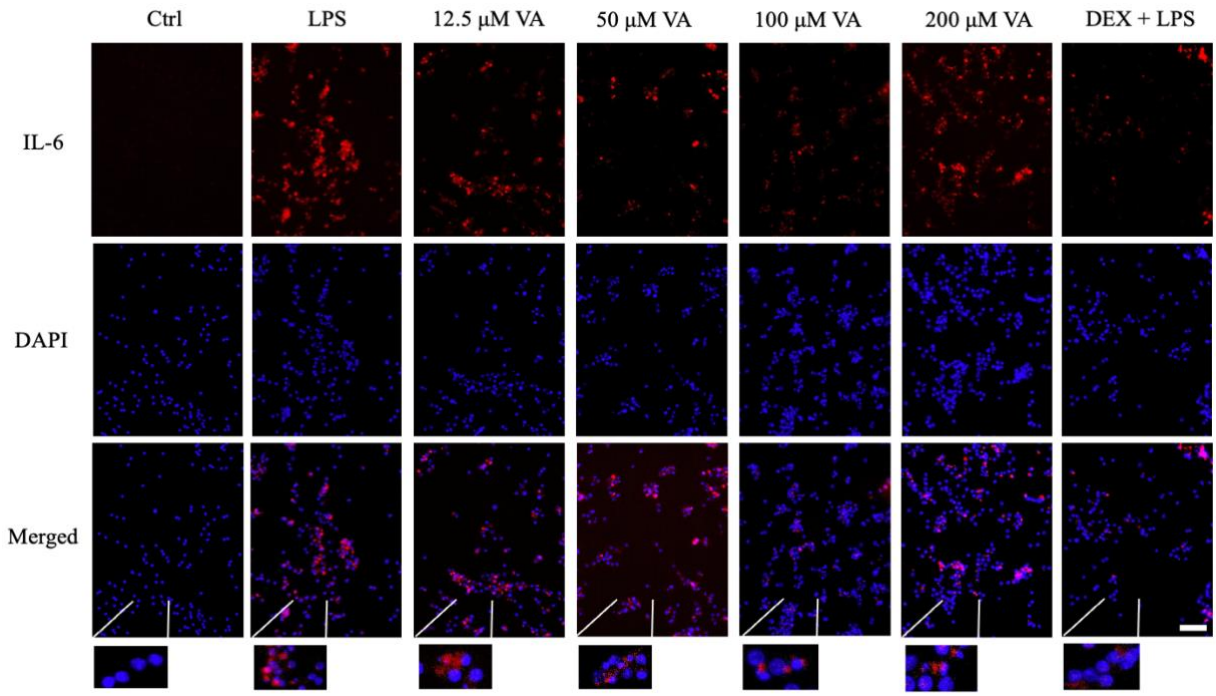

Figure 20. Immunofluorescent staining was performed to observe the effects of vanillic acid treatment on IL-6 production. DAPI was used as a nuclear marker. The upper middle and bottom panels indicate IL-6 (red), DAPI (blue) and merged images, respectively. Bar, 90 μm.

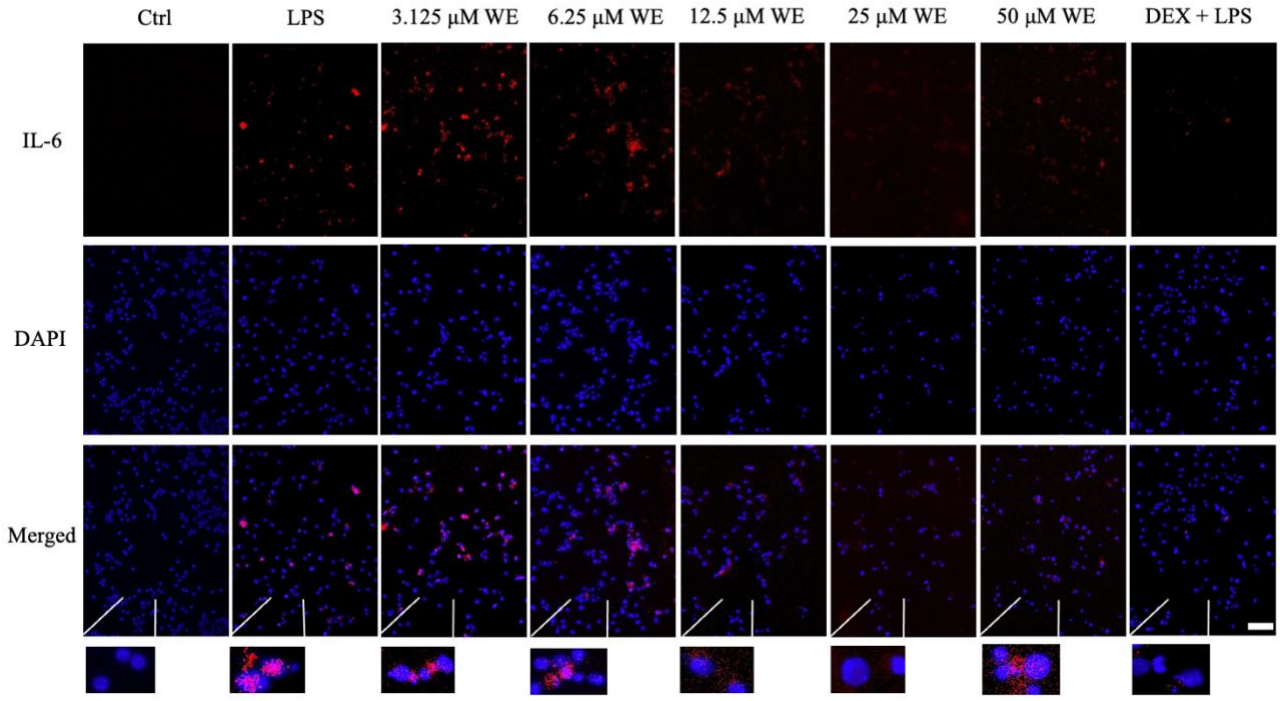

Figure 21. Immunofluorescent staining was performed to observe the effects of water extract treatment on IL-6 production. DAPI was used as a nuclear marker. The upper middle and bottom panels indicate IL-6 (red), DAPI (blue) and merged images, respectively. Bar, 90 μm.

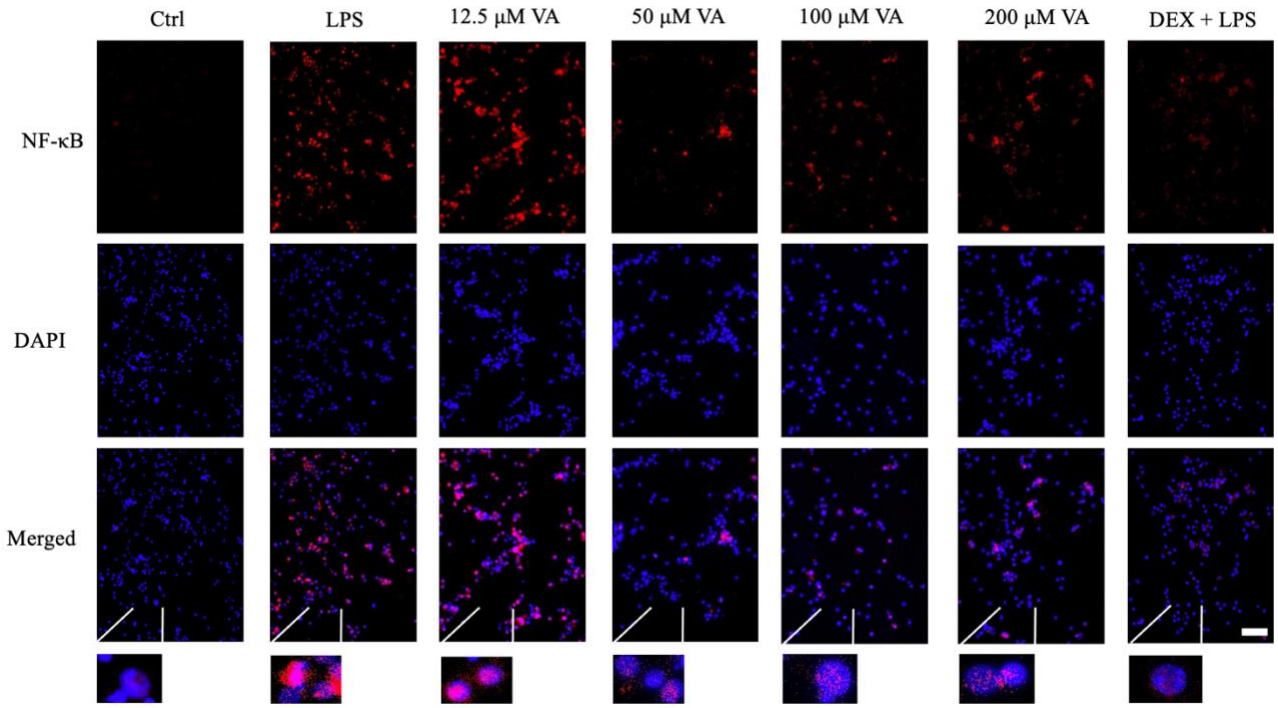

Figure 22. Immunofluorescent staining was performed to observe the effects of vanillic acid treatment on NF-κB production. DAPI was used as a nuclear marker. The upper middle and bottom panels indicate NF-κB (red), DAPI (blue) and merged images, respectively. Bar, 90 μm.

6. DISCUSSION

In this study, we investigated the role of novel water and methanol extracts derived from *Amomum villosum* Lour., a traditional Chinese herbal medicine, on RAW 264.7 macrophages and human dermal fibroblasts (HDF), subjected to oxidative stress and cytotoxicity. Our aim was to explore the antioxidant and anti-inflammatory properties of these extracts and their potential therapeutic implications for managing inflammatory conditions, particularly rheumatoid arthritis (RA). Our findings revealed that both water and methanol extracts increased HDF cell proliferation. Furthermore, *A. villosum* extracts showed protective antioxidant effects against H_2O_2-induced oxidative stress and LPS-induced cytotoxicity. Moreover, our water extract demonstrated both inhibitory and stimulatory effects on LPS-stimulated IL-6 production on RAW 264.7 macrophages. Vanillic acid served as a reference standard for method development and establishing a baseline for the extracts. Vanillic acid has been shown to be a major phenolic component of *A. villosum* and a potent antioxidant with well-established anti-inflammatory properties (Kim *et al.*, 2011). Therefore, we chose vanillic acid as a standard to assess the efficacy of our extracts against oxidative stress and inflammation. The effects observed with vanillic acid were comparable to those observed with the extracts.

The pathogenesis of RA is multifactorial and often associated with genetic predisposition, environmental factors and dysregulation of the immune system (Jang *et al*., 2022; Smolen *et al.,* 2018). During infection, pro-inflammatory cytokines like IL-6, contribute to inflammation, joint destruction, synovial hyperplasia and autoimmunity (Kadomoto *et al.*, 2022; Pandolfi *et al.,* 2020; Smolen *et al.,* 2018). IL-6 is involved in the bone erosion of RA, which primarily results from the differentiation and activation of osteoclasts, which are cells responsible for bone resorption. This process is mediated by the interaction between receptor activator of NF-κB (RANK) ligand (RANKL), produced by T cells, and pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1, secreted by macrophages and fibroblast-like synoviocytes (FLSs) within the synovial lining (Kadomoto *et al.*, 2022; Pandolfi *et al.,* 2020). Bone erosion in RA exacerbates joint damage and leads to irreversible structural deformities, contributing to impaired mobility, increased pain and diminished quality of life for affected individuals (Kronzer & Davis, 2021). Dysregulated IL-6 production can lead to chronic inflammation, highlighting its significance in disease progression (Pandolfi *et al.,* 2020).

Amomum villosum Lour. has been studied for its potential therapeutic effects, particularly its antioxidant and anti-inflammatory properties in gastrointestinal disorders, cancer, functional dyspepsia, hypolipidemia and hypoglycaemia (Zhang *et al*., 2024; Gao *et al*., 2023; Cai *et al*., 2021; Yue *et al*., 2021). The medicinal values of *A. villosum* are no doubt due to the bioactive chemical ingredients, including volatile oils and non-volatile compounds such as polyphenols and polysaccharides (Gao *et al*., 2023; Cai *et al*., 2021).

6.1 WATER AND METHANOL EXTRACTS CONTAIN BORNYL ACETATE, VANILLIC ACID AND OTHER POLYPHENOLS

Water and methanol extracts of *A. villosum* were analysed for terpenoids and polyphenols. Through NMR retention time data, four compounds were suspected in our extracts: bornyl acetate, vanillic acid, quercetin and tannic acid (Figures 7, 8, 9 & 10). Among these, vanillic acid was further confirmed, with its percentage calculated by dividing the peak area of the component by the total peak area of the HPLC chromatogram. Vanillic acid emerged as a predominant polyphenolic constituent, constituting 14.5% in the water extract and 20.7% in the methanol extract (Figures 11 & 12). While other components were not thoroughly verified, previous studies have confirmed the presence of bornyl acetate, vanillic acid and quercetin, suggesting that tannic acid may be a potential new component of *A. villosum* (Zhang *et al*., 2024; Zeng *et al*., 2022; Chen *et al*., 2018). Therefore, our findings on the chemical constituents of A. villosum align mostly with previous studies (Zhang *et al.,* 2024; Zeng *et al.,* 2022; Chen *et al*., 2018). However, some variations in the percentage of phenolic compounds in the total yield might occur. Bornyl acetate has been reported to account for 1.6% of the total constituents in A. villosum (Zeng *et al.,* 2022). There is no report regarding the percentage amount of the other constituents we have identified. The yield of chemical compounds is influenced by various extraction factors, such as extraction time, temperature and solvent type. Discrepancies in our results may arise from differences in the extraction method employed, leading to varying levels of constituents that impact overall outcomes. Altering extraction conditions, particularly solvent types, could lead to the identification of additional components. Conversely, we conducted our experiments on a single batch, minimizing the potential for batch-to-batch variations.

Enhancing the bioavailability of these herbal constituents can be achieved by optimizing extraction conditions. Zhang *et al.,* 2024 have already explored this aspect with ethanol extraction focusing on polyphenolic components, yet further investigations are warranted. This variability adds an intriguing dimension to the potentialities of *A. villosum.*

6.2 ENHANCED HDF PROLIFERATION INDUCED BY VANILLIC ACID AND EXTRACTS OF *AMOMUM VILLOSUM* LOUR.

Human dermal fibroblasts (HDF) are important in wound healing and maintaining the structural integrity of the skin. They contribute to the production of collagen, which provides structural support, and other essential components of the extracellular matrix (ECM), including fibronectin, proteoglycans, laminins, metalloproteinases, glycosaminoglycans and prostaglandins. Additionally, activated HDF may participate in skin immunity by secreting various chemokines and cytokines, including TNF-α, IL-6, IL-12, IL-10, IFN-γ and GM-CSF (Merec-Sadowska *et al.,* 2021).

In our study, vanillic acid significantly enhanced HDF proliferation over a 72-h incubation period. Previous research has demonstrated the proliferative effects of vanillic acid in dermal papilla cells by activating the PI3K/Akt/Wnt/β-catenin pathway, important in cell proliferation, survival, differentiation and migration (Kang *et al.,* 2020). In that study, vanillic acid induced the activation of the Wnt/β-catenin proteins, along with proteins such as Cyclin D1, CDK6, Cdc2 p34 and phospho-pRB, which are involved in cell cycle progression (Kang *et al.,* 2020). The mechanism underlying the enhancement of HDF proliferation by vanillic acid may therefore involve the regulation of cell cycle proteins, facilitating cell division and proliferation.

Interestingly, the proliferative effect of vanillic acid was observed specifically in HDF and not in RAW 264.7 macrophages. This differential response could be attributed to the expression patterns of specific receptors or signalling pathways involved in cell proliferation. RAW 264.7 macrophages, being primarily involved in immune response and inflammation, may not express the receptors or signalling molecules necessary to mediate the proliferative effects of vanillic acid. Consequently, we focused our subsequent experiments on HDF to explore the potential therapeutic implications of *A. villosum* extracts in promoting wound healing and skin regeneration.

Nonetheless, the methanol extract of *A. villosum* exhibited higher efficacy compared to the vanillic acid treatment group. However, our water extract exhibited effects similar to those of vanillic acid (Figures 13 & 14). We calculated the molarity of our extracts by aligning the concentration of vanillic acid to account for the percentage concentration in both the water and methanol extracts, as determined by HPLC (Figures 11 & 12). This discrepancy in our results may be attributed to the presence of other polyphenols or bioactive molecules within the extracts, which could contribute to the observed proliferation effect. Additionally, we observed a reduction in HDF cell proliferation between 24 and 48 hours after treatment with extracts from *A. villosum*. This decrease may be

attributed to alterations in cellular signalling cascades and the cells' adaptation to new environmental conditions.

Giacomini Bueno *et al.,* 2014 demonstrated that extracts from *Poincianella pluviosa* containing polyphenols such as gallic acid, also found in *A. villosum* (Zhang *et al*., 2024), enhanced HDF proliferation and tissue regeneration. Similarly, the compound 6-dehydrogingerdione (6-DG) from *Zingiber officinale* (Zingiberaceae) promoted HDF proliferation and migration by enhancing the production of various growth factors. It also reduced MMP-1 expression and restored tissue inhibitor of metalloproteinase-1 (TIMP-1) secretion, while increasing collagen production (Chen *et al*., 2013). Several other studies investigating the effects of polyphenols on HDF have reported similar findings (Merec-Sadowska *et al.,* 2021). Furthermore, Merec-Sadowska *et al.,* 2021 suggested that activator protein (AP)-1 and NF-kB, important in regulating numerous genes involved in cell cycle regulation, cell proliferation, apoptosis and the pathogenesis of inflammation, could also be pivotal targets for future investigations in understanding the molecular mechanisms underlying the effects of polyphenols on HDF proliferation. In conclusion, these results suggest that the synergistic action of various compounds within the extracts may enhance HDF proliferation more effectively than vanillic acid alone by activating various cellular pathways.

Overall, our findings suggest that vanillic acid and *A. villosum* extracts have the potential to enhance HDF proliferation, which could have significant implications for wound healing and tissue regeneration. Further elucidation of the specific molecular mechanisms and bioactive components responsible for this process could facilitate the development of innovative therapeutic approaches aimed at addressing skin disorders, promoting tissue repair and controlling inflammation.

6.3 PROTECTIVE EFFECTS OF *AMOMUM VILLOSUM* LOUR. EXTRACTS AGAINST H_2O_2-INDUCED OXIDATIVE STRESS

The excessive production of reactive oxygen species (ROS) is associated with cellular and tissue pathogenesis, resulting in disturbances in physiological processes and exacerbating the development of various chronic diseases (Djordjevic *et al.*, 2023; Lim *et al.,* 2020; Nakao *et al.,* 2008). Both Nrf2 and NF-κB are recognized as potential regulators involved in the cellular response to oxidative stress (Canton *et al.,* 2021). Hydrogen peroxide (H_2O_2) is a strong oxidant, diffusing easily between extra- and intracellular environments, thus contributing significantly to oxidative stress development (Nakao *et al.,* 2008). To establish a suitable oxidative stress model, we utilized a concentration of 750

μM H_2O_2, which induced approximately 30% cell death (Figure 16A), consistent with previous findings (Goddu *et al.,* 2018; Nakao *et al.,* 2008).

Our investigation revealed that vanillic acid exhibited antioxidative effects against H_2O_2-induced oxidative stress in RAW 264.7 macrophages, with a more pronounced effect observed in HDF (Figure 16B & 17A). The experiment conducted with RAW 264.7 cells did not achieve the desired percentage of cell death following H_2O_2 stimulation to assess the desired effectiveness of vanillic acid treatment. However, a discernible trend is still observable (Figure 16B). These findings align with previous studies that have highlighted vanillic acid's potent antioxidant properties to scavenge ROS and prevent oxidative damage to cellular components (Lim *et al.,* 2020; Kim *et al.,* 2011).

Furthermore, our water and methanol extracts exhibited protective effects against H_2O_2-induced oxidative stress at relatively low concentrations in both RAW 264.7 macrophages and HDF. Notably, the protective effect of the extracts on HDF appeared to be greater than that observed in RAW 264.7 macrophages (Figure 16C & 17B). The protective effects of *A. villosum* extracts are likely mediated through their antioxidant properties and modulation of intracellular signalling pathways. For example, previous studies have shown that oxidative stress inhibits the Nrf-2 pathway in HDF cells (Buranasudja *et al.,* 2022; Shi *et al.,* 2021). Notably, *A. villosum* is known to contain several other anti-inflammatory compounds and antioxidants, such as quercetin, protocatechuic acid, catechin and bornyl acetate (Zhang *et al.,* 2024). These additional bioactive components may synergistically contribute to the observed effects against oxidative stress by each attenuating different molecular pathways.

A key attribute of macrophages is their considerable adaptability, enabling them to swiftly alter their functional characteristics through polarization in response to signals from the intricate tissue microenvironment, which aids in their self-protection (Kadomoto *et al.*, 2022; Mosser *et al.*, 2021). Additionally, macrophages possess robust mechanisms to neutralize H_2O_2 and maintain cellular homeostasis, thereby preventing oxidative damage (Canton *et al.,* 2021). These mechanisms involve the expression of antioxidant enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx), which effectively neutralize ROS and mitigate oxidative stress (Kanwugu & Glukhareva, 2023; Goddu *et al.,* 2018). Activation of the Nrf2/HO-1 pathway is demonstrated to be crucial in this process (Figure 23) (Kadomoto *et al.*, 2022; Canton *et al.,* 2021; Goddu *et al.,* 2018). Moreover, studies on *A. villosum* have shown that a norditerpene exhibits antioxidant activity by activating the Nrf-2/HO-1 pathway in RAW 264.7 cells following H_2O_2 stimulation (Gao *et al.,* 2023). Interestingly, H_2O_2-induction has not been shown to enhance NF-κB

concentration in RAW 264.7 cells unlike other cell types such as neutrophils (Nakao *et al.,* 2008), indicating a cell-specific response. In conclusion, the protective effect of our extracts on RAW 264.7 may be influenced by the macrophages' ability to neutralize ROS, potentially attenuating the therapeutic efficacy of our extracts.

6.4 THE ANTI-APOPTOTIC EFFECT OF VANILLIC ACID AND *AMOMUM VILLOSUM* LOUR. EXTRACTS ON LPS-INDUCED CELL TOXICITY

The exposure to LPS triggers macrophage activation via TLR4, leading to robust production of proinflammatory cytokines such as IL-6, IL-1 and TNF-α, through the NF-κB pathway (Pandolfi *et al.,* 2020). These pro-inflammatory cytokines are vital in regulating the inflammatory response and promoting immune cell recruitment to the site of infection or injury (Kadomoto *et al.*, 2022; Na *et al.*, 2019). LPS has been extensively investigated concerning its role in driving the inflammatory response in macrophages (Lim *et al.,* 2020; Kim *et al.,* 2010). In our study, LPS induction at 1.0 µg/mL resulted in a significant cell death rate of 42.8% (Figure 18A), consistent with previous findings (Cao *et al.*, 2019). Consequently, we elected to employ LPS at this concentration for our subsequent *in vitro* experiments. While vanillic acid demonstrated notable protective effects at concentrations of 100 and 200 µM, limited time constraints allowed only one repetition of triplicates, precluding statistical analysis (Figure 18B). Notably, dexamethasone, a well-known anti-inflammatory medication, exhibited similar efficacy to vanillic acid at 200 µM (Figure 18B).

Moreover, water and methanol extracts exhibited varying effects in LPS-stimulated RAW 264.7 cells. The water extract, particularly at concentrations of 25 and 50 µM (equivalent to vanillic acid), demonstrated toxicity, possibly exceeding the therapeutic window (Figure 18C).

Vanillic acid has been demonstrated to possess anti-inflammatory properties in RAW 264.7 macrophages, through modulation of the NF-kB pathway (Kim *et al.*, 2011). Vanillic acid was proven to inhibit the degradation of IκB-α molecules in LPS-induced inflammatory models, thereby preventing the translocation of p65 into the nucleus of murine macrophages for transcriptional activation. This leads to the downregulation of expression of TNF-α, prostaglandin E2 (PGE2), cyclooxygenase-2 (COX-2), nitric oxide (NO) and IL-6 by stimulating caspase-1 (Figure 23) (Kim *et al.*, 2011).

Additionally, LPS-induced RAW 264.7 cells exhibited oxidative stress and activation of Nrf2/HO-1 pathway, resulting in decreased levels of proinflammatory cytokines, NO and

prostaglandins (Figure 23) (Lim *et al.,* 2020; Gao *et al.,* 2023). While the exact components responsible for these effects were not identified in both studies, quercetin is known to exhibit similar properties (Lim *et al.,* 2020). We propose that our extracts could likely exert their anti-inflammatory effects through pathways identical to those described above. However, further studies are required to establish an appropriate dosage and confirm these hypotheses.

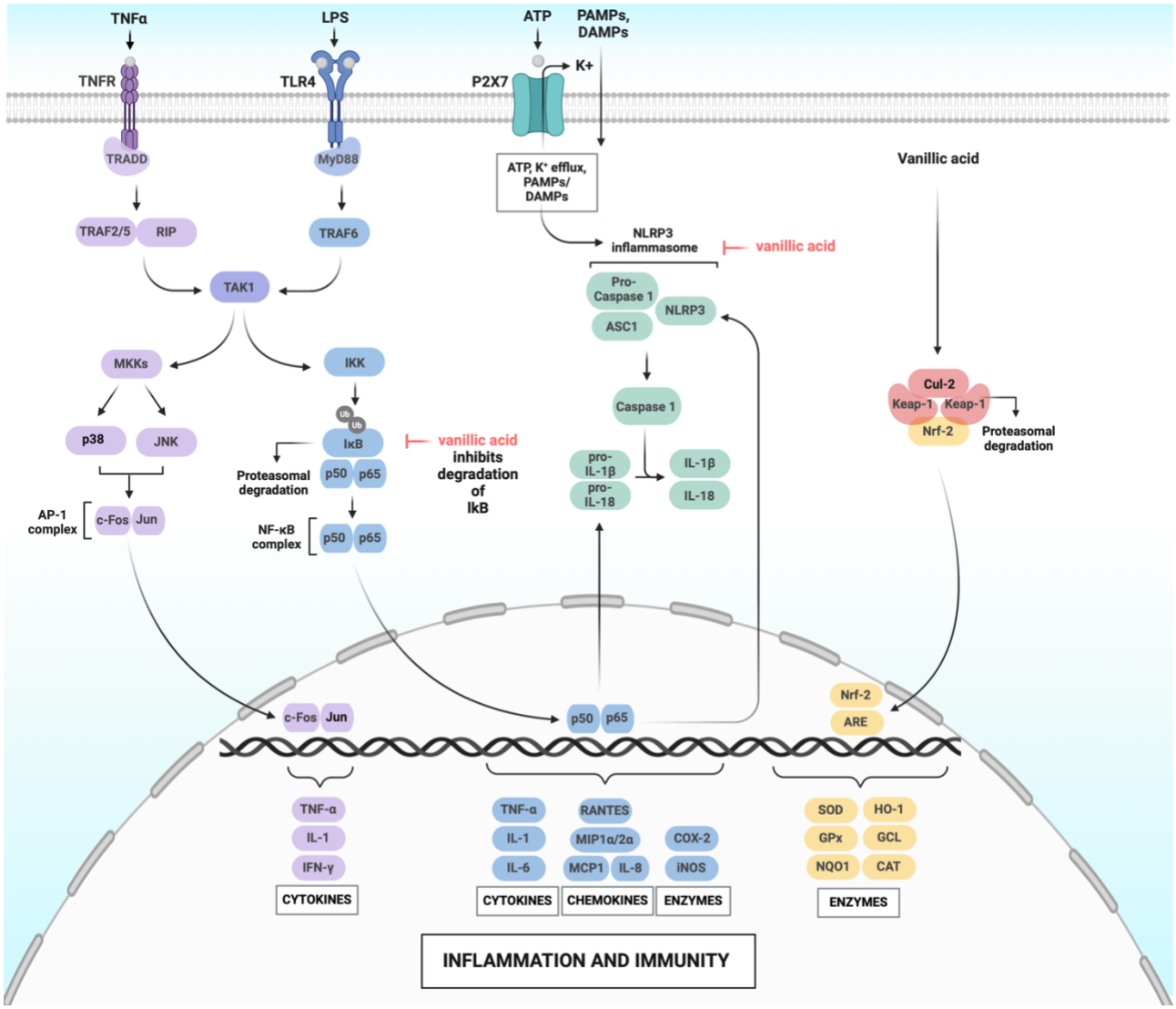

Figure 23. Inflammation pathways and the anti-inflammatory activity of vanillic acid (modified with BioRender from Kanwugu & Glukhareva, 2023; Kaur *et al*., 2022; Canton *et al.,* 2021; Ma *et al.,* 2021).

TNF, tumour necrosis factor; TNFR, tumour necrosis factor receptor; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; TLR, Toll-like receptor; IKK, IκB kinase; IκB, inhibitor of NF-κB; NF-κB, nuclear factor-kappa B; COX-2, cyclooxygenase-2; iNOS, Inducible nitric oxide synthase; Cul-2, Cullin-2; Keap-1, Kelch-like ECH-associated protein 1; Nrf2, Nuclear factor-erythroid 2 p45-related factor 2; SOD, Superoxide

dismutase HO-1, Heme oxygenase 1; GPx, Glutathione peroxidase; GCL, Glutamate-cysteine ligase; NQO1, NAD(P)H quinone dehydrogenase 1; CAT, Catalase.

6.5 VANILLIC ACID INHIBITS IL-6 PRODUCTION IN LPS-STIMULATED RAW 264.7 MACROPHAGES

IL-6 and NF-κB serve as a pivotal mediator in the inflammatory response and the pathogenesis of autoimmune diseases (Pandolfi *et al.,* 2020). Therefore, in the present study, we aimed to identify the extent to which vanillic acid and water extract from *A. villosum* protect RAW 264.7 macrophages against cytotoxicity. This was accomplished by evaluating the levels of inflammation markers, namely IL-6 and NF-κB, subsequent to the induction of cellular stress through LPS stimulation. Our findings showed a significant increase in cytokine production in RAW 264.7 cells upon treatment with LPS, as compared to unstimulated cells. This heightened cytokine production is a typical response mediated by the activation of TLR4 upon exposure to LPS, which leads to macrophage polarization toward the M1 phenotype and subsequent proinflammatory activity (Kadomoto *et al.,* 2022). However, pre-treatment with vanillic acid resulted in a significant reduction in IL-6 levels in RAW 264.7 macrophages exposed to LPS (Figure 19A). These results support that vanillic acid possesses anti-inflammatory properties and can mitigate LPS-induced inflammatory injury by modulating NF-κB signaling pathway, corroborating previous studies (Figure 23) (Kim *et al.,* 2011).

Furthermore, water extract treatment at various concentrations exhibited differential effects on IL-6 production in LPS-stimulated RAW 264.7 macrophages. While concentrations of 3.125, 6.25, and 50 μM (equivalent to vanillic acid) of water extract led to increased IL-6 production, a concentration of 25 μM (equivalent to vanillic acid) significantly attenuated IL-6 production (Figure 19B). These findings suggest that our LPS stimulation may not have been adequate, warranting further repetition of the experiments to validate the effect of our water extract. This inadequacy was evidenced by the lack of a robust increase in IL-6 production, which could potentially be resolved with either a higher LPS concentration or a longer incubation time. Additionally, optimizing the method used to detect IL-6 levels may also be necessary to ensure accurate measurements and capture subtle changes in cytokine expression.

NF-κB production was significantly inhibited by vanillic acid (50 μM) or dexamethasone pre-treatment (Figure 19C). Interestingly, other concentrations of vanillic acid did not yield the same effect. This observation, when compared to our findings regarding IL-6 production, suggests that the therapeutic window for vanillic acid could be relatively narrow. Alternatively, the lack of effect may

also be attributed to inadequate LPS stimulation due to similar reasons described previously. Unfortunately, we did not have sufficient time to replicate these experiments or to assess the effect of extracts on NF-κB production.

6.6 OVERALL LIMITATIONS

The study has several limitations, including those related to the choice of cell models, extraction methods, the depth of mechanistic understanding and the necessity for animal studies. The study primarily utilized RAW 264.7 macrophage cell models and HDF cells to assess anti-inflammatory effects. While these models are relevant, they may not fully represent the complexity of inflammatory conditions *in vivo.* Additionally, using only one cell type (HDF) to represent the target tissue in RA might not capture the full spectrum of inflammatory responses observed in the disease.

In addition, the study employed water and methanol extraction methods, but other methods could yield different results. Especially, we did not address the potential polysaccharide effects of *A. villosum*, and the extraction method we used might have caused polysaccharides to precipitate out.

Moreover, the study evaluated the anti-inflammatory and antioxidant effects of the extracts but did not fully elucidate the underlying molecular mechanisms. Further investigation into specific pathways and molecular targets would provide a deeper understanding of how the extracts exert their effects. The study also focused on a limited set of cytokines, such as IL-6, without exploring other important mediators of inflammation, such as TNF-α.

While *in vitro* studies provide valuable insights, they may not accurately reflect the complex physiological conditions *in vivo*. Thus, extrapolating the results to clinical settings should be done cautiously. Therefore, conducting animal studies to validate the findings *in vivo* would provide more robust evidence of the extracts' efficacy and safety. Addressing these limitations in future studies would strengthen the scientific understanding of the therapeutic potential of *A. villosum* extracts in managing inflammatory conditions like RA.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

Our findings demonstrate that vanillic acid, as well as water and methanol extracts from *Amomum villosum* Lour. exhibit potent antioxidant and anti-inflammatory properties, effectively reducing oxidative stress and cytotoxicity in RAW 264.7 macrophages and HDF cells. We tentatively suggest that these beneficial effects are attributed to the bioactive compounds present in the extracts. Importantly, to the best of our knowledge, our investigation represents the first exploration of *A. villosum's* effects on HDF cells, expanding our understanding of its pharmacological profile. We are also the first to report that vanillic acid enhances HDF proliferation. Additionally, we found that *A. villosum* might contain tannic acid, adding to its potential therapeutic properties

Our study sheds light on the potential therapeutic benefits of vanillic acid and *A. villosum* extracts in alleviating oxidative stress, a key factor in RA (Kaur *et al*., 2022). Elevated lipid peroxidation levels observed in RA are associated with reduced antioxidant system efficacy and disease activity (Djordjevic *et al.*, 2023). Moreover, lipid peroxidation and oxidized low-density lipoproteins (LDL) likely contribute to accelerated atherosclerosis in RA patients (Schett *et al.*, 2018). Furthermore, persistent inflammation fosters lipolysis and systemic fatty acid release, contributing to dyslipidaemia development (Djordjevic *et al.*, 2023). Therefore, the hypolipidemic effects of *A. villosum* could also potentially help treat dyslipidaemia in RA (Kim *et al.,* 2022).

Vanillic acid has shown efficacy in reducing inflammation in knee osteoarthritis, a condition also characterized by synovitis, by mitigating caspase-1, ASC and inflammasome NLRP3, and decreasing IL-1β and IL-18 levels (Figure 23). In addition, vanillic acid alleviated pain-related behaviour and downregulated pain mediators in fibroblast-like synoviocytes (FLSs). Reduced synovial fibrosis was also observed in the animal experiments (Ma *et al.,* 2021). Both vanillic acid and *A. villosum* offer promising strategies for reducing inflammation in RA by inhibiting pro-inflammatory cytokines such as IL-6. However, it's important to acknowledge the limitations seen in conventional RA medications, as achieving a thorough blockade of pro-inflammatory markers may not always be optimal and controlled decreases may be necessary in certain cases (Pandolfi *et al*., 2020).

Current research on *A. villosum* components has increasingly focused on the non-volatile components (Zhang *et al*., 2024; Kim *et al*., 2022; Liu *et al*., 2022; Lim *et al*., 2020; Zhang *et al*., 2013), with new polysaccharides being isolated and purified, suggesting further avenues for

exploration (Liu *et al*., 2022; Zhou *et al*., 2021). Moreover, studies on the pharmacological effects of other plant parts, such as rhizomes and leaves, remain relatively scarce (Gao *et al*., 2023).

Future research directions should explore the synergistic effects of different components within *A. villosum* extracts and potential interactions with existing RA medications. Herbal medicines belonging to TCM are known for their minimal adverse effects, ease of administration and combability with modern therapeutics, which could help decrease dosage and mitigate possible side effects of current medications (Tang *et al*., 2008).

Elucidating the exact mechanism by which our water and methanol extracts exert their antioxidative effects in the present study could be achieved by testing the molecular pathways, potentially with specified inhibitors. Additionally, investigating different cytokines, such as TNF-α, or conducting more *in vitro* inflammatory studies would be beneficial. Assessing the effects of these extracts on RA-related chronic inflammatory diseases *in vivo* would also be a valuable next step. Overall, our study contributes to the growing body of evidence supporting the therapeutic potential of *A. villosum* in managing inflammatory conditions, paving the way for further research and clinical exploration in this area.

8. ACKNOWLEDGMENTS

Foremost, I owe sincere gratitude to my supervisor, **Dr Belal Chami**, who welcomed me into his group despite my limited background in this field. You patiently accommodated my perfectionism and remained unfazed by my Finnish straightforwardness and honesty. I appreciate your ability to infuse humour into our daily interactions while still maintaining a high level of efficiency in our work. Thank you for making it possible for me to come back to Sydney.

I'm deeply grateful to, **Guanqing (Milissa) Huang**, for developing the extraction method and producing the extracts, as well as the data for Figures 13, 14 and 17B. Thank you for your guidance, support and for always pushing me to do my best.

Special thanks to **Kangzhe (Steven) Xie**, **Ella Verley** and **Angelique Dunn** for always being so kind and helpful in the lab. Thank you, **Steven**, for always answering all my questions even outside your responsibilities. Thank you, **Ella**, for not making me do those stool aliquotes after all.

I extend my sincere gratitude to my co-supervisor, **Päivi Tammela**, for generously dedicating your time to review my thesis and for patiently assisting me with every aspect of my work. Your reassuring kindness and encouragement have been instrumental in helping me navigate through this process.

Grateful acknowledgments to the thesis reviewers, **Mikaela Grönholm** and **Yvonne Holm**, for generously dedicating your valuable time to evaluate this study.

Thank you, **Kaiser Hamid**, **Colin Duke** and **Wilson Tran**, from Sydney Pharmacy School for your help with NMR and HPLC. Thank you also to **Milissa** for her significant contributions to this.

Thanks to everyone in CPC for making the days lighter with interesting conversations, lunch breaks outside and good banter. Thanks to these mates for all the activities outside of work.

Warm thanks to **Tiina Immonen**, our TRANSMED programme director, for her invaluable guidance and support throughout my master's studies. I am grateful for her role in fostering a sense of community within TRANSMED, making it feel like a family. **Tiina's** kindness and cheerful character significantly eased the challenges of the past two years.

Hearty thanks to **all my friends** who have supported me, even if they might not fully understand what I'm studying. Thank you, **Mom and Dad**, for your unwavering support throughout my academic journey. Thank you for reminding me that I don't always need to get the best grade and for supporting and helping me in my move to Australia (twice) and coming to visit me here. Thank you for loving me unconditionally.

9. REFERENCES

Aziz, NH., Farag, SE., Mousa, LA., Abo-Zaid, MA. (1998). Comparative antibacterial and antifungal effects of some phenolic compounds. *Microbios,* 93(374):43–54.

Buranasudja, V., Muangnoi, C., Sanookpan, K., Halim, H., Sritularak, B., Rojsitthisak, P. (2022). Eriodictyol Attenuates H2O2-Induced Oxidative Damage in Human Dermal Fibroblasts through Enhanced Capacity of Antioxidant Machinery. *Nutrients*, 14(12):2553.

Cai, RB., Yue, XY., Wang, YL., Yang, YY., Sun, DJ., Chen, LX. (2021). Chemistry and bioactivity of plants from the genus Amomum. *Journal of Ethnopharmacology*, 281:114563.

Canton, M., Sánchez-Rodríguez, R., Spera, I., Venegas, FC., Favia, M., Viola, A., Castegna, A. (2021). Reactive Oxygen Species in Macrophages: Sources and Targets. *Frontiers in Immunology*, 12:734229.

Cao, Y., Chen, J., Ren, G., Zhang, Y., Tan, X., Yang, L. (2019). Punicalagin Prevents Inflammation in LPS-Induced RAW 264.7 Macrophages by Inhibiting FoxO3a/Autophagy Signaling Pathway. *Nutrients,* 11(11):2794.

Caplan, A. (1953). Certain unusual radiological appearances in the chest of coal-miners suffering from rheumatoid arthritis. *Thorax*, 8(1):29–37.

Chen, CY., Chiu, CC., Wu, CP., Chou, YT., Wang, HM. (2013). Enhancements of Skin Cell Proliferations and Migrations via 6-Dehydrogingerdione. *Journal of Agricultural and Food Chemistry*, 61(6):1349–1356.

Chen, LX., Lai Y-F., WX., Cai, J., Hu, H., Wang, Y., Zhao, J., Li, SP. (2020). Comparison of volatile compounds in different parts of fresh Amomum villosum Lour. from different geographical areas using cryogenic grinding combined HS-SPME-GC-MS. *Chinese Medicine*, 15:97.

Chen, N., Sun, G., Yuan, X., Hou, J., Wu, Q., Soromou, LW., Feng, H. (2014). Inhibition of lung inflammatory responses by bornyl acetate is correlated with regulation of myeloperoxidase activity. *Journal of Surgical Research*, 186(1):436–445.

Chen, Z., Ni, W., Yang, C., Zhang, T., Lu, S., Zhao, R., Mao, X., Yu, J. (2018). Therapeutic Effect of Amomum villosum on Inflammatory Bowel Disease in Rats. *Frontiers in Pharmacology*, 20(9):639.

Chiang, LC., Ng, LT., Chiang, W., Chang, MY., Lin, CC. (2003). Immunomodulatory activities of flavonoids, monoterpenoids, triterpenoids, iridoid glycosides and phenolic compounds of Plantago species. *Planta Medica*, 69(7):600–604.

Choi, Y., Choi, JK., Jang, YH., Lee, S., Lee, S., Choi, JH., Kim, S. (2017). Anti-inflammatory effect of Amomum xanthioides in a mouse atopic dermatitis model. *Molecular Medicine Reports,* 16(6):8964–8972.

Chuang, TY., Cheng, AJ., Chen, IT., Lan, TY., Huang, IH., Shiau, CW., Hsu, CL., Liu, YW., Chang, ZF., Tseng, PH., Kuo, JC. (2017). Suppression of LPS-induced inflammatory responses by the hydroxyl groups of dexamethasone. *Oncotarget*, 8(30):49735–49748.

Cojocaru, M., Cojocaru, IM., Silosi, I., Vrabie, CD., Tanasescu, R. (2010). Extra-articular Manifestations in Rheumatoid Arthritis. *Maedica*, 5(4):286–91.

Crowson, CS., Matteson, EL., Myasoedova, E., Michet, CJ., Ernste, FC., Warrington, KJ., Davis, JM. 3rd, Hunder, GG., Therneau, TM., Gabriel, S.E. (2011). The lifetime risk of adult-onset rheumatoid arthritis and other inflammatory autoimmune rheumatic diseases. *Arthritis & Rheumatology,* 63(3):633–639.

Darsih, C., Windarsih, A., Damayanti, E., Amiru, VA., Indrianingsi, AW., Marfu'ah, S., Sujarwo, W. (2023). Antibacterial and Angiotensin I-Converting Enzyme (ACE) Inhibition Activities of Essential Oil from Java Cardamom (Amomum compactum) Fruit. *Indian Journal of Microbiology*, 63(3):263–271.

Dedmon, LE. 2020. The genetics of rheumatoid arthritis. *Rheumatology,* 59(10):2661–2670.

Djordjevic, K., Milojevic Samanovic, A., Veselinovic, M., Zivkovic, V., Mikhaylovsky, V., Mikerova, M., Reshetnikov, V., Jakovljevic, V., Nikolic Turnic, T. (2023). Oxidative Stress Mediated Therapy in Patients with Rheumatoid Arthritis: A Systematic Review and Meta-Analysis. *Antioxidants (Basel),* 12(11):1938.

Dong, L., Zhang, S., Chen, L., Lu, J., Zhao, F., Long, T., Wen, J., Huang, J., Mao, Y., Qi, Z., Zhang, J., Li, L., Dong, Y. (2023). In vivo anti-hyperuricemia and anti-gouty arthritis effects of the ethanol extract from Amomum villosum Lour. *Biomedicine & Pharmacotherapy*, 161:114532.

Fraenkel, L., Bathon, JM., England, BR., St Clair, EW., Arayssi, T., Carandang, K., Deane, KD., Genovese, M., Huston, KK., Kerr, G., Kremer, J., Nakamura, MC., Russell, LA., Singh, JA., Smith, BJ., Sparks, JA., Venkatachalam, S., Weinblatt, ME., Al-Gibbawi, M., Baker, JF., Barbour, KE., Barton, JL., Cappelli, L., Chamseddine, F., George, M., Johnson, SR., Kahale, L., Karam, BS., Khamis, AM., Navarro-Millán, I., Mirza, R., Schwab, P., Singh, N., Turgunbaev, M., Turner, AS., Yaacoub, S., Akl, EA. (2021). 2021 American College of Rheumatology Guideline for the Treatment of Rheumatoid Arthritis. *Arthritis Care & Research*, 73(7):924–939.

Gao, C., Zhu, M., Xu, W., Wang, Y., Xiong, L., Sun, D., Sun, M., Lin, Y., Li, H., Chen, L. (2023). Chemical constituents from the stems and leaves of Amomum villosum Lour. and their anti-inflammatory and antioxidant activities. *Bioorganic Chemistry*, 131:106281.

Giacomini Bueno, F., Pier Panizzon, G., Souza de Leite Mello, EV., Lechtenberg, M., Petereit, F., Palazzo de Mello, JC., Hensel, A. (2014). Hydrolyzable tannins from hydroalcoholic extract from Poincianella pluviosa stem bark and its wound-healing properties: Phytochemical investigations and influence on in vitro cell physiology of human keratinocytes and dermal fibroblasts. *Fitoterapia,* 99: 252–260.

Goddu, RN., Henderson, CF., Young, AK., Muradian, BE., Calderon, L., Bleeg, LH., Fukuto, JM., Lin, J. (2018). Chronic exposure of the RAW246.7 macrophage cell line to H2O2 leads to increased catalase expression. *Free Radical Biology and Medicine,*126:67–72.

Guimarães, CM., Gião, MS., Martinez, SS., Pintado, AI., Pintado, ME., Bento, LS., Malcata, FX. (2007). Antioxidant activity of sugar molasses, including protective effect against DNA oxidative damage. *Journal of Food Science,* 72(1):C039–C043.

Heilmann, J., Brun, R., Mayr, S., Rali, T., Sticher, O. (2001). Minor cytotoxic and antibacterial compounds from the rhizomes of Amomum aculeatum. *Phytochemistry,* 57(8):1281–1285.

Huang, J., Fu, X., Chen, X., Li, Z., Huang, Y., Liang, C. (2021). Promising Therapeutic Targets for Treatment of Rheumatoid Arthritis. *Frontiers in Immunology*, 12:686155.

Huong, LT., Dai, DN., Thang, TD., Bach, TT., Ogunwande, IA. (2015). Volatile constituents of Amomum maximum Roxb and Amomum microcarpum C. F. Liang & D. Fang: two Zingiberaceae grown in vietnam. *Natural Product Research*, 29(15):1469–1472.

Itoh, A., Isoda, K., Kondoh, M., Kawase, M., Kobayashi, M., Tamesada, M., Yagi, K. (2019). Hepatoprotective effect of syringic acid and vanillic acid on concanavalin a-induced liver injury. *Biological and Pharmaceutical Bulletin*, 32(7):1215–1219.

Jang, S., Kwon, EJ., Lee, JJ. (2022). Rheumatoid Arthritis: Pathogenic Roles of Diverse Immune Cells. *International Journal of Molecular Sciences*, 23(2):905.

Kadomoto, S., Izumi, K., Mizokami, A. (2021). Macrophage Polarity and Disease Control. *Internal Journal of Molecular Sciences,* 23(1):144.

Kamchonwongpaisan, S., Nilanonta, C., Tarnchompoo, B., Thebtaranonth, C., Thebtaranonth, Y. Yuthavong, Y., Kongsaeree, P., Jon Clardy. (1995). An antimalarial peroxide from Amomum krervanh Pierre. *Tetrahedron Letters,* 36(11):1821–1824.

Kampa, M., Alexaki, VI., Notas, G., Nifli, AP., Nistikaki, A., Hatzoglou, A., Bakogeorgou, E., Kouimtzoglou, E., Blekas, G., Boskou, D., Gravanis, A., Castanas, E. (2004). Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells: potential mechanisms of action. *Breast Cancer Research*, 6(2):R63–R74.

Kang, JI., Choi, YK., Koh, YS., Hyun, JW., Kang, JH., Lee, KS., Lee, CM., Yoo, ES., Kang, HK. (2020). Vanillic Acid Stimulates Anagen Signaling via the PI3K/Akt/ β-Catenin Pathway in Dermal Papilla Cells. *Biomolecules & Therapeutics (Seoul),* 28(4):354–360.

Kanwugu, OS. & Glukhareva, TV. (2023). Activation of Nrf2 pathway as a protective mechanism against oxidative stress-induced diseases: Potential of astaxanthin. *Archives of Biochemistry and Biophysics,* 741:109601.

Kaur, J., Gulati, M., Singh, SK., Kuppusamy, G., Kapoor, B., Mishra, V., Gupta, S., Arshad, MF. Porwal, O., Jha, NK., Chaitanya, MVNL., Chellappan, DK., Gupta, G., Gupta, PK., Dua, K., Khursheed, R., Awasthi, A., Corrie, L. (2022). Discovering multifaceted role of vanillic acid beyond flavours: Nutraceutical and therapeutic potential. *Trends in Food Science & Technology*, 122:187–200.

Kim, HR., Antonisamy, P., Kim, YS., Lee, G., Ham, HD., Kwon, KB. (2022). Inhibitory effect of Amomum villosum water extracts on α-glucosidase activity. *Physiological and Molecular Plant Pathology*, 117:101779.

Kim, MC., Kim, SJ., Kim, DS., Jeon, YD., Park, SJ., Lee, HS., Um, JU., Hong, SH. (2011). Vanillic acid inhibits inflammatory mediators by suppressing NF-κB in lipopolysaccharide-stimulated mouse peritoneal macrophages. *Immunopharmacology and Immunotoxicology*, 33(3):525–532.

Kim, MS., Ahn, EK., Hong, SS., Oh, JS. (2016). 2,8-Decadiene-1,10-Diol Inhibits Lipopolysaccharide-Induced Inflammatory Responses Through Inactivation of Mitogen-Activated Protein Kinase and Nuclear Factor-κB Signaling Pathway. *Inflammation,* 39(2):583–91.

Kim, SJ., Kim, MC., Um, JY., Hong, SH. (2010). The beneficial effect of vanillic acid on ulcerative colitis. *Molecules*, 15:7208–7217.

Kim, YS., Kim, HR., Antonisamy, P., Lee, YR., Lee, G., Jung, HJ., Kwon, KB. (2022). Amomum villosum Lour. Fruit extract mitigates hyperlipidemia through SREBP-2/LDLR/HMGCR signaling in high-cholesterol diet-fed mice. *Journal of King Saud University - Science*, 34(7):102230.

Klareskog, L., Rönnelid, J., Saevarsdottir, S., Padyukov, L., Alfredsson, L. (2020). The importance of differences; On environment and its interactions with genes and immunity in the causation of rheumatoid arthritis. *Journal of Internal Medicine,* 287(5):514–533.

Konappa, N., Udayashankar, AC., Krishnamurthy, S., Pradeep, CK., Chowdappa, S., Jogaiah, S. (2020). GC-MS analysis of phytoconstituents from Amomum nilgiricum and molecular docking interactions of bioactive serverogenin acetate with target proteins. *Scientific Reports*, 10(1):16438.

Kronzer, VL., Davis, JM. 3rd. (2021). Etiologies of Rheumatoid Arthritis: Update on Mucosal, Genetic, and Cellular Pathogenesis. *Current Rheumatology Reports*, 23(4):21.

Kumar, G., Chauhan, B., Ali, M. (2014). Isolation and identification of new phytoconstituents from the fruit extract of Amomum subulatum Roxb. *Natural Product Research,* 28(2):127–133.

Lee, JA., Lee, MY., Seo, CS., Jung, DY., Lee, NH., Kim, JH., Ha, H., Shin, HK. (2010). Anti-asthmatic effects of an Amomum compactum extract on an ovalbumin (OVA)-induced murine asthma model. *Bioscience, Biotechnology & Biochemistry*, 74(9):1814–1818.

Li, S., Ding, X., Zhang, H., Ding, Y., Tan, Q. (2022). IL-25 improves diabetic wound healing through stimulating M2 macrophage polarization and fibroblast activation. *International Immunopharmacology,* 106:108605.

Lim, DW., Choi, HJ., Park, SD., Kim, H., Yu, GR., Kim, JE., Park, WH. (2020). Activation of the Nrf2/HO-1 Pathway by Amomum villosum Extract Suppresses LPS-Induced Oxidative Stress In Vitro and Ex Vivo. *Evidence Based Complementary Alternative Medicine*, 3:2837853.

Lim, DW., Kim, H., Park, JY., Kim, JE., Moon, JY., Park, SD., Park, WH. (2016). Amomum cardamomum L. ethyl acetate fraction protects against carbon tetrachloride-induced liver injury via an antioxidant mechanism in rats. *BMC Complementary Medicine and Therapies*, 16:155.

Liu, H., Zhuang, S., Liang, C., He, J., Brennan, CS., Brennan, MA., Ma, L., Xiao, G., Chen, H., Wan. S. (2022). Effects of a polysaccharide extract from Amomum villosum Lour. on gastric mucosal injury and its potential underlying mechanism. *Carbohydrate Polymers*, 294:119822.

Lotfi, N., Thome, R., Rezaei, N., Zhang, GX., Rezaei, A., Rostami, A., Esmaeil, N. (2019). Roles of GM-CSF in the Pathogenesis of Autoimmune Diseases: An Update. *Frontiers in Immunology*, 10:1265.

Lu, CL., Wang, LN., Li, YJ., Fan, QF., Huang, QH., Chen, JJ. (2022). Anti-hyperglycaemic effect of labdane diterpenes isolated from the rhizome of Amomum maximum Roxb., an edible plant in Southwest China. *Natural Product Research*, 36(10):2570–2574.

Lu, S., Zhang, T., Gu, W., Yang, X., Lu, J., Zhao, R., Yu, J. (2018). Volatile Oil of Amomum villosum Inhibits Nonalcoholic Fatty Liver Disease via the Gut-Liver Axis. *BioMed Research International*, 3589874.

Luo, D., Zeng, J., Guan, J., Xu, Y., Jia, RB., Chen, J., Jiang, G., Zhou, C. (2022). Dietary Supplement of Amomum villosum Lour. Polysaccharide Attenuates Ulcerative Colitis in BALB/c Mice. *Foods,* 11(22):3737.

Luo, JG., Yin, H., Fan, BY. and Kong, LY. (2014). Labdane Diterpenoids from the Roots of Amomum maximum and Their Cytotoxic Evaluation. *Helvetica Chimica Acta*, 97:1140–1145.

Ma, Z., Huang, Z., Zhang, L., Li, X., Xu, B., Xiao, Y., Shi, X., Zhang, H., Liao, T., Wang, P. (2021) Vanillic Acid Reduces Pain-Related Behavior in Knee Osteoarthritis Rats Through the Inhibition of NLRP3 Inflammasome-Related Synovitis. *Frontiers in Pharmacology,* 11:599022.

McInnes, I. B., & Schett, G. (2011). The pathogenesis of rheumatoid arthritis. *New England Journal of Medicine*, 365(23):2205–2219.

Merecz-Sadowska, A., Sitarek, P., Kucharska, E., Kowalczyk, T., Zajdel, K., Cegliński, T., Zajdel, R. (2021). Antioxidant Properties of Plant-Derived Phenolic Compounds and Their Effect on Skin Fibroblast Cells. *Antioxidants (Basel),* 10(5):726.

Mosser, DM., Hamidzadeh, K., Goncalves, R. (2021). Macrophages and the maintenance of homeostasis. *Cellular and Molecular Immunology*, 18(3):579–587.

Na, YR., Stakenborg, M., Seok, SH., Matteoli, G. (2019). Macrophages in intestinal inflammation and resolution: a potential therapeutic target in IBD. *Nature Reviews Gastroenterology &* Hepatology, 16(9):531–543.

Nakao, N., Kurokawa, T., Nonami, T., Tumurkhuu, G., Koide, N., Yokochi T. (2008). Hydrogen peroxide induces the production of tumor necrosis factor-α in RAW 264.7 macrophage cells via activation of p38 and stress-activated protein kinase. *Innate Immunity*, 14(3):190–196.

Okada, Y., Eyre, S., Suzuki, A., Kochi, Y., Yamamoto, K. (2019). Genetics of rheumatoid arthritis: 2018 status. *Annals of the Rheumatic Diseases*, 78(4):446–453.

Okada, Y., Wu, D., Trynka, G., Raj, T., Terao, C., *et al*. (2014). Genetics of rheumatoid arthritis contributes to biology and drug discovery. *Nature*, 506(7488):376–381.

Pandolfi, F., Franza, L., Carusi, V., Altamura, S., Andriollo, G., Nucera, E. (2020). Interleukin-6 in Rheumatoid Arthritis. *International Journal of Molecular Sciences,* 21(15):5238.

Roh, SE., Woo, JA., Lakshmana, MK., Uhlar, C., Ankala, V., Boggess, T., Liu, T., Hong, YH., Mook-Jung, I., Kim, SJ., Kang, DE. (2013). Mitochondrial dysfunction and calcium deregulation by the RanBP9-cofilin pathway. *The FASEB Journal,* 27(12):4776–4789.

Schalkwijk, J., Van der Berg, W., Van de Putte, L., Joosten, L. (1986). An experimental model for hydrogen peroxide–induced tissue damage. Effects of a single inflammatory mediator on (peri)articular tissues. *Arthritis & Rheumatology,* 29(4):532–538.

Schett, G., Neurath, MF. (2018). Resolution of chronic inflammatory disease: universal and tissue-specific concepts. *Nature Communications*, 9(1):3261.

Shi, X., Cheng, W., Wang, Q., Zhang, J., Wang, C., Li, M., Zhao, D., Wang, D., An, Q. (2021). Exploring the Protective and Reparative Mechanisms of G. lucidum Polysaccharides Against H2O2-Induced Oxidative Stress in Human Skin Fibroblasts. *Clinical Cosmetic and Investigational Dermatology*, 14:1481–1496.

Sinitha, K., & Thoppil, JE. (2017). Evaluation of antioxidant, anti-inflammation and chemical composition of methanolic extract of Amomum masticatorium Thwaites (Zingiberaceae). *Internal Journal of Pharmacognosy*, 4:413–418.

Smolen, JS., Aletaha, D., Barton, A., Burmester, GR., Emery, P., Firestein, GS., Kavanaugh, A., McInnes, IB., Solomon, DH., Strand V., Yamamoto, K. (2018). Rheumatoid arthritis. *Nature Reviews Disease Primers,* 4:18001.

Tang, J-L., Liu, B-Y., Ma, K-W. (2008). Traditional Chinese medicine. *The Lancet*, 372(9654):1938–1940.

The Wellcome Trust Case Control Consortium. (2007). Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*, 447(7145):661–678.

Toledano, E., Candelas, G., Rosales, Z., Martínez Prada, C., León, L., Abásolo, L., Loza, E., Carmona, L., Tobías, A., Jove,r JÁ. (2012). A meta-analysis of mortality in rheumatic diseases. *Reumatologia Clinica,* 8(6):334–41.

Toumi, Hechmi. (2022). ed. *Rheumatoid Arthritis.* London: IntechOpen, Print.

Wannaporn, C., Nuntawong, N. (2015). Diterpenes and kawalactone from the rhizomes of Amomum uliginosum J. Koenig. *Biochemical Systematics and Ecology*, 63:34–37.

Weyand, CM., Hicok, KC., Conn, DL., Goronzy, JJ. (1992). The Influence of HLA-DRB1 Genes on Disease Severity in Rheumatoid Arthritis. *Annals of Internal Medicine*, 117(10):801–806.

WHO: https://www.who.int/news-room/fact-sheets/detail/rheumatoid-arthritis, Read 7.11.2023.

Yue, J., Zhang, S., Zheng, B., Raza, F., Luo, Z., Li, X., Zhang, Y., Nie, Q., Qiu, M. (2021). Efficacy and Mechanism of Active Fractions in Fruit of Amomum villosum Lour. for Gastric Cancer. *Journal of Cancer*, 12(20):5991–5998.

Zeng, Y., Muhammad AK., Wei, H., Lin, D., Xiomeng, Y., Xia, L., Xiaying, P., Yang, J. (2022). Chemical Constituents of Functional Food Amomum villosum to Combat Human Diseases. *Current Chinese Science,* 2:57–67.

Zhang, D., Li, S., Xiong, O., Jiang, C., Lai, X. (2013). Extraction, characterization and biological activities of polysaccharides from Amomum villosum. *Carbohydrate Polymers,* 95(1):114–122.

Zhang, JS., Cao, XX., Yu, JH., Yu, ZP., Zhang, H. (2020). Diarylheptanoids with NO production inhibitory activity from Amomum kravanh. *Bioorganic & Medicinal Chemistry Letters,* 30(8):127026.

Zhang, M., Shuai, XX., Zhi, W., Dai, TT., Wei, CB., Li, Y., He, JJ., Du, LQ. (2024). Characterization, antioxidant and antitumor activities of phenolic compounds from Amomum villosum Lour. *Frontiers in Nutrition*, 11:1327164.

Zhang, TT., Lu, CL., Jiang, JG. (2015). Antioxidant and anti-tumour evaluation of compounds identified from fruit of Amomum tsaoko Crevost et Lemaire. *Journal of Functional Foods,* 18(Part A):423–431.

Zhou, S. (1993). Cultivation of Amomum villosum in tropical forests. *Forest Ecology and Management*, 60(1–2):157–162.

Zhou, Y., Qian, C., Yang, D., Tang, C., Xu, X., Liu, E. H., Zhong, J., Zhu, L., Zhao, Z. (2021). Purification, Structural Characterization and Immunomodulatory Effects of Polysaccharides from Amomum villosum Lour. on RAW 264.7 Macrophages. *Molecules*, 26(9):2672.